DETECTION OF STAPHYLOCOCCAL ENTEROTOXIN B BY AFFINITY RADIOIMMUNOASSAY

Dissertation for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY
NARONG NIYOMVIT

1976

This is to certify that the

thesis entitled

Detection of Staphylococcal Enterotoxin B
by Affinity Radioimmunoassay

presented by

Narong Niyomvit

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Food Science

K. J. Meeker
Major professor

Date Nov. 24, 1976

O-7639

ABSTRACT

DETECTION OF STAPHYLOCOCCAL ENTEROTOXIN B
BY AFFINITY RADIOIMMUNOASSAY

By

Narong Niyomvit

The objective of this investigation was to develop a rapid, sensitive method for detection of staphylococcal enterotoxin B (SEB). Antibody to enterotoxin B was covalently attached to Sepharose 4B by use of cyanogen bromide activation. The antibody gel was diluted with Sephadex G-25, and 2 ml packed volume of the diluted gel was placed in a small column. Assays were carried out by sequentially pumping sample, ^{125}I-labeled SEB, 0.05 M sodium phosphate buffer saline (pH 7.5) containing 0.15 M NaCl, and 0.13 M NH_4OH (pH 10.5) containing 0.15 M NaCl through the column. The amount of ^{125}I-labeled SEB removed from the column with the pH 10.5 solution was related to the amount of SEB in the sample. After washing with the pH 10.5 buffer, the column was ready for reuse. A single column could be used for over 100 determinations.

In buffer solutions, the minimum SEB concentration which could be detected was 1.2 ng/ml. When SEB was

added to reconstituted nonfat dry milk and hamburger, the minimum detection levels were 2.2 ng/ml and 6.3 ng/g, respectively. The sample of milk required no treatment prior to assay. Hamburger required only centrifugation and filtration through Nucleopore filters to remove fat from the sample. The results of this investigation showed that the affinity radioimmunoassay technique which was developed can be utilized for the detection of SEB.

DETECTION OF STAPHYLOCOCCAL ENTEROTOXIN B BY AFFINITY RADIOIMMUNOASSAY

By

Narong Niyomvit

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science and Human Nutrition

1976

DEDICATION

To my parents

ACKNOWLEDGMENTS

The author very sincerely wishes to express his deepest appreciation to Dr. Roger F. McFeeters for his excellent guidance, encouragement, and individual assistance throughout the doctoral program toward the completion of this dissertation.

The same appreciation is extended to Dr. J. R. Brunner, Dr. K. Stevenson, Dr. L. L. Bieber, and Dr. A. H. Tucker for their assistance and suggestions.

Last but not least the author wishes to express his gratitude to the Government of Thailand and Kasetsart University for providing the leave of absence and financial support for this program. The Department of Food Science and Human Nutrition of Michigan State University has provided ample facilities and excellent cooperation for the whole program.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

INTRODUCTION

The acute illness caused by staphylococcal enterotoxins is fairly common in the United States and in other countries of the world, especially in the developing areas. Five enterotoxins, designated types A, B, C, D, and E, have been identified. They are produced by various strains of Staphylococcus aureus. Man is considered the main reservoir of enterotoxic staphylococci. They are frequently inhabitants of the skin and mucosal surfaces of the upper respiratory tract. In fact, the nasal passages constitute the principle sites of bacterial multiplication from which the organism can be transferred readily to food by sneezing. Food handlers with skin lesions or contaminated hands may also innoculate food.

The number of Staphylococcus aureus organisms may increase during processing, storage, and distribution of foods. The extent of such growth depends upon a variety of environmental conditions. Some of the more important environmental factors are temperature, length of time held at any given temperature, and associated growth of other organisms. Growth of Staphylococcus

aureus to high populations may lead to production of enterotoxin and subsequent food poisoning. The enterotoxins are resistant to heat and low pH. In general, enterotoxins formed in food products will not be inactivated by subsequent processing.

Convenient methods for detection of staphylococcal enterotoxin in food are important to regulatory agencies and the food industry in order to prevent contaminated food from reaching consumers. Improved detection can also aid research into the mechanisms of enterotoxin production in food and evaluation of new techniques to prevent enterotoxin formation. Considerable efforts have been made to develop rapid, sensitive methods for measurement. The method used for detection must be sensitive enough to assure safety. A method which can reliably detect 0.1-0.2 μg/100 g of food is considered adequate since it is thought that a minimum of 1 μg of enterotoxin is required to produce symptoms in man (Bergdoll et al., 1976). If enterotoxin is detectable in a food at any level, it is not considered safe for consumption.

Several methods to determine the presence of enterotoxin in foods have been developed. The biological assays available are not sufficiently sensitive to detect the small amounts of enterotoxin usually present in foods. The first successful method for detection of enterotoxin in food was the immunodiffusion method developed by

Casman and Bennett (1965). The method has been successful in determining if food contains enterotoxin. However, the sensitivity of the method is relatively low, it is laborious, and the cost per determination is high. Several investigators have attempted to modify this method to shorten the time required to obtain results and to make it equally sensitive for all types of food (Bergdoll et al., 1976). In recent years new methods for detection of enterotoxin have been developed. These include solid-phase radioimmunoassay (RIA) (Johnson et al., 1971; Collins et al., 1973), and reversed passive hemagglutination assay (RPHA) (Silverman et al., 1968). However, a high degree of operational skill is required to perform these assays and the mechanical manipulation required is not readily automated. Solid-phase radioimmunoassays are sufficiently sensitive for detection of enterotoxins so that the high degree of purification and concentration required by the Casman and Bennet procedure is not necessary. However, it was thought that a new approach to solid-phase radioimmunoassays could provide significant improvements over previously used procedures.

The objective of this project was to investigate an alternative approach to radioimmunoassay of staphylococcal enterotoxin B. This approach provides the following advantages: (1) multiple use of antibody,

(2) relatively rapid analysis compared to the Casman and Bennett procedure, and (3) a system that is better adapted to automated analysis than conventional radio-immunoassay techniques.

LITERATURE REVIEW

General Characteristics of Staphylococci

Staphylococci have long been known to be associated with man and animals. Although they can initiate a wide variety of infections, food poisoning caused by staphylococci is strictly an intoxication. Symptoms of staphylococcal food poisoning begin 1-6 hours after ingestion of contaminated foods and consist initially of salivation and nausea. This is quickly followed by vomiting, abdominal cramping, and diarrhea. The mortality rate is very low or nil (Jay, 1970).

Characteristics of Staphylococcus aureus

The bacteria causing staphylococcal food poisoning are classified under the name of Staphylococcus aureus. Only certain strains of Staphylococcus aureus can produce enterotoxin. Cultures of Staphylococcus aureus are facultatively anaerobic, gram positive, nonmotile, nonphotosynthetic, and do not form spores. They typically occur in grape-like cluster and the individual cells are approximately 0.8 to 1.0 μm in diameter.

Staphylococcus aureus is mesophilic in nature and can grow at a temperature as low as 6.7 C (Angelotti et al., 1961). In general, these organisms have an optimum growth temperature around 37 C. They are capable of growth over a pH range of 4.5 to 8 with an optimum pH of 4.8 to 7.6 (Nickerson & Sinskey, 1974). These organisms also possess a high degree of tolerance to compounds such as tellurite, mercuric chloride, neomycin and sodium azide, all of which have been suggested agents for isolation of staphylococci.

Distribution of Staphylococcus aureus in Foods

Staphylococcus aureus have been found in many types of commercial foods. Silverman et al. (1961) recovered staphylococci from commercial shrimp. Walker et al. (1961) also isolated these organisms from cheese and showed that they died off during the normal aging of these products. These organisms have also been isolated from cheddar cheese ranging from 50 to 220,000 per gram (Donnelly et al., 1964). Coagulase-positive staphylococci have been found in vegetables, market meat, poultry and other foods of animal origin (Munch-Peterson, 1963; Splittstoesser et al., 1965).

The type of foods most frequently cited as having caused staphylococcal intoxications are ham and ham products, chicken products, especially chicken salad,

potato salad (Munch-Peterson, 1963), vegetable products (Splittstoesser et al., 1965) and bakery goods such as eclairs, which have custard fillings. Milk and ice cream have sometimes been implicated as vehicles of staphylococcal poisoning.

Staphylococcal Enterotoxins

Types of Enterotoxin

Five immunologically distinct types of enterotoxin have been identified and designated with letters A (Casman, 1960), B (Bergdoll et al., 1959a), C (Bergdoll et al., 1965), D (Casman et al., 1967), and E (Bergdoll et al., 1971). Some strains of Staphylococcus aureus produce only one type of enterotoxin such as Staphylococcus aureus 196E (ATCC 12565) which produces enterotoxin A (Casman et al., 1963) and Staphylococcus aureus 243 (ATCC 14458) which produces exclusively enterotoxin B. These are the type strains for these enterotoxins. Others produce two or more enterotoxins. Two different enterotoxin C's from different staphylococcus strains were found (Borja & Bergdoll, 1967; Avena & Bergdoll, 1967) and classed as C_1 and C_2 on the basis of their different isoelectric point, 8.6 and 7.0, respectively. In the study of staphylococci isolated from 305 cultures from outbreaks, 49% were enterotoxigenic, with 62.4% of these produced enterotoxin A, 17.4% enterotoxin C, 11.4% enterotoxin B, and 8.7% A, B, or C in combination

(Hall, 1968). Type B was the first to be purified (Schantz et al., 1965). Most experimental toxicity data have been derived from work with this enterotoxin due to the fact that it is the one most easily obtained. Relatively little information exists on type D and E which were the latest to be identified.

Factors Affecting Staphylococcal Enterotoxin Production in Foods

It has been found that large numbers of Staphylococcus aureus organisms must be present in food to cause enterotoxin production. It has been estimated that 1 to 4 μg of type A enterotoxin would be required to cause symptoms (Casman & Bennett, 1965). Foods which caused staphylococcal poisoning were found to have staphylococcal counts of 50×10^6 to 200×10^6 per gram of food. The number necessary to produce enough enterotoxin to induce food poisoning depends on such factors as the nature of the food substrate, its pH, holding temperature, and the characteristics of the strain in question. The number and type of competing microorganisms present were very important. It was shown that staphylococci were unable to compete well with normal food-borne bacteria in both fresh and frozen foods (Troller & Frazier, 1963).

Nature of Staphylococcal Enterotoxins

All enterotoxins purified to date appear to be simple proteins that contain relatively high levels of lysine, aspartic acid, glutamic acid and tyrosine among the 18 different amino acids making up the peptide chain. Enterotoxin B was found to contain 239 amino acids residues with a molecular weight of 28,366 (Huang & Bergdoll, 1970b). The sequence has been determined by Huang and Bergdoll (1970b). The molecular weights of enterotoxin A, C_1, and C_2 are 34,700 (Chu et al., 1966), 34,100 (Borja & Bergdoll, 1967), and 34,000 (Avena & Bergdoll, 1967), respectively. The study of amino acid sequences of these enterotoxins is currently in progress (Robern et al., 1975).

Stability of Staphylococcal Enterotoxins

Staphylococcal enterotoxins are unique among bacterial toxins in that they are very heat resistant. Davison and Dack (1939) found the potency of enterotoxin could be only gradually decreased by prolonged boiling or autoclaving.

Purified enterotoxin A is relatively heat labile compared to enterotoxin B. A decrease of 50% in the reaction of enterotoxin A with its specific antibody was observed when it was heated at 60 C in 0.05 M sodium phosphate buffer, pH 6.85, for 20 minutes (Chu et al.,

1966). This result could not be compared to those obtained by Denney et al. (1966). They found that heat inactivation at this temperature for enterotoxin A, based on cat emetic response, to be 11 minutes (F^{48}_{250} = 11 min.) or F^{46}_{250} = 8 min. when monkeys were employed. The heat inactivation at 60 C was believed to be due to enterotoxin aggregation. Hilker et al. (1968) reported that crude enterotoxin A, 21 µg/ml in veronal buffer, pH 7.2, required heating at 100 C for 130 minutes to reduce the enterotoxin concentration to less than 1 µg/ml.

The biological activity of enterotoxin B was retained after heating 16 hours at 60 C at pH 7.3 (Schantz et al., 1965). It could not be inactivated by pasteurization or spray drying of milk as currently practiced (Read & Bradshaw, 1966). Satterlee and Kraft (1969) showed that enterotoxin B was more heat sensitive at 80 C than at 100 or 110 C. They also showed that thermal inactivation was more pronounced at 80 C than at either 60 or 100 C when heated in the presence of meat proteins. Reed and Bradshaw (1966) found that raw milk, containing 30 µg of SEB per ml, needed 37.6 minutes to inactivate 99% or more of SEB (F = 37.6 min.) at 121 C.

The biological activities of enterotoxin C_1 did not change when it was heated at 60 C for 30 minutes, but

the solution became turbid when heating was continued for 60 minutes (Borja & Bergdoll, 1967). The reaction of enterotoxin C_2 with its anti-enterotoxin was reduced to about 20% of normal when it was heated at 100 C for 1 minute (Avena & Bergdoll, 1967).

In summary, enterotoxin A is the most sensitive to heat, while enterotoxin B is the least sensitive. Purified enterotoxins are more sensitive to heat than the crude toxins.

Also, native enterotoxins were resistant to proteolytic enzymes such as trypsin, chymotrypsin, rennin, and papain. Pepsin destroys their activity at a pH of 2, but is ineffective at higher pH values (Bergdoll, 1966; Chu et al., 1966).

Toxic and Antigenic Sites of Enterotoxins

Enterotoxins contain only two residues of half-cystine which are cross-linked to form a cystine residue. The half-cystine residues in enterotoxin A and B are at positions 92 and 112 (Huang & Bergdoll, 1970a) which creates a cystine loop of 20 amino acid residues. It is believed that this cystine loop is involved in the activity of the enterotoxin (Dalidovicz et al., 1966; Bergdoll & Robbins, 1973). It was suggested that amino acids adjacent to cysteine at position 112 may be involved in the active site because these amino acids

are alike for enterotoxin A and B (Bergdoll & Robbins, 1973). The residues near to the half-cystine residue at position 92 in enterotoxin may comprise the major antigenic site since there are several tyrosyl residues. Most lysyl residues, which are evenly distributed on the surfaces of molecule, are not thought to be involved in either the toxin or antigenic sites (Spero et al., 1971). The biological activity of enterotoxin A was found to be destroyed when tyrosyl residues were modified (Chu et al., 1966). The normal positive charge of the enterotoxin is thought to play an important role in both emesis and in the reaction of enterotoxin with its specific antibody (Jay, 1970).

Production and Purification of Staphylococcal Enterotoxins

Enterotoxin may be readily produced in the laboratory by cultivation of toxigenic strains in Brain Heart Infusion broth (Bergdoll, 1970).

Several methods have been proposed for production of enterotoxins. These methods include the sac-culture technique of Casman and Bennett (1963), the semisolid plate technique of Casman and Bennett (1963), and cellophane-over-sold-agar-plate of Hallander (1965). Factors that affect the enterotoxin production such as pH, incubation temperature, water activity, salt, sodium

nitrate, glucose, streptomycin, acriflavine and Tween 80 were reviewed by Bergdoll (1970).

The appearance of enterotoxin in a culture medium was studied by Lilly et al. (1967). They reported that enterotoxin B appeared in the culture medium as early as 6 hours and increased through the stationary phase. This was confirmed by Morse et al. (1969) who also observed that pH of the medium increased during the releasing period. They suggested that enterotoxin was not preformed and released by change in pH but rather by the result of catabolic repression. Markus and Silverman (1968, 1969) worked with Staphylococcus aureus and found that 95% of enterotoxin B was released during the latter part of the exponential phase of growth. They showed that the presence of enterotoxin was dependent upon de novo protein synthesis.

Enterotoxin A was found to be a primary metabolite for it was secreted mainly in the exponential phase of growth (Markus & Silverman, 1969).

Purification of enterotoxin B was first done by Bergdoll et al. (1959b). Schantz et al. (1965) developed a simplified purification procedure which resulted in a 50-60% recovery of enterotoxin B.

The purification of enterotoxin A is somewhat different from those used for enterotoxin B. A method

for purification of enterotoxin A with 30-35% yield of toxin was developed by Chu et al. (1966).

Casman et al. (1967) used the method developed by Schantz et al. (1965) to purify enterotoxin D. The authors suggested that highly purified enterotoxin D was obtained, but no proof was presented. Food Research Institute laboratories purified enterotoxins C, D, and E by methods similar to those employed for A (Bergdoll, 1970).

Production of Enterotoxin Antibodies

The preparation of antibody for each enterotoxin may be different in some detail by each investigator. Casman and Bennett (1964) prepared antibody to enterotoxin A by injecting the rabbits at weekly intervals, using the following amounts: 0.18, 0.36, 0.72, 2.8, 5.6, 11.2 µg intravenously, 400 µg subcutanously, and also 88, 260 and 1760 µg mixed with aluminum phosphate. After an interval of 1 month, the rabbits were injected intravenously with 500 µg of enterotoxin A and after 7 days, bled from a marginal ear vein. The injection was repeated three times at monthly intervals, removing approximately 60 ml of blood at each bleeding.

Nace and Spradlin (1962) prepared antibody to enterotoxin B by injecting a series of purified enterotoxin B, 0.005, 0.01, 0.04, 0.2 and 1.0 mg, at

approximately 1-week intervals. After five weeks, 2 to 3 mg enterotoxin were injected and bleeding began 8 days later. Four to six bleedings of approximately 50 ml each over a 3-week period were made from an ear vein. Booster injections of 2 to 3 mg of enterotoxin can be given every 5 weeks indefinitely with 4 to 6 bleedings after each injection.

Methods for Detection of Enterotoxins

Enterotoxin detection in foods is still a problem for food investigators because of the lack of sensitive methods for the detection and assay. The current procedures for detection of staphylococcal enterotoxins are laborious and time consuming.

Biological Methods

Kittens (Hammon, 1941) and young rhesus monkeys (Surgalla et al., 1953) have been used successfully in many laboratories for detecting enterotoxin in culture filtrates. The cat method requires the inactivation of substances that may give symptoms similar to those caused by enterotoxin. The most reliable biological assay of the enterotoxin is the feeding of young rhesus monkeys because only enterotoxins cause emesis in these animals. However, the high cost of the animals and the fact that they become resistant to the enterotoxin after a few tests severely limits use of this procedure.

A number of studies have been undertaken to identify enterotoxin with biological activities other than emetic action on animals. For example, Milone (1962) investigated the effect of enterotoxin on tissue cultures and Schaeffer et al. (1966) looked for effects on human embryonic intestinal cells. Both studies showed negative results.

Serological Methods

Enterotoxins are proteins which can induce antibodies when injected into animals. The enterotoxins can then form a precipitate when mixed with their specific antibodies. This reaction is the basis for serological techniques for enterotoxin detection. The precipitate formed can be observed either in a solution (precipitation reaction) or in a gel (gel diffusion technique). Although the reaction of enterotoxin with anti-enterotoxin does not necessarily indicate biological activity of enterotoxins, in most instances the correlation between the two is sufficient to justify using the immunological reaction to assay for enterotoxin (Bergdoll, 1970).

Precipitation in gel. The first precipitation technique for enterotoxin detection was introduced by Surgalla et al. (1952). However, the sensitivity of the method, 1-2 μg/ml, was too low for detection of enterotoxin in foods.

Bergdoll et al. (1959b) introduced another method called double gel diffusion tube. The method involves the placing of a layer of agar between the antibody-agar and antigen layers. The antibody and enterotoxin will diffuse toward each other through the agar gel and form a precipitate line when they come into contact. The position of the line is dependent on the relative concentration of the enterotoxin and antibody. The assay can detect enterotoxin as low as 0.05 µg/ml. However, the time required to detect a precipitate with this small amount is up to 21 days (Hall et al., 1965).

Bergdoll et al. (1965) used an Ouchterlony Plate technique to detect enterotoxin in unknown materials. Wells were made 2 to 4 mm apart. Antibody is placed in the center well and control and unknown enterotoxins are alternated in the outer wells. The method is useful for detection of 5 to 20 µg/ml enterotoxin, but the smaller amounts require 2 to 3 days to obtain precipitation lines.

Casman and Bennett (1963) developed a microslide technique for enterotoxin detection. It required 1 to 3 days to develop precipitin lines. A minimum of 0.1 µg/ml of enterotoxin could be detected. In order to detect 0.1 to 0.2 µg of enterotoxin per 100 g of food, enterotoxin in the food extract must be partially purified and concentrated. The first method for purification and concentration of enterotoxin from food was developed by

Casman and Bennett (1965). The method involved a rather lengthy procedure requiring several days to reduce the extract to 0.1 to 0.2 ml. However, the microslide technique was an effective procedure for detecting enterotoxin in foods at sufficiently low concentrations which would cause illness. Zehren and Zehren (1968), for example, successfully identified contaminated cheese vats so that enterotoxin-free cheese could be sold. Reiser et al. (1974) modified this microslide method so that the result could be obtained on the third day. They used Amberlite CG-50 ion exchanger to absorb enterotoxin directly from the partially treated food extract (extraction at pH 4.5 then, fat was removed with $CHCl_3$, pH 7.5). This eliminated an overnight concentration step. Trypsin was used to eliminate the interfering contaminants. The microslides were held at 37 C to reduce developing time.

In the Laurell electroimmunodiffusion procedure (Gasper et al., 1973) antiserum was mixed with agarose gel. Samples of 1.5 to 10 ng of enterotoxin in 10 µl buffer (0.15 to 1.0 µg/ml) were transferred from 4 mm wells into gel by electrophoresis. The precipitation cones formed were made visible by staining with thiazine red in acetic acid. The method was not sensitive enough for quantitative measurement of enterotoxin present in foods because it fails to produce visible precipitin lines at sufficiently low concentrations. The method also

required 1 to 8 hours for electrophoresis and another 2 to 5 hours for developing a visible cone.

Precipitate reaction. Several methods have been developed to detect enterotoxin directly in food extracts so that the concentration, purification, and microslide diffusion steps could be eliminated.

A fluorescent antibody technique was introduced by Genigeorgis and Sadler (1966). Specific antibodies to enterotoxin were conjugated with fluorescin isothiocyanate and used to precipitate the enterotoxin in samples. The precipitate was collected on Millipore filter membranes, washed, and impression smears were made on a slide. The sensitivity of this method was about 1 μg/ml.

Morse and Mah (1967) developed a hemagglutination inhibition test. Enterotoxin was coupled with sheep erythrocytes. The sensitized erythrocytes were added to various combinations of enterotoxin-antibody. Hemagglutination occurred only if enterotoxin was insufficient. The method was as sensitive as microslide test, but it still required several hours for the reaction to be complete.

Silverman et al. (1968) proposed the reversed passive hemagglutination method for determination of enterotoxin in foods. The procedure was based upon the adsorption of enterotoxin antibody to tannic acid treated

sheep red blood cells followed by agglutination of the sensitized cells with enterotoxin. The sensitivity of the method was 0.1 μg/100 g of food without concentration of the extract. It required 1 to 2 days to obtain the result. There were two major problems for this method. First, the antibody in some preparations was not adsorbed or coupled to red blood cells. Secondly, nonspecific agglutination of sensitized cells by food extracts sometimes occurred, particularly in meat extracts.

Radioimmunoassay (RIA). The technique of radioimmunoassay was first developed for measurement of hormones (Berson et al., 1956), but its use has been expanded rapidly during the past decade to other areas (Skelley et al., 1973). The procedure was based on the competitive inhibition by unlabelled hormone of the binding of labelled hormone to a specific antibody (Yalow & Berson, 1959). The competition required at least two reagents, a specific antibody and labelled antigen. The reaction complex formed between antibody and antigen was almost always soluble at the concentrations employed. Therefore, it was necessary to separate bound from free antigen after reaction. Separation techniques included electrophoretic and chromatoelectrophoeretic methods (Yalow & Berson, 1961; Hunter & Greenwood, 1964), gel filtration (Genuth et al., 1965), nonspecific precipitation of Ag*Ab (Grodsky & Forsham, 1960; Odell et al., 1965),

immunoprecipitation of Ag*Ab complex (Skom & Talmage, 1958), solid-phase adsorption of Ag* (Herbert et al., 1965; Lau et al., 1966) and solid-phase antibodies (Isojima et al., 1970; Catt et al., 1968). For some compounds, a number of different RIA procedures have been developed that differed primarily in the technique used to separate bound from free antigen. The physical manipulations and the care required to maintain an accurate RIA technique made it essential to have skilled technicians for these assays. In addition, the incubation of samples often required considerable time. Catt et al. (1967) attached antibody to an insoluble polymer such as polystyrene, or bromoacetyl cellulose. The use of antibody in this procedure allowed rapid removal of the free radioactive antigen by washing the solid phase with water after the completion of immune reaction. In principle, a single separation procedure would serve for all radioimmunoassays. The technique became known as the "solid-phase radioimmunoassay."

Johnson et al. (1971) used the radioimmunoassay for quantitative measurement of crude and purified enterotoxin B. A polystyrene tube was used as a supporting medium. The tubes are first sensitized with antibody, followed by incubation with purified or crude enterotoxin and ^{125}I-enterotoxin B mixture. After a 4-hour incubation at 37 C, the buffer was added and the supernatant was

counted to determine the amount of unbound labelled enterotoxin. From the relationship of unbound enterotoxin and amount of standard enterotoxin, a standard curve was obtained.

Collins et al. (1972) used the same inhibition test for detection of enterotoxin B, but bromoacetylcellulose (BAC) was used instead of polystyrene tubes. The method is somewhat different from those of Johnson et al. (1971). The antibody-BAC must be first diluted in order to bind 50% of fixed amount of ^{125}I-enterotoxin, followed by incubation with the mixture of unlabelled and labelled enterotoxin for 15 minutes at room temperature. Then, the inhibition curve is obtained. Collins et al. (1973) used this method for detection of enterotoxin A. Enterotoxin A at the level of 0.01 μg/ml could be detected in a variety of media such as ham, milk products, crab meat, and custard. No interference was found with any media or food products tested.

Later, Bukovic and Johnson (1975) used solid-phase radioimmunoassay for enterotoxin C in milk, cheddar cheese, custard, and ham salad. The assay was sensitive to 1 to 10 ng of enterotoxin per g of food.

A comparative study of serological detection methods for enterotoxin was done by Bennett et al. (1973). The result of the study is as follows: (1) The RPHA method lacks the desired specificity and is limited by

its reproducibility and variations in test interpretation. It also requires high quality antigen. (2) The microslide test and radioimmunoassay are very consistent. The microslide method does not require purified antibody, while purified enterotoxin is important in the successful interpretation of radioimmunoassay results.

Radiolabelling of Staphylococcal Enterotoxin

The requirement for radiolabelling of enterotoxin is that high specific activity be achieved without loss of antigenic reactivity. This means that the labelling procedure must be mild enough to maintain the conformation of functional groups of enterotoxin. In addition, the intrinsic radioactivity of the labelled antigen should not cause rapid loss of activity. Several methods have been introduced. Most of them used ^{125}I or ^{131}I. ^{125}I has been used extensively in labelling protein because of its high specific activity, long half life (60 days), and relatively low cost. It also emits low energy gamma rays and absence of β-radiation diminishes the potential for autodestruction of the labelled antigen (Galskov, 1972).

For iodination of enterotoxin, many factors must be considered. These include (1) damage to the labelled enterotoxin incured during labelling and purification

and (2) damage during the subsequent storage (Skelley et al., 1973).

The iodination is easy to perform. It is usually carried out at room temperature at pH 7.5 in phosphate buffer. It usually requires about 20-30 minutes for iodination, and a separation of the iodinated protein (Skelley et al., 1973). For iodination, enterotoxin must be highly purified. The reaction time, amount of oxidizing agent and pH must be optimized and the separation of labelled enterotoxin must be done immediately after labelling.

Iodine monochloride (McFarlane, 1958), lactoperoxidase (Marchalonis, 1969) and chloramine-T (Hunter & Greenwood, 1962) have been used as oxidizing agents. Iodine monochloride does not work well with less than 2-5 mg of protein (Freeman, 1959). Lactoperoxidase was used successfully to iodinate immunoglobulin to low specific activity with radioactive iodine (Marchalonis, 1969). Thorell and Johansson (1971) used this method to iodinate insulin and found that the damage was very low. Chloramine-T has been widely used, especially when high specific activities are required (Freeman, 1967). The method is suitable for small quantities of protein and is so rapid that the damage to protein due to radiation is minimized.

Upon the addition of chloramine-T, the redox potential rises slowly. The potential determines the ratio of I_2 to I^- which in turn determines the amount of H_2OI^+ ion. The available H_2OI^+ ion then replaces H^+ ion on a tyrosine ring to form mono- or di-iodotyrosine. This replacement occurs without the alteration of protein (Greenwood & Hunter, 1963). At the end of the reaction period, the potential is lowered to the starting level by sodium metabisulphite. A carrier, bovine serum albumin (BSA), is usually added to the reaction mixture immediately after sodium metabisulphite.

Purification is almost always necessary in order to free enterotoxin from unreacted iodine and from damaged components (Skelley, et al., 1973). This step must be done immediately following iodination, otherwise, excessive adsorption on glassware may occur. The addition of BSA following iodination aids in purification as well as in preventing enterotoxin loss by adsorption. The purification can be done by gel filtration (Johnson et al., 1971) or by dialysis (Collins et al., 1972). The gel filtration method has advantages of rapidity and is, therefore, the method of choice.

Chloramine-T was used to iodinate enterotoxins A and B successfully by Johnson et al. (1971) and Collins et al. (1972). Chloramine-T was found to have an adverse effect on the stability of some enterotoxin A preparations.

Collins et al. (1972) also investigated the microdiffusion method developed by Gruber and Wright (1967), using gaseous ^{125}I, and found no deleterious effects on the enterotoxin A molecule because there was no direct contact between reactants and protein. It yields a product of lower specific activity than those iodinated with Chloramine-T. Labelled enterotoxin could be used for approximately 2 months without changing its antigenicity.

Kauffman and Johnson (1975) studied the stability of ^{125}I-labelled enterotoxins A, B, and C at two specific activities, 4 and 40 mCi per μg of protein. They found that enterotoxin with high specific activity showed extensive dissociation of ^{125}I when stored at different temperatures, including -23 C. In comparison, enterotoxin with low specific activity did not show a significant loss of ^{125}I when stored at -23 C for as long as 2 months. Enterotoxins with low or high specific activity formed aggregates immediately upon labelling. Aggregate formation increased in high specific activity enterotoxins during storage at -23 C. Enterotoxin with low specific activities showed no significant increase in aggregate formation, even after 2 months at -23 C. The aggregate forms of the enterotoxin were either devoid of antigenic activity in solid-phase radioimmunoassay or they possessed significantly reduced antigenic activity. Therefore, if the labelled enterotoxin is to be stored for a

considerable time, it should be quickly frozen and stored at -23 C or lower.

Coupling of Proteins to Agarose Gels

Methods for coupling of enzymes, antigens, antibodies, and other biologically active compounds to solid supports, especially agarose, have been greatly expanded in recent years (Cuatrecasas & Anfinsen, 1971). These developments have made affinity chromatography a major technique for protein purification.

The solid supports used for this purpose may be a hydrophobic polymer, such as polystyrene, or hydrophilic, such as cellulose or cross-link dextran. Agarose is considered to be a good supporting medium because it exhibits good flow rate properties which are retained after coupling. It is also mechanically and chemically stable to the conditions of coupling and to varying conditions of pH, ionic strength, temperature, and presence of denaturants which are needed for adsorption and elution (Cuatrecasas & Anfinsen, 1971).

A method for coupling protein or peptides to carbohydrates was introduced by Axen et al. (1966). The method involves two steps: (1) the formation of reactive intermediate by treating a polysaccharide for a short period with aqueous cyanogen bromide under alkaline conditions and (2) coupling the intermediate with

protein or peptides in neutral or preferably slightly alkaline aqueous medium.

Porath et al. (1967) activated agarose at pH 11 by using 25 mg of cyanogen bromide and 50 g of agarose. The activated product was then used for coupling by adding 0.1 M sodium hydrogen carbonate and protein. The mixture was stored at 5 C for 20 hours with a constant stirring. Cuatrecasas (1970) modified this method by using 50 to 300 mg of cyanogen bromide per ml of packed Sepharose. The reaction was run for 8 to 12 minutes at 20 C, pH 11. It was recommended that the amount of protein should be 20 to 30 times greater than the amount which was to be coupled.

This activation procedure has some disadvantages. It lacks a well-defined end point and is difficult to reproduce. In addition, wide fluctuations in pH are difficult to avoid during titration. Addition of cyanogen bromide as a solid requires that this volatile lacrimatore be weighed in a closed vessel in fume hood. March et al. (1974) activated Sepharose by adding cyanogen bromide, dissolved in acetonitrile, to beads suspended in a solution of sodium carbonate. The necessity for titration and the use of a pH meter during the reaction were eliminated. Activation for 1 minute resulted in coupling capacities comparable to those obtained with the titration method.

Porath et al (1967) found that a protein-agarose gel was very stable. No loss in activity of an enzyme occurred after storage in 0.01 M acetate buffer, pH 4.1 for 2 months at room temperature. This gel also tolerated relatively extreme conditions such as 0.1 M NaOH and 1 M HCl for 2-3 hours, 6 M guanidine-HCl and 8 M urea solution for prolonged periods (Cuatrecasas, 1970). This degree of stability allows the use of denaturants to elute specifically bound protein or to wash a column in preparation for reuse.

MATERIALS AND METHODS

Materials

Staphylococcal enterotoxin B (SEB) was obtained commercially from Makor Chemicals Ltd., Jerusalem, Israel. The powder contained about 2 mg sodium phosphate per mg of protein and was readily soluble in aqueous solutions. Polyacrylamide electrophoresis at pH 4.5 showed a major band that accounted for about 95% of the total protein and one minor band. The supplier claimed that the SEB was at least 95% pure.

The antibody to SEB was also obtained from Makor Chemicals Ltd. It was in a powder form, which was reported to contain 0.25 mg of antibody per mg of solid.

Other chemicals used were all reagent grade. NaCl, KSCN, KI, $Na_2S_2O_5$, and urea were obtained from Mallinckrodt Inc., St. Louis, Mo. Bovine serum albumin (BSA), Sephadex 4B-200 and Sephadex G-25-150 were obtained from the Sigma Chemical Co., St. Louis, Mo. Chloramine-T was purchased from Eastman Kodak Co., Rochester, N.Y.

Coupling of Enterotoxin Antibody to Sepharose 4B

The coupling of SEB antibody to Sepharose 4B was done by the method of March et al. (1974). Five grams of Sepharose 4B were washed with deionized water 2 to 3 times to remove preservative. The washed Sepharose was then mixed with 5 ml of water and 5 ml of 2 M sodium carbonate. The mixture was stirred slowly at the beginning but more vigorously when 0.25 ml of an acetonitrile solution of cyanogen bromide was added (2 g CNBr in 1 ml of acetonitrile). The slurry was stirred for 2 minutes and filtered under vacuum on a coarse-sintered funnel. The activated gel was washed in succession with 50 to 60 ml each of 0.1 M sodium bicarbonate, pH 9.5, water and 0.05 M sodium bicarbonate buffer, pH 7.5. The washed gel was transferred into a plastic bottle containing 100 µg of SEB antibody dissolved in 5 ml of phosphate buffer. The mixture was stored at 4 C for 20 hours for the coupling reaction. After coupling, the beads were washed with dissociating agent (0.13 M NH_4OH containing 0.15 M NaCl) and 0.05 M phosphate buffer containing 0.15 M NaCl (PBS). This antibody gel was stored at 4 C for over 18 months in 0.05 M phosphate buffer, pH 7.5 without losing antibody activity.

Iodination of Enterotoxin

Iodination with ^{125}I was performed by a modification of the method of Greenwood and Hunter (1963). Solutions of chloramine-T, $Na_2S_2O_5$, and KI were prepared on the same day of iodination in 0.05 M sodium phosphate buffer with 0.15 M NaCl (PBS), pH 7.5. Carrier-free $Na^{125}I$, free from preservative or reducing agents, was obtained in 0.1 N NaOH from ICN Pharmaceutical Inc., Irvine, Calif. at a concentration of 40 mCi per ml. Fifty µg of SEB were dissolved in 50 ml of PBS and transferred into a vial containing 1 mCi ^{125}I. The iodination was begun by addition of 50 µl of 0.26 mg/ml chloramine-T. After 45 sec., the reaction was stopped by addition of 50 µl of 0.26 mg/ml $Na_2S_2O_5$. This was followed by addition of 50 µl of 2% KI, 100 µl of 2% BSA, and 150 µl of transfer solution (10 g/l KI and 100 mg/l bromphenol blue in 16% sucrose solution).

The solution was transferred to a Sephadex G-25 column. The vial was washed once with 70 µl of transfer solution which contained 8% sucrose. This was also placed on the column.

Separation of labelled SEB from the reaction mixture was done by gel filtration using a 1.25 x 15 cm Sephadex G-25 column equilibrated with PBS. Prior to application of sample to the column, 1.0 ml of 2% BSA solution was passed through the column to minimize the

adsorption of SEB to the glass. Fractions (1.0 ml) were collected in test tubes containing 1.0 ml of 2% BSA. The fraction with the highest count, containing approximately 0.35 µg/10 µl, was saved for further use. The ^{125}I-SEB was stored at 4 C. It was used for at least 6 weeks after iodination.

Preparation of Antibody Column

The antibody column was made by the Glass Laboratory, Chemistry Department, M.S.U. It was made of 1 cm O.D. diameter glass tube 7.5 cm long with a 4.5 cm stem (Figure 1). The column had a 7/15 ground glass joint for convenient connection of tubing.

Sepharose-AEB was mixed with well-washed Sephadex G-25 in proper proportions (1:5 to 1:30) (volume by volume). Both Sepharose-AEB and Sephadex G-25 were put into slurry form, 1 part of gel with 1 part of PBS, before pipetting. Gel of about 2 ml was packed into the column (about 4 cm in the column). Before the column was used for SEB assays, the irreversible binding sites in the column had to be saturated. The column was washed with 25 ml of dissociating agent, used in the subsequent assay, 50 ml of PBS, and finally incubated overnight with 20 µg SEB. Then, the column was washed with 15 ml of dissociating agent and 30 ml of PBS. Using the assay sequence system (Table 5), the predetermined amount of

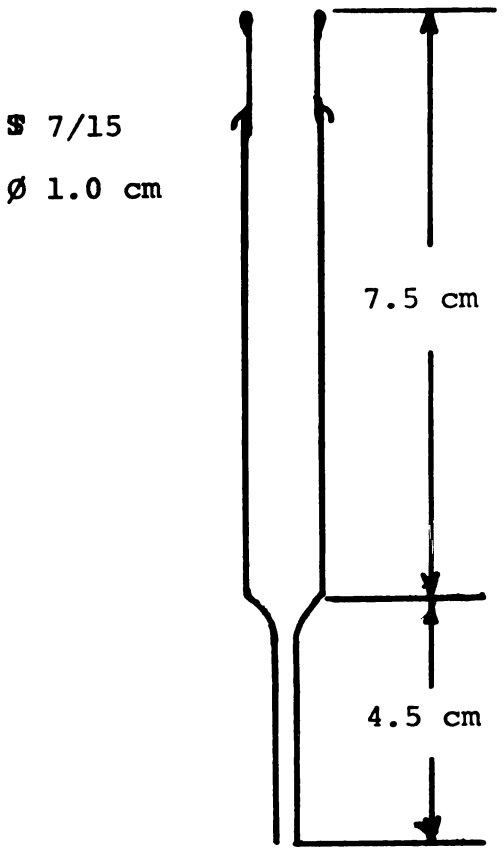

Fig. 1. Column used for enterotoxin B determination.

labelled SEB was run through the column at least 4 times or until a constant count was obtained.

Measurement of Activity

Radioactivity was measured with either a Packard Model 2001 Tricarb Scintillation Spectrometer (gamma ray counter) or a Packard Model 3310 Tricarb Scintillation Spectrometer (liquid scintillation counter). A 0.5-ml sample of NH_4OH eluate was added to either 10 ml of distilled water or 10 ml of counting cocktail (Research Products International Corp., Elk Grove Village, Ill.) depending upon whether the gamma-ray counter or the liquid scintillation counter was used. Samples were neutralized with 0.1 N acetic acid before counting to reduce chemiluminescence.

Foods Used in Recovery Study

Measured amounts of SEB (1.5 to 10 ng/ml of milk or 1.5 to 12 ng/g of hamburger) were added to reconstituted nonfat dry milk and hamburger and then stored in a refrigerator for 24 hours prior to analysis to allow equilibration of SEB in the food samples.

Inoculation of Reconstituted Nonfat Dry Milk with Staphylococcus aureus

Reconstituted nonfat dry milk was prepared by dissolving 9.6 g of nonfat dry milk in 100 ml of PBS, then divided into two portions, 100 and 500 ml. Milk was

pasteurized at 145 F for 30 minutes and, after cooling to room temperature, inoculated with Staphylococcus aureus Strain 196E (producing enterotoxin A) and Strain 243 (producing SEB) to 100 and 500 ml samples, respectively. The inoculated milk samples were incubated at 37 C for 20 hours.

Measurement of Enterotoxin B in Reconstituted Nonfat Dry Milk

Reconstituted nonfat dry milk was adjusted to pH 7.5 with 2 N NaOH. This milk was applied to the column directly without any further treatment.

Measurement of Enterotoxin B in Hamburger

Hamburger containing PBS was homogenized without further dilution at high speed (setting at speed 7) in a Sorval homogenizer. After centrifugation and removal of fat, the supernatant liquid was examined for SEB content directly. In the experiment, 10 grams of hamburger were homogenized for 45 seconds in 10 ml of 0.15 M NaCl after adjusting the pH to 7.5 with 2 N NaOH. The homogenate was centrifuged at 32,800 x g for 20 minutes. The supernatant liquid was decanted into a beaker and stored in a refrigerator for about 30 minutes or long enough to solidify the fat. The aqueous portion was removed and filtered in succession through Nucleopore filters with

0.8 and 0.4 μm pore sizes (Nucleopore Corporation, Pleasanton, CA). The filtrate was used for SEB determination.

RESULTS

Development of an Affinity Radioimmunoassay Procedure

Radioimmunoassays usually require mixing and incubation of antigen and antibodies in a liquid phase followed by some procedure for separation of bound and free antigen. Some solid-phase assays have been developed in which the antibody is attached to an insoluble support. This simplifies separation of bound and free antigen, but the physical operations are similar.

The objective of this investigation was to develop a calibrated antibody affinity column which could provide sensitivity comparable to other RIA procedures, but which was reusable, allowed rapid assays and which could be automated if necessary.

The basic starting concept for this assay is shown in Figure 2. A reusable column is prepared with antibody covalently attached to the gel (A). Enterotoxin from the sample is selectively bound by antibody (B), then labelled enterotoxin is put through the column in an amount sufficient to saturate the remaining antibody sites and the excess is washed off the column (C). The

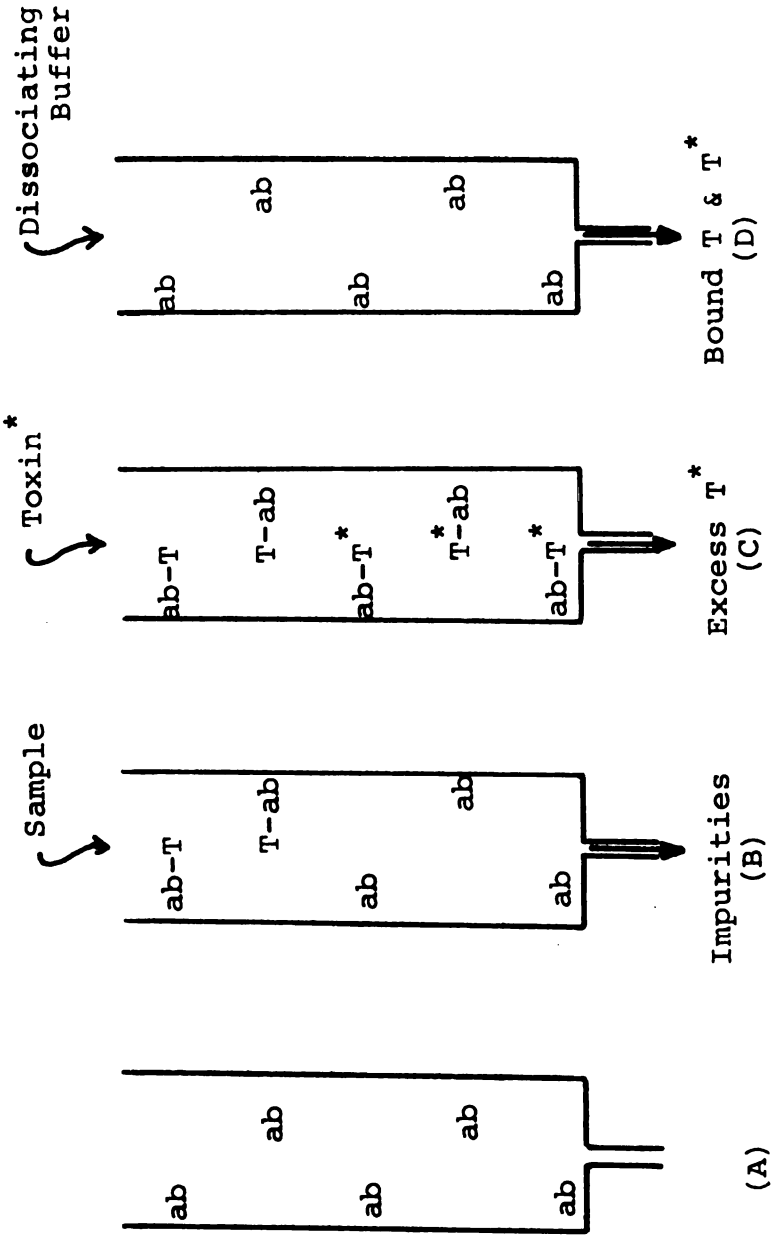

Fig. 2. Basic concept for affinity radioimmunoassay.

bound SEB is removed by elution with dissociating agent (D). The labelled SEB eluted from the column would be inversely proportional to the amount of SEB in the original sample.

In order to achieve this goal, six steps were required:

(1) Application of sample to the anti-enterotoxin column;

(2) Removal of impurities from the column;

(3) Addition of labelled SEB;

(4) Removal of unbound labelled SEB;

(5) Elution of bound SEB; and

(6) Re-equilibration of the column for another sample.

Appropriate conditions had to be developed for suppression of nonspecific binding, specific binding of SEB, removal of impurities, addition of labelled SEB, elution of bound SEB, analysis of bound labelled SEB, and column regeneration in order to obtain a quantitative assay procedure. The following steps were investigated in the development of an assay for SEB.

(1) Selection of a buffer for removal of unbound SEB and other impurities;

(2) Selection of a dissociating agent for removal of bound SEB;

(3) Development of equilibration conditions for the antibody column;

(4) Determination of the amount of labelled SEB required for a specific column;

(5) Suppression of nonspecific binding of SEB;

(6) Evaluation of dose-response curves and the factors involved in maintenance of consistent assays.

When the conditions for the first step were determined, that step was used to study the second condition and so on. Agarose was used for immobilization of antibody. However, to achieve acceptable flow properties of the antibody columns, the agarose was diluted 1:10 with Sephadex G-25. Later, a 1:15 dilution was used to reduce the amount of labelled SEB required.

Binding of Enterotoxin, Removal of Impurities, and Removal of Unbound Labelled Enterotoxin

A single buffer was required to accomplish these three elements of the assay. It was necessary to minimize tailing, provide conditions of pH and ionic strength which would allow specific binding of SEB to antibody while preventing nonspecific binding of SEB. Phosphate buffer of 0.05 M pH 7.5 was used initially because it had been used successfully for detection of SEB by Casman and Bennett (1965) and Johnson et al. (1971).

To study the effectiveness of buffers in removing unbound, labelled SEB 20 µl of labelled SEB was added to a 1:10 dilution antibody-gel column. The column was then washed with aliquots of 0.05 M phosphate buffer containing 0.15, 0.30, and 0.50 M NaCl. Using a peristalic pump, a flow rate of 150 ml/hr was used. Fifteen 1.5 fractions were collected and 0.5 ml was taken for measurement of radioactivity. The column was then eluted with 25 ml of 2 M NH_4OH, pH 11.5 to remove the bound SEB. The column was re-equilibrated with 50 ml of 0.05 M phosphate buffer. Figure 3 shows the elution patterns of unbound labelled SEB. Phosphate buffer containing different concentrations of NaCl was used to increase the ionic strength of the buffer to decrease tailing of the elution. Figure 3 shows that tailing was reduced as the concentration of NaCl increased. However, 0.5 M NaCl appeared to precipitate labelled SEB because the sum of counts of all fractions was much lower than that of the control without NaCl. This did not occur with 0.15 or 0.30 M NaCl in the phosphate buffer. NaCl of 0.15 M was selected for use in the assay system because more SEB was expected to bind to antibody in the column at this concentration. Figure 3 shows that 30 ml of PBS was sufficient to remove unbound, labelled SEB. Therefore, this volume was selected for the assay.

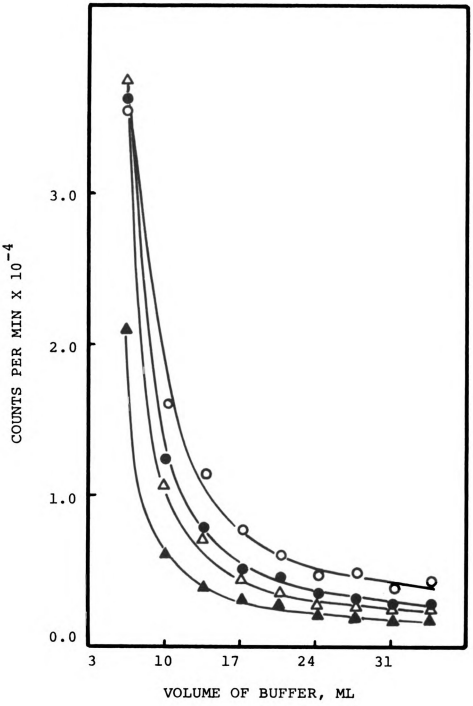

Fig. 3. Effect of NaCl on elution of unbound labelled SEB. Symbols: O—O, 0.05 M phosphate buffer; ●—●, 0.05 M phosphate buffer with 0.15 M NaCl; Δ—Δ, 0.05 M phosphate buffer with 0.30 M NaCl; ▲—▲, 0.05 M phosphate buffer with 0.50 M NaCl.

Selection of Dissociating Agent

In this experiment, the dissociating agents investigated were: 8 M urea, pH 3 and 10, 3 M KSCN pH 8, 6 M guanidine-HCl, pH 3, 1.0 and 2.0 M NH_4OH pH 11.25 and 11.5, respectively.

The column and method used in this experiment were the same as those described in the previous section except 30 ml instead of 50 ml PBS was used to remove impurities and unbound labelled SEB from the column. Bound labelled SEB eluted from the column was collected in 1.5-ml fractions when the flow rate was less than 80 ml/hr. For flow rates greater than 80 ml/hr, 3.5 ml fractions were collected. The radioactivity in each fraction was determined by counting with a gamma-ray counter. The column was re-equilibrated for the next sample by washing the column with 50 ml of 0.05 M PBS.

Figure 4 shows the elution pattern of the three buffers. The buffer containing KSCN was not effective for removal of bound SEB. The greatest dissociation of bound labelled SEB was obtained with 8 M urea, pH 3. It also showed the least tailing. Urea in pH 10 buffer was much less effective than in the pH 3 buffer for removal of labelled SEB. Six molar guanidine-HCl was only slightly inferior to urea. The greatest degree of tailing was observed with 2 M NH_4OH (Figure 4). However, when flow rates were considered, 8 M urea and 6 M

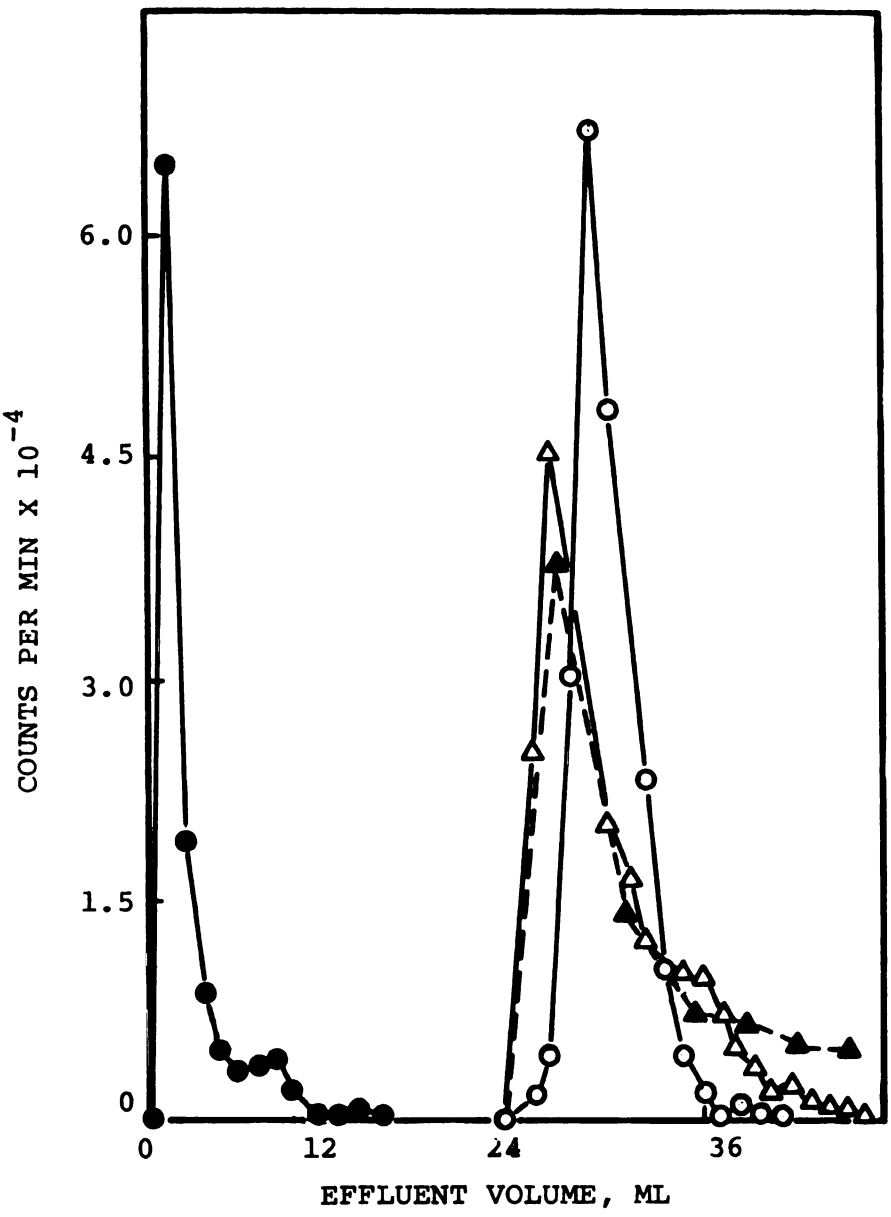

Fig. 4. Comparison of 8 M urea, pH 3.0; 6 M guanidine-HCl, pH 3.0; and 2 M NH_4OH, pH 11.5 for removal of bound ^{125}I-enterotoxin from antibody column. Symbols: O—O, urea; Δ—Δ, guanidine-HCl; ▲—▲, NH_4OH; ●—●, PBS.

guanidine-HCl could not be pumped at rates higher than 40 ml/hr because of their high viscosity. Thus, NH_4OH was selected for further investigation because a flow rate of up to 75 ml/hr could be used with a 1:5 to 1:30 gel dilution.

Improvement of Dissociating Ability of NH_4OH

It was necessary to reduce the tailing of the NH_4OH dissociating buffer to take advantage of its superior flow rate. The addition of NaCl to NH_4OH was investigated as a means for improving the performance of the buffer. The same assay was followed as described in the preceding section with the substitution of various dissociating agents containing NH_4OH. Figure 5 shows that the addition of 0.3 M NaCl increased the amount of SEB dissociated and reduced tailing. Figure 6 shows the effect of different NaCl concentrations on tailing. It was found that 0.15 M NaCl gave the optimum suppression of tailing. Figure 6 also shows that 15 ml of 1.0 M NH_4OH containing 0.15 M NaCl was sufficient to remove the bound SEB under the conditions used in this study.

Optimum Concentration of NH_4OH

Using the assay sequence described above, NH_4OH of different concentrations, each containing 0.15 M NaCl, was passed through the column repeatedly. Fifteen ml of

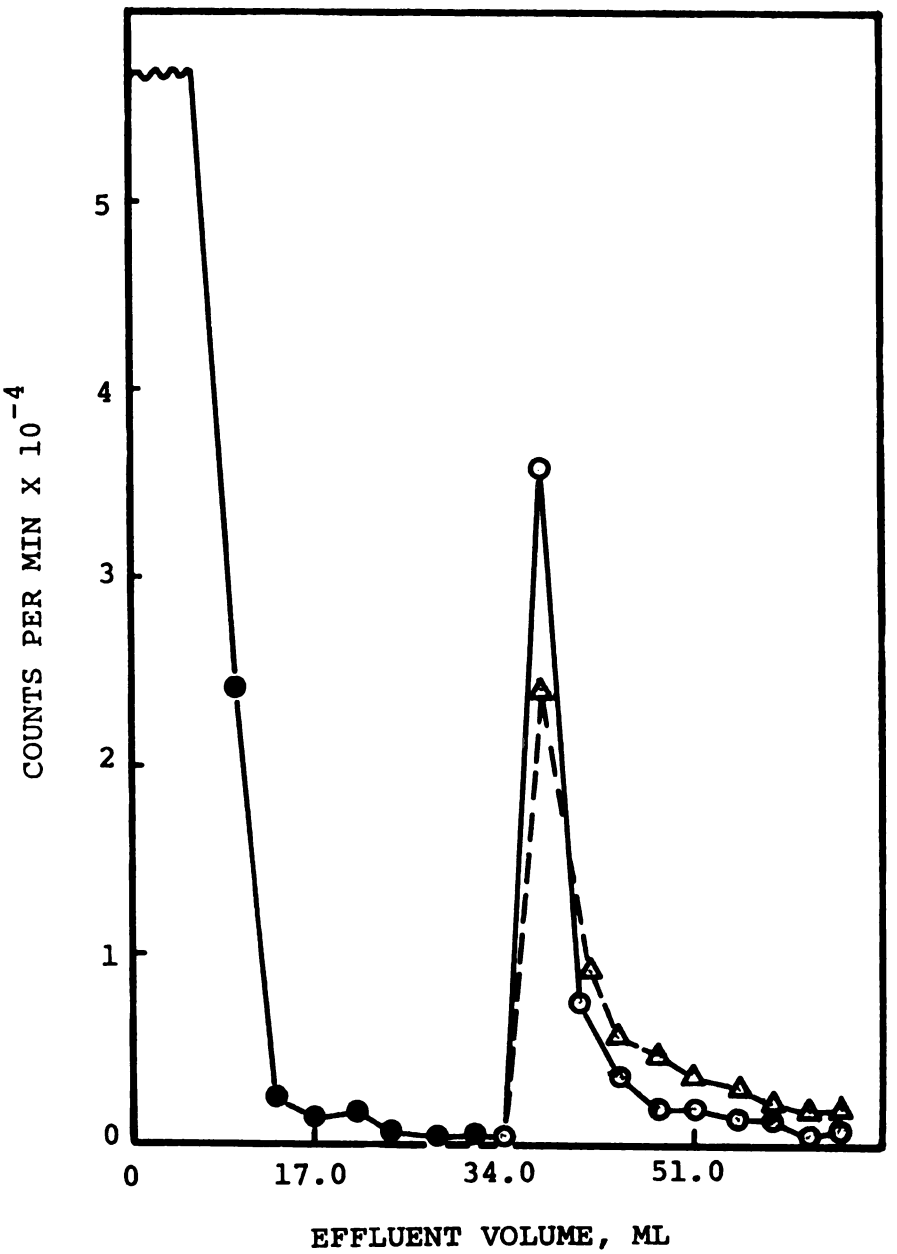

Fig. 5. Comparison of elution patterns for removal of bound labelled SEB with pH 11.5, 2 M NH_4OH with and without NaCl added. Symbols: ●—●, washing with PBS to remove impurities and unbound labelled SEB; Δ—Δ, 2 M NH_4OH without NaCl; O—O, 2 M NH_4OH with 0.30 M NaCl.

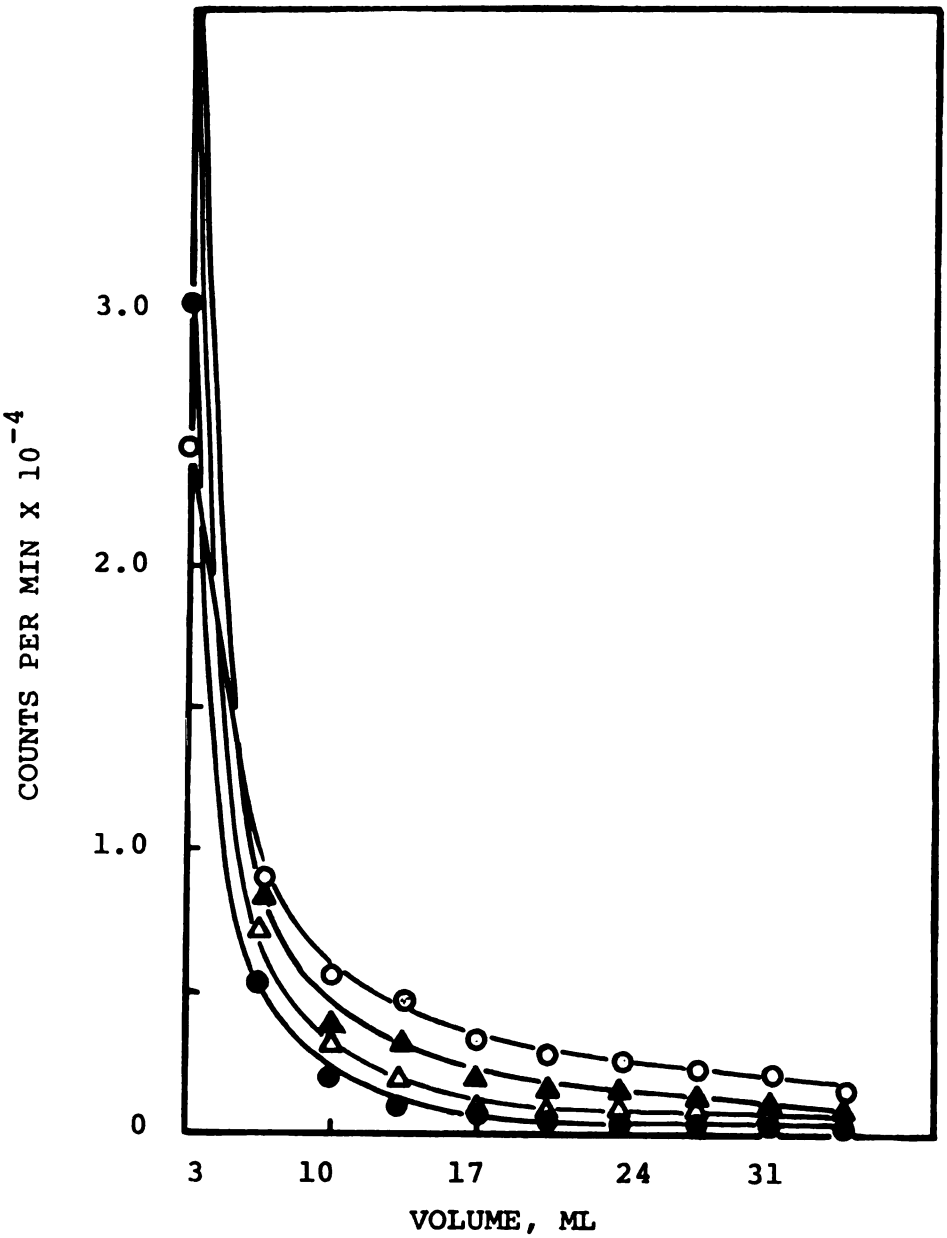

Fig. 6. Elution patterns for removal of bound labelled SEB with different concentrations of NaCl. Symbols: O—O, 1.0 M NH_4OH; ●—●, 1.0 M NH_4OH with 0.15 M NaCl; Δ—Δ, 1.0 M NH_4OH with 0.30 M NaCl; ▲—▲, 1.0 M NH_4OH with 0.50 M NaCl.

NH_4OH-eluate was collected and 0.5 ml was taken for radioactivity measurement using a gamma-ray counter. The NH_4OH concentrations were 0.13, 1.0, and 2.0 M. Figure 7 shows the relationship between the binding capacity and the number of cycles. It was found that the binding capacity was not stable with 1.0 and 2.0 M NH_4OH. The loss of binding capacity of the column was accompanied by formation of gel clumps and a decline of the flow rate. This instability of the column may be due to either the denaturation of the antibody in the column or disintegration of gel. The loss of binding capacity was not observed with 40 samples for buffer containing 0.13 M NH_4OH. Therefore, this concentration was selected for the assay system.

Equilibration of the Column

After elution of bound SEB with NH_4OH, the column must be prepared for the next sample by replacing the dissociating buffer with pH 7.5, PBS. It was necessary to determine the volume of phosphate buffer required for equilibration. The procedure used in this experiment was to equilibrate 1:10 dilution antibody-gel with 0.05 M phosphate buffer pH 7.5 containing 0.15 M NaCl. Twenty µl of labelled SEB were run through the column, followed by washing unbound SEB with 30 ml PBS. Bound enterotoxin was eluted with 15 ml of 0.13 M NH_4OH containing 0.15 M NaCl. The column was then washed with PBS and 3.5-ml

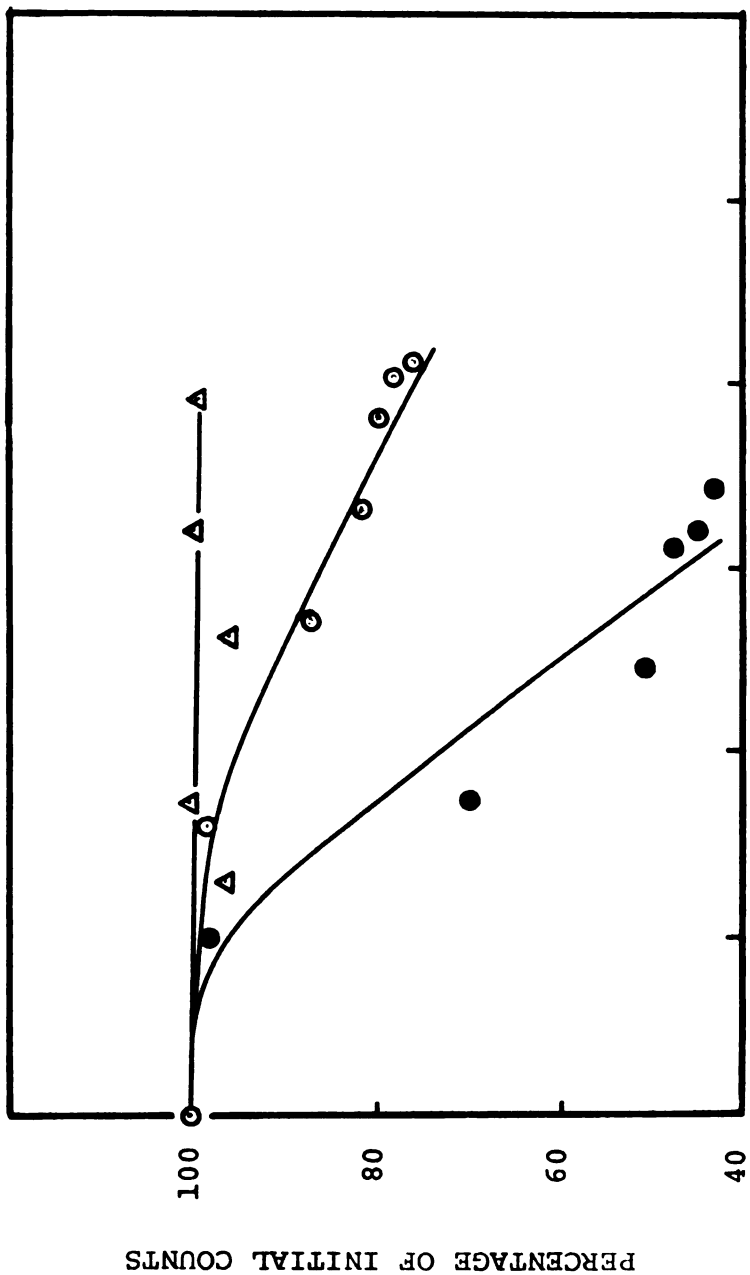

Fig. 7. Counts of controls run repeatedly when different concentrations of NH_4OH were used as dissociating agent. Symbols: Δ——Δ, 0.13 M NH_4OH, pH 10.5; O——O, 1.0 M NH_4OH, pH 11.25; ●——●, 2.0 M NH_4OH, pH 11.5.

fractions were collected. The pH and the presence of NH_4^+ were determined for each fraction with Nessler's reagent. The results are shown in Table 1. The pH reached 7.5 by fraction 3, but ammonium ion was detected until fraction 8. This result indicated that at least 30 ml of PBS were required to wash off NH_4OH.

Table 1

Equilibrating the Antibody Column with PBS

Volume of Buffer, ml	pH	NH_4^+
3.5	10.25	++++
7.0	7.8	++++
10.5	7.5	+++
14.0	7.5	+++
17.5	7.5	++
21.0	7.5	++
24.5	7.5	+
28.0	7.5	-

Time Required for Contact Between Enterotoxin and Antibody

It was necessary to have a reasonable time of contact between antigen and antibody in order to obtain sufficient antigen binding. For the assay, it was important to determine the contact time to have a sufficient count in order to do the assay. In experiments with 1:10 dilution-gel column, the following sequence was used: 40 µl labelled SEB, 30 ml of 0.05 M PBS, 15 ml of 0.13 M NH_4OH containing 0.15 M NaCl and 30 ml of 0.05 M PBS. Fifteen ml of NH_4OH-eluate was collected

and 0.5 ml was taken for a radioactivity measurement using a liquid scintillation counter. Table 2 shows that increasing the contact time from less than one minute (labelled SEB was passed through the column and washed off immediately) to 5 minutes increased the count rate by about 39%. A further 20% increase in bound antigen was observed if the contact time was increased to 10 minutes. In order to maximize the antigen binding, 10 minutes was selected as a contact time for labelled SEB. Twenty minutes was used for samples because it was thought that high levels of impurities in food samples might interfere to some extent with SEB binding. Consistent assays were obtained using this time for binding. However, further investigation is required to optimize this factor in the assay.

Amount of Labelled Enterotoxin Required for the Assay Procedure

The amount of labelled SEB used in the assay system must be high enough to saturate antibody binding sites. Using the assay sequence mentioned in the preceding section, 10, 20, 30, and 40 µl of labelled SEB were applied to the column with a contact time of 10 minutes. Fifteen ml of NH_4OH-eluate were collected and counted. Figure 8 shows the relationship between the amount of labelled SEB applied to the column and the radioactivity that was subsequently eluted. There is a

Table 2

Effect of Contact Time on Binding of Labelled SEB

Contact Time Min.	Count/min.[a]	Mean, $\bar{X}$	Standard Deviation, s
< 1[b]	2,215 2,769	2,492	-
5	3,900 3,452 3,543	3,452	237
10	4,348 4,355 3,956 3,944	4,150	232

[a]The count was done by liquid scintillation spectrometer, Packard Model 3310, using 0.5 ml of 0.13 M NH_4OH eluate in 10 ml of counting cocktail 3a70.

[b]Labelled enterotoxin was passed through the column and washed off immediately with PBS.

Table 3

Effect of Contact Time on Binding of SEB to Its Antibodies in the 1:15 Dilution Antibody-Gel Column (The result obtained from incubation of 0.68 ml of 2 ng SEB [in PBS] per ml with different contact time, using 40 µl of labelled SEB, 30 ml of PBS for washing impurities and unbound labelled SEB and 15 ml of 0.13 M NH_4OH [with 0.15 M NaCl] for elution)

Contact Time, Min.	Counts Per Min. of NH_4OH Eluate[a]	Mean, $\bar{X}$	Standard Deviation, s
Control[b]	3,696 3,702 3,624	3,674	43.4
15	3,629 3,540 3,637	3,602	53.8
20	3,623 3,583	3,603	-
30	3,613 3,682 3,576 3,494	3,591	78.3

[a]Average count from 5-minute counting of 0.5 ml NH_4OH eluate in 10 ml counting cocktail 3a70, using liquid scintillation spectrometer, Packard Model 3310.

[b]Without SEB in the sample.

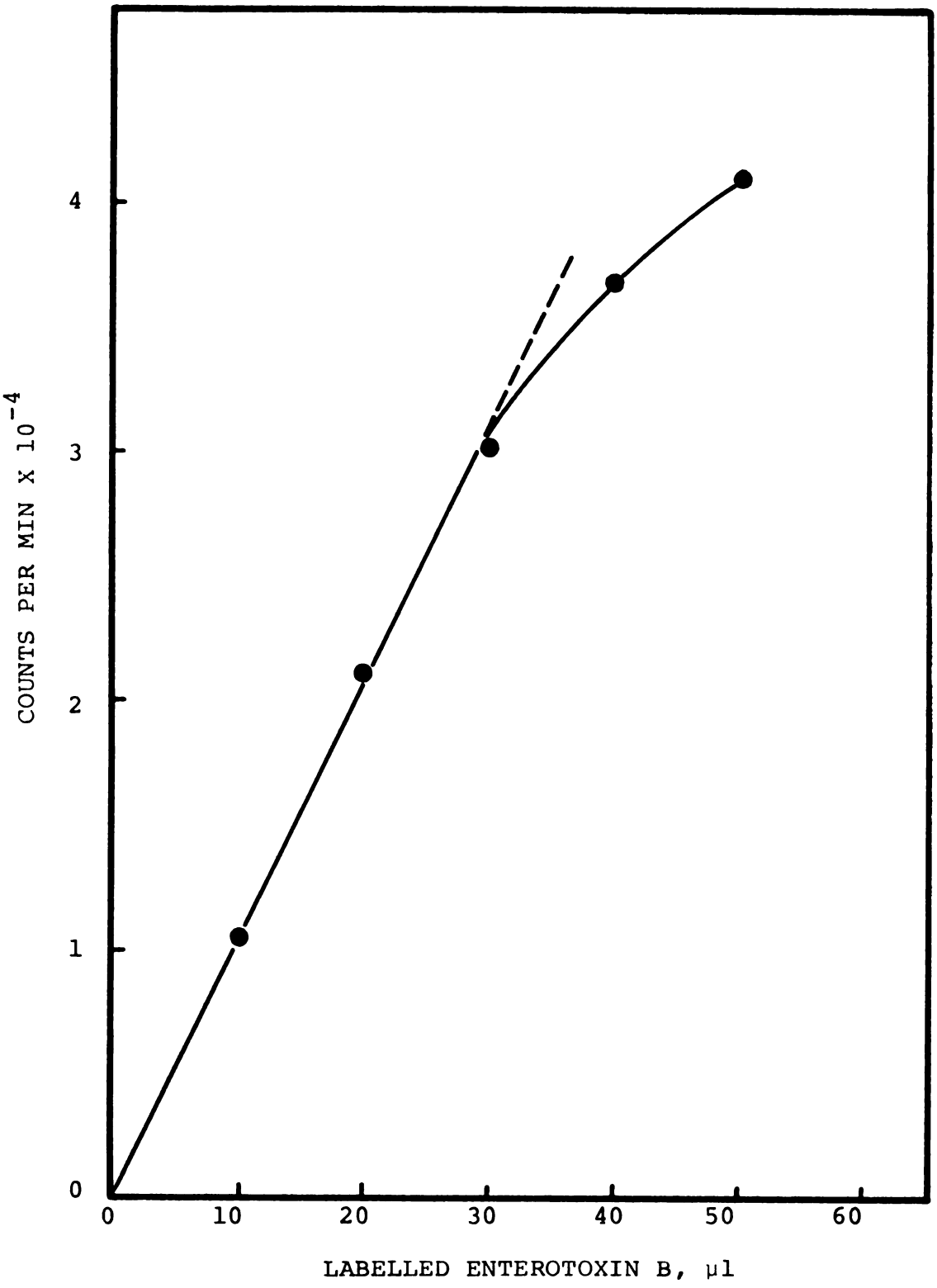

Fig. 8. Relationship between the amount of bound and added labelled SEB in the column 1:15 dilution antibody-gel.

linear increase in the radioactivity bound with up to 30 µl. In the measurement of SEB in samples applied to the column it was found that 40 µl of labelled SEB solution was required to observe a response for samples with low enterotoxin concentrations.

Suppression of Nonspecific Binding

If nonspecific binding of SEB to the gel or glass column occurred to a large extent, it could lead to false-positive errors with low levels of SEB. The addition of an inert protein has been used to suppress nonspecific binding to glass or gel (Greenwood & Hunter, 1963). An experiment was designed to suppress nonspecific binding of SEB to the column. Two ml of Sephadex G-25 were packed into a column and eluted with dissociating agent followed by PBS. One-half ml of BSA (5 to 20 mg/ml) was passed through the column. This was followed by 10 ml of PBS, 40 µl of labelled SEB, 30 ml of PBS, and finally eluted with 15 ml of 0.13 M NH_4OH containing 0.15 M NaCl. The activity was counted using 0.5 ml of NH_4OH-eluate.

The above experiment was repeated using 0.5 ml of 20 mg/ml BSA up to the step before adding the labelled SEB. Instead of adding only labelled SEB, a mixture of labelled SEB and BSA (40 µl of labelled SEB:0.65 ml of BSA) was added to the column. The BSA concentrations

used were 5, 10, and 20 mg/ml. Then the column was washed with PBS and eluted with dissociating agent. The NH_4OH was counted as before.

Table 4 shows that nonspecific binding of labelled SEB is 2.28% of that of the control (column 1:15 dilution antibody gel) when no protein was added.

It decreased to 1.4% when BSA was increased to 20 mg/ml. The suppression of nonspecific binding was more effective when protein is also present in a sample. It was reduced to about 1% in the sample containing 20 mg/ml BSA.

Table 4

Suppression of Nonspecific Binding

Treatment of Column mg/ml BSA	Add Labelled Enterotoxin	Enterotoxin Bound %
0	in buffer	2.28
5	"	1.97
10	"	1.76
20	"	1.44
20	in BSA, 5 mg/ml	1.23
20	in BSA, 10 mg/ml	1.12
20	in BSA, 20 mg/ml	1.01

Assay Sequence

The investigations of factors involved in this affinity radioimmunoassay led to development of the assay procedure shown in Table 5. A series of 7 steps were required which took a total of 73 minutes. The column

Table 5

Assay Sequence for Enterotoxin B

	Step	Time Required Min.
1.	Addition 0.5 ml of 2% BSA and followed by washing with 10 ml 0.05 M phosphate buffer saline	5
2.	Addition of sample and incubation	20
3.	Wash off the impurities and adsorbed substances with 5 ml 0.05 M phosphate buffer saline, pH 7.5	2
4.	Addition of 40 µl of labelled enterotoxin	10
5.	Wash off unbound labelled enterotoxin with 30 ml, 0.05 M phosphate buffer saline, pH 7.5	12
6.	Elution of bound enterotoxin with 15 ml 0.1 M NH_4OH, pH 10.5	12
7.	Wash off NH_4OH with 30 ml 0.05 M phosphate buffer saline, pH 7.5	12
	Total	73

was treated with 0.5 ml of 2% BSA prior to sample application. After a sample was applied to the column and incubated for 20 minutes, the column was washed with pH 7.5 PBS to remove impurities from the column without removal of bound SEB. Labelled SEB was added so it would bind to those antibody molecules that did not react with SEB in the sample. The labelled SEB which did not bind was removed by another PBS wash. The bound SEB, both labelled and unlabelled, was then eluted with dissociating NH_4OH. The radioactivity of the eluted SEB was inversely proportional to the amount of SEB in the sample. The dissociating NH_4OH was removed and the column prepared for the next sample by equilibration with PBS.

In the manual procedure used during development of the assay, steps 1 (BSA), 2 and 4 were done with gravity flow. A flow rate of 150 ml/hr was used for steps 1 (PBS), 3, 5 and 7, and a 75 ml/hr flow rate was used for step 6.

Dose-Response Curve

Dose-response study was done using the procedure outlined in Table 4. The concentrations of SEB used were 1.5, 3.0, 4.5, 6.0, 7.5, 9.0, and 10.5 µg/ml. These solutions were run through a column in which the antibody-gel was diluted 1:15 with Sephadex G-25. A sample of 0.68 ml and contact time of 20 minutes were used for all concentrations. Fifteen ml of NH_4OH-eluate

were collected and a 0.5 ml sample was taken for activity measurements. The count with no SEB in the sample control was the average of four determinations. Duplicate samples were run for each of the other SEB samples. Figure 9 shows the relationship between the SEB level and the counts per minute of bound SEB. The same data are shown in Figure 10, but the radioactivity is expressed in terms of the percentage of the counts bound in the control assays.

The slope, intercept, and correlation coefficient for the least square regression of the curve plotted in Figure 10 were calculated and found to be -0.821%/ng SEB, 100.1%, and 0.986 respectively.

Factors Affecting the Dose-Response Curve

Dilution of antibody gel with Sephadex G-25 reduced the antibody concentration in the column. This, in turn, affected the dose-response curve. Figure 11 shows the effect of variation of labelled SEB concentration from 20 to 40 µl when a column of 1:15 dilution was used. At a low SEB level, no apparent response is observed with 20 µl labelled SEB. A response is observed when the labelled SEB is increased to 40 µl. An improved response was obtained when the dilution of the antibody gel with Sephadex G-25 was increased from 1:5 to 1:15.

For the effect of pH, there is little evidence that antigen-antibody complex exhibits a significant pH

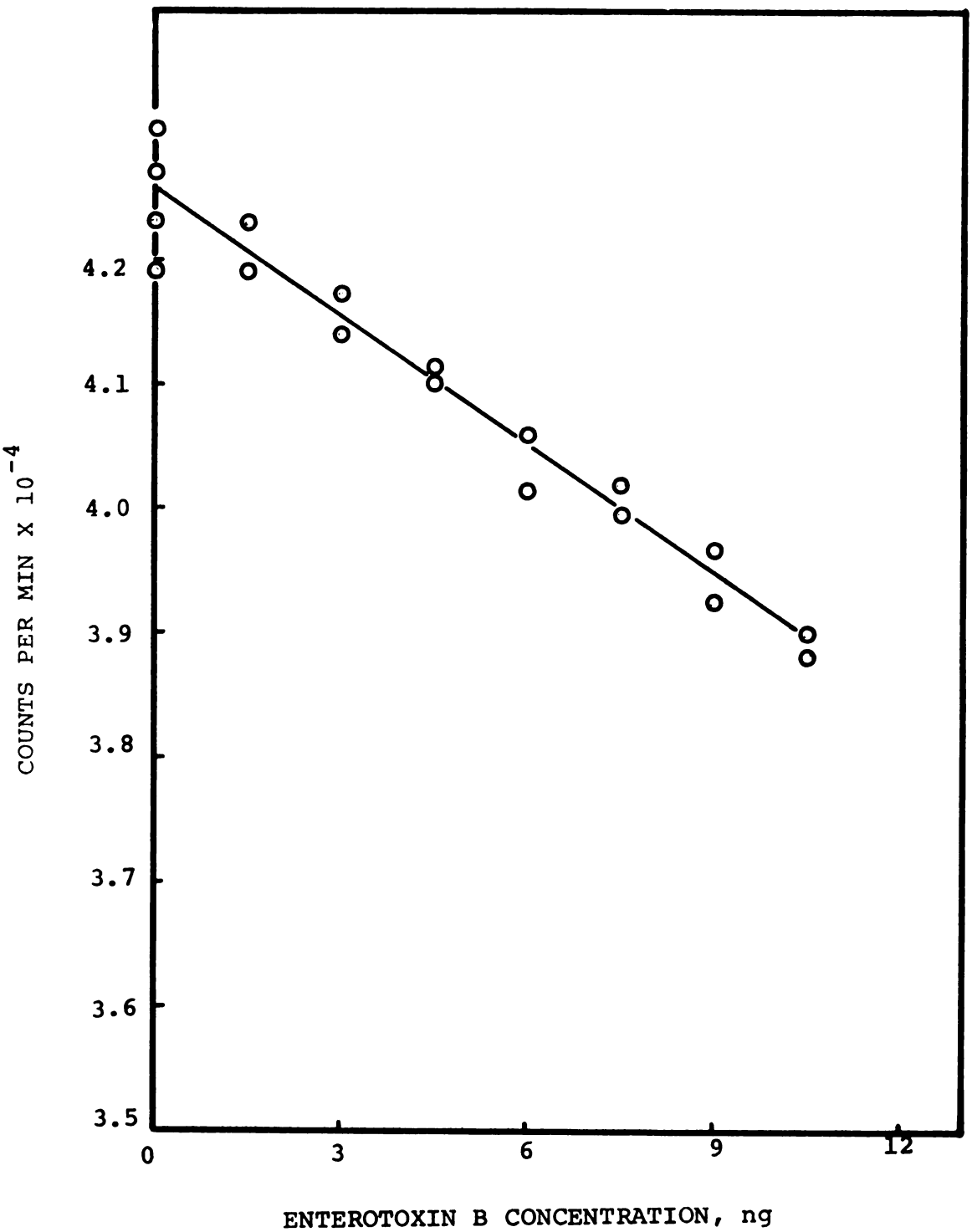

Fig. 9. Standard curve of SEB in PBS, showing the relationship between the enterotoxin level and counts per minute.

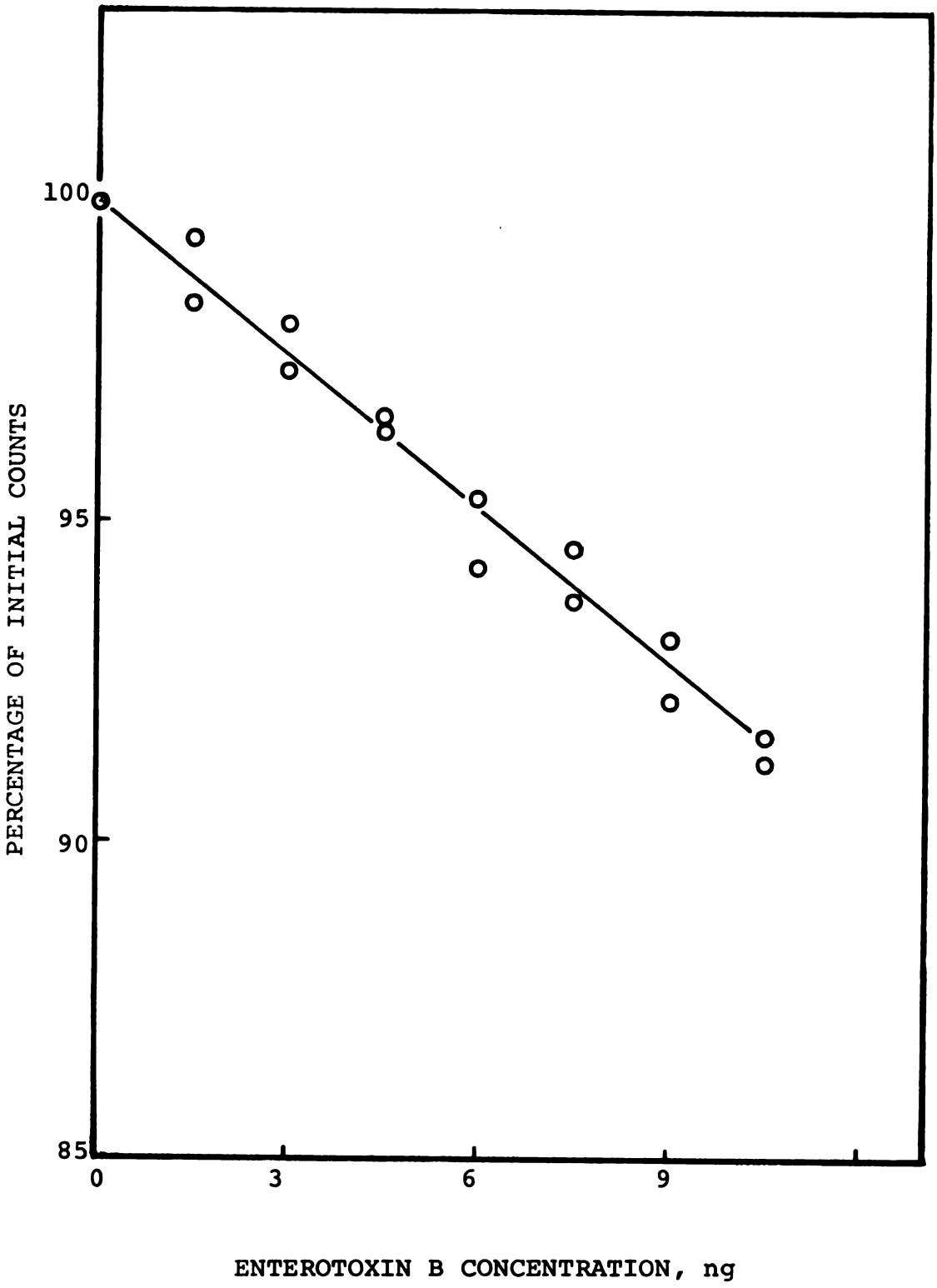

Fig. 10. Standard curve of SEB in PBS, showing the relationship between the enterotoxin level and percentage of initial counts.

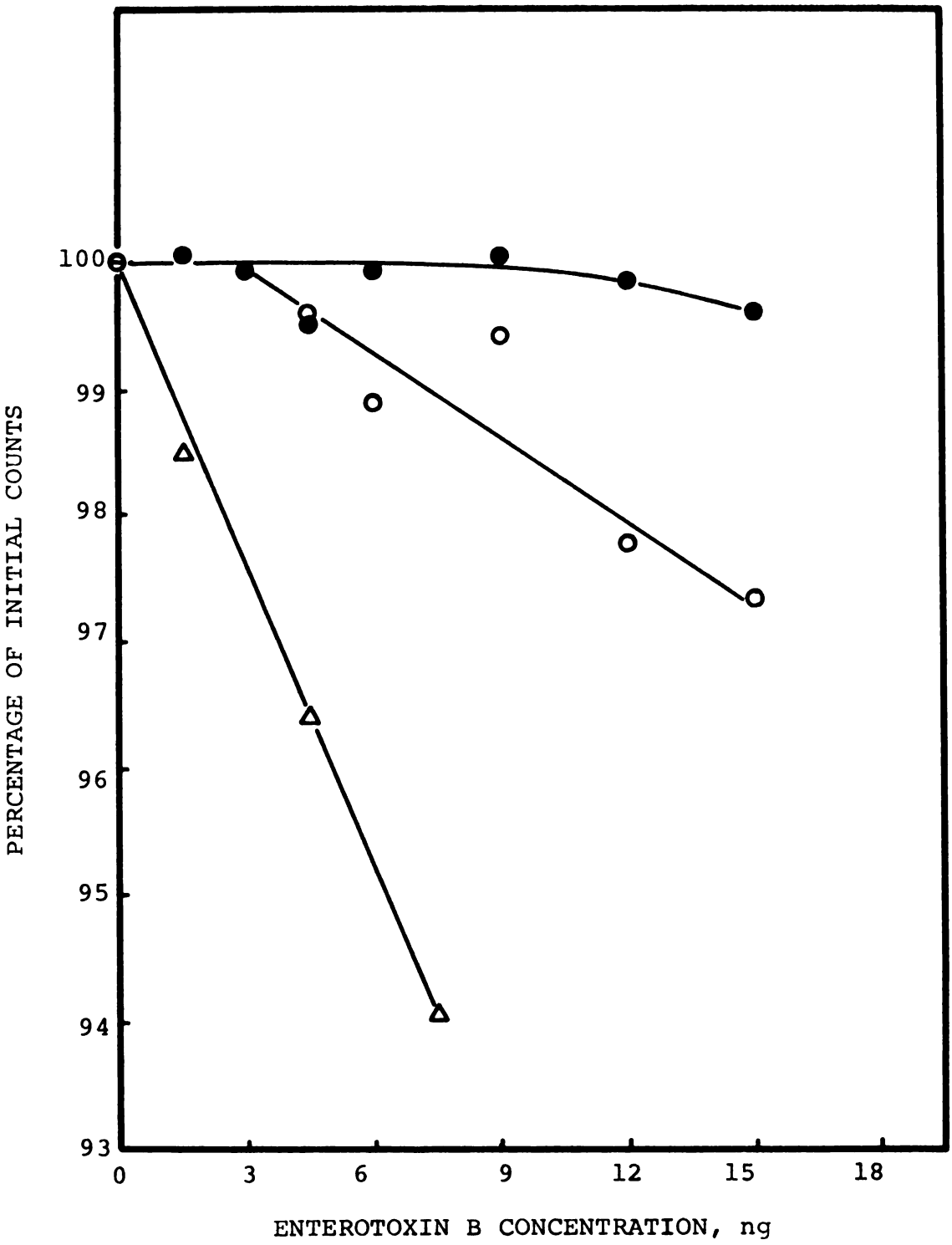

Fig. 11. Effect of gel dilution and concentration of labelled SEB on the dose-response curve. Symbols: ●—●, 1:5 dilution antibody-gel column with 20 µl labelled SEB; O—O, 1:5 dilution antibody-gel column with 40 µl labelled SEB; Δ—Δ, 1:15 dilution antibody-gel column with 40 µl labelled SEB.

dependency in the range of 7 to 8 (Berson & Yalow, 1973). But it was necessary to maintain a constant pH for both standard and unknown samples in order to maintain equilibrium of the column.

The ionic strength of the buffer system is very important for antigen-antibody complexes. High concentrations of NaCl may inhibit reactions (Berson & Yalow, 1973). If the salt concentration of the unknown sample differs from that of standards, differential inhibition of the immunochemical reaction will give erroneous results. In this experiment, when 0.68 ml of reconstitute nonfat dry milk prepared in 0.05 M phosphate buffer containing 0.30 M was used as a sample, the count of the eluate increased 5.45%.

Validation of the Assay Procedure

To validate an assay, one must demonstrate SEB in the sample and standard (purified enterotoxin) react with antibody identically and that under the conditions of assay, other substances in the sample do not affect the determination. Enterotoxins A and B were produced in milk samples inoculated with Staphylococcus aureus strains 196E (ATCC 13565) and 243 (ATCC 14458), respectively. Authentic SEB samples were also obtained from the Food and Drug Administration. The ability to detect SEB from these samples was checked.

From the experiment, it was found that the antibody column can be used to detect SEB prepared by FDA and produced in the inoculated milk. No cross-reaction was observed with enterotoxin A from FDA. The experiment also showed no cross-reaction occurred between substances in the inoculated medium (milk inoculated with Strain 196E) and antibody in the column (Table 6).

Detection of Enterotoxin B in Foods

Detection of Enterotoxin B in Nonfat Dry Milk

Measured amounts of SEB were added to reconstituted nonfat dry milk. The concentrations used were 1.5, 3.0, 4.5, 6.0, 7.5, 9.0, and 10.5 ng/ml. Then, the pH of the milk was adjusted to 7.5 with 2 N NaOH. The samples were run using the procedure described in Table 5.

Figure 12 shows the relationship between the counts per minute and the SEB concentration. In Figure 13 the data are expressed in terms of the percentage of the control counts. The second standard curve has a slope, intercept, and correlation coefficient of -0.793%/ng SEB, 100.2%, and 0.972, respectively.

The amount of SEB in an unknown sample can be determined directly from this standard curve if a sample is prepared in the same way as samples used in preparing a standard curve.

Table 6

Validation of the Assay Procedure

Sources of Enterotoxin	Percentage of Initial Count	Concentration of SEB, ng/ml	
		Added	Found
Enterotoxin B from FDA	93.44	8.0	8.1
Enterotoxin B from milk inoculated with S. aureus Strain 243 (ATCC 14458)	91.67	Unknown[a]	7300[b]
Enterotoxin A from FDA	99.93	1000	ND[c]
Enterotoxin A from milk inoculated with S. aureus Strain 196E (ATCC 13565)	100.09	Unknown[a]	ND[c]

[a]No enterotoxin was added.

[b]Milk was diluted 1,000 times before the determination.

[c]Not detectable.

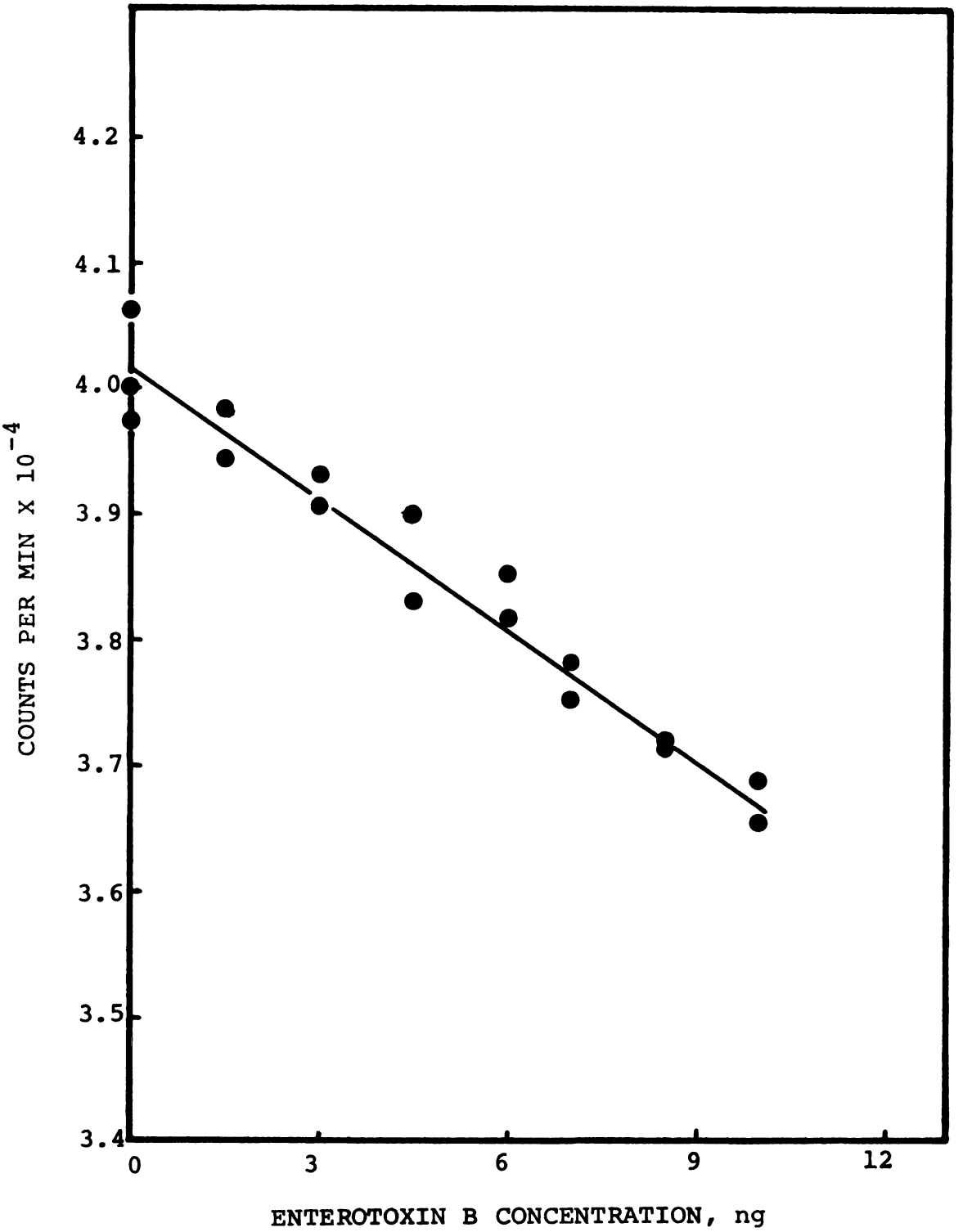

Fig. 12. Standard curve of SEB in reconstituted nonfat dry milk, showing the relationship between the enterotoxin level and counts per minute.

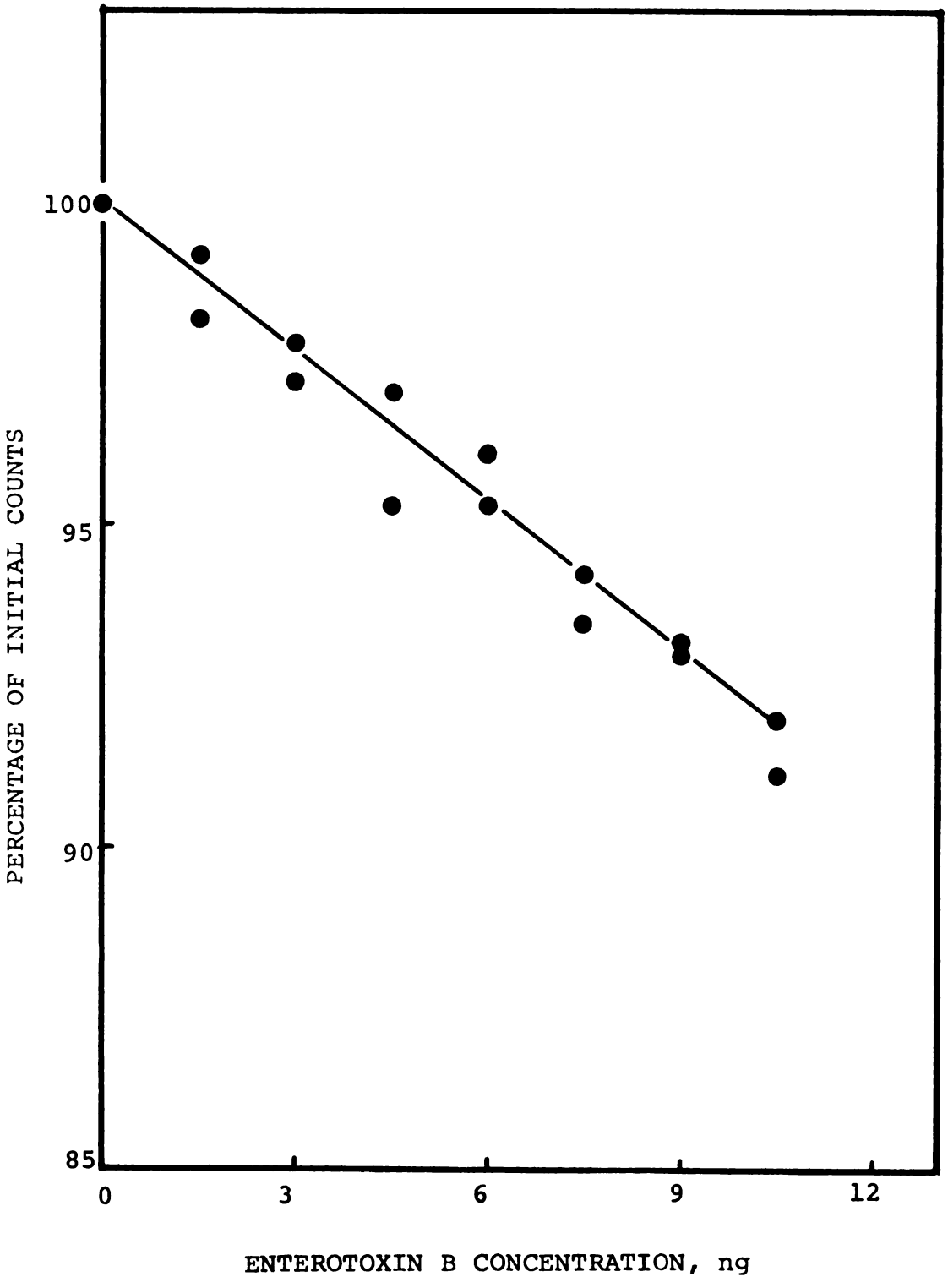

Fig. 13. Standard curve of SEB in reconstituted nonfat dry milk, showing the relationship between the enterotoxin level and percentage of initial counts.

Detection of Enterotoxin B in Hamburger

Ten grams of hamburger were mixed with 10 ml SEB solution (SEB was dissolved in 0.05 M PBS). The concentration of SEB was so prepared that 1 g of hamburger contained 2, 4, 6, 8, 10, and 12 ng. The pH of the hamburger was adjusted to 7.5 with 2 M NaOH. Several re-adjustments were required to be sure that the pH of the mixture was 7.5. After 24 hours, the SEB in the samples was extracted (see MATERIALS AND METHODS) and 0.68 ml was run through the column for SEB determination. Two curves were constructed in the same way as for reconstituted nonfat dry milk (Figures 14 and 15). The slope, intercept, and correlation coefficient for the linear portion of the curve were found to be -1.568%/ng SEB, 106.5%, and 0.969, respectively. The amount of SEB in the sample can be calculated directly by use of this standard curve.

Determination of D-Value for Enterotoxin Denaturation in Reconstituted Nonfat Dry Milk

Reconstituted nonfat dry milk was inoculated with Staphylococcus aureus Strain 243. After 20 hours of growth, the whey portion (pH 5.2) was separated from the curd (formed during incubation) and centrifuged at 32,800 x g for 20 minutes. Ten ml of this aqueous solution were pipetted into Thermal Death Time (TDT)

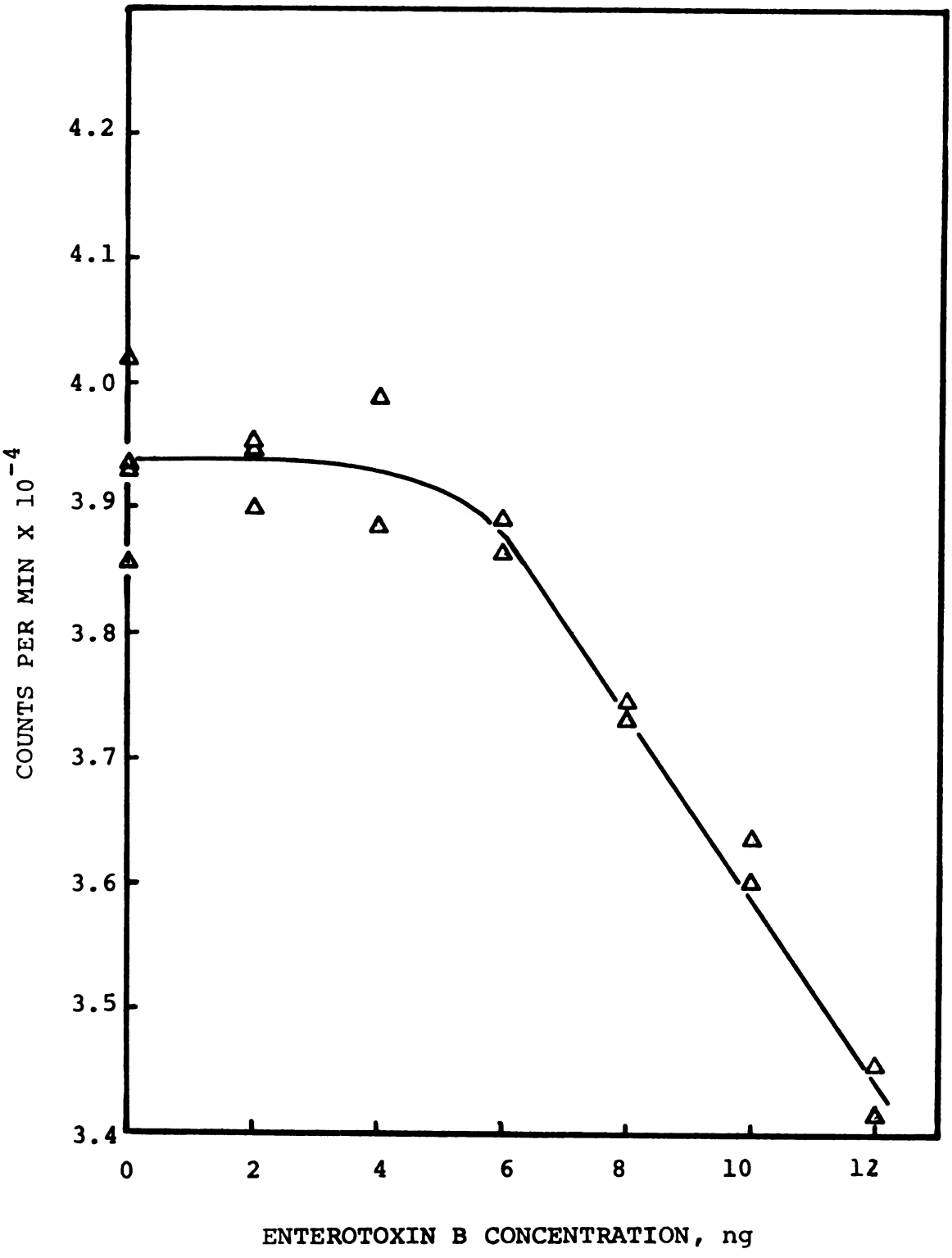

Fig. 14. Standard curve of SEB in hamburger, showing the relationship between the enterotoxin level and counts per minute.

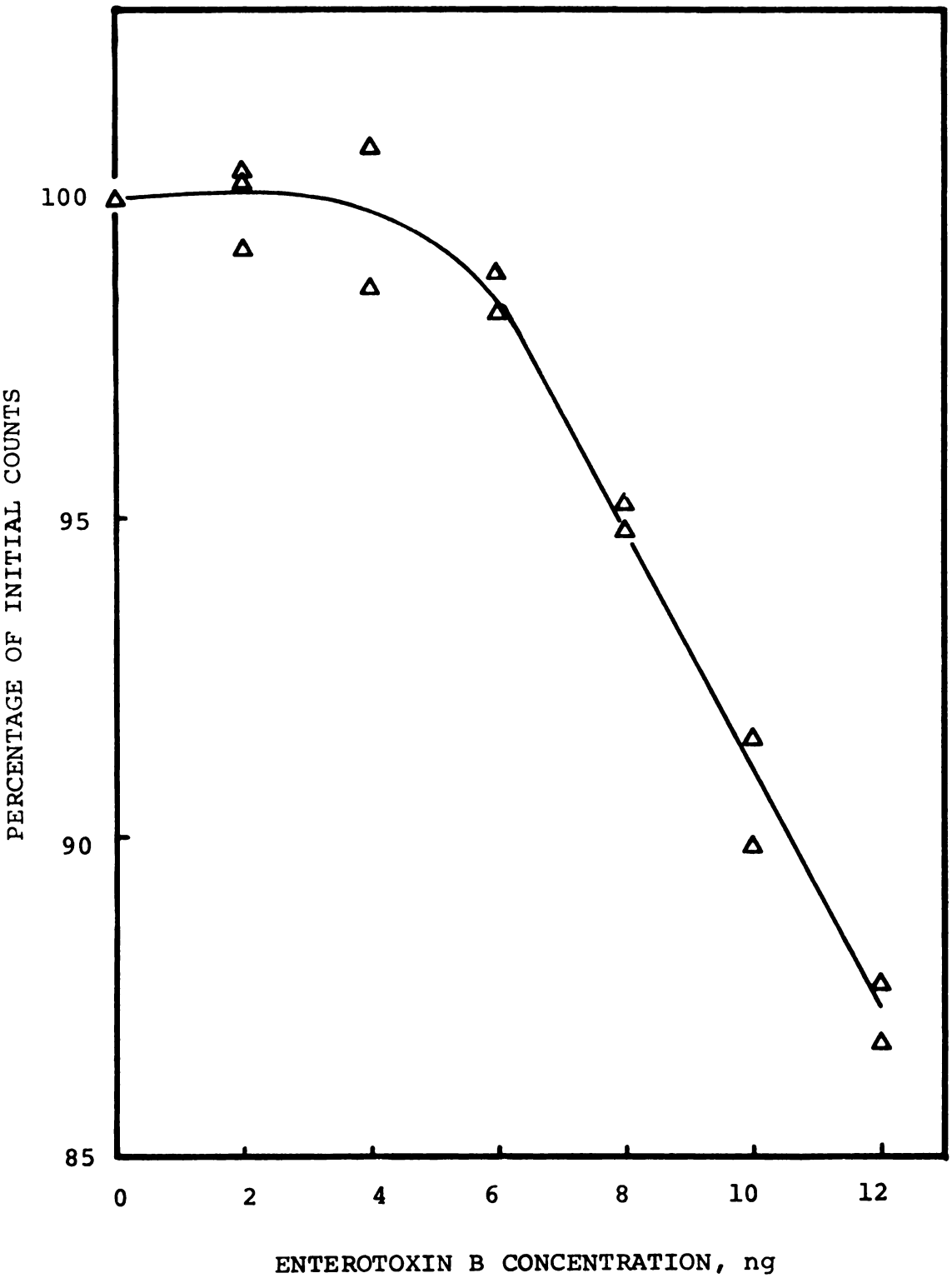

Fig. 15. Standard curve of SEB in hamburger, showing the relationship between the enterotoxin level and percentage of initial counts.

cans. The cans were heated at 121 C (250 F) in a miniature retort for 2, 5, 10, and 15 minutes (uncorrected for heating lag) and cooled immediately with water to room temperature. The pH of heated milk was adjusted to 7.5 with 2 M NaOH. The samples were centrifuged at 32,800 x g for 20 minutes and analyzed for SEB remaining using the standard curve for milk (Figure 13). Figure 16 shows the relationship of the amount of SEB found and time for each treatment. The correlation coefficient was 0.9969. The D-value, calculated as the reciprocal of the slope, was 10.4 minutes.

Sensitivity of the Assay

The sensitivity of the assay was determined by "the least-detectable dose" method (Hunter & Greenwood, 1964; Feldman & Robard, 1971). The least detectable dose was defined as the lowest concentration of unlabelled SEB which results in a significant change in the response. Significant change was defined as the mean of the standard deviation of the response at the point where the unlabelled SEB is zero, multiplied by a "significant value" of Student's *t*.

Table 7 shows the calculated values for the least-detectable dose of SEB in PBS, reconstituted nonfat dry milk and hamburger. For a standard curve of SEB in PBS using four samples, the mean value of the counts for control (no enterotoxin was added) was 42,568, and the

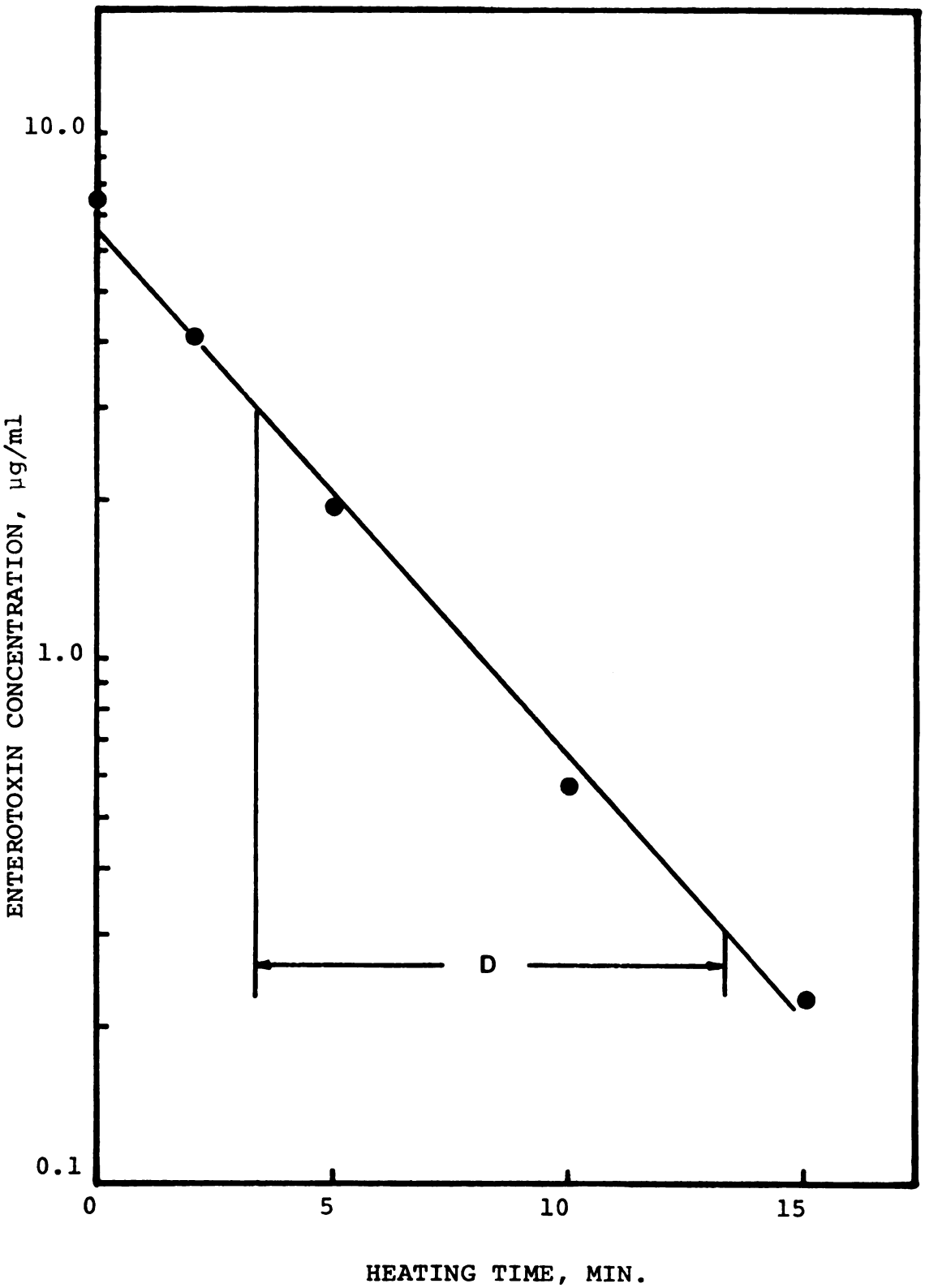

Fig. 16. Heat inactivation of SEB in reconstituted nonfat dry milk at 121 C, pH 5.2.

Table 7

Sensitivity of SEB Assay

Standard Curve	Number of Sample, n	Mean, $\bar{X}$	Standard Deviation, s	Significant Change at 95% Confidence[a]		The Least-Detectable Dost, ng/ml
				cpm	%	
Buffer	4	42,568	509	399	0.94	1.2
Milk	3	40,122	480	701	1.75	2.2
Hamburger	4	39,378	923	724	1.84	6.3

[a]The t for significance at 0.05 level are 2.920 and 2.353 for n = 3 and n = 4, respectively.

standard deviation, s, was 509. The significant change at 95% confidence was 399 counts per minute (significant change = $ts/\sqrt{n-1}$), or 0.94% reduction in count. This corresponded to a minimum SEB concentration of 1.2 ng/ml for detection. The least-detectable dose response for milk and hamburger were found to be 2.2 and 6.3 ng/ml, respectively.

Reproducibility

For the evaluation of reproducibility of the assay, a toxin concentration near the midpoint of the assay range (0 to 12 ng/ml) was selected. Enterotoxin at a concentration of 6 ng/ml in PBS was run four times during a two-week period when 64 samples, including meat and milk, were measured. The average level detected was 5.9 ± 0.4 ng/ml. Six consecutive runs were done with a different batch of ^{125}I-SEB using the standard curve in Figure 10. A concentration of 6.5 ng/ml of SEB was used. The average level detected in this experiment was found to be 6.6 ± 0.4 ng/ml. When the data from these 10 determinations were combined and expressed as the percentage SEB recovered, the average was 100.4 ± 5.7% (Table 8).

Table 8

Reproducibility of the Assay

Concentration of SEB Added ng/ml	Concentration of SEB Found ng/ml	Mean ng/ml	Standard Deviation	Percentage of Added EB Measured	Mean Recovery from 10 Samples %	Standard Deviation %
6.0	6.4	5.9	0.4	106.7	100.4	5.7
	5.5			92.2		
	5.8			95.9		
	6.1			101.6		
6.5	7.2	6.6	0.4	111.2		
	6.7			103.5		
	6.2			95.1		
	6.6			101.5		
	6.5			99.4		
	6.3			97.1		

DISCUSSION

An affinity radioimmunoassay has been developed for the measurement of staphylococcal enterotoxin B in buffer solution and in food products. The principle of the assay is similar to the solid-phase radioimmunoassay reported by Catt et al. (1967). However, the ability to reuse the immobilized antibody and the potential for adapting the column technique for automated analysis systems are significant advantages of this procedure.

SEB was used in the development of the affinity radioimmunoassay procedure because the SEB and its antitoxin are commercially available. In addition, SEB is stable under the experimental conditions which were employed.

Agarose 4B was used to bind antibody since it was more porous than the 6B derivative and has greater capacity than the 2B gel. Antibody was readily attached to agarose 4B with a general cyanogen bromide coupling procedure (March et al., 1974). The immobilized antibody was stable to extended refrigerated storage and to a variety of experimental conditions. The functional

stability of agarose-AEB complex allows repeated assays with a single column with the procedure given in Table 5. One column has been used for assay of over 100 samples without measurable loss of its antibody activity.

Most antibody preparations which are used for RIA show marked heterogeneity of binding affinity for the antigen (Parker, 1976). During the initial experiments to bind and dissociate SEB from antibody columns, it was not possible to dissociate low levels of SEB from the column. It was found that before a new batch of antibody gel could be used for the column it had to be saturated by overnight treatment with an excess of SEB. Twenty micrograms of SEB were sufficient for saturation. Either purified SEB or a crude SEB preparation concentrated from a culture filtrate was suitable for this purpose. The column was then eluted with dissociating agent to free those antibody binding sites with sufficiently low affinity that antigen could be dissociated in the assay. After this procedure, which had the effect of permanently saturating the high affinity binding sites, low levels of SEB could be attached and removed from the column repeatedly.

The dissociation of antigen was improved with higher NH_4OH concentrations. This had the effect of increasing the capacity of the antibody column. However, 1 M and 2 M NH_4OH resulted in significant loss of binding

capacity upon repeated use (Figure 7). Clumping of the gel was observed with repeated use of these solutions. Microscopic examination showed fragmentation of the gel beads. The column was stable for over 40 cycles with respect to SEB binding capacity and physical structure when the NH_4OH concentration was reduced to 0.13 M. The fraction of the antibody binding sites which dissociated with this lower level of NH_4OH was reduced, but it was still sufficient for the range of SEB concentrations for which the assay was intended. Therefore, 0.13 M NH_4OH, pH 10.5, containing 0.15 M NaCl was chosen as the dissociating agent.

The buffer for equilibration and removal of unbound impurities from the antibody column (steps 2, 4, and 6 in Table 5) had to be chosen to provide conditions which allow specific binding of SEB, prevents nonspecific binding and minimizes tailing during elution of unbound labelled SEB from the column (step 4). A pH 7.5 phosphate buffer was chosen since this was known to provide suitable conditions for antigen-antibody reactions in the slide diffusion technique (Casman & Bennett, 1965) and in solid phase radioimmunoassays for SEB (Johnson et al., 1971). Sodium chloride was added to the phosphate buffer to reduce tailing during elution of unbound SEB. The results in Figure 3 show a reduction in tailing as the NaCl concentration was increased. However, previous work

with affinity chromatography (Robinson et al., 1972) indicated that improved binding of antigen should occur at lower NaCl concentrations. Therefore, 0.15 M NaCl was selected for subsequent experiments because it resulted in a significant reduction in tailing. In addition, the nonspecific binding of labelled SEB at this salt concentration was only 2.3%.

A salt concentration of 0.15-0.20 M was found by Casman and Bennett (1965) to be effective for extraction of staphylococcal enterotoxins from food samples. For radioimmunoassay, samples for analysis should have the same pH and ionic strength as that of the equilibrated column for maximum sensitivity and accuracy. Therefore, an assay system with 0.15 M NaCl should minimize the work required to prepare a sample for analysis.

The nonspecific binding of labelled SEB to the gel and column was reduced by treatment of the column with 0.5 ml of a 2% BSA solution prior to application of a sample.

The flow rate for steps 1, 3, 5, and 7 (Table 5) when PBS was pumped through the column was 150 ml/hr. It was found with a 1:5 to 1:30 antibody gel that 150 ml/hr was the maximum flow rate which could be maintained. Higher rates resulted in compaction of the gel and a gradual decline in flow rate. A 75 ml/hr flow rate for the dissociation agent was chosen to allow a longer

contact time between dissociating agent and the SEB-antibody complexes. Further experiments are required to determine whether this flow rate might be increased without changing the elution characteristics.

Once the assay procedure described in Table 5 had been developed, it was necessary to evaluate the specificity and accuracy of the assay. Dose-response curves were obtained for the commercial SEB added to PBS, reconstituted nonfat dry milk and hamburger. The data obtained from the dose-response study were plotted in two ways. Figures 9, 12, and 14 show the counts per minute measured in the NH_4OH-eluate as a function of enterotoxin concentration in the samples. The milk and buffer curves (Figures 9 and 12) were straight lines over the range of concentration measured. The hamburger samples (Figure 14) did not show a significant decline until the SEB concentration was 6 ng/g of hamburger. However, above 6 ng/g, a linear relationship was observed with a slope greater than that found for PBS or milk samples. Figure 17 shows that standard curves prepared in reconstituted nonfat dry milk and hamburger are always lower than that prepared in PBS. The lower count of labelled SEB bound with milk indicates some interference by impurities in the sample. In hamburger samples, the lack of response at low SEB levels suggests the possibility that a factor(s) may be present in the sample

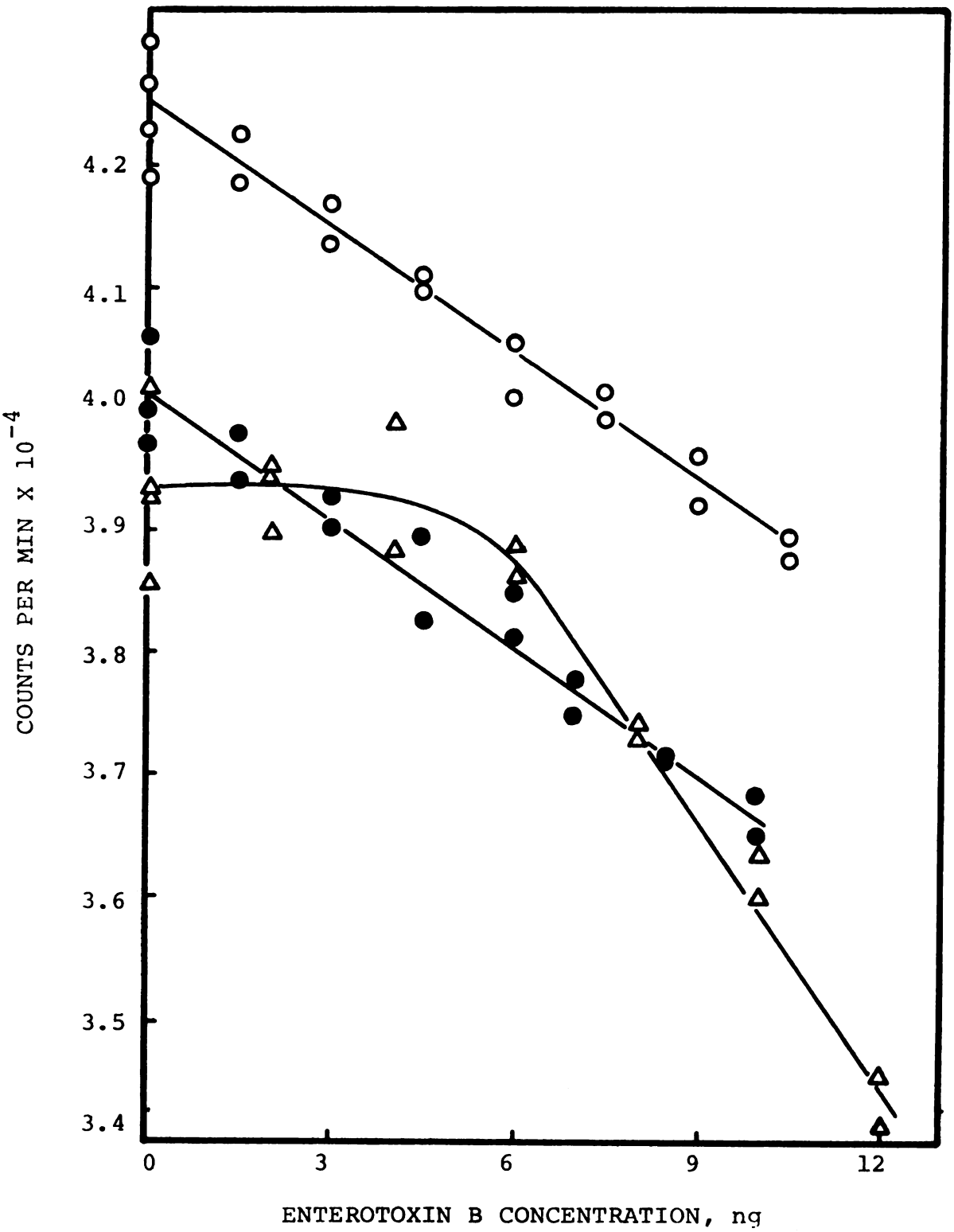

Fig. 17. Comparison of standard curves of SEB in PBS, reconstituted nonfat dry milk and hamburger, showing the difference when the plots are based on counts per minute. Symbols: O—O, PBS; ●—●, reconstituted nonfat dry milk; Δ—Δ, hamburger.

which binds SEB so it cannot be extracted. One of the possible factors that might bind SEB at low levels would be antibody since Bergdoll (1972) found that enterotoxin antibodies were usually present in animals in a minute amount. After all these substances were neutralized with the added SEB, the excess SEB began to appear in the extract.

The data were also calculated in terms of the percentage of the average counts obtained in the specific products with no enterotoxin. These results are shown in Figures 10, 13, and 15. This method for plotting the results was found to be preferable when samples are done over a period of several days or weeks because at a given SEB concentration the counts per minute measured declined as the ^{125}I decayed. However, the activity calculated as percentage of a control sample run on the same day did not change. Therefore, a single standard curve could be utilized for a period of several weeks if necessary. In addition, it can be seen in Figure 18 that the dose response curve for the SEB in PBS and milk are nearly coincident when expressed on a percentage basis. Therefore, it may be possible to use a single standard dose-response curve for several food products provided that it is first established that the curves are linear over the range of concentrations which are measured.

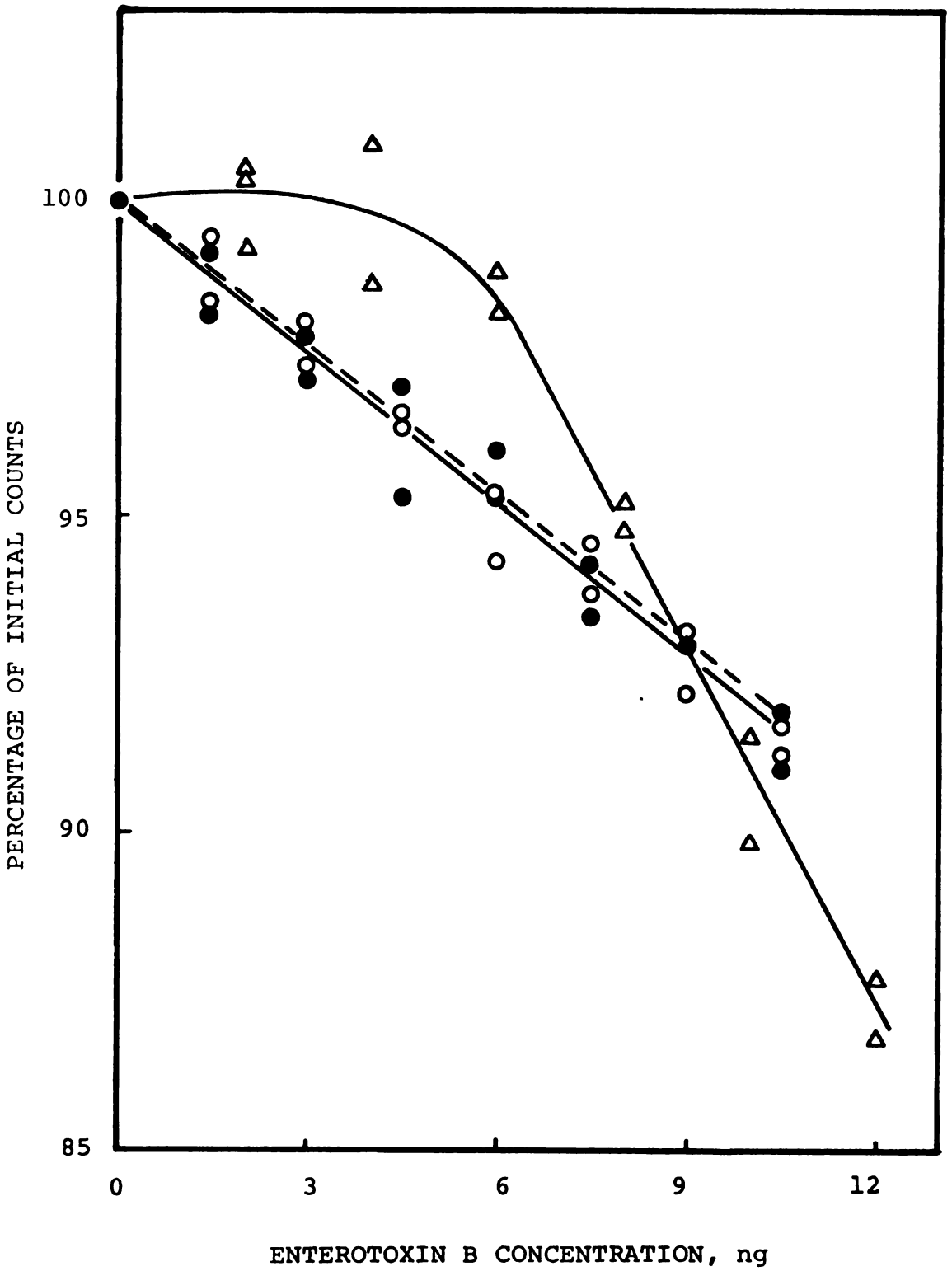

Fig. 18. Comparison of standard curves of SEB in PBS, reconstituted nonfat dry milk and hamburger, showing the difference when the plots are based on percentage of initial counts. Symbols: O—O, PBS; ●—●, reconstituted nonfat dry milk; Δ—Δ, hamburger.

The minimum detectable concentration for SEB was calculated according to the method of Feldman and Rodbard (1971). The calculated levels were 1.2 ng/ml for buffer, 2.2 ng/ml for reconstituted nonfat dry milk, and 6.3 ng/g for hamburger. These levels are within the range for practical SEB detection in food suggested by Bergdoll et al. (1976).

Table 9 shows data on several assay techniques which have been used for detection of staphylococcal enterotoxins. The minimum detection levels of the assay system developed are lower than those found by Collins et al. (1973) for RIA of enterotoxin A in ham, milk products, crab meat, and custard. The levels were comparable to the minimum detectable levels of both enterotoxin A and B reported by Johnson et al. (1973) in a variety of foods. When the time required for preparation of a sample is taken into consideration, affinity and solid-phase radioimmunoassays require the least preparation time. They did not require complicated and lengthy purification steps like other methods. Food was extracted with PBS at pH 7.5 and centrifuged to remove food particles. The supernatant obtained was used for SEB determination directly without any further treatment. Table 9 shows that both affinity and solid-phase radioimmunoassays require approximately one hour for preparation of a sample while other methods require 2 to 48

Table 9

Minimal Detectable Level and Approximate Time for Extraction and Assay of SEB by Various Quantitative Method

Method	Minimal Detectable Level of SEB, ng/ml	Time Required, Hour	
		Extraction	Assay
Affinity radioimmunoassay	1.2	1	1 1/4
Solid-phase radioimmunoassay:			
Collins et al. (1973),	10[a]	1	22
Johnson et al. (1973)	1	1	22
Reversed passive hemagglutination assay (RPHA) (Silverman et al., 1968)	1.5	2	12-24
Double gel-diffusion tube test (Hall et al., 1965)	50	48	170
Microslide technique:			
Casman and Bennett (1965),	100	72	48-72
Reiser et al. (1974)	100	48	18
Laurell electroimmunodiffusion (Gasper et al., 1973)	150	?[b]	2-5
Oudin single gel-diffusion tube test (Weirether et al., 1966)	1000	2	20-24

[a]For enterotoxin A.

[b]Under investigation.

hours. Although RPHA method does not require concentration step, it does require a step for precipitation of protein in a sample. A great number of false positive assays may be obtained due to nonspecific agglutination of the sensitized cells if protein precipitation is not done.

Affinity and solid-phase radioimmunoassays appear to offer higher sensitivity and shorter sample preparation times than other techniques. When these two methods are compared, affinity radioimmunoassay requires a shorter time for assay of a sample. This is because the affinity radioimmunoassay does not require the reaction to go to equilibrium. The time required for the reaction of labelled SEB with its antibodies is just long enough to get a constant count under the assay conditions.

Good reproducibility of the SEB affinity radioimmunoassay was found both with samples run consecutively, and with samples measured over a two-week period when other samples were run in between the samples used to check the reproducibility. For the 10 determinations shown in Table 8, a mean of 100.4% of the added SEB concentration was measured. The standard deviation was 5.7%. Once a standard curve was obtained, it could be used for at least several weeks even when the batch of ^{125}I-SEB was changed. Johnson et al. (1973) checked the recovery of both enterotoxin A and B from condensed

milk. They used six samples for each enterotoxin, in which 1 to 75 ng of enterotoxins were added to 1 ml of milk. The average percentage recovery was 87.2 with SEB and 90.7 with enterotoxin A. Standard deviations of the recovery were 29.8 and 20.5% for enterotoxin B and A, respectively.

The specificity of the column was evaluated by using purified enterotoxin A and B obtained from the Food and Drug Administration and milk samples in which the A and B enterotoxins were produced by Staphylococcus aureus strains 196E and 243, respectively. No SEB was detectable with either of enterotoxin A samples even though the purified enterotoxin was used at nearly 500 times the minimum detectable level of SEB. According to data provided with the FDA purified SEB, a concentration of 8 ng/ml was used as a sample. Based upon the standard curve developed with commercial SEB, a concentration of 8.1 ng/ml was measured. SEB was also detected at a level of 7,300 ng/ml in the milk sample. These data indicated no cross reactivity of the anti-SEB column with enterotoxin A and ability to accurately measure SEB from different sources. Similar result concerning about cross reactivity for enterotoxin A and B has been reported by Bergdoll and Robbins (1973).

A D-value of 10.4 minutes was measured for thermal denaturation of SEB in pH 5.2 milk at 121 C.

The fact that this is similar to the D-value of 9.4 minutes in pH 6.4 to 6.6 milk using slide immunodiffusion (Reed & Bradshaw, 1966) is a further indication that the affinity radioimmunoassay technique gives results which are comparable to those obtained using other assay methods.

Further improvement of the affinity radioimmunoassay procedure should be possible. One of the important areas of improvement would be to use only those antibodies with an affinity for SEB in the optimum range for this method. If the highest affinity antibodies were removed, it would not be necessary to presaturate new columns with SEB. Removal of the lowest affinity antibodies might make the point of column saturation more distinct. In addition, it might reduce the time required for sample incubation so that consistent binding of SEB to the optimum affinity sites can be obtained.

To obtain only antibodies with SEB affinities in the working range for the assay, it may be possible to reverse the procedure used in the assay. A preparative column with immobilized SEB could be prepared. Antibody to SEB would be put through the column at the same pH and ionic strength that is used for SEB binding in the assay. After washing to remove low affinity antibodies and impurities, the antibody could be eluted with 0.13 M NH_4OH containing 0.15 M NaCl. This would remove the

proper antibody fraction, but leave the very high affinity antibodies attached to the column.

CONCLUSIONS

An affinity radioimmunoassay has been developed for staphylococcal enterotoxin B. The assay is sensitive, reproducible, and accurate for SEB measurements in phosphate buffer, reconstituted nonfat dry milk, and hamburger. A column of antibody gel has been used for over 100 assays without detectable loss of antibody activity. The assay is relatively simple to run and it is rapid, requiring about 1 1/4 hr per sample, compared to the immunodiffusion method (Casman & Bennett, 1965), or a solid-phase RIA procedure with comparable sensitivity which required an 18-hr incubation period (Johnson et al., 1973).

The procedure developed is not well adapted to large numbers of manual assays because each column would require at least 2 channels of an accurate peristalic pump. However, it may be useful for small numbers of manual assays, since it appears that a calibrated column could be prepared and used as required. The major potential of the procedure would appear to be the development of an integrated autoanalyzer system in which several columns could be processed simultaneously.

The assay does not depend upon any properties peculiar to enterotoxin B. It should be possible to develop similar assays for other toxins, hormones, or other compounds for which suitable antibodies and stable binding proteins are required.

BIBLIOGRAPHY

BIBLIOGRAPHY

Angellotti, R., M. J. Foter, and K. H. Lewis. 1961. Time-temperature effects on salmonella and staphylococci in foods. Am. J. Pub. Hlth. 51: 76-88.

Avena, R. M., and M. S. Bergdoll. 1967. Purification and some physiochemical properties of enterotoxin C. Staphylococcus aureus strain 361. Biochem. 6: 1474-1480.

Axen, R., and J. Porath. 1966. Chemical coupling of enzymes to cross-linked dextran (Sephadex). Nature 210: 367-369.

Bennett, R. W., B. A. Keosyan, S. R. Tatini, H. Thota, and W. S. Collins. 1973. Staphylococcal enterotoxin: A comparison study of serological detection methods. Canadian Institute of Food Science and Technology J. 6: 131-134.

Bergdoll, M. S. 1966. Immunization of rhesus monkey with enterotoxin B. J. Infect. Disease 116: 191-196.

Bergdoll, M. S. 1970. Enterotoxins. In Microbial Toxins Vol. III, edited by T. C. Montie, S. Kadis, and S. J. Ajil. Academic Press Inc., New York, N.Y. pp. 265-296.

Bergdoll, M. S. 1972. Enterotoxin. In The Staphylococci, edited by J. O. Cohen. Wiley-Interscience, New York, pp. 301-331.

Bergdoll, N. S., C. R. Borja, and R. M. Avena. 1965. Identification of a new enterotoxin as enterotoxin C. J. Bacteriol. 90: 1481-1489.

Bergdoll, M. S., C. R. Borja, R. Robbins, and K. F. Weiss. 1971. Identification of enterotoxin E. Infection and Immunity. 4: 593-595.

Bergdoll, M. S., R. Reiser, and J. Spitz. 1976. Staphylococcal enterotoxin detection in food. Food Technol. 30(5): 80-84.

Bergdoll, M. S., and R. N. Robbins. 1973. Characterization of type of staphylococcal enterotoxins. J. Milk Food Technol. 36: 610-612.

Bergdoll, M. S., H. Sugiyama, and G. M. Dack. 1959a. Staphylococcal enterotoxin. I. Purification. Arch. Biochem. Biophys. 85: 62-69.

Bergdoll, M. S., M. J. Surgalla, and G. M. Dack. 1959b. Staphylococcal enterotoxin. Identification of a specific precipitating antibody with enterotoxin neutralizing property. J. Immunol. 83: 334-338.

Berson, S. A., R. S. Yalow. 1973. Radioimmunoassay. In Peptide hormones, edited by S. A. Berson and R. S. Yalow. American Elsevier Publishing Company, Inc., New York, N.Y., pp. 84-120.

Berson, S. A., R. S. Yalow, A. Baumann, M. A. Rothschild, and K. Newerly. 1956. Insulin-^{131}I metabolism in human subject: Demonstration of insulin binding globulin in the circulation of insulin treated subjects. J. Clin. Invest. 35: 170-190.

Borja, C. R., and M. S. Bergdoll. 1967. Purification and partial characterization of enterotoxin C produced by Staphylococcus aureus strain 137. Biochem. 6: 1467-1473.

Bukovic, J. A., and H. M. Johnson. 1975. Staphylococcal enterotoxin C: Solid-phase radioimmunoassay. Appl. Microbiol. 30: 700-701.

Casman, E. P. 1960. Further serological studies of staphylococcal enterotoxin. J. Bacteriol. 79: 849-856.

Casman, E. P. and R. W. Bennett. 1963. Culture medium for production of staphylococcal enterotoxin A. J. Bacteriol. 86: 18-23.

Casman, E. P. and R. W. Bennett. 1964. Production of Antiserum for staphylococcal enterotoxin. Appl. Microbiol. 12: 363-367.

Casman, E. P. and R. W. Bennett. 1965. Detection of staphylococcal enterotoxin in food. Appl. Microbiol. 13: 181-189.

Casman, E. P., R. W. Bennett, A. E. Dorsey, and J. A. Issa. 1967. Identification of a fourth staphylococcal enterotoxin, enterotixin D. J. Bacteriol. 94: 1875-1882.

Catt, K. J., H. D. Niall, and G. W. Tregear. 1966. Solid-phase radioimmunoassay of human hormone. Biochem. J. 100, 31c.

Catt, K. J., H. D. Niall, C. W. Tregear, and H. G. Burger. 1968. Disc solid-phase radioimmunoassay of human lutienizing hormone. J. Clin. Endoclinol. Metab. 28: 121-126.

Catt, K. J., and G. W. Tregear. 1967. Solid-phase radioimmunoassay in antibody-coated tubes. Science. 158: 1570-1571.

Chu, F. S., K. Thadhani, E. J. Schantz, and M. S. Bergdoll. 1966. Purification and characterization of staphylococcal enterotoxin A. Biochem. 5: 3281-3289.

Collins, W. S., A. D. Johnson, J. F. Metzger, and R. W. Burnett. 1973. Rapid solid-phase radioimmunoassay for staphylococcal enterotoxin A. Appl. Microbiol. 25: 774-777.

Collins, W. S., J. F. Metzger, and A. D. Johnson. 1972. A rapid solid-phase radioimmunoassay for staphylococcal B enterotoxin. J. Immunol. 108: 852-856.

Cuatrecasas, P. 1970. Protein purification by affinity chromatography, derivation of agarose and polyacrylamide gel. J. Biol. Chem. 245: 3059-3065.

Cuatrecasas, P., and C. B. Anfinsen. 1971. Affinity chromatography. In Methods in enzymology, Vol. XXII edited by W. B. Jakoby. Academic Press, Inc., New York, pp. 345-378.

Dalidowicz, J. E., S. J. Silverman, E. T. Stefanye, and L. Spero. 1966. Chemical and biological properties and alkylated staphylococcal enterotoxin B. Biochemistry 5: 2375-2381.

Davison, E., and G. M. Dack. 1939. Some chemical and physical studies of staphylococcal enterotoxin. J. Infect. Dis. 64: 302-306.

Denny, C. B., P. L. Tan, and C. W. Bohrer. 1966. Heat inactivation of staphylococcal enterotoxin A. J. Food Sci. 31: 762-767.

Donnelly, C. B., L. A. Black, and K. H. Lewis. 1964. Occurrence of coagulase positive staphylococci in cheddar cheese. Appl. Microbiol. 12: 311-315.

Feldman, H., and D. Rodbard. 1971. Mathematical theory of radioimmunoassay. In Principles of comparative protein binding assays, edited by W. D. Odell and W. H. Daughaday. J. B. Lippincott Co., Philadelphia, pp. 158-173.

Freeman, T. 1967. Trace labelling with radioiodine. In Handbook of experimental immunology, edited by D. M. Weir. F. D. Davis Company, Philadelphia, pp. 579-607.

Freeman, T., C. M. E. Mathews, A. S. McFarlane, H. Benhold, and E. Kallee. 1959. Albumin labelled with ^{131}iodine in an analbuminaemic subject. Nature 183, 606.

Galskov, G. 1972. Radioimmunochemical corticotropin determination. Acta Endocrinol 69 Suppl. 162, 5.

Gasper, E., R. C. Heimsch, and A. W. Anderson. 1973. Quantitative determination of type A staphylococcal enterotoxin by Laurell electroimmunodiffusion. Appl. Microbiol. 25: 421-426.

Genigeorgis, C., and W. W. Sadler. 1966. Immunofluorescent detection of staphylococcal enterotoxin B. II Detection in foods. J. Food Sci. 31: 441-449.

Genuth, S., L. A. Frohman, and H. E. Leboritz. 1965. A radioimmunological assay method for insulin using insulin-^{125}I and gel filtration. J. Clin. Endoclinol. Metab. 25: 1043-1049.

Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of ^{125}I-labelled human growth hormone of high specific radioactivity. Biochem J. 89: 114-123.

Grodsky, G. M., and P. H. Forham. 1960. An immunochemical assay of total extractable insulin in man. J. Clin. Invest. 39: 1070-1079.

Gruber, J., and G. G. Wright. 1967. Iodine-131 Labeling of purified microbial and antigens by micro-diffusion. Proc. Soc. Exp. Biol. Med. 126: 282-284.

Hall, H. E. 1968. Enterotoxin and enzyme production by a selected group of Staphylococcus aureus cultures. Bact. Proc., pp. 77-78.

Hall, H. E., R. Angelotti, and K. H. Lewis. 1965. Detection of staphylococcal enterotoxin in food. Health Lab. Sci. 2: 179-191.

Hallander, H. O. 1965. Production of large quantities of enterotoxin B and other staphylococcal toxins on solid media. Acta Pathol. Microbiol. Scand. 63: 299-305.

Hammon, W. McD. 1941. Staphylococcal enterotoxin: an improved cat test, chemical and immunological studies. Am. J. Public Health. 31: 1191-1198.

Herbert, V., K. S. Lau, C. W. Gottlieb, and S. J. Bleicher. 1965. Coated charcoal immunoassay of insulin. J. Clin. Endocrinol. Metab. 25: 1375-1384.

Hilker, J. S., W. R. Heilman, P. L. Tan, C. B. Denny, and C. W. Bohrer. 1968. Heat inactivation of enterotoxin A from Staphylococcus aureus in veronal buffer. Appl. Microbiol. 16: 308-310.

Huang, I-Y., and M. S. Bergdoll. 1970a. The primary structure of staphylococcal enterotoxin B. II. Isolation, composition, and sequence of chrymotryptic peptides. J. Biol. Chem. 245: 3511-3517.

Huang, I-Y., and M. S. Bergdoll. 1970b. The primary structure of staphylococcal enterotoxin B. III. The cyanogen bromide peptides of reduced and aminoethylated enterotoxin B, and the complete amino acid sequence. J. Biol. Chem. 245: 3518-3525.

Hunter, W. M., and F. C. Greenwood. 1962. Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature 194: 925-926.

Hunter, W. M., and F. C. Greenwood. 1964. A radio-immunoelectrophoretic assay for human growth hormone. Biochem J. 91: 43-56.

Isojima, S., O. Naka, K. Koyama, and H. Adachi. 1970. Rapid radioimmunoassay of human luteinizing hormone using polymerized anti-human chorionic gonadotrophin as immunoadsorbent. J. Clin. Endocrinol. Metab. 31: 692-699.

Jay, J. M. 1970. Modern food microbiology. D. Van Nostrand Company, New York, pp. 194-207.

Johnson, H. M., J. A. Bukovic, and P. E. Kauffmann. 1973. Staphylococcal enterotoxin A and B: Solid-phase radioimmunoassay in food. Appl. Microbiol. 26: 309-313.

Johnson, H. M., J. A. Bukovic, P. E. Kauffman, and J. T. Peeler. 1971. Staphylococcal enterotoxin B: Solid-phase radioimmunoassay. Appl. Microbiol. 22: 837-841.

Kabat, E. A. 1976. Structural concepts in immunology and immunochemistry. Holt, Rinehart and Winston. New York, pp. 204, 223.

Kauffman, P. E., and H. M. Johnson. 1975. Stability of ^{125}I-labelled staphylococcal enterotoxins in solid-phase radioimmunoassay. Appl. Microbiol. 29: 776-779.

Lau, K. S., C. W. Gottlieb, and V. Herbert. 1966. Preliminary report on coated charcoal immunoassay of human chorionic "growth hormone prolactin" and growth hormone. Proc. Soc. Rxp. Biol. Med. 123: 126-131.

Lilly, H. D., R. A. McLean, and J. A. Alford. 1967. Effect of curing salt and temperature on productions of staphylococcal enterotoxin. Bact. Proc., p. 12.

March, S. C., I. Parikh, and P. Cuatrecasas. 1974. A simplified method for cyanogen bromide activation of agarose for affinity chromatography. Anal. Biochem. 60: 149-152.

Marchalonis, J. J. 1969. An enzymatic method for the trace iodination of immunoglobulins and other proteins. Biochem. J. 113: 299-305.

Markus, Z., and G. J. Silverman. 1968. Enterotoxin B production by nongrowing cells of Staphylococcus aureus. J. Bacteriol. 96: 1446-1447.

Markus, Z., and G. J. Silverman. 1969. Enterotoxin B synthesis by replicating and non-replicating cells of Staphylococcus aureus. J. Bacteriol. 91: 506-512.

Milone, N. A. 1962. Study on the use of tissue culture for bioassay of staphylococcal enterotoxin. J. Food Sci. 27: 501-507.

McFarlane, A. S. 1958. Efficient trace-labelling of protein with iodine. Nature 182, 53.

Morse, S. A., and R. A. Mah. 1967. Microtiter hemagglutination-inhibition assay for staphylococcal enterotoxin B. Appl. Microbiol. 15: 58-61.

Morse, S. A., R. A. Mah, and W. J. Dobrogosz. 1969. Regulation of staphylococcal enterotoxin B. J. Bacteriol. 98: 4-9.

Munch-Peterson, E. 1962. Staphylococci in food and food intoxication. J. Food Sci. 28: 692-710.

Nace, G. W., and P. Spradlin. 1962. Bleeding rabbits. Turtex News 40: 26-29.

Nickerson, J. T., and A. J. Sinskey. 1974. Microbiology of foods and food processing. American Elsevier Publishing Co., New York, p. 247.

Odell, W. D., J. F. Wilber, and W. E. Paul. 1965. Radioimmunoassay of thyrotropic in human serum. J. Clin. Endocrinol. Metab. 25: 1179-1188.

Odell, W. D., G. A. Araham, W. R. Skowsky, M. A. Hescox, and D. A. Fisher. 1971. Production of antisera for radioimmunoassays. In Principles of competitive protein-binding assays, edited by W. D. Odell, W. H. Daughaday. J. B. Lippincott Co., Philadelphia, pp. 57-76.

Parker, C. P. 1976. Radioimmunoassay of biologically active compounds. Prentice-Hall Inc., New Jersey, p. 43.

Porath, J. R. Axen, and S. Emback. 1967. Chemical coupling of protein to agarose. Nature 215: 1491-1492.

Read, R. B., and J. G. Bradshaw. 1966. Staphylococcal enterotoxin B thermal inactivation in milk. J. Dairy Sci. 49: 202-203.

Reiser, R., D. Conaway, and M. S. Bergdoll. 1974. Detection of staphylococcal enterotoxin in foods. Appl. Microbiol. 27: 83-85.

Robern, H., M. Dighton, Y. Yano, and N. Dickie. 1975. Double-antibody radioimmunoassay for staphylococcal enterotoxin C_2. Appl. Microbiol. 30: 525-529.

Robinson, P. J., P. Dunnill, and M. D. Lilly. 1972. Factors affecting scale-up of affinity chromatography of β-galactosidase. Biochim. Biophys. Acta. 285: 28-35.

Satterlee, L. D., and A. A. Kraft. 1969. Effect of meat and isolated meat proteins on the thermal inactivation of staphylococcal enterotoxin B. Appl. Microbiol. 17: 906-909.

Schaeffer, W. I., J. Gabliks, and R. Calitis. 1966. Interaction of staphylococcal enterotoxin B with cultures of human embryonic intestine. J. Bacteriol. 91: 21-26.

Schantz, E. J., W. G. Roessler, J. Wagman, L. Spero, D. A. Dunnery, and M. S. Bergdoll. 1965. Purification of staphylococcal enterotoxin B. Biochem. 4: 1011-1016.

Silverman, S. J., A. R. Knott, and B. Howard. 1968. Rapid, sensitive assay for staphylococcal enterotoxin and a comparison of serological methods. Appl. Microbiol. 16: 1019-1023.

Silverman, G. J., J. T. R. Nickerson, D. W. Duncan, N. S. Davis, J. S. Schacter, and M. M. Joselow. 1961. Microbial analysis of frozen raw and cooked shrimp. I. General result. Food Technol. 15: 455-458.

Skelley, D. S., L. P. Brown, and K. P. Besch. 1973. Review radioimmunoassay. J. Clin. Chem. 19: 146-186.

Skom, J. H., and D. W. Talmage. 1958. Nonprecipitating insulin antibodies. J. Clin. Invest. 37: 783.

Spero, L., H. M. Jacoby, J. E. Dalidowicz, and S. J. Silverman. 1971. Gaunidination and nitroguanidination of staphylococcal enterotoxin B. Biochem. Biophys. Acta. 251: 345-354.

Splittstoesser, D. F., G. E. R. Hervey, and W. P. Wettergren. 1965. Contamination of frozen vegetables by coagulase-positive staphylococci. J. Milk and Food Technol. 28: 149-151.

Surgalla, M. J., M. S. Bergdoll, and G. M. Dack. 1952. Use of antigen-antibody reactions in agar to follow the progress of fractionation of antigenic mixture: application to purification of staphylococcal enterotoxin. J. Immunol. 69: 357-365.

Surgalla, M. J., M. S. Bergdoll, and G. M. Dack. 1953. Some observations on the assay of staphylococcal enterotoxin by the monkey-feeding test. J. Lab Clin. Med. 41: 782-788.

Troller, J. A., and W. C. Frazier. 1963. Repression of Staphylococcus aureus by food bacteria. II. Causes of inhibition. Appl. Microbiol. 11: 163-165.

Thorell, J. I., and B. G. Johanson. 1971. Enzymatic iodination of polypeptides with ^{125}I to high specific activity. Biochim. Biophys. Acta. 251: 363-369.

Walker, G. C., L. G. Harmon, and C. M. Stine. 1961. Staphylococci in colby cheese. J. Dairy Sci. 44: 1272-1282.

Weirether, F. J., E. E. Lewis, A. J. Rosenwald, and R. E. Lincoln. 1966. Rapid quantitative serological assay of staphylococcal enterotoxin B. Appl. Microbiol. 14: 284-291.

Yalow, R. S., and S. A. Berson. 1959. Assay of plasma insulin in human subjects by immunological methods. Nature 148: 1648-1649.

Yalow, R. S., and S. A. Berson. 1961. Immunological specificity of human insulin: Application to immunoassay of insulin. J. Clin. Invest. 40: 2190-2198.

Zehren, V. L., and V. F. Zehren. 1968. Examination of large quantities of cheese for staphylococcal enterotoxin A. J. Dairy Sci. 51: 635-644.