The use of an enzyme immunoassay for the detection of salmonellae in foods

Scott Arthur Minnich
Iowa State University

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THE USE OF AN ENZYME IMMUNOASSAY FOR THE DETECTION OF SALMONELLA IN FOODS

Iowa State University

Ph.D. 1981

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The use of an enzyme immunoassay for the detection of salmonellae in foods

by

Scott Arthur Minnich

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

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INTRODUCTION

Clinical diagnosis of salmonellosis is a relatively straightforward procedure, since these pathogens, when present in an infected individual, usually are found in large numbers and in an undebilitated state. In contrast, detection of salmonellae in foods and feeds tends to involve a long, tedious, and expensive series of manipulations. This is especially true in processed foods which have undergone various processing treatments such as heating, drying, or freezing. Although there is ample evidence in the literature that salmonellae can survive such treatments, they are generally reduced to low numbers and often they are sublethally injured. Mishandling of such products prior to consumption poses a major health threat. Detection methodologies formulated to determine food safety with respect to salmonellae must, therefore, insure that resuscitation and recovery of undebilitated cells occur. This necessitates inoculation of foods into a nonselective medium followed by selective enrichment to insure their recovery. For example, the Food and Drug Administration's (FDA, 1976) recommended pure culture analysis for salmonellae detection advocates the pre-enrichment of food samples in a nonselective medium followed by selective enrichment and selective plating. Total analysis time, using this method, requires four to five days and
involves considerable expense.

Efforts to reduce the time and cost of Salmonella analysis have primarily focused on the development of immuno-fluorescence and enrichment serology procedures. Although these are effective screening techniques, problems with high rates of "false positives" and the subjective interpretation of results are hindrances in their routine application.

Krysinski and Heimsch (1977) advocated the use of an enzyme-labeled antibody method for salmonellae detection, based on results that they obtained in a model study. An indirect test was used and Salmonella typhimurium was the test strain. Their enzyme-labeled assay technique (ELAT) made use of cellulose acetate membrane filters as a solid support matrix to which cells were fixed. Cells were spotted on membranes by suction, fixed and exposed to anti-S. typhimurium H antibody. Following removal of unbound antibody, the membranes were exposed to an anti-antibody coupled to peroxidase. After removal of unbound antibody conjugate, the membranes were developed by immersion in peroxidase substrate; positive S. typhimurium cultures were identified by the formation of a chromogenic reaction product. This reaction could be detected visually because of the amplification effect of the enzyme.
Minnich (1978) further demonstrated the potential of the ELAT by expanding it to cover a wide variety of salmonellae serotypes. Commercial polyvalent antiserum was found to be suitable for the test after the removal of IgM, the primary immunoglobulin class elicited by cross-reactive O antigens. Though problems were encountered with the use of peroxidase-antibody conjugates, Minnich (1978) concluded that a further refined ELAT would have definite advantages over existing methods of salmonellae testing. This study was undertaken to further develop the enzyme immunoassay and then compare its performance against methods in current use.
LITERATURE REVIEW

The Salmonella Problem in the United States

Salmonellosis was credited in a report prepared in 1969 by the National Academy of Sciences (Foster, 1969) as being one of the most significant communicable diseases in the United States. Over the past decade, salmonellosis has continued to be a problem for industry as well as for regulatory officials and the public. The number of cases per annum continues to rise, as reflected by increased reports of confirmed salmonellae isolates from humans recorded by the Center of Disease Control; these have ranged from 22,000 to 33,000 per year (Gregg, 1980). Though the actual number of cases is unknown, it is conservatively estimated that only 1% of cases are reported (Gregg, 1980, Foster, 1969). The increased incidence of salmonellosis can be attributed, in part, to improved surveillance measures. These include: better cultural methods for isolation and identification of salmonellae, improved reporting procedures, and better communication between state and federal health services (Foster, 1969). Yet, irrespective of these factors, evidence clearly indicates that the true incidence of the disease as well as the opportunity for outbreaks has increased over the past twenty years (Foster, 1969, Gregg, 1980, Ryder et al., 1976).

A variety of contributing factors is responsible for the
increased incidence of salmonellosis. Changes in eating habits, shifting from home prepared meals to communal eating establishments such as common meals at schools, restaurants, and other mass eating establishments, have occurred. Consumption of raw or slightly heated products of animal origin might lead to salmonellosis. Mass production of processed foods, increased consumption of poultry and poultry products, and increased association of salmonellosis with pets also are involved (Lamm et al., 1972, Gailbraith, 1962). Improved food hygiene measures, which may eliminate or reduce a product's natural flora that otherwise might inhibit the growth of salmonellae or cause contaminated food to be discarded due to spoilage, also might play a role in the incidence of salmonellosis. An increased use of antibiotics, which may contribute to the prolongation of the carrier state in both humans and animals, and the feeding of contaminated feedstuffs to animals which serve as the primary reservoir hosts for these pathogens, could contribute to increased incidence of this disease (Foster, 1969).

Although the National Academy of Sciences recommended increased budgeting for salmonellae research, deficiencies in these efforts are evident (Foster, 1969, Purchase, 1979); with tighter fiscal budgeting expected because of inflation and funding cutbacks, these deficiencies can be expected to become more pronounced. For example, Purchase (1979) reported
that little or no research is being conducted on poultry feed production, a major source of flock contamination. Furthermore, limited research is being done on hatcheries and breeding flocks, on vertical transmission, and on the sampling of dead embryos and infertile eggs for salmonellae. Of the approximately 11,000 scientists working in federal and state agricultural research, less than 13 scientist years per annum involve research directed towards salmonellosis in the poultry industry, and only 7 scientist years are used by state experimental stations and universities (Purchase, 1979). Yet, poultry products are a major contributor to the incidence of salmonellosis in humans. A recent study by Swaminathan et al. (1978b), indicated that 14% of poultry products tested at the retail level were positive for salmonellae.

An even smaller effort is devoted to research on mammalian salmonellosis (Purchase, 1979). Unless this trend is reversed, hopes for control and/or proposed eradication programs are minimal. Perhaps one deterrent to serious control measures is the estimated cost to benefit ratio. Economists in Canada have concluded that approximately $300 million per year would be required to eradicate salmonellae from poultry and to maintain flocks essentially free of salmonellae while the benefits would be only $23 million per year (Finn and Mehr, 1978). Similar studies applied to the poultry industry of the U.S., which generates $7 billion annually in revenue,
would increase poultry costs anywhere from 5 to 50% (U.S.D.A., 1977, cited in Purchase, 1979). This of course, would ultimately be financed by the consumer. Such an eradication program, however, might prove worthwhile when one considers that American consumers pay an annual $1.2 billion in medical costs due to salmonellosis (U.S.D.A., 1978).

Salmonellosis is a major economic problem in the U.S.; the $1.2 billion spent each year in medical costs does not include lost work income because of work absence, surveillance and preventative measures such as routine stool culturing of food handlers, recall of contaminated products, and litigation expenses incurred in civil suits. One recall during 1967, involving chocolate candy, cost the manufacturer an estimated $5 million (Foster, 1969). This estimate did not include losses resulting from marred product prestige when the public was informed of the contamination.

In addition, costs associated with nonhuman salmonellosis are substantial. Loss of weight and morbidity among livestock and poultry, surveillance expenses, and expenditures associated with destroying infected animals all have significant economic impacts on industry and the consumer. Rothenbacker (1965) reported that 26 Michigan farms experienced a mortality rate of 32.6% in calves caused by Salmonella infections over a 20-month period. Other expenditures include drug and veterinary bills, decreased production of milk and
eggs, and general loss of consumer confidence. One final consideration is the possible governmental costs which can conceivably be foreseen as a result of more liberal interpretation of consumer protection laws. Although mortality, due to salmonellosis, is estimated to be less than 1%, this reflects a substantial number of deaths if indeed 2 million cases occur annually. It is conceivable that a consumer might attempt to file suit against the federal government for damages caused by the death of an individual following consumption of U.S.D.A.-inspected and approved meat or poultry. This agency currently does not include screening for salmonellae in meat inspection although zero tolerance standards, with respect to salmonellae contamination, are required by the Food and Drug Administration (FDA).

In summary, salmonellosis is of extreme importance from both the medical and economic perspectives. The ultimate eradication or control of this disease will be achieved only by improved surveillance methods and increased funding of basic research. Even though the derived benefits for eradication will not exceed the costs of such a program, some believe that practical application of research findings and progressive use of technology could achieve ultimate eradication of salmonellosis at a minimal cost to the producer and consumer (Purchase, 1979).
Physiological, Biochemical and Environmental Characteristics of the Genus *Salmonella*

The genus *Salmonella* is comprised of Gram-negative, peritrichously flagellated bacilli that conform to the definition of the tribe *Salmonellae* and family Enterobacteriaceae. Urease is not produced, malonate and gelatin are not utilized, and growth is inhibited by potassium cyanide. Furthermore, acid is not produced in Jordan's tartrate medium and the amino acids lysine, arginine, and ornithine are decarboxylated. Inositol and dulcitol are utilized by numerous strains. Importantly, sucrose, raffinose, salicin, and lactose are not fermented. Inactivity on the latter carbohydrate, lactose, is a primary factor that distinguishes *Salmonella* from the "coliform group". Occasionally, aberrant strains are encountered, necessitating a circumspect identification versus focusing on a single factor or rigid group of factors (Edwards and Ewing, 1972). For example, 0.5% of *Salmonella* isolates ferment lactose (Edwards and Ewing, 1972).

The ability of salmonellae to survive adverse environmental conditions results in a problem in many processed foods. Frequently contaminated foods include poultry, meats, egg products, and a wide variety of processed foods which utilize their components (Foster, 1969, Poelma and Silliker,
1976). Salmonellae survive a variety of harsh treatments commonly employed in food processing. These include freezing, drying, high salt concentration (low water activity), and acidic conditions. Generally, salmonellae can grow between 7 to 45°C and can survive a pH range from 4.2 to 9.0 (Edwards and Ewing, 1972). Enkiri and Alford (1971) demonstrated that the frequency of isolation of various Salmonella spp. is related to their resistance to freezing and drying. Serotypes most commonly involved in human salmonellosis showed high resistance to freezing. Frozen contaminated meat samples contained viable salmonellae after 10 weeks of storage. Similar results were obtained with dried cells stored at 10°C. Digirolamo et al. (1970) likewise demonstrated the ability of salmonellae to survive freezing.

Stability was pronounced in Pacific oysters, a food commodity frequently implicated in food-borne disease due to the ability of bivalves to concentrate, by filtration, pathogenic organisms during feeding. Spray-drying under high temperatures, as employed in powdered milk processing, was shown by Miller et al. (1972) to reduce numbers of viable Salmonella, but was not sufficient to destroy all cells. Survival of salmonellae is enhanced by low water activity; these organisms can survive indefinitely in meats, bone meal, and egg whites below moisture levels of 10 to 12% (Foster, 1969, Crumine and Foltz, 1969). Abuse of these products prior to consumption,
therefore, poses a major foodborne disease hazard. Crumine and Foltz (1969) demonstrated the ability of _S. montevideo_ to survive on wheat for 28 weeks at 13% relative humidity. Survival time decreased as the moisture content increased.

High salt concentrations generally do not affect salmonellae until the concentrations exceed 9% and then the death rate is slow and temperature-dependent (Prost and Rieman, 1967). Alford and Palumbo (1969) demonstrated that these pathogens retained viability for up to 70 days in fresh ground pork at 10°C, pH 5, and 8% NaCl. Prost and Rieman (1967) showed that numerous strains survived for up to 8 weeks in salami sausage kept at room temperature with a 23% brine concentration. Low pH and high temperatures were not sufficient to significantly reduce _Salmonella_ numbers in fermented Thuringer sausage incubated at 46°C at a pH of about 5.3; in some samples Goepfert and Chung (1970) demonstrated multiplication of salmonellae. Furthermore, refrigeration of this product reduced the numbers of organisms present, but was not sufficient to insure complete destruction or even low levels of contamination. Gamma radiation resistance in the salmonellae has also been demonstrated. Epps and Isziak (1970) demonstrated that salmonellae lost virulence when exposed to ionizing radiation but that surviving cells were still pathogenic. Licciardello et al. (1969) subjected four _Salmonella_ serotypes to either 15 or 20 cycles of
irradiation and culture; reduced virulence was observed in only one serotype.

Serology

There are currently an estimated 1,700 strains of Salmonella. Serology is the foundation of their classification. Two systematic approaches to serological differentiation are currently in use: the system proposed by Edwards and Ewing (1972) and that developed by Kauffmann and White (Kauff, mann, 1966). Both schemes rely on the same classification criteria but evolve them into separate designs.

Edwards and Ewing (1972) maintain that differences in antigenic composition of otherwise biochemically similar groups do not warrant separate species status. Therefore, the genus Salmonella is divided into three species: S. typhi, S. choleraesuis, and S. enteritidis. The majority of the 1,700 serotypes are placed in the latter species. Proper identification of an isolate is exemplified by the serotype "worthington", which under the described classification is properly denoted S. enteritidis ser Worthington.

In contrast, Kaufmann (1966) proposed that antigenic differences among the salmonellae constitute sufficient genetic variability to justify speciation based on serological constitution. This schema divides the genus into four major groups (I-IV) which can be interpreted as subgenera. Group I
contains biochemically typical strains, groups II and III contain aberrant strains, and group IV contains the genus Arizona. Within each group, antigenically distinct strains are given species status. Thus, in this system, the aforementioned serotype is simply denoted S. worthington.

Complete antigenic analysis of the genus involves the typing of three main antigens (Edwards and Ewing, 1972). These include the "O" or somatic antigens, the "H" or flagellar antigens, and the "Vi" antigen which is associated with the capsule. Analysis of O and H antigens are sufficient to identify the serotype and are routinely done, with the exception of S. typhi where the Vi antigen is an important characteristic.

The O antigens are heat stable; they are phospholipid-polysaccharide (LPS) complexes associated with the cell wall. The nature of the terminal groups and the order in which they occur in the repeating units of the polysaccharide chain render specificity to this group of antigens (Edwards and Ewing, 1972, and Landy and Weidanz, 1964). These antigens are utilized to group the Salmonella, with the different groups being designated alphabetically. O antigens are routinely detected by slide agglutination with appropriate test sera. It is important to note that O antigens are shared throughout the Enterobacteriaceae; therefore, antisera prepared against
whole-cell preparations of *Salmonella* often cross-react with other genera in the family (Edwards and Ewing, 1972, Aleksic and Rhode, 1972). A further crucial point is the nature of the immune response elicited by these antigens. In studying the O antigens of *S. enteritidis*, Landy and Weidanz (1964) and Landy et al. (1965) demonstrated that the primary immunological response to O antigens was the formation of immunoglobulin M (IgM). Immunoglobulin G (IgG) was formed only when large doses were administered to animals on a daily basis, and even then, specific IgG antibody against the O antigen never exceeded 1% of the serum antibodies.

The H antigens are heat labile and composed of flagellin (protein) subunits with a molecular weight of 40,000 daltons (Ada et al., 1964, Edwards and Ewing, 1972, Nossal et al., 1964). Flagellin is extremely antigenic and it is not uncommon to obtain antibody titers in excess of 1:100,000 (Weil and Saphra, 1953). In contrast to the O antigens, H antigens are specific for the genus *Salmonella* and, thus, antisera prepared with purified flagella will not cross-react with other genera, except for *Arizona*. Phase variation is another characteristic of H antigens and refers to the reversible variation of H antigen determinants. This phenomenon is unique to the genera *Salmonella* and *Arizona*. Phases are denoted as phase I (specific phase) and phase II (nonspecific
phase) and are represented by small Roman letters and Arabic numerals, respectively. Not all serotypes are diphasic; thus, some may contain identical H antigens with a different O antigen complement. These latter types are generally contained in the groups having "g" or "m" flagellar antigens. In a recent report, Silverman and Simon (1980) and Simon et al. (1980) determined that a site-specific inversion of a controlling element was responsible for flagellar phase transition in Salmonella. This variation of antigenicity presumably allows these organisms to evade the host immune response. H-antigenic analysis is routinely performed by tube agglutination of formalized cells in the presence of specific antibody at 50°C. The reaction occurs rapidly, giving a floccular, loosely knit, aggregate of cells which is macroscopically visible (Edwards and Ewing, 1972, Sperber and Deibel, 1969).

Antisera Production and Antibody Purification

One problem with the serological identification of salmonellae is that cross-reactions with related members of the Enterobacteriaceae are often encountered. The commercial production of high titer anti-H sera involves the immunization of animals with formalized whole cells; thus, the resulting antisera contain both H and O antibodies. In an attempt to
produce antisera of high titer and free of O antibodies, Aleksic and Rhode (1972) immunized rabbits with purified flagellin. These workers utilized the method of Martinez (1963) to purify flagellin, and harvested antisera contained high H titers free of O antibodies. However, when this method was used by Minnich (1978) and Stahl (1979) residual O antibodies were found in antisera preparations.

Fey (1979) and Fey and Suter (1979) recently reported an alternative method for the production of high titer H antisera. This method involved the solubilization of flagellin by harvesting whole cells in 0.05N HCl. Flagella were efficiently stripped from the cells; the cells were then removed by centrifugation. The resulting supernatant, containing soluble flagellin, was neutralized and the protein was precipitated with ammonium sulfate. The flagellin thus prepared was sufficiently pure to elicit high titered H antisera of 12,800 to 51,200 and O titers of less than 50.

Svenungsson and Lindberg (1977, 1978a, 1978b, 1979) and Svenungsson et al. (1979) have also been successful in producing high titer, specific anti-Salmonella antisera. They synthesized O antigens (these O antigens not being found in other enterics) and coupled them to bovine serum albumin (BSA) prior to use in immunization. Antisera produced by using synthetic antigens were group-specific and performed favorably in the detection of clinical isolates; these
results are discussed later. Their approach shows great potential and should be put to practical use.

An alternative method for the procurement of specific H antisera devoid of contaminating O antibodies was used by Minnich (1978). Realizing the nature of the immune response elicited by these two types of antigens (e.g., flagellin stimulates the production of primarily IgG and LPS elicits primarily IgM) serum was fractionated by gel filtration. The IgG fraction did not agglutinate heated cells whereas, the whole serum preparations did, indicating that gel filtration successfully removed crossreacting antibodies. Since the fractionation of antiserum by gel filtration substantially diluted the preparation and lowered the titer, an alternative method for fractionation would be desirable.

The use of *Staphylococcus* protein A (SPA) affinity chromatography has been used by a number of researchers (Hjelm et al., 1972, Swaminathan et al., 1978a). SPA, a protein of approximately 42,000 daltons derived from the cell wall of selected staphylococci, has an affinity for mammalian IgG (Bjork et al., 1972, Hjelm et al., 1975). The binding site for the protein is located in the Fc region of the immunoglobulin. Each SPA molecule can bind two IgG molecules at neutral pH conditions; subsequent dissociation is achieved by lowering the pH to 3.0. Several extensive reviews of the properties and
applications of SPA are available (Biberfeld et al., 1975, Goding, 1978).

Salmonella Detection Methodologies

The FDA's adoption of zero tolerance with respect to Salmonella in foods has stimulated food industries to subject their raw materials and products to close scrutiny. Many of the various procedures for salmonellae identification developed will be reviewed here; however, it should be noted that all procedures basically fall into two categories. The first category consists of procedures that utilize conventional pure culture isolation; the second category includes those procedures that employ indirect evidence of Salmonella contamination. In both categories, samples are cultured in pre-enrichment and/or selective enrichment media. The complexity of the various procedures is due primarily to the fact that the salmonellae are generally present in low numbers with respect to competing microflora (Polema and Silliker, 1976). The culturing steps and their respective purposes will be reviewed before focusing upon specific methods found in the literature.
Pre-enrichment

This step generally involves inoculation of the sample into a nonselective medium in order to recover injured cells (F.D.A., 1976, Polema and Silliker, 1976). Many processed foods are subjected to treatments that reduce the number and viability of contaminating organisms. These include freezing, heating, desiccation and exposure to preservatives, high osmotic pressures, and pH extremes. Various "recovery" media are employed in pre-enrichment; these include lactose broth, lactose broth containing 0.6% Tergitol 7, nutrient broth, lauryl tryptose broth, and mannitol purple sugar broth. Lactose broth is the pre-enrichment medium of choice, and although most salmonellae are unable to ferment this sugar, they reproduce in large numbers as the pH is dropped by other microorganisms. The recommended sample to pre-enrichment medium ratio is 1:10 with subsequent subculturing into selective broth by using a 10% (v/v) inoculum (F.D.A., 1976, Polema and Silliker, 1976).

Selective enrichment

The selective enrichment process is intended to permit increases in the population of salmonellae while inhibiting the growth of other organisms present in food samples or pre-enrichment cultures. A variety of media have been formulated for the selective enrichment of salmonellae. The most commonly
employed media are: tetrathionate broth (TT), selenite cysteine (SC) broth, brilliant green (BG) broth, selenite brilliant green (SBG) broth, SBG-sulfadiazine (SBGS) broth, MacConkey's broth, GN broth, BG plus bile salts, magnesium chloride malachite green broth, neutral red lysine iron broth, and strontium chloride broth (Polema and Silliker, 1976).

Generally, unprocessed foods suspected of having high salmonellae counts, e.g., meats, poultry, and frog legs, can be inoculated directly into selective media, although pre-enrichment of these samples will yield higher salmonellae concentrations. Cox and Mercuri (1978) recently compared the recovery of four Salmonella serotypes from unprocessed chicken carcasses by using four selective enrichment media. They concluded that direct selective enrichment is practical, but care should be exercised in the choice of selective media. Both SC and SBG were effective in the recovery of low levels of salmonellae (ca. 20 cells per carcass) whereas, SBGS and TT were ineffective.

Combined pre-enrichment and selective enrichment methods have been examined to determine the feasibility of reducing analysis time. Sveum and Hartman (1977) reported the use of time-release capsules containing tetrathionate or selenite added to pre-enrichment broths. Their results indicated that the method was practical and saved analysis time and materials.
Sveum (1978), in a related study, also determined that the addition of these sterile selective agents into pre-enrichment cultures, following a specified time, was effective. Catalase and pyruvate have also been shown to aid in the recovery of sublethally injured cells. Martin et al. (1976), Raymond et al. (1978), and Hartman (1979) demonstrated increased recoveries of salmonellae and/or coliforms by the addition of one or both of the above components. The ability of pyruvate and catalase to enhance the repair of injured cells is attributed to their $\text{H}_2\text{O}_2$-degrading activity. Heating of cells results in the impairment of catalase function, presumably via denaturation, which causes death of cells due to the accumulation of toxic $\text{H}_2\text{O}_2$. Although more work is required, the possibility of adding catalase and pyruvate to selective enrichment media and thus sidestepping the requirement of pre-enrichment, or reducing pre-enrichment time, shows promise.

Incubation of selective cultures varies with the product under examination; temperatures used are generally in the 35 to 45 C range. The use of high temperature incubation has been endorsed for quite some time. Rodet in 1889, as cited by Harvey and Price (1979), reported a method to isolate S. typhi from contaminated drinking water by using an incubation temperature of 44.5 C. Confirmatory studies by Vincent in 1890 (Harvey and Price, 1979) demonstrated better
success at a temperature of 42 C. Harvey and Thompson (1953) determined that temperatures above 43 C were inhibitory to *S. typhi* and *S. pullorum* when selenite F broth was used but were suitable for the majority of salmonellae serotypes. Other studies have demonstrated the advantages of high temperature incubation (Chau and Huang, 1976, and Harvey and Price, 1979); however, success of these selection techniques varies with the materials examined and the serotype encountered. When employment of higher temperatures is used under well-defined conditions, greater purity of salmonellae growth is generally obtained, thus, simplifying and reducing total analysis time. Yet, since research has not been conducted with a wide spectrum of materials, the majority of enrichment protocols recommend a conventional 35 or 37 C incubation. An excellent review concerning this aspect of salmonellae recovery has been compiled by Harvey and Price (1979).

Subculturing times also vary with the product, although 18 to 24 hr is standard. Hobbs (1962) recommended an incubation period of 72 hr, and others have recommended 8, 24, and 72 hr (Polema and Silliker, 1976).
Pure Culture Techniques

Pure culture methods follow selective enrichment, with isolation of colonies on selective media containing a nutritive agar base. A wide variety of selective and differential media has been developed for the isolation of salmonellae. These employ agents that allow for a rapid differentiation of suspect salmonellae colonies based on the inhibition of nonsalmonellae and the fermentation of various carbohydrates and/or the production of $H_2S$. Decarboxylation of lysine is also a criterion for differentiation in some plating media. Selective agents commonly used for salmonellae recovery include bile salts, sulfonamides and antibiotics, dyes (e.g., brilliant green) and heavy metals.

Common bile salts media include *Salmonella-Shigella* (SS) agar and MacConkey agar. The specific bile-derived compound desoxycholate is utilized in desoxycholate-citrate (DC) agar and xylose-lysine desoxycholate (XLD) agar. Hektoen-enteric (HE) agar employs a combination of bile salts and desoxycholate. The incorporation of citrate into SS and DC agars increased the selectivity of desoxycholate, as demonstrated by Leifson (1935). Likewise, Shanson (1975) demonstrated an increase selectivity of bile salts media with the addition of various heavy metals.

Sulfonamides have been incorporated into BG agar
formulations, enhancing the selectivity of this medium (Moats, 1981, in press). Novobiocin, which inhibits the growth of Proteus, has also been used in various media for increased selectivity of salmonellae (Hoben et al., 1973, Moats, 1978, Shanson, 1975).

Differentiation based on lactose and sucrose fermentation has long been used as a criterion for salmonellae detection. SS and MacConkey's agars employ lactose, whereas HE also contains salicin. Salmonellae, the majority of which do not ferment these carbohydrates, produce an alkaline reaction on these plating media in contrast to the "coliform group" which produce acid fermentation products from both lactose and sucrose. Recent formulations for these media also include iron and thiosulfate to detect H₂S production. Iron and thiosulfate are ingredients in XLD and HE agars and two modifications of BGS developed by Moats and Kinner (1976), tryptic soy xylose lysine (TSXL) agar and tryptic soy BG agar. Selectivity in these latter two media is greatly enhanced because the only organisms other than Salmonella that produce alkaline-H₂S reactions around colonies are Proteus spp. and slow lactose-fermenting Citrobacter spp.

Since lactose-fermenting salmonellae are occasionally encountered, media employing xylose and lysine have been developed. These media, LXD and TSXL, are also useful in
differentiating slow lactose-fermenting *Citrobacter* spp.

The selective properties of brilliant green have been utilized in some media, most notably BG agar. However, selectivity of this medium is a problem because the dye is very sensitive to heat, and overcooking of the medium during preparation substantially decreases its selective properties (Moats, 1981, in press). Some workers have consequently recommended that it may be more advantageous to add filter-sterilized brilliant green after heat sterilization of the basal medium (Moats and Kinner, 1974). This emphasizes the requirement for closely following the manufacturer's instructions for rehydration and preparation of selective media to insure consistent and reproducible results. Overcooking as well as extended storage and exposure to light also destroys the selectivity of HE and SS agars (Fagerberg and Avens, 1976, and Read and Reyes, 1968). Interestingly, some authors have reported that salmonellae other than *S. typhi* grow better if BS plates are aged at refrigerator temperatures for a few days before use (Hobbs, 1963 and McCoy, 1962). Incubation temperatures are in the 35 to 37 C range for selective plating media with subsequent incubation of negative plates for an additional 24 hr. All positive colonies from selective plates are confirmed by biochemical testing and serotyping.

The American Association of Analytical Chemists (AOAC) and the FDA recommend a pure culture technique which involves
pre-enrichment for 18 hr, selective enrichment for 24 hr, and plating on selective agars BS, BG, and SS followed by 24 hr of incubation at 35 to 37 C (FDA, 1976). Positive colonies are subcultured on triple sugar iron (TSI) and lysine iron (LI) agar slants which are incubated for an additional 24 hr. Positive tubes are presumptive evidence of salmonellae and are subjected to further cultural and serological testing (e.g., no urease activity and agglutination with polyvalent antisera). Final confirmation is obtained by positive reactions in lysine decarboxylase, phenol red dulcitol, tryptone, KCN, malonate, and MR-VP broths. These additional tests represent an added 24 hr, giving a total analysis time of approximately 5 or 6 days.

The advent and commercial availability of rapid identification kits for the Enterobacteriaceae has reduced the required work load substantially with respect to identification of suspect isolates. A review covering this aspect of salmonellae identification was recently compiled by Hartman and Minnich (1981, in press). The cost of the recommended pure culture analysis as described by the FDA (1976) was estimated by the USDA and the FDA (Cherry et al., 1971) to be approximately $5 to $7 per sample. Today, a sample might cost $15 to $20.

As a result of the high cost and long analysis time associated with pure culture techniques, alternate, rapid
methods of screening food samples have been developed. Reduction of analysis time is of particular importance when monitoring perishable commodities. The primary thrust in this area has been the development of refined immunofluorescent and enrichment serology techniques.

Immunofluorescence

Immunofluorescence (IF) has been applied to the screening of food products following its trial and success in clinical microbiology. Both direct and indirect IF methods have been described. The initial application of IF methods to food and feedstuffs was reported by the Russian workers Arkhangelskii and Kartashova (1962). The first research in the U.S. applying IF to detect Salmonella in foods was performed by Georgala and Boothroyd (1964). They used an indirect test for screening raw meat samples with fluorescein isothiocyanate (FITC) and rhodamine RB-200 conjugated to anti-rabbit goat globulin. The method was promising; however, false positives caused by nonspecific background staining and cross-reactions with nonsalmonellae were a deterrent to widespread acceptance of their method. Gibbs and Hamilton (1971) utilized the indirect method with a polyvalent 0 antiserum which was absorbed with 6 crossreacting organisms; these authors recorded a false-positive rate of 13%. In a further report, Gibbs et al. (1972) compared the direct and indirect IF procedures to
conventional techniques and concluded that the direct method gave fewer false positives, but neither IF procedure gave unequivocal results because of the high rate of false positives encountered when using either IF method. Studies performed since 1970 have primarily involved the direct method. The indirect method has lost favor because it results in higher levels of nonspecificity, increases the chance of washing fixed cells from the slides, involves more time, and requires two serological reagents versus one (Thomason and Wells, 1971).

In other IF studies, Thomason and Wells (1971) employed a polyvalent OH antiserum instead of single O or H sera and found that this method was more accurate in that fewer false-negatives were encountered. Cross-reactions were still a problem; 12% of E. coli and Citrobacter strains tested, and 36% of Arizona strains, were positive. Cross-reactions also occurred with Serratia, Providencia, Pseudomonas, Shigella, and Proteus cultures. Thomason (1976) reported a microcolony technique to aid in the suppression of background fluorescence; however, when this was tested by Mohr et al. (1970) they obtained 62% false positives and 9% false negatives out of 52 culturally positive samples. Fantasia (1969) also used the direct technique, and with 592 samples representing 34 different foods, obtained no false negatives and only 10 false positives. Goepfert and Insalata (1969) reviewed IF
procedures published to that time. They recommended use of improved fixation methods, standardization of conjugation procedures and need for commercially available antisera, as well as further sensitivity studies. They reflected on the difficulties in comparing existing methods and in making further conclusions without the establishment of standard reagents and test protocols. As it now stands, it is "standard" to use polyvalent OH antisera prepared by immunizing rabbits with formalized whole cells (Kauffmann, 1966, Edwards and Ewing, 1972). Other attempted increases in specificity and sensitivity have involved absorption of polyvalent OH sera with cross-reacting nonsalmonellae and centrifugation of enrichment broths to concentrate cells. The former causes loss of specific antibody and the latter increases the numbers of false positives and adds nothing to sensitivity (Thomason, 1976).

In July, 1972, the Center for Disease Control issued its specifications for standard FITC-labeled Salmonella OH antibodies. Thomason and Herbert (1974) published an evaluation of commercial conjugates for IF detection of Salmonella in various foods. Agreement between cultural and IF tests ranged from 65 to 100% and false positives ranged from 0 to 35% depending upon the type of sample. The mean false-positive rate was 9%. Fresh meats and poultry gave particularly high false-positive results which may have been
caused by large numbers of competing enteric organisms that
gave cross reactions. The AOAC adopted an "official first
action" for screening food samples with IF in October, 1974.
"Final action" recommendations were adopted in 1975, and
diagnostic laboratories now have available a standard
screening procedure using IF (Thomason, 1976). Since
standard polyvalent OH sera are commercially available,
this technique is widely used.

The standard IF technique is performed in the following
manner. A multiwell, coated slide is prepared with the
sample, fixed with an alcohol-chloroform-formalin solution
for 3 min and air dried. Dried smears are covered with poly­
valent fluorescent Salmonella antibody conjugate and allowed
to react for 15 to 30 min while preventing desiccation of the
smear. Several rinsings follow, and the slides are examined
for fluorescing cells (Thomason, 1976). A preliminary report
by Munson et al. (1976) determined that the system could be
automated; however, the expense of instrumentation was
prohibitive for widespread use. The AOAC emphasized that IF
should be used only as a screening test backed up by cultural
methods (FDA, 1976). Of considerable importance is the fact
that commercially available antisera will cross react with
other genera in the Enterobacteriaceae (Thomason, 1976).

Critical aspects of the IF method involve both concen­
tration of the organism and microscopy. A minimum requirement
of $10^5$ Salmonella/ml is necessary to yield one fluorescing cell per oil immersion field. Optimal concentration is approximately $2 \times 10^6$ Salmonella/ml or 20 fluorescing cells per field. When the concentration is too great, loss of intensity results due to excess antigen relative to available antibody, implying the need of thin smears or dilution of the sample (Thomason, 1976). Although reports indicate that IF can be as sensitive as cultural procedures, in large scale comparisons the accuracy of the method was low because of problems with background fluorescence, technician error, inadequate enrichment, overdiluted sera, and incomplete coverage of pooled sera (Gibbs et al., 1972, Insalata et al., 1967, and Thomason, 1976). Of major importance is the large number of false positives caused by cross reactions (Gibbs et al., 1976, Insalata et al., 1976, Thomason, 1976). Although the use of H antisera would theoretically reduce the number of false positives, an increased number of false-negative results would possibly be encountered due to diminished fluorescence because flagella are easily sheared from the cell. Swaminathan et al. (1978a) however, obtained better results with a fluorescent antibody conjugate prepared from SPA purified H-IgG in comparing various commercial conjugates for the detection of salmonellae in retail samples of meat and poultry. They obtained fewer false positives as well as reduced nonspecific staining when using their
preparation than when using commercial OH conjugates. Other workers have likewise obtained better results with polyvalent H antisera (Goepfert et al., 1970, Haglund et al., 1964, Harrington and Ellis, 1972, Sjoquist et al., 1966). Swaminathan et al. (1978a) attributed the lack of false positives to the use of polyvalent H antisera. It is interesting to note that these sera were prepared from highly motile whole cells and that the flagellar antisera thus produced contained considerable titers of O agglutinins, i.e., 1:640 to 1:3560 (Goepfert and Hicks, 1969). The purification of IgG may have reduced or eliminated the O antibodies with the concomitant removal of IgM from the serum preparation.

Although IF is widely used, problems with the subjective interpretation of results (i.e., a 1+ versus a 4+ reaction) and the fact that positive reactions must be submitted to cultural confirmation (an additional 4 or 5 days analysis) are major disadvantages of IF detection methods. The added expense and maintenance of a fluorescent microscope is also a consideration.

Enrichment Serology

Sperber and Deibel (1969) developed a Salmonella detection methodology, based on the serology of the specific H antigens, to eliminate the problem of crossreactivity
encountered in the IF techniques. The method, termed enrichment serology (ES), involves an 18 hr lactose broth pre-enrichment, 24 hr selenite cysteine and tetrathionate broth selective enrichments, and 6 hr post-enrichment in M-broth. The presence of salmonellae in the food sample was determined by tube agglutination employing a polyvalent H serum derived from pooled Spicer-Edwards serum. Complete analysis required approximately 50 hr. The test was simple, inexpensive, and as accurate as the AOAC conventional pure culture technique. Furthermore, the technique eliminated the requirement of a fluorescent microscope and reduced most errors of subjective interpretation. Most importantly, false positives were eliminated with this system, except for Arizona which is a pathogen that should also be monitored. Of 1,141 samples tested, 1.2% were false negatives which were attributed to nonmotile strains of Salmonella.

The tube agglutination required about $5 \times 10^7$ salmonellae per ml as did the IF procedures (Sperber and Deibel, 1969). In ES, 0.5 ml of formalized saline (0.6 ml formalin per 100 ml saline) was mixed with 0.5 ml of M-broth culture; H-antisera was added and allowed to react at 50 C. A positive reaction was evidenced by floccular agglutination within 2 hr. Pooled Spicer-Edwards polyvalent H-antisera was employed; although residual O antibodies exist in this serum, cross
reactions are prevented by two mechanisms: (i) the pooled serum is diluted 1:500 or 1:1,000 which substantially decreases the titer of O antibodies, (ii) the formalization of motile Salmonella cells results in flagella that are rigid and extended from the cell surface, providing a physical barrier that prevents attached O antibodies from reacting with cells in close proximity. Thus, the number of false positives was greatly reduced. Barkate (1968) utilized this method in screening certain feeds for Salmonella. Of 1,894 suspected or confirmed samples, Salmonella spp. were detected in 1,141 by using the AOAC pure culture technique, and 1,134 samples by using ES. The ES procedure required 60 hr and was recommended for routine quality control testing.

Mohr et al. (1970) compared the microcolony, rapid IF, conventional IF, and ES methods against cultural procedures. Using 52 contaminated samples, they obtained 0 false negatives and 27 false positives for the conventional IF technique; 1 false negative and 7 false positives for ES, and 4 false negatives and 33 false positives for the microcolony procedure. They recommended ES followed by conventional IF for the analysis of foods.

In summary, enrichment serology is simple, gives few false positives and requires less costly equipment and less technical expertise than IF. Analysis time was greatly reduced compared to conventional procedures, and the cost of materials
was reduced in comparison to IF and pure culture methods.

Other Methods of Salmonella Detection

A variety of other novel methods have been designed to facilitate more rapid recoveries and presumptive identification of salmonellae. Banwart (1967, 1969a,b, and 1971) and Banwart et al. (1968) conducted a number of studies on the efficacy of using a custom-designed glassware apparatus that would select for and differentiate motile enterics. The apparatus, termed a Banwart Jar, consisted of a central chamber from which three U-tubes projected through the container. The U-tubes could be filled with various selective and differential media to register the presence or absence of salmonellae. A low concentration of agar in the tubes facilitated the selection of motile organisms which could be differentiated based on the reactions given in the various media. Banwart and Kreitzer (1969a,b) used this system for the examination of egg noodles, cake mixes, and candies. The combination of mannitol fermentation, $\text{H}_2\text{S}$ production, and motility, with subsequent serological confirmation, was used to detect salmonellae in these samples; results compared favorably with those obtained by using conventional methods. Similar studies were conducted with turkey rolls and fresh chicken parts (Banwart, 1969b), eggs (Banwart et al., 1968) and
frozen poultry products (Banwart, 1971). The system correlated well with existing methodologies; however, the expense of the glassware and difficulty in preparing the system (e.g., addition of agar to U-tubes) rendered it impractical. It has never met with wide acceptance.

Richter and Banwart (1980) recently reported the use of an X-ray microprobe technique to detect salmonellae in foods. The X-ray microprobe can be used to determine the level of elements present in microorganisms. Since the elemental compositions of salmonellae and other enterics are similar, their system measured increased sulfur concentrations due to the specific binding of FITC antibody conjugate. Thus, their system, as described, is a modification of IF. Large scale comparisons with other methods have not been conducted to date.

Swaminathan and Ayres (1978a) developed a membrane-filter disc immobilization technique for the detection of salmonellae in foods. This method involved concentrating cells from selective broths on a membrane filter and the subsequent inversion of the filter pad on a semisolid selective medium. A paper disc saturated with Salmonella polyvalent H antiserum was then placed approximately 2.5 cm from the edge of the filter and the plate containing the system was incubated in a moist chamber at 37 C. Following incubation, the plate
was examined. Motile salmonellae migrated into the surrounding medium until contact was made with the diffusing antisera. The formation of an immobilized line of growth was presumptive evidence for the presence of salmonellae in the sample.

Coagglutination (COA) methods, which rely on the IgG binding property of SPA, have also been applied to the detection of salmonellae. Although reports using this technique have been limited to the detection of clinical isolates, the applicability of the methods to foods is evident and shows feasibility. Svenungsson et al. (1979) utilized COA for presumptive identification of clinical isolates; antisera were made in response to synthetic antigens, as mentioned earlier. Recognizing that cross reaction existed among the Enterobacteriaceae, these authors made synthetic O antigens 2, 4, 8, and 9, coupled them to BSA, and obtained specific antisera to Salmonella groups A, B, C2, C3, and D by injecting the preparations into rabbits. The antisera were then bound to S. aureus Cowan I cells and used in the COA protocol. In one study, out of 416 Salmonella tested, the COA procedure correctly identified all 24 serogroup A strains, 119 serogroup B strains, and 39 serogroup D strains. Agglutination was unexpectedly obtained with 2 of 44 strains belonging to C2, and no agglutinations occurred with 24 nonsalmonellae. The test was 1,000 times more sensitive than IF; the simplicity
of the test was also an advantage. Edwards and Hilderbrand (1976) also used a COA test for the presumptive identification of salmonellae-like colonies on selective plating media. Cowan I cells were treated with a variety of commercial polyvalent antisera. By adding one drop of the treated cells to a colony, an agglutination reaction could be macroscopically observed within 45 sec. Sanborn et al. (1980), in a similar study, determined that the COA method was sensitive, inexpensive, and easy to perform in the diagnosis of infant salmonellosis.

A rapid radiometric method for the detection of salmonellae was recently proposed by Stewart et al. (1980). This novel approach involved the inhibition of $^{14}$CO$_2$ evolution from dulcitol when cells were in the presence of polyvalent H antiserum. The requirement of expensive radioisotopes, scintillation counter, and the need for running duplicate flasks with and without antisera make the system, as proposed, too cumbersome for routine use.

Enzyme Immunoassays

Since the introduction of "tagged" antibodies by Coons et al. (1942), a variety of immunoassays employing labeled antibodies have been developed. These include the use of various fluorescent dyes, ferritin, radioisotopes, and low
molecular weight enzymes. Immunofluorescent procedures have a wide spectrum of application and have, consequently, become one of the best established procedures in microbiology and clinical science. Ferritin-labeled antibodies, introduced by Singer and Schick (1961) have also had wide application in the localization of specific tissue antigens when used with electron microscopy. However, the use of ferritin-antibody procedures is limited by the high molecular weight of the ferritin moiety. Nonetheless, it was an important development because it was the first system using antibodies coupled to other proteins, an imperative step to the subsequent development of enzyme-antibody conjugates. The radioimmunoassay (RIA), developed by Berson and Yalow (1959), is one of the most powerful and sensitive tools in research for the localization and quantification of specific antigens. Yet, the disadvantages of radioisotope use has given impetus to the discovery of alternative methods. The short shelf life of expensive reagents, the need for expensive and complex instrumentation for analysis, and the strict safety measures required for isotope use and waste disposal have limited the use of RIA. Increased public awareness and concern over the use of radioactive material may further limit the use of this technique if more stringent controls are adopted or waste disposal sites become limited.

The introduction of enzyme immunoassays (EIA), which have
the sensitivity of RIA as well as the safety of IF, is a reasonable alternative. Richard Krause, Director of the National Institute of Allergy and Infectious Diseases, stated in 1976 that once the various applications of EIA were recognized, it would come into wide use in both clinical and research laboratories (Krause, 1976). This prediction has been realized as evidenced by the quantities of publications endorsing this technique during the last four years.

The potential for using low molecular weight enzymes as cytochemical staining reagents was recognized by Strauss (1964a) in the study of protein binding sites and the absorption and intracellular degradation of protein. Horseradish peroxidase (HRPO) was injected into test animals and absorption from the blood into various cell types was traced. The HRPO was developed with the substrate benzidine, yielding a blue reaction product that faded rapidly to a brown or yellow. Fading of the product and nonspecific binding of enzyme to cell membranes and nuclei made interpretation of results difficult. Subsequent investigations revealed that the blue reaction product of benzidine could be stabilized by bathing the tissue preparations in a cold alcoholic solution of 70% nitroprusside (Strauss, 1964b). In similar studies, Graham and Karnovsky (1966) used 3,3'-diaminobenzidine as a developing reagent, yielding a tan reaction product that was sufficiently electron dense so that it could be viewed under
the electron microscope. To improve upon this technique, Nakane and Pierce (1967) and Avrameas and Uriel (1966) independently developed a technique whereby histochemically demonstrable enzymes of low molecular weight could be conjugated to specific antibodies by using various bifunctional reagents. Since that time, a cascade of methods employing enzyme-labeled antibodies have been described.

The requirements for enzyme markers were reviewed by Avrameas (1972) and include the following: high specific activity and turnover number, good stability at room temperature, ability to retain enzymatic activity after conjugation, and commercial availability. Table 1 summarizes the most popular conjugation reagents and enzymes used; horse radish peroxidase (HRPO) and alkaline phosphatase (Alk-Phos) are the most routinely employed enzymes. Table 2 contains a summary of these two enzymes, substrates used, and the reaction products. Commercial HRPO has a molecular weight of 40,000 daltons and contains identical subunits as determined by starch gel electrophoresis and amino acid composition (Keilin and Hartree, 1951). The enzyme contains 18% carbohydrate (Theorell, 1951). Alk-Phos, purified from calf intestine, is suitable for EIA because of its low molecular weight and ability to react with a variety of phosphoric acid esters (Voller et al., 1976, Avrameas et al., 1978).

To couple an enzyme to a protein requires a bi- or
Table 1. Conjugating reagents and enzymes employed to produce enzyme-labeled antibodies

<table>
<thead>
<tr>
<th>Conjugating reagents</th>
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<tbody>
<tr>
<td>1. p,p'-difluoro-m,m'-dinitrodiphenyl sulfone</td>
</tr>
<tr>
<td>2. l-ethyl-3-(dimethyl-aminopropyl) carbodiimide</td>
</tr>
<tr>
<td>3. bisdiazotized o-dianisidine</td>
</tr>
<tr>
<td>4. l-cyclohexyl-3-(3-morpholineoethyl) carbodiimide method p-tolulenesulfonate</td>
</tr>
<tr>
<td>5. cyanuric chloride</td>
</tr>
<tr>
<td>6. glutaraldehyde</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. peroxidase</td>
</tr>
<tr>
<td>2. phosphatase</td>
</tr>
<tr>
<td>3. tyrosinase</td>
</tr>
<tr>
<td>4. glucose oxidase</td>
</tr>
<tr>
<td>5. lactate dehydrogenase</td>
</tr>
<tr>
<td>6. α-amylase</td>
</tr>
</tbody>
</table>

Table 2. Chromogenic products produced by most commonly used enzyme-labeled antibodies with specific substrates

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrates</th>
<th>Color produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>peroxidase</td>
<td>3,3'-diaminobenzidine + hydrogen peroxide</td>
<td>Brown</td>
</tr>
<tr>
<td>peroxidase</td>
<td>o-phenylenediamine (OPD)</td>
<td>Orange</td>
</tr>
<tr>
<td>peroxidase</td>
<td>benzidine</td>
<td>Blue</td>
</tr>
<tr>
<td>peroxidase</td>
<td>z-z'-azino-di-(3-ethyl benzothiazolin-sulfone-6)(diammonium salt)</td>
<td>Green</td>
</tr>
</tbody>
</table>
Table 2 (Continued)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrates</th>
<th>Color produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>alkaline phosphatase</td>
<td>naphthol + diazonium salt (fast garnet)</td>
<td>Red</td>
</tr>
<tr>
<td>alkaline phosphatase</td>
<td>p-nitrophenyl phosphate</td>
<td>Yellow</td>
</tr>
</tbody>
</table>

multi-functional reagent. Care in the selection of a conjugation reagent must be exercised to insure that the activity of both the enzyme and antibody are preserved. Various reagents have been successfully employed (Table 1). Nakane and Pierce (1967) used p,p′-difluoro-m,m′-dinitrophenyl sulfone (FNPS) and 1-ethyl-3-(dimethyl-aminopropyl) carbodiimide in their original study. After making extensive comparisons of coupling reagents, Avrameas and Ternynck (1969) concluded that glutaraldehyde was most advantageous due to ease of the procedure and reproducibility of results. The free amino groups of the immunoglobulin and epsilon-N of HRPO lysine and the alpha-N of N-terminal amino acids are the functional groups responsible for the cross-linking of proteins, the reaction being similar to a Schiff-base mechanism. Cross-linking of enzymes with antibody by using glutaraldehyde produces a heterogeneous population of enzymatically and serologically active complexes.
with a theoretical ratio of one to three enzyme molecules per antibody molecule when using the procedure described by Avrameas and Ternynck (1969). Nakane and Kawaoi (1974) developed a modified conjugation procedure to insure greater labeling of the antibody population by first oxidizing the carbohydrate fraction of HRPO with periodate. Aldehyde groups were formed; these would then be available for coupling to amino groups on the immunoglobulin. Available amino groups on the enzyme were blocked with 1-fluoro-2,4-dinitrobenzene to prevent self-coupling of peroxidase molecules. This method insured up to 95% labeling of IgG without significant loss of enzymatic or serological activity. A recent comprehensive review of coupling techniques has been compiled by Avrameas et al. (1978).

Applications of enzyme-labeled antibodies are broad and include the following: localization of specific intracellular antigens (Avrameas and Uriel, 1966, Lawellin et al., 1977a,b, Nakane, 1975, and Nakane and Pierce, 1967), virus and virus antigen localization and identification in cell and tissue culture (Ubertini et al., 1971, and Wicker, 1971), assays for hormones (Weeman et al., 1978), clinical application for the identification of soluble antibodies and antigens (Engwall and Perlmann, 1971, 1972, Voller et al., 1975, 1976), the diagnosis of autoimmune diseases (Pesce et al., 1978), and the identification of
bacteria (Hsu et al., 1979, Krysinski and Heimsch, 1977, Polin and Kennett, 1980, Swaminathan et al., 1978a, and Thoen et al., 1979). The amenability of EIA to automation is also an advantage to its implementation in a variety of routine diagnostic tests as well as research where large volumes of material are to be analyzed.

In their original study, Nakane and Pierce (1967) employed HRPO and Alk-Phos to locate the six hormones of the anterior pituitary gland of male rats. An indirect method was used by first reacting tissue preparations with rabbit antibody for each hormone, followed by application of goat anti-rabbit immunoglobulin labeled with peroxidase or phosphatase to localize the reaction site. Staining was achieved as described by Graham and Karnovsky (1966). This indirect method offered increased sensitivity over a direct technique, due to binding of more conjugate per antigenic site. Nonspecific binding of peroxidase, as encountered by Strauss (1964a), was alleviated; unlike IF, enzyme-labeled antibodies produced a permanent preparation which could be viewed with both the light and electron microscopes. The method was extremely sensitive because of the amplification effect of the enzyme. An added enhancement over IF was that conjugates were stable indefinitely at temperatures below 0 C and for several months at 4 C. Nakane and Pierce (1967) concluded that enzyme-labeled
antibody techniques (ELAT) were as sensitive and more convenient than IF methods. Avrameas and Uriel (1966) obtained similar results when using HRPO-labeled sheep anti-rabbit IgG for the detection of intracellular rabbit gamma-globulin in spleen cells. The simultaneous detection of two or more intracellular antigens was also demonstrated by employing two different enzyme conjugates that produced different reaction products upon development.

In the study of virus antigens in cell culture, Wicker (1971) compared ELAT and IF and determined that the sensitivity of both techniques was equivalent at the light microscopic level. ELAT was superior to ferritin-labeled antibodies when the electron microscope was used. Likewise, Ubertini et al. (1971) obtained better visualization with enzyme-antibody conjugates compared to fluorescent microscopy, while studying reovirus in chimpanzee liver monolayer cultures. These latter research workers indicated endogenous peroxidase(s) in cell culture may lead to difficulty in data interpretation when using peroxidase-conjugated antibodies to detect specific antigens. Consequently, a variety of methods have been developed to inactivate endogenous activity while preserving the integrity of the labeled antibody activity. These include treatment of cell cultures or cell sections with methanol-\text{H}_2\text{O}_2 (Minard and Cawley, 1978), \text{NaN}_3 or other
peroxidase inhibitors.

The next stage in the development of enzyme immunoassays, which has led to the greatest application of the method, was the successful binding of soluble antigens and antibodies to a solid support phase (polystyrene and polyvinyl tubes and microtitration plates) in such a way that immunological activity was retained. This is the basis for the methods collectively termed enzyme-linked immunosorbent assays (ELISA) developed by Engwall and Perlmann (1971, 1972) and Weeman and Schurrs (1971, 1972). In the succeeding decade since ELISA was first introduced, the method has become widely accepted in research and clinical laboratories.

Because of the versatility of ELISA, a number of modified methodologies have been developed. These include: (i) competitive immunoassays in which test sera antibody titers can be quantified by competing for antigen with labeled antibody; (ii) double-antibody sandwich techniques whereby test antigen is allowed to react with its analogous specific fixed antibody followed by the addition of enzyme-antibody conjugate; (iii) a modified double-antibody sandwich ELISA which is similar to (ii) but uses labeled anti-immunoglobulin; (iv) inhibition ELISA which, in principle, is similar to hemagglutination inhibition; (v) the indirect
ELISA used to determine antibody titer and; (vi) solid phase anti-IgM ELISA which has been used specifically in the detection and measurement of IgM for the early diagnosis of viral and bacterial infection.

The original method described by Engwall and Perlmann (1971, 1972) was a sandwich technique for the quantification of specific antibody. Tubes coated with antigen were incubated with test antisera followed by the addition of an enzyme-labeled preparation of anti-globulin. Less than 1 ng of specific antibody per ml of serum was detectable which compared favorably with other methods in routine use. The advantages of ELISA over RIA were also evident: stability and long shelf life of immunological reagents, sensitivity and ease of data interpretation, and inexpensive instrumentation.

Voller et al. (1976) demonstrated that ELISA was as effective as hemagglutination or complement fixation for the detection of antibodies to rubella. Furthermore, their use of microtitration plates (Voller et al., 1975), in place of tubes, decreased the volumes of reagents that were needed and further simplified ELISA methodology. Their work has been confirmed by a number of research workers, and a recent publication by Forghani and Schmidt (1979) reviewed the sensitivity and specificity of ELISA for the detection of
measles and rubella viral antibodies. Voller et al. (1976) have recommended the micro-ELISA technique for routine screening of pregnant women for rubella, cytomegalovirus, toxoplasmosis, and herpesvirus. Benjamin (1977) used an indirect ELISA for the diagnosis of mucotaneous herpes simplex virus infection and concluded that the technique was sensitive and had definite advantages over other methods. Likewise, Harmon et al. (1979) found ELISA highly satisfactory for the detection and serological identification of adenovirus. Currently, a number of manufacturers market a variety of ELISA kits which makes the technique even more amenable to routine use.

ELISA has also been applied to the serodiagnosis of various parasitic diseases. Rutienberg et al. (1975) compared IF to ELISA in the diagnosis of Trichinella spiralis infection and found that the latter was more sensitive. Capron et al. (1975) reviewed the use of HRPO-conjugates in the diagnosis of protozoan (malaria, trypanosomiasis, amoebiasis), helminthic (schistosomiasis, facioliasis, and filarial worms), and nematode (trichinosis, Strongyloides and Ascaris) diseases. The low detection limit of ELISA, its accuracy compared to IF, and its simplicity make it appropriate for immunodiagnosis of human parasitic infections, particularly in underdeveloped countries where these diseases are a problem and rapid simple methods can be used
in less sophisticated laboratories.

Further illustration of the sensitivity of ELISA was demonstrated by Kitagawa and Aikawa (1976) and Lawellin et al. (1977). A conjugation reagent, maleimidobenzoyl-N-hydrosuccinimide ester (MBS) was synthesized by Kitagawa and Aikawa (1976) and used to conjugate anti-insulin immunoglobulin to beta-galactosidase for the assay of insulin. By using this conjugate for EIA, 20 to 800 pg of insulin were detectable in sera, proving that the assay was more sensitive than the analogous RIA. Lawellin et al. (1977a) used ELISA for the quantification of aflatoxin B₁ in biological tissue. This technique permitted the detection of less than 10 pg of the toxin per gram. They used a similar approach to detect aflatoxin in *Aspergillus parasiticus* hyphae by using an indirect technique (1977b).

Other applications of ELISA include the detection of bacterial antigens and toxins. Drow et al. (1979), using an indirect sandwich ELISA, were able to detect *Haemophilus influenzae* type b antigen in clinical specimens from patients with *Hemophilus* meningitis. The technique was sensitive over a range of 1 to 700 ng antigen per ml. They concluded that ELISA was more sensitive than counter-immunoelectrophoresis and simpler to perform than RIA. In a similar study, conducted by Harding et al. (1979), ELISA was 25 times more sensitive than counter-immunoelectro-
phoresis for the detection of *Streptococcus pneumoniae* antigen in samples of cerebral spinal fluid. They concluded that ELISA should be applicable to the diagnosis of pneumococcal infections. ELISA has also been successfully used in the detection of botulinal toxins A and E (Notermans et al., 1978, 1979). Low quantities of toxin were detectable; less than 100 mouse i.p. $LD_{50}$ for type E toxin. The simplicity and rapidity of the method, and the fact that experimental animals were not required, makes it a prospective replacement for conventional assay procedures. Schultz et al. (1979) showed that ELISA was a sensitive, specific, quantifiable assay for *Pseudomonas aeruginosa* exotoxin A. Antigens have also been measured from organisms such as *Vibrio cholerae* (Voller et al., 1979), *Escherichia coli* (Voller et al., 1979), *Brucella* spp. (Thoen et al., 1979), and *Salmonella* (Svenungsson and Lindberg, 1978a,b).

One of the initial problems that made investigators reluctant to use ELISA was the lack of instrumentation for quantitative analyses. Measurement of color intensity required the transfer of the reaction mixture to spectrophotometer cuvettes, a tedious and time-consuming process. With the advent of the micro-ELISA technique, this became an increasing problem because of small sample volumes. Although qualitative confirmation of ELISA tests is adequate in some situations, those systems used to titer antibody or antigen
concentration require quantitative evaluation of color intensity. Ruitenberg and Brosi (1978) reported the use of an automated system adaptable to both macro- and micro-ELISA tests. Even though their analysis was favorable, the expense of the unit was prohibitive for routine use in most laboratories. Clem and Yolken (1978) described a colorimeter for direct measurement of microtitration plates in which absorbance was determined by reading through the plate bottom. They estimated that a microtitration plate with 96 wells could be read in approximately 5 min, and the unit was relatively inexpensive. McMurray and Blanchflower (1979) developed a multichannel probe colorimeter for reading flat-bottom micro-hemagglutination plates. The use of a dipping probe eliminated problems associated with the meniscus and interference by air bubbles.

Since the initial reports on automated systems have been published, a number of scientific instrument manufacturers have marketed units for micro-ELISA analysis. The simplest and least expensive products are manual readers which are colorimeters adapted to read microtitration plates or custom designed cuvettes. Also included are fully automated instruments with washing capability, printers, and computer input and output ports. As of yet, a comprehensive study comparing these commercial systems has not been conducted, and selection of the appropriate instrument should
be weighed in light of the test being used. A list of manufacturers with units commercially available through 1979 is included in a review by Voller et al. (1979).

A more recent development of EIA is the application of chemiluminescence substrates (Avrameas et al., 1978, and Yolken and Stopa, 1979). These substrate reaction products reportedly are more sensitive than those that rely on colorimetry; the fluorogenic products can be quantitated to the picomole range because light, not color, is the parameter that is measured.

EIA as Applied to the Detection of Salmonella

Krysinski and Heimsch (1977) developed an indirect ELAT for the detection of Salmonella in foods. S. typhimurium was used as a model. Mixed broth enrichment cultures were spotted on cellulose acetate membranes via suction by use of a multiwell lucite template fitted to a Millipore filter base. Cells were fixed to membranes by air-drying and exposure to glutaraldehyde, followed by immersion in rabbit anti-S. typhimurium flagellar IgG. Membranes were subsequently washed and then exposed to goat anti-rabbit (GAR) IgG conjugated with HRPO. A washing cycle followed. Subsequent exposure to 3,3'-diaminobenzidine yielded a brown reaction product. Alternatively, benzidine, which was fixed
with nitroprusside to produce a stable blue reaction product, was used on spots containing known levels of *S. typhimurium*. The reaction products were easily read macroscopically due to the magnification effect of the enzyme-substrate reaction. The technique required fewer cells than enrichment serology and thereby reduced analysis time and projected expense. Analysis time was further reduced, and the technique simplified, by the simultaneous testing of 7 samples on each 25-mm diameter membrane. The technique showed promise as a possible routine screening procedure pending the development of an H antisera pool free from cross-reacting O antibodies. When Spicer-Edwards whole antiserum was tested with the ELAT, cross-reactions occurred, indicating that this commercially available serum was unsatisfactory because of the presence of somatic antibodies.

Minnich (1978) expanded the original model system proposed by Krysinski and Heimsch (1977) by determining a practical means of obtaining a wide-spectrum *Salmonella* H antiserum free of O antibodies. A three-fold approach was adopted: (i) formulation of an H antiserum free of O antibodies by performing immunizations with purified flagellin, the combination of H antigens correlating to the determinants in the commercially available Spicer-Edwards antiserum; (ii) absorption of O antibodies in the above pool and the Spicer-Edwards serum with cross-reacting organisms; (iii)
fractionation of both serum pools to obtain purified IgG with the concomitant removal of O antibodies which are primarily IgM. Although some problems were encountered with endogenous peroxidases giving false positives, the third approach, serum fractionation, gave promising results, required the least effort, and showed feasibility. The commercial availability of Spicer-Edwards serum and the ease of its modification for use in the ELAT demonstrated further potential for the application of this technique in the food industry.

Swaminathan and Ayres (1980) also used peroxidase-conjugated anti-H IgG to detect salmonellae in foods. Antisera preparations were purified by using SPA-affinity chromatography to isolate the IgG; the IgG was then coupled to peroxidase by the method of Nakane and Kawaoi (1974). The test involved fixation of cells from selective enrichment broths to slides followed by the application of the enzyme-antibody conjugate. After the removal of unbound conjugate, the slides were flooded with peroxidase substrate and allowed to react for a specified time. Positive reactions were determined by viewing the slides microscopically for stained cells. The advantages noted in this study were the specificity of the conjugates, ease of test performance, and the elimination of the need for a fluorescent microscope, as required in IF.

Three other key studies of EIA methods for the detection
of bacteria, although they did not pertain to Salmonella detection, are relevant to this study. An approach similar to that of Swaminathan and Ayres (1980) was used by Hsu et al. (1979) to identify Bacteroides spp. Thoen et al. (1979) used a direct EIA for characterizing the A and M antigens of Brucella isolates. In this approach, cells were fixed to microtitration plates pretreated with calf serum-glutaraldehyde and then exposed to HRPO-antibody conjugates. Following incubation for 8 min, the wells were washed 8 times to remove unbound conjugate and then exposed to a substrate solution of \( \text{H}_2\text{O}_2 \) and 2,2'-azino-di-(3-ethyl-benthiocoumarin-6-sulfonate) (ABTS) in citric acid buffer. A green reaction product indicated a positive sample. They concluded that the EIA provided considerable savings in time and materials, compared with the conventional tube agglutination test. Furthermore, the procedure was adaptable to automation. A Gilford (Gilford Instrument Co., Inc., Oberlin, Ohio) automatic EIA instrument is used.

Finally, a report which proved very helpful in the Salmonella detection method developed in this dissertation research, was published by Polin and Kennett (1980). Their approach was similar to that of Thoen et al. (1979); however, neither cells nor antibody were fixed to the solid phase. By mixing these two components, allowing them to react, and subsequent removal of unbound antibody by centrifugation,
they obtained highly satisfactory results in the typing of *Streptococcus* spp. The employment of monoclonal antibodies was an added advantage to the system because it substantially increased specificity to the antigens in question.
MATERIALS AND METHODS

Organisms

Bacterial cultures were obtained from the University of Idaho and Iowa State University culture collections. *Salmonella* serotypes utilized in pure culture studies were originally obtained from Robert W. Ryder, M.D., the Bureau of Epidemiology, Enteric Disease Branch, Center of Disease Control, Atlanta, Georgia.

Media

All bacteriological growth media were commercial products (Difco Laboratories, Detroit, Michigan) with the exception of M-broth which was prepared as described by Sperber and Deibel (1969).

Antibody Purification Methods

As discussed earlier, cross reactions among the *Enterobacteriaceae* may be reduced by fractionation of antisera. For the present study, pooled Spicer-Edwards anti- H polyvalent serum was initially fractionated by gel filtration. A Sephadex G-200 column (100X 2.5 cm) (Pharmacia Fine Chemicals, Piscataway, N.J.) was poured following swelling of the resin in phosphate buffered saline (PBS; 8.5 g NaCl, 0.386 g NaH$_2$PO$_4$	extcdotH$_2$O, 1.02 g Na$_2$PO$_4$ in 1 liter
distilled H₂O, pH 7.2). The void volume of the column was determined by using blue dextran while operating with a hydrostatic head pressure of 16 cm. Four-ml volumes of whole serum were passed through the column, and 6-ml fractions were collected by using a type 3402-B Radi Rak fraction collector (LKB Produkter AB, Stockholm, Sweden). Protein elution was monitored by absorbance at 280 nm. Fractions that contained the IgG peak were pooled and concentrated by ultrafiltration with an Amicon filtration apparatus equipped with a P-3 membrane (Amicon Corporation, Lexington, MA).

Since gel filtration is time-consuming and results in a considerable dilution of IgG, an alternative approach of antiserum fractionation was used. Staphylococcal protein A (SPA) was coupled to Sepharose 4B (Pharmacia Fine Chemicals) as described by March et al. (1974) and modified by Swaminathan et al. (1978a). Activation of the Sepharose 4B was carried out by washing 10 ml of slurry with 300 ml of distilled water in a 60-ml sintered glass funnel. One volume (10 ml) of 2 M sodium carbonate was slowly mixed with the washed beads at 4 C and as the stirring rate was increased, 0.05 volume (0.5 ml) of acetonitrile-cyanogen bromide (CNBR) solution (2 gm CNBR/ml of freshly redistilled acetonitrile) was added. This mixture was stirred vigorously
for 1.5 min after which it was poured back into a 60 ml sintered glass funnel. By applying low suction, the slurry was rinsed first with 10 vol (100 ml) of 0.1 M sodium bicarbonate, pH 9.5, followed by 100 ml of 0.2 M sodium bicarbonate, pH 9.5. The resulting moist compact cake of activated Sepharose beads was subsequently transferred to a plastic bottle containing 40 mg of SPA (Sigma Chemical Co., St. Louis, MO) dissolved in 10 ml of 0.2 M sodium bicarbonate, pH 9.5. The combined mixture of SPA and activated Sepharose was stirred slowly on a magnetic stir plate for 20 hr at 4°C. Following the coupling reaction, the slurry was refiltered and suspended in an aqueous solution of 0.05 M 2-aminoethanol in 0.2 M sodium bicarbonate, pH 9.5, to bind all remaining reactive imido groups of the Sepharose with primary amino groups. The beads were then washed with 200 ml of each of the following: 0.1 M acetate, pH 4; 2 M urea; and 0.1 M sodium bicarbonate, pH 10. Each of the above solutions contained 0.5 M NaCl. The slurry was resuspended in PBS, pH 7.2, poured into a 10-cm column, and washed with three volumes of PBS.

Purification of IgG by using SPA affinity chromatography was accomplished by adding 4 to 6 ml of antiserum to the column. Serum proteins and immunoglobulins other than IgG were washed through the column and collected in 6-ml fractions. When absorbance readings at 280 nm dropped below
0.05, 0.1 M Glycine-HCl buffer, pH 3.0, was added to the buffer reservoir and dissociation of IgG was measured spectrophotometrically. Routinely, all IgG peaks were pooled immediately upon elution from the column and dialyzed against PBS, pH 7.2, for 16 to 24 hr to bring the pH back to neutrality.

Determination of Antibody Purity with Sodium Dodecyl Polyacrylimide Gel Electrophoresis (SDS-PAGE)

The purity of SPA-treated Spicer-Edwards IgG (SE-IgG) was monitored by gradient SDS-PAGE. A glycine discontinuous buffer system, as described by Takacs (1979), was used. A ratio of 30:0.8 polyacrylimide to methyl-bis-acrylamide (Biorad, Richmond, CA) was used for gel preparation. Solutions used in SDS-PAGE were prepared as follows:

1. Sample buffer, 2X, pH 6.8;

- 1M Tris-HCl 12.5 ml
- 2-mercaptoethanol 10.0 ml
- Glycerol 20.0 ml
- SDS 4.6 g
- 0.1% bromophenol blue 2.0 ml
- Diluted to 100 ml

2. Electrode buffer, pH 8.3;

- 0.025 M Tris-HCl 3.03 g
- 0.192 M Glycine 14.41 g
- 0.1% SDS 1.0 g
- The volume was adjusted to 1 liter
3. Resolving gel buffer; pH 8.8

0.0375 M Tris
0.1% SDS
0.001 M EDTA

The volume was adjusted to 1 liter

4. Fixing and staining solution:

0.25% coomassie brilliant blue R-250 in methanol: acetic acid: H₂O (5:1:5 v/v/v)

5. Destaining solution:

methanol:acetic acid:H₂O (5:1:5 v/v/v)

The gel chamber was assembled by sandwiching two spacers between two glass plates (12 cm x 20.3 cm) within a plastic bag (Ziploc, 17.8 cm x 20.3 cm). These plates were then sandwiched between an additional two plates and held in place with rubber bands. Degassed resolving buffer containing acrylimide-bis acrylimide solution received 0.1 ml of freshly prepared 10% ammonium sulfate (Biorad, Richmond, CA) and 0.01 ml of N,N',N'-tetramethylethylenediamine (TEMED). Density gradient gels (4 to 12% acrylamide) were prepared by using a density gradient former (Beckman Instruments, Inc., Palo Alto, CA). Gel polymerization was carried out at room temperature. Following solidification, the sample application comb was gently removed before rinsing the gel in resolving buffer. The plastic bag was then cut along the bottom edge and the sandwiched gel was immersed in the electrophoresis chamber; care was taken to insure
that air bubbles were not trapped on the bottom of the gel. Gels were pre-run for 30 min at 200 volts prior to sample application.

Whole rabbit serum, commercial rabbit IgG (Gibco, Grand Island, N.Y.) and SPA-purified IgG at concentrations of 2 mg per ml were dissolved in sample buffer and heated in a boiling water bath for 2 min to dissociate proteins into individual monomers.

By using a microsyringe (Hamilton Co., Inc., Whittier, CA), 5 μl and 10 μl volumes of sample were delivered to the gel wells. Immediately following sample application, the electrodes were connected to the power supply and electrophoresis was begun at 100 volts per gel until all samples migrated into the gel, whereupon the voltage was increased to 150 volts. Current was applied until the tracking dye reached the gel bottom. The gels were then stained with 200 ml of staining solution for 1 hr at 37 C, destained for 1 hr at 60 C, and dried as described by Takacs (1979).

Antibody-enzyme Labeling Methods

Goat anti-rabbit immunoglobulin (GIBCO) was labeled with horse radish peroxidase (HRPO; Sigma) by using the method of Nakane and Kawaoi (1974). Five mg of HRPO was dissolved in freshly prepared 0.3 M sodium bicarbonate buffer, pH 8.1. To this solution 0.1 ml of 1% 2,4,-dinitrofluorobenzene (DNFB)
in absolute ethanol was added to block available E-amino groups on the enzyme. The formation of aldehyde groups on the carbohydrate moiety of HRPO, to be used in subsequent cross-linking with IgG, was carried out by adding 1.0 ml of 0.07 M NaIO$_4$ in distilled water and allowing the oxidation to proceed for 30 min at room temperature. This solution was then dialyzed against 1-liter changes of 0.1 M carbonate buffer, pH 9.5. Five mg of GAR-IgG was added to the treated enzyme and mixed on a magnetic stir plate for 2 to 3 hr at room temperature. Then 5 mg of NaBH$_4$ was added and the solution was held at 4°C overnight. The resulting conjugate was dialyzed against several changes of PBS and applied to a G-200 Sephadex column to separate conjugated antibody from unbound enzyme. The conjugate fraction was pooled and frozen until needed in PBS-1% bovine serum albumin (BSA) in 4-ml amounts.

Alkaline phosphatase (Alk-Phos) labeling of GAR and SPA-purified SE-IgG was accomplished by using the procedure described by Voller et al. (1976). Glutaraldehyde was used as the cross-linking agent to conjugate Alk-Phos type VII (Sigma) to GAR (Gibco) and SPA-purified IgG.

The commercial enzyme preparation, supplied as an ammonium sulfate precipitate, was pelleted by centrifugation and the supernatant was discarded. Then 2 mg of IgG in 1.0 ml of PBS was added to 5 mg of Alk-Phos. This solution
was exhaustively dialyzed at 4 C against PBS, pH 7.2, to remove remaining ammonium sulfate ions. Glutaraldehyde (25% aqueous solution, Sigma) was added to the enzyme-IgG mixture to a concentration of 0.2%, and the solution was gently mixed at room temperature for several hours. Removal of excess glutaraldehyde was accomplished by dialysis at 8 to 14 C against several changes of PBS, whereupon the dialysis bag was transferred to a reservoir of 0.05 M Tris buffer, pH 8.0, and dialyzed against several buffer changes at 4 C. Following this extensive dialysis, the conjugate (2 ml) was diluted to 4 ml with Tris buffer, pH 8.0, containing 1% BSA and 0.2% NaN₃. The conjugates were stored in the dark at 4 C.

EIA with Cellulose Acetate
Membrane Filters

Initially, membrane filters were used as solid phase matrices in attempts to develop an EIA for the detection of salmonellae in foods. Salmonella spp. and control organisms (e.g., Citrobacter freundii, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae and Proteus spp.) were spotted on membranes as described by Krysinski and Heimsch (1977) and Minnich (1978). An indirect system was employed by using anti-H IgG purified by gel filtration or by SPA-affinity chromatography. To reduce analysis time,
however, pooled SE-IgG was labeled with HRPO. Cell deposits were occasionally washed from membranes; therefore, a reduction in washing by using a direct assay might eliminate this problem.

Minnich (1978) determined that endogenous peroxidases and/or catalases would react with HRPO substrate, leading to false positive results. Immersion of spotted membranes in absolute methanol and exposure of cells to formaldehyde vapors did not reduce this activity. In the present study, heat was used in attempts to inactivate peroxidase and catalase activities while at the same time preserving the integrity of the flagellar antigens. Cultures of E. coli B, Salmonella spp., Enterobacter aerogenes, and B. subtilis were incubated for 16 to 18 hr and then heated in a 56 C water bath for various time periods. Cells of each culture were spotted on membrane filters. Salmonella cultures were also monitored for flagellar integrity by using tube agglutination tests. Membrane filters containing cell deposits were dried at room temperature and then immersed in HRPO substrate (0.3% benzidine + 0.03% H₂O₂ in water). A blue reaction product indicated activity of endogenous enzyme whereas, cell spots remaining colorless indicated heat inactivation of these enzymes.

An alternative method for inactivating endogenous peroxidase was also utilized. Minard and Cawley (1978)
determined that inactivation of peroxidase in tissue culture cells could be achieved by exposing cells to high concentrations of H$_2$O$_2$ in absolute methanol. Therefore, membranes containing cells were soaked for 30 min in a solution of 3% H$_2$O$_2$ in absolute methanol and then were run through the EIA procedure as described by Krysinski and Heimsch (1977). The same approach was also tried by using 0.2% NaN$_3$ in absolute methanol.

**EIA Employing Alkaline Phosphatase-Antibody Conjugates**

Because of problems associated with the use of peroxidase assays, a different enzyme label was selected. Alkaline phosphatase was chosen because it had been employed in a variety of ELISA procedures, it was commercially available, and it was relatively inexpensive. To determine whether endogenous phosphatases would interfere with an assay when this enzyme was used, 0.1-ml samples of cultures of several salmonellae and a variety of non-salmonellae were dispensed into microtitration-plate wells and mixed with phosphatase substrate (1 mg/ml of p-nitrophenyl phosphate in diethanolamine buffer, pH 9.6). The plates were incubated for 1/2 hr at 37 C and subsequently checked for the formation of the yellow chromogenic reaction product. The solubility of the reaction product precluded
the use of membrane filters as the solid phase for the EIA; therefore, microtitration plates were used for these studies.

Since the results of the aforementioned study were satisfactory, a variety of cell fixation methods was explored as a means of absorbing bacterial cells to the polystyrene plastic of microtitration plates. The following methods were tried: air drying; air-drying with methanol fixation (Benbough and Martin, 1976); pretreatment of plates with BSA and 25% glutaraldehyde (Thoen et al., 1979); and the use of 0.1% poly-L-lysine. The fixation procedure described by Thoen et al. (1979) gave the greatest success and is described as follows: 0.05 ml of 1% BSA was added to the wells of a microtitration plate and air-dried or dried with the aid of a hair dryer. These plates could be stored at room temperature. Before use, 0.05 ml of 25% aqueous glutaraldehyde was added to each well; after 30 min the plates were washed with distilled water. Then 0.05 ml of selective enrichment medium (tetrathionate or selenite cysteine broth) containing Salmonella spp. and control enterics was dispensed into individual wells and allowed to air dry. With cells fixed in this manner, 0.05 ml of purified anti-H antibody was added and allowed to incubate for 2 hr. Unbound antibody was washed from the wells by using eight rinses of PBS-Tween 20 (0.5% Tween 20 in PBS). GAR-Alk-Phos conjugate was subsequently added and allowed to react for 2 hr at room temperature. After unbound conjugate had been
removed by using the same wash protocol, 0.2 ml of substrate dissolved in diethanolamine buffer, pH 9.6, was added. Plates were incubated at 37 C for 30 min to allow hydrolysis of the substrate and then the results were visually read. The 10% diethanolamine buffer consisted of 97 ml of diethanolamine, 800 ml of water, 0.2 g of NaN₃, 100 mg of MgCl₂6H₂O; 1M HCl was added until the pH was 9.8. The total volume was made up to 1 liter with water. The substrate solution was always made immediately before use by dissolving substrate tablets (p-nitrophenyl phosphate, Sigma) in buffer; one 5-mg tablet was dissolved in 5 ml of diethanolamine buffer.

Salmonella Detection with Simulated Contaminated Foods

To determine the true efficacy of the EIA procedure that had been developed by using pure cultures, studies were initiated on food samples that were inoculated with low numbers of heat-stressed *S. newport*. Cultures of this organism were inoculated into Tryptic Soy Broth (TSB) and incubated at 37 C for approximately 18 hr. A fresh broth culture was then prepared by transferring 0.1 ml of the inoculum to 8 ml of sterile TSB and allowing it to incubate for 5 hr at 37 C to insure that cells were in exponential growth. A cell count was made on the culture in exponential growth by pour-plating appropriate dilutions in Tryptic Soy Agar (TSA). The culture
tube was then immersed in a 52 C water bath for 30 min to stress the cells. The percentages of injury and death were determined by diluting the cultures serially in sterile PBS; spread plates were made on TSA and SS or Eosin Methylene Blue (EMB) agars.

Two experiments were performed, each involving four 25-g samples of fresh lean hamburger. The samples were aseptically weighed and placed in sterile Waring Blender containers. In the first experiment, one sample received 0.1 ml of a \(10^{-2}\) dilution, another received 1.0 ml of the same dilution, and the remaining two remained uninoculated to serve as controls. The second experiment involved a higher temperature heat stress (55 C) and the two test samples received 0.1 ml or 1.0 ml of a \(10^{-4}\) dilution of the heated culture. Each food sample was homogenized for 2 min with 225 ml of lactose broth. The homogenates were dispensed into sterile wide-mouth screw cap jars and placed in a 35 C incubator. These samples were then analyzed by the standard pure culture technique (SPCT) as described in the Bacteriological Analytical Manual (FDA, 1976). Selective enrichment broths also were screened for Salmonella by the EIA; results were quantified by determining the absorbance of the reaction product at 405 nm.
Analysis of Naturally Contaminated Foods

Encouraging results with inoculated food samples with EIA led to the testing of naturally contaminated foods. However, it was noted in the previous study that fixed cells occasionally were eluted during the washing regime; thus, a direct label was prepared, SE-IgG conjugated to Alk-Phos. By reducing the required wash steps in a direct procedure vs. an indirect method, it was felt that the problem caused by loss of cells during washing could be circumvented.

Naturally contaminated foods were obtained from both private and governmental laboratories as listed in Table 3. These samples were analyzed by EIA and SPCT. Samples for EIA

Table 3. Sources of naturally contaminated foods

<table>
<thead>
<tr>
<th>Food product</th>
<th>Source</th>
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<tbody>
<tr>
<td>Active dried yeast</td>
<td>A. H. Schwab, Deputy Director Minneapolis Center of Microbiological</td>
</tr>
<tr>
<td></td>
<td>Investigations</td>
</tr>
<tr>
<td></td>
<td>Department of H.E.W.</td>
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<td></td>
<td>Food and Drug Administration</td>
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<tr>
<td></td>
<td>Minneapolis, Minnesota 55401</td>
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<tr>
<td>Diet-all apricot spice condiment</td>
<td>Dr. W. H. Andrews Food and Cosmetics Microbiology Branch,</td>
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<td></td>
<td>Food and Drug Administration</td>
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<td></td>
<td>Washington, D.C. 20204</td>
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<tr>
<td>Chocolate candy</td>
<td>Dr. G. Gabis</td>
</tr>
<tr>
<td></td>
<td>Siliker Laboratories</td>
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<tr>
<td></td>
<td>1304 Halsted St.</td>
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<tr>
<td></td>
<td>Chicago Heights, Illinois 60411</td>
</tr>
<tr>
<td>Chicken and pork products</td>
<td>Local retail food markets, Ames, Iowa</td>
</tr>
</tbody>
</table>
were subjected to pre-assay enrichment by inoculating tubes containing 9 ml of M-broth with 0.1 ml of either tetrathionate or selenite cysteine selective enrichment broth. M-broth cultures were incubated 4-6 hr and analyzed by the EIA. Routinely, the plating media employed for isolating *Salmonella* via the conventional technique were SS, BG, and BS. Salmonellae-like colonies were transferred to Triple Sugar Iron agar (TSI) and Lysine Iron agar (LIA) tubes and incubated for 24 and 48 hr. Isolates giving positive reactions in the above two media were checked for purity and then characterized biochemically by using the Minitek system (BBL, Cockeysville, MD). Isolated organisms conforming to *Salmonella* characteristics were mixed with pooled Spicer-Edwards polyvalent antiserum for serological confirmation by tube agglutination as described by Sperber and Deibel (1969). A total of 40 food samples were examined with the direct EIA.

**Studies Utilizing Indirect EIA and Centrifugation Washes**

Inconsistent results in the previous study caused by variations in cell fixation to plastic microtitration wells consequently lead to the adoption of a modified procedure described by Polin and Kennett (1980). This approach eliminated the requirement of cell fixation and allowed for the rapid washing of cells by centrifugation directly in
microtitration plates or cuvette paks (Gilford Inst., Inc., Oberlin, OH). Quantitative results were also recorded in these studies by using a manual EIA reader (Gilford) and an automatic Titertek instrument (Flow Laboratories, Inc., McLean, VA). The Gilford unit utilizes custom EIA cuvettes which are made in sets of ten wells, 50 wells per pack (see Figure 1). These are especially amenable to the type of analysis described because absorbance readings are made through the side of the cuvette versus the bottom as is the case with readers designed for microtitration plates, e.g., the Flow instrument. Thus, absorbance of light by the sheet of bacterial cells is less of a problem than when readings are made of light that must pass through a layer of cells.

An indirect EIA procedure was used in a series of tests. Pure cultures were examined first, followed by tests on naturally contaminated foods. A 0.1-ml sample of post-enrichment M-broth culture was dispensed into a well of a flat-bottom microtitration plate (Micro-Elisa flat bottom, Cooke Engineering, Alexandria, VA) or cuvette pack (Gilford); three wells were used per sample, two as replicates and the third as a control to monitor washing efficiency and/or the expression of endogenous phosphatase activity. Two test wells of the triplet received 0.1 ml of SPA-purified SE-IgG at a maximum dilution that still gave a positive tube agglutination test as determined beforehand with known salmonellae. The control
Figure 1. Gilford cuvettes and conventional microtitration plates that were utilized in this study.
well received 0.1 ml of antibody diluent (2% polyvinyl pyroli-
done, 1% BSA, and 0.6% formalin in PBS). Incubation at room
temperature for 1 hr was followed by three 5-min centrifugation
washes at 2500 rpm. A universal model UV International
centrifuge equipped with a rotor for centrifuging microtitra-
tion plates was used. Gilford cuvette packs were centrifuged
by making a plastic spacer to hold the packs in the micro-
titration plate centrifugation holders. Washings to remove
unbound antibody were done with PBS-Tween 20 (pH 7.2, 0.5%
Tween 20); cells were gently resuspended after each centrifu-
gation. After the last wash, 0.1 ml of GAR-Alk-Phos (Sigma),
at a 1/1000 dilution in PBS-Tween 20, was added to each well.
The plates were incubated at room temperature for 1 hr, and
unbound conjugate was removed by a repeated wash cycle as
outlined above. Phosphatase substrate was added (0.2 ml per
well) and the plates (cuvette packs) were incubated at 37 C
for 30 min. The absorbance of enzyme product was read at
405 nm and recorded.

A total of 98 food samples was analyzed. The naturally
contaminated food samples that were used included active dried
yeast, Diet-all apricot spice condiment, chocolate candy,
and a variety of pork and poultry products purchased at
local retail markets. Sources of the first three food
products are listed in Table 3. Results obtained by using
the EIA procedure were compared with results that were
obtained by using three other methods of *Salmonella* detection: IF, ES, and the SPCT. In addition, pre-enrichment broths were tested by EIA to determine the efficacy of using an EIA screening procedure very early in the isolation schema. This was done by adding 0.1 ml of pre-enrichment medium to 10 ml of M-broth and incubating the tubes at 37 C for 4 to 6 hr. These samples were then subjected to the EIA protocol described for examination of the selective enrichments.

Immunofluorescence tests were performed according to Thomason (1976) by using commercial polyvalent OH fluorescein isothiocyanate conjugates (Clinical Sciences, Whippany, N.J.). Fluorescing cells were graded on a 1+ to 4+ scale; a 4+ reaction was a strongly fluorescing cell that contained stained flagella. As with the previous studies, all isolates giving positive TSI and LIA reactions were characterized biochemically by using the Minitek system (BBL). Isolates that conformed to the biochemical definition of the genus *Salmonella* and gave a positive tube agglutination test were sent to B. O. Blackburn, D.V.M. at the National Veterinary Service Laboratory, Ames, Iowa for serological identification.
Sensitivity Thresholds of EIA

Studies with *S. anatum*, *S. schwarzengrund*, and *S. rubislaw* were conducted to determine the sensitivity of the EIA. Serial 10-fold dilutions of overnight M-broth cultures of the above organisms were made in PBS. A total cell count was determined by pour-plating appropriate dilutions on TSA. Concomitantly, 0.1 ml amounts of dilutions $10^{-1}$ through $10^{-7}$ were dispensed in microtitration plate wells and subjected to EIA analysis. A control organism (*E. coli* B) was also used to determine background levels of absorbance. Results were recorded by visual observations of the yellow reaction product of Alk-Phos.

EIA Sensitivity Using a Fluorogenic Substrate

Yolkèn and Stopa (1979) reported that the use of 4-methyl umbelliferyl phosphate (MUP) as Alk-Phos substrate increased the sensitivity of EIA 10 to 100-fold. MUP (Sigma) was used at a concentration of $10^{-4}$ M in diethanolamine buffer, pH 9.6, supplemented with $10^{-2}$ M MgCl$_2$·7H$_2$O. An overnight broth culture of *S. anatum* was diluted as described in the previous study and a total count determined. EIA analysis of 10-fold dilutions were also performed and the microtitration plates were exposed to long-wave ultraviolet light to detect fluorescence of the substrate reaction product, 4-methyl umbelliferone.
RESULTS

Antiserum Fractionation

Figure 2 depicts a typical gel filtration profile of polyvalent anti-H on a Sephadex column. The void volume of the 85 cm x 2.0 cm column was 120 ml. IgG, intermediate in molecular weight with respect to albumin and IgM, eluted in the central peak. An Ouchterlony double diffusion assay was used to detect the presence of IgM. No perceptible line of precipitation was observed between wells containing re-concentrated IgG and anti-rabbit IgM.

Figure 3 depicts a typical fractionation of Spicer-Edwards antiserum by using SPA affinity chromatography. The affinity chromatography column (10 cm x 1 cm) was operated at a hydrostatic head pressure of 30 cm and a flow rate of 25 to 30 ml per hour. The void volume was approximately 10 ml. SPA affinity chromatography possessed certain advantages for IgG purification, including speed of the procedure, the relatively large amounts of serum that could be fractionated at a time, and the low dilution factor of material due to the small size of the column. From 15 ml of antiserum, 60 mg of purified IgG were obtained. This was either stored in PBS with 0.2% NaN₃ or lyophilized and stored at -20 C. An overnight dialysis of the pooled fractions of IgG in PBS was sufficient to return the pH to neutrality.
Figure 2. Gel filtration elution profile of whole Spicer-Edwards antiserum. High molecular weight proteins, including IgM, elute in the first peak. The second peak contains IgG and other intermediate molecular weight proteins. The third peak consists of small molecular weight proteins, primarily albumin. Fractions 21-29, which contained IgG, were pooled and concentrated by ultrafiltration.
Figure 3. A typical serum fractionation profile via SPA affinity chromatography is depicted in this illustration. Whole serum is applied to the column and all serum proteins other than IgG are washed through the column with PBS. When the absorbance is less than 0.05 as indicated by "a", 0.1 M glycine-HCl buffer pH 3.0 is added to the buffer reservoir. As the pH drops to 3.0, IgG is eluted. Fractions 15 to 18 were pooled and used in subsequent EIA analysis. The column is "regenerated" by flushing out the glycine-HCl buffer with PBS, pH 7.2
IgG Purity Check by SDS-PAGE

SDS-polyacrylamide gradient gels were prepared and used to monitor the purity of SPA-purified IgG. Figure 4 is a comparative run of Spicer-Edwards whole serum, commercial goat anti-rabbit IgG, and SPA-purified IgG. As can be seen from the electrophoresis patterns, the IgG that was purified by SPA absorption and elution resulted in two protein bands representing the light and heavy chains of the molecule. These purified IgG preparations demonstrated agglutinating activity against all Salmonella cultures tested. No IgM was detectable by Ouchterlony double-diffusion against IgM. Titers ranged from 1:40 to 1:100, depending, of course, on the amount of material that was placed on the column.

Enzyme-antibody Labeling

As previously mentioned, initial studies were conducted with horseradish peroxidase as the labeling probe. The method of Nakane and Kawaoi (1974) was used to produce goat anti-rabbit peroxidase (GAR-PER) conjugates. This method was developed to eliminate some of the problems associated with protein-protein conjugation encountered when glutaraldehyde was used as a crosslinking agent (glutaraldehyde couples the free amino groups such as the epsilon-amino groups of
Figure 4. SDS-PAGE gel comparing: (A) Spicer-Edwards whole serum, (B) commercial goat anti-rabbit IgG, (C) SPA purified S.E. IgG. The two bands in slot C represent the heavy and light chain of the immunoglobulin molecule.
lysine); the result is a heterogeneous population of high molecular weight conjugates of enzyme and antibody, antibody and antibody, and enzyme coupled to enzyme. The procedure developed by Nakane and Kawaoi (1974) capitalizes on the carbohydrate moiety of HRPO. Amino groups are blocked on the enzyme with DNFB and the carbohydrate is oxidized to produce aldehyde groups. Aldehyde groups are then available to react with the free amino groups of added IgG molecules; the reaction is mediated by sodium borohydride. Figure 5 is a typical elution profile obtained from a Sephadex G-200 gel filtration column; low molecular weight, unbound HRPO eluted first, followed by the IgG-HRPO conjugate. This procedure generally resulted in approximately 95% binding efficiency of IgG with HRPO and a ratio of 2 to 3 HRPO molecules per IgG molecule.

Alkaline phosphatase (Alk-Phos) labeling of IgG was also conducted. The procedure of Voller et al. (1976) was used. This one-step conjugation method utilized glutaraldehyde as the crosslinking agent. Glutaraldehyde reacts readily and irreversibly with the epsilon-amino groups of lysine present in proteins, but the mechanism for this reaction is not well-understood. In a number of studies (Avrameas et al., 1978) little or no free antibody was detected after the coupling reaction, and between 60-70% of the initial enzyme activity was present. The immunological reactivity of conjugates prepared in this manner is difficult to determine because of
Figure 5. This illustration depicts a gel filtration profile of conjugated antibody and free unconjugated peroxidase after using the conjugation procedure described by Nakane and Kawaoi (1975). Enzyme-antibody conjugate elutes first followed by the low molecular weight unbound peroxidase. Peroxidase absorbs at 430 nm, and protein at 280 nm.
the heterogeneous population of high molecular weight mole-
cules produced. No attempt was made to separate conjugated
GAR-Alk-Phos from unbound Alk-Phos.

During the course of these studies, GAR-Alk-Phos conjugates
became commercially available and one source (Sigma Chemical,
St. Louis, MO) was tested and found to be highly satis-
factory. These conjugates were prepared from GAR-antibodies
isolated by immunospecific purification to remove essentially
all goat serum proteins including immunoglobulins that do not
bind specifically to rabbit IgG. The enzymatic activity of
the preparations used was 300 units/ml, and they were used at
the recommended dilution of 1:1,000.

EIA Results Utilizing
Membrane Filters

Initial studies were performed in attempts to combine
the model study of Krysinski and Heimsch (1977) and the
results obtained by Minnich (1978) into a workable Salmonella
schema suitable for routine screening of foods. Obstacles
encountered in the two previous studies included the require-
ment of H-specific antiserum devoid of cross-reacting O anti-
bodies and a means of inactivating intracellular peroxidases
and/or catalases which developed the peroxidase substrate
leading to false-positive reactions. Fractionation of the
commercial anti-H antiserum circumvented cross-reaction
problems encountered in the earlier investigation. A variety of methods to eliminate endogenous peroxidases activity were used and will be described in some detail.

First of all, cells spotted on membranes were exposed to absolute methanol, formaldehyde vapors, 30% \( H_2O_2 \) in absolute methanol, and \( NaN_3 \). Intracellular peroxidases were not completely inactivated by any of these treatments. Another approach, which appeared at first to solve this particular problem, involved the use of heat. Recent reports in the literature (Hartman, 1979, Martin et al., 1976, and Raymond et al., 1978) indicated that catalase and pyruvate, when added to selective media, increased the recovery of heat-stressed cells. These data suggested that essential catalases and/or peroxidases were susceptible to heat; if these enzymes (or pyruvate, which degrades \( H_2O_2 \)) were not supplied exogenously, the cells succumbed to toxic build up of peroxide. Based on this hypothesis, cultures of salmonellae, \textit{Citrobacter freundii}, \textit{Bacillus subtilis}, and \textit{E. coli} B were inoculated into TSB or M-broth, incubated overnight, and then immersed in a 56 C water bath. Samples (0.1 ml) of each culture were spotted on membranes after 0, 2, 10, and 15 min of heat exposure. The membranes were subsequently air-dried and immersed in peroxidase substrate. Cell deposits displayed decreasing quantities of color with increasing heat-exposure times. This suggested that
the enzymes were being denatured and that a heat treatment prior to EIA analysis could circumvent the problems encountered with false positives.

Pure culture studies were initiated by using an indirect EIA procedure and a 10 min, 56 C, heat treatment of all test organisms. A 1:40 dilution of SPA-purified IgG was used to treat the membranes, and three rinse cycles (30 ml of PBS per rinse) were used to remove unbound antibody. Filter membranes were subsequently immersed in diluted GAR-PER conjugate, washed, and exposed to peroxidase substrate. Upon immersion in substrate, all cell deposits immediately turned blue, indicating that peroxidase activity was present. Positive tests included *B. subtilis* cells, which possess no serological identity with the *Enterobacteriaceae*. These results suggested that: (i) the conjugate was nonspecifically absorbing to cells, (ii) the washing protocol was not sufficient to remove unbound conjugate, or (iii) endogenous peroxidases still expressed themselves, indicating that variables other than heat treatment were involved. Increased washing of membranes resulted in the elution of the cells; therefore, a direct label was prepared with SPA-purified IgG conjugated to HRPO. This was done to reduce overall washing requirements. Membranes tested with the direct EIA by using 90 ml of PBS to remove unbound conjugate, however, yielded the same results; all cell spots reacted with substrate.
In attempts to define the variables involved in this problem, 1.0-ml samples of formalized, heat-treated cell suspensions of salmonellae, Enterobacter aerogenes, E. coli, and B. subtilis were reacted with equal volumes of diluted Spicer-Edwards IgG-HRPO (SE-IgG-HRPO). These broth suspensions were incubated at 50°C for 1 hr and then were subjected to three consecutive washes with PBS in a centrifuge to remove unbound conjugate. A 0.1-ml portion of supernatant from each culture was added to 1.0 ml of peroxidase substrate to check for residual enzyme-antibody conjugate. These tubes failed to produce a color reaction, indicating that all unbound conjugate was removed. The cells were resuspended in 1.0 ml of PBS, spotted on membranes, dried, and immersed in substrate. All cultures gave positive reactions; however, the chance observation was made that salmonellae spots reacted first, followed by color development on control cells spots. This suggested that an intermediate enzyme reaction product might be reacting with intracellular enzymes which were inadequately inactivated by heat. Alternatively, the conjugate might have been sufficiently nonspecific that it reacted with a variety of serologically unrelated organisms.

To test the former hypothesis, heat-treated E. aerogenes and B. subtilis cells were spotted on two sets of membranes. Each set was immersed in two separate, fresh, preparations of benzidine-\(\text{H}_2\text{O}_2\) substrate. No color reaction developed as
was expected based on the results of initial inactivation studies. However, when 0.05 ml of conjugate or diluted free enzyme was added to one set of membranes in substrate solution, color developed immediately in the cell spots. When cell suspensions of the same cultures were either boiled or autoclaved, no color developed, even in the presence of exogenous peroxidase. The results of this set of experiments seemed to signify that prior heating of cells did not completely inactivate cellular peroxidases. Furthermore, cellular permeability to benzidine apparently was decreased. Hence, an intermediate reaction product of benzidine may have been generated, either by exogenous addition of enzyme or conjugate bound to salmonellae, and permeated bacterial cells.

Free radicals generated when peroxidases act on substrate (White et al., 1978 and the present research), may have been responsible for problems encountered with the use of IgG-HRPO. This problem was not pursued further, and the adoption of a conjugate employing Alk-Phos was used in all further studies because the problems associated with endogenous peroxidases appeared insurmountable from a practical perspective.
EIA Analysis with Alkaline Phosphatase Conjugates

The problems encountered with a peroxidase antibody label necessitated the adoption of an alternative enzyme probe. Alkaline phosphatase, derived from calf intestines, has been utilized for many ELISA studies and was chosen for use in subsequent EIA experiments for Salmonella detection. Initial studies to determine if intracellular phosphatases would interfere with the system were conducted first. Washed cell suspensions (0.10 ml) of a variety of test organisms were incubated with 1 ml of phosphatase substrate (p-nitrophenyl phosphate in diethanolamine buffer, pH 9.6) for 1 h and observed for a yellow reaction product. No color was observed with the exception of a 3-day-old culture of E. aerogenes C3000. Overnight suspensions of this organism were negative, implying that potential problems might arise in older cultures if phosphate became limiting.

Unlike the peroxidase substrate reaction product, the alkaline phosphatase product (p-nitrophenol) is soluble and would not remain localized on cell spots fixed to membranes. Consequently, membrane filters were not a feasible solid phase matrix because there was no way of determining which spot(s) was giving a positive reaction. Microtitration plates were adopted, therefore, for use as a solid phase.

Fixation of cells to the wells of the microtitration
plates was a difficult problem. Polystyrene plastic tubes and microtitration plates have the characteristic of binding protein irreversibly at a high pH (Voller et al., 1979), and these are routinely employed in a variety of ELISA procedures. When my studies were initiated, the only report found in the literature involving the binding of bacterial cells to polystyrene microtitration plates was a method by Benbough and Martin (1976); a 30-second exposure to absolute methanol was used. I used this method in pure culture studies; however, later I discovered that air-drying was just as effective. Undoubtedly, cells were eluted in the EIA protocol, but sufficient numbers were generally present to be detected. Some wells containing known positives occasionally gave negative results; thus, replicates of five or more wells per sample were required to assure valid data. Table 4 contains a composite of these initial studies. Ten replicates per sample were used, and the alkaline phosphatase substrate was pooled to determine the absorbance in 1-ml spectrophotometer cuvettes.

To reduce the number of cells eluted during washing of microtiter plates, a direct label was prepared by using SPA-purified IgG and Alk-Phos. Pure culture studies with the direct EIA (data not shown) were encouraging, although the low titer of the conjugates, which were used at a 1/20
Table 4. Absorbance values obtained by using the indirect EIA on pure cultures

<table>
<thead>
<tr>
<th>Organism</th>
<th>Absorbance(^a) (405 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. newport</td>
<td>1.10</td>
</tr>
<tr>
<td>S. sandiego</td>
<td>0.65</td>
</tr>
<tr>
<td>S. senftenberg</td>
<td>1.40</td>
</tr>
<tr>
<td>S. tennessee</td>
<td>1.45</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>1.40</td>
</tr>
<tr>
<td>C. freundii</td>
<td>0.40</td>
</tr>
<tr>
<td>E. coli B</td>
<td>0.56</td>
</tr>
<tr>
<td>E. coli K12</td>
<td>0.54</td>
</tr>
<tr>
<td>E. aerogenes</td>
<td>0.33</td>
</tr>
</tbody>
</table>

\(^a\)Absorbance values represent the pooled substrate solution from 10 samples, 0.2 ml per sample.

dilution, was a concern from an economic standpoint.

The next step in developing an EIA for food testing was to analyze food samples inoculated with salmonellae. The objective was to determine the efficacy of the EIA with mixed cultures of food contaminants as well as to determine the effectiveness of the procedure when various selective media (lauryl sulfate, tetrathionate, and selenite cystine) were used. Fresh hamburger samples were inoculated with
heat-stressed *S. newport* by using a method described by Sveum (1978). An overnight broth culture of *S. newport* was held at 52 C for 30 min; this purportedly results in a 2-log (99%) reduction of viability with approximately 70 to 80% sublethal injury to survivors. Cell injury was monitored by the concomitant plating of cells on both TSA (nonselective) and EMB (selective) agars. In actuality, the first experiment resulted in only a 20% kill; 14% of the surviving cells were sublethally injured.

Of the four hamburger samples initially analyzed, 1 sample received approximately $1.4 \times 10^5$ *S. newport*/gram and the other sample received approximately $1.4 \times 10^4$ cells/gram. The other two samples were not inoculated, thus serving as controls. The results are shown in Table 5. These inocula were higher than desired; therefore, the next attempt involved a 30-min heat treatment at 55 C. Also a $10^{-4}$ dilution of cells was used to insure that low numbers of cells were used for inocula of the meat samples.

The second attempt at inoculating hamburger with heat-stressed cells gave negative results. The heat treatment reduced the total count to approximately $5 \times 10^4$ cells/ml, and 1 ml of a $10^{-4}$ and $10^{-5}$ dilution of this culture was used. Comparative counts on TSA and SS agars indicated that 84% of the survivors were sublethally injured. The meat sample that received the highest concentration of cells received
Table 5. Absorbance values obtained from selective enrichment cultures of hamburger with and without added S. newport

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tetraethionate</th>
<th>Selenite cysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculated</td>
<td>1.4</td>
<td>0.64</td>
</tr>
<tr>
<td>Inoculated</td>
<td>1.2</td>
<td>0.62</td>
</tr>
<tr>
<td>Uninoculated</td>
<td>0.48</td>
<td>0.80</td>
</tr>
<tr>
<td>Uninoculated</td>
<td>0.45</td>
<td>0.62</td>
</tr>
<tr>
<td>Control^</td>
<td>0.21</td>
<td>0.42</td>
</tr>
</tbody>
</table>

^Controls consisted of the two selective enrichment media inoculated with E. coli B.

about 0.25 S. newport cells/gram. All four samples, inoculated and controls, gave negative results with both the EIA and standard pure culture method.

Although these experiments were less than ideal with respect to inoculum size, they nonetheless pointed out two important considerations. First, selenite cysteine gave aberrant results, presumably caused by color interference; the red precipitate of selenite cysteine broth absorbed at 405 nm. Second, the normal flora of these meat samples did not interfere with the assay. Hence, the test demonstrated potential for actual food analysis.
Rather than continue studies with simulated salmonellae-contaminated foods, a variety of naturally contaminated foods was obtained from industry and governmental laboratories; they are listed in Table 3. Forty samples were analyzed by using the direct EIA method. In contrast to the aforementioned simulated contamination studies, a 4 to 6 hr post enrichment of selective media in M-broth was used (Sperber and Deibel, 1969) to eliminate the interference caused by the red precipitate in selenite cysteine broth. Samples were simultaneously analyzed, except where noted, by EIA, IF, ES, and the standard pure culture technique (SPCT) for comparative purposes. The fixation protocol of Thoen et al. (1979) was also used to bind cells to microtitration plates. The EIA results were determined by visual observation of the yellow reaction product of Alk-Phos. A composite of the results obtained for these forty samples appears in Table 6.

Two problems were encountered in these analyses of naturally contaminated foods. First, inconsistent results were obtained in the washing regime that was used to remove unbound conjugate. In the second set of dried yeast samples, unbound conjugate apparently was not removed sufficiently, because the control wells, which received 0.05 ml of E. coli B, gave relatively strong background reactions. Second, there were continued problems of cells sloughing from the microtitration plate wells. Four to five replicates of each sample
Table 6. Numbers of positive results obtained when naturally contaminated foods were examined by EIA, SPCT, IF, and ES

<table>
<thead>
<tr>
<th>Sample type</th>
<th>No. of samples</th>
<th>EIA(^a)</th>
<th>IF</th>
<th>ES</th>
<th>SPCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Spice condiment)</td>
<td>8</td>
<td>3</td>
<td>N(^b)</td>
<td>N</td>
<td>3</td>
</tr>
<tr>
<td>Spice condiment</td>
<td>6</td>
<td>3</td>
<td>4</td>
<td>N</td>
<td>3</td>
</tr>
<tr>
<td>Spice condiment</td>
<td>6</td>
<td>3</td>
<td>5</td>
<td>N</td>
<td>2</td>
</tr>
<tr>
<td>Spice condiment</td>
<td>8</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>(Dried yeast)</td>
<td>6</td>
<td>1</td>
<td>N</td>
<td>N</td>
<td>1</td>
</tr>
<tr>
<td>Dried yeast</td>
<td>6</td>
<td>6</td>
<td>N</td>
<td>N</td>
<td>0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>40</td>
<td>19</td>
<td>-</td>
<td>-</td>
<td>12</td>
</tr>
</tbody>
</table>

\(^a\)EIA = enzyme immunoassay, SPCT = standard pure culture technique.

\(^b\)N = analysis not performed.

...were run, since some cell deposit(s) eluted from the wells. Even though the overall EIA results compared favorably with the SPCT, with the exception of the last set of dried yeast samples, confidence in obtaining consistent results was questionable because of the problems indicated above. A more reliable cell fixation process was required.

The use of poly-L-lysine as a fixative was examined in attempts to improve the adherence of bacterial cells to micro-
titration plates. The results were inconsistent and are dis­
cussed only briefly. Microtitration plates were filled with
a 0.1% aqueous solution of poly-L-lysine (Sigma, St. Louis,
MO) and held at 4 C overnight. The poly-L-lysine solution was
poured off and the plates were rinsed with PBS. M-broth cultures
were then dispensed into the wells and dried with a blow
dryer. Although this method resulted in adequate fixation of
cells, it also required an additional 2-hr incubation of wells
with 1% BSA to bind remaining poly-L-lysine sites, after drying
of the cells. Without BSA treatment, conjugate bound to the
wells, leading to false-positive reactions.

EIA Analyses Using Unfixed Cells
Washed by Centrifugation

Polin and Kennett (1980) described an EIA procedure to
identify group B streptococci in which cells and antibody
were added to microtitration plate wells, neither being fixed
to a solid phase. Removal of unbound antibody was achieved
by three 5-min washings of cells. Each time the cells were
sedimented by centrifugation and the supernatants were dis­
carded. When this same approach was adopted to the EIA for
salmonellae screening, consistent results were obtained with
both pure cultures and naturally contaminated food samples.
Quantification of results was achieved by using either a
Gilford manual EIA reader (model 25066X9, Gilford Inst. Lab,
Oberlin, OH) or a Flow laboratory Titertek multiscan EIA reader (Flow Lab., McLean, VA).

Table 7 contains results obtained during pure culture studies and use of the Gilford instrument. Duplicate samples were run with each culture, and a control cuvette well was employed to monitor washing of unbound antibody and conjugate antibody. This latter well received no specific anti-H IgG, but only had exposure to GAR-Alk-Phos. Table 8 contains the results of a similar study conducted with the Flow Titertek instrument.

Comparative analyses of 98 food samples were conducted by using IF, ES, SPCT and the quantitative EIA that was developed as described in the preceding pages. Also, the pre-enrichment medium was monitored with the EIA by inoculating 0.1 ml of each 18- to 24-hr pre-enrichment medium into 10 ml of sterile M-broth. The M-broth subcultures were incubated for 4 to 6-hr at 37 C and then were analyzed by EIA. Tables 9 through 15 are results from each of the experiments; the composite results appear in Table 16. Two criteria were used to score positive EIA samples; from pure culture studies and the initial food samples examined it was noted that controls varied, with respect to absorbance, from 0.060 to 0.380. Hence, an absorbance above 0.400 was one criterion that was used to conclude that a sample was positive. With a few food samples, the difference between the control and replicate
Table 7. Absorbancies obtained when pure cultures were analyzed by an indirect EIA with centrifugation washing

<table>
<thead>
<tr>
<th>Organism</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample 1</td>
</tr>
<tr>
<td>S. anatum</td>
<td>0.874</td>
</tr>
<tr>
<td>S. blockley</td>
<td>2+</td>
</tr>
<tr>
<td>S. bredeney</td>
<td>2+</td>
</tr>
<tr>
<td>S. cerro</td>
<td>2+</td>
</tr>
<tr>
<td>S. heidelberg</td>
<td>2+</td>
</tr>
<tr>
<td>S. illinois</td>
<td>2+</td>
</tr>
<tr>
<td>S. kaapstad</td>
<td>1.920</td>
</tr>
<tr>
<td>S. miami</td>
<td>2+</td>
</tr>
<tr>
<td>S. paratyphi B</td>
<td>2+</td>
</tr>
<tr>
<td>S. rubislaw</td>
<td>2+</td>
</tr>
<tr>
<td>S. salinatus</td>
<td>2+</td>
</tr>
<tr>
<td>S. sandiego</td>
<td>2+</td>
</tr>
<tr>
<td>S. semftenberg</td>
<td>2+</td>
</tr>
<tr>
<td>S. thompson</td>
<td>2+</td>
</tr>
<tr>
<td>S. zuerich</td>
<td>2+</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>0.237</td>
</tr>
<tr>
<td>Enterobacter freundii X539A</td>
<td>0.254</td>
</tr>
<tr>
<td>Enterobacter freundii</td>
<td>0.153</td>
</tr>
<tr>
<td>Escherichia coli 2B14</td>
<td>0.337</td>
</tr>
</tbody>
</table>

\(^a\) Gilford Manual EIA reader used to measure absorbancies.
\(^b\) Control wells received no S.E.-IgG, but did receive GAR-Alk-Phos.
\(^c\) N-analysis not performed.
Table 8. Absorbancies obtained when pure cultures were analyzed by an indirect EIA\textsuperscript{a} with centrifugation washing

<table>
<thead>
<tr>
<th>Organism</th>
<th>Absorbance (405 nm)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample</td>
<td>Control</td>
</tr>
<tr>
<td>S. anatum</td>
<td>1.008</td>
<td>0.027</td>
</tr>
<tr>
<td>S. rubislaw</td>
<td>1.271</td>
<td>0.030</td>
</tr>
<tr>
<td>S. senftenberg</td>
<td>1.339</td>
<td>0.070</td>
</tr>
<tr>
<td>S. zuerich</td>
<td>1.249</td>
<td>0.025</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>0.255</td>
<td>0.012</td>
</tr>
<tr>
<td>Escherichia coli B</td>
<td>0.159</td>
<td>0.015</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The Flow Titertek instrument was used to measure absorbancies.

samples was perceptible even though the samples gave O.D. values of less than 0.400. Therefore, a difference of 0.200 O.D. units between control and sample was considered a borderline positive. Three of the food products tested (Diet-all spice condiment, active dried yeast, and chicken giblets from a local retail market) were naturally contaminated with \textit{Salmonella}. Figures 6 and 7 demonstrate positive and negative samples using microtitration plates and Gilford cuvettes. These isolates were identified by using the minitek identification system; the biochemical reactions of these three \textit{Salmonella} isolates are indicated in Table 17.
Figure 6. This Gilford microcuvette depicts positive and negative test samples. Sample 1 (wells 1-3) and sample 2 (wells 4-6) are positive tests as indicated by the chromogenic reaction product. Sample 3 (wells 7-9) is negative. Wells 3, 6, and 8 are control wells to monitor washing efficiency; they received Alk-Phos conjugate but no S.E.-IgG. Three samples were generally run with each cuvette which allowed for the testing of 15 samples per cuvette pak. The cuvettes were washed and could be reused.
Figure 7. Microtitration plate depicting positive and negative samples. Samples (3 wells per sample) were added to vertical columns 1, 3, 5, 7, 9, and 11. Wells in rows C and H that received test material were used as controls. Of the 12 samples tested with this plate, 3 were strongly positive.
Each isolate was sent to the National Animal Disease Service Laboratory, Ames, Iowa, for serological analysis. Serological typing indicated that the foods were contaminated with *S. minnesota*, *S. heidelberg*, and *S. rubislaw* (Tables 9, 11, 14, 15).

Of great interest is the success obtained with the EIA when monitoring pre-enrichment broths. All positive confirmed samples were originally identified as positive from pre-enrichment media subcultured for 4 to 6 hr in M-broth. Furthermore, in many instances, a higher O.D. value was obtained from the pre-enrichment broths versus the O.D. values determined from subcultures of selective media. Of even greater interest was the fact that all EIA "false positives" were obtained from the same food lots that were shown to be actually contaminated, by the SPCT.

**Sensitivity Threshold of the EIA with p-nitrophenyl Phosphate**

Results obtained by using three *Salmonella* cultures for sensitivity threshold determinations indicated that approximately $1 \times 10^5$ cells per ml gave a positive reaction that was sufficiently strong for visual discrimination of positive versus negative results. *S. rubislaw* gave a weekly positive reaction at the level of $1 \times 10^4$ cells per ml.
Table 9. Comparative analysis of twelve 25-g samples of spice condiment (EIA absorbances were determined with the Gilford instrument)

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Pre-Tet</th>
<th>Tet</th>
<th>s.c.</th>
<th>IF Tet</th>
<th>s.c.</th>
<th>ES Tet</th>
<th>s.c.</th>
<th>SPCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (+)</td>
<td>0.774</td>
<td>0.829</td>
<td>0.562</td>
<td>4+</td>
<td>4+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2 (+)</td>
<td>0.905</td>
<td>1.45</td>
<td>0.791</td>
<td>4+</td>
<td>4+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3 (+)</td>
<td>0.899</td>
<td>0.901</td>
<td>1.020</td>
<td>4+</td>
<td>4+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4 (+)</td>
<td>0.672</td>
<td>0.933</td>
<td>0.850</td>
<td>4+</td>
<td>4+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5 -</td>
<td>0.386</td>
<td>0.104</td>
<td>0.215</td>
<td>-</td>
<td>1+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6 (+)</td>
<td>0.732</td>
<td>1.05</td>
<td>1.263</td>
<td>4+</td>
<td>4+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7 (+)</td>
<td>0.911</td>
<td>0.546</td>
<td>0.987</td>
<td>4+</td>
<td>4+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8 (+)</td>
<td>0.538</td>
<td>0.619</td>
<td>0.827</td>
<td>4+</td>
<td>4+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9 -</td>
<td>0.288</td>
<td>0.126</td>
<td>0.150</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10 (+/-)</td>
<td>0.346</td>
<td>0.182</td>
<td>0.201</td>
<td>3+</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11 (-)</td>
<td>0.137</td>
<td>0.69</td>
<td>0.215</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12 (+)</td>
<td>0.866</td>
<td>1.085</td>
<td>0.966</td>
<td>4+</td>
<td>4+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\) Pre-enrichment EIA analysis.

\(^b\) Post-enrichment EIA analysis from tetrathionate and selenite cysteine.

\(^c\) Immunofluorescence analysis from tetrathionate and selenite cysteine; fluorescing cells were graded on a 1+ to 4+ scale.

\(^d\) Post-enrichment enrichment serology analysis; tube agglutinations were graded on a plus/minus scale.

\(^e\) Positive isolates were confirmed biochemically and by tube agglutination with polyvalent antiserum; isolates were serotyped and determined to be S. rubislaw.

\(^f\) Positives identified by pre-enrichment EIA analysis; sample 10 was considered borderline positive since it gave an absorbance greater than 0.20 above the control.
Table 10. Comparative analysis of twelve 50-g samples of chocolate candy (EIA absorbancies were determined with the Gilford instrument)

<table>
<thead>
<tr>
<th>Sample&lt;sup&gt;a&lt;/sup&gt;</th>
<th>EIA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>IF</th>
<th>ES</th>
<th>SPCT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-</td>
<td>Tet s.c.</td>
<td>Tet s.c.</td>
<td>Tet s.c.</td>
</tr>
<tr>
<td>1</td>
<td>0.234</td>
<td>0.062</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.243</td>
<td>0.050</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>0.270</td>
<td>0.113</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>0.367</td>
<td>0.046</td>
<td>N</td>
<td>3+</td>
</tr>
<tr>
<td>5</td>
<td>0.207</td>
<td>0.045</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>0.263</td>
<td>0.046</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>0.278</td>
<td>0.049</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>0.184</td>
<td>0.058</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>0.218</td>
<td>0.054</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>0.299</td>
<td>0.101</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>0.173</td>
<td>0.064</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>0.237</td>
<td>0.047</td>
<td>N</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>See Table 8 for explanation of abbreviations.

<sup>b</sup>N = absorbancies were not determined, since usual examination indicated that all samples were negative.
Table 11. Comparative analysis of twenty-six 25-g samples of active dried yeast (EIA absorbancies were determined with the Gilford instrument)

<table>
<thead>
<tr>
<th>Sample</th>
<th>EIA</th>
<th>IF</th>
<th>ES</th>
<th>SPCT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Tet</td>
<td>s.c.</td>
<td>Tet</td>
</tr>
<tr>
<td>1</td>
<td>0.044</td>
<td>Na</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.252</td>
<td>N</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>0.041</td>
<td>N</td>
<td>N</td>
<td>3+</td>
</tr>
<tr>
<td>4</td>
<td>0.104</td>
<td>N</td>
<td>N</td>
<td>3+</td>
</tr>
<tr>
<td>5</td>
<td>0.312</td>
<td>N</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>0.292</td>
<td>N</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>0.165</td>
<td>N</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>0.253</td>
<td>0.039</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>0.225</td>
<td>0.221</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>0.216</td>
<td>0.015</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>0.350</td>
<td>0.157</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>0.152</td>
<td>0.063</td>
<td>N</td>
<td>1+</td>
</tr>
<tr>
<td>13</td>
<td>0.222</td>
<td>0.071</td>
<td>N</td>
<td>3+</td>
</tr>
<tr>
<td>14</td>
<td>0.123</td>
<td>0.075</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>0.050</td>
<td>0.021</td>
<td>N</td>
<td>3+</td>
</tr>
<tr>
<td>16</td>
<td>0.226</td>
<td>0.063</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>17^b</td>
<td>1.262</td>
<td>0.605</td>
<td>N(+)</td>
<td>4+</td>
</tr>
<tr>
<td>18</td>
<td>0.095</td>
<td>0.078</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>0.212</td>
<td>0.057</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>0.256</td>
<td>0.037</td>
<td>N</td>
<td>2+</td>
</tr>
<tr>
<td>21</td>
<td>0.241</td>
<td>0.021</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td>0.206</td>
<td>0.08</td>
<td>N</td>
<td>2+</td>
</tr>
<tr>
<td>23</td>
<td>0.164</td>
<td>0.045</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>0.334</td>
<td>0.064</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>0.250</td>
<td>0.055</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td>0.377</td>
<td>0.071</td>
<td>N</td>
<td>-</td>
</tr>
</tbody>
</table>

^aAbsorbancies were not determined, since visual examination indicated all were negative except sample 17 (sc).

^bThe isolate was identified as _S. minnesota_.


Table 12. Comparative analysis of twelve 25-g samples of pork sausage and liver (absorbancies were determined with the Flow TiterTek instrument)

<table>
<thead>
<tr>
<th>Sample</th>
<th>EIA Pre</th>
<th>EIA Tet</th>
<th>IF Tet s.c.</th>
<th>ES Tet s.c.</th>
<th>SPCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.230</td>
<td>0.115</td>
<td>N</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.252</td>
<td>0.178</td>
<td>N</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>0.131</td>
<td>0.001</td>
<td>2+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>0.270</td>
<td>0.044</td>
<td>N</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>0.287</td>
<td>0.001</td>
<td>N</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>0.208</td>
<td>0.092</td>
<td>N</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>0.354</td>
<td>0.014</td>
<td>2+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>0.332</td>
<td>0.001</td>
<td>2+ 3+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>1.206</td>
<td>0.959</td>
<td>N(+)</td>
<td>4+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>0.290</td>
<td>0.098</td>
<td>N</td>
<td>2+ 2+</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>0.200</td>
<td>0.001</td>
<td>N</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>0.308</td>
<td>0.047</td>
<td>N</td>
<td>2+ 2+</td>
<td>-</td>
</tr>
</tbody>
</table>

aN indicates visual determination done; sample 9 was positive by visual examination.

Sample 9 was inoculated with 1 loopful of an overnight culture of S. anatum to serve as a positive control.
Table 13. Comparative analysis of twelve 25-g samples of chicken wings (absorbancies were determined with the Flow Titertek instrument)

<table>
<thead>
<tr>
<th>Sample</th>
<th>EIA Pre-Tet</th>
<th>EIA s.c.</th>
<th>IF Tet s.c.</th>
<th>ES Tet s.c.</th>
<th>SPCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.239</td>
<td>0.191</td>
<td>0.151</td>
<td>1+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.124</td>
<td>0.124</td>
<td>0.148</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>0.242</td>
<td>0.164</td>
<td>0.220</td>
<td>1+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>0.319</td>
<td>0.177</td>
<td>0.194</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>5</td>
<td>0.215</td>
<td>0.167</td>
<td>0.148</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>0.279</td>
<td>0.133</td>
<td>0.151</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>0.238</td>
<td>0.183</td>
<td>0.131</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>0.208</td>
<td>0.102</td>
<td>0.105</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>0.261</td>
<td>0.079</td>
<td>0.098</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>0.191</td>
<td>0.156</td>
<td>0.092</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>11</td>
<td>0.230</td>
<td>0.186</td>
<td>0.142</td>
<td>1+</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>0.178</td>
<td>0.167</td>
<td>0.136</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 14. Comparative analysis of 25-g samples of chicken giblets (samples 1-6) and ground pork (samples 7-12) (absorbancies were determined with the Flow Titertek)

<table>
<thead>
<tr>
<th>Sample</th>
<th>EIA</th>
<th>IF</th>
<th>ES</th>
<th>SPCT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Tet</td>
<td>s.c.</td>
<td>Tet</td>
</tr>
<tr>
<td>1</td>
<td>0.245</td>
<td>0.087</td>
<td>0.244</td>
<td>-</td>
</tr>
<tr>
<td>2(^a)</td>
<td>0.451</td>
<td>0.143</td>
<td>0.408</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>0.303</td>
<td>0.094</td>
<td>0.147</td>
<td>-</td>
</tr>
<tr>
<td>4(^a)</td>
<td>0.462</td>
<td>0.347(^b)</td>
<td>0.387</td>
<td>4+</td>
</tr>
<tr>
<td>5</td>
<td>0.204</td>
<td>0.077</td>
<td>0.235</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>0.208</td>
<td>0.076</td>
<td>0.259</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>0.267</td>
<td>0.083</td>
<td>0.129</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>0.267</td>
<td>0.150</td>
<td>0.090</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>0.198</td>
<td>0.098</td>
<td>0.159</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>0.259</td>
<td>0.089</td>
<td>0.162</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>0.181</td>
<td>0.076</td>
<td>0.225</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>0.173</td>
<td>0.052</td>
<td>0.314</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) Were culturally confirmed as positive; the isolate was S. heidelberg.

\(^b\) In sample 4, the EIA test on s.c. was considered positive because the absorbance was greater than 0.2 O.D. units above that of the control.
Table 15. Comparative analysis of twelve 25-g samples of chicken giblets (samples 1, 2, 5, 6, 9, 10, 12)\(^a\) and pork liver (samples 3, 6, 7, 8, 11) (absorbancies were determined with the Flow Titertek)

<table>
<thead>
<tr>
<th>Sample</th>
<th>EIA</th>
<th>IF</th>
<th>ES</th>
<th>SPCT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Tet</td>
<td>s.c.</td>
<td>Tet</td>
</tr>
<tr>
<td>1</td>
<td>1.236(^b)</td>
<td>0.608</td>
<td>0.391</td>
<td>4+</td>
</tr>
<tr>
<td>2</td>
<td>0.216</td>
<td>0.177</td>
<td>0.169</td>
<td>2+</td>
</tr>
<tr>
<td>3</td>
<td>0.217</td>
<td>0.173</td>
<td>0.114</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>0.816(^b)</td>
<td>0.157</td>
<td>0.1315</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>0.497(^b)</td>
<td>0.185</td>
<td>0.594</td>
<td>3+</td>
</tr>
<tr>
<td>6</td>
<td>0.348</td>
<td>0.156</td>
<td>0.123</td>
<td>3+</td>
</tr>
<tr>
<td>7</td>
<td>0.230</td>
<td>0.152</td>
<td>0.219</td>
<td>3+</td>
</tr>
<tr>
<td>8</td>
<td>0.180</td>
<td>0.334</td>
<td>0.140</td>
<td>2+</td>
</tr>
<tr>
<td>9</td>
<td>0.350</td>
<td>0.382</td>
<td>0.246</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>0.139</td>
<td>0.197</td>
<td>0.230</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>0.336</td>
<td>0.144</td>
<td>0.110</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>0.587(^b)</td>
<td>0.507</td>
<td>0.225</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\)Chicken giblets were obtained from the same package as those samples in Table 13.

\(^b\)Indicates positive samples from pre-enrichment EIA.
Table 16. Composite of the comparative analysis results obtained from 98 food samples reported in Tables 9 through 15

<table>
<thead>
<tr>
<th>Category</th>
<th>EIA</th>
<th>IF</th>
<th>ES</th>
<th>SPCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>20</td>
<td>13</td>
<td>23</td>
<td>13</td>
</tr>
<tr>
<td>Negative</td>
<td>78</td>
<td>85</td>
<td>75</td>
<td>85</td>
</tr>
<tr>
<td>False positive</td>
<td>7</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>False negative</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) Pre = positives identified from pre-enrichment.
\(^b\) Post = positives identified from post-enrichment.

Table 17. Biochemical reaction profiles of three Salmonella isolates from naturally contaminated foods

<table>
<thead>
<tr>
<th>Test or substrate</th>
<th>Isolate 1</th>
<th>Isolate 2</th>
<th>Isolate 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂S</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arginine</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lysine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ornithine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lactose</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ONPG</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Raffinose</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Arabinose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Citrate</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Malonate</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Inositol</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ducitol</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Indole</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methyl red</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Voges-Proskauer</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Poly H antiserum</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>agglutination</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Sensitivity Threshold of EIA with a Fluorogenic Substrate

Yolken and Stopa (1979) recently reported that the use of 4-methyl umbelliferyl phosphate (MUP) increased the sensitivity of an ELISA technique 100-fold for detection of rotavirus. I conducted one experiment with MUP substrate by using the indirect EIA developed for Salmonella detection. 

S. anatum was used. Visual observation of diluted S. anatum indicated that approximately $10^4$ cells per ml gave a reasonably strong reaction (versus the background level of fluorescence) to detect a positive result. More importantly, on the basis of visual discrimination, fluorogenic reactions were easier to read than was the yellow color produced when p-nitrophenyl phosphate was used as the substrate.
DISCUSSION

A number of novel techniques for Salmonella detection in foods have appeared in the literature over the past two decades. The acceptance of a new procedure, however, requires that it demonstrate decided advantages over existing methods. Considerations of sensitivity of the method, technical expertise and time required for analysis performance, cost effectiveness, and amenability to automation are factors that should be addressed. The initial ELAT model for Salmonella detection developed by Krysinski and Heimsch (1977) demonstrated such potentials. By using filter membranes as a solid phase detection matrix, simultaneous multisample analysis requiring little technical expertise and expense was shown to be feasible. Furthermore, the lack of a requirement for specialized equipment, and the macroscopic determination of results were added advantages of the procedure. The major obstacle foreseen in developing the method further for the detection of all salmonellae was the requirement of a polyvalent antiserum free of cross-reacting O-antibodies. Minnich (1978) demonstrated the further potential of the ELAT by showing that commercial polyvalent Salmonella antisera could be modified (by purification of IgG via gel filtration) to eliminate the problems associated with cross-reactions among other members of the Enterobacteriaceae. Although problems were
encountered with interpretation of results because of the expression of intracellular peroxidases, it was believed that the potential of the technique, particularly its possible adaptation for automation, warranted further investigation.

The present study was undertaken to further test the possibility that an enzyme immunoassay for Salmonella could be applied to food analysis. The main objectives were to confirm, by further experimentation, the observation that serum fractionation to remove IgM (the primary immunoglobulin class formed against O antigens) resulted in a reduction of cross reactions. In addition, a means of inactivating interfering cellular peroxidases that made data interpretation difficult when using peroxidase-antibody conjugates was to be determined. Finally, assuming that the above objectives met with success, a comparative study using naturally contaminated foods was to be made to judge the performance of the EIA versus existing methods of Salmonella detection currently in use.

To reduce the quantity of IgM in antibody preparations for use in the EIA, gel filtration was initially utilized. Studies with IgG purified in this manner supported the earlier contention of Minnich (1978) that cross-reactions could be eliminated or reduced by removal of IgM. However, the time required for serum fractionation by this means, as well as the dilution of IgG and resulting requirement of reconstitution,
were definite disadvantages. Consequently, the use of SPA affinity chromatography for IgG purification was investigated and found to be highly satisfactory. Even though the use of this method is initially capital intensive because setting up the column involved approximately a $400 investment, the column could be used repeatedly without loss of performance. The durability of the SPA column was demonstrated by the fact that it has been in use for over two years, has been put through over twenty-five cycles of serum fractionation and has survived two cold room failures necessitating repouring of the slurry. There was no apparent loss of performance. The rapidity of fractionation was an added advantage. Relatively large amounts of whole serum (4 to 8 ml), when applied to the column one morning yielded purified IgG ready for use by the next day. In contrast, gel filtration generally required 24 to 30 hr for serum to pass through the column and the IgG peak had to be reconcentrated before use. Furthermore, gel filtration, using a 2.5 x 100 cm column, was limited to 4-ml amounts of whole serum; otherwise, the resolution of peaks became a problem. A 6-ml quantity of Spicer-Edwards antiserum, when applied to an SPA column, resulted in a 12-ml fraction of purified IgG that was directly used for EIA analysis at a 1:20 dilution. This was calculated to be sufficient for over 10,000 EIA tests.

Investigations undertaken to find a means of negating
the effect of interfering endogenous peroxidases when using HRPO-conjugates were unsuccessful. A variety of peroxidase inactivation methods were examined. These included the use of formaldehyde, absolute methanol, 30% \( \text{H}_2\text{O}_2 \) in methanol, 0.2% \( \text{NaN}_3 \) in methanol, and heat denaturation. As discussed previously, heating cultures prior to analysis appeared in preliminary studies to solve the problem; no color reaction developed when membranes that contained cells heated for 10 min at 56°C were immersed in substrate. However, false positives were obtained in later tests, and their occurrence eventually lead to the conclusion that heat "inactivation" was actually an alteration in permeability to substrate. Once the peroxidase reaction was initiated in the substrate bath, either by the presence of HRPO-conjugate bound to *Salmonella* or the addition of free enzyme, all cell deposits reacted. The continued use of HRPO-conjugates was abandoned. Interestingly, this problem was not addressed by Krysinski and Heimsch (1977); however, these authors did indicate that nonspecificity of the test was encountered when commercial Spicer-Edwards antiserum was employed. They attributed the nonspecificity to cross-reactions caused by the presence of O antibodies. In retrospect, they may have observed a combination of cross-reactions and expression of cellular peroxidases. Likewise, Swaminathan and Ayres (1980) did not encounter this problem (personal communication, B. Swaminathan, July, 1979)
in their slide test to detect Salmonella by using HRPO-conjugates. In this instance, the slide fixation procedure might have inactivated the enzymes in question.

Due to the problems with the use of HRPO-conjugates, alkaline phosphatase-labeled antibodies were substituted for HRPO conjugates in the EIA protocol. Bacterial phosphatases are inducible enzymes and thus are produced when inorganic phosphate becomes limiting. The experiments designed to detect interference from these endogenous enzymes indicated that they would not be a problem unless cultures were examined after extended incubation. C. freundii C3000 did react with phosphatase substrate after 3 days of incubation, but was not a problem if 18 to 24 hr cultures were examined.

In contrast to peroxidase, alkaline phosphatase did not form an insoluble reaction product that remained localized on membrane filters. Hence, microtitration plates were used in place of cellulose acetate filters for the solid phase. A new series of problems subsequently arose with respect to the fixation of bacterial cells to the plates. Polystyrene and polyvinyl microtitration plates and tubes have been routinely employed in a variety of ELISA techniques because protein will bind irreversibly to these plastics at a high pH. In pure culture studies, it was determined that air-drying or air-drying coupled with a brief treatment with methanol was adequate for the fixation of bacterial cells. However, it was
also found that known *Salmonella* samples occasionally failed to react in some replicate wells, indicating that sloughing of the cells was occurring. In an attempt to circumvent this problem, a direct assay (S.E.-IgG coupled directly to Alk-Phos) was instituted to reduce the number of rinse cycles that would be required. Also, the fixation procedure described by Thoen et al. (1979) was adopted. Although these changes met with success in both pure culture studies and the analysis of simulated and naturally contaminated foods, inconsistency in results ascribed to fixation variables resulted in an overall lack of confidence in the procedure.

Even though inconsistent results in these studies raise questions regarding the reliability of the technique, important observations were brought to light that warrant further discussion. First of all, the potential of the EIA was demonstrated in its specificity; no cross-reactions were observed in pure culture studies or mixed culture situations in the actual analysis of food samples. Second, quantitation of results by measuring the absorbance of the substrate reaction solution was shown to be feasible, thus, increasing the ease of data interpretation. This latter point is in contrast to IF where the subjective interpretation of observed fluorescing cells may vary with the technician. Finally, these initial studies pointed out potential problems when examining selective enrichment media. The inoculated-food studies indicated that tetra-
thionate broth could be examined directly, whereas the red precipitate of selenite cysteine broth interfered with both visual and quantitative determinations of results. Hence, the requirement of post-enrichment in M-broth, as described by Sperber and Deibel (1969), was adopted for subsequent analyses.

Inconsistency in the fixation of cells to microtitration plates, particularly in actual food analysis, could not be surmounted. Other reports of EIA procedures applied to bacteria (Thoen et al., 1979) have dealt with the serological identification of isolates in pure culture. As my studies with pure cultures of Salmonella confirmed, analysis of isolates that were fixed by air-drying was feasible, due presumably to the high numbers of organisms used. Thoen (Charles O. Thoen, personal communication, September, 1979) also found that the fixation procedure that he initially developed was not necessary; simple air-drying adequately fixed enough cells for his analysis. Sloughing of Brucella spp. from plates occurred, but to what extent this occurred had not been examined.

To circumvent the fixation problem, the procedure by Polin and Kennett (1980) for Streptococcus identification was adopted with slight modification. This EIA procedure did not require fixation of cells or antibody to the solid phase; both were simply mixed and allowed to react, then unbound antibody
was removed by centrifugation washing. The system described by these authors required pretreatment of microtitration plate wells with 1% gelatin in tissue culture medium. Presumably, the gelatin covered binding sites on the solid phase and eliminated the possibility that specific antibody or enzyme-antibody conjugates would bind during the analysis and cause a high reaction-product background. I found that this pretreatment was unnecessary with the *Salmonella* EIA test because control wells used to monitor washing of conjugate gave little or no background absorbance. When a method modified after Polin and Kennett (1980) was used, highly satisfactory results were obtained in a comparative study of EIA and three other methods of *Salmonella* detection.

The most surprising results that I obtained were those obtained with EIA analysis of pre-enrichment media. Sensitivity studies indicated that the EIA could detect $10^4$ to $10^5$ *Salmonella* cells/ml. Therefore, it was reasoned that if salmonellae reached this concentration in the pre-enrichment medium they may be detectable at the earliest stage of analysis. Since most food samples are routinely pre-enriched at a 10% w/v ratio, a 4 to 6-hr post-enrichment in M-broth was deemed necessary to dilute out food particles which would potentially cause problems in absorbance readings or nonspecifically bind antibody. This short post-enrichment was sufficient to yield turbid cultures. M-broth was selected since it is slightly
selective for salmonellae because the primary carbon sources are citrate and mannose. Another reason that M-broth was the medium of choice is because it was formulated to enhance flagellation of cells, the antigens of concern in the EIA test. Of the 98 food samples examined, pre-enrichment EIA detected 20 positive samples. The SPCT, which is used as the standard to judge performance of screening tests, detected only 13 positive samples; all 13 were included in the 20 positives by EIA. The isolates from the SPCT were confirmed biochemically and serologically. Likewise, the EIA and ES procedures using post-enrichment in M-broth from selective enrichment media, correlated with the SPCT; the same 13 samples gave positives. The high false positive rate with IF was probably due to the fact that fresh meats were examined and these contained large numbers of other Enterobacteriaceae. Excessive numbers of false positive reactions has routinely been a problem of the IF procedure in the examination of fresh meats (Thomason, 1976).

Based on the number of positives from SPCT, the EIA pre-enrichment EIA had a "false positive" rate of approximately 7%. However, what is most interesting is the fact that the 7 sub-samples that were judged false positive by pre-enrichment EIA were obtained from food samples from which other sub-samples contained salmonellae. For example, the 16 chicken giblet samples (Table 15) came from the same package; four were
identified as positive from pre-enrichment EIA and only two of these were confirmed positive by ES, IF, SPCT, and selective enrichment EIA. Of further interest is the comparison of O.D. values from the two samples ultimately shown to be positive. Sample 1 gave a very strong reaction (O.D. = 1.230) whereas, the selective enrichment EIA average O.D. value for tetrathionate was 0.610 and selenite cysteine was 0.391, a borderline positive. Similarly, sample 5, shown to be positive by pre-enrichment EIA, was negative in tetrathionate and positive for selenite cysteine. These results suggest that selective enrichment may be too selective against some salmonellae. The two samples shown positive by pre-enrichment EIA might have contained a serotype that failed to replicate in the selective enrichment media. This is a reasonable hypothesis since the requirement for using two selective enrichment media is based on the knowledge that different serotypes differ with respect to sensitivity to selective agents. Hence, with guarded enthusiasm, I conclude that EIA pre-enrichment analysis may be substantially more sensitive than SPCT. If this supposition is borne out by further studies, this may be the most important observation made during the present investigation.

Pre-enrichment EIA testing warrants further investigation because the advantages of being able to directly examine pre-enrichment media are manyfold. First of all, is the advantage of savings in time. Food samples set up in the
morning for pre-enrichment can be incubated for 24 hr and used to inoculate M-broth post-enrichment media tubes the next morning. These can be incubated for 4 to 6 hr, and results from EIA analysis would be available by late afternoon. In short, only two working days would be sufficient, a significant savings in time over the 5 to 7 days presently required to perform a Salmonella analysis by SPCT, or three working days required by the proposed EIA procedure on enrichment cultures. Secondly, examination of pre-enrichment media would result in half the required number of samples for examination; once selective enrichment has been used, the sample number is doubled because both tetrathionate and selenite cysteine broths must be examined. Last of all, the economics of such a procedure are evident; a considerable savings of media and materials as well as technician time would be realized.

Definite advantages of the EIA procedure over other screening methods are also worthy of mention. The sensitivity of the EIA is equivalent to or better than the lower limit of IF which required $10^5$ salmonellae/ml (Thomason, 1976). Tube agglutination, as performed in ES, requires an optimal concentration of approximately $10^7$ salmonellae/ml. Furthermore, problems in IF are occasionally encountered when high concentrations of salmonellae are present (Thomason, 1976); decreased fluorescence of cells is obtained due to the high concentration of antigen. This would not be a problem in EIA
analyses because bound enzyme-antibody conjugates would produce the same amount of end product whether it was concentrated on few cells or diluted out with excess antigen. In addition, the EIA results are quantitative and the reaction product is stable; in contrast, IF results are subjective and the fluorescence fades rapidly once the slides are exposed to ultraviolet light. Specificity of the EIA was also demonstrated. The use of purified IgG decreased the rate of cross-reactions. IF, however, utilizes polyvalent OH conjugates and gives a mean false positive rate of 9% (Thomason, 1976). Last of all, the amenability of EIA to automation is a great advantage. The system proposed allows for rapid and simultaneous multisample analysis. Automated ELISA systems are currently in use, and the adaptation of the Salmonella EIA test to present commercial equipment could be accomplished readily.

As far as disadvantages are concerned, the Salmonella EIA analysis has one drawback. This involves the use of centrifugation washings to remove unbound antibody. Consequently, the requirement of a centrifuge adapted for the use of microtitration plates may be a deterrent to adoption of the procedure. Furthermore, the final EIA test described was an indirect procedure; thus, two centrifugation steps were used. The development of a direct test, which was shown to be feasible in this study, would reduce the centrifugation washing requirement by one half; however, a reliable means of fixation of
cells to the plates is needed.

With respect to the instrumentation used in this study, both the Flow and Gilford units were satisfactory. I preferred the Gilford unit. Gilford cuvettes contain deeper wells which hold 0.5 ml. Therefore, by adding 0.3 ml of washing buffer and gently shaking the cuvettes, cells could be rapidly and thoroughly resuspended. In contrast, microtitration plates hold a maximum of 0.25 ml, and resuspension of cells was more difficult. Occasionally, the use of wooden applicator sticks was required to resuspend cell pellets in the plates, which made their use somewhat more cumbersome than the Gilford cuvettes. Also, variability in background absorbance was slightly more pronounced with the Flow instrument than with the Gilford instrument. The microtitration-plate EIA reader makes absorbance readings through the bottom of the plate and therefore an uneven distribution of cells over the plate bottom resulted in this variance. In contrast, the Gilford unit reads absorbance through the side of the cuvette and absorbance by the cells was less of a problem. This variability could be further decreased by centrifugation of the Gilford cuvettes prior to reading because the cells tended to resuspend, to a limited extent, during the 30-min incubation with substrate. Finally, the difference in the cost between the two units is worthy of consideration. The Gilford unit sells for approximately $2500 versus approxi-
mately $11,000 for the Flow instrument. However, the fact that the Flow unit has printout capabilities and a computer input port is included in the instrument are considerations. Laboratories analyzing lower numbers of samples would probably not require as sophisticated a unit as the Flow instrument, and the manual Gilford unit would suffice. Laboratories with higher sample loads might find fully automated units (such as the Flow instrument) more advantageous.

In summary, the proposed use of a Salmonella EIA for the detection of these pathogens was shown to be as accurate as other methods currently in use. The EIA is sensitive, specific, requires little expertise to perform, and allows for the simultaneous multisample analysis of food products. Results can be read visually or, provided the instrumentation is available, quantitatively. Furthermore, the preliminary results of this study indicate that the EIA analysis may be more sensitive when applied to the direct screening of pre-enrichment media. Though confirmatory tests are required to validate this latter contention, the savings in time and expense would be of great benefit to industry and regulatory agencies should this point be proven true. Furthermore, the use of commercial polyvalent antiserum and enzyme-antibody conjugates was shown to be feasible; hence, the EIA did not require the manufacture of specific reagents. Also, the successful production of direct anti-H enzyme conjugates was
demonstrated, showing that a direct system is feasible.

Areas of future consideration should include the development of a direct EIA system to reduce analysis time. This may be somewhat of a problem because of the low titer of commercial antisera that are presently available; the direct studies in this dissertation determined that direct antibody-enzyme conjugates had to be used at relatively low titers. Higher titer antisera would be desirable. More experimentation is also required to determine a reliable fixation protocol to circumvent the requirement of centrifugation, the major disadvantage of the proposed EIA technique. Alternatively, the direct assaying of flagella, which can easily be stripped from salmonellae by blending, sonication, or acid treatment, may prove a profitable avenue of research. Detached flagellar protein and subsequent fixation to microtitration plates with conventional ELISA protocol (binding at high pH) might circumvent cell fixation problems. Lastly, the employment of fluorogenic substrates, which was only briefly addressed in this study, shows promise of increased sensitivity. Their use should be examined further.
1. An enzyme immunoassay (EIA) was developed to detect salmonellae in foods.

2. It was determined that alkaline phosphatase was the most appropriate enzyme marker to use in conjugate preparations. Horseradish peroxidase substrate reacted with cellular peroxidases leading to "false positives" and difficulty in result interpretation when peroxidase conjugates were used.

3. The use of staphylococcal protein A affinity chromatography was found to be a highly efficient and effective method for the purification of Spicer-Edwards IgG and the concomitant removal of IgM. The removal of IgM, the primary immunoglobulin class elicited by cross-reactive O antigens, was determined to increase the specificity of the EIA.

4. The EIA was sensitive and specific. Concentrations of $10^5$ salmonellae per ml gave clearly positive visual and quantitative results when using p-nitrophenyl phosphate as the substrate. One experiment employing the fluorogenic substrate, 4-methyl umbelliferyl phosphate, indicated that a concentration of $10^4$ S. anatum cells per ml was sufficient for a positive identification. Specificity was demonstrated; a variety of nonsalmonellae were tested, all of which did not react or did so minimally.
5. In the comparative analysis of 98 food samples, the EIA was equivalent in the performance of enrichment serology and standard pure culture technique. It was found to be more specific than the immunofluorescent technique.

6. The EIA was also shown to be just as effective in the analysis of pre-enrichment media following a short (4-6 hr) post-enrichment in M-broth. Furthermore, it was determined that the EIA may be more sensitive since higher numbers of positive subsamples were obtained from pre-enrichment analysis of known salmonellae contaminated foods. This was the most significant finding and shows great potential with respect to savings in analysis time and expense.

7. Based on the results of this study, the EIA analysis of foods was determined to be a promising alternative method for detecting salmonellae. It is a sensitive and specific technique, requires little technical expertise to perform, and allows for the simultaneous multisample analysis of products. The amenability of the procedure to automation and the demonstrated use of colorimetric quantification using two commercial readers demonstrate specific advantages over other methods currently in use.
Indirect EIA Procedure for the Detection of Salmonellae in Foods

1. Add 0.1 ml of broth culture to cuvette or microtiter well. Routinely, three wells are used per sample; two for replicate analysis and one well for a control.

2. To the test wells add 0.1 ml of a diluted Spicer-Edwards (Difco) purified IgG preparation. Purification of IgG using staphylococcal protein A affinity chromatography is a rapid and simple procedure (see Swaminathan et al., Appl. Environ. Microbiol. 35:911-919). Serum is applied to the column and washed through with PBS, pH 7.2. Once the absorbance (280) drops below 0.05, the buffer is changed to 0.1 M glycine-HCl pH 3.0 and the IgG elutes from the Sepharose. An overnight dialysis of the IgG peak against PBS is sufficient to bring the pH back to neutrality. Sepharose with bound protein A can be purchased ready made from a variety of commercial suppliers, e.g., Pharmacia or Sigma. The IgG is titered beforehand with several Salmonella serotypes; the highest dilution giving a visible agglutination is used in the EIA. The IgG is diluted in PVP-BSA-PBS (2.0 g polyvinyl pyrrolidone-40, 0.5 g bovine serum albumin, 0.6 ml formalin in 100 ml of phosphate buffered saline, pH 7.2). Diluent without IgG is added to the control well.

3. Incubate plates or Gilford cuvette paks at room temperature for 1 hr and wash with PBS-Tween 20 (0.5% Tween 20
in PBS) three times to remove unbound antibody. Washing is accomplished by centrifugation of plates for 5 min at 2,500 rpm. Care should be taken to insure that cells are thoroughly resuspended after each wash.

4. Add 0.1 ml of diluted goat anti-rabbit alkaline phosphatase conjugate to all three wells. Incubate plates for 1 hr and remove unbound conjugate as above. Sigma conjugate preps are used at a 1:1000 dilution and were determined to be highly satisfactory for use in this EIA technique. The control well will determine washing efficiency or nonspecific binding of conjugate to cells or plates.

5. Each well then received 0.2 ml of alkaline phosphatase substrate. Substrate is prepared immediately before use by adding 5 mg (one tablet of Sigma phosphate substrate 104) to 5 ml of diethanolamine buffer, pH 9.8. Diethanolamine buffer consists of 97 ml of diethanolamine, 800 ml of water, 0.2 g NaN₃, 100 mg of MgCl₂ 6H₂O; 1 M HCl is added until the pH is 9.8 after which the volume is brought to 1 liter.

6. The plates with added substrate are incubated for 30 min at 37 C; the production of a chromagenic reaction product (yellow) indicates a positive sample. Results can be determined visually or (provided that instrumentation is available) quantitatively.
LITERATURE CITED


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Of making many books there is no end, and much study wearies the body. Now all has been heard; here is the conclusion of the matter: Fear God and keep his commandments, for this is the whole duty of man (Ecclesiastes 12:12-13).