Production of Staphylococcal Enterotoxins A, B, and C in Colloidal Dispersions

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Larger amounts of enterotoxin were produced when Staphylococcus aureus S-6 was grown under still (nonshaken) conditions in a medium that was a paste or gel than were produced in a liquid dispersion with the same colloidal ingredient or in control basal broth (4% NZ Amine-NAK containing 50 μg of thiamine per 100 ml and 1 mg of niacin per 100 ml). Four colloidal ingredients were used which had been previously demonstrated to not support enterotoxin production in buffer. The effect of the type of dispersion occurred earlier than that of the colloidal ingredient, but interactions were found. This effect was not observed when the cells were grown with aeration (shaken). Four other strains of S. aureus followed a similar pattern for enterotoxins A, B, and C, although liquid and paste with cornstarch and carrageenan were the only media compared to the control broth. Enterotoxins A and B were produced earlier by S. aureus S-6, and much greater quantities of enterotoxins were produced for all strains when incubated shaken.

Most foods in which Staphylococcus aureus have multiplied and produced enterotoxins and which, when consumed, cause food poisoning are complex colloidal systems. The physical state, independent of the nutrient contribution, may affect the production of enterotoxin.

The studies which have been reported previously have been focused on the production of high levels of enterotoxins. Casman and Bennett (2) observed greater enterotoxin A production in semisolid brain heart infusion plates than in liquid; however, Šimkovičová and Gilbert (13) did not find large amounts of enterotoxin with this method, although, for one of two strains for which data are reported, there was a slight increase over that produced in broth alone. Neither of these two studies offers a direct comparison of effect of colloidal state itself. Membrane culture methods have been recommended (1, 2, 7, 10) and may exert a surface effect as well as permit dialysis.

In foods, the physical state of the substrate may exert an effect upon bacterial growth or enterotoxin production, or both, by changes in aeration, adsorption of metabolic products or nutrients, buffer action, alteration in rate of diffusion, alteration in available water, or changes in the bacterial dispersion and possibly related differences in the growth pattern. However, the complex environment is difficult to study. Therefore, in the present study, numbers of colony-forming units and enterotoxin levels were compared in shaken and still cultures which had been varied in physical state by the addition of single colloidal ingredients to basic broth.

MATERIALS AND METHODS

Strains. The S. aureus S-6 culture, a strain that produces enterotoxins A and B, obtained from M. S. Bergdoll (Food Research Institute, Madison, Wis.), was preserved on porcelain beads (9) for use in the major portion of the study. Enterotoxin B-producing strain 243, enterotoxin A-producing strains 265-1 (from R. W. Bennett, Food and Drug Administration, Washington, D.C.) and 13N-2909 (a high producing mutant strain from S. J. Silverman, Fort Detrick, Frederick, Md.), and enterotoxin C-producing strain 361 (from M. Bergdoll) were used in a follow-up study which compared the NAK broth control with liquid and paste states produced with cornstarch and carrageenan.

Colloidal ingredients and dispersions. Liquid, suspension, paste, and gel with each of four different colloids were made with 4% NZ Amine-NAK (Sheffield Chemical, Union, N.J.) supplemented with 50 μg of thiamine per ml and 1 mg of niacin per ml. In a limited study with two replications, 3% NZ Amine-NAK plus 3% PHP (a pancreatic digest of casein; Mead, Johnson, International, Evansville, Ind.) re-
placed the 4% NAK. The colloidal ingredients were cornstarch (Corn Products Refining Co., Argus, Ill.), agar (Noble special agar, Difco), carrageenan (Gelcarin, Marine Colloids, Inc., Springfield, N.J.), and low methoxyl pectin (LMP) (unstandardized product with no compounds added, Sunkist Growers, Corona, Calif.). Proportions used are given in Table 1. The final pH of each was 6.8. (Predetermined amounts of 0.1 N NaOH were added at the time of initial preparation or later, depending upon the colloid.) The pH was checked on random lots during the experiments. Consistencies of the dispersions (Table 1) were determined in standardizing procedures and at intervals during the experimental period. For liquid and paste, the Brookfield viscometer was used; for gels, the percentage sag was measured by taking the height of the gel before and 1 min after removal from the container. Water activity (a_w) in each medium was calculated from the value for water potential at 27 °C measured in duplicate samples by a thermocouple psychrometer (C-51 Sample Chamber, Wescor, Inc., Logan, Utah), standardized with KCl solutions of predetermined osmotic pressure. Preparation and storage conditions were carefully standardized for all replications.

Fifty-milliliter amounts of each medium and of the NAK broth control were put into 300-ml triple-baffled shake flasks (Bellco Glass, Inc., Vineland, N.J.) for shaken samples and into plain 250-ml Erlenmeyer flasks for still samples and covered with gauze-cotton closures secured with clips.

Dispensers with starch made by adding weighed quantities of cornstarch to basal medium which had been preheated to 80 °C were heated to 90 °C with constant stirring. Samples of 50 ml were autoclaved for 15 min at 121 °C. For suspensions, 15 medium pears (approximately 1.8 g) of tapioca (Manhattan Adhesives Corp., Brooklyn, N.Y.) were added to each flask before autoclaving. Agar and carrageenan were dispersed by heating in the basal medium, and were then distributed in 50-ml fractions. After being autoclaved for 15 min, the flasks were held at room temperature until pastes and gels were set (2 h for agar, 1 h for carrageenan). Pastes for both still and shaken samples were then shaken for 2 h.

The use of LMP differed from that of the other colloids in that it was necessary to make a slurry of LMP in 95% ethanol before addition to the basal medium. Samples were calculated to be 50 g after adjustment of pH and calcium ion concentration. After 1 min of autoclaving at 121 °C, the pH of each was adjusted to 6.8 with precalculated volumes of sterile 0.1 N NaOH. Sterile CaCl_2 (5 ml) was added to the paste (0.05 M CaCl_2) and gel (0.10 M CaCl_2) dispersions. For suspensions thickened with agar, carrageenan, and LMP, 15 6-mm lengths of glass tubing (Exax Raschig Rings, Kimble Products, Toledo, Ohio) were added to each flask before autoclaving.

Inoculation. Media, which were 24-h-old and had been held at 37 °C a minimum of 15 h either shaken or still, were inoculated with 0.5 ml of a dilution of a 24-h NAK broth culture to give 10^6 organisms per ml. In the NAK-PHP series, two levels of inoculum, 8 x 10^4 and 6 x 10^6 colony-forming units (CFU)/ml, were compared. All flasks, except gels, were swirled to mix; the inoculum was spread over the surface of the gel. After inoculation, triplicate flasks of control NAK broth and duplicate flasks of liquid, suspension, and paste dispersions were incubated at 37 °C for both still and shaken (gyroratory water bath shaker, New Brunswick Scientific Co., New Brunswick, N.J.; 200 rpm) samples. All gel samples were incubated still.

Sampling. Sampling was done at 5, 8, and 24 h. For all of the samples except the gels, 6-ml samples were removed at each of the times. A different gel sample was used at each sampling time. Samples were diluted 2:1 with NAK broth and then mixed by manual shaking except for the use of mechanical blending for cornstarch samples. CFU were determined by direct plating of appropriate dilutions on plate count agar (Difco). The sample was then heat treated at 50 °C for 10 min to kill the staphylococci and was cooled, the pH was determined, and the sample was centrifuged for 30 min at 1,200 x g to obtain the supernatant fraction for enterotoxin studies. Starch samples were frozen and thawed twice before centrifugation to facilitate separation of a liquid phase. Enzymatic hydrolysis of agar, starch, and LMP dispersions before centrifugation did not increase enterotoxin recovery or detection in inoculated samples.

Enterotoxin assay. Three methods of varying sensitivity were used for the determination of enterotoxin B, and two for enterotoxins A and C. Crowley's micro-slide gel double-diffusion technique as modified by Casman et al. (3) was used to estimate enterotoxin levels prior to Oudin assay and for samples containing too little enterotoxin to quantitate by the single gel-diffusion method. Samples giving negative results were concentrated ninefold with Aquadisc (Calbiochem, Los Angeles, Calif.) and retested. The detection limit for the micro double-diffusion technique was 0.2 µg/ml.

The reversed passive hemagglutination technique (RPH) adapted to a micro scale by Silverman et al. (12) was used to analyze those concentrated samples which were negative for enterotoxin B with the micro double-diffusion technique. Controls used in all cases included nonsensitized cells and uninoculated suspensions. Since the limit of detection of this method was approximately 0.0007 µg/ml, a negative RPH sample was considered to be negative for enterotoxin. Development of this technique for enterotoxin A was unsuccessful, nor was the method used for enterotoxin C.

The Oudin single gel-diffusion method as described by Hall et al. (6) was used for quantitative assay for the three enterotoxins. The sample which had been dialyzed against 4% NAK broth was placed on top of the prepared agar column, and the tube was incubated in a glass-walled water bath at 30 °C. Measurements of the migration of the precipitate band were taken at 24, 48, and 72 h by using a cathetometer with a short-focus telescope. The K value (slope) was then compared to that obtained with known concentrations of enterotoxin (diluted in 4% NAK, pH 7.4). The minimal concentration of enterotoxin for which this method could be used reliably was 4 µg/ml.
The purified enterotoxins A and B used for these assays were provided by E. J. Schantz (Fort Detrick, Frederick, Md.) and for enterotoxin C, by M. S. Bergdoll. Part of the antisera for enterotoxins A and B and all of that for enterotoxin C were provided through the courtesy of M. S. Bergdoll; much of the antisera for types A and B was produced in the investigators' laboratory. The titers of the antisera from both sources were the same.

Statistical analyses. The experiment with strain S-6 was a replicated split-plot design with colloidal ingredients as main plots. One of the four colloids was chosen at random for each day's experimentation in each of two replications. Duplicate samples for each dispersion, and triplicate samples of the NAK broth, were analyzed with respect to CFU, pH, and enterotoxin. Shaken and still samples were analyzed separately at 5, 8, and 24 h. Least significant differences were used to test for differences between each ingredient-dispersion combination. Quantitative measures of enterotoxin were available for 8- and 24-h shaken samples and 24-h still samples. A logarithmic transformation was applied to CFU. Correlations were studied for the 8 and the 24 h data for shaken and the 24-h data for still samples.

RESULTS

For all of the physical states included, shaking during incubation greatly increased the total amount of enterotoxin at the end of 24 h as has been reported by others (4) and also resulted in earlier detectable enterotoxin. For the samples which were incubated in a thin layer without shaking, the physical state of the inoculated dispersions influenced the yield of enterotoxin. In general, pH and CFU followed trends in enterotoxin levels. Enterotoxin levels are expressed in the text on the basis of the minimal concentrations detectable by the assay methods if below that for which direct quantitation was possible. Data are presented on an unconcentrated basis in Tables 2, 3, and 4. The statistical analysis for the S-6 study is summarized in Table 5.

The dispersions varied in viscosity (Table 1) depending upon the concentration of the colloidal ingredient, except for those with LMP in which the viscosity was controlled by the calcium level and carrageenan paste/gel which was controlled by physical treatment after sterilization. The $\alpha_w$ was above 0.99 for all; the lower in the range of 0.996 to 0.992 being those made with NAK + PHP. Gels with NAK had an $\alpha_w$ of 0.995.

Shaken samples, strain S-6: enterotoxin levels. After 5 h of incubation, most shaken samples contained about 0.2 $\mu$g per ml, and all but the carrageenan suspensions had at least

### Table 1. Composition and consistency at time of inoculation of colloidal dispersions

| Colloidal ingredient | Dispersion | Concentration in basal medium (%) | Apparent viscosity (CP)* |
|----------------------|------------|----------------------------------|------------------------|
|                      |            |                                  | Shaken | Still  |
| Cornstarch + pearl tapioca for suspension | Liquid | 1.4 | 13 | 40 |
|                      | Suspension | 1.4 | 220 | 180 |
|                      | Paste | 3.5 | 400 | 2,100 |
|                      | Gel | 5.3 |           | 11*  |
| Low methoxyl pectin + Raschig rings for suspension | Liquid | 1.8 | 20 | 18 |
|                      | Suspension | 1.8 | 20 | 24 |
|                      | Paste | 1.8 | 89 | 510 |
|                      | Gel | 1.8 |           | 32*  |
| Agar (Noble), + Raschig rings for suspension | Liquid | 0.085 | 13 | 6 |
|                      | Suspension | 0.085 | 6 | 8 |
|                      | Paste | 0.45 | 550 | 720 |
|                      | Gel | 0.50 |           | 9*   |
| Carrageenan + Raschig rings for suspension | Liquid | 0.1 | 10 | 37 |
|                      | Suspension | 0.1 | 10 | 48 |
|                      | Paste | 0.5 | 1,100 | 2,300 |
|                      | Gel | 0.5 |           | 7*   |

* Centipoises, determinations made with a Brookfield Viscometer.
* For gels, viscosity was not determined. Gel strength was estimated by measuring the percentage of sag from the height of the gel before and 1 min after removal from the container.
* Exax Raschig rings (Kimble Products, Toledo, Ohio).
* Five milliliters of 0.05 M CaCl$_2$ added as part of liquid for pastes; 5 ml of 0.10 M CaCl$_2$ for gels.
### Table 2. Colony-forming units of S. aureus S-6, pH, and enterotoxins A and B in control and colloidal dispersions incubated with shaking at 37°C for 5, 8, and 24 h

| Medium               | 5 h               | 8 h               | 24 h              |
|----------------------|-------------------|-------------------|-------------------|
|                      | CFU/ml pH | Entero- toxin B micro-slide* concn* | CFU/ml pH | Entero- toxin B (μg/ml) concn* | CFU/ml pH | Entero- toxin B (μg/ml) concn* |
|                      |            | +/−               |            | +/−               |            | +/−               |
| Control: NAK broth   | 8.3 × 10⁶   | 6.8 19/5 5/0      | 1.3 × 10⁹ | 7.9 20 17/7 7/0    | 5.1 × 10⁶ | 8.7 170 24/1       |
| Cornstarch + NAK     |                 |                   |              |                   |              |                   |
| Liquid               | 1.3 × 10⁶   | 6.8 0/4 4/0       | 1.3 × 10⁶ | 7.7 17 4/0         | 9.1 × 10⁶ | 8.8 160 4/0        |
| Suspension           | 2.1 × 10⁶   | 6.8 0/4 4/0       | 5.1 × 10⁶ | 7.0 14 4/0         | 2.4 × 10⁶ | 8.4 180 4/0        |
| Paste                | 1.2 × 10⁶   | 6.8 2/2 2/0       | 5.7 × 10⁶ | 7.2 12 4/0         | 1.6 × 10⁶ | 8.6 220 4/0        |
| Low methoxyl pectin  |                 |                   |              |                   |              |                   |
| + NAK                |                 |                   |              |                   |              |                   |
| Liquid               | 5.8 × 10⁶   | 6.7 4/0           | 5.1 × 10⁶ | 7.5 16 2/2 2/0     | 2.5 × 10⁶ | 8.0 210 4/0        |
| Suspension           | 3.8 × 10⁶   | 6.7 4/0           | 6.3 × 10⁶ | 7.5 15 2/2 2/0     | 2.7 × 10⁶ | 7.5 210 4/0        |
| Paste                | 1.1 × 10⁶   | 6.8 4/0           | 1.1 × 10⁶ | 7.7 5 2/2 2/0      | 3.2 × 10⁶ | 7.5 120 4/0        |
| Agar + NAK           |                 |                   |              |                   |              |                   |
| Liquid               | 6.6 × 10⁶   | 6.8 2/2 2/0       | 1.9 × 10⁹ | 8.0 20 2/2 2/0     | 1.2 × 10⁹ | 8.7 160 4/0        |
| Suspension           | 5.8 × 10⁶   | 6.8 2/2 2/0       | 1.0 × 10⁹ | 8.1 19 3/1 1/0     | 1.0 × 10⁹ | 8.7 100 4/0        |
| Paste                | 6.9 × 10⁶   | 6.7 2/2 2/0       | 4.0 × 10⁹ | 7.6 11 4/0         | 7.8 × 10⁹ | 8.5 73 4/0         |
| Carrageenan + NAK    |                 |                   |              |                   |              |                   |
| Liquid               | 1.2 × 10⁶   | 6.9 4/0           | 1.1 × 10⁹ | 8.0 25 3/1 1/0     | 1.2 × 10⁹ | 8.7 170 2/2        |
| Suspension           | 1.0 × 10⁶   | 6.9 2/2 0/2       | 1.1 × 10⁹ | 8.0 24 3/1 1/0     | 1.2 × 10⁹ | 7.8 150 3/1        |
| Paste                | 7.1 × 10⁶   | 6.8 4/0           | 1.4 × 10⁹ | 6.8 1 3/1 1/0      | 5.8 × 10⁹ | 8.7 24 4/0         |

* Detection limit for the micro-slide procedure was 0.2 μg/ml in the sample tested; +/− indicates number of positive samples/number of negative samples. * Negative samples were concentrated ninefold, then dialyzed.
### Table 3. Colony-forming units of S. aureus S-6, pH, and enterotoxins A and B in control and colloidal dispersions incubated still at 37°C for 5, 8, and 24 h

| Medium                  | 5 h            | 8 h            | 24 h           |
|-------------------------|----------------|----------------|----------------|
|                         | CPU/ml | pH | Entero- | pH | Entero- | pH | Entero- | CPU/ml | pH | Enterotoxin A micro-slide* concen' (+/-) | pH | Enterotox | (ml/ml) | Enterotoxin B micro-slide* concen' (+/-) | pH | Enterotoxin | micro-slide* concen' (+/-) |
| Control: NAK broth      | 1.5 x 10⁶ | 6.7 | 2/22   | 18/4 | 3/21 | 2.9 x 10⁵ | 6.7 | 0/24   | 8/16 | 12/4 | 0/24 | 4/20 | 1.2 x 10⁵ | 7.2 | 3 | 15/9 |
| Cornstarch + NAK        |             |               |       |      |      |              |     |        |      |      |      |      |          |     |   |      |
| Liquid                  | 6.2 x 10⁷ | 6.8 | 0/4    | 2/2  | 2/2  | 2.7 x 10⁵ | 6.6 | 0/4    | 2/2  | 2/0  | 2/2  | 0/2  | 1.5 x 10⁵ | 7.2 | 5 | 4/0 |
| Suspension              | 3.5 x 10⁷ | 6.7 | 2/2    | 0/2  | 2/2  | 1.0 x 10⁵ | 6.4 | 0/4    | 4/0  | 2/2  | 2/2  | 0/2  | 7.8 x 10⁵ | 8.2 | 29 | 4/0 |
| Paste                   | 8.7 x 10⁷ | 6.8 | 2/2    | 0/2  | 4/0  | 6.3 x 10⁵ | 6.6 | 0/4    | 2/2  | 2/0  | 2/2  | 0/2  | 4.0 x 10⁵ | 8.3 | 38 | 4/0 |
| Gel                     | 4.0 x 10⁷ | 6.8 | 2/2    | 0/2  | 2/2  | 2.9 x 10⁵ | 6.8 | 0/4    | 4/0  | 2/2  | 2/2  | 0/2  | 4.5 x 10⁵ | 7.6 | 47 | 4/0 |
| Low methoxyl pectin     |             |               |       |      |      |              |     |        |      |      |      |      |          |     |   |      |
| + NAK                   |             |               |       |      |      |              |     |        |      |      |      |      |          |     |   |      |
| Liquid                  | 1.2 x 10⁷ | 6.7 | 0/4    | 4/0  | 0/4  | 2.3 x 10⁵ | 6.6 | 0/4    | 2/2  | 1/1  | 0/4  | 2/2  | 4.6 x 10⁵ | 6.9 | 3 | 4/0 |
| Suspension              | 1.0 x 10⁷ | 6.7 | 0/4    | 4/0  | 0/4  | 2.2 x 10⁵ | 6.6 | 0/4    | 2/2  | 2/0  | 0/4  | 0/4  | 6.9 x 10⁵ | 6.9 | 1 | 4/0 |
| Paste                   | 1.8 x 10⁷ | 6.7 | 1/3    | 3/0  | 0/4  | 4.3 x 10⁵ | 6.7 | 0/4    | 2/2  | 1/1  | 0/4  | 0/4  | 3.3 x 10⁵ | 7.4 | 11 | 4/0 |
| Gel                     | 1.9 x 10⁷ | 6.7 | 2/2    | 0/2  | 0/4  | 4.9 x 10⁵ | 6.7 | 2/2    | 0/2  | 0/4  | 0/4  | 0/4  | 6.1 x 10⁵ | 7.4 | 14 | 4/0 |
| Agar + NAK              |             |               |       |      |      |              |     |        |      |      |      |      |          |     |   |      |
| Liquid                  | 3.9 x 10⁷ | 6.7 | 0/4    | 2/2  | 0/4  | 3.2 x 10⁵ | 6.7 | 0/4    | 4/0  | 0/4  | 0/4  | 0/4  | 4.8 x 10⁵ | 7.3 | 3 | 4/0 |
| Suspension              | 2.1 x 10⁷ | 6.7 | 0/4    | 2/2  | 0/4  | 3.2 x 10⁵ | 6.7 | 0/4    | 4/0  | 0/4  | 0/4  | 0/4  | 4.6 x 10⁵ | 7.4 | 4 | 4/0 |
| Paste                   | 6.0 x 10⁷ | 6.8 | 0/4    | 3/1  | 0/4  | 3.0 x 10⁵ | 6.7 | 0/4    | 4/0  | 0/4  | 0/4  | 0/4  | 3.6 x 10⁵ | 7.7 | 6 | 4/0 |
| Gel                     | 5.8 x 10⁷ | 6.8 | 0/4    | 2/2  | 0/4  | 2.8 x 10⁵ | 6.9 | 4/0    | 0/4  | 0/4  | 0/4  | 0/4  | 4.3 x 10⁵ | 7.5 | 23 | 4/0 |
| Carrageenan + NAK       |             |               |       |      |      |              |     |        |      |      |      |      |          |     |   |      |
| Liquid                  | 1.2 x 10⁷ | 6.7 | 0/4    | 4/0  | 0/4  | 6.5 x 10⁵ | 6.5 | 3/1    | 1/0  | 2/2  | 0/2  | 7.8 x 10⁵ | 8.0 | 17 | 4/0 |
| Suspension              | 3.9 x 10⁷ | 6.7 | 0/4    | 2/2  | 0/4  | 5.8 x 10⁵ | 6.5 | 4/0    | 3/1  | 1/0  | 3/1  | 1/0  | 7.3 x 10⁵ | 8.0 | 16 | 4/0 |
| Paste                   | 4.5 x 10⁷ | 6.8 | 0/4    | 4/0  | 0/4  | 1.4 x 10⁵ | 6.6 | 3/1    | 1/0  | 2/2  | 2/0  | 6.6 x 10⁵ | 8.0 | 23 | 4/0 |
| Gel                     | 6.6 x 10⁷ | 6.8 | 2/2    | 1/1  | 0/4  | 2.9 x 10⁵ | 6.9 | 4/0    | 0/4  | 0/4  | 2/2  | 0/2  | 1.5 x 10⁵ | 7.9 | 24 | 4/0 |

* Detection limit for the micro-slide procedure was 0.2 μg/ml in the sample tested; +/- indicates number of positive samples/number of negative samples.

* Reversed passive hemagglutination used for concentrated samples detected 0.0007 μg/ml or above of enterotoxin B, expressed on the basis of the concentrated sample; methodology was not available for the use of this technique for the detection of enterotoxin A.

* Negative samples were concentrated ninefold, then dialyzed.
0.02 μg/ml (Table 2). Enterotoxin A was present at levels of 0.02 μg/ml or slightly greater in about half of the samples.

At 8 h, enterotoxin B levels did not differ (5% level) for the colloidal ingredients. However, samples containing colloids differed from the controls with differences (1% level) demonstrated among the states of dispersion. Enterotoxin B in the control NAK broth and in the colloidal media averaged 19 μg/ml with the exception of pastes. Pastes prepared with carrageenan and LMP averaged 3 μg/ml, whereas those prepared with cornstarch and agar averaged 12 μg/ml. Less efficient aeration may occur but both carrageenan, which gave the thickest paste (Table 1), and LMP, the thinnest, are lower in enterotoxin. A minimum of 0.02 μg/ml of enterotoxin A was present with more than half of the samples having about 0.2 μg (Table 2).

By 24 h the control NAK broth averaged 170 μg of enterotoxin B per ml. Colloidal ingredients and type of dispersion interacted (Table 5). Concentration of enterotoxin was markedly lower in the paste than in the liquid or suspension except for an opposite trend with starch (Table 2). Differences in replications were small except for LMP. A minimum of 0.2 μg of enterotoxin A per ml was present in all samples except for a few isolated carrageenan samples.

Still samples, strain S-6: enterotoxin levels. In still samples, although enterotoxin B was present at 5 h, the amount was generally less than 0.02 μg/ml (Table 3). After 24 h, the

| Strain | Enterotoxin | Medium | Incubation | CFU/ml | pH | Microslide\((+/-)\) | Enterotoxin µg/ml |
|-------|-------------|--------|------------|--------|----|----------------|------------------|
| 243   | B           | Control: NAK broth | Shaken     | 2.4 x 10^9 | 8.8 | 3/0 | 17 (16-18) |
|       |             | Cornstarch + NAK | Still      | 1.5 x 10^9 | 7.2 | 0/4 | 0.12 (0.08-0.18) |
|       |             | Liquid       | Still      | 2.2 x 10^9 | 7.2 | 5/0 | 0.25 (0.2-0.3) |
|       |             | Paste        | Still      | 5.6 x 10^9 | 8.2 | 5/0 | 1.8 (1.7-2.1) |
| 265-1 | A           | Control: NAK broth | Shaken     | 2.7 x 10^9 | 8.7 | 3/0 | 16 (14-40) |
|       |             | Cornstarch + NAK | Still      | 7.6 x 10^9 | 7.2 | 0/4 | 0.1 (neg-0.1) |
|       |             | Liquid       | Still      | 1.9 x 10^9 | 7.2 | 5/0 | 0.38 (0.2-0.6) |
|       |             | Paste        | Still      | 2.4 x 10^9 | 7.9 | 4/1 | 0.43 (0.2-0.6) |
| 13N-2909 | A         | Control: NAK broth | Shaken     | 2.5 x 10^9 | 7.8 | 3/0 | 19 (16-22) |
|       |             | Cornstarch + NAK | Still      | 3.8 x 10^9 | 7.0 | 5/0 | 0.7 (0.6-1.0) |
|       |             | Liquid       | Still      | 3.6 x 10^9 | 6.8 | 5/0 | 0.8 (0.6-1.0) |
|       |             | Paste        | Still      | 3.5 x 10^9 | 7.1 | 5/0 | 1.5 (1.0-3.0) |
| 361   | C           | Control: NAK broth | Shaken     | 8.0 x 10^9 | 9.0 | 3/0 | 73 (70-80) |
|       |             | Cornstarch + NAK | Still      | 6.9 x 10^9 | 7.4 | 6/0 | 1.8 (1.3-2.5) |
|       |             | Liquid       | Still      | 1.3 x 10^9 | 7.4 | 6/0 | 3.1 (2.3-4.3) |
|       |             | Paste        | Still      | 4.2 x 10^9 | 8.1 | 6/0 | 3.9 (3.1-5.2) |
|       |             | Carrageenan + NAK | Still    | 2.1 x 10^9 | 7.9 | 6/0 | 9.9 (5.2-13) |
|       |             | Liquid       | Still      | 8.3 x 10^9 | 8.4 | 6/0 | 37 (32-48) |

* Averages of duplicate samples for each of three replications.
* Detection limit for the micro-slide procedure was 0.2 μg/ml; +/- indicates number of positive samples/number of negative samples.
* Microslides on negative samples (concentrated ninefold and dialyzed) were 4/0 for c, 2/2 for e, and 1/0 for f.
* If quantitation by the single gel-diffusion assay was not possible after concentration of the samples, micrograms per milliliter was estimated from the micro-slide procedure by comparison with enterotoxin dilutions.
colloidal ingredient and type of dispersion each had a significant effect (1% level) (Table 5). Enterotoxin B was significantly lower in the control NAK broth and in liquid states of colloidal dispersions, except for those with carrageenan. Gels had the highest concentration, from 47 μg/ml with cornstarch to 14 μg/ml with LMP as compared to an average of 3 for the control broth (Table 3).

Enterotoxin A production was also less rapid with still incubation (Tables 2 and 3). Only a few samples from both broth and cornstarch contained as much as 0.02 μg/ml at 5 h, but the number was similar to that for samples positive for B; at 8 h, scattered samples contained 0.2 μg/ml or above. After 24 h, nearly all samples had a minimum of 0.2 μg/ml. The effect of colloidal ingredient appeared to be greater than that of type of dispersion.

**Staphylococcal multiplication and pH changes.** When samples were incubated without shaking as compared to shaken, the rate of increase in CFU was less (Tables 2 and 3). The difference was less after 24 h. Differences in pH were not appreciable at 5 h, about 1 pH unit lower at 8 h, and 2 pH units at 24 h, including the gels (Tables 2 and 3). In shaken samples, increase in CFU was rapid from the zero hour count of 10⁴ per ml. The average count in NAK broth was 8.3 × 10⁴ at 5 h and 1.3 × 10⁴ at 8 h. Maximal counts had been reached prior to 24 h. The colloidal ingredient, as compared to the NAK broth, had no significant effect at 5 h but had a slight, although significant (5% level), depressing effect at 8 h. The addition of cornstarch or LMP as the colloidal ingredient had increased the 24-h CFU slightly (significant at the 1% level). The state of dispersion had little effect nor was there a significant interaction between dispersion and colloidal ingredient. Although pH differences were small, effects of the state of dispersion and the interaction with colloids were highly significant statistically. The colloids did not appear to differ until 24 h, when the LMP had the highest average number of CFU (2.8 × 10⁴ as compared to 7.6 × 10⁴ for the control); pH was the lowest, 7.7.

**Other strains.** Four additional strains were compared after 24 h still incubation in liquid and paste states with cornstarch as the colloid.

**Table 5. F values from analysis of variance on data from S. aureus S-6 study for replicated split plot design with colloidal ingredients as main plots**

| Incubation | Factor                  | Source of variation | DF | 5 h     | 8 h     | 24 h    |
|------------|-------------------------|---------------------|----|---------|---------|---------|
| Shaken     | pH                      | Colloidal ingredient (I) | 3  | 0.91    | 3.59    | 14.22** |
|            |                         | Control vs colloidal | 1  | 12.69** | 19.67** | 15.06** |
|            |                         | State of dispersion (D) | 2  | 14.62** | 10.59** | 5.00*   |
|            |                         | I × D                | 6  | 19.88** | 6.11**  | 1.96    |
|            | Colony-forming units    | Colloidal ingredient (I) | 3  | 2.55    | 0.41    | 16.92** |
|            |                         | Control vs colloidal | 1  | 0.15    | 6.30*   | 8.20*   |
|            |                         | State of dispersion (D) | 2  | 0.01    | 4.99*   | 0.72    |
|            |                         | I × D                | 6  | 3.33    | 3.39    | 0.91    |
|            | Enterotoxin (μg/ml)     | Colloidal ingredient (I) | 3  | 0.31    | 0.31    | 7.02**  |
|            |                         | Control vs colloidal | 1  | 12.48** | 3.43**  | 6.43*   |
|            |                         | State of dispersion (D) | 2  | 20.15** | 20.30** | 10.16** |
|            |                         | I × D                | 6  | 2.86    | 2.86    | 10.16** |
| Still      | pH                      | Colloidal ingredient (I) | 3  | 0.51    | 1.71    | 10.99** |
|            |                         | Control vs colloidal | 1  | 3.45    | 1.52    | 33.33** |
|            |                         | State of dispersion (D) | 3  | 2.70    | 25.32** | 6.56**  |
|            |                         | I × D                | 9  | 2.06    | 9.14**  | 49.20** |
|            | Colony-forming units    | Colloidal ingredient (I) | 3  | 2.26    | 9.14**  | 49.20** |
|            |                         | Control vs colloidal | 1  | 1.86    | 18.10** | 36.52** |
|            |                         | State of dispersion (D) | 3  | 5.55*   | 20.33** | 5.68*   |
|            |                         | I × D                | 9  | 2.54    | 2.16    | 3.58*   |
|            | Enterotoxin (μg/ml)     | Colloidal ingredient (I) | 3  |        |        | 11.21** |
|            |                         | Control vs colloidal | 1  |        |        | 20.39** |
|            |                         | State of dispersion (D) | 3  |        |        | 7.78**  |
|            |                         | I × D                | 9  |        |        | 1.41    |

* * Indicates significant at 5% level or above; **, 1% level or above.
* Not quantitated.
little nutritive effect as indicated by no or slight increases in CFU of inoculated staphylococci except possibly for the higher concentration of carrageenan. There was no enterotoxin production when the colloids had been added to buffer to provide the four types of dispersions.

In addition, reducing sugars were determined for the starch dispersions, and then filter-sterilized glucose was added to adjust the control NAK broth and each starch dispersion to the

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**Table 6.** Colony-forming units, pH, and enterotoxin production for four strains of *S. aureus* after 24 h of incubation at 37 C in control and colloidal dispersions with NAK-PHP broth

| Strain | Enterotoxin | Medium | Inoculum (CFU/ml) | Incubation | CFU/ml | pH | Enterotoxin (μg/ml) |
|--------|-------------|--------|------------------|------------|--------|----|-------------------|
| S-6    | B           | Control: NAK-PHP broth | 10<sup>9</sup> | Shaken | 2.2 x 10<sup>10</sup> | 8.9  | 170               |
|        |             | Cornstarch + NAK-PHP     | 10<sup>9</sup> | Shaken | 2.3 x 10<sup>10</sup> | 8.8  | 160               |
|        |             | Liquid                     | 10<sup>9</sup> | Still  | 1.4 x 10<sup>9</sup>  | 7.1  | 3.1               |
|        |             | Paste                      | 10<sup>9</sup> | Still  | 9.0 x 10<sup>8</sup>  | 7.4  | 4.0               |
| 243    | B           | Control: NAK-PHP broth     | 10<sup>9</sup> | Shaken | 2.9 x 10<sup>10</sup> | 9.1  | 23                |
|        |             | Cornstarch + NAK-PHP      | 10<sup>9</sup> | Shaken | 2.4 x 10<sup>10</sup> | 9.0  | 24                |
|        |             | Liquid                     | 10<sup>9</sup> | Still  | 1.0 x 10<sup>9</sup>  | 7.1  | 0.9               |
|        |             | Paste                      | 10<sup>9</sup> | Still  | 6.6 x 10<sup>8</sup>  | 7.0  | 1.2               |
| 256-1  | A           | Control: NAK-PHP broth     | 10<sup>9</sup> | Shaken | 1.1 x 10<sup>9</sup>  | 9.0  | 3.4               |
|        |             | Cornstarch + NAK-PHP      | 10<sup>9</sup> | Shaken | 1.3 x 10<sup>9</sup>  | 8.8  | 1.6               |
|        |             | Liquid                     | 10<sup>9</sup> | Still  | 1.6 x 10<sup>9</sup>  | 7.2  | 0.2<sup>a</sup>   |
|        |             | Paste                      | 10<sup>9</sup> | Still  | 1.6 x 10<sup>9</sup>  | 7.1  | 0.9               |
| 13N-2909 | A       | Control: NAK-PHP broth     | 10<sup>9</sup> | Shaken | 6.6 x 10<sup>8</sup>  | 9.0  | 0.3<sup>a</sup>   |
|        |             | Cornstarch + NAK-PHP      | 10<sup>9</sup> | Shaken | 9.8 x 10<sup>8</sup>  | 8.8  | 0.2<sup>a</sup>   |
|        |             | Liquid                     | 10<sup>9</sup> | Still  | 4.4 x 10<sup>8</sup>  | 7.4  | 0.3<sup>a</sup>   |
|        |             | Paste                      | 10<sup>9</sup> | Still  | 2.2 x 10<sup>8</sup>  | 7.3  | 0.3<sup>a</sup>   |
|        |             | Cornstarch + NAK-PHP      | 10<sup>9</sup> | Still  | 2.9 x 10<sup>8</sup>  | 7.0  | 0.4<sup>a</sup>   |
|        |             | Liquid                     | 10<sup>9</sup> | Still  | 2.1 x 10<sup>8</sup>  | 7.0  | 0.7               |
|        |             | Paste                      | 10<sup>9</sup> | Still  | 2.3 x 10<sup>8</sup>  | 7.8  | 1.2               |
|        |             | Cornstarch + NAK-PHP      | 10<sup>9</sup> | Still  | 9.1 x 10<sup>7</sup>  | 7.0  | 2.6               |

*3% NZ Amine-NAK-3% protein hydrolysate powder plus vitamins. Averages of two replications with duplicate samples of each.

*If quantitation by the single gel-diffusion assay was not possible after concentration of the samples, micrograms per milliliter was estimated from the micro-slide procedure by comparison with enterotoxin dilutions.
level of the highest concentration found (17 mg/ml in the suspension and gel). Enterotoxin B was low in the still broth and liquid dispersion (average of 0.9 μg/ml) and higher in the suspension and gel (20 and 13 μg/ml, respectively).

A second broth, NAK-PHP, which was higher in protein content was used as the basis for liquid and paste cornstarch dispersions (Table 6). The trends were little different from those with NAK broth, that with the higher proportion of cornstarch yielded larger quantities of enterotoxin.

Limitations. It was more difficult to study the still systems because of the low levels of enterotoxin to be determined. If the concentration reported is below 0.4 μg/ml, the value was estimated from dilution comparisons and the limits of detection of the methods used. In recovery studies, it was found that enterotoxin A decreased during the concentration procedure and, therefore, reported concentrations below 4 μg/ml are lower than the original.

DISCUSSION

It is evident that under conditions of forced aeration, there was no increase in enterotoxin production attributable to the addition of the colloidal ingredients. However, under more limited aeration (incubation still), paste and gel dispersions had increased production of enterotoxin. In preliminary investigations, gels surface-inoculated and those inoculated throughout the medium before gelation gave similar levels of enterotoxin. Colony form differs with the consistency of the growth medium; further study may delineate this or other physical factors as being important. Bacterial cells may adhere to colloid particles and thus encounter a different microenvironment (8). Haider et al. (5) in experiments with S. cerevisiae and A. niger observed more efficient utilization of glucose in the presence of montmorillonite. A protective effect has been attributed to several synthetic polymers substituted for part of the serum in media for selected human cell lines (11). Differences in αw appear not to be the cause since all were above 0.99 (14).

Since foods represent colloidal systems and have no active aeration during storage, the production of enterotoxin may be favored by the colloidal matrix. However, not all colloids were equally effective.

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