Agglutinating Antiserum for the Isolation of *Salmonella* with Special Reference to Isolation from Egg Albumin[1]

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Food products that are examined for the presence of *Salmonella* usually contain a mixed bacterial flora; when present, the *Salmonella* may represent only a small fraction of the total microbial population. Selective enrichment broths are used to enhance the possibility of isolating *Salmonella* from these mixed populations. To recover the *Salmonella*, the preferential multiplication of the *Salmonella* in the enrichment broths must result in a numerical ratio of *Salmonella* to "contaminants" which permits the growth of at least one *Salmonella* colony among the total isolated colonies which develop on the plating medium. If the plating medium selectively inhibits some of the contaminants, then *Salmonella* should be recovered from a smaller *Salmonella* to contaminants ratio.

Salmonella may be present in the enrichment cultures even though colonies of these organisms are not found on streaked plates. More successful isolations are obtained when several different selective plating media are used instead of one (Edwards and Ewing, 1955). Duplicate streaked plates may show entirely different results when incubated at different temperatures; for example, 37 and 43 C. If the coliforms present do not grow well at the higher temperature, then the 37 C plate may be negative for *Salmonella* whereas the 43 C plate may have an abundance of *Salmonella* colonies. When mixtures of pure cultures of a *Salmonella* and a coliform are prepared to give a very high coliform to *Salmonella* ratio, no *Salmonella* colonies may be found on the streak plates, although the mixture may actually contain thousands of *Salmonella* per ml.

Any method which would consistently recover these undetected *Salmonella* in the enrichment broth would increase the sensitivity of the isolation procedure. The isolation of *Brucella* from feces without the use of any selective enrichment broth or plating medium has been accomplished by agglutination (Eyre, 1908; Amoss and Poston, 1929, 1930). In these instances, the fecal material contained millions of *Brucella* per gram and the antiserum was used for specifically agglutinating and concentrating the desired organism before plating.

Where only a few *Salmonella* are present in a sample, the agglutination of these organisms by the antiserum would be unlikely. If, however, the sample is incubated in a broth containing *Salmonella* agglutinating antiserum, the multiplication of the *Salmonella* should result in the formation of *Salmonella* aggregates which can be differentially concentrated by appropriate centrifugation. The removal of the supernatant should leave a *Salmonella* enriched sediment which would be more favorable for the desired isolation.

Materials and Methods

The agglutinating antisera used in the experiments involving known *Salmonella* types were prepared in our own laboratory (Edwards and Ewing, 1955), although a few preliminary experiments were performed with commercial antiserum. The antisera were added to the culture medium to a final concentration corresponding to the highest dilution which gave good tube agglutination (1:1000 to 1:4000). The polyvalent *Salmonella* flagellar agglutinating antiserum employed in the dried egg white experiments was kindly supplied by Dr. P. R. Edwards[2] and was used at a final concentration of 1:400 to 1:500, except where noted.

Salmonella strains used for the mixture of pure cultures were stock cultures obtained from several laboratories. Coliforms were isolated from powdered egg whites and were selected for their ability to grow well on the culture media used. The inocula for the experiments with mixtures of pure cultures were prepared so that 0.1 ml of the mixtures contained several million coliforms and decimally decreasing numbers of *Salmonella*. The actual numbers inoculated were calculated from standard plate counts of the 3 to 4-day-old broth cultures. The experiments were usually done in triplicate.

The dried egg whites were supplied by Dr. W. I. Taylor[3] or were egg whites in packaged angel food or chiffon cake mixes. Sterilized egg albumin was prepared by autoclaving in a moisture proof container. The solubility of this egg albumin was slightly less than that of the unautoclaved albumin but still contained the factor(s) which interferes with the isolation of *Salmonella* by the direct enrichment procedure.

Enrichment in selenite F broth supplemented with

[1] This work was supported by contributions from various companies and associations of food industries.

[2] Communicable Disease Center, U. S. Public Health Service, Atlanta, Georgia.

[3] Swift and Company, Chicago, Illinois.

cystine (North and Bartram, 1953) followed by streaking on brilliant green agar was the standard procedure of isolation (Taylor, Silliker, and Andrews, 1958) against which the efficacy of the antiserum method was compared. The antiserum was added to nutrient broth or to lauryl tryptose broth; except for the omission of lactose, the latter is the medium used for the confirmatory procedure in water analysis.

In the isolation of *Salmonella* from egg albumin, six 1-g aliquots of each sample were tested by each method. The addition of this amount to 10 ml of the appropriate broth gave a final concentration of 10 per cent egg albumin and minimized the amount of polyvalent antiserum needed. To attain end points, some high *Salmonella* count egg whites were mixed with other samples which contained many coliforms but no detectable *Salmonella*.

The direct method of isolation consisted of dissolving the 1-g aliquot in 10 ml of the proper broth. The removal of the soluble components of the egg albumin which impairs the selectivity of the enrichment broth in the centrifugation method (Silliker and Taylor, 1958) was accomplished by dissolving the egg albumin aliquot in 10 ml of saline and centrifuging at maximal speed in the International clinical centrifuge (model CL)[4] for 20 min. The supernatant was decanted and the sediment suspended in 10 ml of the appropriate broth for incubation.

When the enrichment was in broth containing antiserum, the cultures were centrifuged after 18 hr incubation at 37 C. Minimal centrifugation was used to pack the agglutinated *Salmonella* sufficiently so that the supernatant could be decanted without losing detectable numbers of *Salmonella*. In our clinical centrifuge this was 30 sec after reaching full speed. The sediments obtained by centrifugation were suspended in a few ml of selenite-cystine broth and streaked on brilliant green agar. More selenite-cystine was added to a total of 10 ml. After 18 hr incubation this "enriched" culture was also streaked.

[4] International Equipment Company, Boston, Massachusetts.

TABLE 1

*Isolation of Salmonella typhimurium from nutrient broth containing phase 1 and 2 flagellar antisera**

Inoculum		Control	Nutrient Broth Containing Antisera		
Coliform	*S. typhimurium*	Selenite-cystine	Supernatant†	Sediment	Sediment incubated in selenite-cystine
5.9×10^6	3.4×10^5	+++	−−−	+++	+++
5.9×10^6	3.4×10^4	+++	−−−	+++	+++
5.9×10^6	3.4×10^3	+++	−−−	+++	+++
5.9×10^6	3.4×10^2	+++	−−−	+++	+++
5.9×10^6	3.4×10^1	+++	−−−	+−+	+++
5.9×10^6	3.4×10^0	+−−	−−−	++−	+++

* Increasing ratio of coliform to *S. typhimurium* in mixed inoculum. Experiment in triplicate. Selenite-cystine broth used as a control.

† Streak of supernatant after 18 hr incubation and centrifugation.

RESULTS

The effectiveness of agglutinating antiserum for separating out the multiplying *Salmonella* cells is shown in table 1. The proper final concentrations of phase 1 and 2 flagellar antisera of *S. typhimurium* were added to nutrient broth tubes which were then inoculated with different ratios of coliform and *S. typhimurium* cells. Selenite-cystine broth tubes were similarly inoculated.

The addition of specific agglutinating antisera makes nutrient broth as effective a medium for isolating *Salmonella* as selenite-cystine broth. The *Salmonella* isolation was not as good when the antiserum was added after the mixed inocula had been first incubated in plain nutrient broth or when the antiserum was added to selenite-cystine broth. Most of the subsequent work was done with lauryl tryptose broth instead of nutrient broth since the former should have specific inhibitory action against the gram-positive rods.

Experiments with other coliforms and *Salmonella* serotypes (*S. oregon*, *S. newington*) indicate that the results in table 1 were not an isolated phenomenon peculiar to the two strains of microorganisms used. Somatic antisera were just as effective as flagellar antisera, but H antisera were preferred since higher titers can be obtained and agglutination is more flocculent than with O antisera.

The presence of egg albumin in the selenite-cystine

TABLE 2

*Improved Salmonella typhimurium recovery from nutrient and lauryl tryptose broths containing phase 1 flagellar antiserum**

Inoculum		Recovery of *Salmonella*					
			10% egg albumin in medium				
Coliform	*S. typhimurium*	Selenite-cystine control	Selenite-cystine	Nutrient broth with antiserum		Lauryl tryptose with antiserum	
				Sediment	Enriched†	Sediment	Enriched†
1.7×10^6	2.6×10^5	+++	+++	+++	+++	+++	+++
1.7×10^6	2.6×10^4	+++	−−−	+++	+++	+++	+++
1.7×10^6	2.6×10^3	+++	−−−	+++	+++	+++	+++
1.7×10^6	2.6×10^2	+++	−−−	+++	+++	+++	+++
1.7×10^6	2.6×10^1	+++	−−−	+++	+++	+++	+++
1.7×10^6	2.6×10^0	−−−	−−−	++−	+−−	+++	−++

* Sterilized egg albumin present in 10 per cent concentration where indicated; selenite-cystine control has no egg albumin. Experiment in triplicate.

† Sediment obtained by centrifugation after 18 hr incubation in antiserum containing broth incubated 18 hr in selenite-cystine broth before streaking.

enrichment medium makes the recovery of *Salmonella* difficult. The experiments described above suggested the possibility that better isolation of *Salmonella* may be possible even in the presence of the inhibitory factors of egg albumin. In this case only the phase 1 antiserum was used since the *S. typhimurium* strain was found to be stable in this phase. Sterilized egg albumin was used in a final concentration of 10 per cent. The direct method of isolation was used (table 2).

In the absence of egg albumin the recovery of *S. typhimurium* from selenite-cystine broth inoculated with a mixture of pure cultures was good. The presence of 10 per cent of sterilized albumin markedly reduced the effectiveness of the cystine selenite broth as a selective enrichment medium. On the other hand, even in the presence of egg albumin, good recoveries were obtained from nutrient broth and lauryl tryptose broth containing agglutinating antiserum.

These results, while suggestive of the sensitivity of the antiserum method for the isolation of *Salmonella* from egg albumin, do not prove that the method will be effective in the isolation of these organisms from commercial powdered egg whites which would contain a mixed flora. Egg albumin samples of diverse sources were, therefore, tested (table 3). The egg albumins were (1) added directly to selenite-cystine, column A; (2) centrifuged in saline and the sediment incubated in selenite-cystine broth (centrifuge method), column B; (3) added directly to lauryl tryptose broth containing polyvalent flagellar antiserum, column C; (4) centrifuged in saline and the sediment incubated in lauryl tryptose broth with antiserum, column F. In methods (3) and (4) the sediment obtained by light centrifugation after 18 hr incubation in the antiserum containing broth was then incubated in selenite-cystine for selective enrichment, columns D and G. Since D and G are the continued analysis of the aliquots started in C and F, respectively, the total number of *Salmonella* isolations from the six aliquots per albumin sample by the direct or centrifugation methods is tabulated in columns E and H. In most instances this is the higher of the figures of columns C and D or F and G but this is not always the case. For example, with egg albumin sample no. 3, three of six cultures are positive in F and G, but *Salmonella* was isolated from four of the 1-g portions tested. This is because one culture positive from the streak of the agglutinated sediment was negative upon enrichment in selenite-cystine broth while the reverse occurred with a different g aliquot.

TABLE 3

Recovery of Salmonella from powdered egg albumin by different methods. Number of Salmonella positive tubes per number of 1-g aliquots tested

Sample No.	Selenite-Cystine		Lauryl Tryptose with Antiserum					
			Uncentrifuged			Centrifuged		
	Uncentrifuged	Centrifuged	Sediment	Enriched*	Combined†	Sediment	Enriched*	Combined†
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
1	1/6	3/6	5/6	6/6	6/6	5/6	6/6	6/6
2	2/6	0/6	6/6	6/6	6/6	4/6	6/6	6/6
3	0/6	2/6	0/6	0/6	0/6	3/6	3/6	4/6
4‡	0/6	2/6	0/6‡	2/6‡	2/6‡	3/6‡	3/6‡	3/6‡
5	0/6	2/6	1/6	2/6	2/6	1/6	2/6	2/6
6	1/6	3/6	1/6	2/6	3/6	2/6	4/6	4/6
7	1/6	3/6	0/6	3/6	3/6	5/6	3/6	5/6
8	1/6	2/6	1/6	3/6	3/6	3/6	4/6	5/6
9	1/6	0/6	1/6	1/6	2/6	1/6	3/6	3/6
10	0/6	0/6	0/6	1/6	1/6	2/6	5/6	5/6
11	0/6	1/6	1/6	2/6	2/6	2/6	3/6	3/6
12	2/6	2/6	2/6	3/6	3/6	4/6	4/6	5/6
Totals...	9/72	20/72	18/72	31/72	33/72	35/72	46/72	51/72

* Sediment obtained from growth in antiserum-containing broth enriched in selenite cystine.

† Total number of aliquots positive for *Salmonella*.

‡ Antiserum at 1:200 dilution instead of 1:400. Antiserum methods were negative for *Salmonella* at 1:400 dilution of polyvalent antiserum. *Salmonella* isolated did not agglutinate at 1:400 but did at 1:200.

The inoculated broths were incubated for 18 hr and streaked on brilliant green agar plates. Suspicious colonies were tested on triple sugar agar slants and identified as *Salmonella* by slide agglutination with polyvalent and then group *Salmonella* antisera. Urea breakdown was tested where indicated.

Comparison of columns A and B supports the results of Silliker and Taylor (1958) that the removal of the soluble components prior to incubation in selenite-cystine broth improves the recovery of *Salmonella* from egg albumin. The χ^2 analyses show that the difference observed would occur by chance in less than 5 times out of 100. The isolation of *Salmonella* from lauryl tryptose broth containing polyvalent flagellar antiserum without initial centrifugation (direct method) is superior to that of the uncentrifuged selenite-cystine method (A vs C) and is comparable to that by the centrifugation method in selenite-cystine broth (B vs C). On the other hand, incubation of the centrifuged sediment from egg albumin in lauryl tryptose containing antiserum is superior to incubation of the centrifuged sediment in selenite-cystine broth (B vs F, 95 per cent confidence limits). Subsequent incubation of this "specifically agglutinated" sediment in selenite-cystine resulted in a slight increase in the number of *Salmonella* recoveries (C vs D and F vs G). The sum of all the positive isolations either by the direct streaking of the agglutinated sediment or by incubation of this sediment in selenite cystine broth is significantly higher than the *Salmonella* isolations achieved from the direct streaking of the "specific sediment" (C vs E and F vs H, 95 per cent confidence limits). The total isolation resulting from the centrifugation and agglutination method is markedly superior to that of the

isolation by the centrifugation method alone (B vs G, 99 per cent confidence limits).

Discussion

The unsatisfactory status of the methodology for the detection of *Salmonella* in foods is evident in the symposium on this topic (Dack, 1955). It is also reflected in the publications concerned with the improvement of selective enrichment broths (Stokes and Osborne, 1955; Osborne and Stokes, 1955). Extensive improvement of selenite F and tetrathionate broths would seem unlikely because of the nature of these media. The selective enrichment property resides in a quantitative difference in the susceptibility of *Salmonella* and coliforms to the ingredients of these media and not in a qualitative difference. The present work on the use of specific agglutinating antiserum is an attempt to utilize a qualitative difference between these groups of microorganisms to improve the isolation of *Salmonella*.

The centrifugation method (Silliker and Taylor, 1958) utilizes a different rationale. The soluble portion of the food products is responsible for upsetting the selective property of the enrichment broths. Thus, the enrichment cultures of the bacteria separated from the soluble portion of food product resulted in significantly greater detection of *Salmonella*. The combination of centrifugation and specific agglutination results in further improvement.

The probable explanation for the recovery of *Salmonella* by the antiserum method is the specific agglutination of the *Salmonella* as they multiply during the incubation in lauryl tryptose antiserum broth. There is a differential concentration of the larger and heavier clumps of *Salmonella* organisms during the incubation and the following minimal centrifugation. The multiplication of the other organisms is inhibited by the lauryl tryptose broth or compensated for by decanting of the supernatant. Thus, there is not only an increase in the total number of *Salmonella* cells but also a relative increase in the ratio of *Salmonella* to coliform as compared to that in the original sample. The streaking of this sediment after suspension should result in a greater chance for the detection of *Salmonella*.

Selective enrichment media of the usual composition can be used for food products if *Salmonella* multiplication is allowed to take place to a sufficiently high level prior to inoculation into the enrichment media (Slocum, in Dack, 1955). The processing and holding of the food product may result in subtle damage to the salmonellae which makes them more sensitive to the toxic effect of the enrichment broths. A preliminary incubation of the sample in a broth containing no toxic agent may facilitate the "recovery" of the *Salmonella* and result in the pre-enrichment suggested by Slocum. While the present work was in progress, North and Slocum (personal communication) have found that the recovery of *Salmonella* from egg albumin can be increased by incubating egg albumin in lactose broth for 24 hr at 37 C and transferring the resulting growth to a selective enrichment broth. The incubation of the egg albumin sample in lauryl tryptose antiserum broth may be comparable to this pre-enrichment procedure with the added advantage of specifically concentrating the agglutinated *Salmonella* in the sediment prior to inoculation of the selective enrichment broth.

Since polyvalent *Salmonella* agglutinating antiserum of adequate titer is not generally available, the use of the agglutination method is limited at the present time. However, the results presented illustrate the uncertainty of the methodology for *Salmonella* recovery currently being employed for the examination of egg white, and presumably other foods.

The application of the agglutination principle for the isolation of other specific microorganisms from a mixed bacterial flora seems possible. Certain difficulties may be encountered. If the samples contain other organisms which settle rapidly, then the method would be less successful. The widespread occurrence of organisms which can produce substances which inhibit the growth of the desired species, for example, antibiotics against *Salmonella* produced by coliforms (Levine and Tanimoto, 1954) would be another limiting factor. If antigens through which the specific agglutination is sought are present in both the species to be isolated and the other common bacteria in the sample, the method would lose its specificity. These possible difficulties were not of importance in the present study of 12 different egg albumin samples.

Summary

The isolation of *Salmonella* from egg white based on the antigenic difference between the *Salmonella* and the other microorganisms present in the inoculum is described. The method depends on the incubation of the sample in a noninhibitory broth containing *Salmonella* agglutinins. The sediment obtained from such a culture by minimal centrifugation contains a ratio of *Salmonella* to other bacteria which is more favorable for the isolation of *Salmonella*. The possibility of using a similar principle for the isolation of other bacteria from a mixed bacterial flora is discussed.

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An Evaluation of the *Lactobacillus viridescens* Assay for Thiamine

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Microbiological assays for thiamine, long in use, have been supplanted almost entirely by a chemical method. Advantages of the microbiological assay over the chemical are sensitivity and ease of sample preparation. The two most frequently used microbiological techniques have employed *Lactobacillus fermenti* 36 (Sarrett and Cheldelin, 1944) and baker's yeast (AOAC, 1945). Recently, Deibel, Evans, and Niven (1957) showed that *Lactobacillus viridescens* could be used. They found this bacterium to be adequately specific for thiamine, and lower concentrations of the vitamin could be detected by this organism than by either *L. fermenti* or by the chemical assay. These investigators prepared samples for assay by the technique described for the thiochrome method (AOAC, 1955), but noted that a simpler extraction procedure might be adequate.

This paper deals with a test of the reliability of the *L. viridescens* assay for thiamine.

EXPERIMENTAL METHODS

Organism. Lactobacillus viridescens was obtained from the American Type Culture Collection as no. 12706, and was kept as a stock culture by weekly transfer in agar stabs as described by Evans and Niven (1951) and by Deibel *et al.* (1957). Stock cultures, inoculum, and assay tubes were incubated at 30 C.

Sample preparation. The extraction procedure described in *Official Methods of Analysis* (AOAC, 1955) for samples to be assayed microbiologically for riboflavin was used.

Assay medium. A commercial assay medium[1] was used exclusively in this study. Double strength assay broth was dispensed in 5-ml amounts into 18 by 150 mm matched Pyrex test tubes. Aliquots of the test solutions were added, and the volume was brought to 10 ml with distilled water. The tubes were covered with aluminum caps and autoclaved at 10 lb for 6 min.

Inoculum. A 16- to 20-hr broth culture of *L. viridescens* (Evans and Niven, 1951; Deibel *et al.*, 1957) was harvested by centrifugation, and the cells were washed once with 0.9 per cent sterile saline. The washed cells were diluted to 70 to 75 per cent transmittancy (580 mμ) with saline, and each assay tube was seeded with one drop of this suspension.

Measurement of response. Growth was measured turbidimetrically[2] at 580 mμ after 16 to 18 hr of incubation at 30 C.

[1] Bacto LV thiamine assay broth, Difco Laboratories, Detroit, Michigan.

[2] A Coleman model 14 spectrophotometer was used. Coleman Instruments, Inc., Maywood, Illinois.

TABLE 1

Recovery of thiamine

Type of Sample	Quantity of Thiamine Found	Thiamine Added	Expected Recovery	Actual Recovery	Recovery
	μg	μg	μg	μg	%
		100.0	100.0	97.2	97.2
		100.0	100.0	98.9	98.9
Macaroni, enriched...	23.8	100.0	123.8	123.5	99.6
Milk, fortified.....	12.1	100.0	112.1	113.7	101.4
Bread, enriched.....	7.8	8.0	15.8	15.1	96.1
Bread, enriched.....	7.5	16.0	23.5	22.4	95.3
Bread, enriched.....	7.4	25.0	32.4	33.6	103.7