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Thermal inactivation of staphylococcal enterotoxin B with changing pH, protein concentration, and ionic strength

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Iowa State University, Ph.D., 1970
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THERMAL INACTIVATION OF STAPHYLOCOCCAL ENTEROTOXIN B WITH
CHANGING pH, PROTEIN CONCENTRATION, AND IONIC STRENGTH

by

Sister Evamonica Mercedita Jamlang, S.Sp.S.

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1970

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INTRODUCTION

The heat stability of the staphylococcal enterotoxins has been a matter of interest to researchers since 1930 when Dack et al. reported that boiling for 30 minutes did not destroy its activity. The fact became even more puzzling when it was learned that the enterotoxins are proteins. Several papers have been published on the heat inactivation of the enterotoxins but the information available could not be correlated because of the differences in the purity, the pH and the initial concentration of the samples. Therefore, this study was undertaken to determine the effects of these conditions on the heat inactivation of a highly purified enterotoxin preparation. After the behavior of the purified enterotoxin has been determined, its behavior when in combination with other proteins or food constituents could be studied and a difference in reaction could be attributed to the presence of these other substances. Satterlee and Kraft (1969) noticed a more rapid loss of the antigen-antibody reaction when partially purified enterotoxin was heated at 80°C than at 100°C. Heating experiments using the purified enterotoxin will reveal whether this phenomenon is characteristic of the enterotoxin or whether it is an effect of a reaction with other substances.

Enterotoxin B was reported to be the most heat resistant of the enterotoxins but enterotoxin A has been the enterotoxin incriminated in almost all the staphylococcal food poisoning incidents that have been investigated. The reasons for the prevalence of enterotoxin A have not been clarified as yet since the enterotoxins are equally toxic when tested

on animals and since conditions for their production do not seem to differ very much. Although this study was undertaken with the purpose of applying the information that will be obtained to foods, enterotoxin B was used because of its greater availability and its greater heat resistance. Since enterotoxin B is the most heat resistant of the enterotoxins, information that could be obtained about its heat resistance could be applied to the other enterotoxins as well.

REVIEW OF LITERATURE

The first significant step toward establishing the presence of enterotoxic substances in staphylococcus cultures was made by M. A. Barber (1914) when he started an investigation after becoming an accidental victim of what seemed to be gastroenteritis. The illness came after he drank cow's milk contaminated with white staphylococcus from the cow's udder. Barber reported that the toxin was produced only after the milk had been allowed to stand at room temperature for some hours. He tested the toxicity of his isolate by drinking cultures of the white staphylococcus and was able to reproduce the symptoms similar to gastroenteritis within a few hours.

In 1930, Dack et al. showed that the toxin was found in sterilized filtrates of the organism. In their case, it was a hemolytic strain of Staphylococcus aureus grown in veal infusion broth and sterilized by the use of an N-Berkefeld filter. This finding established the fact that the enterotoxin was an exotoxin. However, there remained some tendency to confuse staphylococcal food poisoning with a gastrointestinal infection also caused by staphylococcus. The confusion lasted as late as 1948 (Elek, 1959).

Enterotoxigenic Strains of Staphylococcus

Having established the enterotoxic property of staphylococcal filtrates, investigators turned to the questions of which strains were enterotoxigenic and how they could be differentiated from nonenterotoxigenic strains. The first attempts to differentiate strains made use of

cultural characteristics.

Fermentation of different carbohydrates did not prove to be very useful (Stritar and Jordan, 1935; Shaughnessy and Grubb, 1936; Kupchik, 1937). Stone (1935) proposed a specially prepared gelatin medium consisting of 15% gelatin and 3% Difco beef extract which was supposed to be liquefied by enterotoxigenic strains only. Chinn, in 1936, claimed that Stone's medium was not able to separate food poisoning strains from those isolated from infections. All the strains he examined were able to liquefy Stone's medium as long as they were first subcultured on starch agar and incubated for about 35 days. Kupchik's findings (1937) based on animal experiments supported the observation that Stone's medium had no significant differential value.

Chapman et al. (1937) presented a number of cultural characteristics which they found useful in recognizing enterotoxigenic strains of staphylococcus. Included therein were hemolysis of rabbit blood, coagulation of human and rabbit plasma, yellow or orange pigment, orange or violet growths on crystal violet agar, growth on bromthymol blue agar and fermentation of mannitol. On the other hand, Kupchik (1937) stated that chromogenesis of enterotoxigenic strains can vary through all the shades and that nitrate reduction, hemolysin production and liquefaction of gelatin were characteristics common to all staphylococci. Hussemann and Tanner (1949) also failed to find agreement between Stone's reaction, the series of tests proposed by Chapman et al. (1937), the 7.5% NaCl phenol red mannitol agar test and animal tests. Coagulase formation, fermentation of mannitol and pigment formation were of least differential value.

The coagulase test, which has become a classical method for differentiating pathogenic from non-pathogenic staphylococci and which was proposed by Chapman *et al.* (1937) as a means for detecting food poisoning strains, was further examined by Evans and Niven (1950) using 114 strains of staphylococcus. They reported that all of the enterotoxigenic cultures tested (22 strains) were coagulase-positive but that some coagulase-positive strains (8 strains) were not enterotoxigenic. Of the 24 strains of coagulase-negative staphylococcus isolated from foods incriminated in food poisoning incidents, none produced enterotoxin when tested by monkey feeding. More recently, Bergdoll *et al.* (1967b) isolated a coagulase-negative strain that produced vomiting in rhesus monkeys and three other coagulase-negative strains that produced enterotoxin but at very low levels. It has now become the common opinion that enterotoxigenic strains are generally coagulase-positive with few exceptions. No cultural method of differentiation has been accepted.

The commonly known enterotoxigenic strains of staphylococcus are shown in the following table.

Table 1. Common enterotoxigenic strains of staphylococcus

Official designation	Common designation	Origin
ATCC 13565	196E	isolated in 1940 by G.G. Slocum of the U.S. Food and Drug Administration from cooked ham involved in an outbreak in Vancouver, Washington.
None	100	isolated by E. O. Jordan of the University of Chicago in 1932 from a cake responsible for food poisoning.

Table 1. (Continued)

Official designation	Common designation	Origin
ATCC 14459	C-246-3	isolated in 1955 by E. P. Casman of the U.S. Food and Drug Administration from canned shrimp involved in a food poisoning incident.
ATCC 14458	243	isolated in Washington, D.C. in 1954 from a child suffering from "acute non specific diarrhea."
ATCC 13566	S-6	isolated by J. B. Evans of the American Meat Institute Foundation in 1947 from frozen shrimp.
ATCC 19095	137	isolated in 1933 from a leg abscess of a patient in the Albert Merritt Billings Hospital, University of Chicago.
F 4626/62	293 (Casman <u>et al.</u> , 1966) 361 (Bergdoll <u>et al.</u> , 1965a)	isolated by Betty Hobbs in England in 1962 from chicken responsible for a food poisoning outbreak among passengers on a plane.
ATCC 23235	494	isolated from a turkey salad involved in a food poisoning incident in 1964 in Washington, D.C.
None	315	isolated in Texas in 1963 from cooked turkey incriminated in a food poisoning outbreak.

Nature, Classification and Properties of the Enterotoxins

In 1933, Woolport and Dock differentiated the enterotoxin from the staphylococcal hemolysin, dermatotoxin and lethal toxin by the use of

antiserum and monkey feeding tests. They found that the enterotoxin was not neutralized by antiserum effective against the other toxins and that strains which did not produce any of the other toxins caused food poisoning symptoms in monkeys. This finding was further substantiated by Dolman's work (1934) who used human volunteers. He tested on twenty persons culture filtrates of strains that were shown to be highly hemolytic, and none of them showed symptoms of food poisoning. However, since the β -hemolysin was not yet well characterized at this time, speculations still remained concerning the relationship between this toxin and the enterotoxin (Woodward and Slanetz, 1941; Dolman, 1943). Using a strain of staphylococcus that produced only β -toxin and testing in human volunteers, Dolman (1943) disproved the possibility of the enterotoxin and β -toxin being one entity.

Early investigators were divided on whether the enterotoxin was a protein or a carbohydrate (Hammon, 1941; Bergdoll et al., 1952). Reports were also conflicting as to its antigenicity (Dack et al., 1931; Woolpert and Dack, 1933; Minnett, 1938; Dolman and Wilson, 1938, 1940; Hammon, 1941; Dolman, 1944). Some measure of increased tolerance to the enterotoxin had been demonstrated, and Dolman and Wilson (1938) reported a specific flocculation reaction between the enterotoxin and its homologous antibody but no conclusive evidence for passive immunity had been shown.

Neutralization of the enterotoxin by serum from kittens which had acquired increased tolerance was obtained by Dolman et al. (1936) and Davison et al. (1938). The mixture when injected intraperitoneally into kittens did not give a positive reaction. Neutralization was also

demonstrated by Dolman (1944) by intravenous injection of enterotoxin-serum mixtures into cats and by Surgalla et al. (1954) through monkey feeding tests. In 1958, Casman was able to give evidence that the enterotoxin can confer passive immunity. Serum from immunized rabbits protected cats when injected with this serum 3 to 5 minutes before injection of the challenging enterotoxin.

Serological studies made by Bergdoll et al. (1959b) and Casman (1958, 1960) for the purpose of devising an assay method for the enterotoxin revealed the presence of more than one enterotoxin. Using antiserum to the known food poisoning strains 196E, 243 and S-6 and a modification of Ouchterlony's agar diffusion method (1953), Casman found that 196E produced an antigen common to 13 (including S-6 but not 243) of 22 supposedly enterotoxigenic strains whereas strain 243 produced an antibody which reacts only with enterotoxin from 243 and S-6. The 196E type antigen was tentatively named enterotoxin F (food poisoning) and the 243 type antigen, enterotoxin E (enteritis) (Casman, 1960). These two antigens were reported to be heat stable, i.e., did not lose activity when heated at 100°C for 30 minutes. The remaining 8 of the 22 strains examined lost their "enterotoxicity" when heated under the above mentioned conditions. At the 1962 meeting of the American Society for Microbiologists, these designations were changed to enterotoxin A for the 196E type and enterotoxin B for the 243 type (Casman et al., 1963).

In 1965, Bergdoll et al. (1965a) identified a third enterotoxin which they named enterotoxin C. Enterotoxin C was produced by strains 137 and 361. Strain 137 (ATCC 19095) was designated the prototype strain. A fourth

enterotoxin has been recently identified (Casman et al., 1966, 1967).

Enterotoxin D was produced by three strains of staphylococcus; the prototype strain 494 (ATCC 23235), 293 and 315. These three strains did not produce enterotoxins A or B.

The question of whether the enterotoxins were proteins or carbohydrates was settled by Bergdoll et al. (1951, 1952). Partial purification of culture filtrates from strain S-6 produced a substance that had very little carbohydrate (less than 5%) and gave positive ninhydrin reactions. Further work by this group on the partially purified enterotoxin revealed that the enterotoxin was a water soluble protein of molecular weight between 15,000 and 25,000, had an isoelectric point near 8.5, contained a high percentage of lysine and was resistant to trypsin (Bergdoll, 1956).

In 1965, Schantz et al. were able to prepare a highly purified enterotoxin B from strain S-6. This purified preparation gave a single symmetrical peak in ultracentrifugation analysis. Electrophoresis in starch gel with 0.02 M borate buffer at pH 8.6 and in polyacrylamide at pH 4.5 showed two bands. The major band accounted for at least 95% of the toxin solution. A second precipitin band was also visible in its reaction with its anti-serum but only at high concentrations of the enterotoxin. The major precipitin band accounted for 99.5% of the enterotoxin solution. The isoelectric point was at pH 8.6.

Ultracentrifugal studies by Wagman et al. (1965) using sedimentation velocity and diffusion measurements by boundary spreading analysis gave a value of 35,300 for the molecular weight of enterotoxin B while Bergdoll et al. (1965b) using sedimentation velocity and diffusion measurements by

the agar gel method of Schantz and Lauffer (1962) obtained a value of 30,650. From the amino acid composition, Spero et al. (1965) calculated a molecular weight of 35,380.

Chu et al. (1966) succeeded in purifying enterotoxin A from strains 196E, C-246-3 and 100. They reported a molecular weight value of 34,500 from sedimentation velocity-diffusion data and 35,300 from Archibald's approach-to-equilibrium method. The isoelectric point was shown to be 6.8 by means of paper electrophoresis.

In the process of purifying enterotoxin C, Bergdoll's group at the Food Research Institute at Madison, Wisconsin, found that the enterotoxin C from strain 137 differed in electrophoretic mobility from the enterotoxin produced by strain 361. The strain 137 enterotoxin, designated C₁, had an isoelectric point of 8.6 (Borja and Bergdoll, 1967) whereas the strain 361 enterotoxin, designated C₂, had an isoelectric point of 7.0 (Avena and Bergdoll, 1967; Bergdoll et al., 1967a). Enterotoxin D has not yet been purified.

The enterotoxins seemed to be of equal toxicity when injected intravenously into monkeys (see Table 2). For enterotoxin B, which has been the most studied of the enterotoxins since it could be obtained more easily in workable amounts, more accurate figures are available. Schantz et al. (1965) gave the effective intravenous dose as 0.1 μ g per kg. body weight and the oral dose as 0.9 μ g per kg body weight. Some of the properties of the enterotoxins are given in Table 2 as reported by Bergdoll et al. (1967a).

Table 2. Some properties of the enterotoxins

	Enterotoxin			
	A	B	C ₁	C ₂
Emetic Dose (ED ₅₀) (μ g/monkey)	5	5	5	5
Nitrogen Content (%)	16.5	16.1	16.2	16.0
Sedimentation Coefficient ($s_{20,w}^0$), S	3.04	2.78	3.00	2.90
Diffusion coefficient ($D_{20,w}^0$), $\times 10^{-7}$ cm ² sec ⁻¹	7.94	8.22	8.10	8.10
Reduced viscosity (ml/g)	4.07	3.81	3.40	3.70
Molecular weight	34,700	30,000	34,100	34,000
Partial specific volume	0.726	0.726	0.732	0.742
Isoelectric point	6.8	8.6	8.6	7.0
Maximum absorption (m μ)	277	277	277	277
Extinction ($E_1^{1\%}$ cm)	14.3	14.4	12.1	12.1

All the enterotoxins are composed of single polypeptide chains. The terminal amino acids and amino acid compositions are shown in the following tables (Bergdoll *et al.*, 1967a).

Table 3. Terminal amino acids of the enterotoxins

Enterotoxin	N-terminal	C-terminal
A	alanine	serine
B	glutamic acid	lysine
C ₁ (137)	glutamic acid	glycine
C ₂ (361)	glutamic acid	glycine

Table 4. Amino acid composition of the enterotoxins

Amino Acid	No. amino acid residues			
	Enterotoxin A (35,700)	Enterotoxin B (30,000)	Enterotoxin C ₁ (34,100)	Enterotoxin C ₂ (34,100)
Lysine	31	35	38	37
Histidine	7	5	7	7
Arginine	9	5	4	4
Aspartic acid	48	47	53	54
Threonine	22	13	18	20
Serine	16	14	18	19
Glutamic acid	32	22	24	24
Proline	7	7	8	8
Glycine	22	9	18	17
Alanine	11	5	9	8
Half-cystine	2	2	2	2
Valine	18	17	22	20
Methionine	3	8	8	9
Isoleucine	13	9	12	12
Leucine	27	18	20	18
Tyrosine	22	21	21	21
Phenylalanine	12	13	12	12
Tryptophan	3	2	2	2
Amide NH ₂	37	29	36	34
Total	305	252	296	294

The enterotoxins in their native state are resistant to the proteolytic enzymes trypsin, chymotrypsin, rennin and papain. Below pH 2, papain can inactivate the enterotoxins but not at higher pH values (Schantz et al.,

1965; Chu et al., 1966; Bergdoll, 1967). They seem to be very compact molecules which can be unfolded in guanidine hydrochloride or urea and full biological activity can be regained after removal of the guanidine hydrochloride or urea (Dalidowicz et al., 1966; Borja and Bergdoll, 1969). The single disulfide bridge in enterotoxin B can be reduced and reoxidized or alkylated without any alterations in the emetic activity or behavior in immunodiffusion in gel (Dalidowicz et al., 1966).

Assay Methods for the Enterotoxins

Biological methods

In order to prove the enterotoxic property of his staphylococcus isolate, Barber (1914) drank his milk cultures of the organism and tested them on other volunteers. For a while, human volunteers were the only means employed for determining enterotoxicity. The symptoms of the illness were nausea and sudden vomiting 2-4 hours after ingestion of the toxin. Several bouts of vomiting took place within the next few hours with or without abdominal cramps and diarrhea. Rise in temperature, collapse, numbness of extremities, pallor and cold sweats could be manifested. Recovery started after 3-5 hours. (Dolman, 1943; Dennison, 1936).

The first report of a monkey test was made by Jordan and McBroom (1932). They fed filtrates of a 2-3 days' growth to four species of monkeys: red spider (Ateles geoffroyi), black spider (Ateles ater), black howler (Alouata palliata inconsonans) and white face (Cebus capucinus). The filtrates were protosted in human volunteers. The monkeys manifested the symptoms of gastroenteritis. Woolpert and Dack (1933), after 43 feeding

tests in rhesus monkeys, confirmed the validity of the monkey test as a means of assay for highly toxic preparations. Oral administration of such amounts of enterotoxin as usually occur in foods did not produce positive reactions in monkeys. When low concentrations of enterotoxin were injected intravenously, vomiting resulted but at this time Woolpert and Dack were not yet certain about the specificity of such a reaction. An advantage given for the monkey feeding test was that the α - and β -hemolysins do not cause emesis in monkeys when given orally and therefore false positive reactions due to the hemolysins are avoided (Fulton, 1943).

Dolman et al. (1936) suggested intraperitoneal injection into kittens of culture filtrates after the α - and β -hemolysins had been inactivated by the addition of formalin or heating at 100°C for 20-30 minutes. Minett (1938) evaluated Dolman's kitten test together with feeding tests in monkeys, dogs and cats and concluded that the kitten injection test gave the best results. He noticed a great variability in the response of monkeys and cats to oral administration and according to him, dogs reacted only after 24 hours. However, Fulton (1943) declared that the vomiting reaction in Dolman's kitten test was due to the β -lysin which was not sufficiently inactivated by boiling for 20 minutes. He found that strains of staphylococcus which produced β -lysin all gave positive results and that injection of purified α - and β -lysins induced vomiting.

Dogs had been used successfully by Da Silva (1967) for testing the enterotoxigenicity of staphylococci isolated from turkey products. The enterotoxic solutions were injected intravenously and vomiting within 3 hours was reported as a positive reaction.

Robinton (1949) suggested the use of frogs for enterotoxin determination. Reverse peristalsis and spasms within 45 minutes after oral administration of the toxin should be a positive indication of enterotoxin activity. Eddy (1951) evaluated the frog test and attributed the positive response to a non-specific reaction to viscid substances.

Nematodes had also been proposed and found unsuitable for the analysis of enterotoxin (Chang and Hall, 1963). The contractile coiling motion and paralysis after 30-60 minutes of exposure to the test solution was obtained with enterotoxic as well as non-enterotoxic culture filtrates and even with uninoculated medium. It was concluded that all fluids containing 1% or more of protein hydrolysate or 1-2% salts produced irritation and motility changes in nematodes.

Other types of reactions that had been recommended as indicators of enterotoxin activity were the pyrogenic effect on cats or rabbits (Clark and Borison, 1963; Martin and Marcus, 1964; Clark and Page, 1968) and the effect on intestinal transport (Sullivan, 1969). A rise in body temperature of 2.6-3.6^oF was exhibited by cats which received less than the emetic dose of enterotoxin B and a rise of 1.8-3.5^oF in cats which received less than the emetic dose of enterotoxin A (Clark and Page, 1968). Inhibition of the net transport of water, Na, K, Cl, glucose and lactate was produced in the excised sections of the small intestines of rats by incubation in a solution containing 2 mg enterotoxin B per liter (Sullivan, 1969).

Obviously, the difficulties encountered in the use of animal tests were mostly due to the lack of an adequate method for isolating the enterotoxin from the other staphylococcal toxins. Heating, addition of formalin

and the use of antiserum to non-enterotoxigenic strains which was proposed by Davison et al. (1938) were not fully satisfactory and had disadvantages. After the development of purification procedures for the different enterotoxins, animal tests gave more reliable information.

Serological Methods

The use of antigen-antibody reactions in agar was first applied to the enterotoxins as a qualitative study to determine the minimum number of precipitating antigens and to follow the purification procedure of the enterotoxin (Surgalla et al., 1952). The method of Oudin (1952) was used which consisted in the introduction of an antiserum-agar mixture (1:1 v/v) into agar coated tubes of about 3 mm diameter. The agar was made up of 0.6% agar dissolved in 0.85% NaCl containing 1:5000 merthiolate. The tubes were filled two-thirds full with the antiserum-agar solution and this was allowed to solidify. Antigen solutions were layered on top of the solidified agar and the tubes were sealed with parafilm and incubated at 22^o-25^oC. Band migration was measured every 24 hours for three days. The number of migrating bands was interpreted as the minimum number of antigen-antibody systems. Migration rates were used to identify bands from one tube to another. Surgalla et al. (1952) obtained three precipitating systems in their 'purified' enterotoxin sample but they were not able to identify the enterotoxin band.

Bergdoll et al. (1959 a and b), using their purified enterotoxin preparation from strain S-6, obtained a single band in the double gel diffusion method of Oakley and Fulthorpe (1953). Antiserum obtained from

rabbits immunized with this purified toxin exhibited only one kind of antibody. The double gel diffusion test of Oakley and Fulthorpe was similar to the Oudin's test except that the antiserum-agar layer, after solidifying, was overlaid with an equal volume of 0.3% buffered agar and allowed to solidify after which the antigen solution was added. The antigen and antiserum diffused towards each other and formed precipitin bands in the middle agar layer at levels of optimum concentration.

The Oudin gel diffusion test was adapted for a quantitative enterotoxin determination by Bergdoll et al. (1959a). It had been shown (Oudin, 1952; Kabat and Mayer, 1948) that the distance traveled by the band front was proportional to \sqrt{t} ; t being the time of travel. When migration was plotted versus \sqrt{t} , a straight line was obtained whose slope k was proportional to the log of antigen concentration. Bergdoll et al. (1959a) used an antiserum dilution of 1:40 to 1:60. They incubated the tubes at 30°C and measured the migration of the band every 24 hours for three days. A standard curve was determined using known amounts of purified enterotoxin.

Hall et al. (1963) shortened the incubation period from three days to 24 hours. They made use of the postulate of Becker et al. (1951) that the rate of migration of the precipitin band front is directly proportional to antigen concentration in single gel diffusion tubes under the conditions of excess antigen and low antibody concentration. They found a linear relationship between band movement and enterotoxin concentration over a range of concentration of several hundred $\mu\text{g/ml}$ to 1 $\mu\text{g/ml}$. Weirether et al. (1966) reported that good estimates of toxin concentration can be obtained even after only four hours of incubation.

Several variables were shown to influence the rate of migration of the precipitin band--ionic strength and pH of the antigen solution, nature of the diluent, nature of the salts used in the buffer solution and incubation temperature (Hall et al., 1963; Weirether et al., 1966).

The sensitivities of the Oudin and Oakley gel diffusion tests were reported as 1 $\mu\text{g/ml}$ and 0.05 $\mu\text{g/ml}$ respectively for enterotoxin B (Hall et al., 1963) and 0.33 $\mu\text{g/ml}$ and 0.25 $\mu\text{g/ml}$ respectively for enterotoxin A (Read et al., 1965).

The immunodiffusion techniques described above do not measure toxicity per se but they were shown through parallel injection and feeding tests in monkeys to be closely correlated. (Schantz et al., 1965; Read and Bradshaw, 1966; Chu et al., 1969).

The plate test of Ouchterlony (1953) which involves radial diffusion of interacting substances was applied by several investigators to determine the identity or nonidentity of enterotoxins from several cultures (Casman, 1958, 1960; Bergdoll et al., 1959b). Precipitin lines common to enterotoxin filtrates from different strains were thus identified.

Another group of serological methods for enterotoxin determination are the passive or reversed passive hemagglutination and hemagglutination inhibition tests (Robinson and Thatcher, 1965; Brown and Brown, 1965; Morse and Mah, 1967; Johnson et al., 1967; Silverman et al., 1968). In the passive hemagglutination tests, erythrocyte suspensions are sensitized with antigen and estimation of the antibody can be made by direct agglutination of the sensitized cells or by agglutination inhibition in which case the sensitized cells are added to different dilutions of the antibody

mixed with a fixed amount of antigen (predetermined unit dose). Where an excess of antigen is present, no agglutination will occur. Estimation of antigen can also be done by the latter method.

The reversed passive hemagglutination techniques were initiated by Richter et al. (1962) whereby the erythrocytes are sensitized with antibody rather than antigen. Direct estimation of antigen can be done using this method. Bis-diazotized benzidine is used to sensitize the erythrocytes with the antibody.

The hemagglutination techniques mentioned above require only a few hours for the test proper but preparatory operations can take some time. The sensitivity of these tests is comparable to that of the gel diffusion tests (Johnson et al., 1967; Silverman et al., 1968).

A modification of the reversed hemagglutination technique is the latex agglutination method whereby polystyrene latex particles instead of erythrocytes are sensitized with antiserum. The rest of the procedure is similar to that of hemagglutination (Salomon and Tew, 1968).

The fluorescent antibody technique developed by Coons and Kaplan (1950) was applied by several investigators to the detection of staphylococcal enterotoxin B (Friedman and White, 1965; Genigeorgis and Sadler, 1966). The method consists in forming an antiserum to enterotoxin B conjugated with fluorescein isothiocyanate and staining smears of the staphylococcal culture on a microscope slide with different dilutions of the conjugate or precipitating a drop of the toxin solution with two drops of serially diluted conjugate and observing for fluorescence. The latter method is more sensitive and can detect a minimum concentration of 1 $\mu\text{g}/\text{ml}$. Friedman and

White (1965) demonstrated by this method that the enterotoxin in staphylococcal cultures was present outside the cell and loosely bound to the cell surface.

Production Methods

Initially, enterotoxin was produced in the laboratory in plain infusion broth at pH 7.6 by incubation for 48 hours at 37°C (Jordan, 1930). Burnet (1930) stated that greater amounts of enterotoxin could be obtained if incubation was done in an atmosphere containing CO₂. The function of CO₂ was said to be the alteration of intracellular pH independently of that of the environment. In 1935, Jordan and Burrows reported that staphylococci lost their toxigenic property after a few months in veal or beef infusion broth but that this property could be restored temporarily by growing the bacteria in an atmosphere containing 25% CO₂. Successive transfers in starch veal infusion agar restored toxigenic ability for longer periods of time than incubation in CO₂.

The role played by starch was not known but Kelly and Dack in 1936 showed that staphylococci could grow and produce enterotoxin on bread. On the other hand, Stone (1935), in a study of foods incriminated in food poisoning outbreaks, declared that "protein was the only consistent major ingredient of the foods involved."

According to Casman (1938), addition of agar to the medium and incubation in a carbon dioxide-oxygen atmosphere were important for toxin production. In 1940, he reported that broth cultures could equal agar cultures in toxin production if the broth was cultured in shallow layers and rocked continuously during incubation in a carbon dioxide-oxygen atmosphere.

The first attempt at a semi-synthetic medium was made by Dolman and Wilson (1938). They proposed a medium containing Difco proteose peptone, NaCl, K_2HPO_4 , KH_2PO_4 , $MgSO_4$ and $CaCl_2$ at pH 7.4. The medium was used as a 0.3% agar culture. In 1941, Favorite and Hammon proposed a simplified, fully dialyzable medium composed of casein hydrolysate, glucose, thiamine chloride (vitamin B_1) and nicotinic acid. The pH was adjusted to 7.6 and incubation was done in a CO_2 atmosphere on a rotating machine. This medium was patterned after Mueller's (1939) medium for diphtheria toxin production. Earlier Gladstone (1938) had devised a medium for staphylococcal hemolysin production consisting of amino acids, inorganic salts and the growth factors vitamin B_1 and nicotinic acid suggested by Knight (1937). He found that substituting protein hydrolysate for the mixture of amino acids produced better yields.

Casman (1958) did not find the medium of Favorite and Hammon adequate for consistent good yields of toxin. He proposed the addition of calcium pantothenate, l-cystine, tryptophan and $MgSO_4$ for better growth of the organism, the substitution of sodium acetate for glucose, and the addition of iron for better enterotoxin production. The pH of the medium was adjusted to 7.2-7.4.

Chemically defined media, tested by Surgalla and Hite (1946), contained varying amounts of amino acids plus the following substances: $MgSO_4$, $FeSO_4$, $(NH_4)_2SO_4$, KH_2PO_4 , NaOH, glucose, nicotinic acid, thiamine, and calcium pantothenate. They concluded that simplification of the medium decreased growth and hemolysin production and therefore also enterotoxin production which they assumed through qualitative monkey feeding and cat tests to be

parallel to hemolysin production.

The lack of a quantitative assay procedure for the enterotoxins had prevented earlier attempts to evaluate the comparative values of the various media proposed. After the introduction of the gel diffusion method of assay, more definite information could be obtained.

Using the gel diffusion titer, i.e., the highest dilution of toxin giving a line of precipitation, as a measure of the enterotoxin, Casman and Bennett (1963) compared the enterotoxin producing ability of three kinds of media: 1) the medium of Dolman and Wilson (1938), 2) brain heart infusion agar (0.6-0.7% agar) at pH 5.3, and 3) a medium containing a pancreatic digest of casein, glucose, NaH_2PO_4 , thiamine hydrochloride, nicotinic acid and calcium pantothenate at pH 5.8. A pancreatic hydrolysate of casein was found to give better production than an acid hydrolysate. Brain heart infusion medium was superior for the production of both enterotoxins A and B. The initial pH of the medium had an effect on enterotoxin production. The optimal value was found to be pH 6.5-7.0.

The above investigators also designed a cellophane sac culture in which growth and toxin production were confined to the interior of a dialysis tubing suspended in the liquid medium. More highly concentrated enterotoxin preparations which were free of non-dialyzable material were obtained in this manner. Hojvat and Jackson (1969) used this sac assembly but with the brain heart infusion broth inside the sac and a phosphate buffered inoculum outside the sac. The assembly was incubated on a rotary shaker at a speed of 170 rpm. A more sophisticated form of the sac culture is the two-chambered or three-chambered dialysis flask designed by Gerhardt and Gallup (1963) and Herold et al. (1967).

Large scale production of enterotoxins was done at the Food Research Institute, Madison, Wisconsin using media containing a pancreatic digest of casein, either Protein Hydrolysate Powder (PHP) (Mead Johnson and Co.) or N-Z Amine (Sheffield Chemical Co.) or a combination of both supplemented with thiamine and niacin. Incubation was done on a rotary shaker or in deep aerated cultures. Average yields for enterotoxin A were 2-8 $\mu\text{g}/\text{ml}$ and for enterotoxin B, 200-500 $\mu\text{g}/\text{ml}$ (Kato et al., 1966; Bergdoll, 1967). Optimal initial pH of the medium was found to be 6.8 for the production of enterotoxin B. Production of enterotoxin A did not vary significantly within an initial pH range of 5.3-6.8 (Reiser and Weiss, 1969). As the volume of the medium was increased for a fixed container size in shake cultures, the yield of the enterotoxin decreased (Kato et al., 1966).

Mah et al. (1967) determined the minimal amino acids necessary for growth of strain S-6. A mixture of these amino acids when substituted for an equivalent amount of protein hydrolysate in a medium containing inorganic salts and vitamins gave equal growth but only one-seventh of the enterotoxin yield of the more complex medium.

Enterotoxin B synthesis in a protein hydrolysate medium supplemented with niacin, thiamine and calcium pantothenate took place at the post exponential growth phase and was essentially complete before the maximal stationary phase was reached, according to Morse et al. (1969). The pH of the medium was critical in enterotoxin production. Yields were increased at pH 6.4 and above and repressed at pH 5 and below.

Markus and Silverman (1969) proposed the presence of an enterotoxin precursor pool in the cells after 16 hours of growth. Cells harvested after

this period of time and resuspended in two kinds of restricted media; one with a nitrogen source (N-Z Amine) plus phosphate and the other without a nitrogen source but with glucose plus phosphate, produced enterotoxin without the usual lag period and without replication of cells. In the restricted medium containing a nitrogen source, cells harvested after only four hours of growth produced enterotoxin but not in the glucose medium. Chloramphenicol, penicillin and streptomycin which were known to inhibit enterotoxin production in replicating cells (Rosenwald and Lincoln, 1966; Morse et al., 1969; Friedman, 1966) did not prevent its formation by non-replicating cells.

Purification Methods

In the early stage of enterotoxin study, attempts were made to purify the enterotoxin mostly by precipitation methods. Jordan and Burrows (1933) reported that the enterotoxin could be extracted from an acid solution by ethyl ether or chloroform. The results of Davison and Dack (1939) disagreed with this report. They found that the enterotoxin was not soluble in chloroform. They were able to precipitate the enterotoxin by salting out with saturated ammonium sulfate. Hammon (1941) tried a combined ammonium sulfate and alcohol treatment. The ammonium sulfate supposedly separated the hemolysins and dermatotoxin and the alcohol precipitated the enterotoxin. Their final product contained no detectable nitrogen by the micro-Kjeldahl method but 2.0-6.5 mls caused vomiting in kittens.

Purification of enterotoxin B

In 1951, Bergdoll et al. presented a partial purification procedure

consisting in in vacuo concentration of filtrates of strain S-6, dialysis, precipitation with ammonium sulfate, acid, ethanol and methanol. A great part of the contaminating substances (97% by weight) was removed by dialysis. Most of the enterotoxin was precipitated by 75% saturation of ammonium sulfate and completely precipitated at 100% saturation. Acid precipitation was done at pH 3.5.

Adsorption of the enterotoxin B on a column of diatomaceous silica was later added to this procedure after the ammonium sulfate or acid precipitation (Bergdoll et al., 1952). The enterotoxin was eluted from the column with citrate-phosphate buffer at pH 7.8 and ionic strength 0.12. The purified product showed the presence of four antigens in gel diffusion tests and at least two components in electrophoresis.

Ion exchange chromatography with Amberlite XE64 (IRC 50) and starch gel electrophoresis were used to purify further the product obtained above (Bergdoll et al., 1959a, 1961). The final product exhibited only one antigen in Oakley's gel diffusion test and one peak in the ultracentrifuge. A dose of 1 μ g nitrogen caused emesis in monkeys.

Hallander (1963) employed gel filtration of the dialyzed culture filtrates of strain S-6 in Sephadex G-100 and separated three fractions, one of which contained the enterotoxin together with α -hemolysin, leucocidin and fibrinolysin. By increasing the ionic strength of the elution buffer, he could separate α -hemolysin and leucocidin from fibrinolysin but the enterotoxin peak overlapped both of these peaks. Frea et al. (1963) also used gel filtration on a Sephadex G-100 column after dialysis and ethanol precipitation of the culture filtrates of S-6. The toxin-containing

fractions were then subjected to electrophoresis on a column packed one-third of the length with Sephadex G-50 and the rest with Sephadex G-75. Disc gel electrophoresis at pH 8.9 of the purified product and the purified toxin preparation of Bergdoll et al. (1959a) showed an upper major band and a lower minor band in both preparations except that the preparation of Frea et al. had less of the minor band than the sample of Bergdoll et al. The upper band was shown to be the enterotoxin band by immunodiffusion tests. The purified enterotoxin had a minimum effective dose of 0.26 μ g nitrogen per kg body weight when injected intravenously in monkeys.

Baird-Parker and Joseph (1964) used a modification of the procedure of Bergdoll et al. (1959a, 1961) wherein they passed the culture supernatant through two ion exchange columns (Amberlite IRC-50) set in series. This step removed most of the toxin from the supernatant. The eluate was subjected to alcohol precipitation, dialysis and lyophilization. Double gel diffusion tests and ultracentrifugal analysis showed homogeneity. Starch gel electrophoresis using Poulik's discontinuous buffer system of 0.3 M boric acid and 0.05 M NaOH (gel pH 8.68) showed two major bands and a number of minor ones in both their sample and that of Bergdoll et al. (1959a, 1961). Both of the major bands gave precipitin lines in gel diffusion tubes but one band was relatively more toxic when injected intraperitoneally into kittens than the other (Joseph and Baird-Parker, 1965).

A purification procedure devised by Schantz et al. (1965) consisted in adsorption on an ion exchange resin (CG-50) batchwise and then on a column of the same resin and final chromatography on carboxymethyl cellulose (CMC). The eluates were dialyzed after each column treatment and finally

freeze dried. Tests on the purified product for carbohydrate, lipid, nucleic acids, α - and β -hemolysins, apyrase and dermonecrotxin were all negative. Ultracentrifugation and free electrophoresis revealed only one component. Disc gel electrophoresis at pH 4.3 (β -alanine-acetic acid) and Ouchterlony gel diffusion tests showed a second minor band. Purity of the sample is estimated from the Ouchterlony test to be more than 99%. However, starch gel electrophoresis with 0.02 M borate buffer at pH 8.6 had a slower moving component that accounted for 20-30% of the toxin and a faster moving component that accounted for 60-70% of the toxin. Both components were toxic when injected or fed to monkeys. When the ionic strength in starch gel is raised to 0.1 at pH 8.6, the toxin remained as a single band at the origin. It was proposed that the separation into two bands is dependent on ionic strength. When unstained bands were cut out and rerun on starch gel electrophoresis, the faster moving component gave two bands at the same positions as the bands of the original toxin solution whereas the slower moving component gave only a single band with the same mobility. However, Joseph and Baird-Parker (1965) reported that a rerun of their two bands produced a single band for each with unchanged mobilities.

Purification of enterotoxin A

Purification methods for enterotoxin A were only developed recently due to the difficulty encountered in obtaining suitable yields of the enterotoxin in culture filtrates. The normal production yield of the known A producing strains is 2-8 $\mu\text{g/ml}$ of culture supernatant (Kato et al., 1966).

In the initial part of their study, Chu et al. (1966) tried precipitation of the enterotoxin A with trichloroacetic acid (TCA) at pH 2.9-3.0 from strains 196E and C-246-3. The procedure caused denaturation of the enterotoxin and impurities in the precipitated material were difficult to separate. Enterotoxic filtrates from strain 100 were found easier to purify. They developed a purification procedure which involved ion exchange chromatography on two successive columns of carboxymethyl cellulose and gel filtration through Sephadex G-100 and G-75. Dialysis and lyophilization of the eluates were done after each column. The purified enterotoxin A showed homogeneity in ultracentrifugation but double gel diffusion and starch gel electrophoresis according to the method of Smithies (1955) which used a borate buffer of 0.03 M H_3BO_3 and 0.012 M NaOH (gel pH 8.48), showed trace amounts of two other substances. Purity of the preparation was estimated to be at least 95%.

Purification by gel electrophoresis using polyacrylamide was proposed by Denny et al. (1966). The acid gel system (pH 4.3) of Reisfeld et al. (1962) was used. The band containing the enterotoxin was identified by serological tests and the unstained sections of gel columns corresponding to this band were cut out and eluted with β -alanine buffer. The purified enterotoxin A showed a single precipitin line in Ouchterlony gel diffusion plates but no quantitative measurements were made. Tan et al. (1969) modified this method by the use of a sucrose chamber (20% sucrose solution) in the gel column to trap the enterotoxin band. After electrophoresis, the sucrose solution was pulled out with a syringe and frozen or lyophilized. This procedure eliminated the elution of gel sections with buffer. A 40%

recovery of the enterotoxin was reported. These experiments were not done on a preparative scale but suggestions for doing so were made.

Purification of enterotoxin C

The purification procedure for enterotoxin C₁ developed by Borja and Bergdoll (1967) consisted in column chromatography on CM-cellulose and gel filtration, once through Sephadex G-75 and twice through Sephadex G-50. The eluates were dialyzed and lyophilized after each column. The purity of the sample estimated from gel diffusion tests was greater than 99%. Paper electrophoresis using a pH range of 4.4-11.0 gave only a single spot while starch gel electrophoresis at pH 8.7 in 0.1 M Veronal buffer showed the presence of another component.

Enterotoxin C₂ was purified by Avena and Bergdoll (1967). The purification involved chromatography on two successive columns of CM-cellulose, the first using stepwise elution and the second using gradient elution, and gel filtration, twice through Sephadex G-75 and once through Sephadex G-50. The purity of the final freeze-dried product was estimated from double gel diffusion tests as greater than 99%. It showed homogeneity in paper electrophoresis at a pH range of 4.6-9.6 and on agar gel electrophoresis at pH 8.6 and ionic strength 0.1 using Veronal buffer. Immunoelectrophoresis showed a single precipitin line.

Heat Inactivation of the Enterotoxin

The relative stability of the enterotoxins to heat was demonstrated in 1930 by Dack et al. using human volunteers. They found that the toxic activity was not fully destroyed when the staphylococcal filtrates were

heated at 100°C for 30 minutes. Davison and Dack (1939) showed decreasing activity with increasing heating time. Boiling for 30 minutes reduced the potency of the enterotoxin when injected into monkeys from a control value of 77% positive reaction to 33%. After 60 minutes boiling, it was further reduced to 14% positive reaction. Enterotoxic filtrates autoclaved at 15-16 psi for 20 minutes still showed 7% positive reaction.

The hemolysins and dermonecrotxin were known to be labile to heating at 100°C (Parker, 1924; Dolman, 1932; Dolman et al., 1936; Hammon, 1941). Boiling for 30 minutes was therefore used by many investigators as a preliminary preparation of the enterotoxic samples used for animal tests. Some reports stated that β -hemolysin was also relatively stable to heat and that residual activity in the boiled filtrates could be confused for enterotoxin activity (Dolman, 1943; Fulton, 1943; Bigger et al., 1927).

According to Jordan and Burrows (1933), heating the enterotoxin in 0.01 N HCl or 0.01 n NaOH destroyed its activity as measured by monkey feeding tests. Frolov (1966) heated enterotoxic solutions at 100°C for 30 minutes at different pH values and reported that when injected into cats, the symptoms changed from vomiting only to vomiting plus diarrhea to diarrhea only as the pH decreased from 7.0 to 3.8. Enterotoxin heated at pH 3.0 produced no reaction. He suggested the presence of a "vomiting factor" which was thermally stable in neutral media and less stable at low pH and a "diarrhea factor" which was more stable in acid media and less stable in neutral media.

Pure enterotoxin A lost its ability to react with its antibody in gel diffusion tests when a solution of 200 μ g/ml at pH 6.8 was heated for one

minute at 100°C (Chu et al., 1966). Pure enterotoxin B was reportedly more heat resistant than enterotoxin A (Bergdoll, 1967; Chu et al., 1966). It was inactivated, as tested by gel diffusion and cat injection, after 87.1 minutes at 100°C at a concentration of 30 µg/ml and pH 7.2 (Read and Bradshaw, 1966). The activity of pure enterotoxin C was reduced to 20% when heated at 100°C for one minute (Avena and Bergdoll, 1967).

Read and Bradshaw (1966) studied the inactivation of enterotoxin B at temperatures between 96°C and 126.7°C as measured by gel diffusion and intravenous injection into cats. They concluded that the end point of inactivation as measured by gel diffusion was similar to that measured by cat injection. They mentioned that limited studies seemed to indicate that crude enterotoxin B was more heat resistant than the purified toxin. Studies made by Satterlee and Kraft (1969) showed a similar phenomenon for crude and partially purified enterotoxin B. However, they reported that in a ground meat slurry or when in solution with myosin or metmyoglobin, enterotoxin B seemed to be more readily inactivated than in phosphate buffer. They attributed this to a probable binding reaction with meat constituents in addition to the heat inactivation. They also showed that initial inactivation of enterotoxin B was more rapid at 80°C than at 60°C or 100°C.

Enterotoxin A also demonstrated greater heat lability in the pure state than in the partially purified form. Hilker et al. (1968) reported an inactivation time of 130 minutes at 100°C for 21 µg/ml of partially purified enterotoxin A whereas the purified toxin was inactivated in one minute at 100°C at a concentration of 200 µg/ml (Chu et al., 1966).

EXPERIMENTAL PROCEDURE

Enterotoxin Production

Staphylococcus strain S-6 was grown in a culture medium containing 3% Protein Hydrolysate Powder (PHP), a pancreatic digest of casein produced by Mead Johnson & Co., Evansville, Ind.; 3% N-Z Amine NAK, a pancreatic digest of casein produced by Sheffield Chemical Co., Norwich, N.Y.; 0.001% niacin and 0.00005% thiamine. The vitamins were sterilized by filtration through a Seitz filter and added after sterilization of the medium by autoclaving. The pH of the medium was adjusted to 6.5 before sterilization. Inoculum cultures were prepared by inoculating a batch of the medium and incubating for 18 hours at 37°C on a rotary shaker. The culture was dispensed in 4 ml portions in sterilized tubes and frozen. These were thawed as needed and used as a 1% inoculum.

For the production of the enterotoxin, culture media in 600 ml lots contained in Fernbach flasks were inoculated and incubated at 37°C on a rotary shaker at a speed of 280 rpm for 24 hours. After incubation, the bacterial cells were centrifuged and the supernates pooled.

Enterotoxin Assay

The single gel diffusion technique according to Weirether et al. (1966), except for a few modifications, was used to determine enterotoxin concentrations.

Phosphate-buffered saline This diluent was prepared by mixing 85 parts of Solution A and 15 parts of Solution B. Solution A consisted of 11.36 g Na₂HPO₄, 34.0 g NaCl and 0.4 g of merthiolate powder (Thimerosal,

Lilly) brought into solution with distilled water to a final volume of 4,000 ml. Solution B consisted of 2.7 g KH_2PO_4 , 8.5 g NaCl, and 0.1 g merthiolate powder brought to a final volume of 1,000 ml of solution. The mixture of solutions A and B gave a solution which was 0.017 M in phosphate and 0.145 M in NaCl (0.85%) at pH 7.4.

Agar Difco Noble Agar was dissolved in phosphate-buffered saline (by heating for 10 minutes at 121°C) to make a 1% agar solution. The solution was filtered through Whatman No. 1 filter paper in a heated chamber so that the temperature was kept at about 55°C . The filtered agar was dispensed into test tubes in 5 ml portions, allowed to cool and stored at 4°C .

Antiserum The antiserum to enterotoxin B was provided by M.S. Bergdoll of the Food Research Institute, Madison, Wisconsin. It was used in a 1:100 dilution.

Preparation of Gel Diffusion Tubes To prepare 40 gel diffusion tubes, a 5 ml portion of the agar was melted and kept at 48°C . For a 1:100 antiserum dilution, 0.1 ml of the antiserum was mixed with 4.9 ml of heated (48°C) phosphate-buffered saline. The melted agar was added to the antiserum solution and mixed thoroughly. Gel diffusion tubes of about 5 mm in diameter and 5 cm long were filled with 0.25 ml of the antiserum-agar solution by means of a syringe. The tubes were allowed to cool and stored in a humidified chamber to prevent drying of the agar.

Assay Procedure The antiserum-agar in the gel diffusion tube was layered with 0.1 ml of the antigen test solution. The diluent for all antigen solutions was phosphate-buffered saline with 2% NaCl (0.486 M

total NaCl). The open end of the tube was sealed with parafilm and the tube was incubated in a water bath at 30°C for 24 hours. After this time, the precipitin band migration was measured using a Bausch and Lomb 7X eyepiece equipped with a scale graduated in 0.1 mm. The tube and eyepiece were held against a shielded fluorescent light at an oblique angle. Using purified enterotoxin B supplied by M.S. Bergdoll of the Food Research Institute in concentrations of 10, 20, 40, 60, 100, 150 and 200 µg/ml, a standard curve was obtained by plotting band migration in mm against log concentration of enterotoxin. A single batch of antiserum and therefore one standard curve was used throughout the whole study.

Phosphate Assay

The amount of phosphate salts in solution was determined by a colorimetric method. To 0.1 ml of the sample were added 2 ml of 2.5% ammonium molybdate solution in 5N H₂SO₄, 2.9 ml H₂O and 0.2 ml of the reducing agent solution containing 2% 1-amino-2-naphthol-4-sulfonic acid, 12% NaHSO₃ and 1.2% Na₂SO₃. The absorbance at 760 mµ was read after 30 minutes. A standard curve was made using known amounts of phosphate.

Preparation of Ion Exchangers

Amberlite CG-50 ion exchange resin was washed and equilibrated with phosphate buffers according to the method described in the Methods in Enzymology, Volume 1, pp. 113-115.

Carboxymethyl cellulose was prepared for use by first soaking in 0.5 M NaOH for 30 minutes to 1 hour. It was rinsed with distilled water and filtered through a Buchner funnel. This process was repeated until

the washings registered a pH of 8.0. The washed cellulose was soaked in 0.5 M HCl for 30 minutes to 1 hour. It was repeatedly washed with water until the pH of the wash water was neutral. The cellulose was stirred in distilled water and then allowed to settle for 30 minutes. The finer particles that had not settled within this time were decanted off. The washed cellulose was finally equilibrated with the appropriate buffer.

Purification Procedure

The purification method developed by Schantz et al. (1965) was followed with slight modifications. The cell free culture fluid was diluted with 8 volumes of distilled water, and the pH adjusted to 6.4 with dilute HCl. Approximately 9-10 liters of culture fluid was purified at one time. Amberlite CG-50 resin which had been equilibrated with 0.05 M phosphate buffer at pH 6.4 was added to the diluted supernatant at an amount of 2 grams dry weight per liter of solution. The resin was stirred in the culture supernatant for 30 minutes and then allowed to settle. Most of the clear liquid was decanted off and the resin plus the remaining solution was poured into a column (3 x 40 cm). The resin was washed with one column volume of water and the enterotoxin was eluted at a flow rate of 2 ml/min with 0.5 M sodium phosphate at pH 6.8 containing 0.25 M NaCl. The eluate was collected in 10 ml portions. The absorbance of the fractions at 280 m μ was measured using a Zeiss spectrophotometer (Carl Zeiss, Germany). Fractions with absorbance greater than 1.0 were pooled, and the combined solution was analyzed for enterotoxin concentration by the gel diffusion test and for total protein content by the Biuret method.

The pooled fractions were evaporated to half the volume in a rotary evaporator with a bath temperature of 30°C. The evaporated solution was dialyzed against distilled water to a phosphate content of 0.01 M. Enterotoxin and total protein content of the dialyzed solution were determined. The enterotoxin was reabsorbed on a column of CG-50 resin which had been equilibrated with 0.05 M phosphate buffer at pH 6.8. Twenty grams of resin (dry weight) was used per gram of protein. The resin was washed with water until there was no absorbance at 280 m μ and the enterotoxin was eluted with 0.15 M phosphate buffer at pH 8.9 with a flow rate of 2 ml/min. The eluate was collected in 5 ml fractions. The fractions having an absorbance greater than 1.0 were pooled and analyzed for enterotoxin and protein. The solution was evaporated to half its volume in a rotary evaporator and then dialyzed against distilled water to a phosphate content of 0.01 M. The enterotoxin and protein content of the dialyzed solution were measured.

The enterotoxin was adsorbed on a column of CM-cellulose which had been equilibrated with 0.01 M phosphate buffer at pH 6.2. Twenty grams of cellulose (dry weight) was used per gram of protein. The column was washed with the equilibration buffer and eluted with a linear gradient phosphate buffer from 0.02 M at pH 6.2 to 0.07 M at pH 6.8 at a flow rate of 1 ml/min. The volume of each buffer was 300 mls. The fractions containing the protein peak were collected and assayed for enterotoxin and protein concentrations. The solution was evaporated to half its volume and dialyzed for enterotoxin and protein content and then freeze-dried.

The freeze-dried material was stored at -5°C .

Disc Gel Electrophoresis

Polyacrylamide disc gel electrophoresis was used to determine the purity and homogeneity of the purified enterotoxin preparation as well as to detect differences between heated and unheated enterotoxin solutions.

Gel Systems

The three kinds of gel systems used were: 1) the cationic system of Reisfeld et al. (1962) with a running gel containing 7.5% acrylamide at pH 4.3; 2) the anionic system of Ornstein and Davis (Chemical Formulations for Disc Gel Electrophoresis, Canal Industrial Corporation, Bethesda, Md.) with a running gel containing 7.0% acrylamide at pH 8.9; and 3) the cationic system of Williams and Reisfeld (1964) with a running gel containing 7.0% acrylamide at pH 7.5.

Preparation of the Gels

A two-stage gel was used which consisted of a small pore separating or running gel and a large pore stacking or spacer gel. The spacer gel contained 2.5% acrylamide.

Glass columns (6.5 cm in length and 5 mm i.d.) were sealed at one end with rubber base caps and set vertically on a level surface. A volume of 0.7 ml of the separating gel was introduced slowly into the tubes by means of a syringe. With a Pasteur pipet, water was layered very carefully on top of the gel solution to a depth of about one-fourth of an inch, taking care not to disturb the gel solution or cause mixing of the two layers. The gel was allowed to polymerize for 45 minutes at room temperature. After this time, the water layer was drained off and 0.20 ml of the spacer

gel solution was introduced into the columns. Water was again layered slowly on top of the spacer gel solution and the tubes were placed next to a fluorescent light source to polymerize the spacer gel. Fifteen minutes was allowed for polymerization.

The sample, as a 20% sucrose solution, was introduced on top of the spacer gel. Approximately 0.05 ml of the spacer gel solution was layered on top of the sample solution to serve as anticonvection seal. The tubes were again exposed to fluorescent light for about 15 minutes to polymerize the gel seal.

Electrophoresis

Electrophoresis was performed using a Canalco Model 12 Electrophoresis Apparatus (Canal Industrial Corporation, Bethesda, Md.). Twelve samples could be electrophoresed at one time. The columns were inserted into the upper chamber with the separating gel end downward. The base caps were removed carefully from the columns. This was done by first allowing an air leak to form before pulling the caps away. To prevent the formation of air spaces at the ends of the columns, a drop of the electrode buffer was touched to the end of the gel with a pipet. The upper and lower chambers were filled with the electrode buffer and a trace of tracking dye was added to the upper chamber buffer. Methyl green was used as tracking dye for the cationic system and bromphenol blue for the anionic system at pH 8.9. For the system at pH 7.5, no suitable tracking dye was found since an electrophoresis time of about 2 hours was needed to obtain good separation of the bands. The electrodes were connected to a Canalco

Constant Rate Source, Model 1400 and a current of 5 mA per column was used. For the gel systems at pH 4.3 and 7.5, the electrodes were reversed so that the upper chamber was connected to the positive electrode and the lower chamber was connected to the negative electrode.

As soon as the tracking dye reached the end of the separating gel or after 2 hours in the case of the system at pH 7.5, the current was shut off. The columns were removed from the apparatus and chilled in ice for a few minutes to facilitate the removal of the gel. The gel columns were removed from the glass tubes by rimming the ends under water with a syringe needle and pushing the gel out with air from a rubber bulb. The gel columns were immediately immersed in Amido-Schwarz stain which was prepared by dissolving 0.55 g of Amido-Schwarz dye in 7.5% acetic acid to make a final volume of 100 ml. The gel columns were left in the staining solution overnight.

The gels were destained using the same electrophoresis apparatus. One end of the destaining tubes was sealed with a moistened dialysis membrane stretched across the opening and secured around the sides with a rubber band. The gels were removed from the staining solution, rinsed with water and placed inside the tubes with the separating gel towards the sealed end. The tubes were filled with 7.5% acetic acid solution and then inserted into the upper chamber. The upper and lower chambers were filled with sufficient amounts of 7.5% acetic acid to cover the electrodes. Current of 12.5 mA was applied to each column. When the gel columns were fully destained, the current was turned off. The destained gels were kept in 7.5% acetic acid.

Estimation of Enterotoxin Purity

Disc gel electrophoresis was used to estimate the purity of the enterotoxin preparation. Different amounts of the purified enterotoxin were electrophoresed to determine the minimum amount at which the first band becomes visible and that at which a second band starts to appear. The ratio of the two concentrations was used to estimate the purity of the sample.

Estimation of Size Difference between Major and Minor Band

The molecular size of the second band seen in gel electrophoresis of the enterotoxin was estimated by means of a procedure derived from the method proposed by Hedrick and Smith (1968). The gel system at pH 4.3 was used except for the ratio of N, N'-Methylene-bisacrylamide (Bis) to acrylamide in the separating gel which was kept constant at 1:30. The acrylamide concentration of the separating gel was varied between 6-15%. At the end of the electrophoresis run, the dye front was marked by inserting a plastic bristle through the gel. The migration rates of the two bands were measured in mm. relative to the dye front and expressed as the ratio of protein migration to dye migration. The logarithm of these values were plotted against gel concentration.

Determination of the Stability of the Enterotoxin in Buffer Solutions

Purified enterotoxin was dissolved in phosphate buffers of varying ionic strengths at pH 6.4 to obtain a concentration of 100 $\mu\text{g}/\text{ml}$. The phosphate concentrations used were 0.05, 0.08, 0.12 and 0.15 M corresponding

to ionic strengths of 0.06, 0.10, 0.15 and 0.20 respectively. The solutions were kept in a 30°C water bath and analyzed for enterotoxin content every 24 hours for three days.

Heat Inactivation

Varying the Temperature

A solution of enterotoxin in 0.08 M phosphate buffer (I=0.10) containing 100 µg of enterotoxin B per ml of solution was prepared. Melting point capillary tubes (1.6-1.8 x 90 mm) were filled about half full with the enterotoxin solution using a syringe with a 5 in. needle. The open end of the capillary tube was sealed with a propane torch without raising the temperature of the solution.

The heating temperatures used were 60°, 70°, 80°, 90° and 100°C. The capillary tubes were immersed in an oil bath set at the desired temperature. The time required for the liquid inside the tubes to reach the temperature of the bath (come-up-time) was predetermined with the use of a thermocouple connected to a potentiometer. Duplicate tubes were taken out at the come-up-time and at different times during the heating period and the study at each temperature was done twice. The tubes were immediately plunged into ice water when removed from the oil bath. After cooling, the heated solutions were analyzed for enterotoxin. A sample was kept at 30°C during the entire heating time as a control.

Varying the pH

Enterotoxin solutions containing 100 µg/ml in acetate buffer at pH 4.5 and phosphate buffer at pH 7.5 were prepared. In all cases the ionic

strength was fixed at 0.10. The enterotoxin was subjected to heat inactivation at 70°C and 100°C as previously described.

Varying the Ionic Strength

Phosphate buffers (pH 6.4) of the following molarities: 0.015, 0.038, 0.08, 0.15, 0.38, 0.62 and 0.80 corresponding to ionic strengths of 0.02, 0.05, 0.10, 0.20, 0.50, 0.80 and 1.0 respectively were used to prepare enterotoxin solutions containing 100 µg/ml. These solutions were heated at 70°C for 8 minutes, cooled and then analyzed for enterotoxin.

Enterotoxin solutions (100 µg/ml) in acetate buffer at pH 4.5 were treated likewise. The buffers had acetate concentrations of 0.04, 0.08, 0.14, 0.20, 0.28 and 0.34 M equivalent to ionic strengths of 0.01, 0.02, 0.05, 0.07, 0.10 and 0.12 respectively.

Varying the Initial Concentration

Different amounts of enterotoxin were dissolved in buffer to produce solutions of the following approximate concentrations: 20, 50, 100, 150 and 300 µg/ml. Solutions were made in both phosphate buffer (I=0.10 at pH 7.5) and acetate buffer (I=0.02 at pH 4.5). These solutions were heated at 70°C for 10 minutes and analyzed for enterotoxin content after cooling.

Dog Injection Test for Toxicity

Heated enterotoxin solutions were injected intravenously into dogs to determine the actual toxicity of the solutions. The emetic dose was first determined using unheated enterotoxin B. Three dogs were used per sample

and each dog was used only once. Half an hour before injection, the dogs were fed with fresh hamburger meat. The enterotoxic solutions were injected through the cephalic vein and vomiting within 3 hours was reported as a positive reaction.

RESULTS

Enterotoxin Production

The maximal enterotoxin production obtained with the method described in the experimental procedure was 300 μg of enterotoxin B per ml of supernatant. Less production of enterotoxin was obtained when decreased concentrations of PHP and N-Z Amine were used. It was found that aeration was an important factor in the production of enterotoxin B. Incubation on a gyratory shaker at a speed of 280 rpm gave better results than incubation on a magnetic stirrer or aeration by a slow stream of air. The use of bigger containers for the same volume of medium also increased aeration and therefore production. Fernbach flasks containing 500-600 ml of medium or 1 liter flasks with 150-200 ml of medium produced 260-320 μg of enterotoxin per ml of culture fluid.

The sac culture method used by Hojvat and Jackson (1969) in which a dialysis sac containing double strength brain heart infusion broth was suspended in 0.2 M phosphate buffer (pH 7.0) containing the inoculum and incubated for two days on a gyratory shaker was also tried. It gave a maximal yield of 200 $\mu\text{g}/\text{ml}$ when the pH of the brain heart infusion broth was adjusted to 6.5. However, this method was not suitable for large scale production.

It was noticed that the inoculum cultures gradually lost their ability to produce enterotoxin during frozen storage. After a year in frozen storage, enterotoxin production decreased to an average of 50 $\mu\text{g}/\text{ml}$. At least two successive transfers in brain heart infusion broth were needed

to restore the initial enterotoxin producing ability of the bacterial culture.

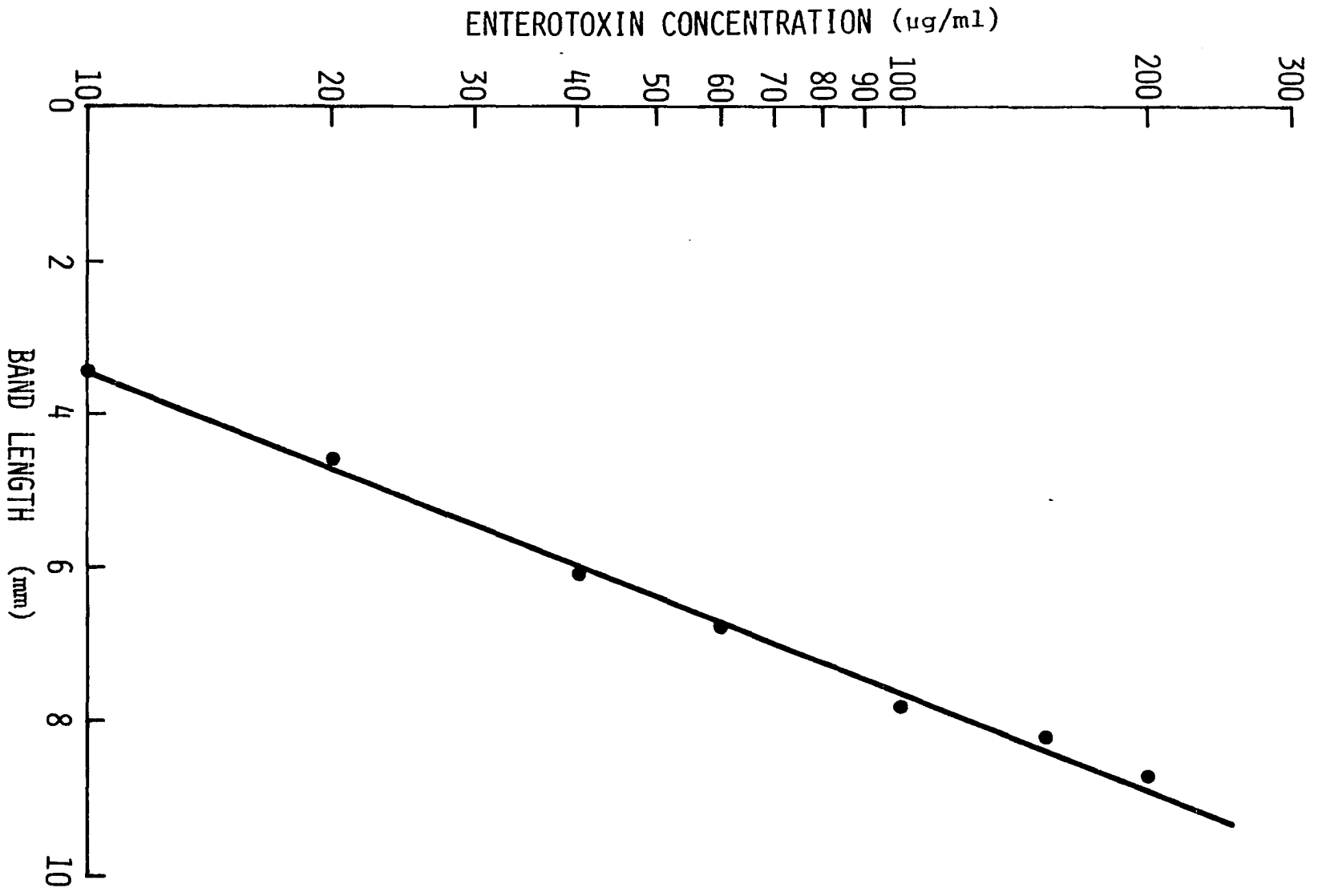
The initial pH of the PHP - N-Z Amine medium was adjusted to 6.5 before sterilization. After 24 hours of incubation, the final pH of the bacterial culture was 8.2. The cells were usually centrifuged right after the incubation period and the supernatant fluid stored in the cold until needed. The pH of the culture fluid was adjusted to 6.4 just before the purification procedure. When the pH adjustment was done before storing, the pH returned to 8.2 after four days with a decrease in the enterotoxin activity. When stored at pH 8.2 for the same length of time, the enterotoxin activity and the pH remained constant.

Enterotoxin Assay

The standard curve obtained with the use of standard enterotoxin solutions is shown in Figure 1. The points corresponding to 150 and 200 $\mu\text{g/ml}$ showed a tendency to be displaced from the straight line connecting the points. Care was therefore taken that migration values in all further determinations fell within the linear part of the curve or below 8 mm. Sufficient dilution of the antigen was done whenever necessary. Duplicate samples usually gave measurements that differed only by 0.1 mm.

Freeze-dried standard enterotoxin and antiserum when hydrated were stored frozen for increased stability since no change in activity of either the enterotoxin or the antiserum was found after a single freezing and thawing. The antiserum solution was frozen in 0.5 ml portions and only one portion thawed at a time. The highest dilution of antiserum that gave

Figure 1. Standard curve for the single gel diffusion assay for enterotoxin using an antiserum dilution of 1:100. Band length was measured after 24 hours at 30°C



adequately visible precipitin bands was 1:100.

Ionic strength was shown to be a factor affecting the rate of migration of the precipitin band (Weirether et al., 1966). The ionic strength of the enterotoxin test solutions was therefore kept at 0.40 or above. The phosphate-buffered saline diluent containing an additional 2% NaCl had an ionic strength of 0.524.

The prepared gel diffusion tubes were kept in a humidified chamber at room temperature. Storing in the cold caused shrinkage of the gel which in turn caused leakage of the antigen solution between the gel and the tube walls.

Enterotoxin Purification

A volume of 9-10 liters of diluted culture fluid or approximately 300 mg of enterotoxin was purified at one time. Gel diffusion tests on the culture supernate showed two precipitin bands, the lower one of which was the enterotoxin band. The first ion exchange procedure adsorbed more than 95% of the enterotoxin from the culture fluid. The toxin was usually eluted after 60-70 mls of eluate, and the pooled fractions had a volume of approximately 90 mls. The average yield of the first column was 80%. Most of the contaminating substances were removed by this process, and the pooled fractions showed an average specific activity of 0.40 mg of enterotoxin (as measured by gel diffusion) per mg of protein (as measured by biuret test).

Dialysis of the pooled fractions against distilled water to reduce the salt content to 0.01 M brought about an 80-85% loss of the enterotoxin

activity accompanied by the formation of a white precipitate. Attempts to regain the activity by dissolving the precipitate in 8M urea and 6M guanidine hydrochloride were unsuccessful. Gel electrophoresis at pH 4.3 of the urea and guanidine hydrochloride solutions of the precipitate gave a number of bands, the darkest of which had the same migration as the pure enterotoxin. Recovery of the activity from the gel was very low. When the gel section of the enterotoxin band was eluted with the electrophoresis buffer, less than 1% of the activity was recovered. Even with the use of a sucrose chamber in the gel (Tan et al., 1969), only 4% recovery was made from an electrophoresis of the pure enterotoxin.

Since α -hemolysin is known to precipitate easily from solution, blood agar plate tests were made on the pooled fractions before and after dialysis. The pooled fractions before dialysis showed a small amount of lysin on blood agar plates while the dialyzed solution after removal of the precipitate was free of lysin.

A similar loss in activity was incurred when the crude culture fluid and purified enterotoxin were dialyzed against water. However, when dialysis of the supernate was done against NaCl at a concentration of 0.18 M or greater, the loss in activity was very small even after 8 hours of dialysis. The same was true for dialysis against 3% PHP solution.

To minimize the enterotoxin loss during dialysis, the pooled fractions were evaporated to half their volume on a rotary evaporator using a water bath temperature of 30°C. The vacuum was adjusted to prevent foaming of the solution. The loss of activity during the evaporation process depended on how well the foaming was controlled. Dialysis against water was done using

a predetermined minimum time necessary to reduce the salt content to 0.1 M. In this way the loss of activity was reduced to 20-30%.

The enterotoxin peak from the second column appeared after 10-15 mls of eluate and was contained in 60-70 mls of buffer. The overall yield after the second column was 30-40%. The specific activity of the pooled fractions was 0.50-0.70.

A typical elution plot from the third column is shown in Figure 2. The toxin peak appeared after approximately 250 mls of eluate and had a volume of about 150 mls. An average overall purification yield of 25% was obtained. The enterotoxin solution was approximately 0.01 M in phosphate when freeze-dried which accounted for the 75-80% salt content of the freeze-dried material. When dissolved in water the specific activity of the solution was 0.91 mg of enterotoxin (measured by gel diffusion) per mg of protein (measured by biuret test) and 1.6 mg of enterotoxin (measured by gel diffusion) per mg of protein (measured by the absorbance at 280 and using an extinction coefficient of 14 for a 1% enterotoxin solution). Bergdoll's purified enterotoxin B gave a specific activity of 1.0 using the latter method.

Test for Purity

Disc gel electrophoresis at pH 4.3 of the purified enterotoxin gave a faster moving major band and a slower moving minor band (Figure 3). The electrophoresis run lasted 50-60 minutes. The major band was about 8 mm behind the tracking dye front with a polyacrylamide concentration of 7.5%. The minor band started to appear when 278 μ g of enterotoxin was applied to

Figure 2. Typical elution plot of the CM-cellulose column. Tubes 44 to 74 were pooled for dialysis and freeze drying

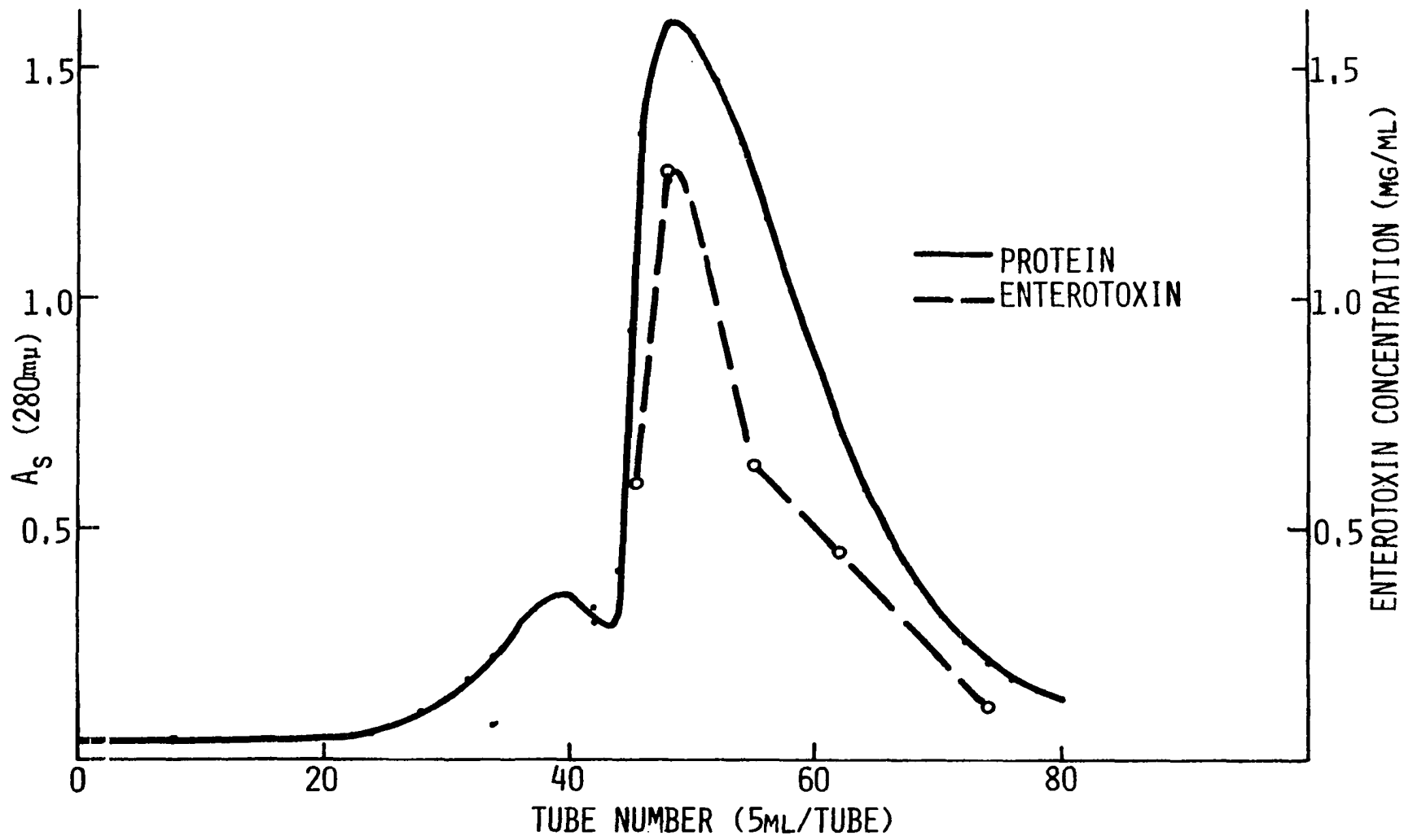
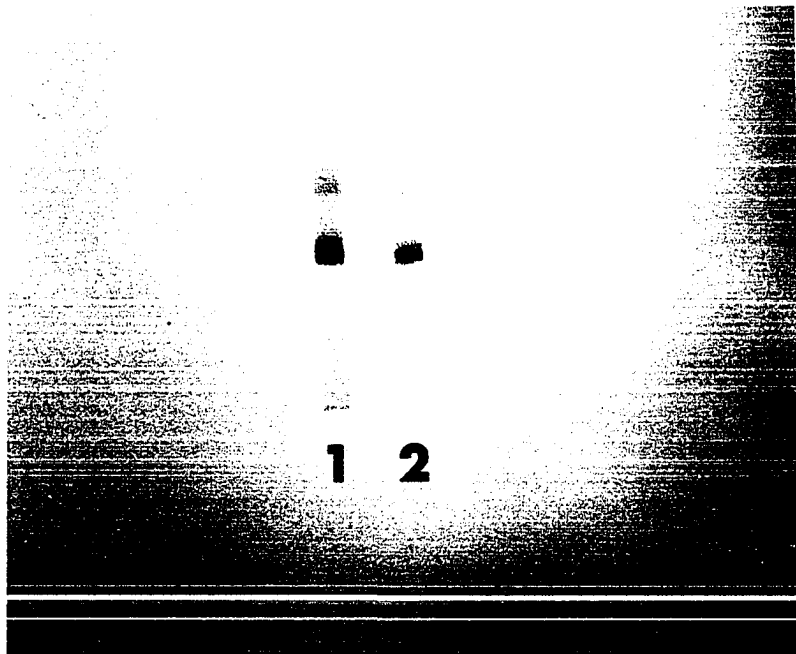


Figure 3a. Disc gel electrophoresis of enterotoxin B at pH 4.3. Running time, 50 min.

1. Gel containing 15 μg of enterotoxin
2. Gel containing 25 μg of enterotoxin supplied by M. S. Bergdoll, Food Research Institute, Wisconsin

Figure 3b. Disc gel electrophoresis of enterotoxin B at pH 7.5. Running time, 2 hrs.

1. Gel containing 15 μg of enterotoxin
2. Gel containing 7.5 μg of enterotoxin



the gel whereas the major band was already visible at an amount of 0.7 μg . The ratio of minor band protein to major band protein was therefore 1:400 thus the purity of the sample was estimated as greater than 99%.

Electrophoresis experiments on the lyophilized enterotoxin after it had been stored for some time showed an increase in the intensity of the minor band per unit weight of enterotoxin with increasing storage time. A densitometer trace showed an approximate doubling of intensity within a period of one month.

Electrophoresis of the purified enterotoxin using the system of Williams and Reisfeld (1964) at pH 7.5 gave a faster moving major band and a slower moving minor band which was more intense and wider than the minor band seen in the gels at pH 4.3 with equal amounts of enterotoxin (Figure 3). A densitometer trace showed three peaks composing the minor band. It took two hours of electrophoresis time to obtain satisfactory separation of major and minor bands. No suitable tracking dye was found for this gel system.

The system of Ornstein and Davis at pH 8.9 gave three bands plus a dark band that stayed at the top of the separating gel. This system was not used for further experiments since the enterotoxin appeared to be precipitating at the pH of the separating gel which is close to the isoelectric point of the enterotoxin.

Estimation of Size Difference between the Major and Minor Band

The size of the minor band protein was estimated in relation to the major band protein which was considered to be the pure enterotoxin. The migration of the two bands in electrophoresis at pH 4.3 with increasing

polyacrylamide concentration was measured. A plot of the logarithm of the migration rates relative to the dye front (R_m) against gel concentration gave two lines that intersect near 0% gel concentration (Figure 4). This is an indication that the major and minor band proteins are size isomeric proteins.

At a gel concentration of 15%, a third band was observed about 1 mm ahead of the major band. Figure 5 shows the gels at 12% and 15% polyacrylamide concentrations.

Stability of the Enterotoxin in Phosphate Buffer

In 0.10 and 0.15 M phosphate buffer solutions, enterotoxin with an initial concentration of 200-250 $\mu\text{g/ml}$ remained constant for three days. In 0.05 M phosphate, it was stable for one day but decreased 2.5% after the second day and 16.2% after the third day. In 0.02 M phosphate, the enterotoxin activity decreased after only six hours and had a 20% decline after three days.

Heat Inactivation

Varying the Temperature

Figure 6 shows the loss of activity with time at different temperatures. The time of heating as shown includes the come-up-time. The come-up-time was found to be 40 sec at 100°C, and initial samples were taken at 40 sec at all temperatures. Activity as measured by gel diffusion tests was almost completely gone after 20 minutes at 70°C and 15 minutes at 80°C but it took 60 minutes at 100°C and 90 minutes at 90°C to decrease the

Figure 4. Plot of the relative migration rates
(relative to the dye front) of major
and minor bands in gel electrophoresis
at pH 4.3

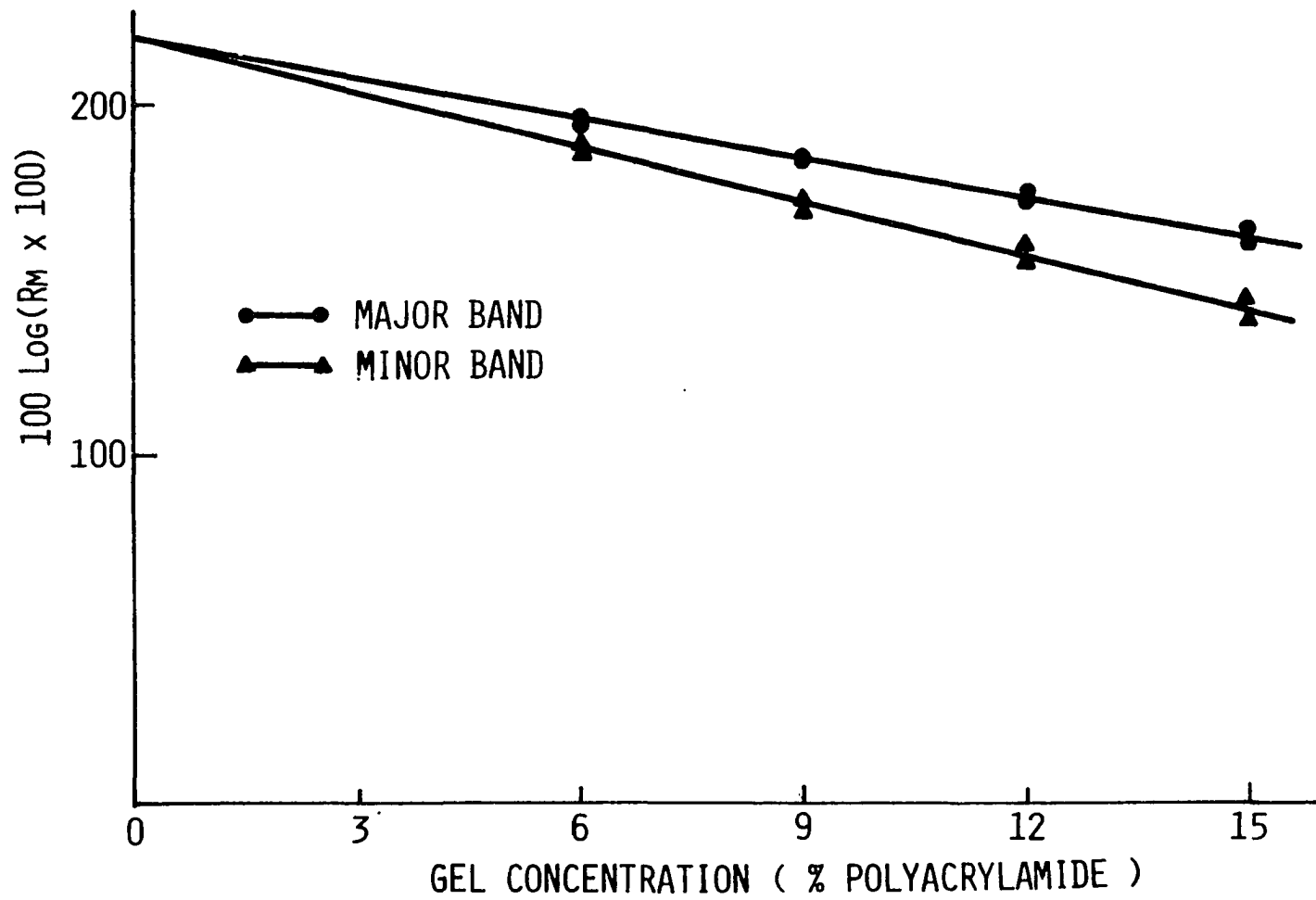


Figure 5. Disc gel electrophoresis of enterotoxin B at pH 4.3 using (A) 12% polyacrylamide gel (B) 15% polyacrylamide gel. Running time, 50 min.

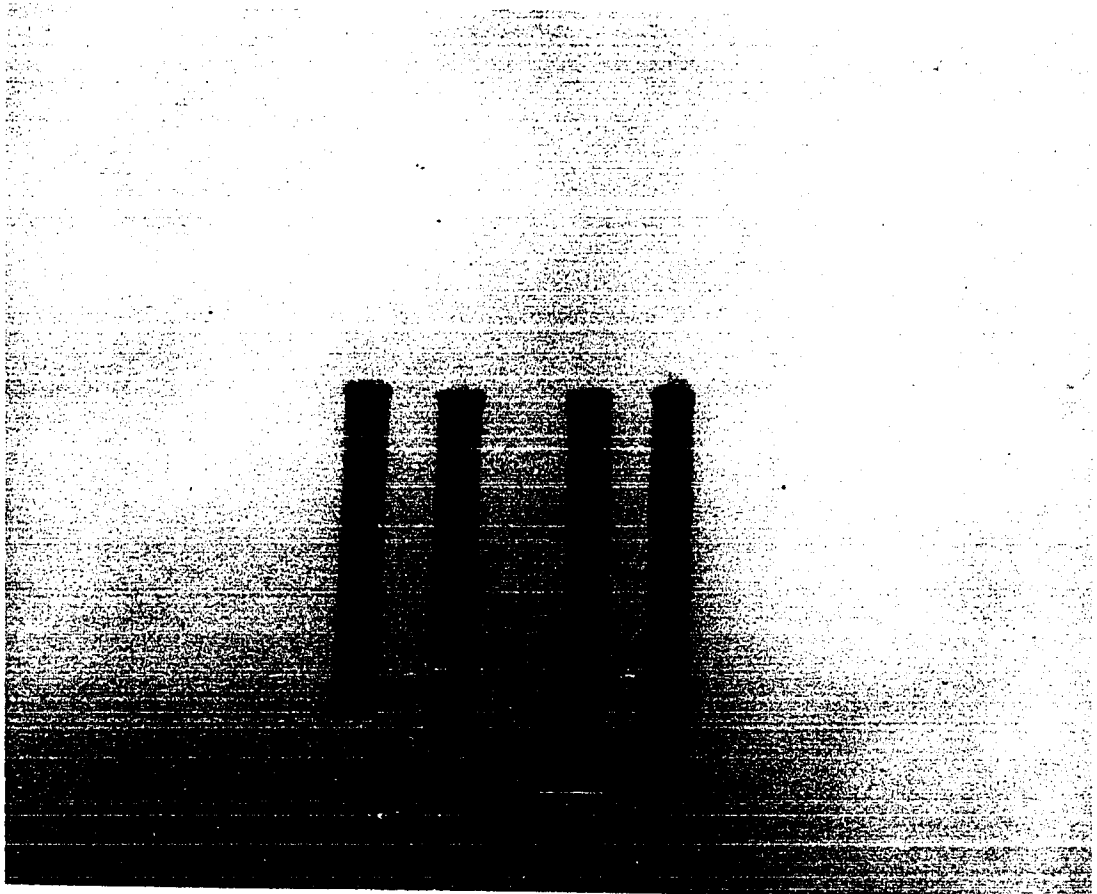
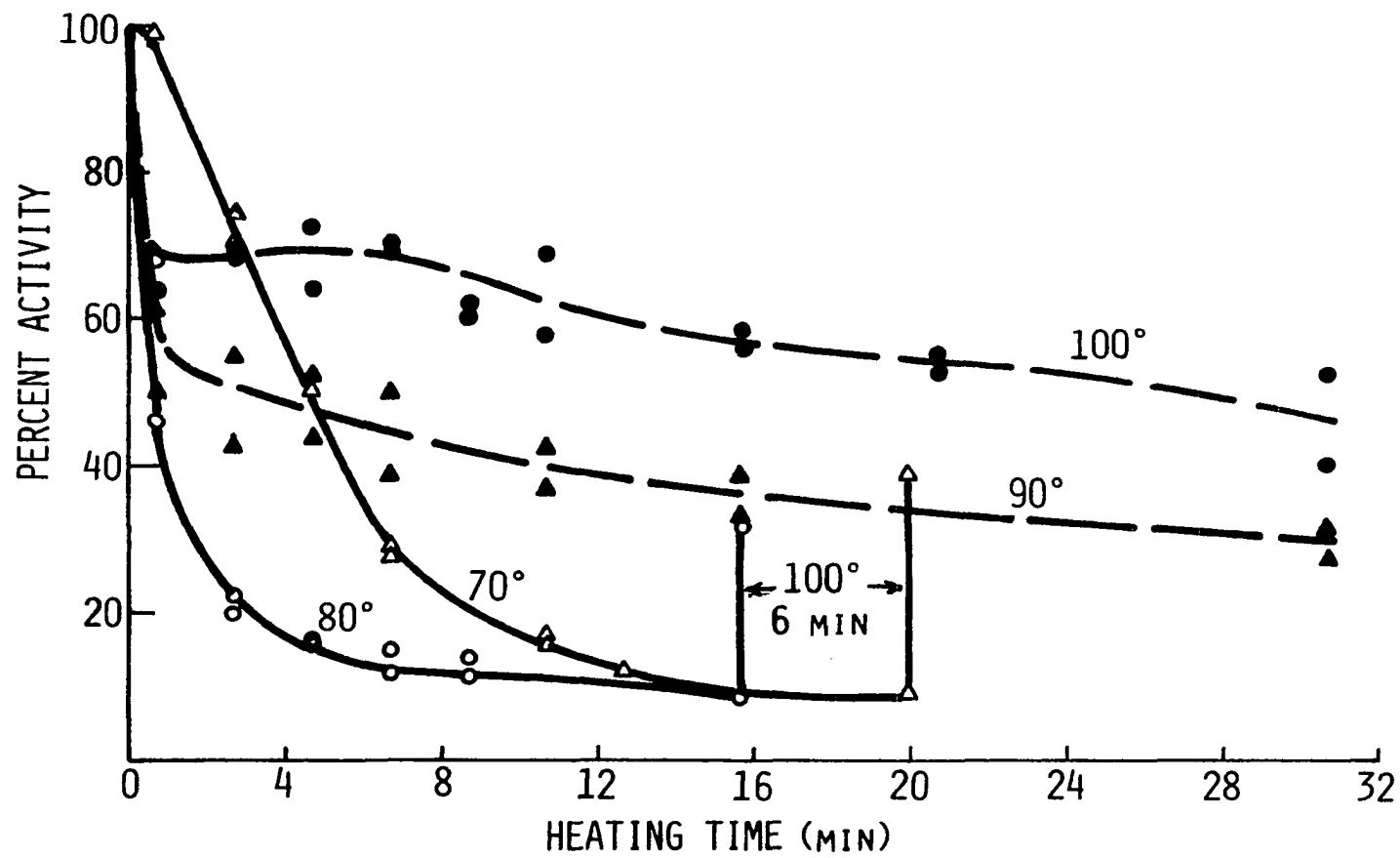


Figure 6. The inactivation of enterotoxin B with time at different temperatures. Conditions were: pH, 6.4; initial concentration, 100 $\mu\text{g}/\text{ml}$; ionic strength, 0.10. Samples heated at 70°C and 80°C were reactivated to the extent shown by heating at 100°C



activity to the same level. The heated solutions at 70° and 80°C were visibly turbid. The last 10% of the activity took a relatively long time to be inactivated and a residual activity still remained after 20 minutes at 70°C and 15 minutes at 80°C. Control samples which were kept at 30°C during the entire heating period did not show a decrease in activity.

Failure to immerse the tubes immediately in ice water after the heating time caused a rapid loss of activity at 100° and 90°C. The inactivation curves at these temperatures were displaced downward by 20-30%.

When the solutions inactivated at 70° and 80°C were heated at 100°C for 6 minutes, about 35-40% of the activity was restored. The solutions were also less turbid.

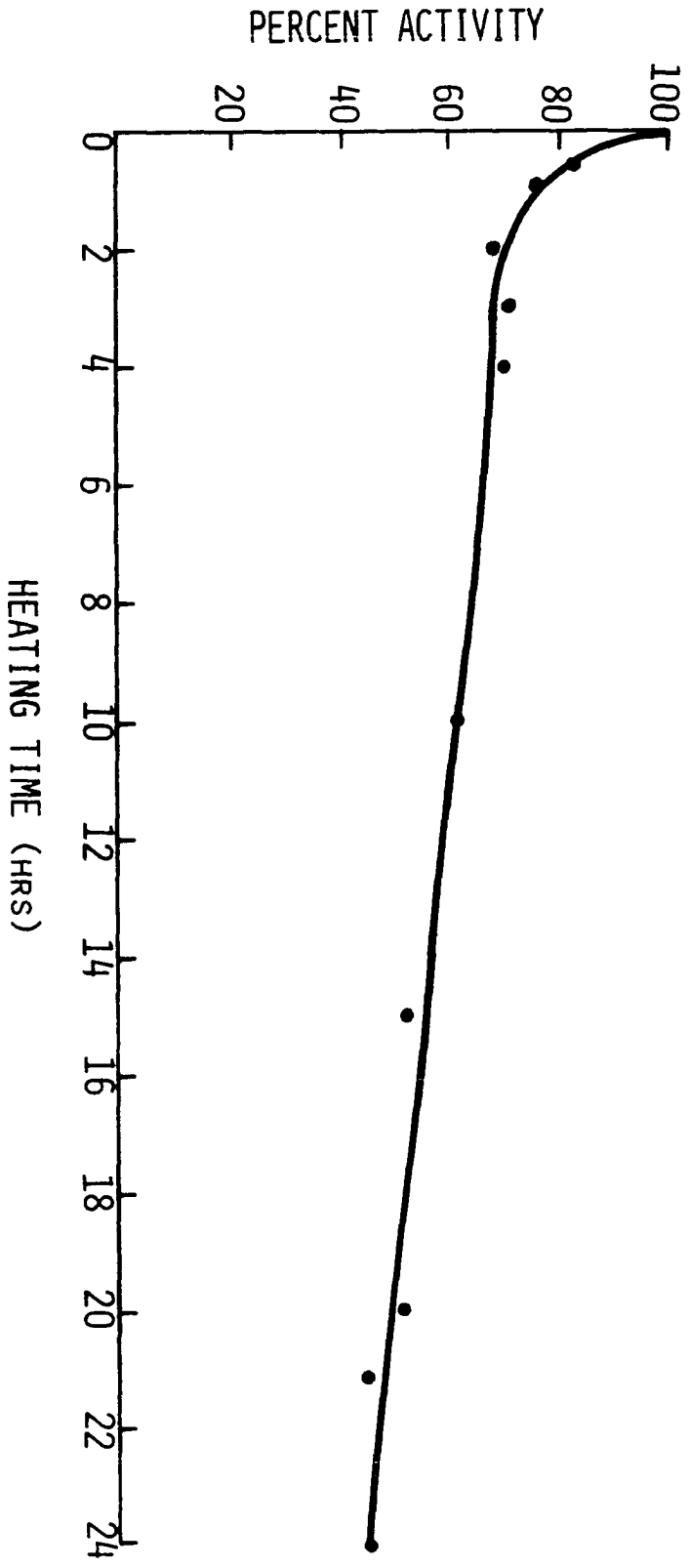
Heating at 60°C produced a slow inactivation (Figure 7). The most rapid decrease was seen during the first two hours wherein about 30% of the activity was lost. After 24 hours, the solution was still 50% active.

Gel electrophoresis at pH 4.3 of the enterotoxin solutions heated at 60°C for 24 hours, 70°C for 10 minutes and 100°C for 10 minutes showed only the major band at an intensity that corresponded to the amount of activity observed in gel diffusion tubes. Electrophoresis at pH 7.5 of the enterotoxin heated at 100°C for 10 minutes still showed the minor band.

Varying the pH

The temperature study was done at pH 6.4. When the pH was changed to 4.5 and 7.5, no considerable change was seen in the inactivation curves at 70°C as long as the ionic strength was maintained at 0.10. However, when heated at 100°C, the enterotoxin at pH 7.5 showed a very rapid loss of

Figure 7. The inactivation at 60°C of enterotoxin B
at pH 6.4; initial concentration, 100 µg/ml;
and ionic strength, 0.10



activity between 10 and 15 minutes (Figure 8). The 50% remaining activity after 10 minutes of heating was completely lost within the following 5 minutes. The inactivated solution, unlike the solutions heated at 70° and 80°C, was not turbid. Control samples kept at 30°C did not decrease in activity during the heating period.

Gel electrophoresis at pH 4.3 of the enterotoxin heated at 100°C for 10 minutes at pH 7.5 showed only one band corresponding to the major band of the unheated toxin. The intensity of the band correlated with the amount of activity as measured by gel diffusion tests. The unheated toxin in acetate buffer (pH 4.5) showed the same two bands as the toxin in phosphate buffers (pH 6.4 and 7.5).

A persisting residual activity was noticed during the heating at 70°C (see Figure 8b). To determine whether this activity was due to redissolving precipitate, the precipitate was centrifuged before the gel diffusion test. The residual activity remained at the same level as in the heated sample which still contained the precipitate.

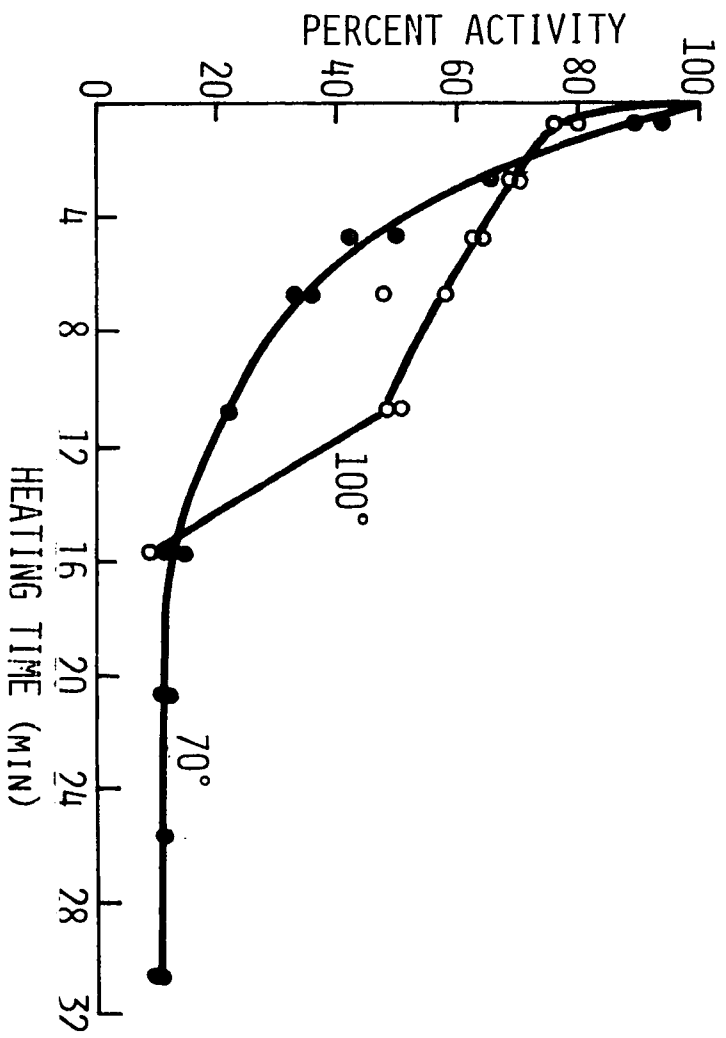
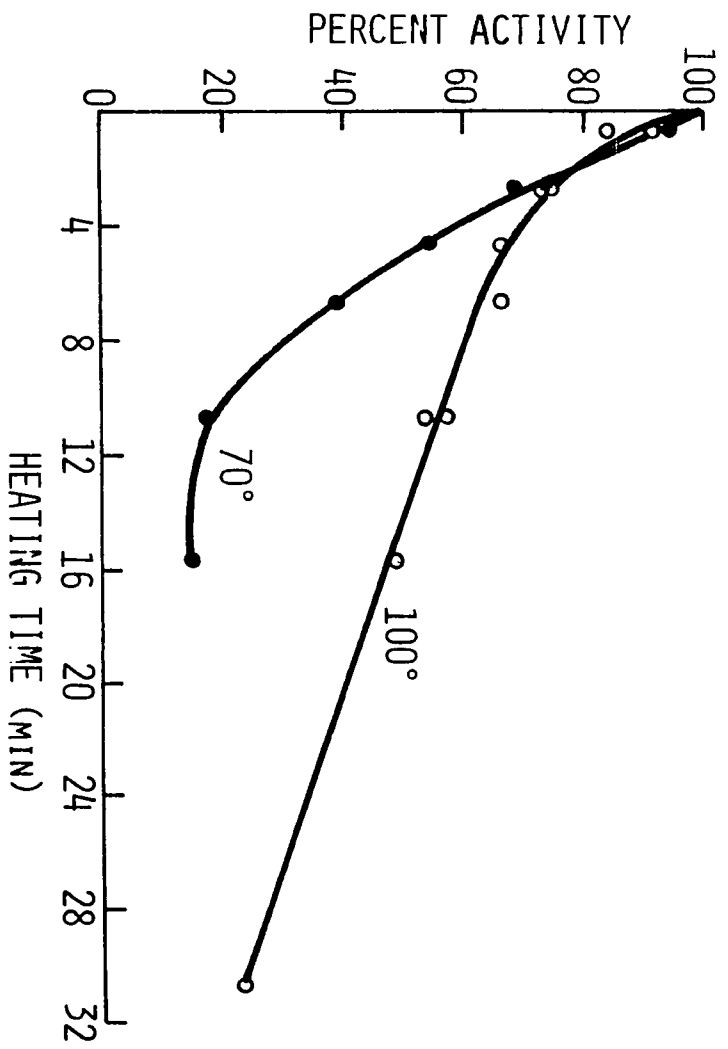
Varying the Ionic Strength

The effect of increasing the ionic strength at pH 6.4 was a gradual increase in the amount of remaining activity in solution after 8 minutes of heating at 70°C (Figure 9a). The increase in activity was only 15% between 0.10 and 1.0.

At pH 4.5, the ionic strength of the solution had an effect opposite to that observed at pH 6.4. With increasing ionic strength, there was increasing inactivation after 8 minutes of heating. A difference of 30%

Figure 8a. The heat inactivation of enterotoxin B at pH 4.5; initial concentration, 100 $\mu\text{g/ml}$; and ionic strength 0.10

Figure 8b. The heat inactivation of enterotoxin B at pH 7.5; initial concentration, 100 $\mu\text{g/ml}$; and ionic strength 0.10



remaining activity was observed between 0.02 and 0.10 (Figure 9b).

The inactivation of the enterotoxin at pH 4.5 and ionic strength 0.02 M was followed at 70° and 100°C. In contrast to the behavior of the solution at an ionic strength of 0.10 M, the inactivation of the 0.02 M solution at 70°C was very slow. After 30 minutes of heating, 45% of the activity still remained (Figure 10). There was 11% residual activity after 80 minutes of heating.

At 100°C, the initial inactivation was more rapid than at 70° but after 20 minutes of heating up to 80 minutes, the residual activity in both solutions had similar values. After 30 minutes of heating, a second shorter band appeared in gel diffusion tubes and was present also in the 60 and 90 minute heated solutions with the same band migration. However, it did not appear in the enterotoxin solution of ionic strength 0.10 and pH 4.5.

Varying the Initial Enterotoxin Concentration

At pH 4.5, an initial concentration of 80-100 µg/ml showed a minimum of inactivation when heated at 70°C for 8 minutes. As shown in Figure 11, a rapid drop in residual activity accompanied the increase in initial concentration. The inactivation of the solution containing 250 µg/ml was measured at different heating times. The inactivation curve was similar to that of the solution at pH 7.5 with an initial concentration of 80-100 µg/ml.

The effect of initial concentration at pH 7.5 was not as great as that at pH 4.5. The concentration which gave a minimum of inactivation was 60 µg/ml.

Figure 9a. The effect of ionic strength on the percent activity remaining in solution after 8 minutes of heating at 70°C and pH 6.4. Initial concentration, 100 µg/ml

Figure 9b. The effect of ionic strength on the percent activity remaining in solution after 8 minutes of heating at 70°C and pH 4.5. Initial concentration, 100 µg/ml

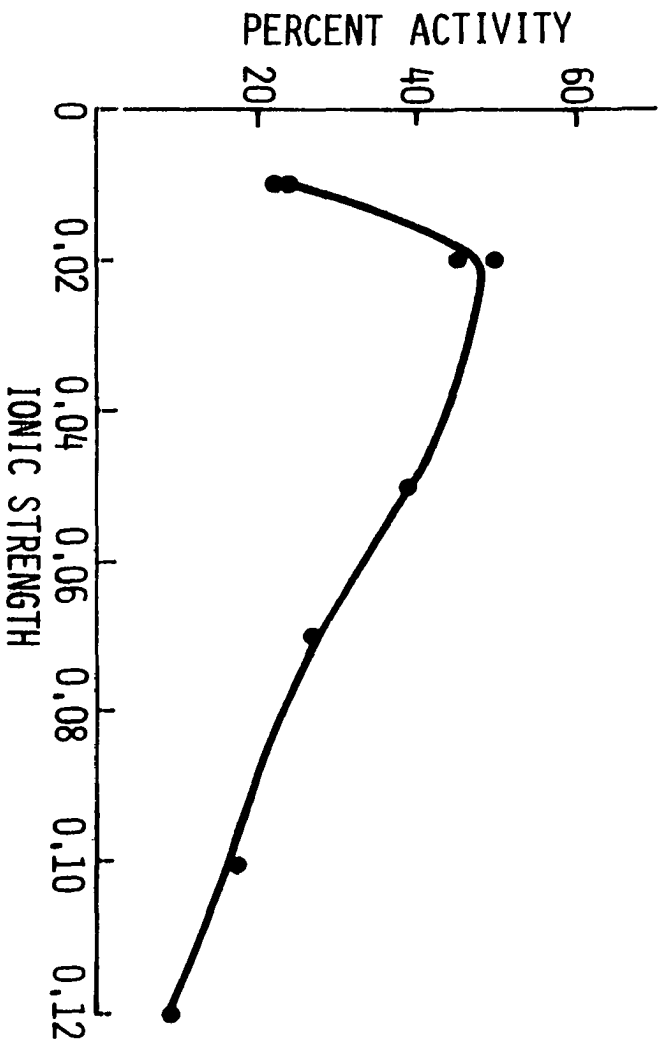
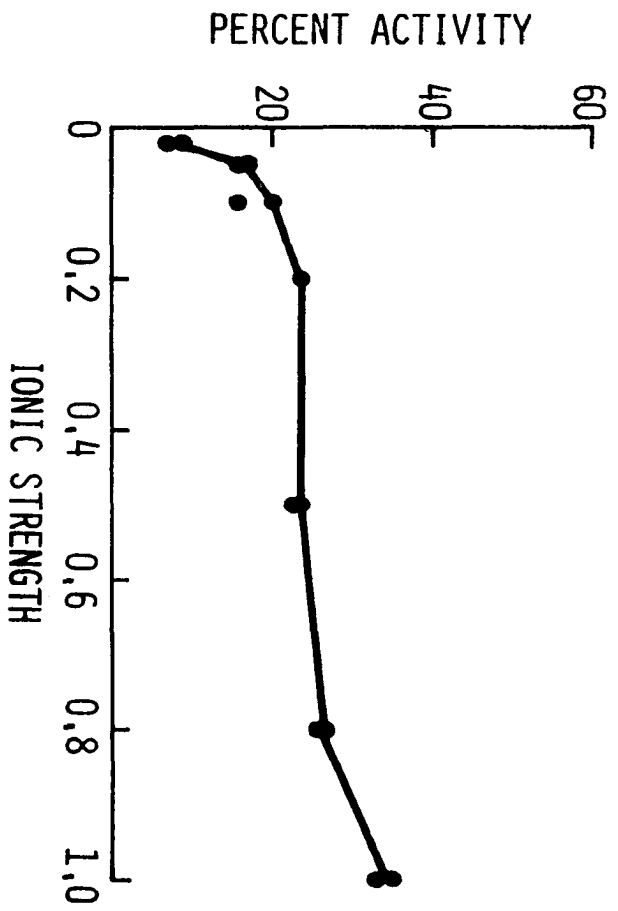


Figure 10. The heat inactivation of enterotoxin B at pH 4.5: initial concentration, 100 $\mu\text{g/ml}$; and ionic strength, 0.02

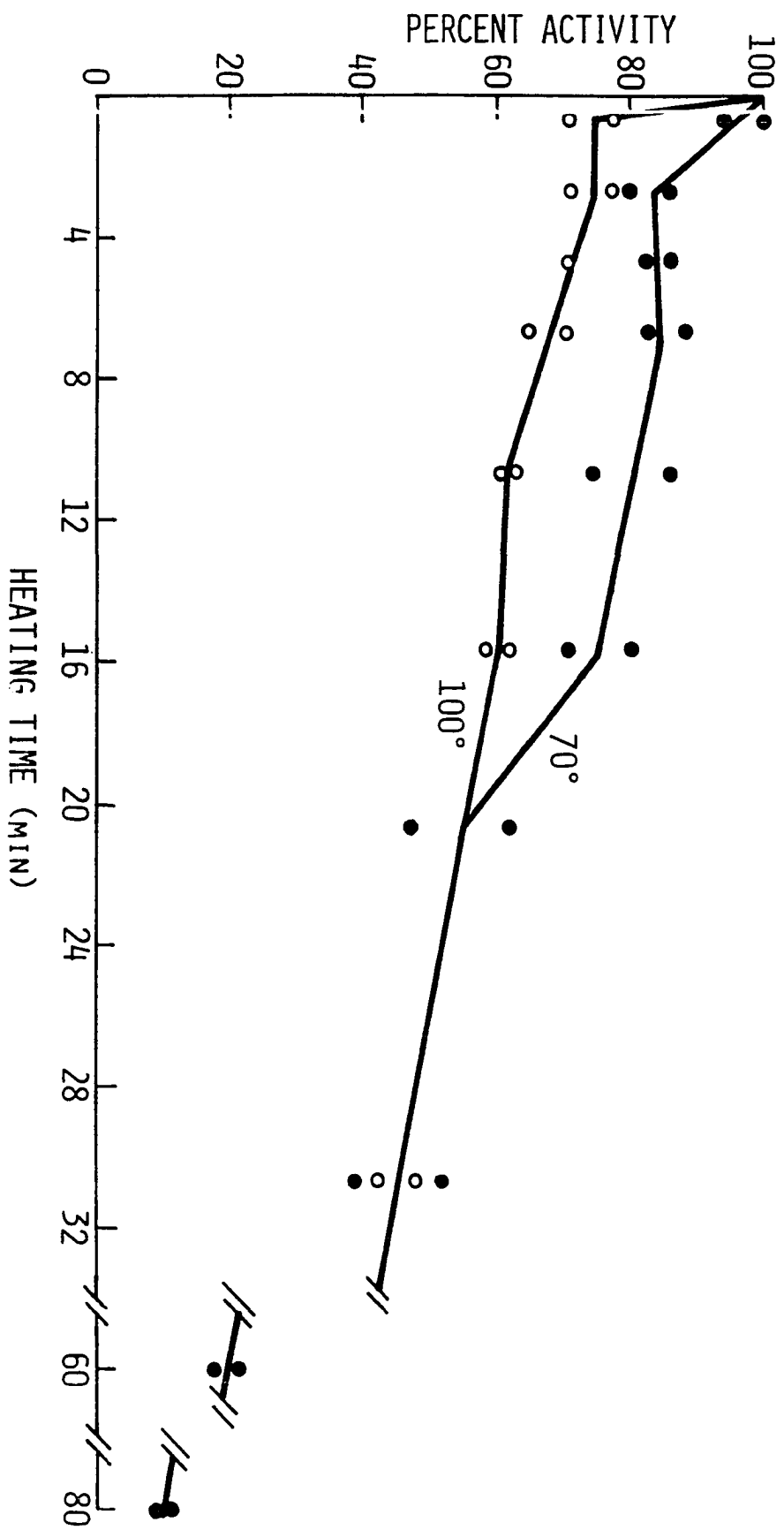
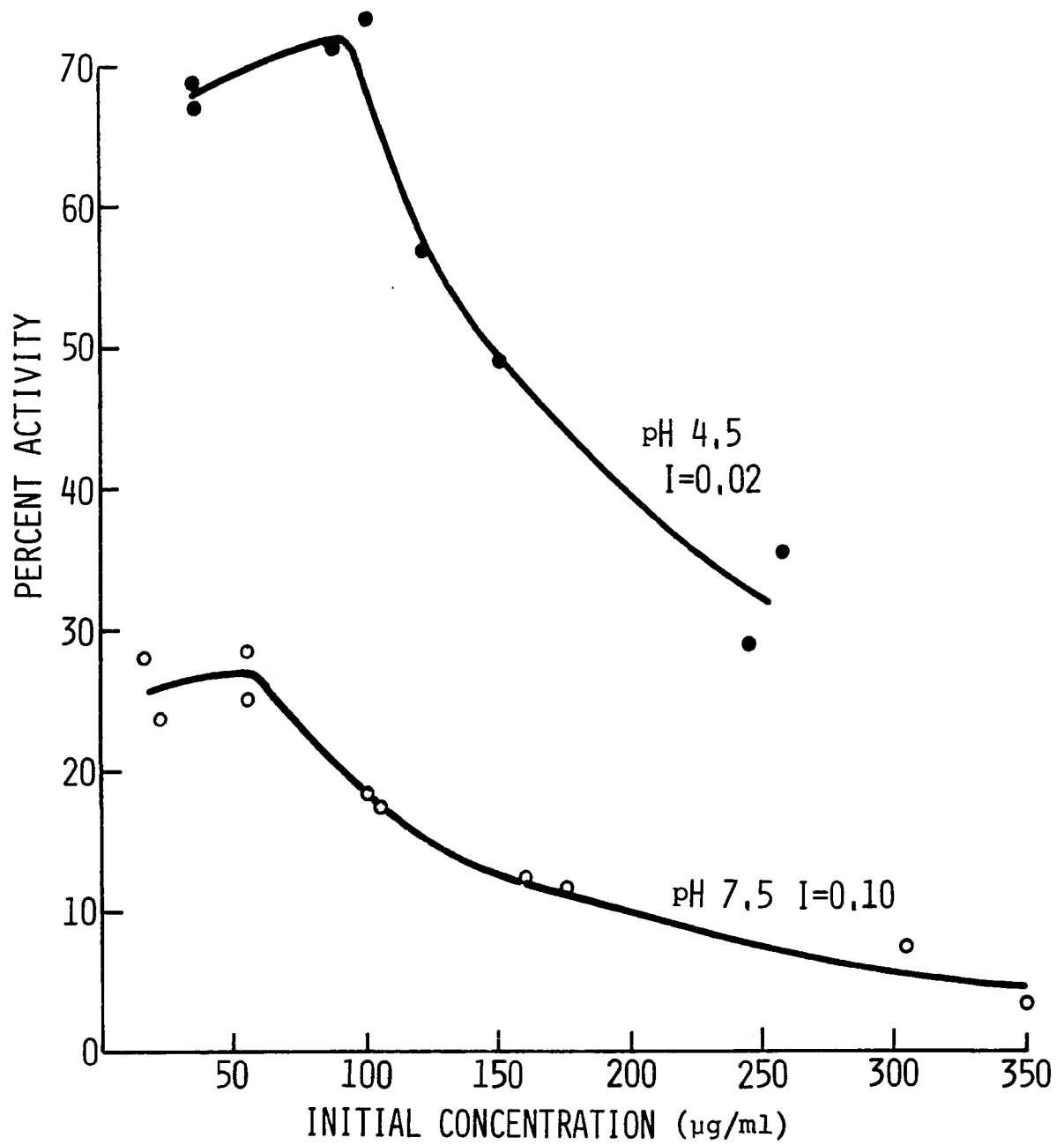


Figure 11. The effect of initial concentration of enterotoxin B on the percent activity remaining in solution after 10 minutes of heating at 70°C



Toxicity in Dogs

Three different dosages of the pure enterotoxin--0.10, 0.30, and 0.50 μg per kilogram of body weight--were tested using a single dog for each dose. All the dogs reacted positively. The dose of 0.30 $\mu\text{g}/\text{kg}$ of body weight was tested on another set of three dogs. A 100% positive reaction was obtained.

It was noted that the dogs were able to develop resistance to the enterotoxin after the second injection and possibly even after the first injection or within a period of 2-4 days. At the third injection, purified enterotoxin at a dose of 0.3 $\mu\text{g}/\text{kg}$ body weight failed to produce any reaction. Fresh dogs were used thereafter for each sample.

The enterotoxin solution at pH 6.4 which had been heated at 70°C for 15 minutes gave a negative reaction in three dogs. The heated solution was given at a dose of 0.30 $\mu\text{g}/\text{kg}$ based on the initial enterotoxin activity before heating. All the dogs showed sluggishness an hour after injection which lasted for about 2-3 hours but there were no other reactions.

The enterotoxin which had been inactivated at 70°C for 15 minutes and then reheated at 100°C for 6 minutes gave a positive reaction in two out of three dogs. One of the dogs reacted with vomiting an hour after the injection. This was followed by two more bouts of vomiting within the next two hours. The dog had recovered after five hours. The second dog had diarrhea two hours after the injection but no vomiting. The third dog did not show any reaction. The heated solution was given at a dose of 0.5 $\mu\text{g}/\text{kg}$ based on the enterotoxin activity before heating. The samples were given the heat treatment about 1 hour prior to injection.

The enterotoxin at pH 7.5, which had been heated at 100°C for 15 minutes and showed no turbidity and a complete loss of activity in gel diffusion tests, was tested on a single dog. A dose of 0.3 µg/kg based on the enterotoxin activity before heating was given to the dog. The dog reacted with vomiting and diarrhea in 1-1/2 hours after the injection.

DISCUSSION

Enterotoxin Production

The culture medium and incubation conditions suggested by Kato et al. (1966) were used in this study to produce enterotoxin B. From 50 mls of medium in a 250 ml Erlenmeyer flask, Kato et al. (1966) obtained 480 μg of enterotoxin B per ml while Reiser and Weiss (1969) reported a production yield of 255 $\mu\text{g}/\text{ml}$. Under the same conditions, we obtained only 200 μg of enterotoxin per ml. When 500-600 mls of medium in Fernbach flasks or 150-200 mls in 1 liter flasks was used, the enterotoxin production was increased to 300 $\mu\text{g}/\text{ml}$. Evidently, aeration is important in obtaining good enterotoxin production. Bubbling air into the medium or incubation on a magnetic stirrer did not seem to supply as much aeration as shaking on a gyratory shaker. The use of baffles in the dialysis flask of Herold et al. (1967) increased the production from 300 $\mu\text{g}/\text{ml}$ to 1100 $\mu\text{g}/\text{ml}$. The sac culture of Hojvat and Jackson (1969) which reportedly produced 1000 $\mu\text{g}/\text{ml}$ in two days gave us only 200 $\mu\text{g}/\text{ml}$. In this method, about 50 mls of culture supernate was obtained from 100 mls of medium.

The unusually high production yields of enterotoxin that have been reported might have resulted from a screening of the cells before inoculation. Sugiyama et al. (1960) showed that when a culture of the staphylococcal strain S-6 was streaked on a plate of antiserum-agar, the colonies that developed produced halos of antigen-antibody precipitate of different sizes. This indicated that cells of a single strain vary in their capacity to produce enterotoxin. Colonies which gave the biggest halos in antiserum-

agar plates could be picked and used for enterotoxin production. Kato et al. (1966) used this technique for increasing their production of enterotoxin A.

The loss of the enterotoxin producing ability with storage of the staphylococcal culture was noticed by Jordan and Burrows in 1935. Their cultures were maintained in veal or beef infusion broth. Successive transfers in starch veal infusion agar restored the toxigenic property of their bacterial cultures, and they attributed the recovery to the presence of starch in the medium. In our study, the enterotoxin producing ability of our inoculum cultures was restored by two successive transfers in brain heart infusion broth. The process of successive transfers probably served as a selective method which separated the actively growing cells rather than being an effect of medium constituents.

The final pH of the bacterial culture after 24 hours of incubation was 8.2. The enterotoxin in the centrifuged culture fluid seemed to be stable at this pH whereas adjustment of the pH to 6.4 brought about a loss of about 50% of the enterotoxin activity within four days and a return of the pH to 8.2. Enzymes present in the culture supernate might have been activated when the pH was decreased to 6.4. The enterotoxins are resistant to proteolytic enzymes but the other proteins in solution could have undergone proteolytic attack. Treatment of the culture fluid with urea and subsequent removal of the urea by dialysis gave a solution which showed 30% more enterotoxin activity than it had before the addition of urea. This was an indication that the enterotoxin had undergone a conformational change probably through hydrogen bonding but it could still refold to its

original conformation when these bonds were broken.

Enterotoxin Assay

In the standard curve for the single gel diffusion test, as the concentration of enterotoxin was increased, there was a tendency for the corresponding points on the plot to be displaced from linearity toward shorter band migration. When a great excess of antigen is applied on gel diffusion tubes, the antigen diffuses at a faster rate through the agar and the antibody present at each level meets with a large excess of antigen so that they combine in a ratio greater than the equivalence ratio. A band is thereby produced which is shorter than that which would have formed if the antigen and antibody combine at equivalence ratio.

The lowest dilution of antiserum that gave a visible precipitin band was used in order to satisfy the conditions for a linear relationship between the antigen concentration and the distance of band migration (Becker et al., 1951). The diffusion of antigen into the antibody layer should approach a true diffusion, thus, the concentration of antibody should be low enough in order that the diffusion of the antibody towards the antigen and the amount of precipitate formed as antigen combines with antibody can be considered to be negligible. In this way, the migration of the leading edge of the band can be considered as the free diffusion of a fixed concentration of antigen.

The amount of antigen solution on top of the agar column was fixed at 0.1 ml. Whether the length of the antigen layer has any effect on the migration of the precipitin band is a question that has not been fully

resolved. There are evidences to support both sides of the issue (Becker et al., 1951).

The ionic strength of the antigen solution affects the precipitin reaction. With increasing ionic strength, the amount of antibody that precipitates is reduced due to a shift in the equilibrium between the reactants and a consequent change in the equivalence ratio (Kabat and Mayer, 1961). Therefore when the ionic strength of the solution is increased, less antibody reacts with the same amount of antigen, and a longer band will be obtained in gel diffusion tubes. Weirether et al. (1966) showed that at ionic strengths below 0.4 in terms of NaCl, band migration increased rapidly with increasing ionic strength but between 0.4 and 1.0, the diffusion rate was constant. The antigen diluent used in our assay procedure contained 0.486 M NaCl and 0.017 M phosphate at pH 7.4 or a total ionic strength of 0.524. The diluent usually composed 80% of the antigen solution on top of the agar gel so that ionic strength could be maintained at 0.40 or above.

In the preparation of the gel diffusion tubes, Weirether et al. (1966) used tubes which were coated with agar and then dried. According to Preer and Telfer (1957), uncoated tubes can be used without danger of leakage of the antigen solution between the gel and the tube walls as long as the gel tubes were not stored in the cold. Long storage of the gel tubes at room temperature might be detrimental to the antiserum but in these experiments, the tubes were used within two to three days after preparation.

Enterotoxin Purification

In the purification procedure of Schantz et al. (1965), the culture

supernate which was obtained from a medium containing 1% PHP and 1% N-Z Amine was diluted with two volumes of water. Since the culture medium used in this study contained 3% PHP and 3% N-Z Amine, the culture fluid had to be diluted with eight volumes of water.

The first ion exchange column gave an average yield comparable to the yield obtained by Schantz et al. (1965). However, the loss of activity encountered during dialysis brought the overall yield down to 25% which is lower than the 50-60% yield reported by Schantz. The recovery of the enterotoxin from the precipitate formed in the dialysis sack was attempted by the use of urea and guanidine hydrochloride. According to Dalidowicz et al. (1966), enterotoxin B can be dissolved in urea and guanidine hydrochloride and full biological activity can be recovered after these reagents had been removed by dialysis. The precipitate obtained during dialysis of the enterotoxin solution was soluble in both urea and guanidine hydrochloride but the solutions, after dialysis, did not show any activity in gel diffusion tests. When the crude bacterial supernate was treated similarly, 68% of the activity was recovered from the urea solution and 36% from the guanidine hydrochloride solution.

The dialysis precipitate probably contained other substances besides the enterotoxin since gel electrophoresis of the urea and guanidine hydrochloride solutions showed a number of bands. Lysin must be one of the components of the precipitate as shown by the blood agar plate test. Electrophoresis of the pure enterotoxin B in urea or guanidine hydrochloride gave only two bands which shows that the other bands from the precipitate could not have been due to denatured enterotoxin species.

At low ionic strengths, it appears that the enterotoxin forms aggregates. The presence of other charged particles in solution in the form of salts or other proteins seem to stabilize the enterotoxin in solution. When the solution contains a sufficiently high concentration of enterotoxin, aggregation is also lessened. The enterotoxin is probably aggregating through the formation of hydrogen or hydrophobic bonds. Since the enterotoxin molecule has an excess of positive charges, the molecules will tend to repel each other. However, in a solution of low enterotoxin concentration and low ionic strength, the orientation of the hydrophobic regions towards the interior of the molecule and away from the charged solvent is relaxed and thus the enterotoxin molecules are probably in a looser type of configuration. Formation of intermolecular hydrogen or hydrophobic bonds could take place unless the enterotoxin concentration is low enough that the distances between molecules do not allow this to happen. The pooled fractions from the first ion exchange column have a sufficiently high concentration of enterotoxin (7-11 mg/ml) to allow aggregation to take place. When the enterotoxin concentration is raised sufficiently by evaporation, the repellent forces between the charged molecules may be stronger due to reduced distance between them and this may serve to keep the hydrophobic regions oriented toward the interior of the molecule and therefore prevent or diminish aggregation.

Nature of the Minor Band in Gel Electrophoresis

Schantz et al. (1965) estimated the purity of their enterotoxin preparation with the use of the Ouchterlony test. Two other precipitin bands

appeared at higher concentrations of the enterotoxin. The amount of dilution it took for the major and minor bands to disappear were determined, and the ratio of the two values was found to be 1:200. The purity of the sample was thus estimated to be greater than 99%. In our study, disc gel electrophoresis was used instead of gel diffusion. Electrophoresis at pH 4.3 should be able to detect any contaminating protein that has an isoelectric point above pH 4.3 and is present at a total concentration of at least 0.6-0.7 μg in the sample solution. The ratio of the amount of minor band protein to that of major band protein was 1:400. Schantz reported that more than 95% of their purified toxin formed the major band in gel electrophoresis using the same conditions. Their electrophoresis experiments in starch gel using 0.02 M borate buffer at pH 8.6 produced two components, the major band accounting for 60-70% of the enterotoxin and the minor band accounting for 20-30%. A rerun of the major band protein produced two bands in the same relative positions as those of the original solution. When the ionic strength was raised to 0.1 M, the toxin remained at the origin as a single band at pH 8.6 and travelled 1-2 cm from the origin at pH 7.0. They concluded that the separation into two components takes place at low ionic strength within the gel. Both components were reported to be toxic to monkeys.

Joseph and Baird-Parker (1965) conducted electrophoresis experiments at pH 8.5-8.68 and toxicity determinations on enterotoxin B and reported that the major band is three times more toxic than the minor band and that the two proteins probably differ only in charge and toxicity.

Chu (1968) mentioned the presence of a small amount of dimer in their

purified enterotoxin which was prepared according to the method of Schantz et al. (1965). He reported that the isoelectric point of the dimer was at pH 6.8.

The results of this study show that the minor band protein is a size isomer of the major band protein. Hedrick and Smith (1968) devised a method of differentiating between a family of charge isomeric proteins and a family of size isomeric proteins. They used disc gel electrophoresis at a separating gel pH of 7.9. The ratio of N,N'-Methylenebisacrylamide (Bis) to acrylamide in the separating gel had to be maintained at 1:30 to obtain reproducible results. The ratios of the migration rates of the bands to the migration rate of the tracking dye were plotted against the gel concentration. Charge isomers, or proteins that have the same mass but different net charge gave lines that were parallel while size isomers or proteins that have the same charge but different mass gave lines that intersected at or near 0% gel concentration. Non-isomeric proteins gave lines that intersect at some other points besides 0% gel concentration. As the gel concentration is increased, the mobilities of charge isomers are retarded equally since they have the same molecular weight thus giving lines of the same slope whereas size isomers are retarded differently, the isomer having a greater molecular weight being retarded to a larger extent than the isomer with lower molecular weight, thus giving lines with different slopes. Since size isomers have the same charge to mass ratio, the lines will intersect near 0% gel concentration. Figure 4 shows that the major and minor bands obtained in gel electrophoresis of enterotoxin B are size isomers. The minor band protein is probably the dimer mentioned

by Chu (1968).

At 15% gel concentration, a faint third band appeared ahead of the major band. This could be an indication of the presence of a contaminating substance which is similar to the enterotoxin in charge and size since it did not separate from the major band within the range of 6-15% gel concentration. The fact that in the 15% gel it migrated faster shows that it has a slightly lower molecular weight than the enterotoxin. On the other hand, the presence of another band in the gel need not indicate heterogeneity. Cann and Goad (1964) had shown that a number of proteins interact with uncharged constituents of acetate or carboxylic acid buffers at pH values lower than their isoelectric points giving a second peak in moving boundary or zone electrophoresis. Since the electrophoresis of the enterotoxin was conducted at an acid pH using β -alanine-acetic acid buffer, there is a possibility that the enterotoxin interacted with the undissociated acetic acid.

The gel system at pH 7.5 gave a major band and what appeared to be one minor band which was relatively wider and more diffuse in appearance than the minor band in the pH 4.3 gels. A densitometer trace showed three different peaks within the minor band. If these peaks correspond to polymers of enterotoxin B, it follows that under the conditions of the electrophoresis at pH 7.5, polymerization of the enterotoxin is enhanced. The minor band at pH 7.5 also appears at lower concentrations of the enterotoxin than at pH 4.3 (see Figure 3). The gel system at pH 7.5 uses Tris-veronal buffer with an ionic strength of 0.0003 while the system at pH 4.3 uses β -alanine-acetic acid buffer with an ionic strength of 0.012.

Therefore the enhanced polymerization of the enterotoxin could be due to an effect of pH or ionic strength or both. When the enterotoxin was stored at pH 7.5 for two days and subjected to electrophoresis at pH 4.3, no difference was observed from the enterotoxin stored at pH 4.3. If the increase in polymerization is an effect of pH, this is an indication that rapid reversal of the polymerization reaction can take place by a change to a more acid pH.

The amount of the minor band protein in gel electrophoresis at pH 4.3 was seen to increase with storage of the freeze-dried enterotoxin. The purified enterotoxin B obtained from M. S. Bergdoll contained a considerable amount of the minor band protein. Electrophoresis of 25 μ g of the toxin gave a dark minor band (Figure 3a). Since Bergdoll's group reported that their enterotoxin preparation contained at least 95% of the major band toxin, the increase in the percentage of the minor band could have developed during storage. A slow dimerization of the enterotoxin must be taking place during storage of the freeze-dried powder.

Heat Inactivation

Varying the Temperature

The rapid decrease in activity at 70^o and 80^oC must be due primarily to the accompanying aggregation of the enterotoxin. Part of the activity was recovered from the aggregated toxin when this was heated at 100^oC for six minutes. A similar behavior has been reported for staphylococcal hemolysin by Arrhenius (1907), for Bacillus cereus lecithinase by Chu (1949) and for Clostridium perfringens lecithinase by Smith and Gardner

(1949). The latter attributed the phenomenon to complex formation involving calcium or magnesium ions. This complex seemed to be formed at 65°C and dissociated at 100°C with consequent recovery of enzyme activity. Since the lecithinase used in their experiments was partially purified lecithinase, no definite conclusions were made.

In the heat aggregation of globular proteins, a distinction could be made between the aggregation that takes place at lower temperatures of molecules still in their native state or slightly modified and the aggregation at higher temperatures, of denatured molecules (Jaenicke, 1967). The former type of aggregation can be reversed at higher temperatures, especially in proteins which have no disulfide bonds, due to the lower bond energies involved but the latter type of aggregation is generally irreversible since intermolecular hydrophobic, ionic and disulfide bonds have been formed.

The aggregation of the enterotoxin at 70° and 80°C must involve molecules, a part of which still retain their native state or at least the immunologically active configuration so that subsequent release of the monomers by heating at 100°C restored 35-40% of the initial activity in gel diffusion test.

Gel diffusion tests do not measure directly the toxicity of the enterotoxin but they have given parallel results when the toxicity was tested in monkeys (Schantz et al., 1965; Chu et al., 1969). The loss of activity in gel diffusion when the enterotoxin was heated at temperatures between 96° and 127°C was reported to be closely correlated with the loss of emetic activity in cats (Read and Bradshaw, 1966). Since the enterotoxin which

had been heated at 70° and 80°C contained aggregated particles which could not diffuse into the agar, gel diffusion could not show whether the aggregated particles have lost their antibody recognition site. Even if the immunologically active site were still intact, the actual toxicity of the aggregated toxin would still have to be tested. Although gel diffusion and animal tests have hitherto been reported to be closely correlated which means that the toxic site and the immunologically active site are quite closely related, still the inactivation of one active site might not always bring about the inactivation of the other. The toxicity of the aggregated enterotoxin was therefore tested by intravenous injection into dogs. The dose at which it was administered (0.3 µg/kg body weight based on the activity before heating) was such that if the aggregated particles should retain full activity, it should give a 100% positive reaction and even if its activity should decrease to one-third of the initial activity, it should still show a positive reaction. However, if the remaining activity would be equal to that which was measured by gel diffusion which was 10% of the initial activity, the reaction in dogs would be negative. A negative response was obtained from three dogs. This shows that the aggregated enterotoxin does not cause emesis when administered intravenously.

Oral administration of the aggregated enterotoxin might give a positive reaction since the enzymes of the digestive tract could be capable of releasing the monomers in an active form. On the other hand, the monomers might be more labile to proteolytic attack after having undergone heat treatment.

The enterotoxin which had been inactivated at 70°C and then reheated at 100°C for 6 minutes and which showed a recovery of 35-40% of the activity was also tested in dogs. The heated toxin was given at a dose of 0.5 µg/kg of body weight, based on the initial activity, so that with 40% remaining activity, the effective dose would be 0.2 µg/kg which should still be emetic in dogs. A positive reaction was obtained in two out of three dogs which indicated that the toxicity of the aggregated particles had also been restored by heating at 100°C together with the immunological activity.

The inactivation curves at 90°C and 100°C showed a very sharp decrease in activity during the come-up-time. This amount of decrease must depend on how long the temperature stays between 70°C and 90°C. Capillary tubes were used to shorten this lag time. Initially, thermal death time tubes were used which required a come-up-time of 4-1/2 minutes during which time, 80% of the activity disappeared. A slight recovery of the activity could be noticed after a few minutes of heating at 100°C after which the activity started to decrease again. It took 60 minutes for the activity to drop to 10% of the initial activity. Read and Bradshaw (1966) reported an inactivation time of 87.1 minutes at 100°C for pure enterotoxin B at pH 7.2 and initial concentration of 30 µg/ml. The inactivation curves at 90°C and 100°C are a measure of the true denaturation of the enterotoxin molecule unlike the decrease in activity at 70°C and 80°C which was due to aggregation of the slightly denatured or undenatured molecules.

The rate of cooling has also an effect on the activity of the solutions heated at 90°C and 100°C. When the capillary tubes were not immersed immediately in ice water, the inactivation curve at 100°C was displaced

20-30% lower than the curve shown in Figure 6. The inactivation curves at 60°, 70° and 80° were not affected by the heating lag and the cooling rates.

Gel electrophoresis at pH 4.3 of the solutions heated at 100°C for 10 minutes, 70°C for 10 minutes and 60°C for 24 hours showed only a decreasing enterotoxin band but did not reveal the presence of any denatured or transformed enterotoxin. The migration of the single band seen in the gel was the same as the migration of the major band of the unheated enterotoxin solution since the electrophoresis of samples containing both the heated and unheated toxin gave only two bands. The minor band from the solution heated at 100°C for 10 minutes was still visible in gel electrophoresis at pH 7.5.

Varying the pH

The change of the pH of the solution did not seem to affect the inactivation of the enterotoxin at 70°C within the pH range 4.5 to 7.5. However, it has a considerable effect on the inactivation at 100°C. At pH 7.5, the enterotoxin was very rapidly inactivated between 10 and 15 minutes of heating while the portion of the inactivation curve below 10 minutes did not differ from that at pH 4.5. The optimal pH for heating at 100°C seems to be 6.4 which has the least decrease in activity after 30 minutes of heating.

The inactivated enterotoxin at pH 7.5 did not undergo aggregation. When injected into a dog at a dose of 0.3 µg/kg body weight based on the initial activity, it caused emesis. The solution is therefore still toxic although it has lost its ability to react with its antibody. Gel

electrophoresis at pH 4.3 of the solutions heated for 10 and 15 minutes both gave only a single band which appears to have the same migration as the major band of the unheated enterotoxin.

As the pH is changed from 4.5 to 6.4 to 7.5, the isoelectric point of the enterotoxin is approached and the net positive charge on the molecule decreases. Chu et al. (1969) found, through chemical modifications of the amino groups in enterotoxin B, that the positive charges on the molecule are essential to maintain the conformation that is biologically active and that they play a role in the binding of the antigen to its antibody. When the positive charges were decreased by acetylation and succinylation of the amino groups, both the emetic activity and the ability to bind with the antibody were reduced. In our experiments, where no chemical modification has been induced but a modification brought about by heating in the presence of an increased amount of negative charges in solution, only the ability to react with the antibody was lost. The conformation of the molecule that is necessary for toxicity is still maintained possibly due to the fact that the positive charges that contribute to maintaining the toxic site were not affected by the change in the pH of the solution. This shows that one biologically active site could be inactivated without causing the inactivation of the other.

Chu et al. (1969) stated that a decrease in net positive charges caused expansion of the molecule. The heating of this expanded molecule at 100°C probably brought about the changes that led to the loss of the immunologically active site of the enterotoxin. At or near pH 6.4, which seems to be the optimal pH, the amount of net positive charges is probably

the normal amount necessary for the compact conformation of the molecule. When the pH is further decreased to 4.5, an increase in inactivation is again noticed at 100°C. The excess of positive charges probably produces repulsion between residues within the molecule which again causes expansion. Part of the difference between pH 4.5 and 7.5 with regard to extent of inactivation might be due to a specific ion effect. These changes in the conformation of the molecule are not noticed when the molecules are not subjected to heat treatment. In the unheated enterotoxin, both the emetic and antibody recognition sites are active within the pH range 4 to 10 (Schantz *et al.*, 1965). A similar effect of pH was reported by Frolov (1966) in which crude enterotoxin solutions were heated at 100°C for 30 minutes and injected into cats. When the pH of the solution was 7.0, only vomiting was observed in the cats; at pH 4.5, vomiting and diarrhea occurred and at pH 3.8, the cats had only diarrhea. This could indicate that the reaction was most severe at pH 4.5 but that at pH values above and below 4.5, the activities of the heated solutions were less.

Varying the Ionic Strength

The effect of varying the ionic strength of the enterotoxin solution at pH 6.4 was a gradual decrease in the amount of inactivation when the toxin was heated for 8 minutes at 70°C. This result is in accord with the usual effect of ionic strength on proteins in solution. The presence of charged particles in solution serves to orient the molecules such that the polar regions are oriented towards the solvent molecules and the non-polar or hydrophobic regions are oriented away from the solvent. This orientation

contributes to the stability of the molecules in solution. The increase of ionic strength increases the stability up to a certain point when further addition of charged particles start to "salt-out" the protein from solution. At low ionic strengths, the molecule can be unfolded more easily than at high ionic strengths. Heating will therefore produce more denaturation in the low ionic strength solutions. In the case of enterotoxin B, a plateau exists between ionic strengths of 0.1 to 0.8 within which no considerable effect of ionic strength can be seen. However, below 0.1 and above 0.8, the effect of ionic strength is more pronounced.

At pH 4.5, the effect of varying the ionic strength of the solution was contrary to that observed at pH 6.4. When ionic strength was decreased below 0.10, inactivation at 70°C was decreased. It seems to be an effect of both the pH and the ionic strength since the solution at pH 6.4 and ionic strength 0.02 also underwent considerable inactivation. One possible explanation for this phenomenon might be that if at pH 4.5 the molecules are in an expanded state due to an excess of positive charges, this expansion of the molecule might expose some hydrophobic regions to the high ionic strength solvent in which case it will tend to seek a lower energy conformation by bonding with the hydrophobic parts of other molecules and form intermolecular hydrophobic bonds with consequent aggregation of the particles. In a low ionic strength solution, the repulsion between the solvent and the exposed hydrophobic areas is not as great as in the higher ionic strength solution and therefore the tendency to seek other hydrophobic regions is not as great. In Figure 9b, the conditions just described might exist between ionic strengths 0.02 and 0.12. With

ionic strengths below 0.02, the molecules might be in a more relaxed conformation due to lack of opposite interactions from the solvent and thus be free to form intermolecular bonds.

A second precipitin band was noticed after 30 minutes of heating the enterotoxin solution at pH 4.5 and ionic strength 0.02 at 100°C. This second band did not appear in the solution of ionic strength 0.10 when heated under the same conditions nor in the solution of ionic strength 0.02 when heated at 70°C for 30 minutes although the remaining activity of the latter solution equaled that of the solution heated at 100°C for 30 minutes. It could not be due to a redissolving precipitate since the heated solution is clear. Since the band length remained constant from 30 minutes to 90 minutes of heating, there is a possibility that it is due to an intermediate state of the enterotoxin as it undergoes transformation from the native state to the denatured state. The amount of enterotoxin at the intermediate state remains constant. At this state, the enterotoxin is probably still reactive with its antibody but its diffusion rate has changed so that a second band appears in gel diffusion.

Varying the Initial Enterotoxin Concentration

As mentioned earlier, increasing the concentration stabilizes a protein in solution due to an increased interaction between molecules. However, the decreasing distance between molecules is also favorable to the formation of intermolecular bonds when the intramolecular bonds are disrupted, in this case by heat. In Figure 11, the two curves show a similar trend. An optimal concentration is obtained at which there is a minimum

of inactivation. At higher concentrations, aggregation is favored by a decreased distance between molecules while at lower concentrations inactivation is increased due to a decreased stability of the molecules in solution.

At pH 4.5 and ionic strength 0.02, the effect of an increase in concentration is greater than at pH 7.5 and ionic strength 0.10. It is understandable that at a low ionic strength, the addition of a certain amount of charged particles creates a greater effect than the addition of the same amount to a solution which already contains a large amount of charged particles. However, a specific ion effect might be also involved.

At an initial enterotoxin concentration of 250 $\mu\text{g/ml}$, the effect of the low ionic strength of the solution was counterbalanced by the high concentration so that the extent of inactivation equaled that of the solution at pH 7.5 and ionic strength 0.10 with an initial concentration of 80-100 $\mu\text{g/ml}$.

Dog Injection Tests

The results of the dog injection tests have been discussed earlier. The use of dogs as the test animals gave satisfactory results except that the use of a dog was limited to one experiment due to the development of resistance to the enterotoxin. Under ordinary conditions, immunity to a soluble antigen will develop only over a period of several weeks. The dogs used in these tests were stray dogs which were chosen after observation for a certain period of time for disease. Being stray dogs, there is great probability that they had been challenged previously with the enterotoxin

so that a single injection was sufficient to increase the amount of antibodies in the animal.

SUMMARY AND CONCLUSIONS

The heating of aqueous solutions of purified enterotoxin B under varying conditions of temperature, pH, ionic strength and initial concentrations showed three different kinds of changes in the properties of the enterotoxin. These changes can be enumerated briefly as: 1) a low temperature aggregation which can be reversed by heating at higher temperatures, 2) loss of both the toxic and the immunological activity of the enterotoxin, and 3) loss of only the immunological activity with retention of the emetic activity.

The first kind of change occurs at 70° and 80°C in all conditions of pH, ionic strength and initial concentration except under the conditions of a low ionic strength at pH 4.5. The aggregation is attributed to the formation of intermolecular noncovalent bonds which could be disrupted by higher temperatures so that when heated at 100°C for a few minutes, the monomers could be released and part of the activity recovered. In this kind of aggregation, the monomers had not yet undergone any considerable denaturation. The aggregation is favored by a low ionic strength, except at pH 4.5, and a low initial concentration.

The loss of the emetic activity, as tested by intravenous injection into dogs, and the loss of the reaction with the antibody accompanied both the aggregation reaction at lower temperatures and the actual denaturation of the enterotoxin at higher temperatures. There is a possibility that oral administration of the aggregated enterotoxin to animals might give a positive reaction but this test was not performed in this

study.

The loss of the reaction with the antibody without loss of emetic activity was noticed in the enterotoxin solution at pH 7.5 when heated at 100°C for 15 minutes.

The purified enterotoxin undergoes a polymerization reaction, probably a dimerization, during storage in solution and in the freeze-dried state. This polymerization reaction is favored by low ionic strength and a pH value that approaches its isoelectric point (7.5 and above). There are indications that the polymerization is rapidly reversed by a change to conditions of higher ionic strength and more acid pH. The dimer composes the second band seen in gel electrophoresis of the enterotoxin at pH 4.3 with a gel containing 7.5% acrylamide.

In view of the results of this study, the immunological tests for the enterotoxin cannot be used as the sole indicator of biological activity especially in studies involving changes in the enterotoxin molecule. Even animal injection tests need to be supported by parallel feeding tests which most closely approach the actual mode of attack of the enterotoxin.

The presence of an optimum pH value in the inactivation of the enterotoxin at 100°C might indicate that the pH of foods would have an effect on the length of thermal processing necessary to inactivate any enterotoxin that may be present. Since this study was done using purified enterotoxin, the actual results might be different when the enterotoxin is combined with food constituents.

If the dimer which had been identified in this study is the same second band seen in gel electrophoresis by a number of investigators, then its

toxicity is only one-third of that of the monomer. This would mean that at the conditions favoring the formation of the dimer, the enterotoxic sample will be less active.

The fact that the enterotoxin is relatively heat resistant as compared to other proteins still remains. The phenomenon of lower temperature aggregation and reversal of aggregation by higher temperatures had been reported for several proteins but at a much lower temperature scale. Whether heating at 70° or 80°C could actually inactivate the enterotoxin faster than heating at 100°C still needs clarification for enterotoxin in foods and for enterotoxin that is fed rather than injected.

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