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Applicability of Aeration and Delayed Addition of Selenite to the Isolation of Salmonellae

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The effect of aeration combined with the delayed addition of selenite on the lag period of several strains of salmonellae and other enterobacteria is reported. A procedure has been developed involving shaking of the sample in a basal medium for 4 hr at 37 C, adding selenite and cystine, and continuing shaking for 20 hr. Confirmation by selective plating, biochemical tests, and serology gave results comparable to the standard lactose pre-enrichment method with the saving of 24 hr and elimination of one set of media. Confirmation by fluorescent-antibody tests showed that fewer positive fluorescent stains were obtained from the aerated procedure than from the lactose pre-enrichment procedure. Precautions in the application of this procedure are discussed.

The standard procedure for the isolation of salmonellae from foods is a time-consuming and laborious method involving pre-enrichment, enrichment, selective plating, biochemical tests, and finally serology (3, 4). The search for more rapid and sensitive techniques has led to a proliferation of methods which propose new and modified media (8, 11, 12), new equipment (1), and accelerated serology through the use of fluorescent microscopy (5, 7) and polyvalent antisera (14, 16). In the standard procedure and in most of the modifications that have been suggested, the different stages are incubated for 18 to 48 hr without agitation (3, 4).

Although aeration to accelerate aerobic growth has been used widely in the fermentation industry and in growth studies, its potential for speeding up the isolation of salmonellae has received only sporadic attention. After the preliminary report of our investigation appeared (J. A. Alford and N. L. Knight, Bacteriol. Proc., p. 1-2, 1969), two other studies came to our attention. One of these (N. F. Insalata and F. C. A. Sunga, Bacteriol. Proc., p. 2, 1969) indicated that aeration is useful for accelerating detection of salmonellae by fluorescent microscopy, and the other (2) showed that aeration of chocolate and chocolate-containing products produces substances inhibitory to salmonellae outgrowth.

The present investigation was undertaken to determine whether aeration would alter the sensitivity of either *Escherichia coli* or salmonellae to selenite and to determine whether a short delay in the addition of selenite could be used to combine pre-enrichment and enrichment in a single flask. From these studies, an accelerated procedure combining aeration and delayed selenite addition was developed and tested on several food products.

MATERIALS AND METHODS

Cultures, media, and chemicals. Most of the cultures used in this study were obtained from Alice Moran, Consumer and Marketing Service, U.S. Department of Agriculture, Beltsville, Md. A few were from our own stock culture collection originally obtained from several sources. The culture media and ingredients were from Difco and BBL; the sodium acid selenite was from BBL. The usual formula for selenite-cystine broth (3), adjusted to *p*H 7.0, was used throughout this investigation unless noted otherwise. To facilitate the delayed addition of selenite or selenite-cystine to the media, a solution of these ingredients was prepared, its *p*H was adjusted to 7.0, and then it was sterilized by filtration through a membrane filter.

Incubation. Most of the flasks and jars involving aeration were incubated on a rotary shaker operated at 200 rev/min in a forced air chamber at 37 C. Nonaerated samples were incubated on a shelf in the same incubator (model G-27; New Brunswick Scientific Co., New Brunswick, N. J.). A few studies were also done on rotary or reciprocating water bath shakers (model D-1, Aminco, Silver Spring, Md., and models R-77 and G-77, New Brunswick Scientific Co.).

Growth curves. Growth rates of several cultures were determined in selenite-cystine broth and in the same medium with selenite and cystine omitted (basal). A 1% inoculum of an 18- to 21-hr nutrient

broth culture was added to 35 to 50 ml of medium contained in a 300-ml Erlenmeyer flask equipped with a side-arm tube (Bellco Glass, Vineland, N.J.). Optical density was determined on a Beckman model B spectrophotometer at 550 nm.

Enumeration of salmonellae in foods. Samples of egg albumen, meat and bone meal, poultry, and artifically contaminated ground pork were examined by the three-tube, three-dilution most-probable-number (MPN) method (4). The size of the sample varied according to the level of contamination expected with a 1,10 (w/v) ratio of sample to medium maintained, except for a few heavily contaminated samples for which the ratio was smaller. The levels of salmonellae contamination ranged from 1 per 30 g to 100+ per g; most of the samples contained 1 to 10 salmonellae per g. All samples, except egg albumen, were mixed with test medium in a Waring Blendor for 1 min. Appropriate portions or dilutions of the sample were distributed into sterile flasks or tubes under a laminar-flow sterile air hood. Samples of egg albumen were weighed, added to the broth, and shaken by hand. For the examination of sausage and sausage ingredients, single 10-g samples, rather than portions of a single larger sample, were examined in each of the methods being tested.

After enrichment by the procedure under investigation, the samples were examined for salmonellae by streaking on Brilliant Green Agar (BGA), picking colonies to Triple Sugar Iron Agar (TSIA), and confirming serologically with *Salmonella* O antiserum (poly A to I), O group antisera A to E, and H antiserum, poly a to z. All antisera were from Difco.

Lactose pre-enrichment. The Bacteriological Analytical Manual (BAM) procedure of the Food and Drug Administration (3) was used as the standard method for comparison.

Fluorescent microscopy. At the time of streaking onto BGA, a loopful of the selective medium also was used to prepare slides for fluorescent microscopy. The procedure was that used by the National Communicable Disease Center (*personal communication*), with *Salmonella* polyvalent O globulin, groups A to H, fluorescein-conjugated, as prepared by the Sylvana Co., Millburn, N.J.

RESULTS

Effect of aeration, incubation temperature, and selenite concentration on length of lag. The prolonged lag in stationary cultures of *E. coli* as compared to salmonellae when grown in the presence of selenite is the basis for the use of selenite enrichment medium (9). There is little or no lag differential without selenite. To determine whether aeration affects this lag differential, *Salmonella typhimurium*, *S. derby*, *S. enteritidis*, *S. tennessee*, and three strains of *E. coli* were grown in stationary culture with and without added selenite. In no instance was the difference in lag with and without selenite affected by aeration.

Galton, Morris, and Martin (4), in reviewing the effect of temperature of incubation on the sensitivity of the enrichment medium, indicated that increasing the temperature from 37 to 42 C had been found useful by some investigators whereas others had found it decreased sensitivity. Higher levels of selenite than the usual 0.4% have also been investigated (17). To determine whether aeration combined with one or both of these changes would enhance selectivity, *S. blockley* and *E. coli* were inoculated into replicate flasks of selenite-cystine broth containing 0.4 and 0.8% selenite and incubated at 37 and 42 C. The results in Table 1 show that doubling the selenite concentration in aerated cultures had a greater effect on *S. blockley* than on *E. coli*, and raising the incubation temperature shortened the lag phase of *E. coli* more than that of *S. blockley*. When higher selenite concentration was combined with higher temperature, the selective action of selenite was decreased even

TABLE 1. *Effect of increasing temperature and selenite concentration on length of lag of S. blockley and E. coli*

Culture	0.4% Selenite		0.8% Selenite	
	Lag period at		Lag period at	
	37 C	42 C	37 C	42 C
	hr	*hr*	*hr*	*hr*
S. blockley	4	5	8	>14
E. coli	8	5	8	11

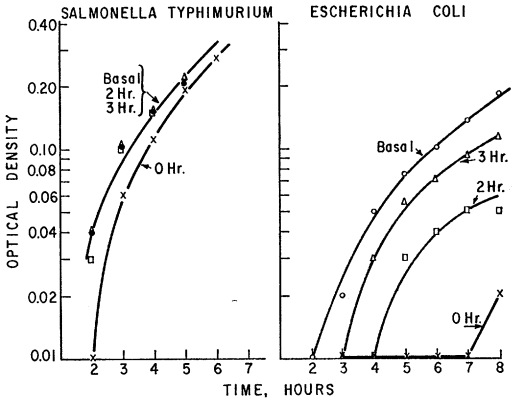

FIG. 1. *Effect of time of addition of selenite and cystine to basal medium on growth of several Enterobacteriaceae. Curves similar to those of S. typhimurium were obtained with S. enteritidis, S. blockley, S. oranienburg, S. tennessee, Proteus species, and a strain of the Arizona group. Curves similar to those of E. coli were obtained with S. choleraesuis and S. choleraesuis var. kunzendorf.*

more. For subsequent experiments, therefore, 0.4% selenite and 37 C were used.

Effect of delayed addition of selenite on growth. Since pre-enrichment is recognized as a useful procedure for improved isolation of salmonellae (3, 4), the possibility of combining pre-enrichment and enrichment in the same flask by delayed addition of selenite was investigated. With most salmonellae, as well as other enterobacteria which are not inhibited by selenite, the time of addition of selenite to the culture had little effect on growth (Fig. 1). With *E. coli*, a definite interruption of growth was evident for as long as 3 hr; *S. choleraesuis* was quite similar to *E. coli* in its response.

Determination of optimal conditions for aerated procedure. The studies with pure cultures of salmonellae and *E. coli* indicated that aeration and delayed addition of selenite might be useful in shortening the procedure for salmonellae isolation. Therefore, a series of combinations of time of selenite addition and length of incubation were tested on dried egg albumen to select the best combination. A series of MPN determinations showed that maximum sensitivity was obtained when cystine was added along with selenite after 4 hr rather than being in the medium from the beginning (Table 2). Table 3 shows that pre-enrichment with aeration for 4 hr, followed by addition of selenite-cystine with continued aeration for 18 to 20 hr, gave the highest percentage of positive samples. No differences in sensitivity were noted with this procedure within the *p*H range of 6.5 to 7.5.

Comparison of methods. A limited number of samples of various food products, some naturally contaminated and others artificially contaminated and frozen, were examined by the aeration method and compared to the standard selenite-cystine enrichment procedure with and without pre-enrichment. Although an insufficient number of positive samples have been examined to permit a significant statistical evaluation, the data in Table 4 indicate that the shortened procedure can give results comparable to the BAM procedure. Near the end of the study, the ES procedure of Sperber and Deibel (16) was published. To explore the possibility of using aeration and delayed selenite addition in conjunction with their procedure, a series of samples of pork sausage and sausage ingredients were examined by four methods: (i) BAM procedure, (ii) M broth of ES procedure inoculated from the selenite of BAM procedure, (iii) aerated procedure, and (iv) M broth of the ES procedure inoculated from the aerated selenite and examined after 6 hr (total 28 to 30 hr). Of the 100 samples examined, 16 were positive by the BAM procedure, 17 by the ES procedure, 12 by the aerated

TABLE 2. *Effect of time of addition of cystine to aerated selenite medium on the isolation of salmonellae from dried egg albumen*

Trial	Cystine added[a]		Lactose procedure[b]
	Initially	After 4 hr	
	MPN/g	*MPN/g*	*MPN/g*
1	0.6	110	110
2	0.4	9.3	21
3	0.4	9.3	21
4	0.4	9.3	9.3
5	0.6	9.3	9.3
6	0.4	9.3	9.3

[a] In both instances, selenite was added at 4 hr.
[b] Lactose pre-enrichment standard procedure (BAM).

TABLE 3. *Comparison of efficiency of recovery of salmonellae from food products by several experimental methods*

Per cent of samples positive by	
Experimental method[a]	Lactose pre-enrichment[b]
0 (A)	67
0 (B)	67
17 (C)	82
33 (D)	82
33 (E)	50
50 (F)	67
50 (G)	67
65 (H)	65

[a] The experimental methods compared are indicated by the letters in parentheses following the data. These methods were as follows, with confirmation on BGA plates, TSIA, and serology. (A) selenite-cystine broth shaken for 6 hr at 37 C; 12 samples used. (B) selenite-cystine broth shaken for 6 hr at 42 C; 12 samples used. (C) basal medium shaken for 6 hr at 42 C; add selenite-cystine and incubate stationary for 18 hr; 6 samples used. (D) Basal medium shaken for 6 hr at 42 C; add selenite-cystine and incubate in a shaker for 18 hr; 6 samples used. (E) Lactose pre-enrichment for 24 hr stationary at 37 C; inoculate selenite-cystine broth, shaken for 6 hr at 37 C; 6 samples used. (F) Basal medium shaken for 4 hr at 37 C; add selenite-cystine, and incubate for 18 to 20 hr stationary; 18 samples used. (G) Basal medium shaken for 21 hr at 37 C; inoculate into selenite-cystine broth and shake for 6 hr; 12 samples used. (H) Basal medium shaken for 4 hr at 37 C; add selenite-cystine and shake for 18 to 20 hr; 17 samples used.
[b] Standard BAM procedure (3).

TABLE 4. *Comparison of methods for isolation of salmonellae from food products*

Method	No. of samples	Per cent of samples with MPN		
		A > B	A = B	A < B
Aeration[a] and pre-enrichment[b]	27	26	52	22
Aeration and selenite-cystine[c]	25	28	60	12
Pre-enrichment and selenite-cystine	23	39	48	13

[a] Aerate for 4 hr, add selenite-cystine, and aerate for 20 hr; BGA, TSIA, serology.
[b] BAM procedure (3).
[c] No pre-enrichment, otherwise same as footnote *b*.

TABLE 5. *Comparison of aerated procedure with lactose pre-enrichment as a source of sample for fluorescent-antibody (FA) staining*

Source of sample	No. of samples[a]	
	+	−
Aerated[b]	91	89
Pre-enriched[c]	97	83

[a] Seven of the aerated and six of the pre-enriched FA-positive samples were not confirmed by biochemical and serological tests; seven of the aerated and six of the pre-enriched FA-negative samples were confirmed.
[b] Basal medium was shaken for 3 to 4 hr; selenite-cystine was added and shaken for 20 to 21 hr.
[c] Lactose broth pre-enrichment, 24 hr; selenite-cystine broth, 24 hr.

procedure, and 23 by the aerated-M broth procedure.

Fluorescent-antibody microscopy. The data in Table 5 show that about 10% fewer positive fluorescent stains were obtained from the aerated procedure than from the standard pre-enrichment procedure. Most of this difference occurred in samples with very low counts.

DISCUSSION

Cells normally resistant to selective agents such as selenite often become sensitive to them when exposed to sublethal heating or drying. The presumed function of pre-enrichment is to allow these injured cells a chance to begin active growth in a noninhibitory medium (4). Ordal and coworkers (6, 15) have shown that repair of heat-injured *Staphylococcus aureus*, as far as ability to multiply in a limiting medium is concerned, can occur without cell multiplication. If the major inhibitory effect of selenite on injured salmonellae disappears by the time the cells have undergone one or two multiplications, then delaying the addition of selenite for this length of time should minimize its effect on salmonellae without affecting its inhibition of coliforms. The data presented here support this conclusion but show that this delay should be minimal since there is little inhibition of *E. coli* once it is growing logarithmically.

The mechanism of inhibition of *E. coli* is not clear although it has been investigated (13, 17). The lack of inhibition of actively growing cells is probably not the result of accumulation of some selenite-detoxifying product within the cell, since cells that were in the stationary phase for only a few hours were selenite-sensitive (e.g., an 18-hr culture was used as the inoculum to show the prolonged lag). We are presently investigating the mechanisms of action of both selenite and tetrathionate.

The lack of any negating effect of aeration on salmonellae outgrowth in a selenite medium or on the toxicity of selenite to *E. coli* indicates that the accelerated rate of growth and higher cell populations in aerated cultures should be useful in isolating salmonellae. The procedure developed in this investigation eliminates the inoculation of one set of media as well as saving at least 24 hr when compared to the standard pre-enrichment procedure. The limited data on sausages indicate that a combination of aeration and delayed selenite addition with the M broth of the ES procedure may provide a further possibility for shortening the time required for testing.

Two precautions should be recognized in the application of aeration and delayed selenite addition. First, variations in the time required for injured cells to repair themselves are more critical in this procedure than in the BAM procedure. Lengthening or shortening the time of addition of selenite to the medium may be necessary, since the inhibitory effect of added selenite decreases when the culture begins active multiplication. Secondly, the report that toxic compounds may be formed from some foods when aerated (e.g., chocolate) indicates a need to explore this possibility for each food. Workers at the University of Wisconsin (*personal communication*) have indicated that the procedure of aeration and delayed selenite addition lacked sensitivity in their examination of samples with very low numbers of salmonellae and large numbers of competing organisms. Although details of their study are not available, competition with other organisms as well as composition of the food and

availability of shaker-incubator space may limit the usefulness of the method. Nevertheless, for quality control in which time and media preparations are important factors, aeration and delayed addition of selenite may be combined to give a useful procedure.

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