APPLIED MICROBIOLOGY, May, 1965
Vol. 13, No. 3


# Influence of Temperature on Bacterial Infection of the Hen's Egg[1]

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Received for publication 16 December 1964

ABSTRACT

BOARD, R. G. (Iowa State University, Ames), AND J. C. AYRES. Influence of temperature on bacterial infection of the hen's egg. Appl. Microbiol. **13**:358–364. 1965.—Temperature of incubation had a marked effect on infection of eggs in which the air cells had been inoculated with a washed suspension of *Serratia marcescens*. There was no evidence of bacterial multiplication or spoilage in eggs held at 10 C for 42 days. Multiplication occurred in the shell membranes of eggs held at 30 or at 37 C when the yolk made contact with these membranes, and continued in the contents of the egg, at which time the first signs of spoilage appeared. In a few eggs, very large populations were present in the shell membranes and in the albumen. In eggs inoculated with *Pseudomonas fluorescens* and held at 10 C, bacterial multiplication occurred in the shell membranes in the first 7 days of incubation. These populations did not appear to change in size in the 7- to 14-day period of incubation. Renewed multiplication and concomitant spoilage of the contents was observed in many of the eggs thereafter.

When eggs are held in the temperature range of 15 to 30 C, there is a lag of 10 to 20 days after bacterial penetration of the shell and before the recovery of appreciable numbers of viable organisms from or the development of macroscopic changes in the contents (Zagaevsky and Lutikova, 1944; Gillespie and Scott, 1950; Bigland and Papas, 1953; Stokes, Osborne, and Bayne, 1956; Orel, 1959; Fromm and Monroe, 1960; Garibaldi and Bayne, 1960). Recent observations (Brooks, 1960; Board, 1964) indicate that, because of the antimicrobial defense of the albumen, bacterial multiplication is confined to the shell membranes during this period and that this confinement persists until the yolk makes contact with the shell membranes. The work to be described was undertaken with the objective of determining whether or not the temperature of incubation influenced the duration of this lag.

## MATERIAL AND METHODS

*Organisms.* *Serratia marcescens* 2G12 was used in the main part of this work, but five strains of *Pseudomonas fluorescens* were used for comparative purposes. The last-mentioned organisms had been isolated from rotten eggs or chicken carcasses. Stock cultures in nutrient broth (Difco) were stored in a domestic refrigerator at 4 C, and, for experimental purposes, an organism was subcultured on three occasions immediately before use. All cultures were grown in nutrient broth at 30 C, and after 18 hr the cells were harvested by centrifugation, twice washed in 0.067 M Sørensen's phosphate buffer (*p*H 7.2), and resuspended finally in distilled water.

*Eggs.* Eggs were produced by an unmated flock of White Leghorns and stored at 10 C for 4 days prior to inoculation. All eggs were candled, and those of poor internal quality were not used. The eggs weighed between 50 and 60 g.

*Inoculating and sampling.* The method has been fully described elsewhere (Board, 1964). Briefly, 0.1 ml of an inoculum containing about $10^4$ organisms per milliliter was injected into the air cell of eggs, and the latter, with the air cell either uppermost or downwards, were incubated at 10, 30, or 37 C. At frequent intervals, five eggs were randomly selected, and the numbers of viable organisms were determined (i) in the inner membrane of the air cell, by placing 0.02 ml of dilutions prepared from the comminuted membrane on nutrient agar, and (ii) in the albumen, by preparing nutrient agar pour plates containing 1 ml of appropriate dilutions.

## RESULTS

In preliminary experiments, attention was given to the influence of temperature and media on the growth of *S. marcescens* 2G12. Good growth occurred on slopes of nutrient agar (Difco) incubated at 10, 30, or 37 C. The results

[1] Journal Paper No. J-5007 of the Iowa Agricultural and Home Economics Experiment Station, Ames. Project No. 1392, Center for Agricultural and Economic Development cooperating.

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obtained with albumen or a mixture of yolk (1 volume) and albumen (1 volume) are summarized in Fig. 1. Extensive bacterial multiplication took place in the mixture during 48 hr of incubation at all three temperatures. With albumen alone, there was a notable reduction in the number of viable organisms during incubation at 10 or 37 C but not at 30 C. The multiplication which occurred in the latter instance was negligible when compared with that in the mixture. Results similar to these were obtained by Ayres and Taylor (1956), who studied bacterial growth in both the intact and the broken-out egg. It is pertinent, also, to note the results of Sharp and Whitaker (1927). These workers observed a rapid decline in the number of viable organisms in albumen inoculated with young broth cultures of gram-negative bacteria incubated at 37 C. This decline did not occur when thermally denatured albumen adjusted to *p*H 9.4 was used. This evidence indicated that the ability of gram-negative bacteria to survive in an unfavorable environment of egg albumen may be influenced by temperature.

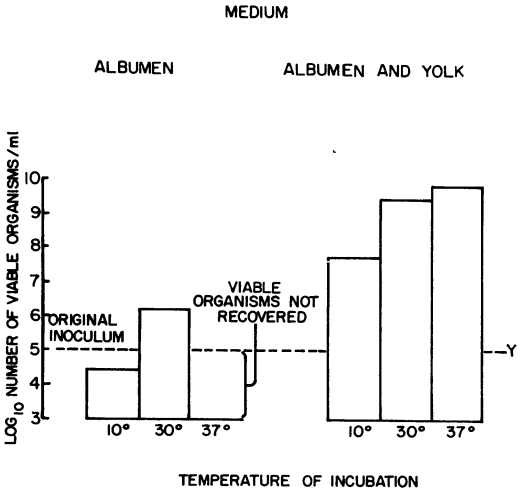


FIG. 1. *Influence of temperature on the growth during 48 hr of Serratia marcescens 2G12 in albumen alone and a mixture of yolk (one volume) and albumen (one volume). Viable organisms not recovered at 37 C; y, size of the original inoculum.*

The changes occurring in the population of *S. marcescens* in the inner membrane of the air cell of eggs held, with their air cells uppermost, at 10, 30, or 37 C are summarized in Fig. 2. The number of viable organisms in the inner membrane of eggs held in a refrigerated (10 C) display cabinet did not change appreciably during the 42-day period of observation. Viable organisms were rarely recovered from 1 ml of albumen, and the eggs did not manifest signs of spoilage.

A different sequence of events was obtained with eggs held at 30 C in a bacteriological incubator. A slight increase in the number of viable organisms was noted in the 5 days after inoculation, and very large populations were present both in the shell membranes and the albumen on and after the 10th day of incubation (i.e., when the eggs were 14 days of age). This gross contamination was observed at a time when it was noted that the yolk was making contact with the inoculated inner membrane of the air cell. Evi-

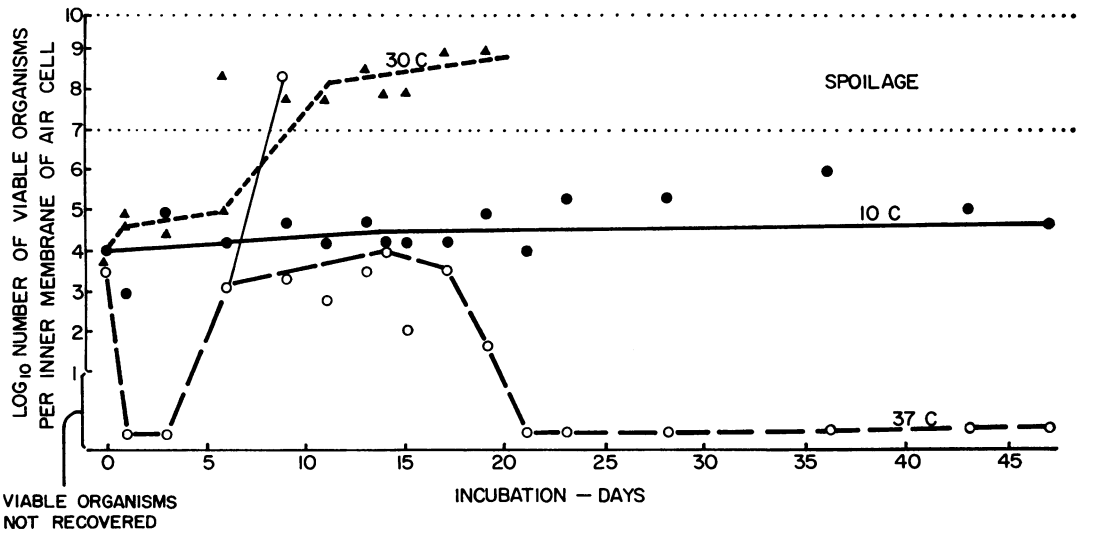


FIG. 2. *Changes in the size of the populations of Serratia marcescens 2G12 in the inner membrane of the air cell of eggs held at 10, 30, or 37 C. Each point is the average obtained from five eggs.*

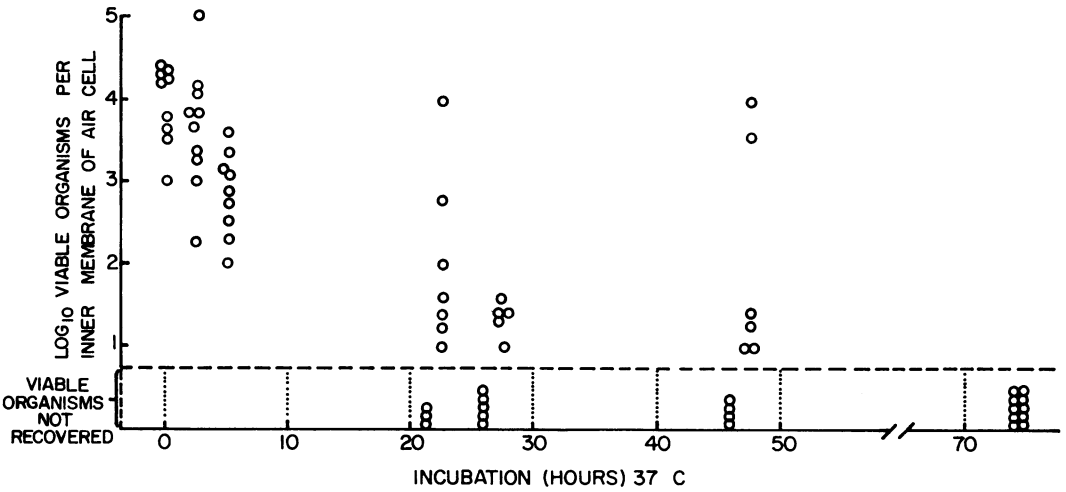


FIG. 3. *Changes in the populations of Serratia marcescens in the inner membrane of the air cell of eggs held at 37 C.*

dence of this union was obtained during candling of the eggs as well as from inspection of the broken-out eggs. In the latter instance, the pigment produced by *S. marcescens* was present on the inner membrane of the air cell and on the surface of the yolk at the place of their union. On further incubation, the pigment became disseminated throughout the white and over the entire surface of the yolk. This sequence of events is essentially the same as those described elsewhere (Brooks, 1960; Board, 1964), and it was concluded that a secondary phase of bacterial multiplication, together with manifestations of spoilage, was induced by the union of the yolk and the inoculated shell membranes.

Yet another sequence of events was observed in eggs held at 37 C. There was a sharp decline (Fig. 2) in the number of viable organisms in the inner membrane of the air cell in the 2 days following inoculation. The data summarized in Fig. 3 and 4 indicate that this was due to migration of organisms from the membranes and the death of the migrants in the albumen. Thus, on the 2nd day after inoculation, it was not possible to recover viable organisms from either the shell membranes or the contents of the majority of eggs, and this situation prevailed until the 5th to 7th day of incubation. At this time, the yolk made contact with the inoculated membrane of the air cell, and this was associated with the recovery of viable organisms from the shell membranes and, to a lesser extent, from the albumen. In exceptional instances, populations of a million or more viable organisms were found in the inner membranes of the air cell as well as in 1 ml of the albumen, and the contents of the eggs were stained throughout with pigment produced

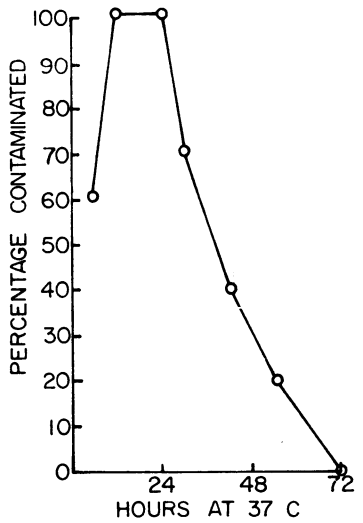


FIG. 4. *Incidence of contamination of the albumen of eggs, incubated at 37 C, in which the air cell had been inoculated with Serratia marcescens. Ten eggs were examined on each occasion.*

by *S. marcescens*. In the majority of eggs, however, populations in the inner membrane of the air cell reached a size of 100 to 10,000 viable organisms, but very few organisms were recovered from the albumen. This level of contamination was a regular feature of all the eggs examined after 1 to 3 weeks after inoculation, yet none of the eggs exhibited signs of spoilage. After this period, viable organisms were not recovered from the shell membranes or from the albumen.

These results confirm observations of Wolk,

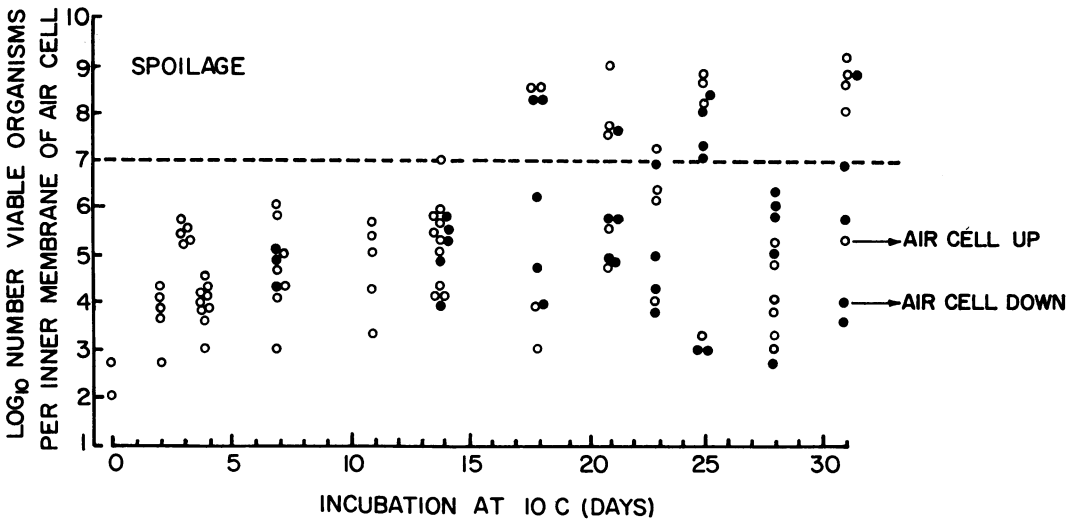


FIG. 5. *Changes in the size of the populations of Pseudomonas fluorescens No. 8 in the inner membrane of the air cell of eggs held at 10 C. Results obtained from experiments 11 and 12 have been combined.*

McNally, and Spicknall (1950) and of Ayres and Taylor (1956) that temperature influences the course of bacterial infection of the hen's egg. Further, the effect of temperature has been noted in eggs infected with *Aeromonas liquefaciens* (Miles and Halnan, 1937), *Proteus vulgaris* (Board, 1962), and other common contaminants of rotten eggs (Ayres and Taylor, 1956; Board et al., 1964). The results obtained with eggs held at 10 C are of particular importance because of the widespread use of refrigerated display cabinets by retailers of eggs. It was for this reason that several strains of *Pseudomonas fluorescens* were used in an extensive investigation of the course of infection in eggs stored at this temperature.

The results given in Fig. 5 and Table 1 were obtained with *P. fluorescens* No. 8, but essentially the same results were given by four other strains of this species. The eggs were inoculated with 100 to 1,000 organisms, and it will be seen from Fig. 5 that the number of viable organisms in the inner membrane of the air cells of the majority of eggs had increased to $10^4$ to $10^6$ by the 7th day of incubation and that the albumen of many of the eggs (Table 1) contained viable organisms. This level of contamination was found in eggs examined on the 14th day after inoculation with but one exception. In the latter instance, the membranes of the air cell contained $10^7$ viable organisms, and the albumen, which was colored with the pigment produced by *P. fluorescens*, had $10^9$ viable organisms per milliliter. The incidence of spoiled eggs increased during further incubation, but, at the same time, a decline in the levels of contamination was noted in eggs which did not manifest signs of infection. These

TABLE 1. *Recovery of viable organisms from the albumen of eggs in which the air cells had been inoculated with Pseudomonas fluorescens No. 8**

| Eggs held at 10 C | Eggs held with their air cells | $Log_{10}$ number of viable organisms recovered from 1 ml of albumen of egg no. | | | | |
|---|---|---|---|---|---|---|
| | | 1† | 2 | 3 | 4 | 5 |
| *days* | | | | | | |
| 2 | Uppermost | − | + | + | − | − |
| 3 | Uppermost | − | − | − | + | + |
| 4 | Uppermost | + | + | + | − | + |
| 7 | Uppermost | + | + | − | NT | NT |
| | Downwards | + | + | + | NT | NT |
| 14 | Uppermost | + | + | − | 9.9‡ | − |
| | Downwards | − | − | − | − | − |
| 21 | Uppermost | 7.8‡ | 7.2‡ | 7.2‡ | 3.4 | 1.4 |
| | Downwards | 8.6‡ | − | − | − | 8.2‡ |
| 28 | Uppermost | − | − | + | + | + |
| | Downwards | + | + | − | − | − |
| 31 | Uppermost | 2.7‡ | −‡ | 3.3‡ | 8.2‡ | − |
| | Downwards | 6.9‡ | 2.9 | 2.5 | 3.5‡ | 8.5‡ |

* These results were obtained from experiment no. 11 (of Fig. 5).

† Symbols: + = fewer than 30 organisms present in 1 ml of albumen; − = no viable organisms recovered; NT = not tested.

‡ The pigment produced by the organism was present in the inner membrane of the air cell or albumen.

trends were found in eggs held with their air cells uppermost as well as in those held in the reverse position. No explanation can be offered to account for the development of large microbial populations and symptoms of spoilage. It has been reported (Board, 1964) that the development of gross contamination and spoilage of the contents

of eggs held at 27 C was due to a union of the yolk and the shell membranes; essentially the same phenomenon (Fig. 2) was noted in eggs inoculated with *S. marcescens* and held at 30 or 37 C. In eggs inoculated with *P. fluorescens* and held at 10 C, there was no evidence of a union of the yolk and shell membranes. The only feature of the results in marked contrast to those obtained with *S. marcescens* or those reported elsewhere (Board, 1964) was the early and persistent contamination of the albumen of the majority of eggs.

## DISCUSSION

The results give additional support to the concept (Gillespie and Scott, 1950) that there are two distinct phases in the course of infection after bacterial penetration of the shell of the hen's egg. During the first phase, bacterial multiplication of a limited extent is confined to the shell membranes, and migrants from this source do not become established in the albumen (Brooks, 1960; Board, 1964). This confinement can last for 10 to 20 days in eggs held at 15 to 30 C (Zagaevsky and Lutikova, 1944; Gillespie and Scott, 1950; Bigland and Papas, 1953; Stokes, Osborne, and Bayne, 1956; Orel, 1959; Fromm and Monroe, 1960; Garibaldi and Bayne, 1960), provided that iron has not been included with the inoculum (Brant and Starr, 1962; Garibaldi and Bayne, 1962*a*, *b*). The confinement is terminated when the yolk makes contact with the shell membranes; this induces a second phase of bacterial multiplication in the contents of the eggs, and, when chromogenic and proteolytic organisms are present, the first signs of spoilage appear (Board, 1964).

In view of the evidence obtained in the present investigation and that presented elsewhere (Sharp and Whitaker, 1927; Ayres and Taylor, 1956; Garibaldi, 1960), temperature appears to influence the pattern just described in three ways. It influences (i) the rate of bacterial multiplication during the phase in which this is confined to the shell membranes, (ii) the coordinated workings of the various components of the antimicrobial defense of the albumen, and (iii) the onset of the secondary phase of bacterial multiplication by virtue of the effect it has on the rate of deterioration of the internal quality of the egg.

Although it has been shown repeatedly that common contaminants of rotten eggs can multiply in a mineral salts solution containing shell membranes (Stuart and McNally, 1943; Stokes and Osborne, 1956; Elliott and Brant, 1957; Garibaldi and Stokes, 1958), recent observations (Board, J. Appl. Bacteriol, *in press*) suggest that the membranes are not a rich source of readily available bacterial nutrients. This view has received support from the observations (Board, 1964) that the rate and extent of bacterial multiplication in the shell membranes in situ is influenced greatly by the nature of the substances present in the extraneous materials deposited on the membranes along with organisms. The present investigation has shown that the behavior of microorganisms on the shell membranes in situ is influenced also by temperature and by inherent properties of an organism. *P. fluorescens*, well known for its ability to grow at low temperatures, multiplied in the membranes of eggs held at 10 C, whereas *S. marcescens* did not, even though it had been demonstrated (Fig. 1) that the strain used in the present investigation would grow in a yolk-albumen mixture at this temperature. This is perhaps another example of the well known phenomenon of one condition (i.e., the lack of nutrients in the membranes) assuming importance when another (temperature) is approaching the limits of tolerance of an organism.

The antimicrobial defense of the albumen has long been recognized, and many components have been described, mainly as the result of investigations not directly concerned with the microbiology of eggs. The following unsatisfactory features can be listed when the albumen is considered as a medium for microbial growth: the lytic and flocculating action of lysozyme (Laschtschenko, 1909; Salton, 1957), the alkaline reaction (*p*H 9.6) of the albumen (Healy and Peter, 1925; Sharp and Whitaker, 1927), the antitryptic activity of ovomucoid (Balls and Swenson, 1934; Lineweaver and Murray, 1947), the combination of biotin with avidin (Eakin, Snell, and Williams, 1940), the combination of riboflavine with an uncharacterized protein (Rhodes, Bennett, and Feeney, 1959), the chelation of iron by conalbumin (Schade and Caroline, 1944; Feeney and Nagy, 1952; Garibaldi, 1960), the low content of nonprotein nitrogen (Haines, 1939), and the contributing inhibitions of other factors (Matsushima, 1958; Rhodes, Bennett, and Feeney, 1960). Such an interpretation as this has received wide acceptance by egg microbiologists, although its implications have not been tested experimentally except in the case of conalbumin and its role in retarding the manifestations of spoilage in eggs infected with certain common contaminants of rotten eggs (Garibaldi, 1960; Garibaldi and Bayne, 1962*a*, *b*).

As yet, however, attention has not been given to the influence of temperature on the coordinated workings of the factors just listed, although there is evidence of a general nature (Sharp and

Whitaker, 1927; Ayres and Taylor, 1956) which suggests that it may play an important role. This supposition is supported by the results obtained in the present investigation. It would appear that this facet of egg microbiology is worthy of further exploration, and it is to be hoped that such investigation would link the interests of those who are concerned with the egg as an item of food with those who are interested in the immunity of the chicken embryo during early development.

Much is known concerning the deleterious effects of high storage temperatures on the internal quality of eggs (Wesley and Stadelman, 1959), but only recently has it been suggested (Board, 1964) that there is an interrelationship between the rate of breakdown of the albuminous sac and the rate of development of macroscopic changes in eggs infected with chromogenic or proteolytic bacteria. Further evidence of this relationship was obtained in the present investigation. The onset of spoilage in eggs held at 30 and 37 C coincided with the union of the yolk and the shell membranes; this occurred on the 5th to 7th day in eggs held at 37 C, but not until the 10th to 15th day in eggs held at 30 C.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant EF-00113-09 from the Division of Environmental Engineering and Food Protection.

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