Death of *Staphylococcus aureus* in Liquid Whole Egg Near pH 8

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Incubating and shaking *Staphylococcus aureus* in liquid whole egg causes a decline in viability. During the period of agitation, the natural pH of the egg rises from about 7.2 to between 8.0 and 8.2 as a result of a loss of carbon dioxide. However, if the pH of the egg is prevented from rising, either by not shaking or by addition of a buffer, *S. aureus* will grow. The cause of death is traced to the presence of lysozyme of egg white. Interestingly, the action of lysozyme is not attributable to its bacterial lytic property but, instead, to the basicity of the lysozyme molecule. This conclusion is supported by the fact that the lytic property of lysozyme is known to have its optimal activity near neutrality and by the finding that protamine sulfate, a nonenzymatic basic polypeptide, also caused death of *S. aureus* at pH 8.0 but not at 7.0. It was postulated that the rise in pH renders the bacterial cells more negatively charged, so that in the presence of positively charged molecules like lysozyme or protamine sulfate a complex is formed, agglutinating the cells.

The hen's egg, as laid, is well protected from microbial invasion by the physical barrier formed by the shell and its membrane. However, a considerable quantity of egg finds its way into commercial channels in the form of broken out liquid whole egg, which is mainly used in the institutional and baking trades. In this form, the egg is highly susceptible to infection by both pathogenic and nonpathogenic microorganisms. For this reason, the U.S. Department of Agriculture requires that all egg products be pasteurized to insure a *Salmonella*-free product. It is not known whether a process designed to eliminate *Salmonella* is sufficient to provide a product free of the more heat-resistant pathogen, *Staphylococcus aureus* (2, 17). Therefore, the ability of *Staphylococcus* to survive and grow in liquid whole egg should be ascertained. *Staphylococcus* is gram positive and would probably be lysed by lysozyme, one of the many substances in egg white inhibitory to bacterial growth (7). But, because lysozyme is capable of complexing with many substances (11, 15, 16), its lytic activity in liquid whole egg could conceivably be reduced. In addition, *S. aureus* exhibits varying degrees of susceptibility to lysis by lysozyme. Thompson and Khorazo (19) found that those strains of staphylococci which produce an orange pigment, ferment mannitol, and produce coagulase are more resistant to lysozyme than are strains which lack these characteristics.

In contrast to the abundant data on the microbiology of shell eggs (for the bacteria in and the mechanism of entry into shell eggs, see review by Board [3]), data on the ability of the various genera of bacteria to infect and grow in commercial liquid whole egg are less numerous. To our knowledge, the only study on the capability of *Staphylococcus* to grow in liquid whole egg is that of Gibbons et al. (8) who inoculated sterile egg meat with *Salmonella, Staphylococcus*, and *Streptococcus* and found that there is little danger of multiplication if liquid egg were kept at 45 F or under and for no longer than 12 h. During a study to provide additional data on the fate of *S. aureus* inoculated into liquid whole egg it was found that, after a lag of a few hours, some strains decreased dramatically in viable counts and then increased only after a period of more than 8 h. This report describes results of a study undertaken to explain this observation.

**MATERIALS AND METHODS**

**Cultures.** Two coagulase-positive strains of *S. aureus*, carried in our culture collection for more than 5 years as lyophilized preparations, were used in this study. Both strains were reisolated on *Staphylococcus* medium no. 110 (Difco). One strain, S30A, was
originally obtained from R. V. Stone of the Los Angeles Department of Health and the other strain, SG8A, from Cutter Laboratories, Berkeley, Calif. Mutant M6 was isolated after allowing strain S30A to incubate in shaken liquid whole egg for 24 h and streaking out on Chapman Stone medium (Difco). The *Salmonella typhimurium* TM-1 was also from our culture collection.

Media. *Staphylococcus* medium no. 110, Chapman Stone medium, yeast extract (YE) and nutrient broth were all products of Difco. Trypticase soy broth (TSB) and Trypticase soy agar (TSA) were purchased from Baltimore Biological Laboratories and, when supplemented with 2% YE, yielded TSB-YE and TSA-YE, respectively.

**Coagulase test.** The coagulase test was that described in directions furnished with coagulase plasma (Difco) using cells grown in brain heart infusion broth (Difco).

**Preparation of liquid whole egg and egg yolk.** Shells, eggs, less than 1 week old, were immersed in 70% (vol/vol) ethyl alcohol for about 15 min. The eggs were then removed, drained, and evaporated free of alcohol. They were cracked aseptically on a sterile breaking knife, and the contents were slowly blended in a sterile Waring blender for about a minute so that air was not excessively incorporated into the egg mix. The blended eggs were stored at −20 °C and thawed as needed. When separate yolks were required they were separated from freshly broken eggs and used immediately. The total viable count in aseptically broken eggs (determined using TSA-YE) was less than 10^3/ml and, even after incubation for 7 h at 34 °C, was only about 10^2/ml, at which time the count on Chapman Stone medium was negative.

**Preparation of inoculum.** Cultures were grown in 50 ml of TSB-YE in 250-ml Erlenmeyer flasks shaken at 34 °C overnight. The cells were harvested by centrifugation, washed twice, and resuspended in sterile demineralized distilled water. The resuspended cells were diluted to a population of about 50 × 10^4 cells/ml and 1 ml of this suspension was used to inoculate 50 ml of TSB-YE medium.

**Incubation of bacteria in liquid whole egg or egg yolk.** The inoculated egg was incubated at 34 °C ± 0.5 °C in an incubator-shaker (New Brunswick Scientific, New Brunswick, N.J.) operated at 150 to 200 rpm with a rotary motion describing a 1-inch (ca. 2.54 cm) circle. Unshaken egg was incubated in small (2 ounces [ca. 0.609 liters]) screw-capped prescription bottles filled to capacity (65 ml) to minimize pH change through loss of CO₂. Samples (1 ml) of egg were removed aseptically at periodic intervals for viable count and pH determinations. Iron, when specified, was added as FeSO₄·7H₂O to give a final concentration of 750 μg of Fe/ml, which is 10 times the amount needed to saturate the conalbumin in the egg. Lysozyme, conalbumin, ovalbumin, and water were added to egg yolk equivalent to that found in whole egg (14). Lysozyme was isolated according to Alderton and Fevold (1). Ovalbumin was purified as described by Kelwick and Cannan (9). The conalbumin was a diethylaminoethyl cellulose preparation of S. Man-deles (unpublished data). Salmon protamine sulfate, grade 1, from Sigma Chemical was used at the same level as lysozyme on a weight basis.

**Estimation of viable cells.** The samples were diluted in TSB-YE and 0.1 ml of the appropriate dilution was spread, in duplicate, on Chapman Stone agar plates and TSA-YE plates. After incubation at 37 °C for 24 to 48 h, the number of colonies were counted.

**RESULTS**

**Growth of *S. aureus* in shaken whole egg.** The viable counts during incubation of three strains of bacteria incubated in liquid whole egg shaken at 34 °C are shown in Fig. 1. Although both strains of *S. aureus* grew slightly during the first 2 h of incubation, their viable numbers then declined with strain S30A being more drastically affected than strain SG8A. In fact, strain SG8A recovered and showed an increase of about two doublings by 7 h. The *Salmonella* culture, on the other hand, grew rapidly throughout the 7 h of incubation resulting in about 11 to 12 doublings in numbers.

**Effect of agitation on pH and growth of *Staphylococcus* in liquid whole egg.** In contrast to the kill observed in shaken whole egg, strain S30A incubated in whole egg at 34 °C without agitation showed a slow increase in viable count (lower part of Fig. 2). The pH of the unshaken culture remained at its initial value of 7.2 (upper part of Fig. 2), whereas that of the shaken culture rose continuously and reached 7.9 by 7 h of incubation. This rise in pH was probably due to the loss of dissolved CO₂ caused by the agitation and the constant renewal of surfaces.

**Effect of preincubation.** As demonstrated above, there was a lag of 2 h before the viable count of S30A decreased in whole egg shaken at 34 °C. To determine if this lag could be abolished or reduced, whole egg was preincubated with shaking at this temperature to allow the pH to rise before inoculation. Although the pH of the preincubated egg had already risen to 7.8 at the time of inoculation, an additional 2 h of incubation, equivalent to control, was still required before any reduction in viable count occurred.

**Growth at near-neutral pH.** If the pH of the shaken whole egg was controlled by buffering at pH 7.0 with 0.1 M phosphate (Fig. 3), S30A not only did not die during the incubation but grew at a rate comparable to that of *Salmonella* (compare with Fig. 1). In the unbuffered control the pH reached 8.1 and the characteristic growth inhibition was seen. Similar results were observed when the pH was maintained near-
Effect of iron addition. Conalbumin, a protein present in egg white, forms a chelate with iron which is more stable at higher pH (5). Therefore, the observed growth inhibition of strain S30A in the shaken whole egg could have been due to iron limitation of growth brought about by the pH rise. Although the addition of a stoichiometric excess of iron (750 μg/ml) stimulated the growth of S30A, it did not eliminate the inhibition.

Lysozyme effect. Thus far we have established that the growth inhibition occurs at an alkaline pH in whole egg, but not in other media such as nutrient broth or TSB. Furthermore, S30A is able to grow prolifically in unsupplemented egg yolk at pH 8.2 (Table 1). Therefore, the inhibitory factor must be present in egg white, which upon a rise in pH causes the reduction in cell numbers. Accordingly, various components of egg white were added to egg yolk (in concentrations equivalent to that found in egg white) to determine their effect on growth of *S. aureus* S30A. The data in Table 1 show that addition of ovalbumin, either alone or together with conalbumin, had no significant effect on the growth of S30A. However, addition of 0.27% (wt/vol) lysozyme, either alone or together with ovomucin, resulted in a decrease in viable count characteristic of that of shaken whole egg.

Isolation of mutants. When *S. aureus* S30A was incubated for 24 h in the shaken whole egg, the population reached numbers in excess of $10^6$ cells/ml. These, presumably, were mutants which not only survived the destructive action of the shaken whole egg but multiplied therein. When several of these mutants were isolated and tested for growth on shaken whole egg, the death observed with the parent culture S30A did not occur, but instead slow growth was observed. The rate of growth of one of the mutants, tested in TSB at pH 8.1, did not differ significantly from that of the parent culture S30A.

 neutrality by periodic addition of 1 N HCl (not shown). Finally, cells of S30A, adapted to a high pH by prior growth in TSB buffered with 0.1 M tris(hydroxymethyl)aminomethane (initial pH 8 and final pH 7.6 at time of harvest), did not grow in the shaken whole egg. This experiment clearly demonstrated that the inability to grow in shaken whole egg was not due to the high pH, but to some factor present in whole egg which was inhibitory only at high pH.

**Fig. 1. Growth of bacteria in liquid whole egg shaken at 34°C.** *Staphylococcus aureus* strain S30A (□), *S. aureus* strain SG8A (△), and *Salmonella typhimurium* strain TM-1 (○).

**Fig. 2. Viability of Staphylococcus aureus S30A (lower graph) and pH change of liquid whole egg (upper graph) during incubation at 34°C under shaking (○) and stationary (△) conditions.
was likewise resistant to 0.27% lysozyme in egg yolk even at pH 8.3 (Table 2). The lysozyme effect on S30A can be simulated with protamine sulfate, both with respect to concentration and to pH dependency (Table 3). Also, the addition of lysozyme to a nutrient broth resulted in inhibition of growth of S30A (Table 4). In this case, a lower concentration of lysozyme than

| Strain S30A | 0 h     | 7 h     |
|-------------|---------|---------|
| pH 7.1, 0.27% lysozyme | 1.6 × 10⁷ | 2.0 × 10⁷ |
| pH 8.1, 0.27% lysozyme | 1.6 × 10⁷ | 6.0 × 10⁴ |
| pH 8.1, 0.135% lysozyme | 1.3 × 10⁷ | 6.0 × 10⁴ |
| pH 8.1, 0.027% lysozyme | 1.2 × 10⁴ | 2.0 × 10⁴ |

| Mutant M6 | 0 h | 7 h |
|-----------|-----|-----|
| pH 8.3, 0.27% lysozyme | 4.5 × 10⁴ | 2.8 × 10⁴ |

* The lysozyme was added to 18 g of egg yolk diluted with 32 g of sterile distilled water to give the final concentration specified.

| PROTOTYPE | PH 6.8, 0.27% protamine sulfate | 0 h | 7 h |
|-----------|---------------------------------|-----|-----|
| pH 7.9, 0.27% protamine sulfate | 1.0 × 10⁴ | <1.0 × 10⁴ |
| pH 7.8, 0.135% protamine sulfate | 1.8 × 10⁴ | 1.6 × 10⁴ |
| pH 7.8, 0.027% protamine sulfate | 1.5 × 10⁴ | 1.1 × 10⁴ |

* Protamine sulfate was added to 18 g of egg yolk diluted with 32 g of sterile distilled water to give the final concentration specified.

Table 3. Effect of pH and concentration of protamine sulfate on growth of S. aureus S30A in egg yolks at 34°C

Data in Table 2 show that at pH 8.1 reducing the lysozyme concentration to one-half (0.135%) reduces the lysozyme effect, whereas reducing the concentration to one-tenth (0.027%) or keeping lysozyme at 0.27%, but adjusting the pH to 7.1, abolished the effect. One mutant, M6, which had been isolated from overnight incubation of whole egg and had been shown to be resistant to the shaken whole egg inhibition,
that found in whole egg was sufficient to bring about the inhibition, but an alkaline pH was still a necessity.

**DISCUSSION**

The foregoing experiments definitely establish that lysozyme inhibits the growth of *S. aureus* S30A in liquid whole egg if shaken, but not when incubated statically. This effect was brought about in liquid whole egg by the presence of lysozyme and the alkaline shift in pH resulting from the loss of CO₂. The kinetics of the decrease in viable count suggests that lysozyme acts not as a lytic agent, but probably exerts its effect by virtue of its highly alkaline isoelectric point. This postulate is supported on the one hand by the ability of protamine sulfate, a highly basic polypeptide, to replace lysozyme and on the other hand by the fact that the pH optimum for the lytic activity of lysozyme is 6 to 7 (18). This is considerably lower than that associated with the action observed here. Whether lysozyme functions by interacting with the cells directly or by interacting with some component in the medium essential for the growth of *Staphylococcus*, thereby making it unavailable to the organism, is not clear. The fact that preincubation of the whole egg for 2 h prior to inoculation did not eliminate the lag period for the expression of the inhibitory effect indicates that contact with the cells is necessary. The ability of lysozyme to agglutinate bacteria has already been reported (6, 10, 18, 20), but is not as well recognized as its lytic property. Lysozyme also forms complexes with many acidic substances (11, 12, 16). Protamine sulfate, likewise, has been reported to be inhibitory to bacteria under alkaline conditions but not at neutrality (4, 13). For these reasons, it is concluded that the interaction between lysozyme and cells reported here is the probable mechanism of killing and growth inhibition.

In view of the high isoelectric point (pH 10.5 to 11.0) of lysozyme (5), its charge likely would not be affected appreciably by the change in pH involved here, but the increase in pH may cause a change in the charge of some critical area either on or in the cell, thus facilitating the formation of complexes with the positively charged lysozyme. However, until more definitive results are obtained, the mode of action of the lysozyme remains a question.

If lysozyme does interact with *Staphylococcus* by an agglutination reaction, one must conclude then that the surface charges of *Staphylococcus* S30A must be quite different from that of *Salmonella* (and perhaps even extended to gram-positive and -negative cells). One may also surmise that the mutants which are resistant to the lysozyme effects are probably altered with respect to their surface charge.

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