

Application of Enzyme-Linked Immunosorbent Assay for Detection of Staphylococcal Enterotoxins in Food

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ABSTRACT

The enzyme-linked immunospecific assay (ELISA) is equally as sensitive as the radioimmunoassay (RIA) for detecting staphylococcal enterotoxins (SE). The substitution of an enzyme in ELISA for I-125 in RIA results in a more stable reagent and enables quantitation spectrophotometrically or, with appropriate enzymes, semi-quantitation by visual estimation. Assay procedures identical in principle to RIA are employed with, of course, the necessity to avoid the presence of enzyme inhibitors. To date, nanogram quantities of SEA, SEB and SEC have been successfully measured in food extracts. In common with RIA, sensitivity is decreased by the presence of food materials.

Application of affinity chromatography utilizing antibody-antigen reactivity to assay for staphylococcal enterotoxins has enabled quantitation in the nanogram to picogram range. By separating the desired antigen (toxin) immunologically rather than through physical techniques, specificity and sensitivity are increased dramatically. Two of these assays, radioimmunoassay (RIA) and enzyme-linked-immunospecific-assay (ELISA) or, as the latter is also designated by some, enzyme immunoassay (EIA) are widely employed in clinical and metabolic studies. They are both specific and relatively rapid assays and, while they are closely related in principle, both possess a number of distinct attributes due to the conjugate marker used for quantitation - iodine-125 (I-125) for RIA and an enzyme for ELISA.

Substitution of an enzyme for radioactive iodine, though, has increased the versatility for detecting or assaying staphylococcal enterotoxins. In fact, ELISA has already demonstrated its applicability for assaying agents either causing or resulting from infections, and identifying serum antibodies, antigens, drugs and metabolites.

Both RIA and ELISA are sensitive, specific and rapid, but both suffer from the problem of decreased sensitivity due to non-specific adsorption (Table 1). Food constituents can significantly contribute to this problem and in certain instances should be removed or neutralized (1,4,5,7,9).

The ELISA assay, by eliminating the need for I-125, utilizes equipment common to most laboratories by replacing a gamma or scintillation counter with a spectrophotometer. Moreover, if desired, some substrate-enzyme reactions can be evaluated visually. The enzyme conjugate, unlike I-125, is relatively stable and can be stored at -20 C for over 1 year.

TABLE 1. *A comparison of RIA and ELISA.*

IN COMMON WITH RIA	ADVANTAGES OF ELISA
Specificity	No radioactive compounds
Sensitivity	Only commonly used laboratory equipment needed
Rapidity	Stable enzyme conjugates
Non-specific adsorption	Capability for visually evaluating a positive reaction

For both techniques, highly purified homologous antisera and antigen must be employed to achieve maximal sensitivity. The usual range of sensitivity for assaying enterotoxins in foods by either RIA or ELISA varies from 100 pg for enterotoxin C (SEC); to less than 1 ng per g of enterotoxins A(SEA), B(SEB), D(SED) or E(SEE) (6,12,13,14). In the authors' laboratory, sensitivity for detecting SEA and SEB by RIA increased from 0.5 ng per ml of extract to less than 0.1 ng per ml of extract by substituting a more highly purified IgG immunoglobulin fraction for a relatively crude antibody preparation.

The principles for ELISA are identical with those of RIA allowing, of course, for the presence in ELISA of the enzyme conjugate. A number of applied and theoretical treatments of RIA (15,16) are also applicable to ELISA and can serve as a useful guide for developing assays.

PROCEDURES FOR ELISA

The sequential and competitive procedures for the direct ELISA assay are illustrated in Fig. 1. Although not essential (7), antibodies are generally attached to a support material, usually by direct adsorption onto a plastic surface although other procedures, such as first adsorbing bovine serum albumin (BSA) to a surface, fixing the BSA with gluteraldehyde and then absorbing the antibody to the BSA, could be used (13). Various plastics including polystyrene, polypropylene and polyvinyl chloride have been evaluated and the most suitable plastic appears to be polystyrene (2). Successful assays, as seen below, have been achieved with such varied forms as test tubes, microtitration plates and plastic beads.

To minimize non-specific adsorption by sites not covered by the antibody, either or both BSA or/and Tween 20 is/are added as blocking agents. The antigen (enterotoxin) is added and incubated at some specific temperature range. When extended incubations are used to achieve maximum sensitivity, it might be necessary to

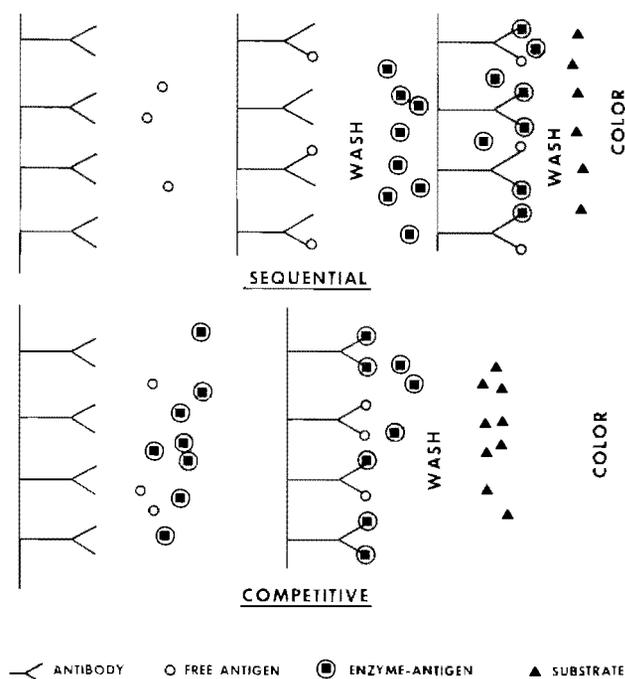


Figure 1. Direct ELISA assays.

add a preservative such as sodium azide. This can present a problem for peroxidase is inhibited by sodium azide, and we have found that merthiolate is also inhibitory. Stiffler-Rosenberg and Fey (14), using 0.02% azide in BSA buffer, apparently found that sodium azide did not seriously interfere with their peroxidase activity. Sodium azide does not interfere with the activity of alkaline phosphatase.

After antigen-antiserum binding and washing, the complex is exposed to an enzyme-homologous-antigen conjugate. The enzymes of choice are usually either peroxidase or alkaline phosphatase, although Morita and Woodburn (7) have employed β -amylase. Procedures for conjugation of these enzymes are usually those of Engvall and Perlmann (3) or Nakane and Kawaoi (8). Peroxidase is an inexpensive enzyme and more stable than alkaline phosphatase, but as seen below, both can be equally sensitive for assaying staphylococcal enterotoxins.

In the direct ELISA, the conjugate will associate with the unoccupied sites. This reaction is relatively rapid, as short as 1 h, but greater sensitivity has been noted when longer incubation times - as much as 18 h-are used (13). Unlike its use in RIA, BSA is usually omitted in ELISA since it was found to interfere with the rate of binding of the conjugate to the antibody site. After washing, the substrate is added, the chromogen allowed to develop and its intensity is read in a spectrophotometer. The substrates commonly used for peroxidase are 5-aminosalicylic acid (purple-brown) or α , α azino-di (3-ethyl benzthi azoline-6-sulfonate) and for alkaline phosphatase it is p-nitro-phenylphosphate. A terminator, usually sodium hydroxide, is used to stop the enzymatic reaction.

In the direct competitive method, both the free and conjugate antigens are allowed to compete at the same

time for the available antibody binding sites. It is apparent that the competitive method eliminates one binding step and a washing, but it is as accurate as the sequential procedure.

The use of the sandwiching technique is also possible with ELISA (Fig. 2). Either the antibody or the antigen can be assayed in the double antibody sandwich. The antigen, capable of binding two homologous antibodies, can be assayed after binding on the adsorbed antibody by, in turn, binding a conjugated homologous antibody.

In the indirect sandwich technique, homologous antibodies can be assayed by the use of a conjugated antibody. For example, if enterotoxin rabbit antisera was to be titrated, it could be adsorbed onto homologous enterotoxin molecules and in turn assayed by the use of conjugated anti-rabbit goat antisera.

In our laboratory at NARADCOM, we have employed both the direct competitive or sequential procedures for both RIA and ELISA. Some typical assay curves are presented in Fig. 3 to demonstrate the sensitivity that can be obtained using either phosphatase or peroxidase labeled antigen for SEA or SEB. The sensitivity of the assay for these toxins in buffer was less than 0.5 ng per ml.

Recent publications on the application of ELISA to assaying staphylococcal enterotoxins in foods reflect the versatility of this method (7,10,13,14). Saunders and Bartlett (13) used a simple extraction procedure to assay for SEA by the sandwich technique, using spiked extracts of hot dog, milk and mayonnaise. They employed microtitre plates and reacted them with glutaraldehyde to increase their capacity for adsorbing anti-SEA. The conjugated anti-SEA enzyme was peroxidase and the range for their assay was 2.5 to 100 ng per ml. Their data indicate a sensitivity for detecting SEA varying from 3.2 ng per ml in a 1 to 3-h test to 0.4 ng per ml with a 20-h test period. They used

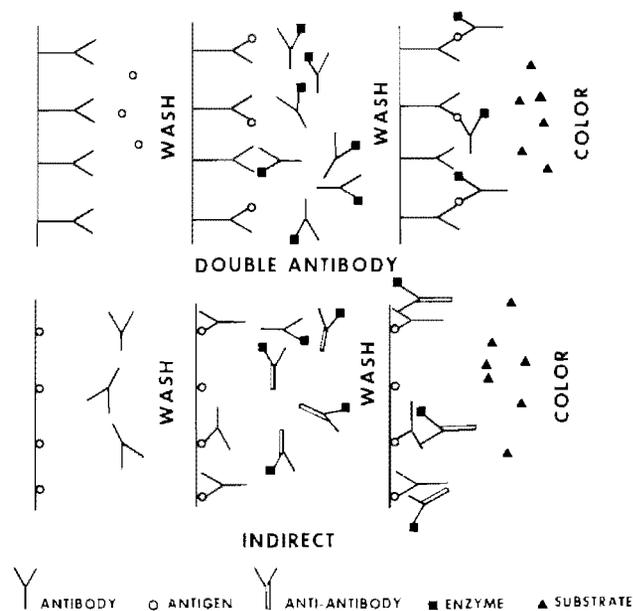


Figure 2. Sandwich techniques used in the ELISA.

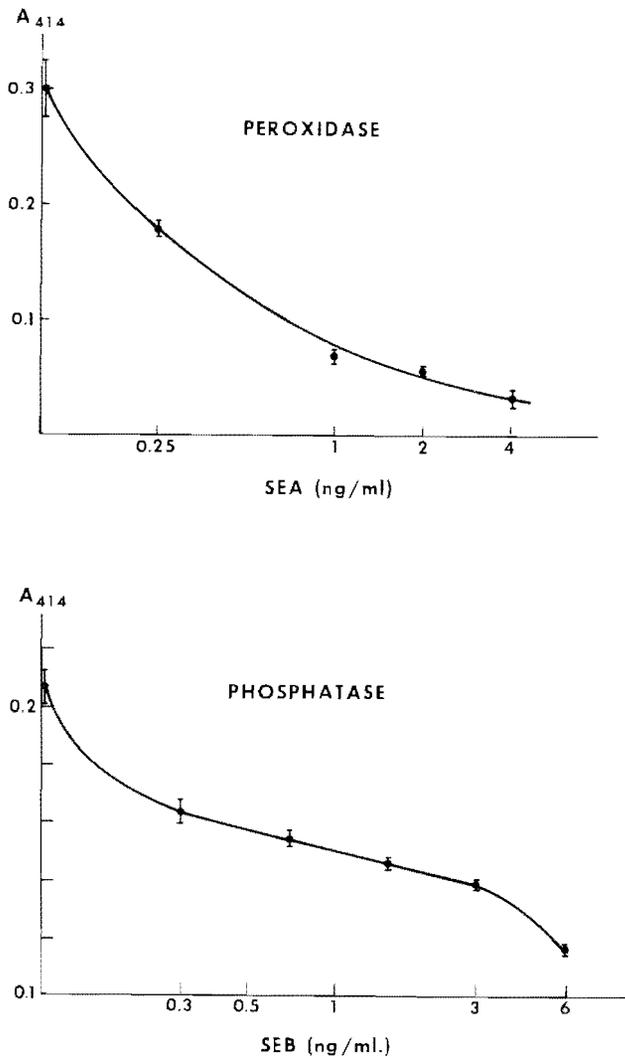


Figure 3. The use of peroxidase and phosphatase in the ELISA of SEA and SEB.

sodium azide in the 20-h test, apparently with no ill effect on peroxidase activity. Their sensitivity, assuming a sample size in each well of the microtiter plate of 0.05 ml, corresponds to 20 ng per well - a remarkable demonstration of the sensitivity of this technique. They did experience difficulty, shared by both RIA and ELISA - a decrease in percentage recovery as the concentration decreased. For a mayonnaise extract spiked with 10 ng per g, 94% of the added SEA was recovered but recovery decreased to 84% for a sample containing 2.5 ng per g. With a spiked hot dog extract, recovery decreased from 94% for a 10-ng-per-g extract to 72% for a 2.5-ng-per-g-extract.

Stiffler-Rosenberg and Fey (14) have taken a different approach in assaying for enterotoxins. Using polystyrene balls coated with enterotoxin SEA, SEB or SEC, they could assay sample volumes of up to 20 ml. The enzyme used in their conjugate was alkaline phosphatase and they employed the direct assay technique. Their procedure for assaying cheese extracts required approximately 24 h for completion. They employed 0.02% sodium azide as a preservative and used both BSA and Tween 20 as

blocking agents. A sensitivity of 1 ng per ml was achieved with a 1 ml sample and 0.1-0.05 ng per ml when a 20-ml sample was used (Table 2). No cross reactions were noted between SEA, SEB or SEC although they did note some interference by cheese extracts - most likely due to non-specific adsorption (11).

TABLE 2. Sensitivity of the ELISA using polystyrene balls.^a

Enterotoxin	Concentration of enterotoxin detected (ng per ml)	
	1 ml Extract	20 ml Extract
A	1	0.1
B	1	0.1
C	1	0.05

^aFrom the data of Stiffler-Rosenberg and Fey (14).

Morita and Woodburn (7) adapted the "homogenous enzyme immune assay" to foods. In this technique, the free antigen (enterotoxin) competes with the enzyme-linked antigen for the homologous antibody. Presence of the antibody adjacent to the antigen-enzyme conjugate interferes with enzyme activity and is a measure of the presence of free toxin in the sample. The enzyme of choice in this study was β -amylase, which is not produced by *Staphylococcus aureus*. Their data indicate a sensitivity in excess of 10 ng per ml. Interference due to food constituents, resulting in high blank values, were found.

LIMITATIONS

One should not assume that the sensitivity for spiked food extracts encountered in the literature can be indiscriminately applied to any food sample. As stated above, when analyzing particular classes of foods, one can expect, and many have noted, an appreciable variation in non-specific adsorbance. The suggestion has been made that standard curves be performed on representative food items at the same time as the unknown has been made, but this adds greatly to an assay effort. Moreover, there are few or no data to indicate that this approach, when applied to foods obtained from a wide variety of sources, will result in the required accuracy. It is also not sufficient to derive a value for sensitivity obtained by comparing the results from a spiked extract to those of a buffer control. Pober and Silverman (11) discussed this problem for RIA and attempted to minimize it by pretreating the antibodies with a food extract. Notermans et al. (10), using the ELISA sandwich technique, enjoyed only limited success in minimizing interference from food extracts due to non-specific adsorbance by reacting the homologous antibody with culture filtrates from nontoxic strains of *S. aureus*.

In the authors' laboratory, we have taken a somewhat different approach in an attempt to eliminate, as much as possible, nonspecific adsorbance and derive an analytical technique capable of acting as a standard procedure. The procedure we use (4) in this effort is shown in Fig. 4, and uses affinity chromatography to separate the toxin from the food extract. The toxin in the supernatant fluid was recovered on an immunosorbent

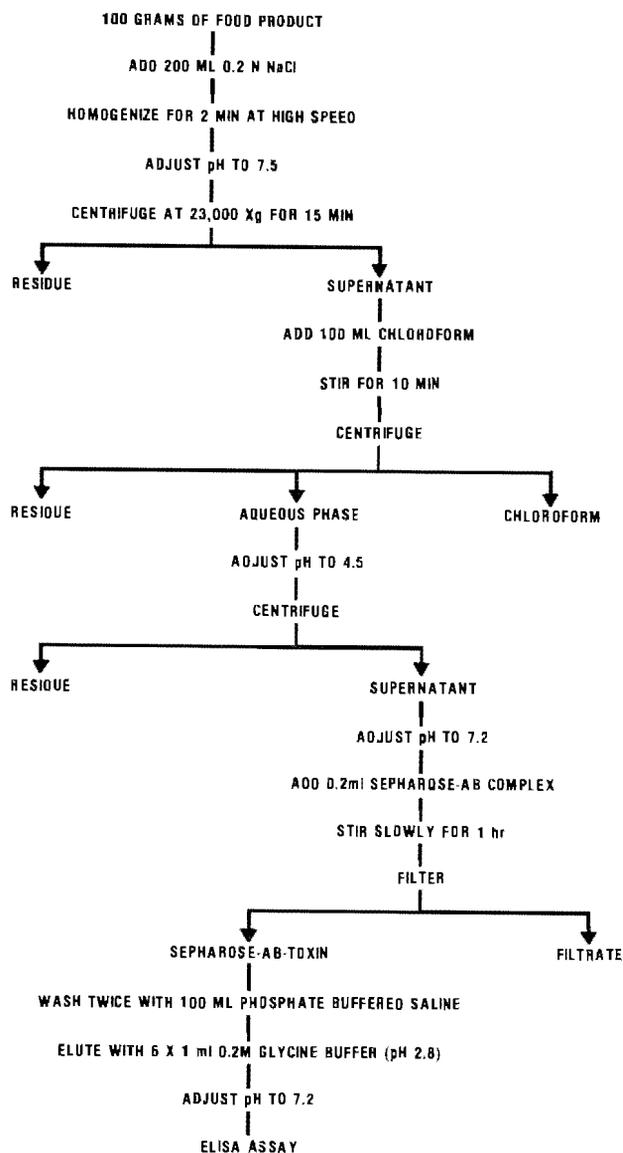


Figure 4. Schematic for the removal of compounds in food capable of interfering with the ELISA.

affinity gel consisting of sepharose conjugated to a homologous antibody. After shaking with the gel for 1 h at room temperature, the gel was separated from the supernatant fluid and washed with phosphate buffered saline solution (0.1 M phosphate, pH 7.4). The toxin was then eluted from the gel with glycine buffer (0.2 M + 0.5 M NaCl, pH 2.8) and recovered by the ELISA technique.

A direct sequential ELISA, using a polystyrene tube as the solid matrix is used. The antibody adsorbed into the polystyrene surface is highly purified homologous rabbit IgG anti-SEA or anti-SEB suspended in 0.1 M carbonate buffer (pH 9.6). Adsorption occurs overnight at ambient temperature and the tubes are then stored at 0-2 C until needed at which time the tubes are drained of their antibody solution. The sample (1 ml) is then incubated at room temperature for 18 h and the enzyme-labeled toxin reacted with its substrate for 4 h.

In Fig. 5, the results of ELISA assay of spiked extracts

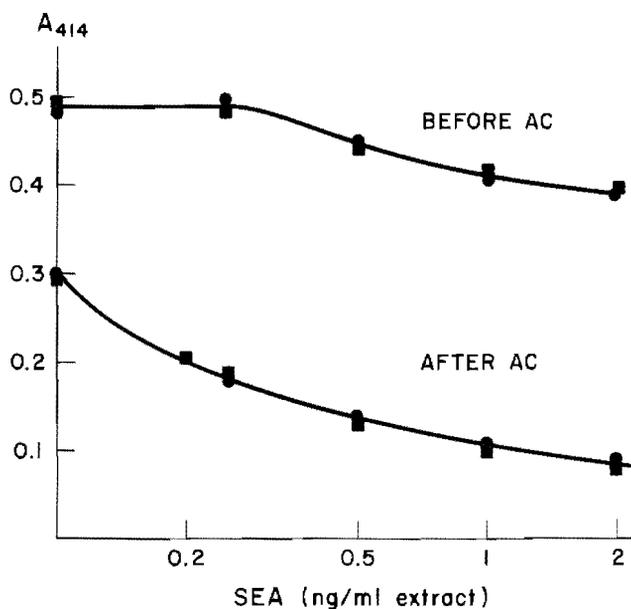


Figure 5. The effect of removing interfering compounds from food on the ELISA.

of milk and cheese both before and after extraction by affinity chromatography are shown. The non-specific interference due to food constituents has been drastically reduced, and a sensitivity of 0.25 ng SEA per ml of extract was obtained. The standard curves derived from milk and cheese were the same, indicating that immunological techniques are more efficient than physical techniques, and can, if verified, simplify the requirements for deriving standard curves for quantification.

This technique, though, is time-consuming and tedious and studies are underway to shorten the procedure. Recovery, presently near 30%, must also be increased, but this recovery level is considerably higher than that reported by Niskanen and Lendroth (9) for the extraction techniques required for the micro-slide assay.

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REFERENCES

- Bergdoll, M. S., R. Reiser, and J. Spitz. 1976. Staphylococcal enterotoxins - detection in food. *Food Technol.* 30:80.
- Bullock, S. L., and K. W. Walls. 1977. Evaluation of some of the parameters of the enzyme-linked immunospecific assay. *J. Infect. Dis.* 136:Suppl. S279.
- Engvall, E., and P. Perlmann. 1972. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-linked anti-immunoglobulin in antigen-coated tubes. *J. Immunol.* 109:129.
- Genigeorgis, C., and J. K. Kuo. 1976. Recovery of staphylococcal enterotoxin from foods by affinity chromatography. *Appl. Environ. Microbiol.* 3:274.
- Johnson, H. M., J. A. Bukovii, and P. E. Kaufmann. 1973. Staphylococcal enterotoxins A and B: Solid-phase radioimmunoassay in food. *Appl. Microbiol.* 26:309.
- Miller, B. A., R. F. Reiser, and M. S. Bergdoll. 1978. Detection of staphylococcal enterotoxins A, B, C, D and E in foods by radio-

cont on p. 413

Participation reflect this goal. These objectives are: to direct the public's views to Agency decisionmakers so that their views can impact on administrative and regulatory policies and to advise the public of how their views assisted the Agency in reaching final decisions and policies.

Following the close of the Food Labeling Hearings, the Office of Consumer Affairs assisted the Agency in formulating a Food Labeling Plan. This plan reflects the consumer views that were submitted to FDA during these hearings. Part of this plan will provide the basis for a proposed regulation that is scheduled to be published in the *Federal Register* sometime early this fall (1979).

Consumer participation at the Food Labeling Hearings provided the Agency with the support and guidance necessary to establish revised policies in numerous important areas. In addition, public views enabled FDA to state its intentions to support legislation in areas that are presently beyond the scope of its authority.

The Food Labeling Hearings did not provide all of the answers necessary for development of improved food labeling. The consumer response, however, was sufficient in some areas to indicate that a revision in policy was desirable and for FDA to be able to state its intention to consider alternatives to current policies. The Agency plans to address several areas of concern raised at these hearings through studies, experimentation and other means.

Public participation at the Food Labeling Hearings exerted a significant impact on the Agency's food labeling policies — and it is important that consumers know this. This is the final stage of good communication and effective public participation. As soon as the proposed food labeling regulation is published in the *Federal Register*, the Office of Consumer Affairs plans to send the proposal to all of the consumers who testified at the hearings or sent written comments to the Agency. Approximately 10,000 consumers will receive the food labeling proposal.

IN CONCLUSION

Regardless of whether it is the industry, health professionals or consumers, the ultimate goal of any group that interacts with FDA is to have its particular point of view reflected in Agency policy or final regulations. Heretofore consumers have not consistently expressed their views to FDA during the decisionmaking process. As a result, the Agency did not have access to consumer views when attempting to resolve an issue or establish a certain regulatory policy. When such a situation occurs, the courtroom usually becomes the first rather than the last forum for discussing differences and considering alternative approaches to a problem. One of the benefits of public participation is that diversity and controversy are actively sought and openly addressed at the early stages of the decisionmaking process.

Keo and Silverman, *con't from p. 407*

- immunoassay, using staphylococcal cells containing protein A as immunoadsorbent. *Appl. Environ. Microbiol.* 36:421.
7. Morita, T. N., and M. J. Woodburn. 1978. Homogeneous enzyme immune assay for staphylococcal enterotoxin type B. *Infect. Immun.* 21:666.
 8. Nakane, P. K., and Kawaoi. 1974. Peroxidase-labeled antibody, a new method of conjugation. *J. Histochem. Cytochem.* 22:1084.
 9. Niskanen, A., and S. Lindroth. 1976. Comparison of different purification procedures for extraction of staphylococcal enterotoxin A from foods. *Appl. Environ. Microbiol.* 32:455.
 10. Notermans, S., H. L. Verjans, J. Bol, and M. van Schothorst. 1978. Enzyme linked immunosorbant assay (ELISA) for determination of *Staphylococcus aureus* enterotoxin type B. *Health Lab. Sci.* 15:28.
 11. Pober, Z., and G. J. Silverman. 1976. Modified radioimmunoassay determination for staphylococcal enterotoxin B in foods. *Appl. Environ. Microbiol.* 33:620.

12. Robern, H., M. Dighton, Y. Yano, and N. Dickie. 1975. Double-antibody radioimmunoassay for staphylococcal enterotoxin C₂. *Appl. Microbiol.* 30:525.
13. Saunders, G. C., and M. L. Barlett. 1977. Double-antibody solid phase enzyme immunoassay for the detection of staphylococcal enterotoxin A. *Appl. Environ. Microbiol.* 34:518.
14. Stiffler-Rosenberg, G. and H. Fey. 1978. Simple assay for staphylococcal enterotoxins A, B and C: Modification of enzyme-linked immunosorbant assay. *J. Clin. Microbiol.* 8:473.
15. Zettner, A. 1973. Principles of competitive bindings assays (saturation analysis) I. Equilibrium techniques. *Clin. Chem.* 19:699-705.
16. Zettner, A., and P. E. Daly. 1974. Principles of competitive binding assays (saturation analysis). II Sequential saturation. *Clin. Chem.* 20:5-14.

Kaplan and Zalkind, *con't from p. 409*

inspection schedules cannot be derived. Too much is unknown or unmeasurable about establishment characteristics and sanitation. However, our arguments strongly support the case that substantial improvements can be made by changing the current widespread practice of constant inspection frequencies. By incrementally varying inspection frequencies in proportion to sanitation deficiencies (and in inverse proportion to inspection costs if the differences are significant enough to warrant it), observing subsequent changes in sanitation scores, and again varying the inspection frequencies, a superior inspection schedule can be successively approached.

REFERENCES

1. Bader, M., E. Blonder, J. Henricksen, and W. Strong. 1978. A study of foodservice establishment sanitation inspection frequency. *Am. J. Public Health* 68:408-410.
2. Baumol, W. J. 1972. Economic theory and operations analysis, 3rd ed. Prentice Hall, Englewood Cliffs, N.J. 626 pp.
3. Cox, C. F., and R. Wilson. 1979. Arlington, Virginia revamps food program, raises sanitation level. *J. Environ. Health* 41(4):208-210.
4. Kaplan, O. B., and A. El-Ahraf. 1979. Relative risk ratios of foodborne illness in foodservice establishments. *J. Food Prot.* 42:446-447.
5. Zaki, M. H., G. S. Miller, M. McLaughlin, and S. Weinberg. 1977. A progressive approach to the problem of foodborne infections. *Am. J. Public Health* 67:44-49.