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Enzymatic Detection of the Growth of *Staphylococcus aureus* in Foods[1]

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A specific method has been developed for the extraction and measurement of staphylococcal nuclease in foods in which *Staphylococcus aureus* has grown. The method was used to compare staphylococcal growth with nuclease production in foods under varying conditions of temperature, aerobiosis, and competition from other microorganisms. It was concluded that the nuclease is produced under any conditions that permit growth of *S. aureus*, and little or no interference with the test was encountered either from mixed, natural populations or from a variety of pure, laboratory cultures. Nuclease and enterotoxin A production were shown to vary in synchrony for the 234 (Casman) strain of *S. aureus*, and the sensitivity of the enzymatic detection of nuclease was comparable to the sensitivity of serological detection of enterotoxin A. It was found that 15 min at 121 C was required to reduce the nuclease activity in slurries of contaminated ham below the level present in the unheated slurry. The extraordinary heat resistance of the nuclease permits its detection even in foods heated subsequent to the growth of *S. aureus*. The nuclease analysis requires about 3 hr to complete and requires no unusual equipment or reagents.

A possible enzymatic means for detecting food contamination by *Staphylococcus aureus* was suggested by the fact that nearly all pathogenic staphylococci produce a unique and remarkably heat-stable nuclease (9, 11). Specific detection of this enzyme in food stuffs would provide *prima facie* evidence of contamination by *S. aureus*. The suitability for human consumption of any food in which *S. aureus* has grown is questionable, and, in addition, since the enterotoxigenic staphylococci would also be detected in this way [perhaps 30% of all pathogenic strains are also enterotoxigenic (5)], identification of such contaminated foods would also identify those capable of causing staphylococcal food poisoning.

The object of our investigation has been to develop a method for detecting staphylococcal nuclease in foods that would be sensitive, specific, and relatively simple.

MATERIALS AND METHODS

Culture methods

The *S. aureus* strain used in most of these studies, 234 (Casman), produces enterotoxin A and was obtained from E. Casman, Division of Microbiology, Food and Drug Administration, Washington, D.C. Its nuclease production is below the mean of five strains of bovine and human origin with which it has been compared.

The culture was stored on Trypticase Soy Agar slants (BBL) at 4 C; bimonthly transfers were incubated at 37 C for 24 hr.

For production of nuclease and enterotoxin, 10 ml of Trypticase Soy Broth in a 100-ml flask was inoculated and shaken at 37 C for 18 hr. A 1-ml amount of this was used to inoculate 50 ml of a completely dialyzable broth (7) in a 500-ml flask. The broth was modified from the original formula by the omission of mannitol and tryptone. After 2 hr of shaking at 37 C, the 50 ml was used to inoculate 450 ml of the same broth in a 4-liter, heavy-walled flask equipped for gassing. An 80% O_2-20% CO_2 atmosphere was established, and the flask was shaken at 37 C, the atmosphere being replaced with the same gas mixture at 6 hr. After 10 hr of incubation, chloroform was added to the culture, and it was held at 4 C for 16 hr. The cells were removed by centrifugation, and the supernatant fluid was used as starting material for toxin purification.

To count *S. aureus* in foodstuffs, the material was homogenized for 1.0 min in sterile 0.05 M phosphate buffer (*p*H 6.8), and the homogenate was serially diluted in the same buffer. Samples of the dilutions were spread on either TPEY medium (BBL) or the medium described by Baird-Parker (1). Replicate counts on the two media were comparable; conse-

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quently, the TPEY medium was more frequently used as it is simpler to prepare. Other details of the counting techniques are discussed in connection with the experiments in which they were employed.

Enterotoxin purification. A 1,800-ml amount of culture supernatant fluid was brought to 40% saturation with $(NH_4)_2SO_4$; the precipitate was collected by centrifugation at 12,000 × *g* and was discarded. The $(NH_4)_2SO_4$ concentration was raised to 80%, and the precipitate collected by centrifugation was redissolved in a minimal volume of a solution of 0.01 M sodium phosphate brought to *p*H 6.8 with NaOH and to a conductance of 1.5 millimhos with NaCl. The redissolved material was dialyzed overnight against the same solution at 4 C. Its volume was then brought to 25 ml by osmo-dialysis against a high-molecular-weight cellulose derivative (Aquacide II, Calbiochem, Los Angeles, Calif.).

This reduced volume was applied to a 25-cm column of cellulose phosphate (Schleicher & Schuell Co., Keene, N. H.) prepared as described elsewhere (7). The column was eluted by use of a NaCl gradient produced by a nine-chambered device (16). Chambers 1, 3, and 5 were loaded with distilled water. Chambers 2, 4, and 6 were loaded with 0.01 M sodium phosphate adjusted to *p*H 7.0, 8.0, and 9.0 with NaOH and to 9.0, 8.0, and 15.0 millimhos, respectively, with NaCl. The resulting complex gradient had a negative inflection point at v/V = 0.3 and conductivity = 3.5 millimhos, and a positive inflection point at v/V = 0.7 and conductivity = 5.0 millimhos. The enterotoxin eluted at approximately 4.5 millimhos.

The enterotoxin was located and assayed by serological procedures described in the next section. The two peak fractions (10-ml total) yielded single precipitation lines by gel double diffusion against anti-enterotoxin A antisera, kindly supplied by E. P. Casman, and against homologous antisera from rabbits. The material in the peak fractions gave a line of identity with reference enterotoxin A, also provided by E. P. Casman. Combined, the peak fractions contained about 3 mg of protein. A portion of the combined peak fractions was concentrated by osmo-dialysis, and a volume containing 150 μg of protein was applied to a strip of Schleicher & Schuell 2043-a paper. The paper was saturated with sodium phosphate buffer (*p*H 8.2, conductivity = 1.5 millimhos), and a gradient of 3 v per cm was applied for 12 hr. The paper was dried at 105 C for 20 min and developed with bromophenol blue (Beckman Technical Bulletin 6095A). A single line appeared showing cathodic migration.

Serological methods. A 5-ml amount of the purified enterotoxin solution was saturated with chloroform and homogenized with 5 ml of Freund's complete adjuvant (Difco) for 2 min in a Sorvall Omnimixer. The homogenate contained approximately 150 μg of protein per ml.

Rabbits were injected with 0.5 ml of the homogenate intraperitoneally and subcutaneously. Simultaneously 0.5 ml of the purified enterotoxin solution (approximately 300 μg of protein per ml) was injected into each hind footpad. Peak antienterotoxin titers were obtained in 18 to 21 days.

Gel double-diffusion analyses of the sera were made by use of microscope slides layered with 0.8% agarose (Fisher Scientific Co., Pittsburgh, Pa.) containing 1.0% NaCl and 0.1% sodium azide. Wells were cut by use of an LKB 6866A gel punch and were filled with 8 μliters of antigen solution or antiserum.

Chemical and enzymatic analyses. To analyze for nuclease activity, a stock solution containing 1.5 mg of denatured calf thymus deoxyribonucleic acid (DNA; Calbiochem) per ml was prepared by boiling for 5 min and cooling quickly. This was stored frozen.

Stock solutions of 0.15 M CaCl and 0.17 M glycine buffer adjusted to *p*H 8.6 with NaOH were also prepared.

To analyze an enzyme solution, 0.200 ml of the substrate and 0.200 ml of glycine buffer were mixed in a heavy-walled glass centrifuge tube, and 0.025 ml of the $CaCl_2$ solution was then added. The mixture was brought to 37.00 ± 0.02 C in a water bath, the enzyme in 0.075 ml of solution was added, and the reaction mixture was incubated for 30 min.

The reaction was terminated by adding 0.5 ml of ice-cold 7% $HClO_4$, followed by 3.0 ml of ice-cold distilled water. The mixture was immediately centrifuged for 15 min at 10,000 × *g* at 4 C, and the supernatant fluid was decanted into a 1 cm^2 silica cuvette.

The blank was prepared in the same way, but the $HClO_4$ was added immediately after adding the enzyme. Blanks were mixed just before adding $HClO_4$ to the reaction mixtures so that the length of time the unhydrolyzed DNA was exposed to the $HClO_4$ in both reaction mixtures and blanks was comparable and was as short as possible.

The 260-mμ absorbance of the supernatant fluids was determined in a Beckman DU spectrophotometer. One unit of nuclease activity was considered to be that amount producing an optical density difference of 1.0 between the reaction mixture and the blank.

Protein was assayed by the method of Lowry et al. (14).

Conductivities were determined by use of a Serfass conductance meter and a cell with a constant of 1.0125 at 25 C.

RESULTS

Extraction of nuclease from foodstuffs. To compare the efficiency of different extraction techniques, 120 g of ham salad, potato salad, or cream pie was mixed with 30 ml of *S. aureus* 234 culture supernatant fluid. A 50-g amount of the mixture was homogenized for 2.5 min in a 1:1 (w/w) mixture with distilled water by use of a Sorvall Omni-Mixer operating at its maximal rated speed. Samples from the resultant homogenate were either adjusted to different *p*H values with 1 N NCl or NaOH, or were brought to 60% saturation with $(NH_4)_2SO_4$ (calculated on the basis of the total volume of the homogenate, previous experience having shown that the nuclease was not precipitated from culture supernatant fluids at this concentration) or to 1% with protamine sulfate.

The percentage actually found of the amount theoretically present in the supernatant fluids ranged from 1.4% with protamine sulfate to 24.0% when 60% saturation with $(NH_4)_2SO_4$ was used. Since the $(NH_4)_2SO_4$ treatment gave the best recovery and was the simplest to perform, it was the extraction method used in all subsequent tests.

However, replicate variability was high with this method, and it was found that NH_4^+ carried over into the reaction mixture was affecting the apparent reaction rate. To avoid this effect, the possibility of precipitating the nuclease from the $(NH_4)_2SO_4$ solution with trichloroacetic acid and redissolving it in NH_4^+-free solution was investigated.

The activity in culture supernatant fluids of the 234 strain, a second food poisoning strain (also obtained from E. P. Casman), the Wood 46 strain, and five strains each of bovine and human origin obtained from our departmental culture collection was determined, and a volume of each sufficient to contain 1.0 unit of nuclease activity was brought to 100 ml with the sterile production broth. Enough $(NH_4)_2SO_4$ was added to each of these 100-ml samples to yield 60% saturation. They were held for 2 hr and then centrifuged. The precipitate was discarded. The supernatant fluid was treated with 0.05 volumes of cold 3.0 M trichloroacetic acid, and the resultant precipitate was collected by centrifugation at 10,000 $\times$ *g* for 15 min. The precipitate was taken up in 5.0 ml of distilled water, transferred to a heavy-walled, glass centrifuge tube, and reprecipitated with 0.05 volume of 3.0 M trichloroacetic acid. The precipitate was collected by a second centrifugation at 10,000 $\times$ *g* for 15 min, and the supernatant fluid was discarded. The *p*H of the precipitate was adjusted to 8.0 to 8.5 by use of 2.0 N NaOH and *p*H 6.0 to 8.5 indicator paper. The centrifuge tube was temperature-equilibrated in a water bath; substrate, cofactor, and buffer were added; and the nuclease activity was measured in the usual way.

The mean recovery of the initial activity after the two trichloroacetic acid precipitations was 94.6%, with a standard deviation of 4.3%. It was thus feasible to use trichloroacetic acid precipitation, since the recovery of the nuclease activity from the $(NH_4)_2SO_4$ was essentially quantitative.

To assess the effect of trichloroacetic acid precipitation upon replicate variation due to NH_4^+, 47.5 g of ham salad was mixed with 2.5 ml of *S. aureus* 234 culture supernatant fluid. The mixture was brought to a volume of 100 ml with distilled water and homogenized as before. Samples (5 ml) of this slurry were treated with $(NH_4)_2SO_4$. The precipitate was removed by centrifugation, and the nuclease activity was precipitated twice with trichloroacetic acid and then measured.

The mean nuclease activity was 0.28 units with a standard deviation of 0.02 units (a standard deviation value only slightly greater, on a percentage basis, than that of an equal number of replicate assays of the culture supernatant fluid), representing 23% recovery of the nuclease activity added to the slurry in the culture supernatant fluid. The trichloroacetic acid precipitation step thus reduced the variability between replicate analyses and, in addition, increased the sensitivity of the test by making it possible to concentrate the nuclease from relatively large volumes of $(NH_4)_2SO_4$ extract.

To compare the effectiveness of the $(NH_4)_2SO_4$ extraction-trichloroacetic acid precipitation in recovery of nuclease activity from foods other than ham salad, a variety of foodstuffs was mixed with *S. aureus* 234 culture supernatant fluid in the ratio of 47.5 g of foodstuff to 2.5 ml of supernatant fluid. The mixtures were then homogenized and analyzed (Table 1).

The amount of activity recoverable is obviously related to the type of food being analyzed, but for a given type of food the level of recovery is reproducible. Generally, starchy foods gave the highest recovery, meats an intermediate recovery, and fat-rich foods the lowest recovery.

To establish that the level of $(NH_4)_2SO_4$ used was optimal for nuclease extraction and that it could extract nuclease actually produced in the food by *S. aureus*, a protocol closely similar to that used by Casman et al. (5) was used. Slices of boiled ham were obtained from a local market

TABLE 1. *Nuclease activity recovered by $(NH_4)_2SO_4$ extraction of foodstuffs contaminated with known amounts of the enzyme*

Foodstuff[a]	Percentage of added nuclease activity recovered	
	Mean	SD
Potato salad	90	4
Poultry stuffing	71	18
Frozen precooked haddock fillet	32	16
Chocolate eclair	28	6
Sliced ham	26	8
Egg salad	20	7
Cream pie	17	5

[a] Ten replicate samples of each foodstuff were analyzed.

and were aseptically recut into 10 × 10 × 0.5 cm slabs. A 1-ml amount of a saline dilution of a 24-hr culture of *S. aureus* 234 containing approximately 250,000 colony-forming units was spread uniformly over one surface of the slice with a bent glass rod. Uninoculated control slices were similarly spread with sterile saline. The slices were incubated in large petri dishes at 37 C for 36 hr, small amounts of sterile water being added as necessary to keep the dish humid and prevent desiccation of the slice.

After incubation, 50-g samples were cut from the slices and homogenized in distilled water containing enough $(NH_4)_2SO_4$ to yield the desired final concentrations. The resulting homogenate was divided into halves, one-half to act as the blank, and was analyzed for nuclease. The quantities found by the different treatments were as follows: 40% $(NH_4)_2SO_4$, 0.22 units per g; 50% $(NH_4)_2SO_4$, 0.50 units per g; 60% $(NH_4)_2SO_4$, 0.67 units per g; 70% $(NH_4)_2SO_4$, 0.00 units per g.

Direct homogenization of the food in 60% $(NH_4)_2SO_4$ was the method of extraction used thereafter.

Association of staphylococcal nuclease production and growth in foods. The method of nuclease analysis described in the first section was applied to establishing the degree of correlation between staphylococcal growth and production of nuclease in inoculated food. In addition, it was desired to know how this relationship was affected by the growth of competing microorganisms and by anaerobiosis, both conditions being likely to occur during staphylococcal development in foods.

To study staphylococcal growth in the absence of a competing microflora, ham slices were autoclaved for 10 min at 15 psi prior to inoculation with *S. aureus*. For studies on the effects of competing microorganisms, the autoclaved slices were recontaminated by superimposition upon an unautoclaved slice for 30 min at 4 C.

Autoclaved ham slices, with and without reintroduction of the normal microflora, were inoculated with *S. aureus* 234 and incubated aerobically and anaerobically at 25 C. Anaerobic samples were incubated in a catalytically deoxygenated atmosphere of H_2.

At the end of the desired incubation period, 50-g samples were homogenized with an equal weight of distilled water and enough $(NH_4)_2SO_4$ to yield 60% saturation. The homogenates were held at 4 C for 2 hr, and then were centrifuged at 10,000 × *g* for 15 min. The supernatant fluid was decanted, and 0.05 volume of 3 M trichloroacetic acid was added. After 15 min, this was centrifuged at 4 C. The precipitate was taken up in 5 ml of distilled water and distributed equally between two of the centrifuge-reaction tubes; 0.12 ml of 3 M trichloroacetic acid was added to each. After 15 min, both tubes were centrifuged, the supernatant fluids were discarded, and the nuclease activity of the precipitates was determined (Table 2).

The production of nuclease in the ham slices inoculated with *S. aureus* 234 only was clearly demonstrable and was greater anaerobically than aerobically. This was an unexpected finding, since nuclease production under anaerobic conditions in broth culture is markedly depressed. However, it has been a reproducible observation.

The normal microflora greatly stimulated the nuclease yield aerobically, perhaps by creating an anaerobic micro-environment in the growth area on the ham. Anaerobically, the normal microflora caused an average reduction of about 50%. Nuclease activity, however, was still obviously easily demonstrable.

Growth and nuclease production were measured together in inoculated ham slices incubated aerobically and anaerobically at 25 and 35 C (Table 3).

The plate counts and the appearance of nuclease correlated well in the inoculated slices, with increasingly close correlation when the plate counts indicated populations of 10^7 per g or higher. The first nuclease activity was detected when the plate count indicated approximately 3,000 *S. aureus* cells per g. This sensitivity was 10-fold more than that expected on the basis of nuclease yield per cell in pure, aerated broth cultures. It suggested that the plating method

TABLE 2. *Effect of competing microflora and anaerobiosis upon the production of nuclease by Staphylococcus aureus 234 in sterile ham slices incubated at 25 C for 36 hr*

Material spread on ham surface	Nuclease activity			
	Air atmosphere		H_2 atmosphere	
	Units/g[a]	SD[a]	Units/g[a]	SD[a]
Sterile saline	0.01	0.01	0.01	0.01
Normal microflora	0.00	0.00	0.00	0.00
S. aureus 234	0.04[b]	0.02	0.61	0.14
S. aureus 234 plus normal microflora	0.55	0.12	0.29	0.06

[a] Ten 50-g ham slices were analyzed.

[b] Significantly greater, at the 1% level, than the nuclease activity in ham either spread with sterile saline or recontaminated with the normal microflora.

TABLE 3. *Staphylococcal counts and production of nuclease in inoculated (Staphylococcus aureus 234) and uninoculated ham slices held aerobically and anaerobically at 25 and 35 C*

Temp of incubation	Atmosphere	Incubation time	Inoculated slices		Uninoculated slices	
			S. aureus[a] (log colony count/g)	Nuclease[a]	*S. aureus*[a] (log colony count/g)	Nuclease[a]
C		*hr*		*units/g*		*units/g*
25	Air	0	<2.00	0.00	<2.00	0.00
		4	<2.00	0.00	<2.00	0.00
		8	2.86	0.00	<2.00	0.00
		12	3.72	0.05	<2.00	0.00
		24	6.50	0.08	<2.00	0.00
		48	7.65	0.16	<2.00	0.00
	H_2	0	<2.00	0.00	<2.00	0.00
		4	<2.00	0.00	<2.00	0.00
		8	3.44	0.03	<2.00	0.00
		12	5.21	0.07	<2.00	0.00
		24	7.05	0.11	<2.00	0.00
		48	9.12	0.24	<2.00	0.00
35	Air	0	2.26	0.00	<2.00	0.00
		8	3.84	0.02	<2.00	0.00
		16	9.36	0.38	<2.00	0.00
		24	9.80	0.41	3.42	0.04
		40	8.11	0.48	6.65	0.09
	H_2	0	<2.00	0.00	<2.00	0.00
		8	3.54	0.04	<2.00	0.00
		16	9.52	0.34	<2.00	0.00
		24	9.68	0.36	3.62	0.05
		40	9.78	0.39	4.91	0.05

[a] Five replicate analyses of a 50-g ham slice were made at each time and a mean value was calculated.

was recovering 10% or less of the *S. aureus* cells actually present in the ham. Further evidence for this was obtained in other experiments (reported in a later section).

For each sample with a plate count of 10^7 staphylococci per g, or greater, the mean and standard deviation of the five replicate nuclease determinations was calculated, and the standard deviations of all such samples were then averaged, yielding a mean standard deviation of 8.6% with a standard error of 0.9. The plate count replicates, treated in the same way, showed a mean standard deviation of 19% with a standard error of 4.2.

The variation in the enzymatic method was thus lower than that of the plate count method, although the variation in the latter was higher than that shown in the data of Crisley et al. (8) on the recovery of seeded staphylococci from sterile foods with the use of the TPEY medium. However, these authors also noted that staphylococcal recovery on TPEY medium is affected by increasing contamination of the foodstuff with other microorganisms, the actual, and more realistic, circumstance of the experiment reported in Table 3.

It can also be seen in Table 3 that some of the uninoculated slices of ham were naturally contaminated with *S. aureus*—probably during the commercial slicing and packaging—at a level requiring 16 to 24 hr of incubation at 35 C to become detectable.

Effect of incubation temperature on nuclease production. To assess the effect of incubation temperature on nuclease production, ham slices were inoculated in the usual manner and, with uninoculated controls, were incubated aerobically and anaerobically at 4 and 44 C.

At 4 C, up to 8 days of incubation produced neither appreciable growth nor nuclease production.

At 44 C, borderline nuclease activity (0.01 units per g) was detectable within 24 hr in anaerobically, but not in aerobically, incubated samples. Plate counts indicated 6.0×10^5 *S. aureus* per g anaerobically and 9.0×10^5 aerobically.

After 48 hr, activity was detectable in both aerobic and anaerobic samples (0.02 and 0.03 units per g, respectively). The anaerobic plate count was 2.1×10^6 per g at this time, but the aerobic plate count had fallen to less than 10^2 per g, suggesting that the viable staphylococci had been competitively suppressed between 24 and 48 hr, whereas accumulated nuclease activity persisted.

Although the actual levels of nuclease were lower after incubation at 44 C than at 25 or 35 C, the relative proportion of nuclease to plate count was nearly the same. It thus seems likely that nuclease is produced at any temperature permitting staphylococcal growth.

Heat stability of the nuclease. It was desirable to know the level of thermal stability possessed by nuclease after its production in foodstuffs. This would be an indication of its detectability in foods heated after staphylococcal growth had occurred. It would also provide a test of its identity, providing the stability was high, since no other such markedly heat-stable bacterial nuclease has yet been reported to our knowledge.

Ham slices inoculated with strain 234 were incubated for 24 hr at room temperature and then slurried with twice their weight of distilled water. Samples of the slurry were placed in glass tubes 14 mm in diameter. These were held in an oil

bath at 121 C for various intervals; they were then cooled and analyzed for nuclease.

The nuclease not only was resistant to destruction (Fig. 1) at this temperature, but the apparent activity increased almost fourfold during the first 8 min of heating. Ohsaka, Mukai, and Laskowski (15) also observed an activation of semipurified nuclease held at 100 C, and attributed it to destruction of a thermolabile inhibitor.

It is apparent that extreme thermal stability is characteristic of the nuclease when produced in foodstuffs.

The activation effect was observed in two replications of this experiment and in broth culture supernatant fluids, suggesting that both the sensitivity and specificity of the detection method could be improved by inclusion of a heating step.

Inoculated ham slices were prepared as before and, after 24 hr incubation, were slurried with an equal weight of distilled water and enough $(NH_4)_2SO_4$ to yield 60% saturation. The homogenate was subdivided; the fractions were held for different intervals in a boiling-water bath and then were analyzed for nuclease activity. The results are also shown in Fig. 1.

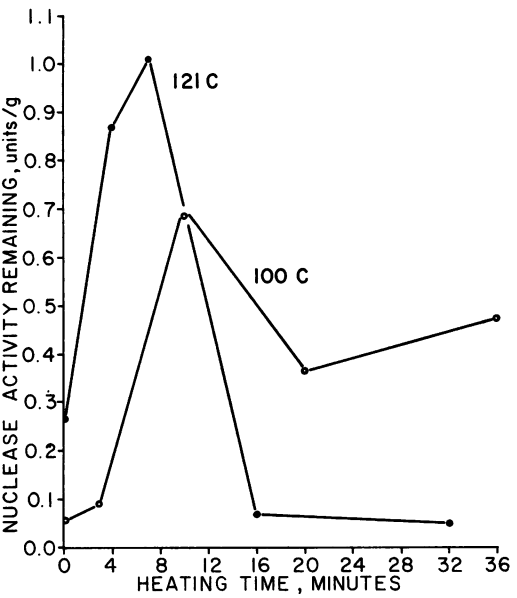

FIG. 1. *Thermal stability of nuclease activity in ham slurries.*

Boiling for 10 min produced a maximal activation, and this boiling step was incorporated into all subsequent analyses.

Noninterference from other bacterial species. The lack of apparent nuclease activity unless *S. aureus* was demonstrably present, even in grossly spoiled foodstuffs, indicated that interference by nucleases, phosphodiesterases, or alkaline phosphatases from microbes other than *S. aureus* was not common. The test conditions —*p*H 8.6, heat-denatured DNA as the substrate, and Ca^{++} as the cofactor—are not optimal, to our knowledge, for any reported nuclease or phosphodiesterase other than the staphylococcal nuclease, and inclusion of heating at 100 C for 10 min in the test routine could be expected to increase further the specificity of the test.

To substantiate this point, ham slices, potato salad, and chocolate eclairs were heavily inoculated with log-phase cultures of a variety of microorganisms that might be anticipated as food contaminants, or that have been reported to produce nucleolytic enzymes. The organisms tested were *Aerobacter aerogenes, Bacillus cereus, B. thuringiensis, Corynebacterium diphtheriae, C. pyogenes, Erwinia carotovora, Escherichia coli, Lactobacillus arabinosus, L. casei, Leuconostoc mesenteroides, Proteus vulgaris, Pseudomonas aeruginosa, Serratia marcesens, Streptococcus faecalis* var. *liquefaciens, S. lactis,* and *S. pyogenes.* The inoculated samples were incubated for 24 hr at 35 C and then were assayed for nuclease, with and without boiling for 10 min.

Only the samples inoculated with *S. faecalis* var. *liquefaciens, C. pyogenes, P. aeruginosa, L. casei,* and *S. marcescens* showed very weak nucleolytic activity. This activity in no case exceeded 3% of that in comparable samples seeded with *S. aureus,* and none of these activities survived boiling.

It was concluded from these results that interference with the test by other bacterial nucleases or deoxyribonucleases is small and that inclusion of the boiling step critically distinguishes the staphylococcal nuclease from these interfering enzymes.

Correlation of nuclease production with enterotoxin production. The study of nuclease production by *S. aureus* 234 at different temperatures had already indicated that, when growth occurred, nuclease production was detectable. However, for the method to be useful in detecting enterotoxigenic strains, nuclease production and enterotoxin production should be shown to vary in synchrony. It was also desirable to know whether the detection of nuclease was as sensitive as the detection of enterotoxin.

S. aureus 234 was grown in shaken broth culture under aerobic (80% O_2-20% CO_2) and anaerobic (80% H_2-20% CO_2) conditions. Previous experience had shown that these conditions result in drastically different productions of α- and β-hemolysins and nuclease, their production being greatly reduced anaerobically.

After 24 hr at 35 C, the culture supernatant fluids were examined for nuclease activity in the usual way, and the enterotoxin end point was determined by extinction titration in the gel double-diffusion system, after 50-fold concentration of the supernatant fluid by osmo-dialysis.

Aerobically, the culture produced 430 units of nuclease and 12 μg of enterotoxin A per ml. Anaerobically, the yields were 28 units and less than 1.0 μg/ml, respectively.

Five 50-g ham slices were inoculated with *S. aureus* 234 and incubated at 35 C for intervals of 10 to 48 hr. Each slice was divided into halves, and one half was analyzed in the usual way for nuclease.

The other half was homogenized, and the homogenate was brought to 40% saturation with $(NH_4)_2SO_4$. After the precipitate was collected and discarded, the supernatant fluid was brought to 85% saturation. The precipitate collected at this level was redissolved in the least volume of 0.1 M phosphate buffer (*p*H 6.8). This was reduced by osmo-dialysis to 0.25 ml, and 0.02 ml of the concentrate, representing about 2 g of the original slice, was tested for the presence of enterotoxin by gel double diffusion.

No precipitin lines appeared, and that this was not due to the procedure was shown by diluting a culture supernatant fluid of the 234 strain until it contained 0.5 μg of enterotoxin per ml and subjecting it to the same procedure. Enterotoxin was readily demonstrable in a volume of the final concentrate that represented about 2 ml (1 μg of enterotoxin) of the diluted starting material.

Casman et al. (7) have shown that the 234 strain inoculated in ham slices and incubated at 30 C for 24 hr produces about 0.1 μg of enterotoxin per g of sliced ham. To detect this level of enterotoxin, they employed column chromatography and osmo-dialysis, following the $(NH_4)_2SO_4$ steps described above. Thus, it was not unexpected that we were unable to detect enterotoxin in an amount of ham that at most should contain about 0.2 μg of enterotoxin, a level below the 1.0-μg sensitivity limit of our slide immunodiffusion procedure. The amount of the concentrate tested, however, represented the maximal amount that could be manipulated in our system without additional steps.

Consequently, the balance of the sample concentrate was subjected to the column chromatographic procedure used to purify enterotoxin. The conductivities of the fractions were determined, and those fractions in which it was anticipated that the enterotoxin would be eluted were concentrated to approximately 20 ml by pervaporation and were reduced finally to 0.05 ml by osmo-dialysis. All of this concentrate was introduced into the well of a diffusion slide by sequential applications of portions, the total representing about 23 g of the ham slice, and a precipitin line against the antiserum was then observed in the halves from the 18-, 24-, 32-, and 48-hr samples, but not in the 10-hr samples.

In the halves used for nuclease analysis, activity was demonstrable in all samples, ranging from 0.04 to 0.50 unit per g in the 10- and 48-hr incubation samples, respectively. Thus, in the 10-hr sample about 10 g of material was necessary for assured demonstration of nuclease, whereas only 0.8 g was necessary in the 48-hr sample.

Detection of nuclease in commercially prepared foodstuff. To assess nuclease recovery from commercial foodstuffs, ham sandwiches were purchased from local restaurants over a period of 2 months. Because the probability of encountering an actual case of dangerous contamination was, presumably, less than 1%, a protocol suggested by our earlier observation of staphylococcal growth when commercially sliced ham was incubated at 35 C was used to increase the probability of positive samples. The sandwiches were placed in a humidified chamber within 30 min of procurement and were incubated aerobically at 35 C. After 16 to 18 hr, they were removed and sliced aseptically into 1-cm cubes; a sample of approximately 10 g was composed by abstracting cubes from all areas of the sandwich. The 10-g sample was homogenized in 90 ml of sterile saline, and the *S. aureus* count was determined by plating. The balance of the sample was homogenized in 60% $(NH_4)_2SO_4$, boiled, and analyzed for nuclease.

As shown in Table 4, 15 of the 48 samples were nuclease-positive, and *S. aureus* could be recovered from 10 of these nuclease-positive samples, but not from any of the nuclease-negative samples.

There were five samples which exhibited nuclease activity but did not yield *S. aureus* on TPEY agar. All had 0.25 unit of activity per g or less (sample 19 with a nuclease activity of 0.21 unit per g, however, did yield *S. aureus* on TPEY agar). Thus, these five either were false positives or the nuclease test was a more sensitive indicator of the presence of *S. aureus* than was TPEY agar. Our earlier results (see *Association of staphylococcal nuclease production and growth in foods*) had suggested that TPEY was recovering 10% or less of *S. aureus* cells present in low numbers in mixed populations. Crisley, Peeler, and Angelotti (8) showed that the re-

TABLE 4. *Staphylococcus aureus colony counts and nuclease activity of commercially prepared ham sandwiches incubated aerobically for 16 to 18 hr at 35 C*

Sample no.[a]	*S. aureus* (log colony count/g)	Nuclease activity (units/g)
16	<2.00	0.17
20	<2.00	0.18
21	<2.00	0.22
8	<2.00	0.23
36	<2.00	0.25
32	4.45	0.34
31	4.62	0.38
24	4.75	0.67
19	5.35	0.21
42	5.52	0.35
9	5.74	0.51
25	6.06	0.47
27	6.20	0.35
45	6.65	0.49
41	6.86	0.42

[a] The samples reported are those that showed nuclease activity of the 48 examined.

covery ability of TPEY agar decreases when the fraction of *S. aureus* in the total population is small. The total population (determined arbitrarily in every 8th sample) of samples 8, 16, 24, 32, 40, and 48, was, respectively, 5.3×10^8, 9.2×10^8, 6.6×10^8, 4.1×10^8, 6.1×10^8, and 4.4×10^8 bacteria per g, exceeding 4×10^8 bacteria per g in every case.

As a test of the supposition that TPEY agar was not recovering *S. aureus* when it was present in the incubated samples in relatively small numbers, a strain isolated from sample 25 was grown for 19 hr at 35 C in Trypticase Soy Broth. The culture was sequentially diluted in sterile broth, and plate counts were made of each dilution in Trypticase Soy Agar. A portion of each dilution was then added to a portion of a homogenate of a commercially prepared ham sandwich incubated and homogenized in the same manner as the samples in Table 4. The homogenate samples receiving *S. aureus* and control portions receiving no addition of *S. aureus* were plated in TPEY agar and a total count was made on Trypticase Soy Agar.

Calculations based on the counts obtained on Trypticase Soy Agar from the broth dilutions of *S. aureus* indicated that the microorganism had been added to the homogenate at levels of 8.3×10^6, 8.2×10^5, 8.4×10^4, 8.8×10^3, 8.8×10^2, and 85 colony-forming units per g of ham.

The control homogenate receiving no addition of *S. aureus* yielded no *S. aureus* colonies on TPEY agar, and the mean of the total plate counts for all the homogenate samples was 6.8×10^8 bacteria per g.

None of the samples receiving less than 8.4×10^4 *S. aureus* cells per g yielded detectable *S. aureus* colonies on TPEY agar. The means of the triplicate TPEY counts on the samples receiving 8.4×10^4, 8.2×10^5, and 8.3×10^6 *S. aureus* cells per g were 3.2×10^4, 4.9×10^5, 6.1×10^6 per g, respectively.

It thus appeared that the ability of TPEY agar to recover *S. aureus* present at less than 10^5 colony-forming units per g of ham was markedly decreased when the total bacterial population was high. Under these conditions, there was a heavy, confluent outgrowth of other species at the lower sample dilutions that interfered with detection of *S. aureus* colonies, a circumstance also noted by Crisley, Peeler, and Angelotti (8).

Such an outgrowth of other bacterial species at the lower sample dilutions was not observed in the experiments with *S. aureus*-seeded ham slices (Table 3), making it possible to account for the detection of lower levels of *S. aureus* in those samples on the basis of a larger *S. aureus* to total count ratio.

To establish why the nuclease content of the samples in Table 4 did not increase in close agreement with increase in colony count as it did in Table 3, the nuclease production ability of the *S. aureus* isolate made from sample 25 was compared to an isolate made from sample 9. Both were grown in broth, seeded onto ham slices, incubated, and analyzed for nuclease and colony-forming units by use of the same procedures that were used for the ham slices described in Table 3.

After aerobic incubation for 24 hr at 35 C, ham seeded with the isolate from sample 25 yielded 0.42 unit of nuclease and 6.4×10^9 colonies per g, whereas the ham seeded with the isolate from sample 9 yielded 0.65 unit of nuclease and 5.1×10^9 colonies per g.

Thus, the isolates differed from each other and from the 234 strain used in the earlier experiment in nuclease productivity. The lack of close agreement between colony count and nuclease activity apparent in Table 4 reflects differences in nuclease productivity among the *S. aureus* strains randomly isolated by the experimental procedure employed.

Consequently, the results with the ham sandwiches provided the second indication that, in large, mixed populations, the nuclease test was actually more sensitive than the selective plating medium. The first was noted earlier in connection with the apparently higher yield of nuclease per

staphylococcal cell in ham slices with a mixed flora than in pure, broth cultures. This would be the expected observation if the actual staphylococcal population in the ham were larger than the plate count indicated.

Six samples of cheese were then obtained from E. P. Casman of the Microbiology Division of the Bureau of Science of the Food and Drug Administration. The samples, identified only by the letters A to F, contained a mixture of samples, some from a lot of kumminost cheese identified as containing staphylococcal enterotoxin by the serological method (Casman, Ann. Meeting Am. Public Health Assoc., 1966) and the rest from cheese lots determined to be free from enterotoxin by the same method (thus containing less than 0.005 μg of enterotoxin A, the sensitivity limit of this serological method).

Triplicate 10-g amounts of each sample were analyzed for nuclease activity. After emulsification in 60% $(NH_4)_2SO_4$, boiling for 10 min, and centrifugation, the solution supernatant to the precipitate and subnatant to the lipid layer was aspirated from the centrifuge tube and used for the subsequent steps of the analysis.

A comparison of the serological and enzymatic analyses is shown in Table 5.

The correlation of nuclease activity with the serological test was good, except for sample A, which was serologically negative but nuclease-positive. Since the cheese-making process is exposed to contamination with bovine-derived strains of *S. aureus*, which frequently do not produce serologically recognizable enterotoxin (5), it is at least as likely that sample A represents a cheese lot contaminated with such a strain as it is that it is a false positive.

The recovery of nuclease activity in the serologically positive samples was unexpectedly low in comparison with the results on ham, and it was found that activity was being carried off with the discarded lipid layer. Preliminary attempts to recover the activity from this layer were unsuccessful and were abandoned when the sample material was exhausted.

TABLE 5. *Comparison of nuclease and enterotoxin analyses of cheese samples*

Sample of designation	Avg nuclease activity	Serological test for enterotoxin
	units/10 g	
B	0.28	+
A	0.21	−
F	0.20	+
E	0.12	+
C	0.03	−
D	0.05	−

DISCUSSION

Measurement of nuclease activity is a sensitive means for detection of foods contaminated with the metabolic products of *S. aureus*. The usefulness of the nuclease test as an indicator of such contamination is increased by the relative rapidity with which it can be conducted (the procedure takes less than 3 hr to complete) and by the fact that it requires no uncommon reagents or equipment.

The kinds of foods to which the procedure has been applied thus far (ham, soft cheese, and potato salad), although limited in number, are representative of quite different types of foodstuffs: meats, dairy products, and vegetables. They are also three foods often specifically implicated in staphylococcal food poisoning.

What constitutes a significant level of nuclease activity in ham from a public health standpoint can be estimated by comparison of nuclease activity with corresponding staphylococcal plate counts and enterotoxin production. In these experiments, whenever 0.34 unit of nuclease, or greater, was detected it was always possible to demonstrate the presence of *S. aureus*. From the comparison of nuclease and enterotoxin production by *S. aureus* 234, production of 0.34 unit of nuclease corresponds to production of 9.5×10^{-3} μg of enterotoxin, an amount likely to be much less than an emetic dose for humans (2). Consequently, detection of 0.34 unit of nuclease indicates staphylococcal contamination with certainty, and, at this nuclease level, it is unlikely that a toxemic concentration of enterotoxin would be missed.

The specificity of the method and lack of interference, even from large, mixed populations of microorganisms, was unexpected. Production of exocellular, nucleic acid-hydrolyzing enzymes is commonly observed in microbial genera: Wagner (18), for instance, reported that 35% of 612 strains of bacteria he examined produced exocellular deoxyribonucleases. Also, at least two other microbial exocellular nucleases have been reported (10, 13, 17), and, of course, a large number of animal and plant nucleodepolymerases, as well as endocellular microbial nucleases, are known (3).

However, the absence of interfering enzymes in pure cultures of microorganisms commonly contaminating foods, or known to be capable of exo- or endonuclease production, as well as their absence from the large, mixed bacterial populations and from the foodstuffs tested, makes it appear that, in the conditions optimal for its activity and in its extraordinary heat stability (reaffirmed in these studies by the results re-

ported in the section *Heat stability of the nuclease*), staphylococcal nuclease is a unique enzyme.

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