The persistence and control of bacterial pathogens in turkey eggs.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy.

by

Colin Baxter-Jones

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Dedication

To my wife, Shelagh.

Contents

SUMMARY

Total bacterial and coliform counts on the shells of turkey eggs were reduced when paraformaldehyde was added to nest boxes containing woodshavings. The number and types of bacteria composing the shell flora of eggs laid in different types of nest box was established. Gram positive cocci predominated on the shells of all nest-laid eggs. Fewer coliforms were isolated from eggs laid on synthetic grass nest pads. Low shell counts (5.5×10^3/egg) were obtained from eggs laid on nest pads regularly cleaned and treated periodically with paraformaldehyde. The synthetic grass was accepted by laying hens.

Eggs were artificially infected by exposing the shells to an inoculum of *S. hadar* using a temperature differential dipping procedure. *S. hadar* was consistently isolated from the contents of infected eggs and from those treated by immersion in warm disinfectant solutions or by fumigation with formaldehyde gas. A marginal pasteurisation ($60^{o}C$, 4 mins.) and an high temperature differential dip (HTD) were fully evaluated with respect to efficacy and also to establish whether they impaired embryonic development. Formalin used in the latter procedure (HTD dip) was embryo-toxic at high concentrations. The use of high temperatures resulted in some embryo mortality. Both methods substantially protected eggs from infection after exposure to large concentrations of *S. hadar*. The test organism was introduced into different sites within eggs. Results indicated that bacteria localised at the shell periphery were destroyed. To be effective it was considered that treatment must precede that phase of the egg infection process involving multiplication at the shell membranes and invasion of the albumen.

A microbiological survey established the contamination rate of normal incubated turkey eggs and those that failed to hatch (3.4% and 10.8% respectively). Dirty floor eggs were more often contaminated. Coliforms, particularly E. coli, formed a significant part of the microflora of incubated eggs and together with micrococci were the predominant groups isolated. Possible relationships between bacterial contamination and hatchability were not considered because of the low isolation rate. The extent of bacterial penetration through the shell of naturally contaminated eggs was assessed. A high percentage of floor-laid eggs and washed floor eggs were invaded by bacteria which could be cultured directly onto solid media from the inner shell membrane.

The incidence of bacterial penetration in soiled eggs was substantially reduced by dipping at high temperature in a solution of alkaline detergent. Very few treated eggs were penetrated by coliforms. Some shell treatments appeared to facilitate bacterial invasion. Egg moulding demonstrated more eggs to be contaminated than either direct or enrichment culture of the egg contents.

A good correlation was observed between the ability of bacteria to successfully infect eggs and their growth in albumen in vitro. Some isolates grew and multiplied in albumen (pH 9.1) at 37°C whereas others multiplied feebly or not at all. An isolate of S. ohio exhibited markedly different growth patterns in turkey albumen from different sources. A possible explanation is suggested. High temperature egg treatment was effective against a range of bacteria artificially introduced into eggs.

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Mrs. Eileen Rowlands must take the credit for successfully tackling my handwriting and typing this thesis. I express my thanks.

My wife, however, receives the final mention, for without her constant encouragement, love and understanding, this thesis would never have been produced.

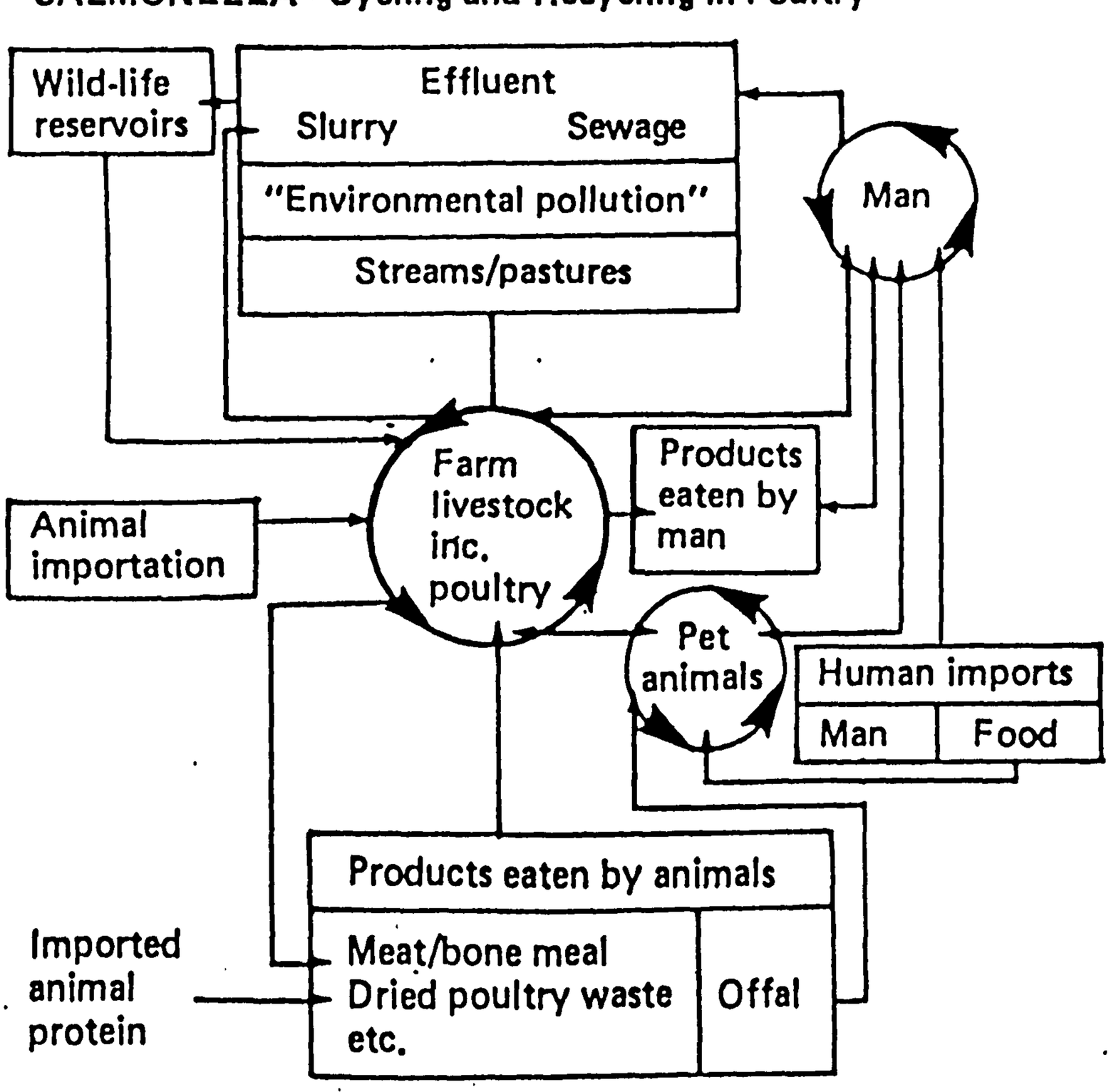
INTRODUCTION Salmonellas and Salmonellosis

Salmonellas other than the avian specific serotypes (S. pullorum and S. gallinarum) do not generally constitute a major problem to the poultry industry.

The disease, salmonellosis, is not of major economic significance or a significant cause of mortality under normal circumstances (5). The organisms however, are commonly associated with outbreaks of bacterial food poisoning in the human population (3). The bacteria responsible for initiating food poisoning cases are harboured by a variety of different foods (151), although poultry meat is one of those most commonly incriminated. A particular problem with turkeys is the often spectacular nature of the outbreaks (2). The size of catering birds often means that large numbers of people become infected in a single outbreak. Improper food handling practices (inadequate defrosting and cooking, together with poor storage conditions) accentuate the problem.

Salmonellosis is an example of a zoonosis with an extremely complex cycle of infection (Fig. 1) involving feedingstuffs, the environmental reservoir, animals and man. Because of the large number of sources of the organism, the control or eradication of the disease in the human population is therefore a very complex issue. Various reports and publications have reviewed the salmonella problem either in its entirety (3) or specifically in relation to the poultry industry(5,113,4,138). Any improvements in the control of this disease will only be achieved by the concerted efforts of a large number of people and organisations who are involved at different stages in the food production chain. This is in part illustrated by the recom-

FIG. 1


SALMONELLA - Cycling and Recycling in Poultry
Wild-life reservoirs
Effluent
Slurry
Sewage
"Environmental pollution"
Streams/pastures
Man
Farm livestock inc. poultry
Products eaten by man
Animal importation
Pet animals
Human imports
Man
Food
Products eaten by animals
Imported animal protein
Meat/bone meal
Dried poultry waste
etc.
Offal

mendations set out in the U.S.D.A. report of 1978 (4) which specifically highlighted: a) the production of animal feedingstuffs b) the primary breeding industry; c) the commercial production units; d) the slaughtering and processing industry and finally e) the consumer.

The ubiquitous nature of salmonella organisms probably makes the complete eradication of salmonellosis in the human and animal populations impracticable (3). A realistic goal would be aimed at achieving control in the animal population by recognising various points in the chain of infection which can be attacked.

The cost of eradication in purely monetary terms far exceeds the benefits derived by the consumer (113,45). As the detection of salmonella organisms in or on a poultry product does not necessarily imply unfitness for human consumption , it is in the end the consumer who must decide whether an eradication programme is worthwhile.

Salmonella and poultry flocks

There are many available data concerning the incidence and dynamics of flock infection within both chicken and turkey flocks. Williams (153) has reviewed the subject in relation to both species. Newly-hatched turkey poults exposed to salmonellae rapidly transmit the organism to other members of the flock housed in litter-floored pens (143). After 14 - 21 days, a high percentage of the flock would typically be found excreting salmonella organisms in their faeces. In one study (30) *S. typhimurium* was detected in the faeces of experimentally infected birds 15 - 22 hours after exposure by the oral route. After a short time, usually the sixth to eighth week after infection, the number of individual birds excreting salmonellae drops markedly (125,143). The intestinal infection referred to is usually asymptomatic.

Occasionally, infection within a flock does not conform to this rather simplified model and the individual bird infection rate does not exhibit this dramatic increase in the early weeks of life. This can be true even when the environment contains large numbers of salmonellae (104). Birds subjected to certain types of stress e.g. following water deprivation can exhibit markedly different shedding patterns (29). The establishment of the intestinal carrier state has been shown experimentally to be influenced by the age of the flock and the size of the inoculum (125,97).

The used pen litter on which chickens are reared can become salmonellicidal(148,105,149). Because of the importance of infected floor litter as a reservoir of infection for young birds, this can obviously influence the transmission patterns within a flock. The number of intestinal carriers of infection could be reduced and the survival

time of the infecting agent in the environment decreased.

Young chicks or poults seeded shortly after hatching, with the normal intestinal microflora of selected adult birds are to a large extent, protected from subsequent infection. The seeding of the gastro-intestinal tract with a protective flora is commonly referred to as "competitive exclusion"(103,118,86). The acquisition of a suitably protective microflora from the environment, rather than by inoculation, may be responsible for altering the dynamics of a flock infection. Infection in older flocks may be abbreviated by this mechanism and, in part, it may explain why it is more difficult to infect older birds with salmonellae.

Adult birds infected with salmonellae, generally show no outward symptoms, but they may serve as intestinal carriers for long periods of time (104). The presence within a laying flock however, of a small number of birds excreting relatively few organisms, can lead to the infection of eggs laid by these hens. Egg contamination, in the cloaca or as a result of faecal contact in the nest box, will be discussed in more detail later.

Salmonella hadar.

This serotype was first isolated, and identified as strain 1281 in the laboratory of the Workers' Sick Fund (Kupat Holim), Haifa, Israel in 1951. Hadar Hàcarmel is a town quarter in Haifa where the patient, from whom the organism was isolated, lived. A new serotype, S. hadar was described by Hirsch et al (76) in 1954.

S. hadar is just one of a number of serotypes of salmonella which are isolated from poultry but it is difficult to consider a single serotype in isolation. Although there are possibly differences between different serotypes and phage types in terms of epidemiology or avian (and or human) pathogenicity, it is true to say that the salmonellas are usually considered as a group of organisms referred to commonly as "paratyphoid salmonellae".

S. hadar achieved prominence because of the increase in the number of human food poisoning cases reported from 1974 - 1979 (124). This serotype was rarely reported in either the human or animal populations before 1971 and the majority of human cases involved individuals recently returned from parts of Africa. 38 strains had been identified by the end of 1971 and there was a progressive increase in the number of isolations thereafter. By the end of 1975, the serotype had established as one of the ten most prevalent serotypes isolated from humans. In 1979, 1600 strains were identified from different food poisoning outbreaks (affecting more than one family or household) reported in this country. In only 91 of these outbreaks was the vehicle of infection identified. Turkey however, proved to be the carrier of infection in 71/91 (78%) of these cases.

Silliker (138) graphically illustrated the increase in the number of S. hadar isolations from the human population in both Canada and the United States. The number of reported isolates was relatively small, although the increase in numbers probably significant. The introduction of S. hadar into Canada and the U.S.A. was associated with the importation of turkey poults from the U.K. between 1977 and 1980 (119). S. hadar was recently isolated from a poultry processing plant in the U.S.A. (35).

Payne and Scudamore (109) related the presence of S. enteritidis and S. hadar within a turkey breeding and rearing establishment in the U.K. to 185 cases of food poisoning between 1967 and 1975. The presence of these serotypes, as suggested earlier, was not responsible for causing significant mortality within the poultry flocks and no clinical disease was observed. Similarly, Gerichter et al (59) associated S. hadar and other serotypes with food poisoning outbreaks in Israel. Poultry were again implicated. This serotype has also been associated with food poisoning outbreaks in Germany (33). There have been sporadic reports of the organism in Central Africa, Nigeria and Poland. S. hadar therefore, has been isolated from the human population in several different countries. Whilst commonly associated with turkeys and latterly to a greater extent, with chickens, S. hadar has been isolated from many different animal species ranging from freshwater aquarium frogs (10) to cattle and horses.

This serotype does not appear to be especially different from others in the way that it has emerged as a significant public health problem. S. virchow provided a similar story. Although first reported in man in 1942 (85), the organism was not reported in animals in the U.K. until 1968. During 1969, 326 incidents were reported and the organism

had become established in poultry. The serotype was not associated with clinical disease in animals, but was often implicated in cases of human food poisoning (111,134). Pennington et al (111) suggested that S. virchow was introduced into poultry via imported contaminated feedingstuffs.

S. agona provides another example and was discussed by Lee (85) and Sojka et al (145). The appearance of this serotype in pigs and poultry and two others (S. indiana and Salmonella 4, 12: d:-:) was again associated with the importation of contaminated feedstuffs (fishmeal from Peru).

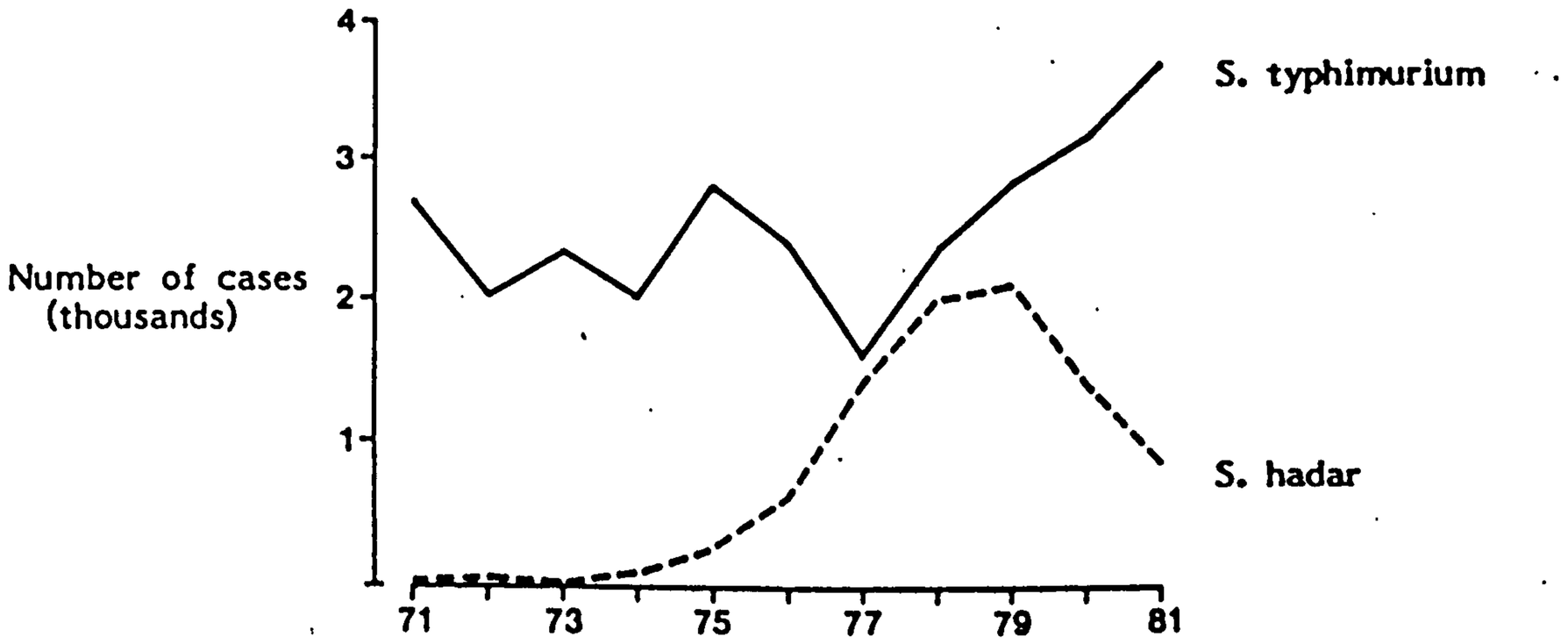
Although the introduction of S. hadar into this country via a similar route has not been officially documented, McCoy (personal communication) has suggested that the serotype was imported into Britain in 1969 in poultry offal. S. hadar was isolated from processed bone meal in Israel (77) as long ago as 1958.

The organism was first isolated from the premises of the primary turkey breeding organisation, where I am engaged in study, in 1973. A single isolation was made from a hatchery fluff sample in the second half of the year. During the remainder of 1973 and the beginning of 1974, 17 isolations were made from feedstuffs used throughout the system. This is again indirect evidence supporting the theory that the organism was introduced via this route.

The number of food poisoning cases in the human population attributable to S. hadar had begun to fall by the middle of 1980 (fig. 2). By the end of 1981, the number of cases in humans had dropped to

FIG. 2

Cases of S. typhimurium and S. hadar infection : 1971-1981



883 (7) and this decrease continued thereafter. The fall in the number of human cases has been paralleled with a decrease in the number of incidents involving animals (6) and this serotype is no longer the most prevalent in domestic poultry species.

The possible routes of infection for a turkey flock

The ubiquitous nature of salmonella organisms ensures that there are many ways in which a flock can become infected. I will consider however, only the primary routes in this brief discussion and these can be listed as follows:-

a) feed

b) environment

c) vertical transmission (egg transmission)

Feed

A comprehensive review of the occurrence of salmonellas in feeds and the importance of animal feedingstuffs in introducing infection into poultry flocks has been presented by Williams (154). Salmonellas are quite commonly isolated from animal (including fish) proteins incorporated into finished poultry feedstuffs. Contamination is not restricted to animal proteins. Raw materials of vegetable origin are sometimes contaminated. Although finished feeds are often pelleted, a process involving heating to high temperatures,the final product can become contaminated from other sources in the mill or at any stage before delivery to the production unit. During a five year epidemiological study of naturally occurring salmonella infection conducted at the Dillon Beach Ranch in California (161), several serotypes of salmonella were introduced onto the premises via contaminated pelleted feed. Four of the eight serotypes introduced onto the ranch were isolated directly from feedstuffs and a fifth was associated with feed.

It has been demonstrated experimentally by Gordon and Tucker (63) that chicks receiving artificially contaminated feed became infected

and shed the organism in their faeces. Egg shell contamination was demonstrated in eggs produced by hens receiving contaminated feed. Chicks hatched from these eggs were also infected.

The environment

Kumar et al (83) studied the dynamics of salmonella infection in turkeys. Three sources of infection were identified; a) breeder flocks and hatchery, b) environment and c) feed. Although the buildings were cleaned and disinfected before the poults were placed, salmonella organisms were isolated from two of the three premises investigated. The flocks subsequently placed in these houses were found to be infected. Similar results were obtained by Hacking (67) in a study of broiler flocks. In addition, new wood shavings laid in the sheds before the birds were placed were also found to be contaminated.

More recently, Morgan - Jones (101) has demonstrated that the inadequate cleaning and fumigation of drinkers within poultry sheds was responsible for spreading contamination to subsequent batches of poults. She also pointed out that salmonella-free feed placed in automatic feeders could become contaminated from other sources, and disseminate infection within a flock.

The importance of water as a vehicle of infection has been noted by several authors (63,101). Dougherty (42) presented similar findings after studying salmonella contamination in broiler flocks. This author considered that contamination arose from two sources

a) resident species present (due to faults in cleaning programmes)

b) species entering the house (e.g. infected wood shavings or litter, feed etc.)

Vermin, including rodents and insects together with pets, farm animals, wild birds and man can also carry salmonella organisms onto a site. The role played by these factors in the epizootiology of salmonella infection is difficult to determine however. Along with contaminated feedstuffs, personnel and equipment were suggested as possible factors being responsible for the introduction of different serotypes onto the Dillon Beach premises (161).

Egg transmission - salmonellas and other pathogens

For convenience, it is possible to divide micro-organisms into two groups, depending upon how they maintain infection between successive generations. This sub-division is not entirely rigid, as some organisms may be adapted to use either method.

Group 1 - enter the egg before oviposition and examples include Salmonella pullorum and Mycoplasma meleagridis. Infection of the ovary and peritoneum can be the result of systemic infection, artificial insemination (73) or contact with adjacent infected tissues as in the case with some viruses.

There is little direct evidence to suggest that this route of infection is important in facilitating the spread of paratyphoid infection. Motile salmonellae have however been isolated from the ovary and peritoneum of infected birds (144). Paratyphoid salmonellas are generally representative of organisms that belong to the other major group of egg pathogens.

Group 2 - These organisms enter the egg at, or immediately after oviposition, as the direct consequence of external shell

contamination. Included in this large group are a number of opportunist pathogens. Some may be responsible for causing disease e.g. pathogenic serotypes of Escherichia coli and others may cause putrefactive changes in eggs, e.g. Pseudomonas aeruginosa. Harry (74) associated the presence of Staphylococcus aureus in the embryonic yolk with shell contamination. In addition to contamination in the nest, he suggested that infection could result from both the handling of incubated eggs and also as a result of shell soiling which occurred during the hatching process. Bierer (11) successfully induced Salmonella typhimurium infection in chicks by spraying the outside of the shells of incubated eggs with this organism.

It has been recognised for a long time that salmonella organisms contaminating the shell exterior, as a result of faecal soiling for example, subsequently infect the egg contents (131). Williams et al (157) demonstrated that a motile Salmonella typhimurium could penetrate the shell and membrane within six minutes of the introduction of an infected faecal cylinder, thus initiating the infection process.

Natural salmonella egg transmission rates tend to be very low however, (Table 1) even when birds are artificially challenged with a particular serotype. It is for this reason that much reliance has been placed on the use of comparative data and experimental models to study the dynamics of egg transmitted salmonella infections.

In fact, most of the present knowledge concerning the course of egg infection has been obtained from studies of the keeping quality of

Table 1

Salmonella isolations from the eggs of naturally or artificially infected laying hens*

No.eggs examined: Naturally infected flocks	Total	%	Salmonella isolations from:	Serotype	Author
3374	224	6.6	shell	S.heidelberg	Smyser et al (141)
	16	0.5	contents		
774	10	5.6	shell	S.thompson	Buxton & Gordon (34
	0	0	contents		
1117	3	0.3	egg	S.typhimurium	Philbrock et al (112
Artificially infected flocks					
1728	0	0	shell and contents	6 serotypes	Mundt & Tugwell(102
622	0	0	shell and contents	S.derby	Mellor & Banwart (92)
141	3	2.1	shell	S.lexington	Forsythe et al (47)
163	3	1.8	contents		
362	8	2.2	shell	S.anatum	
356	41	11.5	contents		
2775	0	0	fresh eggs	S.typhimurium	Olesiuk et al(104)
2772	3	0.1	incubated eggs		
79	-	7.6	shell	S.senftenberg	Cox et al (39)
	0	0	contents		
74		9.5	shell	S.thompson	
		1.4	contents		
79		6.3	shell	S.typhimurium	
		0	contents		

* Data adapted from Hanschke J (71)

infertile table eggs. In particular, this relates to the growth of rot-producing organisms e.g. Pseudomonas aeruginosa in stored eggs. A lot of these data however, are equally applicable to hatching eggs.

The typical course of infection was considered by Gillespie and Scott (61) to be differentiated into three stages: a) contamination and subsequent penetration of the shell, b) colonisation of the membranes and c) infection of the egg contents (albumen and yolk). This view has since received considerable support in the literature. The following discussion will describe the principle factors determining whether or not an egg becomes infected with salmonellae and how various factors can influence the course of infection. These factors can be listed as follows: 1) the laying hen, 2) the environment (of the hen and of the egg), 3) the egg and 4) the micro-organism itself.

The laying hen A laying hen can only infect the surface of an egg, if she carries the organism (as either an intermittent excreter or as a carrier of paratyphoid infection). The main source of bacterial contamination, according to Harry (74), was the egg environment following lay and this would most likely be as the result of contact with contaminated dust and faeces. Mundt and Tugwell (102) artificially infected a small group of laying hens and recovered salmonellae frequently from the egg shells, but not the contents. Salmonella organisms were isolated from the faeces of these birds up to 35 days after the initial challenge. Similar findings were reported by Cox et al (39) when they studied eggs laid over a 10 day period. One must also consider the lower reproductive tract and cloaca as a potential site of infection (common opening to both digestive and urogenital systems). Large numbers of salmonella could be present

in the cloaca of an infected, excreting hen.

The egg environment The available evidence suggests that bacteria deposited on the outside of the shell do not invade the egg unless some agent promotes their translocation through the shell pores. Of the factors considered by Board (21) the suction pressure generated during the cooling of an egg immediately after lay, is probably the most important. Haines and Moran (70) successfully infected eggs by creating a temperature differential between the eggs and the suspending medium in which they were immersed. This experimental procedure also causes the egg contents to contract. Water appears to be an essential agent for microbial penetration. The ability of the shell to prevent the passage of water was considered by Board and Fuller (21) to be important in protecting the egg from bacterial infection. The condition and type of nest litter are therefore important. Heavily contaminated, moist litter was shown by Lorenz et al (87) to increase the production of rotten eggs. The organism responsible for causing the spoilage remained viable as the nests dried, but bacterial numbers rapidly declined. Excessively dirty eggs were identified as a particular problem because the faecal material on the shell presumably retained sufficient moisture to allow microbial penetration.

Smyser et al (141) observed the salmonella contamination rates of egg shells produced by a naturally infected flock. Most of the contamination resulted from eggs becoming infected in the nest. Eggs in contact with the litter for a longer period produced more positive isolations.

Barbour and Nabbut (9) observed that salmonellae were isolated from the contents of eggs laid on the floor of houses containing two chicken breeding flocks. Faeces on the shells of these floor eggs was thought responsible for the massive contamination. A high percentage of the shells of dirty floor eggs in this study were penetrated by other bacteria (22.8 - 25.5%) compared to clean nest eggs (0 - 1.1%).

The method of handling shell eggs after lay can also influence their susceptibility to bacterial contamination. Some specific factors include: a) the method of handling eggs during collection (123), b) the use of contaminated egg trays and boxes (20) c) the procedures adopted for washing eggs (98) and d) the conditions used for storage of eggs before incubation (46).

Because bacteria on the shell can rapidly penetrate the outer structures, it is important to collect and sanitise eggs as soon as possible after they are laid. The hatching egg remains vulnerable to contamination with salmonellae until the chick or poult has hatched. Williams and Dillard (155) did observe however, a decreased potential for S. typhi-murium to penetrate the outer structures of eggs undergoing embryonic development. Schalm (131) implied that in most cases, salmonellae failed to penetrate the shell but infected the chicks at hatch time. Frank and Wright (48) and Buxton and Gordon (34) showed however, that in addition to surviving on the shell exterior, these organisms invaded the egg contents during incubation. The latter authors also demonstrated that cross contamination could occur within a hatching machine. S. thompson was isolated from 21.7% of chicks and eggs which were incubated with a group of contaminated eggs. A subsequent hatch of chicks, in the same machine but after a thorough washing, was also infected. Such reports indicate the importance of maintaining high

standards of hygiene within hatcheries and of minimising the risk of introducing infection from other sources.

During certain periods of the year, late summer especially, the level of salmonella egg transmission has sometimes appeared to increase. This could be related to the ability of paratyphoids to survive in the natural environment or it maybe the result of environmental conditions modifying egg penetration patterns.

Both temperature and relative humidity influence the growth of salmonellae on the egg shell and also the course of infection. Rizk et al (121) observed that these organisms survived better on the shells of eggs stored at low temperatures. Simmons et al (139) reported that salmonellae did not invade the egg shell unless the moisture level was high (97% RH) . At high temperatures (28^{o}C) and in a moist atmosphere, bacteria multiplied extensively in the shell pore/membrane system during a 60 day storage period. The number of bacteria on the shell dropped sharply over the first three days storage but after an initial decline the number of organisms increased, dependant upon temperature, to a peak at 6 - 12 days. Stokes et al (146) artificially infected and stored eggs at a variety of temperatures. The contents of eggs infected with S. oranienburg and held at 1^{o}C for six months, were not infected. The organism was isolated from the shells and membranes of these eggs using an enrichment culture technique. At higher storage temperature the challenge strain was isolated from the contents, usually within seven days of infection. Ellemann (43) showed that salmonellae were isolated from the contents of eggs held at 30^{o}C after four days, whereas no isolations were obtained after two months at 4^{o}C.

Graves and MacLaury (65) studied the bacterial contamination of eggs laid in artificially contaminated nest boxes. These authors related

the incidence of egg contamination to atmospheric temperature and absolute humidity measured whilst the egg was cooling after being laid. The rate at which the egg cools following oviposition may be an important influence on the course of egg infection.

The egg

Some comprehensive review articles have been written on the microbiology of the hen's egg (16,18,26,69) and the non-specific antimicrobial defence system possessed by avian eggs (21,24,147). For the purposes of this brief discussion, the egg can be conveniently divided into two parts:

a) the outer egg structures(shell and cuticle)

and

b) the egg contents (albumen and yolk)

The role of the shell membranes will not be considered. The reader is referred to the review of Board (18).

The outer egg structures

The egg shells of domestic avian species possess a large number of pores traversing the shell which are capped by an organic cuticle. In eggs with undamaged cuticle there is a small variable number of pores which allow the passage of liquid (1) and are commonly referred to as patent pores. It is these pores that allow the passage of bacteria through the shell as elegantly demonstrated by Paton and Ayres (108) and Board and Board (13). The water resistance/repellence offerred by the normal cuticle and shell has been considered one of the two principal components of the physical defence system possessed by avian eggs (19). Vadhera et al (150) noted a higher incidence of rotting in eggs excised from the uterus before the cuticle had been secreted and also in eggs from which the cuticle had been removed chemically. Eggs

with no cuticle were shown by Board and Halls (22) to be more easily invaded by a solution containing carbon black. Further indirect evidence suggesting the importance of the cuticle has been obtained from studies involving the washing of eggs. Haines (68) for example, noted that eggs severely washed or scrubbed and subsequently stored, were more often contaminated with rot-producing organisms.

The importance of an intact shell in reducing the incidence of rotting was noted by Brown et al (28). Cracked eggs with faecal contamination on the shell were shown to have a high incidence of rotting after washing. Cracked artificially infected eggs were heavily contaminated and the infection rate was related only to the numbers of Ps. aeruginosa in the solution used to challenge the eggs. For eggs with intact shells it was first necessary to overcome the water resistance of the shell by imposing a temperature differential. Similar findings were presented by Miller and Crawford (96). After washing, Vadhera et al (150) found more cracked eggs to be contaminated with salmonellas. These organisms were isolated from the contents of cracked eggs by D'Aoust et al (40) quite frequently.

Studies by Orel (106) and Sauter and Petersen (129) have suggested that egg shell quality may be important in protecting eggs from infection. Eggs with better shell quality, using specific gravity as an index, were more resistant to bacterial invasion. Williams et al (157) did not find a relationship between shell thickness and salmonella penetration, but emphasised that cracks and naturally occurring open shell pores were of primary importance in facilitating

shell penetration. In a subsequent study using turkey eggs, Williams and Dillard (156) found white-shelled eggs to be more susceptible than normal brown speckled eggs. Turkey egg shells restrained the passage of salmonellae more than chicken egg shells.

The egg contents

In addition to the physical defence offerred by the outer egg structures, the albumen of domestic avian species possesses anti-microbial properties. The exact role of maternal immunity in protecting the developing embryo from subsequent salmonella infection is not fully understood. Observations made but not reported here suggest that parent hens vaccinated with a heat-killed S. hadar vaccine develop very high antibody titres, as measured by the micro-antiglobulin test. Certainly IgG transmitted to the yolk from the serum of the parent hen could result in some measure of passively acquired resistance being passed to the chick or poult (107). Antibody in the yolk however, would be unlikely to influence the course of egg infection. The exact role of IgA and IgM synthesised locally in the oviduct (122) and secreted into the albumen awaits elucidation. Relatively small amounts of immunoglobulins are present in the white compared to the yolk. In contrast however, and as known since the studies of Laschtshenko (84) in 1909, the albumen of domestic avian species does not support extensive microbial growth. In the most recent review (147), it was suggested that bacterial inhibition resulted from the interplay of several factors. The bactericidal properties of albumen were considered due to the influence of ovotransferrin (conalbumin) and alkaline pH at normal incubation temperature. The small amounts of available nitrogenous compounds in the albumen were also considered important. This non-specific defence system

is discussed more fully later (Chapter 4, Discussion) but clearly represents an extremely effective and comparatively sophisticated mechanism when reviewed in relation to the extremely low contamination rates obtained with naturally clean eggs (26).

The extremely viscous nature of the albumen of fresh eggs probably contributes to the physical defence of the egg against micro-organisms either by ensuring that bacteria within it remain in clumps (61) or by preventing contact of contaminating organisms with the yolk (21).

The micro-organism

The course of infection in eggs infected with paratyphoid salmonellae has been described by several authors (12, 146) and Williams et al (157) have previously summarised much of the previous work on shell penetration by different salmonella serotypes.

It could be argued that infection could be transmitted from one flock to the next without massive contamination of the egg albumen. A single chick or poult infected by the inhalation of a suitably large inoculum at hatch time could subsequently infect the remainder of the hatch after placement. Indeed there would appear no reason why an opportunist pathogen should necessarily establish in the albumen or yolk in large numbers. Such an event would most likely result in the death of the embryo. Extensive microbial growth in the absence of embryonic death could however lead to the transmission of infection within the hatcher. Large numbers of bacteria would probably be present.

There is relatively little information concerning possible differences between salmonella serotypes or phage types and the dynamics of the egg

infection process. Several studies have indicated that different salmonella serotypes can survive on the shell surface for long periods in a variety of different storage conditions (12, 34). Motility was not found necessary to ensure shell penetration by Williams et al (157) who employed contaminated faecal cylinders placed on the shell surface. It is difficult to find many data indicating the number of organisms necessary to ensure penetration. This probably reflects the difficulty of selecting a suitable test system. Stokes et al (146) demonstrated that 4/16 eggs were infected with S. oranienburg following exposure to only 4×10^1 organisms using a temperature differential dipping procedure. The extent of shell contamination may influence the course of infection. Gordon and Tucker (63) demonstrated for example, that the membranes, albumen and yolk of eggs laid by hens receiving heavily contaminated feed were rapidly infected with S. menston. Bacterial proteases were not considered by Garibaldi and Stokes (57) or Board (15) to be important in facilitating microbial penetration of the shell membranes. Brown et al (27) did suggest that enzymes produced by Ps. aeruginosa were involved in the penetration process. These results were open to some question however, because changes in the electron microscopic appearance of the inner membrane may have been as the direct result of bacterial growth after penetration.

Seviour and Board (135) observed after seeding the air cell with a heterogeneous shell flora, that selection occurred within the shell membranes. Temperature was an important factor in establishing which bacterial species became dominant. Simmons et al (139) observed the extensive multiplication of S. anatum and S. heidelberg in the outer egg structures after storage at $23^{o}C$ in a humid atmosphere. As either the temperature or humidity decreased, the number of eggs with high counts within the shell membranes also decreased. If it is assumed

that the antimicrobial properties of albumen are responsible for initially restricting the passage or limiting the growth of organisms within the shell membranes, then the ability of different salmonella serotypes to establish and multiply within the outer egg structure must be considered of prime importance.

At the present time there is only limited information regarding the ability of different serotypes to grow in albumen, although Garibaldi (56) did effect the growth of S. typhimurium in egg white at 28°C after supplementing with iron transport compounds. Harry (72, 74) studied the properties of a number of bacterial isolates which he considered important if an organism was to exhibit pathogenicity. This author found that some organisms, whilst pathogenic in yolk, did not establish in the albumen because they were either non-motile or could not resist the biocidal properties of albumen. Embryonic mortality was associated with the presence of bacteria in the yolk as distinct from the albumen or allantoic fluids. The ability of different serotypes firstly to invade the albumen from the membranes, and secondly to either persist or grow in the albumen, without necessarily causing macroscopic changes in the egg contents, would be worthy of further study.

The Aims and Objectives of the present study

The importance of vertical transmission in the epizootiology of salmonella infection has been discussed. The control of egg transmission must therefore be considered an essential requirement of any programme specifically designed to reduce the prevalence of salmonellas within poultry flocks.

As each chapter of this thesis is preceded by a specific introduction the following paragraphs present only an outline of the approaches made towards solving the problem of how to eliminate a specific microbial pathogen from a turkey hatching egg.

Attempts were made to establish how the bacterial flora of the egg shell could be modified by improving the design and management of nest boxes (Chapter 1). It was thought unlikely that such procedures alone would prevent eggs from becoming infected with salmonellae, rather that the chance of an egg becoming contaminated might be reduced.

Egg treatment provides a most effective means of interrupting the transmission cycle of egg-borne infections. Much emphasis was placed therefore on the development and evaluation of methods designed to reduce the likelihood of turkey poults hatching infected with paratyphoid salmonellae (Chs. 2 and 3).

Consideration was also given to the complex relationships that exist between some bacterial pathogens and fertile eggs. An understanding of the mechanics of the egg infection process could be considered essential to those contemplating the successful elimination of specific organisms. Some of these important considerations, together with

preliminary results obtained with a range of typical egg contaminants are presented in the final chapter (Ch. 4).

CHAPTER I: INTRODUCTION

The influence of the nest environment on the bacterial flora of the egg shell.

Egg transmission arising through the contact of the egg shell with salmonellae, at any stage during or after oviposition, is a major factor influencing the spread and perpetuation of infection (153). There are many available data on the external bacterial contamination of egg shells and some of this information has been presented below. It is included because the naturally occurring egg transmission levels are extremely low and this therefore precludes any work specifically with salmonallae.

The intention of this part of the work was to determine whether it would be possible to improve the general microbiological quality of the egg shell by improving the standard of nest box hygiene or by modifying nest box design. By reducing the total bacterial shell count, or by influencing the types of bacteria composing the shell flora, the chances of a newly laid egg becoming infected with salmonellae could be reduced. The potential benefits are not confined to salmonellae. Rather these organisms should be considered as part of a group of opportunist pathogens which can be transmitted via the egg. These include bacteria capable of producing putrefaction of the egg contents and those which can cause embryonic mortality and clinical disease in newly hatched birds (72).

Harry (74) compared the bacterial flora of egg shells laid in deep litter pens and those laid in battery units and found the former to be contaminated with 15 times the number of aerobic bacteria. He also demonstrated a relationship between the flora on the egg shell and that present in the deep litter that was examined. Total bacterial counts on the shells

examined ranged from 6.2×10^3 - 2.4×10^6 organisms (mean count 3.45×10^5). Apparently clean shells were often found to harbour large numbers of bacteria.

Similar results were reported by Carter et al (37) when wire and litter management systems were compared. Ten times as many bacteria were found on the shells of eggs produced in litter houses and the number of air-borne bacteria was also significantly greater. Quarles (114) concluded that there was a direct relationship between the bacterial environment of the poultry house and egg contamination. He and others later implied (116) that the higher total coliform counts obtained from the air of litter houses were responsible for their subsequent isolation from dead embryos and chicks.

Haines (68) estimated the numbers of viable bacteria on the shell surface and membranes of fresh eggs and found large variations in count between individual eggs. After receipt at the laboratory, no differences were found between eggs laid in batteries and clean eggs laid on the grass of the same farm. Mean aerobic bacterial counts on egg shells varied from 3.5×10^4 - 1.3×10^5, depending upon culture conditions. A heterogeneous shell surface flora was established.

Quarles et al (115) did not show any differences in shell counts between eggs laid in litter and rollaway nest boxes. The eggs produced in pens with litter floors had higher counts irrespective of nest box type.

Rosser (123) demonstrated that the egg shell counts could be affected by the way in which eggs were handled after laying. The mean bacterial count on the shell was reduced by approximately 10-fold when eggs were collected and handled by operators wearing gloves. The shell flora can

therefore be influenced by the method of egg handling employed. Board et al (20) studied the microbiological contamination of egg shells as received at a grading station. Shell surface counts exhibited a marked variability and a range from 10^2 - 10^8 organisms/shell was established. Although the authors observed a correlation between egg soiling and degree of contamination, they were unable to demonstrate that the egg packing materials and boxes were a potential source of contamination. Similarly the egg storage conditions did not exert an influence on egg shell counts.

Moats (99) found an association between the bacterial load on the shell surface and the wash water in which the eggs were immersed. In a subsequent study by the same author (100) the bacterial flora of unwashed eggs was characterised. Again the egg shell counts were highly variable. A range of mean shell counts from 5.6×10^4 - 4.7×10^6 was obtained using a blending method. Total bacterial counts obtained using a rinse method were consistently lower. The differences seen in the bacterial flora of washed and unwashed eggs could possibly be explained if the outside of the shells were contaminated from the equipment (brushes, washwater, conveyors) and materials used for washing.

March (89) was unable to demonstrate a relationship between egg washing procedures and bacterial shell counts. 21.8% of washed egg shells sampled at a grading plant were contaminated with more than 5×10^6 organisms compared to only 8.0% of unwashed eggs. Cracked eggs were shown after washing to have very high bacterial counts.

The level of bacterial contamination on the outside of the shell is therefore influenced by many factors. They include the poultry house general environment, the nest box environment and design, the amount

of organic material on the shell and the method of egg handling during and after collection.

The bacterial load on the shell can also be influenced by the laying hen and it is possible that the egg shell surface itself may exert an effect. The presence or absence of cuticle, shell texture and the number and size of shell pores are perhaps the most obvious factors. Previous workers have often commented on the enormous variability of shell counts obtained from apparently similar eggs.

Graves and MacLaury (65) found a relationship between bacterial isolations from the shell and contents of unincubated and incubated eggs and several environmental parameters. Temperature, absolute humidity and vapour pressure measured at the time the eggs were laid yielded significant correlations.

The work of McNally (90) served to emphasise the need for maintaining nest litter in a clean and dry condition to minimise the amount of spoilage. The frequency of egg collection, which governs the contact time of the egg with the nest litter, could also be important in controlling the amount of surface contamination. Management factors must therefore also be taken into account when considering egg shell bacterial populations.

The work described can be largely divided into two main areas. The first trials were conducted to demonstrate the possibility of controlling the number of bacteria in nest boxes lined with wood shavings. The effect of adding paraformaldehyde prills to the shavings on reducing the bacterial load on the shell was determined.

In the second part of the work, synthetic grass-like materials were used to line the nest boxes and the effect of periodic paraformaldehyde treatment assessed. The nest box design was also considered and rollaway nests were compared to the more conventional flat-bottomed variety. Rollaway nests reduce considerably the contact time between the hen and its egg after laying.

The results reported relate only to the relationship between the treatment used and the microbial flora of the egg shell. Both quantitative and qualitative data are presented. The effects of such treatments on egg production and bird performance are to be reported elsewhere.

MATERIALS & METHODS

Experimental Details

Trial 1

Six litter-floored pens containing 100 birds in a single shed on the same farm were involved. In each pen 25 nest boxes were provided. Each module was of wooden construction with a flat bottom and lined with a layer of woodshavings. The shavings were replenished when necessary.

Two weeks after the birds began to lay, the nests in four pens were treated with paraformaldehyde supplied in the form of small pellets of uniform size and referred to hereafter as prills, (Paraformaldehyde 91 Prills, Messrs. Synthite Ltd., West Bromwich). A calibrated measure was used and a calculated amount was added to the surface layer of shavings. The prills were quickly stirred into the nest material.

Each of the nest boxes in two pens were treated every two weeks with 40 gms. of prills whilst the boxes in another two pens received an 80 gm. dose every month. The first treatments were administered during January 1979 and continued until the end of April 1979. Every two weeks, five samples of nest litter were taken from each pen to provide a viable count.

A sterile scoop was used to transfer surface litter into a sterile plastic bag. Gross faecal material was removed as the samples were collected.

Five freshly laid eggs from each treatment group were sampled at a similar interval. Each egg was removed from the nest by an operator using a paper towel and wearing disposable gloves and was immediately deposited in a Whirl-Pac bag (18 oz. Nasco) containing physiological saline.

The egg shells were rinsed using the method described below.

Trial 2

During July 1979 the nest boxes in five pens at a different farm were treated with paraformaldehyde (40 gms/nest box every two weeks). Another five pens in the same laying shed were not treated and served as controls. 10 eggs from each treatment group were sampled every two weeks for the duration of the trial as above. Nest box litter was not collected.

Further trials were conducted on another site as part of a joint project with the Ministry of Agriculture, Fisheries and Food (MAFF Development Farm Project). The results reported here only relate to those aspects of the trial concerning the microbiological quality of eggs laid in different environments.

Trial 3

Four pens housing approximately 75 birds each and a single adjacent pen containing 100 birds were involved. All pens had litter floors and the housing was of a standard wooden pole-barn type used on all sites. The nest boxes in each pen were specially constructed and each box was equipped with a wooden apron at the front unless otherwise stated. A brief description of the nest boxes used in each pen is given below:-

Pen 1 Wiremesh rollaway nest floor covered with synthetic grass (Astroturf CH4, Monsanto) matting.

Pen 2 Standard flat-bottomed nest covered with Astroturf.

Pen 3 Wiremesh rollaway nest with weld mesh apron replacing standard wooden apron.

Pen 4 Wiremesh rollaway nest

Pen 5 Standard flat-bottomed nest box lined with woodshavings.

The first eggs were laid during June 1980 and the trial finished at the end of August 1980. During this period 20 eggs from each pen were collected every week. The eggs were collected by an operator wearing gloves and were placed onto new paper trays. Between egg collections and until the required number had been collected, the trays were placed inside a plastic bag to prevent extraneous contamination. The trays were then stored in the farm egg room overnight before being transported to the laboratory the following morning.

Each egg was removed from the tray using a clean paper towel and the shell examined to determine the extent of contamination. The eggs were graded as either clean, slightly dirty, or excessively dirty, depending upon the amount of adhering organic material. Clean eggs did not have any visible faecal soiling or staining.

Trial 4

The birds were housed in the same pens used for Trial 3. The trial was conducted over a period from February - July 1981. The nest box types used in the four pens are described below:-

Pen 1 Standard flat-bottomed nest box module covered with Astroturf. The pads were removed from the nests every week and shaken to remove dust and organic material. Paraformaldehyde prills were added to each nest box (25 gms/nest box) after the pads had been cleaned.

Pen 2 as Pen 1 but paraformaldehyde prills were not added.

Pen 3 Wiremesh rollaway nest covered with a different synthetic grass liner (Verdant, Monsanto).

Pen 4 Standard flat-bottomed nest box lined with woodshavings.

20 eggs from each pen were collected on alternate weeks in the manner previously described. After overnight storage in the farm egg room, they were transported to the laboratory the following morning.

Microbiological Analysis

Nest Litter

20 gms. of nest litter were transferred into a sterile polypropylene container. 200 mls. of quarter-strength Ringer's solution (Oxoid Ltd.) was added and the screw cap replaced. Each container was shaken for one minute, allowed to stand for five minutes and then shaken for a further minute. An aliquot was removed and serial decimal dilutions were prepared in physiological saline to allow an estimation of the viable count. The pour plate method was used and the following media employed:

Total bacteria — Blood Agar base containing 7% defibrinated horse blood (Oxoid Ltd.) incubated at 37°C for 48 hours.

Coliform count — Violet Red Bile Agar (VRBA, Oxoid Ltd.), incubated at 37°C for 48 hours.

Salmonella spp — 10 gms. of woodshavings were added to 100 mls. of Buffered Peptone Water (BPW, Lab M Ltd.) contained within a sterile polypropylene jar. The culture was incubated at 37°C for 16 - 18 hours. A 1 ml. aliquot was then transferred into a test tube containing 10 mls. of Muller-Kauffmann Tetrathionate Broth (MKTB, Lab M Ltd.)

The enrichment medium was incubated at 42°C for 48 hrs. and subcultures were made at 24 and 48 hrs. onto Brilliant Green Agar (BGA, Lab M Ltd.). After incubation at 37°C for 24 hrs. the plates were inspected for the presence of salmonella-like colonies.

Moisture content — 25 gms. of each sample were dried in a hot air oven at 105°C for 24 hours, weighed, and the moisture content calculated.

Eggs

A rinse method similar to that described by Gentry and Quarles (58) was used. Each egg was picked up in a clean paper towel and deposited into a Whirl-pac bag (18 oz.) containing 50 mls. of sterile physiological saline at room temperature. The eggs were massaged through the bag for one minute, allowed to stand completely covered for five minutes and rubbed again for one minute. Each egg was then carefully manipulated out of the bag by constricting the polythene underneath.

In Trials 1 and 2 serial decimal dilutions were prepared from the egg rinse fluids and a viable count was obtained using the pour plate method previously described. Total bacterial and coliform counts were obtained during the first trial. For Trial 2 a total bacterial count was obtained and Tryptone Soy Agar (TSA, Oxoid Ltd.) incubated at 37°C for 48 hours was the preferred medium.

The large number of eggs sampled in Trials 3 and 4 required that an alternative procedure be adopted. To obtain the viable count of the egg rinse fluid a dip slide (Uricult, Orion Diagnostica) was totally immersed in the solution. The slide was removed and the excess fluid allowed to

drain off. The slide was re-inserted into its tube and was then incubated at 37°C for 48 hours. The number of colony forming units appearing on the surface of the Cystine-Lactose-Electrolyte Deficient (CLED) medium after 24 and 48 hours incubation was counted. The viable count was obtained by extrapolation from a prepared regression slope.

Identification of bacterial isolates from egg shells (Trials 3 and 4)

Isolates were selected to be representative of types of colonies appearing on the surface of the dip slide. Colour, size, shape and surface appearance were the factors used to differentiate colony type. For each batch of eggs processed, at least two isolates of each colony type were replated to ensure purity. Pure cultures were maintained on nutrient agar slopes.

The isolates were classified into three major groups, using the following properties: morphology, motility, pigment production, Gram stain reaction, production of oxidase, catalase reaction, action on glucose and presence or absence of growth on MacConkey agar. The three major groups recognised were: (i) Gram positive cocci; (ii) Gram positive and Gram variable rods and (iii) Gram negative rods.

Staphylococcus aureus was differentiated from other staphylococci and micrococci. The coagulase test, the presence of haemolytic effects on sheep blood and the reaction on DNAase agar (Oxoid Ltd.) were used to differentiate Staph. aureus as described by De Vriese and Hajek (41).

Bacillus cereus was recognised by the presence of spores and its ability to produce lecithinase in media containing egg yolk (Egg Yolk Emulsion, Oxoid Ltd.) after incubation for 24 hours.

The majority of Gram negative bacteria, including coliforms, were identified using the API 20E system (API Laboratory Products Ltd.). Additional tests described by Gilardi (60) were used to identify non-fermentative Gram negative rods including the pseudomonads.

RESULTS

The mean log. counts obtained from samples of nest litter treated with paraformaldehyde are shown in Table 1.1. Because of the large range in counts observed between samples from the same treatment, it was sometimes impossible to calculate a mean value for that group.

Treatment of nest box litter with either 40 gms. of prills fortnightly, or 80 gms. monthly was effective in reducing the total bacterial count within the litter. The difference in counts between the treated and control groups became more significant as the number of individual doses received by each nest box increased. Unfortunately, there were insufficient data to observe a similar trend for the coliform counts.

There was no significant difference between the two treatment groups (A and B) with respect to the magnitude of the drop in total bacterial count. Paraformaldehyde treatment produced a large fall in bacterial counts. A difference in log. counts of between 3.7 and 4.6 (pens B and A respectively) was observed compared to the counts in the control group on 14th. March.

Total bacterial and coliform populations within the control pen gradually increased. This was probably due to an increase in the amount of soiled litter within the boxes. Salmonellae were not isolated from any of the samples of nest box litter.

Towards the end of this trial, the effect of paraformaldehyde treatment was monitored by observing the egg shell surface. Using an egg rinse technique, the number of aerobic bacteria and coliforms was calculated. The results are expressed in Table 1.2. Coliform counts in the control group were consistently raised from 26th. March to 23rd. April and were

Table 1.1

The effect of adding paraformaldehyde on the total bacterial and coliform counts within samples of nest litter.

Sample Date	Pen treated on sample date	Treatment Group A-('80 gms/month)		Treatment Group B-(40gms/fortnight)		Control	
		1 Total Bacteria	2 Coliforms	1	2	1	2
3.1	A + B	5.06	4.49	6.01	5.85	6.02	4.93
15.1	B	3.94***	NA†	5.23	3.63	6.08	4.40
29.1	A + B	4.08***	1.73***	4.18**	4.13	6.25	5.04
12.2	B	4.11***	NA	3.07***	NA	6.73	5.25
26.2	A + B	3.39***	NA	3.18***	NA	6.79	5.61
14.3	B	1.78***	NA	2.67***	NA	6.34	5.58
26.3	A + B	4.54**	3.77	4.27**	3.12*	6.14	5.38
Standard error of a mean of 5 counts		0.404	0.640	0.608	0.695	0.289	0.508

The figures in the body of the table represent the mean $\log_{10}$ counts/gm. dry weight of nest litter.

* Indicates level of significance of the effect of treatments A and B.

*, ** and *** signify $P < .05$, $P < .01$, and $P < .001$ respectively

† NA - no count available

Table 1.2

The effect of adding paraformaldehyde to nest box litter on the total bacterial and coliform counts on the egg shell surface.

Sample Date	Pen treated on sample date	Treatment Group					
		A		B		Control	
		1*	2†	1	2	1	2
26.3	A + B	5.92	2.61	5.51	2.75	6.32	5.16
11.4	B	3.52	0	3.94	1.36	6.04	5.19
23.4	A + B	5.77	3.71	4.50	1.06	6.96	5.57
30.4	-	4.39	2.61	4.00	1.97	5.44	2.86

The figures in the table represent the mean $\log_{10}$ counts/ egg shell surface.

* Total bacterial counts - LSD between 2 means of 5 = 1.28

† Coliform counts - LSD between 2 means of 5 = 2.45

significantly higher than those on the shells of eggs laid in nest boxes receiving fortnightly treatment on 26th. March ($P < 0.05$), 11th. April ($P < 0.01$) and 23rd. April ($P < 0.001$). A similar pattern was obtained when the total bacterial counts were compared.

The variation in counts obtained between individual eggs necessitated the use of larger treatment groups. In the second trial only one treatment was investigated and 10 eggs from each group were examined. The results are shown in Table 1.3. Total bacterial counts on the shells of treated eggs were consistently lower than those on controls. After the nest boxes had been treated on three occasions there was a significant difference between counts on the shells of control and treated eggs. Mean log. counts for treated pens were significantly lower over the period from 22nd. August to 3rd. October ($P < .01$) but the difference was not significant at the final sampling on 8th. November owing to a significant rise ($P < 0.001$) in the mean for the treated group.

The results obtained from Trial 3 are shown in Table 1.4. Owing to the wide variation between eggs within batches, considerable changes in mean count were needed before any significant effect could be shown. The counts in the table denote the geometric mean colony counts obtained from the dip slide surface of 20 replicates. Figures obtained from eggs laid on wire rollaway nests were not included (pens 3 and 4). Insufficient numbers of eggs were laid in the nest boxes of these pens to allow statistical analysis. The mean increase in count obtained from eggs laid in pens 1 and 2 from the beginning of the trial to week 30 was significant ($P < .01$) and thereafter the mean count from all three pens continued to rise for a further three weeks. The mean shell surface counts obtained varied from $8.3 \times 10^4 - 2.4 \times 10^6$ organisms.

Table 1.3

The effect of adding paraformaldehyde to nest litter on the total bacterial count of the egg shell.

Sample Date	Date of Treatment	Mean $\log_{10}$ bacterial counts* of egg shells from:- Treated Pens	Control Pens
24.7	19.7	3.59	4.44
6.8	1.8	3.77	4.25
22.8	15.8	4.45	5.67
5.9	29.8	3.73	5.26
20.9	12.9	4.15	5.28
3.10	26.9	3.46	5.65
8.11	9.10	5.21	5.88

* LSD between 2 means of 10 = 0.68

Table 1.4

A comparison of the bacterial counts on the surface of egg shells laid in different types of nest box over a 16 week period.

	Synthetic Grass		Wood Shavings	
	Rollaway	Flat-bottomed Nest	Flat-bottomed	
Week No.	Pen No:- 1	2	5	EMS*
26	10.3 (18)	3.96(12)		0.4934
27	5.76	4.23(18)		0.2558
28	10.9	6.83(19)		0.3493
29	11.3	8.21(17)		0.1654
30	27.8	24.6 (16)	17.8	0.4811
31	22.5 (19)	28.9 (17)	27.6(19)	0.2535
32	45.1 (17)	19.7 (19)	11.8	0.3245
33	24.0 (17)	76.0 (18)	29.6	0.3122
34	45.6 (18)	34.7 (19)	55.1	0.3042
35	33.5 (18)	43.8	40.8	0.2395
36	61.1 (18)	43.5	74.1	0.4092
37	20.3	48.2 (16)	89.0	0.3364
38	53.6	44.9 (17)	95.1	0.3980
39	29.7 (15)	59.6 (17)	35.2	0.3328
40	29.3 (12)	55.9 (14)	50.3	0.3602
41	36.1 (17)	111.5 (14)	9.8	0.2730

The figures in the body of the table represent the geometric mean bacterial counts (cfu/dip slide face) obtained from 20 replicates from each of three pens unless otherwise shown (figures in parenthesis).

* EMS = error mean square in one-way ANOVA

The shells of all the eggs were visually inspected and characterised as clean, slightly dirty or excessively dirty. In pens 1, 2 and 5 there were 38.0, 28.2 and 3.2% of eggs respectively with no organic material contaminating the shell. This refers to material either easily removed, or firmly adhering to the surface. Clean and slightly dirty eggs laid in nest boxes of pen 5 were more frequently associated with woodshavings than eggs laid in pens 1 and 2. Relatively few excessively dirty nest-laid eggs were observed irrespective of the type of nest box. The type of bacteria isolated from the shells of eggs laid in the different pens are shown in Table 1.5. Because of the small number of isolates of bacteria other than Gram positive cocci and E. coli, these have been grouped together to allow statistical analysis. This group includes the following bacteria: Klebsiella pneumoniae, Proteus mirabilis and Pseudomonas spp. An exhaustive identification was not attempted because of the large number of isolates involved.

Over the period extending from weeks 30 - 41 there was a highly significant difference between pens 1, 2 and 5 for bacteria in groups 2 and 3. Other differences were less marked. No significant differences were observed between the shell flora of eggs laid in pens 1 and 2. Gram positive cocci composed a dominant part of the microflora of all nest-laid eggs. Fewer coliform bacteria were isolated from the eggs laid on synthetic grass and the microbial flora was less diverse in nature compared to that on the shell of eggs laid in woodshavings.

In Trial 4 the nest pads were regularly shaken during the laying period, to remove organic debris and paraformaldehyde prills were added to the nest box liners of one treatment group. The wiremesh rollaway nest in pen 4 was covered with a different type of synthetic grass (Verdant) which was selected because of its close similarity to natural grass.

Table 1.5

The microbial shell flora of eggs laid in different types of nest box.

Pen No.	No. Eggs	The number of eggs (percentage in brackets) grouped according to the types of bacteria present on the shell:* 1	2	3	4 - 8	9
1	220	78(35.5)	198(90.0)	10(4.5)	3(1.4)	9(4.1)
2	220	65(29.6)	191(86.8)	4(1.8)	1(0.4)	2(0.9)
5	220	53(24.1)	214(97.3)	24(10.9)	7(3.2)	2(0.9)
chi-squared (2df)		6.81	16.0	17.6	5.15	7.69
Level of significance		$p < .05$	$p < .001$	$p < .001$	($p < .10$)	$p < .05$

* The following major groups of bacteria were recognised:-

1 Coagulase positive, Gram positive cocci
2 Other Gram positive cocci
3 E. coli
4-8 Other Gram negative rods
9 Unidentified isolates

The Astroturf pads, on the other hand, had coarse polyethylene blades approximately 10 cms. in length.

The eggs were inspected before processing and the results are presented in Table 1.6. The percentage of clean eggs in pen 4 (14%) was lower than that in pen 3 (X^2, 3 d.f = 74.1, $P < .001$). Pens 1 and 2 had more than twice as many clean eggs compared to pen 3. 67.7 and 71.9% of eggs laid in the nest box of pens 1 and 2 respectively were graded as clean. In pen 4 woodshavings were the main type of material contaminating the shell and a higher proportion of excessively dirty eggs contaminated with either faeces or feathers were observed, compared to eggs laid in the other pens ($X^2 = 29.9$, $P < .001$). There were more slightly dirty eggs laid in pen 3 contaminated with faeces and feathers compared to pens 1 and 2. Relatively few excessively dirty eggs were laid in pens 1, 2 and 3, but rather more were collected from pen 4. Woodshavings adhering to the shell were most frequently the cause of an egg being graded in this category.

The mean bacterial count for pen 1 (Table 1.7) was with one exception (pen 2, week 12), lower than that in all other pens. The difference was very marked compared to pens 3 and 4 ($P < .001$). The mean bacterial count of the rinse fluids did not exceed 10^2 organisms/ml. in pen 1. This was equivalent to an average shell count of less than 5×10^3 bacteria. On week ~~16~~ 14 and from week 20 onwards, the mean counts for pens 1 and 2 were also statistically different ($P < .001$). The mean count for pen 2 was lower than that for pen 3, except on weeks 20 and 26. Similar differences were observed between pens 2 and 4. The mean counts obtained on week 24 were all low. Pens 3 and 4 only differed significantly on week 16 ($P < .01$). The bacterial counts in pens 2, 3 and 4 increased

Table 1.6

The surface contamination of egg shells laid in different types of nest box.

Pen No. and Description	No. Eggs	Contaminating Material	Gross Classification of Egg Shell: Clean	Slightly Dirty	Excessively Dirty
		Straw (St)	-	-	-
1 Synthetic Grass + Paraformaldehyde	396	Faeces (Fa)	-	63(15.9)	1(0.3)
		Feathers (Fe)	-	91(23.0)	1(0.3)
		Clean (C)	268(67.7)	-	-
		St	-	6(1.5)	-
		Fa	-	64(16.4)	1(0.3)
2 Synthetic Grass	391	Fe	1(0.3)	61(15.6)	2(0.5)
		C	281(71.9)	-	-
		St	-	71(18.0)	-
3 Synthetic Grass Rollaway	395	Fa	-	152(38.5)	3(0.8)
		Fe	1(0.3)	203(51.4)	4(1.0)
		C	120(30.4)	-	-
		St	3(0.8)	238(59.6)	81(20.3)
		Fa	-	88(22.1)	20(5.0)
4 Woodshavings	399	Fe	-	146(36.6)	25(6.3)
		C	58(14.6)	-	-

The figures in the table denote the number of eggs (percentage in brackets) classified in the different categories and the association with the type of contaminating material present on the shell.

Table 1.7

The mean bacterial shell counts obtained from eggs laid in different nest boxes measured over a laying period (20 weeks).

Week No.	Mean shell counts*(c.f.u./egg shell) of eggs laid in pen: 1	2	3	4
10	NC †(19)	NC (18)	1.2×10^4	1.7×10^4
12	NC	NC	NC	NC
14	NC	1×10^4	9.0×10^4 (18)	4.0×10^4
16	NC	NC	3.3×10^5	3.4×10^4
18	NC	NC	1.1×10^5	3.5×10^5
20	NC	1.4×10^5 (18)	9.5×10^4	2.6×10^5
22	NC	3.4×10^4 (19)	1.7×10^5	8.0×10^4
24	NC	1.2×10^4	1.4×10^4 (19)	NC
26	NC	1.6×10^5	1.3×10^5	2.9×10^5
28	NC (19)	9.0×10^4 (16)	7.0×10^5	4.3×10^5 (19)

* The mean shell counts in the table were calculated by regressing the geometric mean values (c.f.u./dip slide face) against total aerobic bacterial counts (c.f.u./ml. sample) using a standard regression slope. The mean counts were estimated for each pen using 20 replicates, (if fewer than 20 replicates, the number of eggs is shown in brackets).

† NC = $\leqslant 5 \times 10^3$ c.f.u./egg shell

throughout the trial, although in pen 2 this increase was delayed (week 20). A similar rise was not observed in pen 1. Mean shell counts overall ranged from less than 5×10^3 to 6.5×10^5 organisms. The highest counts were obtained from pens 3 and 4, especially towards the end of the trial.

The characteristic microbial flora of eggs laid in the different pens is shown in Table 1.8. Fewer isolates were obtained from eggs in pen 1 compared to the other pens, so the figures relating the occurrence of a particular group as a percentage of total isolations, are somewhat misleading. In common with previous findings, Gram positive cocci constituted the dominant part of the microflora. In this trial Staph. auerus and Streptococcus spp were differentiated from other staphylococci and micrococci. Of primary interest, however, was the composite group 7 - 11 which included the coliform bacteria. Bacteria in this group occurred on only 7.6% of the shells of eggs laid in pen 1, compared to 16.1, 14.2, and 31.2% in pens 2, 3 and 4 respectively. Eggs laid on synthetic grass had fewer coliforms on their shells.

Table 1.8

The grouping of eggs laid in different nest boxes, according to the types of bacteria present on the shell (weeks 10 - 28).

Types of Bacteria*	Pen No. 1	2	3	4	Chi-squared between pens (3 d.f.)
1	8 (3.6)	11 (1.8)	32 (4.0)	27 (3.3)	5.9
2	160 (72.4)	473 (75.9)	611 (75.7)	583 (71.7)	4.9
3	28 (12.7)	74 (11.9)	88 (10.9)	87 (10.7)	1.0
4	0	1 (0.2)	4 (0.5)	0	-
5 + 6	10 (4.5)	33 (5.3)	44 (5.5)	54 (6.6)	2.2
7 - 11	15 (6.8)	31 (5.0)	28 (3.5)	62 (7.6)	14.5 †
No. eggs	198	192	197	199	
No. isolates	221	623	807	813	

The figures in the table represent the number of isolates (percentage in brackets) obtained from the eggs laid in the different pens.

* Types of bacteria
1 = Staphylococcus aureus
2 = Staphylococci/micrococci (other than Staph. aureus)
3 = Streptococcus spp
4 = Bacillus cereus
5+6 = Other gram positive rods (including Bacillus spp)
7-11 = Gram negative rods (including E. coli)

† Significance of difference = $p < .01$

DISCUSSION

These investigations were conducted to determine whether it was possible to improve the microbiological status of the egg shell after lay. This could be effected by either reducing the bacterial shell count or by changing the composition of the microbial flora on the shell surface. Of special interest was the group of bacteria comprising known or potential egg pathogens, which can subsequently penetrate the shell and its membranes to produce a culminating infection of the egg contents. The paratyphoid salmonellas can be considered as members of this large group of organisms.

The effects of changing egg management practices on the bacterial population within the nest box can be easily determined. Initially, the number of bacteria in the woodshavings lining the nest box was calculated (Table 1.1) but the results were difficult to interpret. This could have been for several reasons. Small amounts of paraformaldehyde could have been carried over into the suspending fluid from which the bacterial counts were obtained. No neutralising agent was included to counteract any residual paraformaldehyde. The range of counts obtained between samples and within a single treatment was very large. Because a relatively small number of samples were collected (five/ treatment pen), the mean counts obtained were markedly increased when a single high count was recorded.

The egg shell was used therefore, to assess the effects of periodically dosing the nest boxes. Available evidence suggests that formaldehyde gas dissipates quite rapidly from the surface of the shell after storage at room temperature(158). Little gas penetrates into the shell membranes or albumen even after eggs are subjected to gas concentrations produced

during fumigation (600 mg/cubic metre). Residual formaldehyde gas on the shell should not have exerted a major influence on the results obtained.

There are several different methods available for estimating the size of the bacterial population on an egg shell (66,58,8) and indeed many variations of each procedure have been used. Whilst it has been shown (66,100)that higher counts are obtained when the entire shell and membranes are mechanically blended, after removal of the contents, a simplified method was sought to enable the processing of large numbers of eggs. A modification of the method described by Gentry and Quarles (58) was used. This method also had the advantage that it was non-destructive.

In these trials the plate counts were obtained on nutrient or selective agars incubated at 37°C. In previous work, a range of temperatures from 20 - 37°C has been used (68,46,37,99,100,66). Haines (68) obtained lower mean shell counts when plates were incubated at 37°C compared to 20°C. The incubation temperature was not found by Moats (99) to influence the number of viable bacteria on the shell surface of eggs that had been processed with two different methods (a shell rinse and a macerate of the shell and membranes).

The higher incubation temperature was selected for this study because it was close to the temperature used for hatching eggs and is also the optimum growth temperature for coliform bacteria. A lower incubation temperature may be more applicable to those interested in studying the fate of eggs in commercial storage conditions. In addition to influencing the viable count, the incubation temperature could also affect the composition of the microbial flora of the egg shell. Such considerations should be borne in mind when interpreting the results of this and other work.

Other factors may also have influenced the shell counts expressed in previous reports. Gentry and Quarles (58) discussed the effects of storing eggs at various temperatures after laying, in relation to the number of viable bacteria remaining on the shell. At 38°C the viable counts obtained from eggs were considerably reduced after 24 hours storage. At 4°C these effects were less noticeable, although by seven days, only 7 - 10% of the original bacterial population remained viable. Haines (68) did not find any differences between the bacterial flora on the shells of eggs sampled immediately on receipt from the farms, or those held for four days at room temperature before sampling. Forsythe et al (46) used eggs that were sampled within eight hours of laying to obtain the mean shell count referred to earlier. However, when eggs were stored in rooms at 25°C with different relative humidities, a slight decrease in shell count was observed during storage. The rate of decrease was slightly greater when eggs were stored at the higher relative humidity (95% RH). A 10-fold reduction in shell surface count was obtained after approximately 10 days storage at 25°C.

Because of the effects of storage on bacterial shell counts, the eggs in these trials were sampled at the earliest possible opportunity. In Trials 1 and 2 the eggs were sampled immediately after removal from the nest boxes at the laying farm. The large number of eggs involved in Trials 3 and 4 required that the procedure be changed. After sufficient eggs had been collected on a particular day, they were stored overnight in the farm egg room. The material was then transported to the laboratory the following morning.

The addition of paraformaldehyde to woodshavings contained in the nest box reduced the total bacterial and coliform counts on the surface of the egg shell (Tables 1.2 and 1.3). This observation confirmed the results obtained by Hodgetts and Dance (78).

The use of synthetic grass materials to line the nest box bases did not markedly reduce the shell counts recorded throughout a 15 week laying period. The bacterial counts on the shells however, were shown to increase over the first five to six weeks during which the nest boxes containing synthetic grass were used. It was thought that this could have been due to the accumulation of dust and organic debris during the early part of the laying period. Eggs laid later in the laying cycle would therefore be exposed to a more contaminated nest environment.

The addition of paraformaldehyde to regularly cleaned synthetic nest pads greatly reduced the bacterial count on the shell surface and the mean shell counts of eggs laid throughout the laying period did not exceed 5×10^3 viable organisms. The regular shaking of the nest liners removed the dust and faeces at the base of the pads and this resulted in between 68 - 72% of the eggs being graded as clean. The cleaning of the liners however did not prevent the shell counts rising during the latter part of the trial in those nest boxes where paraformaldehyde was not added.

The microbial flora on the shells of eggs laid on synthetic grass was slightly different from that obtained on the shells of eggs laid in woodshavings. The typical bacterial flora of the hen's egg shell is shown in Table 1.9. Gram positive bacteria constitute a dominant part of the microflora on the shell of nest clean turkey eggs. The gross composition of the flora is similar to that found on the shells of chicken eggs (68,17, 20,100). There are differences however in the frequency of occurrence of the major groups of bacteria. This could in part be explained by the fact that in the majority of cases, the chicken eggs were obtained from either egg grading or processing plants and not directly from the farm on which the eggs were laid.

Table 1.9

The bacterial flora of the avian egg shell.

The percentage of types of bacteria reported by:-

MICRO-ORGANISMS	Haines (68)	Zagaevsky and Lutikova (160)	Board et al (20)	Moats (100)	Present Study
Gram positive cocci:(total)	25	81	46	72	86
Streptococcus spp		8		8	11
Staphylococci/micrococci (including Staph.aureus)	23	53	46	56	75
Others	2	20		8	
Gram positive rods: (including Bacillus spp, Lactobacillus spp, Coryneform bacteria)	33		5	16	7
Gram negative rods:	38	19	49	13	9
Pseudomonas spp	6		23		1
Flavobacterium spp	3			1	2
Coli-aerogenes	5	19	11	10	3
Others	24		15	1	3
Other micro-organisms (yeasts and moulds)	4			1	

From the 1446 eggs sampled, 3325 bacterial isolates were recognised. Obviously the large number of isolates obtained did not permit an extensive biochemical identification of each isolate. Representative colony types, however, were examined in sufficient detail to allow a classification to be made. In general, eggs laid on synthetic grass had fewer coliform bacteria on their shells (Tables 1.5 and 1.8).

The eggs laid on conventional nest boxes were more often contaminated with woodshavings and a higher proportion of dirty eggs were contaminated with faecal material or feathers. The regular cleaning of the synthetic grass nest pads ensured that organic material was continuously removed. In addition, it was observed that faecal deposits in these boxes were quickly broken down into dry dust which settled to the base of the nest pad. The eggs were supported above this material by the stiff polythene blades of the nest pad and this may have been responsible for reducing the faecal contamination on the shell.

The plastic nest liners, in contrast to woodshavings, do not have the ability to hold large amounts of water. Woodshavings can retain up to 25% or more of their weight as water. The type of bacteria that could survive on the synthetic grass would presumably be influenced by the amount of free water available for growth. It is suggested that bacteria with relatively low a_w requirement would be better suited to this environment. This could also have influenced the type of contaminating flora on the shell surface of eggs laid on synthetic nest pads.

The microbial count on the egg shell in these trials was used as a measure of hygienic production. The majority of bacteria on the shell surface however, (the Gram positive cocci) do not subsequently invade the internal egg structures (Seviour and Board (135)) and it is the Gram negative bacteria, particularly the coliforms and those organisms capable of

putrefying the egg contents, that subsequently establish in the albumen. Only a small percentage of eggs laid on synthetic grass were shown to harbour such organisms on the shell (7.6 - 14.2%).

The predominance of Gram positive cocci necessitates the sampling of large numbers of eggs if a change in the remaining shell flora is to be established. Although this data provides an indication that the microbial flora can be influenced by the nest box environment, confirmation will require further extensive work.

The use of paraformaldehyde in nest litter has been shown by this, and previous studies (78) to dramatically reduce the bacterial load in the nest material and on the egg shell surface. No evidence was obtained however, that this reduction decreased the incidence of shell penetration by specific bacteria. The enumeration of viable bacteria on the shell provides a convenient assessment of hygienic production. A more appropriate measure would require the assessment of true bacterial penetration of the outer egg structures, possibly using pre-selected indicator organisms.

It is believed that this is the first report characterising the microbial flora of egg shells produced on synthetic grass surfaces. Such materials are now gaining more widespread commercial acceptance due primarily to benefits associated with the management of laying birds. When standard procedures have been adopted for the use of these materials i.e. type of grass, method and frequency of cleaning etc, it should be possible to repeat this work. This is at present made difficult because of the large number of possible nest box designs and nest lining materials that are available for study and the fact that each combination must be assessed under farm conditions.

CHAPTER 2 INTRODUCTION

The development of methods to control shell transmitted salmonella infections

The aim of this part of the study was to develop a novel egg treatment method effective against egg transmitted pathogens, particularly paratyphoid salmonellae. Presently used egg sanitation procedures, whilst effective in substantially reducing the number of shell contaminants (see Discussion), do not inactivate bacteria that have already penetrated the shell and underlying membranes.

Gentamycin has been used successfully to eliminate salmonellae in the peripheral egg structures (142) although this antibiotic was not effective when test inocula were introduced directly into the thick albumen. Other workers have obtained similar results. Saif and Shelly (126) successfully treated turkey eggs artificially infected with 25 different salmonella sero-types. 347/429 (80.9%) artificially challenged eggs were demonstrated to be infected, compared to 9/445 (2.0%) treated eggs. Other antimicrobials have been used experimentally (88). These include neomycin, kanamycin and spectinomycin. Carlson and Snoeyenbos (36) inoculated a variety of antibiotics onto the air cell membrane of infected eggs. Even at embryo-toxic levels, the compounds tested failed to eliminate the test organism after it had been introduced directly into the albumen.

An effective egg treatment method for paratyphoid salmonellae would be of considerable benefit, because successive generations of poultry are usually spatially separate. Although such a treatment may not totally eliminate the egg pathogen, the control of vertical transmission could be considered an essential pre-requisite of any salmonella control programme. In this study, antibiotic egg dipping was not contemplated. The long-term

use of antibiotics could not be considered because of the possibility that organisms may be rendered resistant. Using an artificial egg infection model, various non-antibiotic treatment methods were developed and evaluated. These included temperature differential (TD) dipping in a range of disinfectant solutions and a high temperature egg treatment. Both processes share the potential advantage that they can destroy bacteria that have already penetrated the shell. It was intended that they either replace or complement existing egg sanitising methods.

The effects of egg treatment on embryonic development (hatchability) were also considered. The results of these studies will not be reported in detail here, although reference will be made to possible deleterious effects which may ultimately determine the commercial acceptability.

The ability of specific treatments to protect eggs from infection was assessed after the test organism had been introduced via different challenge routes. The size of the inocula was varied over a wide range. A very severe artificial challenge was achieved.

Two egg treatment methods which may eventually prove of value in reducing the level of egg transmitted salmonella infection were developed, evaluated and are fully described.

MATERIALS & METHODS

Eggs

Clean eggs that had been laid in the nest and sanitised at the farm of origin were used throughout. Cracked or soiled eggs were discarded. Egg age at the commencement of individual trials was variable, although usually between 0 - 7 days.

Infecting Solution

The test organism (see Chapter 4) was serially passaged in nutrient broth. Serial decimal dilutions were prepared in physiological saline from an 18 hour broth culture, to provide a suitable inoculum.

Egg Infection Methods - ATD dipping - shell surface exposure (Sal Dip)

For this procedure, the eggs were pre-heated in a 37^{o}C walk-in incubator before being immersed for 2 minutes in the infection solution which was held at ambient temperature (15 - 17.5^{o}C). The infecting solution was prepared by adding a suitable dilution of the test organism to de-ionised water contained within a plastic-lined stainless steel dipping vessel. A viable count was performed on the infecting solution both before and after the eggs had been dipped.

Inoculation of test organism into albumen (Sal Alb.)

The small end of each egg was swabbed with merthiolate (Eli Lilly & Co.). A small hole was drilled through the shell without damaging the underlying membrane. The inoculum suspended in physiological saline was introduced into the outer-thin albumen using a fine

gauge needle (27 g, ½"). Afterwards, the injection site was swabbed with alcohol and the hole was sealed with an adhesive cement (Du Pont).

Egg Treatment

Unless stated otherwise, eggs were held at room temperature for 1 - 2 hours before treatment using one of the methods outlined below:-

a) Hot disinfectant dipping - eggs were immersed in a solution of disinfectant maintained at 40°C for 2 - 3 mins. Disinfectants were used at manufacturers recommended dilutions (see List of Disinfectants)

b) Water temperature differential dip (WTD) - treatment consisted of heating eggs at 40°C for 2 - 3 mins. in water prior to immersion for 5 mins. in a disinfectant solution maintained at ambient temperature.

c) High temperature differential dip (HTD) - eggs were heated in water for 30 - 120 secs. at 60°C (± 1°C) and removed for 15 secs. before dipping in disinfectant solution for 2 mins. at ambient temperature.

d) High temperature dipping - eggs were placed in hot water maintained at 60°C (± 1°C) for different time periods.

e) Pressure differential dip (PD) - eggs placed in disinfectant solution were subjected to a vacuum which was maintained at 15 psi for 6 mins. before being released. Eggs were allowed to equilibrate at atmospheric pressure for 10 mins. whilst still remaining in the solution.

After treatment, eggs were allowed to air dry before being placed in plastic bags for incubation at 37°C for 6 - 8 days.

List of Disinfectants

Trade Name	Compound	Supplier
Micro-Quat	Quaternary Ammonium Compound (QAC)	Microbiologicals Ltd.
38/10 Horticultural Grade Formalin	Formalin (containing 38% w/v formaldehyde)	Microbiologicals Ltd.
Titan Sanitiser 52	Organo-chlorine (Av. chlorine = 2.4%)	Lever Industrial
Halamid	Chloramine (containing 99.3% chloramine) (Av. chlorine = 25%)	Duphar Vet. Ltd.
Tego-51	Ampholytic Surfactant	Th. Goldschmidt Ltd.
Microdine-P	Iodophor	Microbiologicals Ltd.
Cidex	Glutaraldehyde (containing 2% w/v glutaraldehyde)	Arbrook Products Ltd.

Sampling of Eggs

The shell at the small end of each egg was sterilised using merthiolate. . A segment of shell was removed from the small end of the egg using sterile scissors and a sterile cotton wool swab was inserted into the egg contents. The albumen, yolk and the inside surface of the outer membrane were sampled. The egg contents were then discarded and the inside of the egg rinsed clean of albumen with sterile physiological saline. The shell was cut along the long axis and the two halves separated. Sterile forceps were used to peel apart the two shell membranes. A moistened swab was used to sample the inner surface of the outer membrane and the outer surface of the inner membrane. The outer membrane was then completely removed and the interface between the outer membrane and the inside surface of the shell was also swabbed with the same applicator. Particular care was taken to avoid cross-contamination between eggs and within each treatment group.

Culture Methods

Individual swabs were cut off into 10 ml. volumes of Gibco Tetrathionate Broth APHA (Gibco Biocult Ltd.). The cultures were incubated at 42^{o}C for 48 hours. A loopful of culture was streaked onto Brilliant Green agar (Lab M Ltd.) after both 24 and 48 hours. The agar plates were incubated at 37^{o}C for 24 hours before being inspected for the presence of salmonella-like colonies. Presumptive isolations were confirmed using standard biochemical (r/b system, Flow Labs.) and serological tests.

The heat sensitivity of S. hadar

A nalidixic acid resistant strain of S. hadar was used. The organism was cultured in Tryptone Soya broth at 37°C. Suitable dilutions of a twice passaged 18 hour broth culture were inoculated into small screw-capped polycarbonate tubes (1.1cm o.d. x 4 cms) containing either physiological saline or egg albumen. The tubes were pre-warmed in a water bath at 45°C for at least 15 mins. to ensure equilibration and were then transferred to a water bath at the required temperature (55 - 70°C ± 0.5°C). At 30 sec. intervals, individual tubes were removed into an ice bath and allowed to stand for at least 5 mins. so that the contents cooled. Serial decimal dilutions were prepared in physiological saline and 20 µl aliquots were dropped onto the surface of dried Tryptone Soya agar plates using the Miles and Misra (95) technique. The agar contained nalidixic acid (100 µg/ml, Sigma Chemicals Co.). The plates were incubated at 37°C for 48 hours and the number of individual colonies counted..

Preparations of egg albumen

The egg white was harvested aseptically. Each egg was completely immersed in 70% (v/v) ethanol and allowed to dry. The remaining alcohol was removed by flaming the egg shell. The bottom end of each egg was removed with sterile scissors. The albumen was carefully decanted into a sterile beaker with great care being taken to avoid contamination with yolk. The whites of several eggs were collected for each trial. The albumen was pooled and stomached for 30 secs. (Colworth Stomacher) to ensure complete mixing.

Change in albumen pH

When required, the pH of the albumen was adjusted by carefully adding either: a) sterile IN HCl or b) sterile IN NaOH. Supplements were added dropwise with constant mixing to avoid coagulating the albumen. The albumen at the correct pH (7.8 or 9.2) was held at 4^{o}C in a tightly stoppered tube until required.

Inoculation of organisms into albumen

Particular care was taken to ensure that the inoculum was thoroughly mixed with the viscous albumen. This was achieved by repeated sucking up and down using a 1 ml. wide-bore pipette. The same procedure was also used during the preparation of serial decimal dilutions for estimation of the viable count. Separate pipettes were used for each transfer.

RESULTS

Currently used egg sanitising methods (fumigation, QAC dip) were not effective in reducing the egg infection rate following artificial challenge with S. hadar (Table 2.1). WTD formalin treatment resulted in fewer positive isolations of the infecting strain from the egg contents. This method however, was effective only after eggs were artificially infected with relatively small numbers of bacteria. (Table 2.2) These results suggested that salmonellae that had already penetrated the shell could be inactivated by an antimicrobial compound deliberately forced through the small number of open shell pores, using a temperature differential dipping process.

It was evident from the work of Snoeyenbos et al (142) that a marginal pasteurisation process could be used to destroy viable bacteria at, or near, the egg shell surface. To achieve the marginal pasteurisation, eggs were heated in water at a temperature of 60°C (Table 2.3). An additional treatment group was included and consisted of heating eggs at 60°C for 60 secs. immediately followed by immersion in a cold formalin solution (HTD dip). Using this combined treatment, the desired temperature differential across the egg shell was maintained. Heating alone caused a progressive decrease in the recovery of salmonellae. The subsequent formalin dip completely eliminated the bacteria from infected eggs.

A variety of commercially available disinfectants were tested using the HTD treatment method (Table 2.4) and the results were compared to those obtained with formalin. Formalin, Halamid and

Table 2.1

The effect of various shell treatments on the recovery of Salmonella hadar after artificial infection.

Inoculum/ml Dip	Treatment	Salmonella recovery from egg contents	
		No.	%
5×10^2	Controls	24/24	100
	QAC dip (900 ppm)	25/32	78.1
	Formaldehyde fumigation	21/23	91.3
	Formalin dip (2.5% v/v)	24/27	88.9
	WTD formalin (2.5% v/v)	10/37	27.0*

* significantly different ($p < .001$)

Fumigation using paraformaldehyde prills (10 gm/cu.m.) in an automatic cabinet during a 20 min. cycle at 65 - 70°F.

Table 2.2

Salmonella hadar isolations from the egg contents after WTD formalin treatment.

Inoculum/ml Dip	Salmonella isolations from egg contents. Treated	Non-treated
10^2	3/14	11/15
10^3	6/15	14/14
10^4	12/15	13/13

Table 2.3

The effect of heating eggs at 60°C and high temperature differential dip (HTD) on Salmonella hadar in eggs.

Inoculum/ml Dip	Treatment	Salmonella Isolations	
5×10^2	30 secs at 60°C	30/39	(76.9%)
	60 secs	13/39	(33.3%)
	90 secs	7/37	(18.9%)
	HTD formalin(2.5% v/v)	0/40	(0)
	Infected controls	25/37	(67.6%)

the Iodophor (Microdine -P) significantly lowered the egg infection rate. Microdine-P did not prove as effective in further trials. The concentration of the antimicrobial agent (Formalin and Halamid) was found to be extremely important. The efficacy of HTD dipping was directly related to the disinfectant concentration.

Formalin was effective for at least 24 hours following artificial infection (Table 2.5) but the number of salmonella isolations from eggs treated with Halamid began to rise after a few hours. HTD dipping in formalin protected eggs from infection after challenge with large numbers of bacteria ($5.3 \times 10^2 - 1.1 \times 10^6$ /ml. dip). With large inocula a high concentration was required (Table 2.6) to eliminate the infecting strain. Halamid was only effective when the challenge dose was low. As the challenge was made more severe, the treatment efficacy was not maintained.

Other treatment methods were also tested. These included pressure differential (PD) dipping and immersing eggs in disinfectant solutions at $60^{o}C$ for short periods. PD dipping in formalin was very effective in reducing the level of salmonella infection. Hot solutions of disinfectants were apparently only effective if eggs had been infected with small numbers of bacteria (ca. $5 \times 10^2 - 5 \times 10^3$ bacteria/ml. dip).

The effects of egg treatment on hatchability were simultaneously determined. PD dipping in formalin reduced hatchability. An almost linear relationship between the concentration of formalin in the dipping solution and early embryo mortality was observed (0 - 10 days of incubation). In the majority of cases, when the

Table 2.4

The effect of various commercially available disinfectants against eggs infected with Salmonella hadar.

Inoculum/ml Dip	Disinfectant/Treatment	Salmonella Isolations	
		No.	%
1×10^3	Microquat (0.075% v/v)	21/39	53.8
	Titan-52 (0.5% v/v)	13/38	34.2
	Halamid (0.5% w/v)	1/36	2.7
	Tego-51 (0.3% v/v)	8/36	22.2
	Microdine-P (0.5% v/v)	4/35	11.4
	Formalin (2.5% v/v)	1/36	2.7
	60^oC 60 secs in water	12/34	35.2
	Infected controls	46/79	58.2

Table 2.5

The influence of delaying the time after infection to treatment on the isolation of Salmonella hadar from eggs.

Time to treatment after infection with 1×10^3 cells/ml dip (hrs)	Salmonella recovery from egg contents after HTD treatment with:	
	Halamid (0.5% w/v)	Formalin (2.5% v/v)
1	3/17 (17.6%)	1/19 (5.3%)
2	1/17 (5.9%)	0/16 (0)
3	3/18 (16.7)	0/16 (0)
4	3/18 (16.7%)	2/19 (10.5%)
5	5/20 (25.0%)	0/17 (0)
24	9/18 (50.0%)	1/20 (5.0%)
Infected controls	41/57 (71.9%)	

Table 2.6

A dose response curve used to establish the required formalin concentration for the HTD dip.

Formalin Concentration (% v/v)	Isolation of S. hadar from egg contents after challenge with: 10^2/ml dip	10^4	10^6
0.625	5/20	19/20	16/17
1.25	4/17	10/19	17/19
2.5	1/17	6/19	5/18
Controls	15/20	20/20	18/20

eggs were examined, little or no germinal development was evident. HTD formalin treatment also reduced hatchability. Prior to the tenth day of incubation, no adverse effects of treatment were noted, irrespective of formalin concentration and time of heating in hot water ($\leq$ 60 secs.). As formalin concentration increased, so did the number of late dead embryos (10 - 28 days old).

In a further trial, groups of eggs were sequentially treated before incubation. Hatchability on average, was found to be 9 - 10% lower than control eggs.

Heating eggs in hot water for extended periods (60 - 240 secs.) was shown to be very effective in preventing eggs from becoming infected with salmonellae (Table 2.7). No isolations were obtained from eggs originally infected by ATD dipping. Salmonellae introduced into the outer thin albumen were similarly destroyed by the hot water treatment. Whilst such methods have been used to control bacterial growth during the storage of table eggs (see Chapter 3 - Discussion), no reference has been found to the use of similar processes for the treatment of hatching eggs for the specific purpose of eliminating egg-transmitted pathogens.

Corry and Barnes (38)studied the heat resistance of two salmonella serotypes in egg albumen to determine the optimum conditions necessary to ensure their destruction. With commercially prepared egg albumen it is important that the heating process does not cause coagulation of the egg white proteins. This is equally true for hatching eggs and additionally, the process must not cause damage to the blastodisc. The "thermostatisation" procedure of Funk (52) effectively reduced the incidence of rotting in

Table 2.7

The effect of heating eggs for long periods, on the isolation of Salmonella hadar from eggs infected by different routes.

Egg infection method	S. hadar inoculum	Eggs immersed at 60°C for (secs)	Salmonella isolations from egg contents	
			No.	%
Sal Dip	(1.3×10^4/ml)	60	0/12	0
		120	0/12	0
		180	0/12	0
		240	0/12	0
		Control	6/12	50
Sal Alb	(1.6×10^3/egg)	120	1/12	8.3
		240	0/8	0
		Control	5/12	41.7

washed eggs, but also prevented development of the embryo.

Hatchability studies had shown that eggs could be heated at temperatures higher than 60°C for only very short periods and this temperature was considered a realistic maximum. The effect of heating at a variety of temperatures (55 - 70°C) on the survival of S. hadar in saline and egg albumen (pH 7.8 and 9.2) is shown in Fig. 2.1. The organism was more susceptible to heating at 60°C when inoculated into albumen at the higher pH. Corry and Barnes (38) similarly demonstrated increased sensitivity in the albumen of stored eggs, compared to fresh eggs with a lower pH. The thermal sensitivity of a number of different bacteria commonly associated with eggs was also investigated (Fig. 2.2). In albumen (pH 8.2) the survival curves were similar. This suggested that other opportunist egg pathogens may be destroyed by the heat treatment process. Funk (50) used a pasteurisation process (60°C for 10 mins. in oil) to effectively control a number of rot-producing organisms introduced into table eggs destined for storage. Such a severe treatment could not however be applied to hatching eggs.

Different treatment methods were compared (Table 2.8) in a single trial. Heat treatment (60°C for 240 secs.) was extremely effectivein protecting eggs from infection after challenge using both administration routes (Sal Dip, Sal Alb) and compared favourably to the HTD dipping process with either formalin or glutaraldehyde. When the inoculum was delivered into the middle thick or inner thin albumen, rather than close to the shell membranes, heat treatment was only effective when eggs were challenged with small inocula (Table 2.9). This result was not

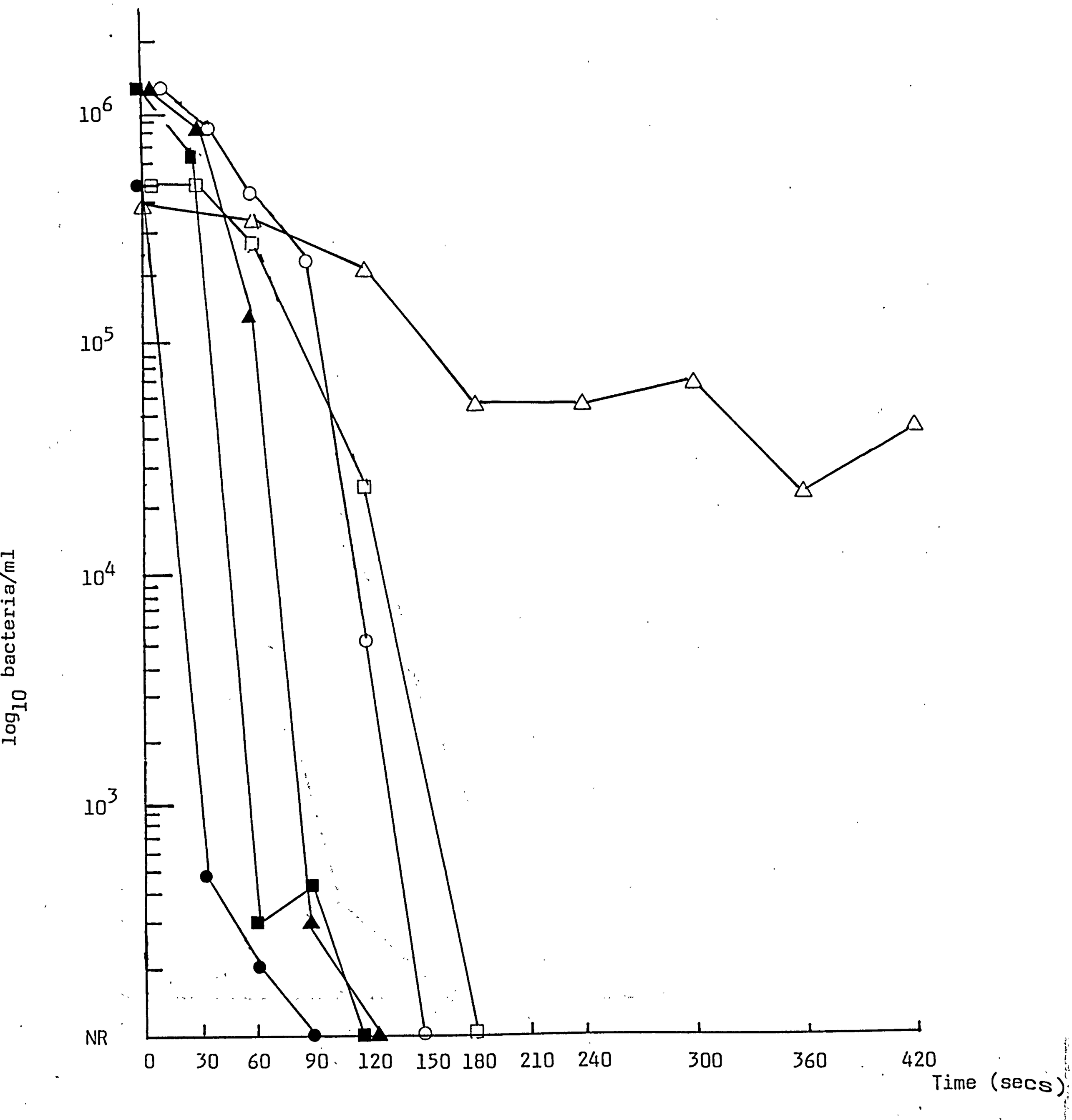


Fig. 2.1 The survival of Salmonella hadar after heating at 55°C (△), 60°C (○), 65°C (■) and 70°C (●) in physiological saline or 60°C in albumen at pH 7.8 (□) and 9.2 (▲).

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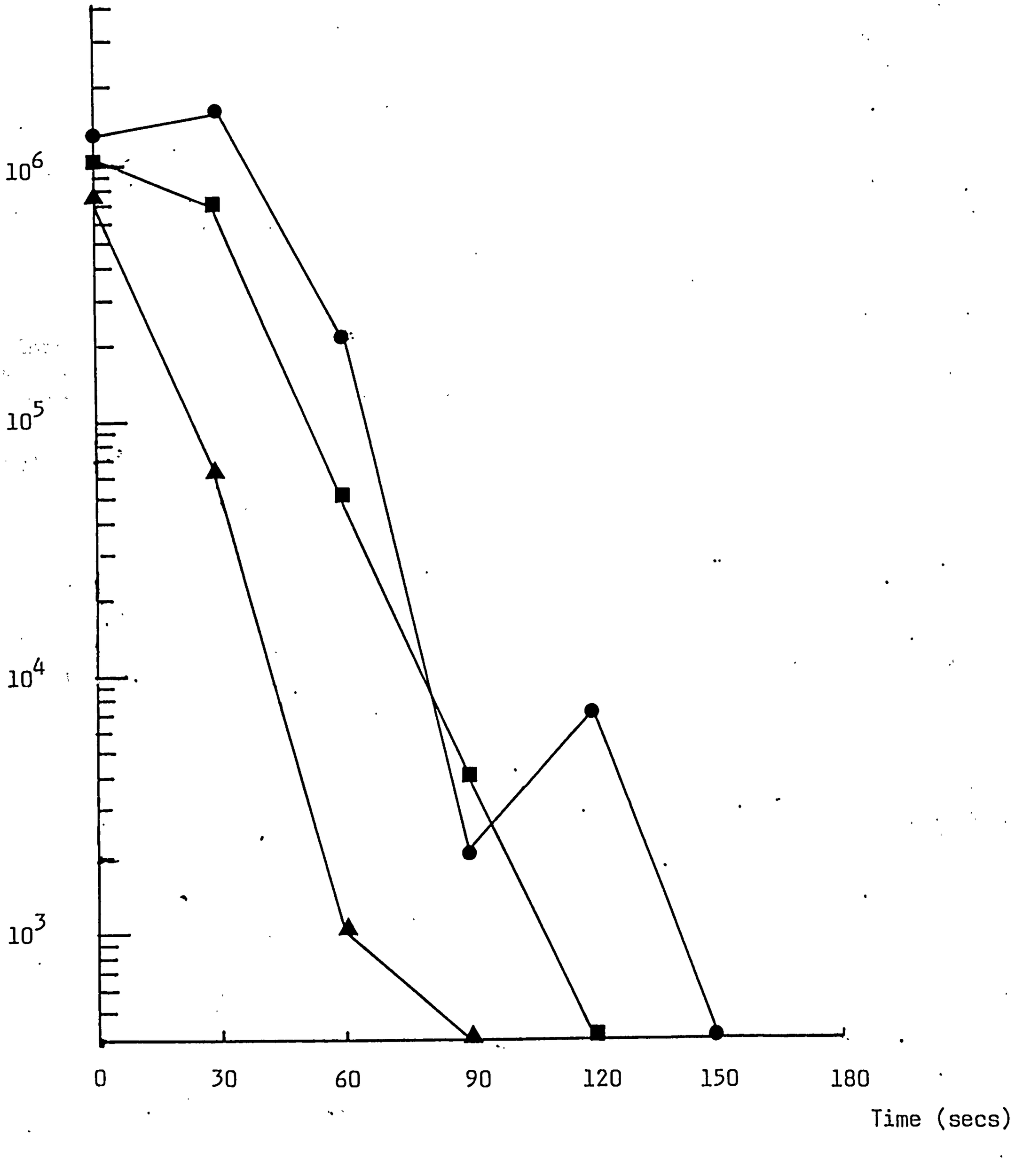


Fig 2.2 The survival of Salmonella hadar (●), Proteus mirabilis (■), and Pseudomonas aeruginosa (▲), in fresh turkey egg albumen (pH 8.2) following heating at 60°C.

Table 2.8

The effect of different egg treatment methods against eggs infected with Salmonella hadar

		Salmonella isolations from egg contents						
			HTD Treatment		Heat Treatment			
Egg infection method	S. hadar inoculum	Control	Formalin (2.5% v/v)	Glutaraldehyde (2% v/v)	60s 60°C	120s	180s	240s
Sal Dip	6.5×10^1/ml	9/18	0/9	0/10	1/10	0/10	0/10	0/10
	7.4×10^3	17/18	2/10	1/9	6/9	0/10	0/10	0/10
	3.2×10^5	18/18	5/10	5/10	5/10	0/10	0/10	0/10
Sal Alb	2.2×10^3/egg	12/19	2/10	6/10	5/9	4/10	0/10	0/10
	2.2×10^5/egg	19/19	7/10	6/9	10/10	7/9	2/10	0/10

Table 2.9

The effect of heat treatment (60°C for 240 secs) after the introduction of Salmonella hadar deep into the albumen.

Inoculum		Time after infection to treatment (hrs)	Salmonella isolations from egg contents. No.	%
(1)	1.0×10^3/egg	2	3/74	4.1
		24	5/72	6.9
		Control	6/25	24.0
(2)	2.8×10^5/egg	2	34/75	45.3
		24	6/71	8.5
		Control	14/21	66.7
(3)	2×10^7/egg	2	73/74	98.6
		24	68/70	97.1
		Control	24/24	100.0

(1) Difference between 2 and 24 hours not significant

(2) Difference between 2 and 24 hours significant - $\chi^2 = 23.1$ ($p < .01$)
2 hours not significantly different from controls - $\chi^2 = 2.19$

(3) No significant differences.

unexpected and suggested that large numbers of bacteria in the albumen would not be destroyed effectively using heat treatment methods. Antibiotic egg dipping (142) was also ineffective after eggs had been artificially challenged by introducing the test organism into the middle thick albumen.

Eggs infected by shell surface contamination rather than by a route more analogous to transovarian transmission, received a substantial measure of protection after artificial challenge. To be effective it was considered that treatment by either hot water or HTD dipping must be applied before the test organism had multiplied in the membranes, prior to invading the albumen in large numbers.

DISCUSSION

Currently used egg sanitation and fumigation practices are extremely effective in controlling the bacterial population on the shell surface. Williams (152) demonstrated that bacterial shell counts could be reduced by 99.85% after fumigation with formaldehyde. Furuta and Sato (53), using artificially infected and soiled eggs, significantly reduced shell bacterial counts, although heavily soiled eggs infected with large numbers of bacteria, were not completely sterilised after fumigation. These authors (54) also reported large reductions in the number of viable bacteria remaining on the shell after dipping in various disinfectant compounds. Gordon et al (62) effectively sterilised the shells of artificially infected eggs following immersion in various germicides at $25^{o}C$.

Hatching eggs are usually sanitised within a few hours after laying to ensure that potential pathogens are killed before they penetrate the shell. Williams et al (157) demonstrated however, that egg penetration by paratyphoid salmonellae could be a very rapid process when conditions were favourable. Using an elegant technique these authors demonstrated that within a matter of minutes following shell exposure, bacteria could be isolated from the membranes beneath the shell. Studies on fumigation by Williams and Siegel (158) illustrated that formaldehyde gas dissipated from the shell within several hours. Little penetration of the shell by the fumigant was observed and the process was not shown to reduce the bacterial contamination in the contents of fresh eggs (117). A number of workers have failed to eliminate salmonellae from eggs using wet sanitising methods after artificially challenging the surface of

the shell. Rizk et al (120) used a number of disinfectant compounds in which to immerse artificially infected eggs. Shell surface counts were significantly reduced but none of the compounds was effective after shell penetration. Similar results were obtained by Peel and Simmons (110).and Frank and Wright (48). These observations implied that the effective control of salmonellae in eggs intended for hatching could only be secured by developing alternative treatment methods. Because salmonella egg transmission rates in naturally infected flocks are extremely low, an artificial infection model was used in the laboratory. ATD dipping was thought to provide a sufficiently good means of challenging eggs to mimic the natural route of egg shell contamination. There is however, enormous variation in the outer shell structures of eggs. This inherent variation was commented upon by Williams et al (157) who advocated the use of large numbers of eggs when studying the kinetics of shell penetration by salmonellae.

The use of temperature differential dipping to secure infection ensured that water containing bacteria would be sucked into any open pores in the shell when the egg contents cooled in the infecting solution. Our own data and those of Board and Halls (22) suggest that only a small proportion of pores are flooded when eggs possess an intact cuticle. The suction generated by the temperature differential is however, sufficient to overcome the water resistance/repellance of the shell (Board (19)). Preliminary results confirmed previous observations that bacteria that had penetrated beneath the shell were not inactivated by either fumigation or wet sanitising (Table 2.1). WTD formalin dipping was more effective because bacteria beneath the shell, close to, or immediately beneath open shell pores, were subjected

to a high concentration of the antimicrobial agent. No data were obtained on the diffusion of formalin within the egg contents, but it is possible that this chemical was selectively bound by the membranes. Only small amounts of solution actually entered the egg as determined by increase in weight after dipping. As the artificial challenge was made more severe, WTD treatment was less effective and an HTD dipping procedure was found to be superior.

The use of heat treatment for hatching eggs prior to incubation has previously been advocated for the control of egg transmitted pathogens. Yoder (159) described a heat treatment process for eggs infected with Mycoplasma gallisepticum and Snoeyenbos et al (142) used a marginal pasteurisation (60°C for 120 secs.) to inactivate paratyphoid salmonellae in the peripheral egg-structures. It is believed however, that the combination treatment consisting of a preliminary heating and a subsequent immersion in cold disinfectant solution has not previously been described. Formalin gave consistently good results when used as the antimicrobial agent and this compound was found superior to other biocides. A Chloramine (Halamid) was also exhaustively studied but the protective effect was not maintained when egg treatment was delayed for more than a few hours following challenge (Table 2.5). This was an important observation because, in some cases, it may prove necessary to treat eggs commercially at a central location and not at the farm on which the eggs are laid. Formalin remained effective for at least 24 hours and, in addition, offered substantial protection to eggs challenged with large numbers of bacteria (Table 2.6).

Dipping eggs in hot water alone caused a progressive decrease in the recovery of salmonellae (Table 2.3). When eggs were immersed for longer periods at high temperature, (2 - 4 mins.) the results were better than those obtained with HTD dipping (Table 2.8). Large inocula introduced directly into the outer thin albumen were destroyed by hot water treatment. A similar protective effect was not demonstrated when large numbers of salmonellae were inoculated into the inner thin albumen adjacent to the yolk sac (Table 2.9). As previously suggested however, egg shell contamination is considered more important than true transovarian transmission in facilitating the vertical spread of paratyphoid salmonellae. Salmonellae within the outer egg structures were consistently eliminated and the majority of artificially infected eggs were protected from subsequent infection. Formalin was shown to be embryo-toxic when used in the HTD and PD dipping systems. Whilst several strains of egg were not adversely influenced by hot water treatment, some deleterious effects were observed. More data are required to establish the optimum treatment conditions so that these procedures could eventually become commercially acceptable for the control of paratyphoid pathogens.

CHAPTER 3 INTRODUCTION

The effects of egg shell treatment in reducing the extent of bacterial penetration in naturally infected eggs.

Because of the many difficulties encountered in detecting naturally transmitted infection, it was necessary to find another way of observing the effects of egg shell treatment. It was decided to look for an alternative indicator organism occurring more frequently in naturally contaminated eggs.

Initial attempts were concentrated on the sampling of eggs that had failed to hatch and contained a dead embryo. The average bacterial contamination rate was calculated and the bacterial contaminating flora established. Eggs that fail to hatch constitute only a small part of the total number of eggs that are incubated (10 - 15%) and it was therefore decided to study the association between bacteria and the live developing embryo. Eggs laid either in the nest or on the floor litter were incubated for 22 - 24 days and their contents examined for the presence of bacteria. Again the typical contaminants were identified.

The relatively low isolation rates obtained during these studies prompted the search of a different technique for evaluating bacterial contamination. Previously all eggs had been sampled by culturing a sample of the embryonic fluids directly onto non-selective agar plates. An egg moulding technique (Board and Board (13)) was used by these authors to determine the actual sites of shell penetration in eggs that had been artificially infected. The method was also used by Smeltzer et al (140) to observe bacterial penetration in naturally infected eggs. Using this method, bacteria that penetrate the shell and membranes, without necessarily invading the albumen, can be observed by direct examination of the inner shell structures.

It was found possible to cultivate bacteria directly from the individual penetration points onto synthetic media. The bacteria present in the outer egg structures were routinely identified. The extent of contamination was assessed by counting the number of discrete bacterial penetration spots.

Different egg shell treatments were evaluated by determining their effects on reducing the number of eggs penetrated by coliform bacteria and also on reducing the total number of bacterial penetration spots. The use of a selective plating medium to culture directly from the inside surface of the inner membrane provided a simple means for the examination of large numbers of eggs. The higher incidence of bacterial contamination observed in eggs sampled in this manner enabled the selection of the most suitable egg treatment method.

The egg moulding technique was also used after eggs had been artificially infected with an antibiotic marked strain of Salmonella hadar. The sensitivity of the technique was compared with that obtained using more conventional methods.

CHAPTER 3 MATERIALS & METHODS

The culture of 10 - 28 day dead embryos

Eggs to be sampled were randomly selected from those that failed to hatch and contained a developing embryo between 10 and 28 days old. They were produced at five different laying farms. Those eggs with evidence of external pipping were not cultured to avoid the possibility of extraneous contamination. The operator wore gloves throughout and care was taken to minimise contamination of the eggs. The air cell end of each egg was sprayed with alcohol. A longitudinal slit was drilled at the air cell end but the shell membranes were not punctured. A sterile disposable plastic loop (Nunc, Denmark) was, inserted and used to sample the embryonic fluids. The material on the loop was immediately transferred to the surface of a solid agar plating medium (Tryptone Soy Agar, TSA, Oxoid Ltd.). The culture plates were incubated at 37^{o}C for 24 - 48 hours.

Identification of bacteria isolated from the egg contents

Only those plates with more than five colonies growing on the surface after incubation for 48 hours, were considered positive. Representatives of each colony type were selected and replated to ensure purity. Cultures were maintained at 4^{o}C on nutrient agar slopes. The large number of isolates examined restricted the number of biochemical tests that could be performed. Gram stains were made and the morphology of the organisms was determined. Motility was demonstrated by microscopic examination of a drop of nutrient broth from the base of the slant.

The isolates were broadly categorised using methods previously described and the following groups were recognised:-

i) Gram positive cocci (group 1)

ii) Gram positive or Gram variable rods (group 2)

iii) Gram negative rods (group 3)

Organisms assigned to Group 1 were classified primarily on the basis of catalase reaction, action on glucose and morphology when observed under the microscope. Catalase negative Gram positive cocci were not further identified.

Isolation of bacteria from 22 - 24 day old embryos

1353 eggs from two laying farms and comprising approximately equal numbers of nest and floor-laid eggs were collected over a period of four consecutive weeks. The nest boxes were of wooden construction and the floors of the boxes were lined with woodshavings. The earth floors of the laying pens were covered with straw litter. The eggs were collected on clean paper trays and transported to the hatchery for grading before being placed in the incubating machines. Depending on the presence and amount of organic soiling, the eggs were classified as either i) clean - no visible soiling; ii) slightly dirty - with less than 5% of the total shell surface soiled and iii) excessively dirty - more than 5% of the shell surface visibly contaminated. In addition, the type of organic contaminating material was recorded. Straw, feathers and faeces were most often present when an egg was soiled.

350 - 400 eggs were incubated on each of four weeks together with other parent stock eggs. After either 22 or 24 days of incubation, the eggs

were removed before the remaining eggs were transferred to the hatching machines. The method used to culture the egg contents has been described above.

Enumeration of bacteria present on the shells of incubated eggs.

A modification of the technique used by Gentry and Quarles (58) and previously described was used to assess the number of viable bacteria on the egg shell surface.

Gloves were worn throughout the experimental procedure and individual eggs were picked up with clean paper towels. The number of bacteria in the egg rinse fluid was assessed using the spread plate method. Serial decimal dilutions of the rinse fluid were prepared in physiological saline. 0.1 ml volumes of each dilution were spread onto the surface of Tryptone Soy Agar. The TSA plates were incubated overnight at 37°C and the number of discrete colonies counted to provide the viable count.

The egg moulding technique

The technique used was described by Board and Board (13). The small end of each egg was swabbed with Merthiolate (Eli Lilly & Co.) and removed with sterile scissors. The contents were allowed to drain from the shell. The eggs were filled with Plate Count Agar (Oxoid Ltd.) containing 2, 3, 5 Triphenyl-tetrazolium chloride (Tetrazolium salt, BDH, 0.01% w/v). After the agar had set, molten paraffin wax was used to seal the hole at the small end of the egg. The eggs were incubated with the air cell end upwards at 37°C for 24 - 48 hours. Where indicated in the text, eggs were incubated for 7 - 10 days at 37°C before the contents were removed and replaced with agar.

The number of individual bacterial penetration spots was recorded, as was their association with morphologically recognisable features on the shell surface. The agar block was examined upon removal to ensure that no unwanted contamination had occurred during processing.

Artificial egg infection

A nalidixic acid marked strain of Salmonella hadar (104) was artificially introduced into eggs using the ATD dipping procedure described previously. In one experiment a blue food colouring dye (Brilliant Blue, Jowett Ltd.) was added to the infecting solution (1% v/v). After infection, the eggs were immersed in a quaternary ammonium disinfectant (QAC, 'San-O-Fec', Whitmoyer, 0.1% v/v) maintained at the same temperature as the eggs. The shell surfaces were allowed to air dry. When the small end of the egg was removed and the contents discarded, the number of dye penetration spots was counted. The eggs were filled with agar containing tetrazolium salt and nalidixic acid (Sigma Chemical Co., 0.01% w/v). After incubation the shell membranes were examined for the presence of dye spots and for evidence of bacterial penetration. Spots associated with the food colouring were often seen in the centre of a patch of reduced formazan dye or superimposed directly on single small bacterial penetration spots.

Isolation of bacteria from the shell membranes

The individual spots of reduced formazan dye were gently rubbed with a moistened sterile cotton wool applicator. The swab was transferred directly onto the surface of a solid plating medium (Brilliant Green Agar, BGA, Lab M Ltd.). BGA plates containing nalidixic acid (0.01% w/v) were incubated at 37^{o}C for 24 - 48 hours. Suspect salmonella-like colonies were identified biochemically and serologically.

High temperature egg dipping

The high temperature egg treatment method has been described previously. Eggs were immersed in water at 60°C (± 0.5°C) for four minutes, within 24 hours of infection. In the majority of experiments, an alkaline detergent (Titan Liquid Trayclean SU976, Lever Industrial) was added to the dipping solution. A solution containing 1.0% v/v alkaline detergent had a pH of 10.5 - 11.0.

Culture of eggs after treatment

The egg moulding technique has been described above. In addition, the egg contents were sampled using a selective enrichment technique. A cotton wool swab was inserted into the egg contents after the small end of the egg had been removed. The swabs were transferred directly into test tubes containing 10 ml. volumes of Tetrathionate Broth APHA (Gibco Europe Ltd.). The samples were incubated at 42°C for 48 hours and subcultures were made onto BGA after 24 and 48 hours. The plates were incubated at 37°C for 48 hours and observed for the presence of salmonella-like colonies.

Naturally infected eggs

These eggs were obtained directly from the producing farms. Because they provided the greatest level of contamination, floor-laid eggs were most often used. These eggs were routinely cleaned and 'sanitised' within 1 - 2 hours of collection in a chlorine-based detergent solution maintained at 43.5°C for 2 - 3 minutes.

One experiment required a pen of 100 laying hens to be established. The nest boxes within this pen were closed so that all eggs were laid on the straw litter covering the earth floor. The birds were not inseminated and infertile eggs were produced. After collection the eggs were held at room temperature on clean paper egg trays in the egg room of the laying site until required. Before being treated, each egg was visually inspected to assess the amount and type of organic soiling on the shell.

Egg treatment methods

Treatment methods involving the use of high temperatures are detailed in the text. A second heating operation was carried out 24 hours later where shown. The eggs were kept covered until they were opened for sampling purposes. Several commercially used egg sanitising methods were also tested. Some involved the dipping of eggs in disinfectant solutions at lower temperatures.

Quaternary Ammonium (QAC) dip

Eggs were immersed in a QAC solution (Microquat, Microbiologicals Ltd., 0.1% v/v) maintained at 40^{o}C for 2 minutes.

Chlorine/detergent wash

Baskets of eggs were placed in the sanitising solution which contained a chlorine based detergent (Nu-San, Wellcome Foundation, 0.33% w/v). The solution was maintained at 43.5^{o}C and the eggs were immersed for 3 - 5 minutes during which time they were agitated.

Fumigation

The eggs were fumigated on plastic trays inside an automatic fumigation cabinet. Solid paraformaldehyde prills (Paraformaldehyde 91 prills,

Messrs. Synthite Ltd., West Bromwich) were heated inside the cabinet to generate the required gas concentration. The fumigation cycle was of a 20 minute duration and the temperature was maintained at 27°C (± 1°).

Warm Alkaline detergent

A solution containing alkaline detergent was maintained at 40°C (± 0.5°C) and the eggs were immersed for 2 minutes. The dip solution was continuously aerated. A stream of air bubbles was generated by passing compressed air through an aquarium-type air block.

QAC/Detergent wash

The eggs were immersed in a stainless steel dip tank containing a QAC detergent solution (Titan Quatdet, Unilever Ltd., 0.1% v/v) for 2 mins. at 40°C (± 0.5°C). The solution was aerated as above.

Assessment of bacterial penetration

The egg moulding technique was used to determine the amount of shell penetration occurring in naturally contaminated floor eggs.

The purple spots on the inner membrane caused by the microbial reduction of tetrazolium salt were individually cultured onto the surface of a nutrient plating medium (TSA). The inner membrane was picked off with sterile forceps and the areas on the outer membrane showing the presence of reduced indicator were similarly cultured. The outer membrane was then removed and any patches of formazan dye on the inside of the shell were gently swabbed.

The bacteria growing on the surface of TSA plates after incubation were identified. The ability of different types of bacteria to penetrate the outer egg structure was established.

Coliforms were isolated readily from the inside surface of the inner membrane. This membrane was routinely cultured in further trials. The whole area of the membrane was rubbed gently with a moistened swab which was transferred to the surface of a Violet Red Bile Agar (VRBA, Oxoid Ltd.) plate. After incubation at $37^{o}C$ for 24 hours, the plates were examined for the presence of typical coliform colonies. An egg was considered to have been penetrated by coliform bacteria if such colonies were observed. If coliforms were not isolated and the inner membrane showed evidence of bacterial penetration, no further attempts were made to identify the contaminating bacteria.

RESULTS

1412 incubated eggs containing an embryo(10 - 28 days old) that had failed to hatch were cultured. The embryonic fluids provided the highest number of isolations when various sites in the same egg were compared (air cell, oesophagus, yolk sac, fluids). Eggs that showed signs of external pipping were not cultured, due to the possibility of extraneous contamination. The incidence of contamination is shown in Table 3.1. 10.8% of all eggs examined contained bacteria which were characterised, as shown in Table 3.2. The majority of positive eggs contained bacteria in pure culture and viable counts of embryo macerates revealed the presence of between 10^3 - 4.5×10^9 bacteria/ml. of fluid.

The two farms yielding the highest isolation rates (B and C) were further investigated. The eggs produced received no form of treatment at the farm and were deliberately not sanitised in an attempt to increase the natural contamination rate. The eggs were incubated within four to seven days of collection and were removed from the incubators after 22 - 24 days. All eggs used in this part of the study were cultured and were not specifically selected for culture because they failed to hatch. Table 3.3 shows the bacterial isolations obtained from both nest and floor eggs produced at the two farms. The mean isolation rate was 3.4% with floor eggs providing three times as many bacteria compared to eggs laid in the nest. Escherichia coli was isolated from 29.8% of all eggs positive on culture. The percentage of isolates identified as Gram positive cocci and rods were 48.9 and 12.8 respectively. Of the 46 organisms obtained from 40 eggs, all but three were assigned to the above groups. Enterobacter cloacae and Pseudomonas spp were isolated from the remaining eggs. The major groups of bacteria isolated were essentially similar to those isolated from eggs that failed to hatch.

Table 3.1

Isolation of bacteria from the embryonic fluids of eggs that failed to hatch and contained a dead embryo.

Farm	No. eggs cultured	No. eggs from which bacteria isolated	Percentage of eggs associated with bacteria
A	365	39	10.7
B	102	17	16.7
C	223	43	19.3
D	602	43	7.1
E	120	11	9.2
Total eggs all Farms	1412	153	10.8

Table 3.2

The types of bacteria isolated from eggs that failed to hatch

Bacteria isolated	Frequency of isolation	Percentage of all isolates
Streptococcus spp.	17	22.4
Staphylococcus spp./Micrococcus spp	12	15.8
Eshericia coli	35	46.1
Enterobacter cloacae	2	2.6
Proteus mirabilis	1	1.3
Other Gram negative rods	5	6.6
Gram positive rods	4	5.3
Total isolations	76	

Table 3.3

The isolation of bacteria from 24 day old incubated hatching eggs containing a developing embryo.

Farm	Eggs collected from	No. eggs from which bacteria isolated	Percentage of eggs associated with bacteria
B	Floor	10/275	3.6
	Nest	5/398	1.3
C	Floor	21/281	7.5
	Nest	10/398	2.5
Total isolations		**46/1352**	**3.4**

The relative frequency of occurrence was different however. Attempts were made to estimate the viable counts on the shell surface of the dirtiest eggs after incubation. In only 9/34 eggs was the shell rinse fluid shown to contain more than 10^2 viable bacteria/ml and in only one instance was the viable count greater than 10^4 bacteria/ml. Gram positive bacilli were most commonly isolated from the shell rinse fluids.

The outside of the egg shells were examined to establish the presence of organic debris. Because floor eggs were included, 18.7% of all the shells graded were excessively dirty. 72.4% of the shells examined that were not excessively dirty were contaminated with either straw, feathers or faeces. In view of the high incidence of organic contamination observed with this group of eggs, a bacterial isolation rate as low as 3.4% was somewhat surprising.

Preliminary studies using the egg moulding technique with floor eggs suggested that shell penetration by bacteria was a more common event (6.9 - 59.3% of eggs showed evidence of penetration) than previous work involving the culture of embryonic fluids had suggested. Eggs produced at Farm C (the same flock used previously) were sampled using this method (Table 3.4).

18% of floor eggs and 48.9% of cleaned and 'sanitised' floor eggs showed evidence of bacterial penetration. Only 5/144 nest eggs (including both non-treated and sanitised eggs) were associated with bacteria.

Table 3.5 shows the results of a trial designed to demonstrate the effect of holding eggs at room temperature before sampling. The maximum egg storage time was three weeks. The overnight floor eggs differ from the non-treated floor eggs in that they remained in contact with the

Table 3.4

Bacterial penetration rates observed using the egg moulding technique.

	Floor-laid eggs		Nest-laid eggs		
Egg shell treatment	Untreated	Chlorine/ detergent wash	Untreated	Sanded* untreated	Sanded QAC dip
No. eggs penetrated by bacteria	9/50	23/47	1/49	2/49	2/46
Percentage	18.0	48.9	2.0	4.1	4.3

* Shell surface lightly sanded to remove organic soiling.

straw litter in the laying pen for 12 - 14 hours. The remaining floor eggs were collected throughout the day at one to two hour intervals. 20.3% of overnight eggs, 18.2% of treated floor eggs and 10.2% of non-treated floor eggs showed evidence of bacterial penetration. Egg storage time did not apparently affect isolation rate, although further work would be necessary to confirm this.

Bacterial penetration on a significant number of occasions was associated with a distinct morphological shell feature e.g. the presence of faecal contamination or the absence of cuticle on a small area of the shell surface. Bacterial penetration was recognised by observing the dye spots caused by the reduction of the dye indicator to its insoluble formazan derivative.

In a similar trial to that conducted by Board and Halls (22) and involving the artificial infection of eggs with S. hadar in a solution containing an aqueous food dye, it was possible to demonstrate that the spots caused by bacterial penetration were coincident with those produced by the deposition of food dye on the shell membranes (Table 3.6) The bacteria penetrated only those pores which allowed the passage of the food dye. Studies with isolated shell membranes showed that a single organism placed on the surface could produce a diffuse pigmented dye spot.

It was found possible, during this experiment, to take a swab from the site of bacterial penetration and transfer the bacteria directly onto the surface of solid plating media. This allowed for the subsequent identification of the contaminating or artificially introduced organisms.

Table 3.5

The effect of prolonging egg storage time on the bacterial penetration of floor eggs.

Egg age at opening (days)	Egg Treatment: Untreated	Overnight floor eggs untreated	Chlorine/ detergent dipped	Chi-squared (2d.f.) between age at sampling
0 - 6	2/29 (6.9)	7/29 (24.1)	5/29 (17.2)	3.23 - NS
7 - 13	2/29 (6.9)	5/30 (16.7)	9/29 (31.0)	5.75 - .10>P>.05
14 - 21	5/30 (16.7)	NS*	2/30 (6.7)	ND

Body of table shows the number of eggs with bacterial penetration (percentage expressed in brackets).

* No sample

Table 3.6

The correlation between bacterial penetration and uptake of food dye after artificial infection.

Salmonella hadar inoculum (organisms/ml)	No. eggs with bacterial penetration*	No. spots on the inner surface of inner membrane	
		Formazan	Food Dye
1×10^2	1/4	1	161
1×10^4	4/4	63	75
1×10^6	4/4	65	64

* S. hadar isolated from inner membrane by culture

The egg moulding technique was used in conjunction with conventional cultural procedures to determine the efficacy of egg treatment following artificial infection with a large dose of S. hadar (ca. 2×10^5 organisms/ml.). On this particular occasion, and subsequently, an alkaline detergent was added to the dip solution to provide a pH of 11.0 - 11.5. Alkaline detergents are quite widely used in egg washing solutions (Buchli (32)) to prevent bacterial contamination and allow water recirculation.

The eggs, 24 hours after infection, were immersed at 60°C for 4 minutes in the detergent solution. Half of the eggs were opened immediately after the shell surfaces had dried. The remainder were incubated for 11 days at 37°C prior to sampling, using both the egg moulding technique and also by selective enrichment of the egg contents. The results are shown in Table 3.7.

The nalidixic acid marked infecting strain was isolated from only those eggs initially infected. All of the infected control eggs were penetrated by S. hadar whilst the selective enrichment of the egg contents and inner membrane demonstrated 11/20 eggs to be positive. Treatment was very effective in reducing the egg infection rate. Similar results were obtained after incubating the treated eggs for 11 days. This suggests that the treatments destroyed the bacteria at the periphery of the egg rather than reducing the number of viable cells or inducing sub lethal damage. The egg moulding method was at least as sensitive as the enrichment method when used to detect the presence of egg infection.

The highest levels of natural egg contamination had been obtained after floor eggs had been immersed in a chlorine-based sanitising solution. To allow an evaluation of improved egg shell treatment methods, 639 'sanitised' floor eggs were sampled over a period of four consecutive

Table 3.7

The effect of dipping infected eggs in hot water containing alkaline detergent on the isolation of Salmonella hadar.

Egg shell treatment	No. eggs	No. eggs with bacterial penetration spots (egg moulds)	S. hadar isolated after culture from: Shell membranes (direct plating)	Egg contents (selective enrichment)
*Infected treated	19	1/19	1	0
Infected non-treated	20	20/20	20	11
†Infected treated	20	1/20	1	0
Non-infected incubated controls	19	1/19	0	0

Eggs infected by ATD dipping in S. hadar (2×10^5 organisms/ml)

* Held for 24 hours before treatment in water at $60^{o}C$ containing alkaline detergent.

† Incubated for 11 days at $37^{o}C$ after treatment.

weeks. All of the eggs originated from the same farm and were dipped in the detergent/sanitiser within 1 - 2 hours of collection. The eggs were then randomised into their respective treatment groups before being transported back to the laboratory in sealed plastic bags. The following morning, the different egg shell treatments were applied. Details of the procedures used are shown in the body of Table 3.8. Between treatments and before sampling, the eggs were stored in bags at room temperature.

The egg moulding technique was used to determine the effects of treatment on bacterial penetration. The inside surface of the shell, the inner surface of the outer membrane and the inside surface of the inner membrane were cultured separately. Initially, all bacteria that were isolated from the sites of dye reduction were identified, but it was later decided only to culture from the inner surface of the inner membrane. The swabs were transferred directly to the surface of solid selective media to allow for the presumptive identification of coliform bacteria. It should be noted that some bacteria, predominantly Gram positive cocci, were usually isolated from the inner surface of the shell only. The dye spots were more clearly visible when the shell membranes were peeled back to expose the shell.

The results of the trial are shown in Table 3.8. The number of eggs penetrated by either coliforms or total bacteria are expressed. 52% of eggs in the control group were contaminated with bacteria, whilst 60 eggs were penetrated by coliforms (36.6%). All of the egg shell treatments significantly reduced the bacterial contamination rate. There appeared however, to be a differential effect against coliform organisms. Only 5/474 (1.1%) treated eggs were positive for this group of bacteria.

Table 3.8

The influence of external shell treatment on total bacterial and coliform penetration of the outer structures of 'sanitised' floor eggs.

Egg shell treatment	Number (percentage in brackets) of eggs with penetration after treatment	
	Total bacteria.	Coliforms
Control	87/166 (52.4)	60/164 (36.6)
60°C 4 mins Hot water	33/171[a] (19.3)	2/171 (1.2)
60°C 4 mins Alk. det.	13/89[ab] (14.6)	0/89 (0)
60°C 4 mins* 60°C 2 mins	27/88[c] (30.7)	3/88 (3.4)
60°C 4 mins* 60°C 4 mins	15/62[abc] (24.2)	0/62 (0)
HTD Formalin (2.5% v/v)	3/64 (4.7)	0/64 (0)

* Second treatment applied after 24 hours.

Treatments with same suffix were not significantly different. No analysis could be performed on coliform penetration results due to small numbers.

E. coli, Ent. cloacae and Ent. aerogenes were common contaminants found within the control eggs. Gram positive cocci and rods were common contaminants of both the treated and control groups. Pseudomonads were occasionally isolated from both groups.

The appearance of the bacterial penetration spots was markedly different in treated and control groups. Those organisms surviving the heating process may have been physiologically impaired. In addition, the number of individual bacterial penetration spots observed in positive eggs was lower in the treated groups.

The relatively small group sizes used in this trial did not enable the selection of a single treatment method. It was known however, that several methods caused a reduction in hatchability. These included HTD formalin dipping and those involving successive heat treatments in water. A large trial was designed therefore to look at the effects of dipping eggs in hot water with added detergent. A pen of 100 birds was selected and the eggs produced were laid exclusively on the floor. The hens were not inseminated, so that all eggs sampled were infertile. Initially, several egg collections a day were organised, but after seven weeks, only one collection was made. In addition, the eggs were moved around the pen on the day they were laid, away from the areas frequented by brooding hens. During the eleventh week of the trial, an additional collection was made. Each day's production was recorded and equally divided into four groups. At stated intervals, two groups of eggs were dipped in an alkaline detergent solution maintained at 60^{o}C for 4 minutes. After dipping, the eggs were allowed to air dry before being sealed in plastic bags for transporting to the laboratory. Half the eggs were incubated at 37^{o}C for 10 days, whilst the remainder were sampled immediately using the egg moulding technique.

The agar filled eggs were incubated for 24 - 48 hours at 37°C and inspected for the presence of dye reduction spots. The number of penetration spots was recorded. The results are shown in Tables 3.9 and 3.10. 27.6% of the control eggs and 14.2% of the treated non-incubated eggs were penetrated by bacteria. The effect of treatment was more marked when the figures obtained for coliform bacteria were compared (19.4% and 4.0% respectively). The same general trends were evident in the incubated groups. The number of penetration spots in those eggs penetrated by coliforms was calculated. 55 spots were counted in 688 treated non-incubated eggs compared to 1489 in 705 control eggs. This represents a 27 fold reduction. The results obtained with incubated eggs were less dramatic, with only a 5 - 6 fold reduction. Mean log. bacterial counts obtained from the shells of control eggs varied from 7.41 - 9.08. Counts obtained from the surface of treated eggs ranged from $10^{3.82}$ - $10^{5.96}$ viable bacteria, a reduction of between 96.98 - 99.98 percent.

The practice of placing eggs in sealed bags during incubation was demonstrated to increase the level of contamination on the shell. This was presumably due to sweating during incubation. Sweating has been shown to be responsible for increasing the microbial populations of the shell and the egg interior (Forsythe et al (46), Fromm and Margolf (49)). When eggs were incubated without being bagged, the shell counts were always lower and the practice was subsequently discontinued. Bacterial penetration rates in treated eggs remained higher however after incubation. This can be explained if it is assumed that the residual organic material on the shell surface provided a focus of infection.

Table 3.9

The dipping of floor eggs in hot solutions of alkaline detergent to control bacterial penetration

	Not Incubated		
	Not Treated	Treated*	Chi-squared between treated and not treated
No. of eggs	659	643	
No. of eggs with penetration by:-			
Coliforms	129 (19.4)	26 (4.0)	73.4 ($p \ll 0.001$)
Total bacteria	182 (27.6)	91 (14.2)	34.8 ($p < .001$)

	Incubated		
	Not Treated	Treated*	Chi-squared
No. of eggs	508	629	
No. of eggs with penetration by:-			
Coliforms	55 (10.8)	18 (2.8)	28.4 ($p < .001$)
Total bacteria	100 (19.7)	94 (14.4)	413 ($p < .05$)

Percentages expressed in brackets.

* Eggs treated by immersing in hot water ($60^{o}C$) containing alkaline detergent, for 4 mins.

Table 3.10

The influence of egg treatment on the number of bacterial penetration spots

	Not Incubated		Incubated	
	Not Treated	Treated*	Not Treated	Treated
No. eggs with bacterial penetration	182/659	91/643	100/508	94/629
No. spots	1814	418	1382	715
No. eggs penetrated by coliforms	130/705	26/688	55/546	18/675
No. spots in eggs penetrated by coliforms	1489	55	972	177

* Eggs treated as in Table 3.9.

The sensitivity of the egg moulding technique was demonstrated by comparing the results obtained using egg moulding, with those obtained with conventional cultural procedures. Over a period of four to five weeks 20/731 (2.7%) eggs were found to contain bacteria when swabs taken from the egg contents were transferred onto solid plating media. On 7/20 occasions bacterial isolation was associated with shell irregularity e.g. a hair crack or small hole. 27.6% of eggs over an equivalent period were shown to be penetrated by bacteria, using the egg moulding method.

Many factors are involved in determining the frequency of egg penetration by bacteria. This can be demonstrated by reference to the results in Table 3.11. Although floor eggs provide a convenient source of material for studying bacterial penetration, they do not necessarily yield consistently high isolation rates. Although it was not the intention at this part of the study to determine the factors affecting bacterial contamination of shell eggs, several interesting observations were made.

The number of eggs penetrated by bacteria decreased between Weeks 2 - 8 (46.9 - 4.3%). At this time the eggs were cleaner and the number of brooding hens within the pen was greater. The eggs were initially collected frequently throughout the day. From Week 9 onwards, only one collection was made and the eggs laid during any particular day were physically distributed about the litter floor away from areas frequented by brooding hens. The eggs, as a result, became more heavily soiled. The effect of this change was to increase the bacterial isolation rate from Week 10 onwards.

Bacterial penetration was also shown to vary from day to day. No success was achieved when attempts were made to correlate bacterial penetration

Table 3.11

The observed variation in bacterial penetration through the shell of floor eggs

	Week during which eggs laid								
	2	4	6	8	10	12	14	16	18
Total bacteria	46.9	22.2	13.5	4.3	33.3	43.5	55.1	63.0	22.2
Coliforms	32.1	14.2	3.7	2.9	29.6	21.3	45.8	48.1	5.6

Figures expressed as a percentage of total eggs with evidence of shell penetration.

with rainfall, relative humidity or age of egg at sampling. The amount of organic material left on the shell was shown to exert an influence however. 31.3% of slightly dirty eggs were shown to be penetrated by coliforms compared to 78.4% of eggs graded as very heavily soiled. These results were more pronounced when the incubated eggs were examined. 50% of very heavily soiled eggs were penetrated, compared to only 14% of eggs with lesser amounts of organic material on the shell surface.

During part of this study, several commonly used egg sanitising methods were evaluated. Eggs collected over a two week period were treated as shown in Table 3.12. All egg treatments increased the number of eggs penetrated by total bacteria. This result was expected for those methods which involved the wetting of the shell surface. The results obtained following fumigation were interesting. It is now believed that sufficient moisture was generated during the inital heating phase of the fumigation process, to facilitate bacterial invasion of the shell.

The results of a further comparative trial are shown in Table 3.13. The treatment of eggs by dipping in hot aqueous solutions of alkaline detergent was shown to be effective in substantially reducing the incidence of bacterial contamination. Nine bacterial penetration spots were observed in this group compared to 203 in the control group. Coliforms were not isolated from the shell and membranes of the treated group. Immersion of eggs in alkaline detergent at $40^{o}C$ and washing in chlorine detergent solution at the same temperature did not significantly reduce bacterial invasion of the shell (χ^2 (2df) = 1.39). Similar results have been obtained using a commercial spray washing machine. The use of a combined QAC and detergent at $40^{o}C$ did limit the extent of bacterial penetration in this trial.

Table 3.12

The influence of external shell treatments on bacterial penetration of floor eggs

	Egg Shell Treatment				
	Non-treated	QAC dip	Formaldehyde fumigation	Chlorine/ detergent wash	Chi-squared between treatments (3 d.f.)
No. eggs	60	63	60	56	
No. with bacterial penetration	11 (18.3)	19 (30.1)	20 (33.3)	11 (19.6)	5.29 NS
No. penetrated by coliforms	9 (15.0)	17 (27.0)	11 (21.7)	4 (7.1)	8.49 $p < .05$

Percentages expressed in brackets.

Table 3.13

A comparison of the effects of different egg shell treatments on bacterial penetration of floor eggs.

	Egg Shell Treatment				
	Non-treated	60°C 4 mins Alk. det.	40°C 2 mins* Alk. det.	QAC* dip	Chlorine/ detergent*
No. eggs	27	26	27	27	23
No. eggs with bacterial penetration	17 (63.0)	5 (19.2)	19 (70.4)	6 (22.2)	18 (78.3)
No. eggs penetrated by coliforms	13 (48.1)	0 (0)	6 (22.2)	1 (3.7)	10 (43.5)
Total no of penetration spots	203	9	218	20	176

Percentage figures expressed in brackets.

* Dip solutions continuously aerated.

DISCUSSION

Because it was impractical to use paratyphoid salmonellas to assess the egg treatment methods, it was necessary to identify an alternative indicator organism. A preliminary survey involved the culture of the embryonic fluids present inside the shell of dead embryos between 10 and 28 days old. 10.8% of samples were shown to contain bacteria. This level of contamination was similar to the 12.7% obtained by Bruce and Johnson (31) using chicken eggs. The contaminating flora was essentially the same although there were differences in the relative proportions of the major groups. Gram positive cocci and coliforms were the predominant organisms and accounted for 84.3% of all isolates. Seviour et al (137) isolated bacteria from 25% of chicken eggs showing no germinal development. 92.6% of the isolates were Gram positive cocci. 7.7% were identified with E. coli compared to the 46.1% obtained in our own studies.

Eggs in this trial were selected for culture primarily because they failed to hatch. Subsequently, untreated nest and floor-laid eggs were cultured in the same manner. Bacteria were isolated from only 3.4% of the eggs cultured. Floor eggs were more frequently contaminated. Although Gram positive cocci were most commonly isolated, 29.8% of the isolates were identified with E. coli. The contaminating flora was therefore similar to that found in eggs that failed to hatch. As in the previous survey, the majority of contaminated eggs contained bacteria in pure culture.

The egg moulding technique developed by Board and Board (13) allowed an assessment of shell penetration by bacteria used to artificially infect chicken eggs. Culture of the embryonic fluids was thought to provide information only about those bacteria which were capable of

penetrating the shell and membranes before eventually establishing a fulminating infection of the egg contents. Many of the bacteria present in the heterogeneous shell surface flora do not subsequently invade and establish in the albumen. Seviour and Board (135) demonstrated that certain Gram negative bacteria achieved dominance in the egg contents following two phases of selection. The first phase occurred at the shell membrane and the second after the egg yolk had made contact with the inner shell membrane following storage. Temperature and relative humidity were important factors affecting the process. The concept invoking two distinct phases of infection was first presented by Gillespie and Scott (61).

It was thought that the egg moulding technique may provide a means of evaluating the frequency of shell penetration by bacteria and it was assumed that the figure obtained would be greater than that observed after culturing the egg contents. The failure of bacteria to subsequently invade the albumen may not be important when it is considered that the embryo, prior to hatching, first contacts and then ruptures the air cell membrane before emerging from the shell. The shell membranes may therefore act as a focus of infection for the developing poult.

Floor eggs with high shell surface counts that had been immersed in a chlorine-based detergent solution were frequently penetrated by bacteria. The level of contamination was significantly worse after the shell surface had been wetted in this manner. In his discussion 'The effects of washing on egg infection ' Board (16) has reviewed several relevant papers from the many written on this subject. Under certain conditions, washing of the shell surface can have deleterious effects. Our own data support this general conclusion.

The egg moulding technique was used to monitor the success of egg treatment following the artificial infection of eggs with a nalidixic acid marked strain of S. hadar. All of the infected eggs showed evidence of bacterial penetration as demonstrated by the presence of reduced dye spots on the inside of the inner membrane. Culture of the egg contents, by selective enrichment in tetrathionate broth, provided fewer positive isolations when eggs were sampled 24 hours after infection. When using the egg moulding technique, it was found possible to culture the infecting organism directly from the membrane at the sites of dye reduction. This enabled the routine identification of bacteria capable of penetrating the shell.

In agreement with Board and Halls (22) a positive correlation was obtained between the presence of reduced formazan and the deposition of synthetic food dye on the shell membranes following artificial infection. This indicated that bacteria could only penetrate shell pores which allowed the passage of the aqueous dye solution. The number of formazan spots on the shell membranes was routinely counted to provide an assessment of the extent of infection.

Because the outside of the shell immediately above the penetration spot could be observed it was often possible to relate bacterial invasion with the presence of a morphologically distinguishable feature. The importance of the intact shell and cuticle in preventing bacterial penetration was evident.

To provide a high egg contamination rate, washed floor eggs were used in early trials designed to assess methods of egg treatment. High temperature egg treatment and temperature differential formalin dipping were effective in reducing total bacterial penetration. There was however, a differential effect of treatment against coliform bacteria.

This group of bacteria invaded 1.1% of treated egg shells compared to 37.6% of control washed eggs. In addition, those spots of reduced tetrazolium dye on the membranes of treated eggs appeared different from those seen in the control group. Such spots were sometimes only clearly visible after the shell membranes had been removed and they were generally very small, with very little pigment being produced. It was thought that egg shell treatment may have adversely affected organisms that eventually penetrated beneath the shell. As a direct result of this observation, eggs in further trials were always incubated for a period before the egg contents were replaced with agar.

High temperature egg treatment was the method selected for further study. A process involving the heating of eggs in either oil or water was described by Funk in 1943 (50). Later (52) in a review he referred to the process as 'thermostabilisation' and detailed the major advantages of the process. These included the destruction of spoilage-causing bacteria. High temperature egg treatment had previously been shown effective against S. hadar used to artificially challenge eggs. (see Chapter 2). Here the effect of dipping naturally soiled eggs in hot water on the extent of bacterial penetration was assessed, using the egg moulding technique. Such a treatment was intended for use with turkey hatching eggs so that the blastodisc was not damaged and embryonic development not seriously impaired.

Artificially or naturally soiled and machine-washed chicken eggs had previously been dipped in either hot oil or water (48.9 - 70^{o}C) for various lengths of time, to either limit or prevent the formation of rots during their subsequent storage (52,133,127,81). The combinations of temperature and time used however, invariably resulted in the

blastodisc being damaged. Goresline et al (64) also reported on the effects of such treatment on egg quality and observed that the stability of the egg white was increased, implying that some structural changes had taken place.

Chicken eggs have also been exposed to very high temperatures for short periods of time. Funk (51) did not find flash pasteurisation (76.6°C for 10 secs.) to be effective in reducing the incidence of bacterial spoilage in table eggs, although Scheibner and Felhaber (132) using a similar treatment regime, substantially reduced the shell surface counts of artificially challenged eggs. Feeney et al (44) immersed eggs in boiling water for 3 secs. after infecting with large numbers of Pseudomonas aeruginosa and prevented subsequent spoilage if the eggs were dipped within 24 hours of infection. The treatment of hatching eggs at such temperatures however, would require the adoption of stringent control measures to avoid ensuing problems in the hatchery. In a more recent study, Sauter (128) treated artificially infected eggs in an immersion type egg washing machine. Washing at 60°C for 3 mins. prevented spoilage during storage, but in common with previous reports, no information was presented relating to the effects of treatments on hatchability. As with the study of Knowles (81), thermostabilisation of the albumen was not achieved using this less severe treatment.

In the present study, an alkaline detergent was used to control the bacterial load within the dip solution and also to facilitate the removal of organic contaminating material on the shell surface of soiled eggs. Various reports had also indicated the increased sensitivity to heat of S. typhimurium and other egg-associated bacteria at elevated pH (79,80). Bacteria on the shell surface may therefore have been more rapidly killed.

Bacterial penetration was quite commonly observed when lightly soiled eggs were evacuated and filled with a non-selective nutrient agar containing an indicator of bacterial gorwth. The egg moulding procedure was used therefore to determine the effect of different egg treatment protocols on the extent of bacterial contamination and subsequent shell penetration.

Dipping infertile soiled eggs in a hot solution of the detergent was extremely effective in reducing total bacterial and coliform penetration. In additon, the shell surface counts were significiantly reduced. Conventional egg treatment methods (fumigation, dipping in warm disinfectant solutions) had little effect on bacterial penetration of soiled eggs compared to the high temperature treatment. These results would be expected because of the failure to eliminate bacteria that have already penetrated beneath the pores of the shell.

The number of floor eggs with evidence of bacterial penetration varied considerably with time during this trial. Graves and MacLaury (65) demonstrated a relationship between bacterial infection of hatching eggs and several environmental factors including temperature and absolute humidity. Although it was possible to show that gross soiling of the shell influenced bacterial penetration, no success met attempts to correlate penetration with rainfall, relative humidity or age of egg at sampling.

CHAPTER 4 INTRODUCTION

Observations on the growth and persistence of selected micro-organisms within turkey eggs.

To determine whether high temperature egg treatment was effective against a range of bacteria commonly associated with eggs, it was necessary to establish a model system similar to that used for S. hadar. Following artificial challenge using an ATD dipping procedure (surface exposure) it became apparent that some micro-organisms did not readily establish in the albumen. The infecting strain could often be isolated from the shell membranes but less often from the egg contents. When the egg moulding technique was employed, the same organisms were shown to penetrate the shell as evidenced by the presence of purple dye spots (reduced formazan) on the inside of the inner membrane.

Tranter (147) investigated the antimicrobial defence system possessed by hen egg albumen. He found that many bacterial species failed to grow in albumen at normal incubation temperatures (39.5^{o}C) and alkaline pH (9.1). Several hatchery-isolated bacteria were observed to survive better than organisms selected from stock collections however. The addition of extraneous iron to albumen so as to fully saturate the ovotransferrin, reversed the inhibitory effects of albumen on Gram negative bacteria but not on Gram positive species. It had previously been suggested by Garibaldi (56) that many egg spoilage pathogens may possess the capability of producing and excreting iron transport compounds which would facilitate their growth in albumen.

A study was made therefore, of the behaviour of a range of organisms initially isolated from the hatchery in turkey egg albumen in vitro. The aim was to indicate whether certain bacteria were better adapted to survive in the relatively inimical environment of the egg white and to

determine whether this influenced their ability to infect artificially challenged eggs.

Small groups of eggs were successfully infected with a range of micro-organisms and treated using the high temperature egg treatment method. The results are fully described.

MATERIALS & METHODS

The organisms used in this study and to inoculate albumen are listed below. Other procedures are fully described in previous chapters.

	Source of isolate
Salmonella hadar (104)	Hatchery
S. ohio	Hatchery
Escherichia coli	Rotten egg
Proteus mirabilis	Rotten egg
Pseudomonas aeruginosa	Rotten egg
Alcaligenes faecalis	Turkey poults
Enterobacter cloacae	Rotten egg
*E. coli 0141	

* This isolate was kindly donated by Dr. H. Tranter, University of Bath.

Cultures were maintained on nutrient agar slopes and sub-cultured every four weeks. The organisms were grown in Tryptone Soya Broth (Oxoid Ltd) at 37°C unless otherwise stated.

Nalidixic acid resistant marker strains were prepared as required by depositing concentrated suspensions of the required organism onto agar plates containing a range of concentrations of the selective agent. Single colonies were picked after an appropriate incubation period and were replated to ensure purity. Nalidixic acid resistant organisms were maintained on nutrient agar slopes containing nalidixic acid (100 or 250 µg/ml).

Preparation of inocula

Initially an overnight culture of the required organism was serially diluted in physiological saline. 1 ml of a suitable dilution (10^{-4})

was added to 9 mls. of albumen so as to provide an initial suspension containing $10^3 - 10^4$ viable organisms/ml. albumen.

Where stated, the inocula were derived from washed cultures. Overnight cultures were centrifuged and the pellet washed in physiological saline. This process was repeated and the twice-washed cells were re-suspended in a small volume of physiological saline. Serial decimal dilutions were prepared from this cell suspension as above.

Incubation of albumen

Albumen of appropriate pH and contained in screw-capped bottles was incubated in a shaking water bath (100 strokes/minute) at $37^{o}C$ for the duration of the experiment. The number of viable bacteria was determined as required, using plate count procedures previously described.

Albumen pH

The pH of the albumen of fresh eggs was adjusted by carefully adding sterile 1M NaOH as previously described. In later experiments the albumen pH was not adjusted. Fresh eggs were held at room temperature for 6 - 7 days and the albumen was then harvested. The pH had reached 9.0 - 9.1 after storage.

RESULTS

Several bacteria did not successfully establish in the albumen of artificially infected eggs (Table 4.1) unless large challenge doses were used. When the egg moulding method was used to detect the presence of the infecting strain within the shell membranes, the majority of eggs demonstrated typical penetration spots. Some isolates e.g. S. hadar and Ps. aeruginosa produced very large diffuse areas of staining on the inner shell membrane (Plate 4.1) whereas others, e.g. P. mirabilis and E. coli produced small, discrete areas of staining (Plate 4.2).

The presence in the infecting solution of extraneous iron (Fe^{2+}) or of an inoculum of S. hadar significantly increased the infectivity of the test organism (Table 4.2). The number of isolations from the egg contents increased.

These observations suggested that the albumen was preventing or limiting the growth of several bacteria, although the inhibition could be overcome by modifying the conditions used to challenge the eggs. It had previously been suggested by Brooks (25) that extensive bacterial multiplication occurred within the air cell membrane following inoculation and after this initial colonisation bacteria subsequently migrated into the albumen. Board et al (14) presented similar findings and stated that extensive bacterial multiplication did not take place in the albumen. These authors considered that organisms within the albumen remained quiescent until they contacted the surface of the yolk.

Some general trends were observed following the inoculation of S. hadar into turkey egg albumen in vitro. A drop in viable count was commonly

Table 4.1

The isolation of micro-organisms from eggs artificially challenged using an ATD dipping procedure.

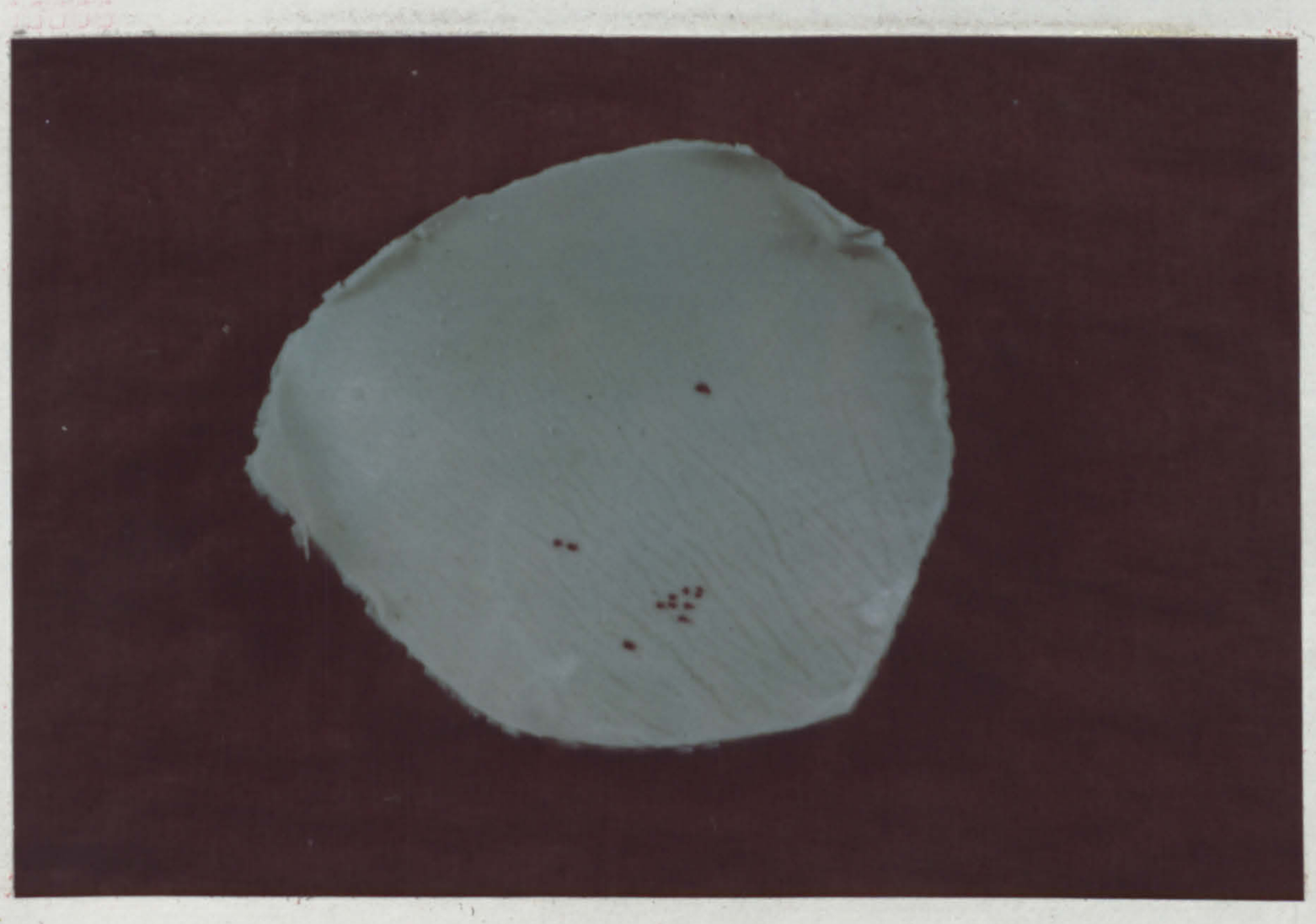
	No. of isolations from egg:					
	Contents	Membranes	Contents	Membranes	Contents	Membranes
Inoculum(Orgs/ml dip):	10^1	- 10^2	10^3	- 10^4	10^5	- 10^6
Organism						
E. coli	*0/6	0	0	1	4	3
Ent. cloacae	3	2	4	4	6	6
P. mirabilis	0	1	0	2	2	4
Alc. faecalis	0	0	1	4	5	5

* 6 eggs were sampled in each case

Plate 4.1

Purple dye spots on the shell membranes of an egg infected with S. hadar and sampled using the egg moulding technique.

Plate 4.2



The typical appearance of the inner shell membrane of an egg infected with E. coli.

Table 4.2

The isolation of micro-organisms from eggs artificially challenged using an ATD dipping procedure.

	Eggs challenged with:					
	Test organism alone (10^3-10^4orgs/ml dip)		Test organism with iron *		Test organism and S. hadar †	
No.isolations from:	Contents	Membranes	Contents	Membranes	Contents	Membranes
Test Organism						
S. hadar	5/6≠	6	6	6	-	-
E. coli	0	4	3	4	3	5
P. mirabilis	2	2	3	1	5	6
Ps. aeruginosa	5	6	6	6	6	6
Alc. faecalis	3	6	6	6	5	6

* Iron added as $Fe_2SO_4.7H_2O$ (10mg/litre)

† The challenge solution was seeded with S. hadar (10^4 orgs/ml) in addition to the test organism.

≠ 6 eggs were sampled in each case.

seen within 7 - 8 hours of incubation at 25°C (usually of 1 or 2 logs.) This was particularly noticeable when the albumen pH was adjusted to 9.1. After seven days incubation however, significant multiplication had taken place. This initial fall in viable count has been noted by previous authors (25,136). At the high pH and with small inocula extended lag times were evident and sometimes no multiplication was observed.

A range of organisms was subsequently inoculated into albumen (Fig.4.1). Using a higher temperature of incubation (37°C), marked differences in growth patterns were observed when the albumen was alkaline. Albumen was bactericidal to S. ohio and bacteriostatic to E. coli and Alc. faecalis. P. mirabilis multiplied only feebly whereas Ps. aeruginosa established in large numbers as at the lower pH. This isolate always multiplied extensively in turkey egg albumen.

The inocula used in this trial were not prepared from washed cultures, although they were diluted at least 10,000 fold to limit the transfer of nutrients from the growth medium to the albumen. There was no appreciable difference between the growth of washed and unwashed cultures. however, when used to seed albumen. It was assumed that significant amounts of iron or simple nitrogenous substances were not being transferred into the egg white and thereby facilitating growth.

The same micro-organisms used above were introduced into albumen harvested from stored eggs collected from a different farm (Fig. 4.2) S. ohio in contrast to the last experiment, successfully established and multiplied to produce a large final population.

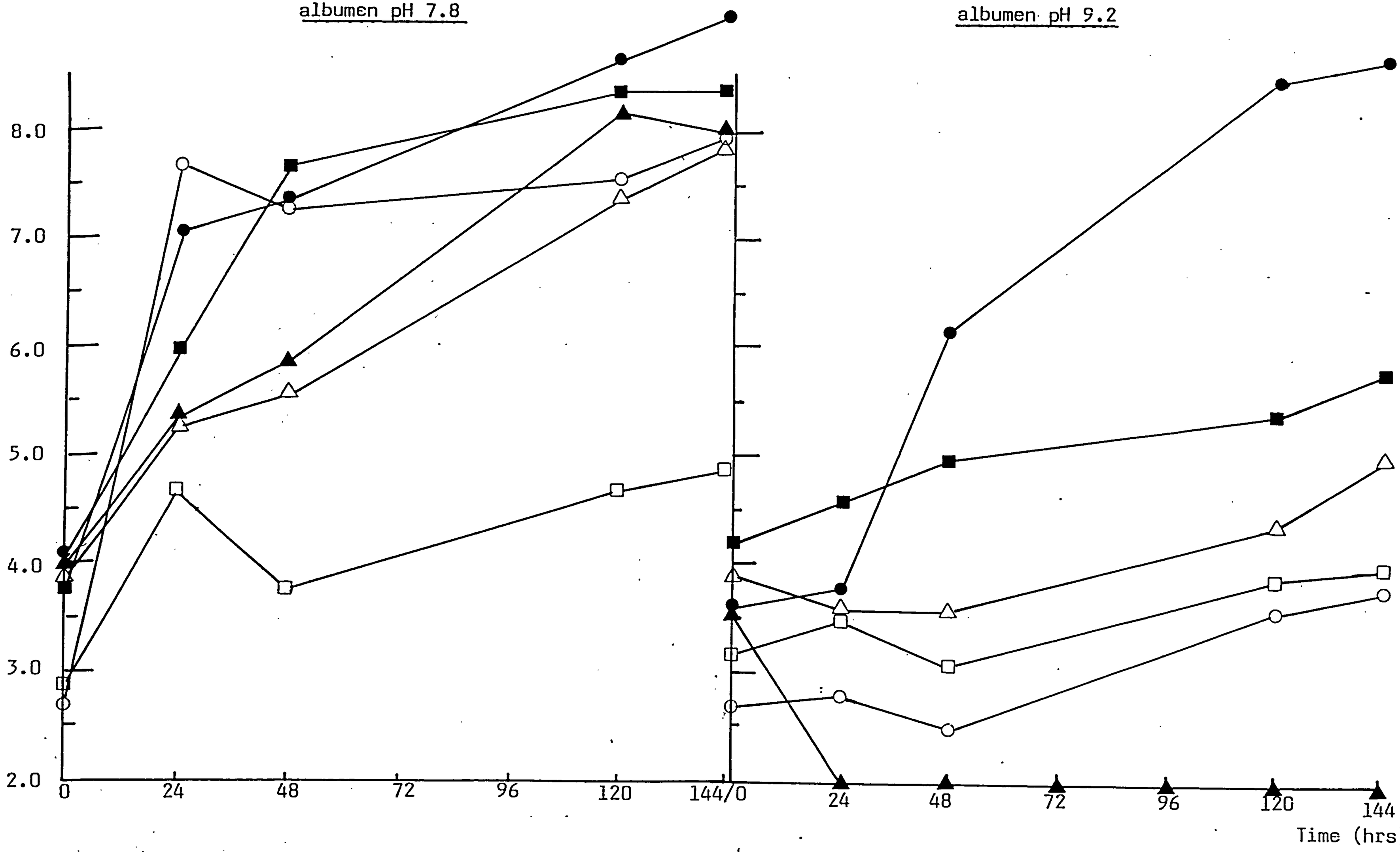
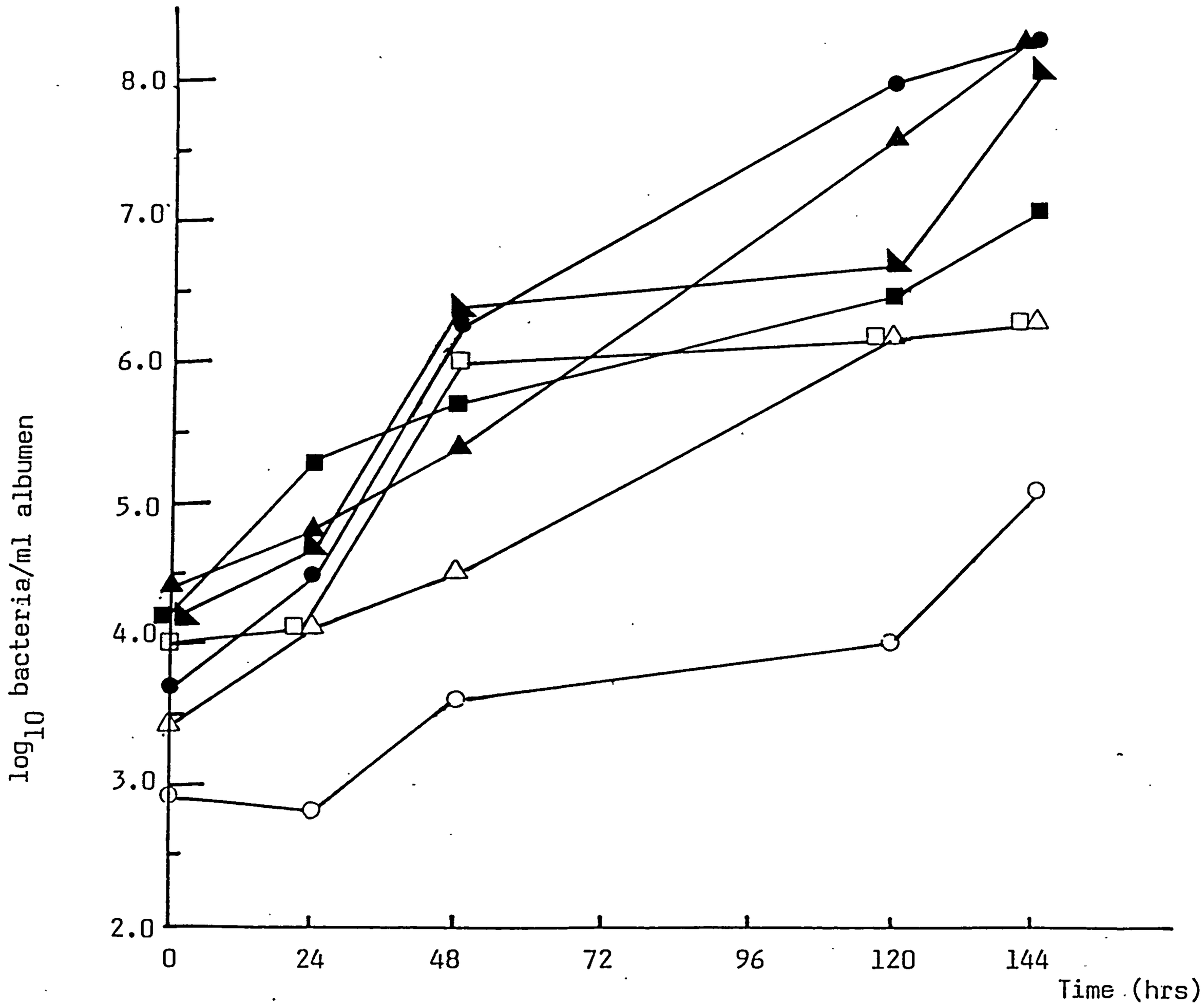


Fig. 4.1 The growth of Pseudomonas aeruginosa (●), Salmonella hadar (■), Salmonella ohio (▲), Escherichia coli (○), Alcaligenes faecalis (□), and Proteus mirabilis (△) in turkey egg albumen (pH 7.8 and 9.2) following incubation at 37°C.



Key to isolates used:-

- ● Pseudomonas aeruginosa
- ■ Salmonella hadar
- ▲ Salmonella ohio
- ◣ Escherichia coli 0141
- ○ Escherichia coli - hatchery isolate
- □ Alcaligenes faecalis
- △ Proteus mirabilis

Fig 4.2 The growth of several bacterial isolates in turkey egg albumen (pH9.1) at 37°C. The albumen was obtained from a different source to that used previously. (Fig. 4.1)

The other organisms demonstrated similar growth patterns. An isolate of *E. coli* (0141) employed by Tranter (147) in a study of hen egg albumen grew and multiplied in turkey egg albumen. As this isolate failed to establish in hen albumen the result suggests possible differences between the ability of turkey and hen albumen to support the growth of some strains of bacteria. A hatchery isolated strain of *E. coli* multiplied very feebly.

Three organisms were later inoculated into albumen collected from eggs laid by the same two flocks. *Ps. aeruginosa* multiplied well in albumen from flock A and B (Fig. 4.3). *S. ohio* did not multiply to any great extent in the albumen from flock B. After an initial fall in viable count, the organism did multiply slightly, but the final population was only in the order of 1 log. higher than the inoculum. Although these results represent only preliminary findings, it would appear that a particular serotype of salmonella exhibited markedly different growth patterns in albumen derived from separate sources.

Small groups of eggs were successfully infected using large challenge doses (10^5 - 10^6 orgs/ml) with an ATD dipping procedure. 24 hours after exposure, high temperature egg treatment (60^{o}C 4 mins. in alkaline detergent) successfully destroyed the test organisms (Table 4.3). No isolations were made from the egg contents of treated eggs.

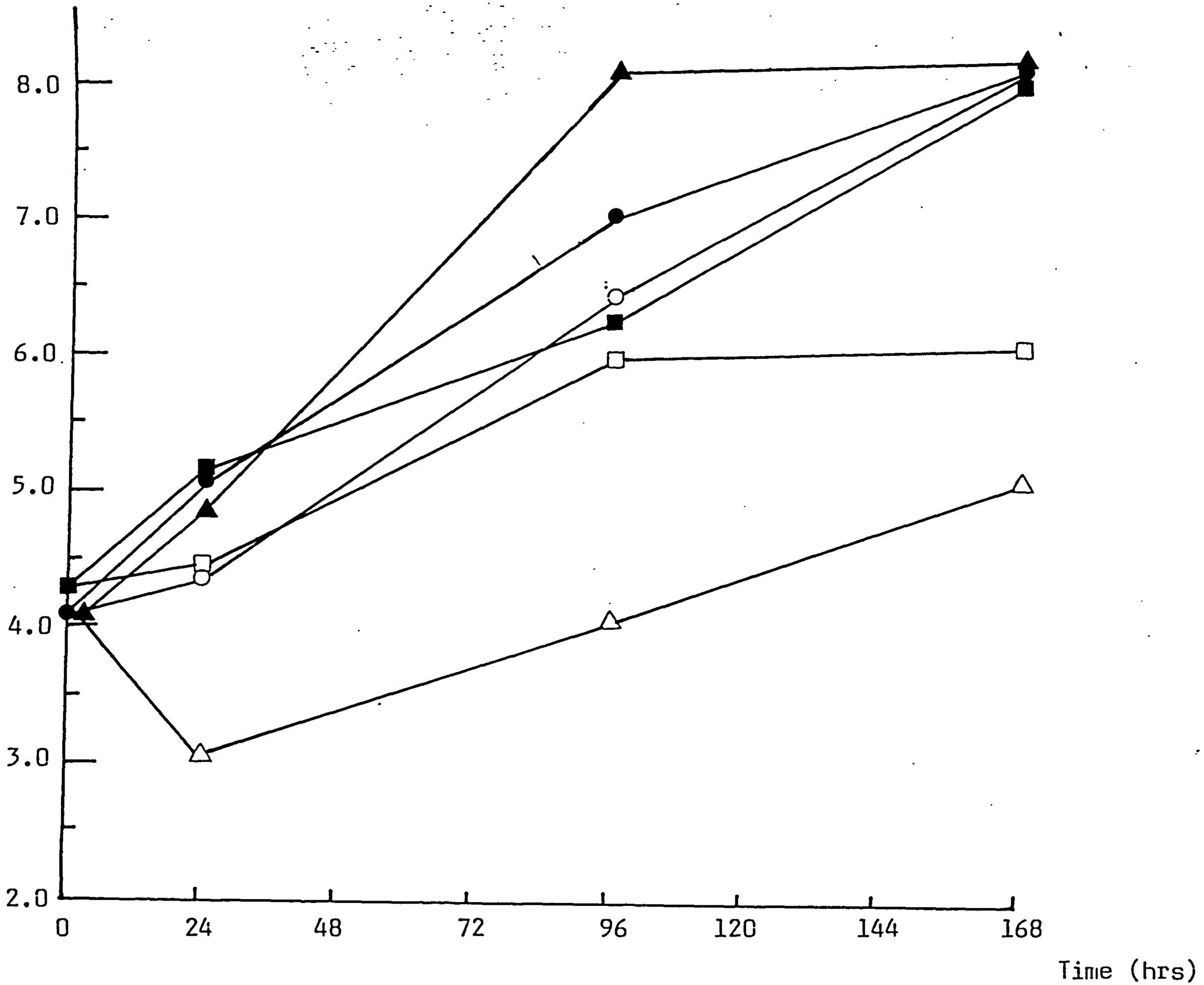


Fig. 4.3 The growth of Pseudomonas aeruginosa (●, ○), Salmonella hadar (■, □) and Salmonella ohio (▲, △) in turkey egg albumen at 37°C. Closed symbols - albumen from eggs laid by flock A, open symbols - flock B

Table 4.3

The effect of high temperature egg treatment on artificially challenged eggs.

Test Organism	Inoculum/ ml dip	No. of isolations of the test organism from the egg contents of: Treated	Control
S. hadar	4.5×10^5	0/6	2/6
E. coli	4.8×10^4	0/6	2/6
P. mirabilis	3.5×10^5	0/6	3/6
Ps. aeruginosa	1.7×10^5	0/6	6/6
Ent. cloacae	8.0×10^4	0/6	4/6
Alc. faecalis	5.5×10^3	0/6	1/6

DISCUSSION

Previous workers have demonstrated that ovotransferrin (conalbumin), an egg white protein capable of chelating iron, exerts a major influence on the growth of micro-organisms in albumen, (Schade and Caroline (130), Garibaldi (55), Board and Halls (23)). Indeed, Board and Fuller (21) considered ovotransferrin to be the principal component of the anti-microbial defence system of the hen's egg. This role had previously been accorded to lysozyme. Gram negative lysozyme-resistant bacteria predominate however, in the contents of eggs that become infected. Board and Hornsey (24) suggested that the primary role of lysozyme maybe in preserving the biological structure of the egg through its association with ovomucin. The albuminous sac, in maintaining the central location of the yolk, could contribute to the physical defence of the egg against microbial invasion. Schade and Caroline (130) demonstrated that microbial inhibition in albumen could be overcome by the addition of iron (Fe^{3+}). Garibaldi (55) completely reversed the growth inhibition observed on Gram negative spoilage bacteria in egg white by saturating the albumen with iron. This author suggested that at high pH (9.1) the stability of the iron-ovotransferrin chelate was increased and that the high pH of albumen itself was not responsible for microbial inhibition. The increase in the pH of egg white during storage is brought about by the loss of CO_2 across the shell (Healey and Peter (75)).

Iron introduced onto the air cell membrane of artificially inoculated eggs has been shown to facilitate microbial growth (Board et al (14)). Similarly, the presence of iron in the water used either to experimentally infect or wash eggs has been shown to influence the course of infection with rot-producing organisms (see Moats (98)). The term 'nutritional

immunity' was used by Kochan (82) to describe the process whereby iron was witheld from bacteria by the host. Garibaldi (56) demonstrated the role of microbial iron transport compounds in vitro in reversing the bacteriostatic effects of ovotransferrin. Tranter (147) also considered the possible role of microbial iron transport compounds in overcoming the antimicrobial defence system of avian egg albumen. This author suggested that the bactericidial nature of albumen resulted from the interplay between ovotransferrin, alkaline pH, temperature of incubation and possibly, the lack of simple nitrogenous substances. The work of Brooks (25) initially suggested that factors other than an adequate supply of iron were important in controlling the growth of bacteria in albumen. This possibility was raised earlier by Haines (69).

Garibaldi (56) studied the effects of iron transport compounds on the growth of S. typhimurium in egg white. He suggested that such compounds may contribute to egg spoilage by facilitating bacterial multiplication in the albumen. Tranter (147) suggested however, that phenolate siderophores (catechols) were not produced in hen egg albumen at high pH (9.2) and at normal incubation temperatures. The possible role of hydroxamate siderophores, most commonly produced by fungi, was not investigated. Several bacterial species produce siderophores belonging to this family e.g. Pseudomonas fluorescens (pyoverdine$_{pf}$) (93,94). It was suggested by this author however, that bacteria capable of producing hydroxamate siderophores may be better suited to survive in the alkaline pH of egg albumen. During the course of the above work, it was observed that several hatchery isolated bacterial species survived better in albumen than cultures derived from stock collections. Ps. fluorescens was one of the organisms studied.

When a range of micro-organisms were used to artificially challenge eggs by surface exposure to suitable inocula, differences were observed in their ability to establish in the albumen. The majority of bacteria studied appeared to penetrate successfully the open shell pores, as demonstrated using the egg moulding technique and it was assumed that their subsequent growth was inhibited by the albumen. The growth of these bacteria on the shell membranes was not studied, although it has previously been suggested that it is here that extensive bacterial multiplication takes place (Brooks (25)) prior to invasion of the albumen. Instead, the organisms were inoculated into turkey egg albumen in vitro and their subsequent development was observed. The majority of previous work has involved the study of hen egg albumen, although Tranter (147) did briefly study the albumen of eggs from domestic poultry and several species of wildfowl.

At high pH (9.1) and after prolonged incubation (6 - 8 days) considerable differences were observed in the growth patterns exhibited by the isolates used. Organisms achieving high populations in egg white (Ps. aeruginosa, S. hadar) successfully established in the albumen of artificially infected eggs. Several isolates did not multiply to any great extent, although on only one occasion was the albumen found to be bactericidal to the inoculum. In albumen from a different source, this isolate of salmonella (S. ohio) grew and multiplied to produce a final population, equivalent to that achieved by Ps. aeruginosa. An isolate of E. coli (E. coli 0141) used by Tranter (147) in his study of hen egg albumen formed a significant population in turkey albumen containing no additional supplements. Further work could establish whether there are major differences between the antimicrobial properties of avian albumen as suggested by this observation.

S. ohio behaved very differently in albumen harvested from eggs produced by different flocks. A specific inhibitory factor could account for this and one possible explanation could be the presence of immunoglobulins (IgA, IgM) in the egg white derived from oviduct secretions. As suggested by Rose and Orlans (122) however, very little is known about the activity of antibody in the albumen due to the relatively small amounts present, compared to the levels of IgG in yolk.

The preliminary results obtained suggested that some bacteria are better adapted to survive and establish in turkey albumen than others. Organisms that successfully establish in the shell membranes, however, without necessarily being able to multiply readily in the albumen, could be equally capable of transmitting infection to the hatched chick or poult. Indeed, Schalm (131) in 1937 suggested that paratyphoid salmonellas could be spread in this manner, with chicks acquiring a respiratory infection at hatch time, directly as the result of shell contamination.

A study of the growth rate of different bacteria in the shell membranes *in situ* compared to albumen *in vitro* may provide useful information. Brooks (25) demonstrated that high counts were obtained in the albumen when the air cell membrane of intact eggs was inoculated with the test organism. He suggested the rate of bacterial multiplication in egg white was unimportant in comparison with the rate of invasion from the heavily colonised membranes.

High temperature egg treatment was effective against a range of bacteria commonly associated with eggs. Funk (50) similarly destroyed a range of artificially introduced bacteria by pasteurising eggs in oil at 60^{o}C for 10 mins. The latter process however, was intended to reduce the

bacterial count of egg products intended for consumption, but not for the specific purpose of eliminating shell transmitted pathogens.

GENERAL DISCUSSION

Salmonella infection can be introduced into poultry flocks via any one of a number of different routes. Egg transmission, arising from shell contact at or shortly after oviposition, can perhaps be singled out because it results in such infection being maintained between generations that are usually spatially separate. To prevent salmonellas and other opportunist pathogens cycling in this manner, due consideration must be given to the egg infection process which is itself influenced by a number of independently variable factors (see Introduction). It is extremely unlikely that under commercial conditions, enough of these factors could be modified sufficiently to influence the spread of infection. The primary aim of this study was to determine whether the chain of infection could be interrupted in an attempt to produce salmonella-free poults from eggs laid by naturally infected flocks.

The influence of changing the nest box environment on the size and diversity of the shell microflora was assessed. The extent of microbial contamination was reduced using synthetic grass nest lining materials, periodically treated with paraformaldehyde rather than woodshavings. It was assumed that if the size of the microbial population on the shell were limited, the chances of an egg eventually becoming infected with a particular micro-organism would be reduced.

Rather than using the total aerobic shell count however, further work could involve the selective enumeration of specific groups of bacteria or indeed a direct assessment of bacterial penetration using methods described in the text. Extremely large numbers of eggs would be required for such a study because the groups of bacteria of major interest form only a small part of the shell microflora (see Table 1.9).

It also became apparent later that clean nest eggs were infrequently penetrated by bacteria, as shown by the egg moulding technique.

A number of methods are used commercially to reduce bacterial contamination on the shells of eggs prior to incubation. These procedures were applied after eggs had been artificially infected using a technique which ensured that at least some viable bacteria penetrated beneath the shell. Naturally infected eggs could not be used because the salmonella infection rate was extremely low.

Neither formaldehyde fumigation nor immersion dipping (in QAC) were effective in preventing S. hadar infection once shell penetration had occurred. Numbers of salmonellae on the shell must have been considerably reduced but viable organisms were consistently isolated from the egg contents of treated eggs. A number of authors (48, 110, 120) have presented similar results.

Hatching eggs have, on occasion, been dipped in antibiotic solutions to secure the control or eradication of certain egg-transmitted microbial pathogens. Vacuum dipping in gentamycin has proved effective against salmonellae. Such compounds would only have limited commercial value if used continuously, primarily because of the likely emergence of resistant strains of bacteria. Their use was therefore not considered and alternative procedures were developed and investigated. Of these a marginal pasteurisation and a high temperature differential disinfectant dipping (HTD) procedure appeared most promising both with artificially challenged and naturally contaminated eggs. The use of a temperature differential in the latter procedure ensured that disinfectant (formalin) was taken up through the small number of open shell pores which provided portals of entry for contaminating bacteria.

Eggs subsequently challenged by dipping in large concentrations of bacteria or administering the inoculum into the outer thin albumen, were substantially protected against salmonella infection. This was not true when the test strain was delivered close to the yolk surface. Salmonellae, however, can generally be regarded as opportunist shell contaminants which only subsequently invade the outer egg structures and proliferate within the egg contents. A suitable treatment applied before extensive bacterial multiplication had taken place in the shell membranes should therefore be effective in substantially reducing the egg infection rate.

The thermostabilisation procedure described by Funk (50) involved the heating of chicken eggs to high temperatures for long periods of time and resulted in the impairment or destruction of the blastodisc. This effect was considered beneficial for eggs destined ultimately for human consumption. The heating procedures described here were intended for use on turkey hatching eggs. If such a procedure were to be of value in controlling egg transmitted infection, any deleterious effects on hatchability must be minimised. Different types of eggs may require different heating conditions. This process however did not arrest embryonic development as much as HTD formalin dipping. Formalin, at high concentrations, was embryo-toxic.

The results obtained in carefully controlled laboratory trials might not have been reproduced under field conditions where many other factors must also be considered. In particular, these relate to the natural level of infection, the influence of the environment on the time required for an organism to penetrate the shell, possible protective effects of organic contaminating material on the shell surface and other management factors, e.g. the length of time the egg remains in contact with the contaminated environment. Naturally

contaminated eggs were treated in various ways and the egg moulding technique (Board and Board (13)) was used to assess the ability of selected treatment methods to prevent bacterial penetration of the shell. Soiled, washed floor eggs were frequently penetrated by coliform bacteria which could be cultured from the sites of dye reduction on the inner shell membrane. By determining the number of eggs penetrated by coliforms or by actually counting the number of discrete penetration spots, an assessment could be made of treatment efficacy.

It was interesting to compare the observed incidence of bacterial contamination using the egg moulding technique with results obtained during a microbiological survey of the egg contents of incubated eggs. Bacteria were isolated from the embryonic fluids of 10.8% of eggs that failed to hatch, a figure similar to that reported by Bruce and Johnson (31). Only 3.4% of non-sanitised eggs incubated for 24 days were infected. Gram negative species, in particular E. coli, formed a significant part of the microflora of incubated eggs, whereas Gram positive bacteria, principally micrococci, predominated on the shell surface. The latter organisms were rarely isolated from within the membranes underlying the shell, although they were often detected beneath the shell. Such a result would tend to support the contention of Seviour and Board (135) who suggested that Gram negative bacteria were selected during the phase of the infection process which occurred when organisms were confined to the shell membranes. This situation would most likely arise because the bacterial growth in the membranes was limited by the presence of inhibitory factors in the albumen (18).

Bruce and Johnson (31) suggested that bacterial contamination was not normally a major factor in determining whether a chick or poult hatched successfully. This view is supported by the present findings. Some micro-organisms are however capable of killing embryos. The presence of opportunist pathogens, rather than those of known pathogenic significance, in close proximity to the shell membranes at hatch time could ensure that poults were exposed to infection prior to or during emergence from the shell. Indeed Schalm (131) proposed a similar mechanism for chicks becoming infected with salmonellae. There would appear no reason to differentiate this group of organisms from a number of other potential pathogens and this in part explains the use of coliforms as indicator organisms to assess the potential value of the egg treatment methods. If this argument is followed it must be debated whether there is a cardinal requirement for potentially pathogenic micro-organisms to establish successfully and to multiply to large populations in the contents of a developing egg. Indeed such an event would most likely result in the appearance of macroscopic changes within the egg (e.g. the formation of rots) or in the death of the embryo. With regard to the vertical transmission of infectious agents, the possibility exists that the outer egg structures provide an important focus of infection for the developing embryo. Some organisms both establish and multiply within incubating eggs and poults from such eggs often hatch despite the presence of large numbers of bacteria. Some salmonellas, other than the non-motile species—specific serotypes, probably behave in this manner.

The egg moulding technique proved a valuable tool for assessing the effects of external shell treatments on bacterial penetration. Cracked eggs and soiled, washed eggs were often contaminated. Such results confirmed the importance to the eggs' natural defence system of the cuticle (by preventing flooding of the shell pores) and the intact

shell as previously discussed. Because each visible dye spot represents the site where a bacterium has traversed the shell, it is possible that the technique may find other useful applications. In particular these could relate to the effect of environmental conditions (temperature and humidity) on the rate and extent of bacterial invasion.

During the course of this study, it was noted that some bacteria failed to infect eggs after artificial challenge. Bacteria could not be isolated successfully from the egg contents after prolonged incubation, although shell penetration was observed in most cases. The inhibition was overcome when the infecting solution was supplemented with iron or simultaneously seeded with an inoculum of S. hadar. This suggested that the antimicrobial properties of albumen were responsible for preventing bacterial growth.

A range of organisms, typical egg contaminants, were inoculated into turkey egg albumen in vitro. Several isolates established and multiplied in alkaline albumen (pH 9.1) at 37°C whereas others did not. A good correlation was observed between the ability of strains to resist the antimicrobial properties of albumen and their ability to successfully infect eggs.

Chicken egg albumen inhibited the growth of Gram positive and negative bacteria under similar incubation conditions (147) although several hatchery-isolated strains appeared better able to resist the inimical growth conditions. A strain of E. coli (E. coli 0141) used in the above study was not killed in turkey albumen and formed a significant population. Further study would be required however, to determine whether there are differences between the antimicrobial properties of albumen from the eggs of chickens and turkeys, bearing in mind that only a limited number of strains have been examined so far.

As previously discussed, the chemical defence offered by the albumen against bacterial infection is presently thought to be non-specific and due to the interplay of conalbumin, pH, incubation temperature and possibly the lack of an adequate supply of nitrogenous substances. The present observation therefore that a serotype of salmonella, S. ohio, behaved very differently in albumen obtained from different sources was extremely interesting and suggested the possibility of a specific element being present in egg white, e.g. locally secreted immunoglobulins (Rose and Orlans (122)).

Whilst obvious differences existed between the growth of bacteria in turkey albumen, such differences may be of secondary importance compared to their ability to colonise the shell membranes. Brooks (25) implied that nearly all the bacteria that accumulated in egg white were simply invaders from the shell membranes. Garibaldi (56) also suggested that some primary egg spoilage pathogens, after colonising the shell membranes, could synthesise and excrete iron transport compounds which would facilitate their growth within the albumen. Although Tranter (147) found no evidence that phenolate siderophores were synthesised by bacteria inoculated directly into hen albumen, it could prove valuable to establish whether the extent of bacterial multiplication within the membranes might influence the subsequent course of infection.

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