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Effect of Temperature on Microbial Proliferation in Shell Eggs[1]

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There are numerous reports in the literature which concern the microbiological spoilage of shell eggs. Serious losses result from spoilage of eggs in marketing channels. In addition, the suspected presence of *Salmonella* and other pathogens has caused concern. It has been demonstrated repeatedly (Haines and Moran, 1940; Gillespie *et al.*, 1950; Lorenz *et al.*, 1952; McNally, 1952; Forsythe *et al.*, 1953) that spoilage bacteria and other microorganisms may gain entrance to the egg content as a result of washing. While the possibility exists that certain bacteria may be deposited within the shell before laying, unless the bird is diseased this source of spoilage is of less consequence than are environmental factors after the egg has been laid. Many types of rots have been observed in intact-shell eggs. Various organisms have been incriminated as causal agents for colorless-, green-, pink-, black-, red-, and mixed-rots (Alford *et al.*, 1950). Among the organisms ordinarily associated with these types of spoilage are *Pseudomonas fluorescens*, *Proteus vulgaris*, and *Serratia marcescens*.

One of the most commonly encountered species of pathogens associated with the shell egg is *Salmonella pullorum*. This organism not only has been identified as the causal agent for white diarrhea of chicks, but has been associated with ovarian infection of pullets and salmonellosis of poultry.

The work reported in this paper was undertaken to determine if the organisms (a) most commonly associated with spoilage of eggs or (b) responsible for disease of chickens would become established and proliferate in the albumen or yolk of eggs that were adequately refrigerated. Organisms used for inoculation were *S. marcescens*, *P. vulgaris*, *P. fluorescens*, and *S. pullorum*. The first three species were chosen because of their prominent role in egg spoilage, while the last was selected because of its significance as a pathogen of poultry.

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EXPERIMENTAL METHODS

Large, clean, unwashed eggs (62 to 65 g) secured from the Iowa State College Poultry Farm within 24 hr after laying were used in these experiments. Ordinarily, they were used immediately. When it was necessary to hold the eggs for a few hr, they were kept at 4.4 C until they could be tested.

Eggs that were to be broken-out were washed manually. Each egg was held lightly by means of sterile rubber gloves or through the use of sterile tongs. The tongs were prepared from 2-foot pieces of ⅛-inch galvanized wire bent sharply in the middle, with a ring at each end into which the egg could be partially inserted. All washing and rinsing solutions were maintained at 43 C to assure that the content of the egg would expand rather than contract. Individual eggs were soaked in 0.5 per cent Kleneg[2] for 2 to 3 minutes and brushed lightly to remove dirt particles. The eggs were then rinsed with sterile distilled water and placed in a 1:1000 Roccal[3] solution for 10 minutes. Excess sanitizing agent was washed from the shell with sterile distilled water, and the egg was dipped in a 95 per cent ethanol solution for 1 minute. After removal from the alcohol, the egg was drained and the residue ignited in order to leave a dry surface. The final flaming of the shell after the free alcohol had drained did not result in any noticeable coagulation of the egg albumen adjacent to the shell. Approximately 90 per cent of the shells sterilized by this procedure were found to be bacteriologically sterile. This method was considered more satisfactory than any of the procedures previously described in the literature (Rettger, 1913; *Standard*

[2] Kleneg—primarily a compound of tetrasodium pyrophosphate, sodium carbonate, trisodium phosphate, polyethylene glycol esters of oleic acid, and trimethyloctadecenyl and trimethyloctadecadienyl ammonium chlorides; manufactured by Armour and Company, Chicago, Ill.

[3] Roccal—primarily a mixture of high molecular weight alkyldimethylbenzyl ammonium chlorides; manufactured by Winthrop-Stearns, Inc., New York, N. Y.

Methods for the Examination of Dairy Products, A.P.H.A., 1948; Gillespie and Scott, 1950).

In earlier experiments, 70 per cent ethanol (by weight) was applied. Price (1939) indicated this concentration to be more germicidal than other amounts of alcohol, but the procedure was abandoned when it was found that such solutions left a moisture film on the shell after the alcohol had been ignited and that any alcohol method, when used without further treatment, failed to yield a sterile shell.

The egg content was removed by a procedure similar to that described by Wolk *et al.* (1950) in which a portion of the shell in the small end of the egg was removed with a sterile forceps and the broken edge of the shell flamed. The egg was then inverted over a sterile beaker and the contents forced out by applying a flame to the air-cell end of the egg. Using aseptic precautions, prepared dilutions of bacterial inocula (as described below) were introduced into the albumen or the yolk of the egg, and the beakers were tightly covered by molding a sheet of sterile aluminum foil over the opening. Samples were stored at 2, 10, and 20 C and analyzed for bacterial flora after 0, 2, 4, 7, 14, 30, and 60 days of storage. In early trials, the lowest storage temperature selected was 0 C, but the 2- to 3-degree fluctuation in the storage cabinet resulted in alternately freezing and thawing part of the albumen. This difficulty was not experienced when a temperature of 2 C was adopted.

For eggs inoculated in the shell, the following procedure was used: The large end of the egg was successively swabbed with Kleneg, sterile water, Roccal solution, and sterile water. After this treatment the shell was protected by covering with a sterile towel and permitted to dry. In order to facilitate inoculation with a hypodermic needle, a hole was drilled through the sterilized portion of the shell using a sterile $\frac{1}{16}''$ bit.

Organisms used for inoculation were *S. marcescens* (ISC 2G3), *P. vulgaris* (ATCC 9920), *P. fluorescens* (NRRL B10), and *S. pullorum* (WURB 3083). To assure that growth would be uniform, each of the species was grown on nutrient agar slants at the optimum temperature for that organism and was transferred daily for 3 successive days before being employed in tests. Since the organisms grew at different rates, cultures of *P. vulgaris* and *P. fluorescens* were used for tests after an incubation period varying from 12 to 14 hr, while *S. marcescens* and *S. pullorum* were used at ages of 14 to 18 hr. The cultures were washed from the slant with sterile Ringer solution. The number of cells of each organism to be inoculated in the egg was determined by direct microscopic count, using a Neubauer counting chamber, and tabulating the bacteria observed in 80 small squares of the slide. These suspensions were diluted to give three levels of inocula approximating 100, 10,000 or 1,000,000 cells. In addition, the inoculum was plated out in nutrient agar fortified with 1 per cent glucose and 0.3 per cent yeast extract to determine the number of viable cells introduced into the egg. In table 1 are shown the comparative numbers of cells as determined by the direct microscopic method and the viable plate count. While duplicate results were not obtained by the two methods, the numbers determined by the direct microscopic count were considered sufficiently precise to provide an approximate estimate of the inoculum.

TABLE 1. *Comparison of numbers of cells as determined by the direct microscopic and agar plate methods*

Organism	Age of cultures (hr)	Number Estimated by	
		Direct microscopic method	Agar plate
Pseudomonas fluorescens	12	100	73
	14	100	101
Proteus vulgaris	13	100	103
	18	100	78
Serratia marcescens	12	100	31
	18	100	70
Salmonella pullorum	12	100	50
	14	100	85

After addition of the inoculum, the small hole made by drilling was sealed with sterile tackiwax and then the egg was placed in storage. The eggs were analyzed at 0, 2, 4, 7, 14, 30, and 60 days of storage by placing the broken-out eggs in a sterile 1 L Waring blendor jar with 200 ml of sterile distilled water and blending for 1 min at a speed provided by setting a Varitran transformer at very low speed (30 to 40 rpm).

Aliquots of this egg-water mixture were plated in fortified nutrient agar and incubated for 3 or 4 days at the optimum temperature for the organism. The experiment was set up in triplicate in order to provide replicates for storage at 2, 10, and 20 C. Each experiment was repeated several times to insure that the results were typical.

Each egg was examined for visible change in color, fluorescence, or turbidity. Observation was made of both white and yolk to determine if the bacterial inoculum had remained localized or had infected all portions of the egg content. Also, a smell test was used to detect off-odor.

RESULTS AND DISCUSSION

At 20 C, eggs inoculated in the yolk spoiled quickly. As may be seen in figure 1, populations of all four of the test organisms exceeded a billion bacteria per egg by the end of the fourth day. Contaminative loads reached levels of approximately 40 to 80 billion per

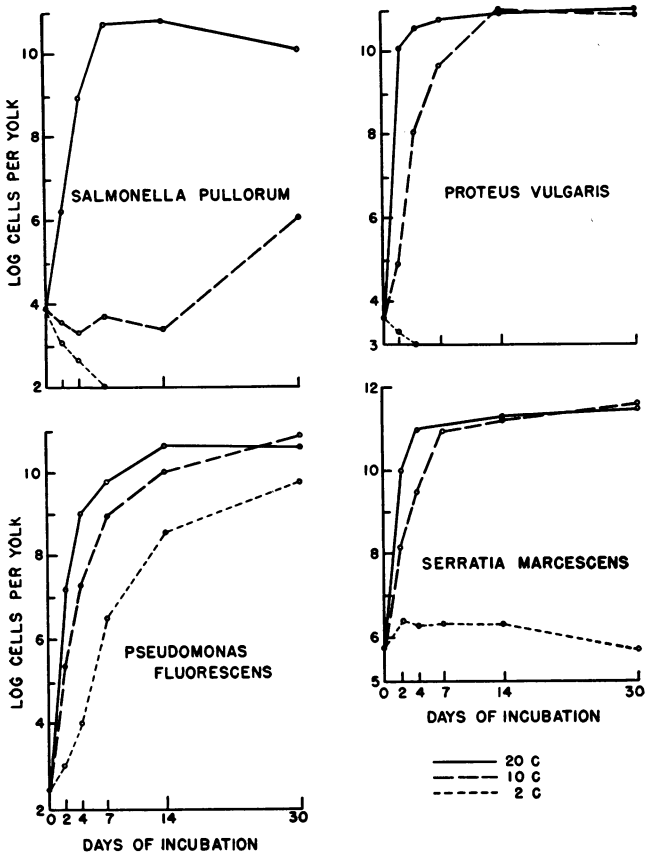

FIG. 1. Relation of storage temperature to number of cells of 4 species of bacteria when inoculated in the yolk of broken-out eggs.

egg for *P. fluorescens*, *S. pullorum*, and *P. vulgaris;* for *S. marcescens*, 250 billion cells were estimated. Numbers of cells of *S. pullorum* did not increase during the first 2 weeks of storage at 10 C, but after 30 days at that temperature had multiplied over one hundred times. At 2 C, populations of *P. vulgaris* and *S. pullorum* decreased; no viable cells of either of these organisms were recovered for 1 and 2 weeks respectively. On the other hand, *P. fluorescens* grew well at 2 C, and at the end of 60 days the populations at 2, 10, and 20 C were estimated to be 38, 76, and 33 billion cells per egg respectively. Numbers of *S. marcescens* did not increase at 2 C, but even at the end of 60 days 4 per cent (22,000) of the original inoculum remained viable.

Numbers of *S. marcescens* and *P. vulgaris* increased rapidly in the albumen of shell eggs and in broken-out egg albumen held at 20 C; *P. fluorescens* and *S. pullorum* populations also increased, but much more slowly (see figures 2, 3, and 4). Total counts for any of the four organisms at 2, 4, 7, 14, 30, or 60 days were much lower when the inocula were introduced in the albumen rather than in the yolk. Proliferation of *P. fluorescens* and *S. pullorum* in albumen was less than one thousandth that of cells inoculated in egg yolk. The difference was least pronounced with *S. marcescens;* with this

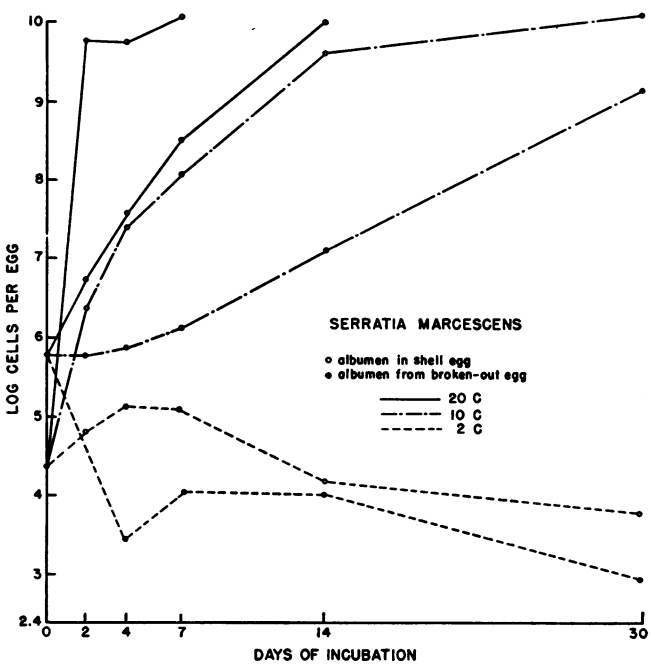

FIG. 2. Relation of storage temperature to proliferation of *Serratia marcescens* inoculated in the albumen of shell and broken-out egg.

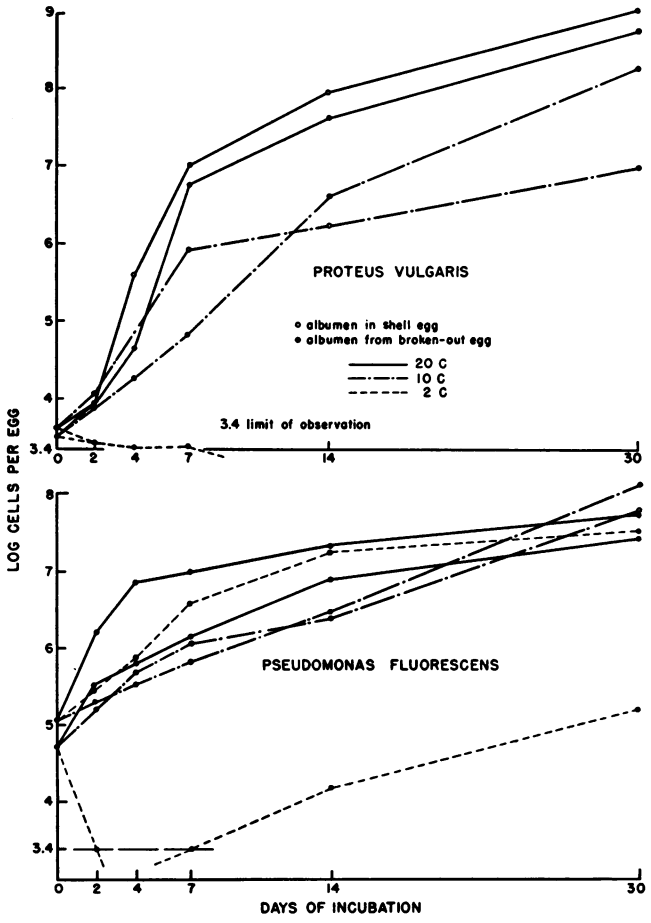

FIG. 3. Relation of storage temperature to proliferation of *Proteus vulgaris* and *Pseudomonas fluorescens* inoculated in the albumen of shell and broken-out egg.

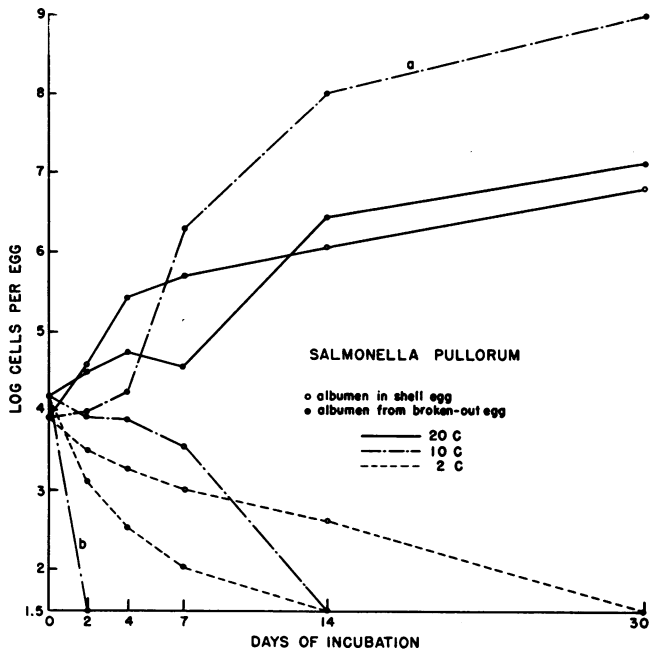

FIG. 4. Relation of storage temperature to proliferation of *Salmonella pullorum* inoculated in the albumen of shell and broken-out egg.

organism the egg was completely overgrown after 14 days at 20 C regardless of where the inoculant was placed.

At 10 C, *S. marcescens* completely overgrew albumen and yolk in the broken-out eggs in 30 days. However, organisms inoculated in the shell did not reach maximum populations at this storage time if the inoculum was placed in the egg albumen rather than in the yolk. No growth of *S. marcescens* at 2 C was observed; instead, some indication was given that the numbers were decreasing at the end of 60 days.

At 20 C, numbers of *P. fluorescens* were in excess of 2 billion per egg by the end of 60 days of storage, but development was much slower and less extensive than it was when inoculated into yolk. With the exception of inocula into the albumen of shell eggs, proliferation of this organism was not diminished at 10 or 2 C when compared to results at 20 C. Also, while there was an initial reduction in the numbers of *P. fluorescens*, inoculated into the shell eggs, the remaining viable cells reproduced at a rate comparable to that at 10 C.

P. vulgaris grew well at 20 or 10 C, but counts were lower in the albumen than in the yolk. Even by the end of 60 days, very little growth took place at 2 C.

Eggs inoculated with *S. pullorum* in the yolk and held at 20 C spoiled during storage, but there was no growth in eggs stored at 10 C or 2 C. At 10 C, growth was less than 1,000,000 organisms per egg at the end of 30 days. When this organism was used as an inoculum and the eggs stored at 2 C, many had less than 50 viable organisms at the end of 60 days' storage.

There is some controversy in the literature concerning the speed with which a bacterial rot develops after infection of the albumen has been established. Stuart and McNally (1943) reported that, in some eggs, *Pseudomonas aeruginosa* penetrated immediately into both albumen and yolk. The incidence of infection of the egg contents reached a maximum after 42 to 48 hr at 23 to 28 C. Gillespie and Scott (1950) considered that, while bacteria slowly attack the shell membrane, once infection of the albumen had been established, subsequent development of a bacterial rot took place rather quickly.

As may be seen in figures 3 and 4, when the albumen of the egg is inoculated, *P. vulgaris* and *P. fluorescens* require 4 to 7 days at 20 C before numbers of these organisms have reached a level that could be detected. With the exception of one lot of eggs, contamination by *S. pullorum* was undetectable by sensory evaluation, even after storage for one month.

It may be observed in figure 4 that one lot of eggs (a) underwent spoilage when stored at 10 C, while the other experiments at 10 C with albumen from broken-out eggs (b) failed to undergo spoilage within 60 days. It may be seen in figure 1 and figure 4 that the storage temperature of 10 C was a rather critical one insofar as *S. pullorum* is concerned. In one set of experiments (a), cells of this organism, after remaining in the initial lag phase for 4 days, reproduced to high numbers. Yolk as well as albumen of many of these eggs were heavily contaminated. In the other tests at 10 C, less than 30 cells of *S. pullorum* were recovered from the egg.

Several explanations may be offered for the more luxuriant growth of bacteria in egg yolk as contrasted to that in egg albumen. Egg albumen contains a number of factors, such as lysozyme, avidin, conalbumin, ovomucoid, ovomucin, and carbon dioxide, which have been shown to inhibit or delay the growth of certain microorganisms. Egg yolk contains most amino acids, vitamins, and other nutrients needed for growth of most microorganisms. Hurley and Ayres (1953) found that, when 10 per cent albumen was added to "¼-" strength Ringer solution, populations of *Escherichia coli*, *Proteus morganii*, or *Alcaligenes faecalis* decreased from that of the original inoculum; in the same type of substrate, *S. pullorum* and *P. aeruginosa* increased in number. On the other hand, when these same organisms were placed in ¼-strength Ringer solution to which 10 per cent whole egg was added, there was prolific growth of all five organisms. While *S. pullorum* and *P. aeruginosa* had multiplied to levels of 2 to 4 million cells per ml in the presence of 10 per cent albumen, from 30 to 200 million cells per ml grew in 18 hr at 37 C when 10 per cent whole egg was added to the ¼-strength Ringer solution.

Visual examination of the eggs failed to reveal evidence of incipient spoilage until the bacterial loads exceeded several hundred million cells per egg. With an

inoculum of only 250 viable cells of *P. fluorescens* into the yolk, fluorescence could be detected by black light[4] on the fourth day of storage at 20 C. The same phenomenon was observed at 7 and at 14 days when the holding temperatures for the egg was 10 and 2 C respectively. In a few instances, a light green to pink discoloration was observed at the same time without special lighting. Fluorescence became more pronounced after longer storage intervals. *P. fluorescens* is the principal cause of spoilage of cold-storage and of washed eggs, and its detection by the ultraviolet candler is of no small importance. However, as is shown in figures 1 and 3, if this species entered the egg proper, it would only be a matter of time until the entire egg becomes spoiled regardless of the refrigerated storage temperature used.

An offensive odor and a muddy grey-brown discoloration was discernible in 2 days in yolks inoculated with 4200 viable cells of *P. vulgaris* and held at 20 C; 7 days elapsed before similar changes were noted in eggs held at 10 C. Red-colored streaks appeared in 2 and 4 days in yolks inoculated with 560,000 viable *S. marcescens* cells and stored at 20 and 10 C respectively. Only a slight off-odor was noted after 7 days of incubation at 20 C in yolk of eggs inoculated with 7900 viable cells of *S. pullorum*. No discoloration or off-odor could be detected in 60 days in yolks of eggs incubated with *P. vulgaris* or *S. marcescens* and stored at 2 C; also, no visual evidence of spoilage was observed in yolks inoculated with *S. pullorum* when the egg product was held at 10 or at 2 C.

Discoloration was not observed at any of the three storage temperatures in egg albumen until the egg had been stored for at least 2 weeks. Ordinarily, from 30 to 60 days of storage were required before the albumen began to fluoresce. This was in marked contrast to the early development of fluorescence in the broken-out yolk. This defect was observed to occur as quickly in eggs stored at 2 C or 10 C as at 20 C. However, it developed in shorter time in the albumen of broken-out eggs than in intact eggs.

Summary

Attempts were made to determine if the spoilage bacteria, *Pseudomonas fluorescens*, *Proteus vulgaris*, and *Serratia marcescens*, and the poultry pathogen, *Salmonella pullorum*, reproduced in the yolk or albumen of broken-out eggs or in shell eggs incubated at 2, 10 or 20 C. The study revealed that only *P. fluorescens* grew at 2 C. Numbers of *S. marcescens* remained about the same at 2 C for 14 to 30 days when stored in yolk, but gradually decreased in albumen; loads of *S. pullorum* and *P. vulgaris* rapidly decreased in both products. All four organisms reproduced when stored in yolk at 10 or 20 C, but proliferation of *S. pullorum* cells was delayed for 14 days. When cells of *S. pullorum* were injected into albumen of broken-out eggs and stored at 10 C, the numbers of organisms decreased with one exception; in one set of experiments growth occurred.

With the exception of *S. marcescens*, the organisms did not become established quickly in egg albumen. Apparently, a number of factors inhibitory to growth prevent extensive growth in albumen, and may even destroy some of the invading bacteria so that the infection remains localized for 2 weeks or longer. When the cells reach the yolk, they reproduce rapidly.

[4] A Vogelite candling lamp (high pressure mercury vapor type) manufactured by Alf McNamee and Sons, Oakland, California was used.

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