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TECHNICAL MANUSCRIPT 246

RAPID QUANTITATIVE SEROLOGICAL ASSAY OF STAPHYLOCOCCAL ENTEROTOXIN B

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Ralph E. Lincoln

SEPTEMBER 1965

UNITED STATES ARMY
BIOLOGICAL LABORATORIES
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DIRECTORATE OF DEVELOPMENT

Project 1C522301A05901

September 1965

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ABSTRACT

A simple, rapid method, based on the Oudin single gel diffusion technique, is described for the quantitative assay of staphylococcal enterotoxin B. The method yields reproducible results without close control of such assay variables as temperature, antiserum dilution, and assay time, provided that the ionic strength is maintained above 0.2 N sodium chloride equivalent.

I. INTRODUCTION

Oudin[1] reviewed the literature on simple diffusion techniques for assay of antigens in which antiserum is incorporated in a tube of soft agar and overlaid with antigen. Upon incubation, a band of precipitate forms at the interface and migrates down into the agar; the rate of migration is directly related to the concentration of the antigen.

The Oudin technique has been applied to the assay of the emesis-producing enterotoxin B from Staphylococcus aureus cultures.[2-6] The assay, as customarily conducted, requires the measurements of diffusion band movement on three successive days while the assay tubes remain immersed in a constant temperature water bath. Standard reference curves are obtained for each lot of antiserum by plotting the length of the precipitate band against the square root of time. A second graph then is made by plotting the slope of the line thus determined against the logarithms of the respective antigen concentrations. A modified Oudin method for the assay of enterotoxin B is described in this report.. Certain variables that affect the test are quantitated.

The method to be described is practical, simple, and rapid. It uses low-cost standard equipment and yields quantitatively reproducible results. The band length is a function of toxin concentration at any time after 4 hr. Therefore, accurate assays of enterotoxin B can be obtained based on the diffusion distance if the band length of standard reference solutions is measured within 30 minutes of measuring the band length of unknown enterotoxin solutions. Close control of variables, i.e., temperature, time of incubation, dilution of antiserum, method of incubation (air or water bath), is not critical because standard reference enterotoxin solutions and unknown toxin samples are all subjected to the same conditions at the same time. Enterotoxin values are simple to calculate and are obtained within 20 to 24 hr. Accuracy, precision, and reproducibility of the assay equal or exceed the conventional simple immunodiffusion techniques described by Oudin[1] and modified by Silverman.[3]

II. MATERIALS AND METHODS

Gel-diffusion tubes containing antiserum specific for enterotoxin B in soft agar are overlaid with known or unknown antigen (enterotoxin B) samples. The length of each zone of precipitation is measured after all tubes have been incubated at 30 C for 20 to 48 hr. A reference curve of known toxin concentration vs. band length is plotted on semi-log paper and a regression line drawn. Values of unknown samples assayed concurrently are then taken directly from the standard reference curve.

A. ENTEROTOXIN B STANDARD

Highly purified, lyophilized, enterotoxin B preparations, used to prepare standards in each group of assays, were supplied by Dr. E.J. Schantz. The amount of purified toxin in a particular sample used as a standard was based on the Kjeldahl nitrogen content multiplied by 6.2. These preparations were derived from S-6 cultures and purified by procedures described by Schantz et al.[8]

B. SPECIFIC ANTISERUM

The specific antiserum used in these studies was rabbit or burro antiserum prepared by Dr. W.G. Roessler as described by Silverman.[3] Antisera were stored at -15 C.

C. PHOSPHATE-BUFFERED SALINE DILUENT

1) Solution A was composed of (i) 11.36 g of Na_2HPO_4 brought to a final volume of 1200 ml; (ii) 34.0 g of NaCl and 0.4 g of powdered merthiolate (Powder No. 20, Thimerosol*) were brought to a final volume of 2800 ml; (iii) both solutions were combined for a total of 4000 ml and stored at 5 C.

2) Solution B was composed of (i) 2.7 g of KH_2PO_4 in a final volume of 300 ml; (ii) 8.5 g of NaCl plus 0.1 g of merthiolate powder in a final volume of 700 ml. Both solutions were combined to make 1000 ml of Solution B and stored at 5 C.

3) A 0.02 M phosphate-buffered saline used to prepare antiserum agar was made by mixing approximately 85 parts of Solution A with approximately 15 parts of Solution B to give a pH value of 7.4. All solutions were prepared in sterile distilled water.

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* Eli Lilly and Company, Indianapolis, Indiana.

D. PREPARATION OF AGAR

Clear agar was prepared by adding 5 g Oxoid agar No. 3 or Ionagar No. 2* or an equivalent agar, to 495 ml of 0.02 M phosphate-buffered saline. After heating for 10 minutes at 121 C, the solution was filtered through Whatman No. 1 paper, then filtered until clear by holding the temperature at 55 C during filtration. After filtration, the agar was cooled to 48 C and dispensed in 20-ml amounts for storage at 4 C.

E. AGAR-COATED ASSAY TUBES

Tubes** of 3 to 5 mm inside diameter and 10 cm long were internally coated with agar to prevent leakage at the glass-agar interface.[1] Also available and more convenient to use are agar-coated, sterile, gel-diffusion tubes*** (approximately 3 mm ID x 10 cm long). Foam-plastic blocks, 0.5 to 2.0 inches thick cut to convenient size are light, convenient holders for small tubes.

F. PREPARATION OF REFERENCE STANDARD TOXIN SOLUTIONS

Reference standards containing 25, 50, 100, 200, and 400 µg/ml of antigen were prepared with purified dried toxin diluted in salt buffer (phosphate-buffered saline plus 2% NaCl). Each assay required a total of 310 µg of dried enterotoxin B based on the Kjeldahl nitrogen content.

G. TITRATION OF ANTISERUM

Antiserum was diluted with phosphate-buffered saline and added to 1% agar to the desired serum dilution and dispensed in tubes. Duplicate tubes were layered with each of five dilutions of reference standards. After incubation at 30 C for 20 to 24 hr, precipitate bands were measured and examined for quality of precipitin zone. Average band-lengths were plotted on semi-log paper against enterotoxin B concentration as shown in Figure 1 to develop a reference standard curve. Suitability of rabbit or burro antiserum was assessed on the basis of both the precipitate band density and sharpness of band (absence of diffuse, fuzzy, secondary, or multiple bands). We used the highest dilution of antiserum that resulted in uniformly dense bands.

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* Consolidated Laboratories, Inc., Chicago Heights, Illinois.
** Scientific Glass Apparatus Co., Silver Spring, Md.
*** Aloe Scientific Co., St. Louis, Mo.

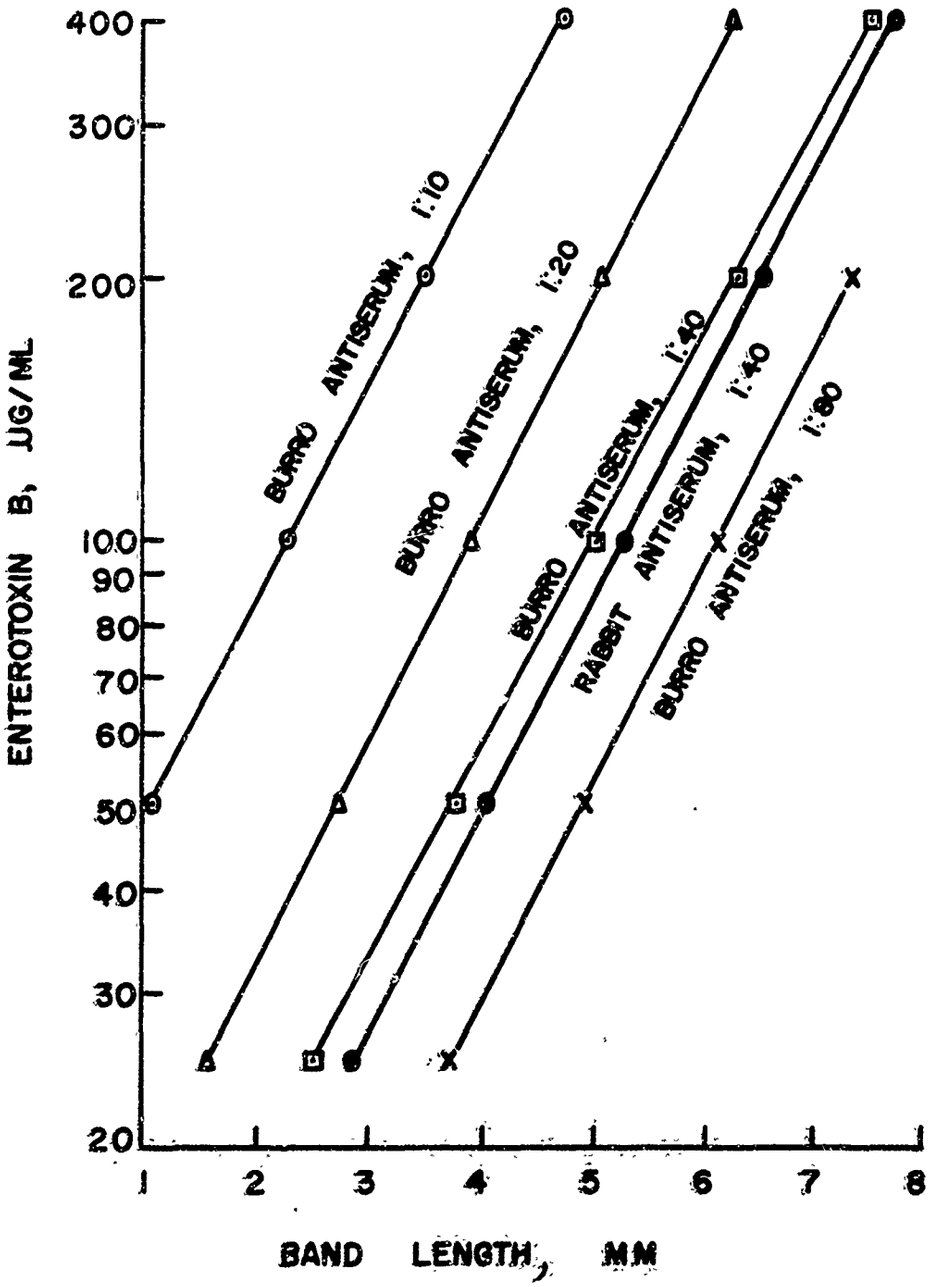

Figure 1. Titration of Antiserum. Effect of dilution on precipitin band length (24-hr reading). Rabbit antiserum, 1:40, shown as typical standard reference curve.

H. PREPARATION OF ANTISERUM AGAR

Ten ml of 1% agar was melted, cooled, and held in a 48 C water bath. For a 1:20 serum dilution, 1 ml of antiserum was added to 9 ml of heated (48 C) phosphate-buffered saline in a large test tube or 100-ml centrifuge tube. After swirling to mix thoroughly, 10 ml of 1% clarified agar was added. Warm Pasteur pipettes were used to fill assay tubes 1/3 to 1/2 full of the antiserum agar. Care was taken to avoid air bubbles in the agar. The tubes, if not used immediately, were stored in a closed, humidified container at 4 C.

I. FILLING GEL-DIFFUSION TUBES WITH TEST ANTIGENS

Assay tubes were overlaid with an amount of test solution approximately equal in depth to the agar below it. All tubes were sealed with modeling clay, plasticine, or corks after filling, and were incubated at 30 C in a water bath or incubator.

J. DIFFUSION-BAND MEASUREMENTS

Routine measurements were made with a binocular microscope equipped with a 9X eyepiece containing a cross-lined disc, a 1X objective, and a mechanical stage with graduated millimeter scales with verniers reading to 0.1 mm. A strong shielded light at an oblique angle to the assay tube was necessary for ease in seeing the band edge. The precipitate band lengths can be measured with calipers; however, this method lacks the desired precision.

K. PREPARATION OF ASSAY SAMPLES

Broth cultures were clarified by centrifugation. They were assayed without dilution or at the several dilutions indicated later in the text.

L. TEST PROCEDURES FOR RAPID OUDIN ASSAY

1) Fix plastic labeling tape to foam-plastic block.

2) Identify tube locations.

3) Fill duplicate gel-diffusion tubes, as described in I, above, "Filling Gel-Diffusion Tubes with Test Antigens," with standard reference toxin solutions and punch into labeled spot in plastic block.

4) Fill antiserum gel-diffusion tubes with appropriate dilution of test samples (antigens) and place tubes in labeled spot in block, one tube per dilution.

5) Stopper all tubes.

6) Place rack of tubes in 30 C incubator for 20 to 24 hr.

7) Measure precipitin band-lengths of reference standard tubes and of sample tubes. If reading time of standard tubes and unknown samples is greater than 30 min, remeasure standard tubes and continue measurements.

8) Plot average band lengths against enterotoxin B concentration of standard reference curve solutions on semilog paper.

9) Calculate potency of test sample by simply taking potency value from reference standard curve and correcting for dilution.

III. RESULTS

A. VARIABLES IN ASSAY

1. Incubation Temperature

Reference standard toxin solutions were assayed at 30, 34, and 37 C to determine the effect of increased temperature. Band migration increased approximately 0.05 mm/deg within this temperature range. Since a relatively constant slope of 1.3 was observed (Fig. 2), no advantage in slope response was gained by increasing the incubation temperature to 37 C. Fluctuation in temperature during incubation was of little importance because standards and unknowns were both subjected to the same environment.

2. Time Elapsed During Reading of Unknown and Standard

The error attributable to band migration during the period of measurements at room temperature was determined with reference standard toxin solution tubes read at 20 to 24 hr. Figure 3 shows the extent of band migration in 4 hr. The longer the elapsed time between reading of the standards and the unknown, the greater the assay error. To reduce this error, reference standard toxin solution tubes were read every one-half hour and the average readings were plotted for calculating the potency for the unknown toxin samples read during each half-hour period. Since the slope of the assay curve increased with time, the error was greatest at the highest potency levels. The maximum assay error when standards were read every half-hour was about ±3.3% at 200 µg/ml, at 25 µg/ml the error was ±1.3%.

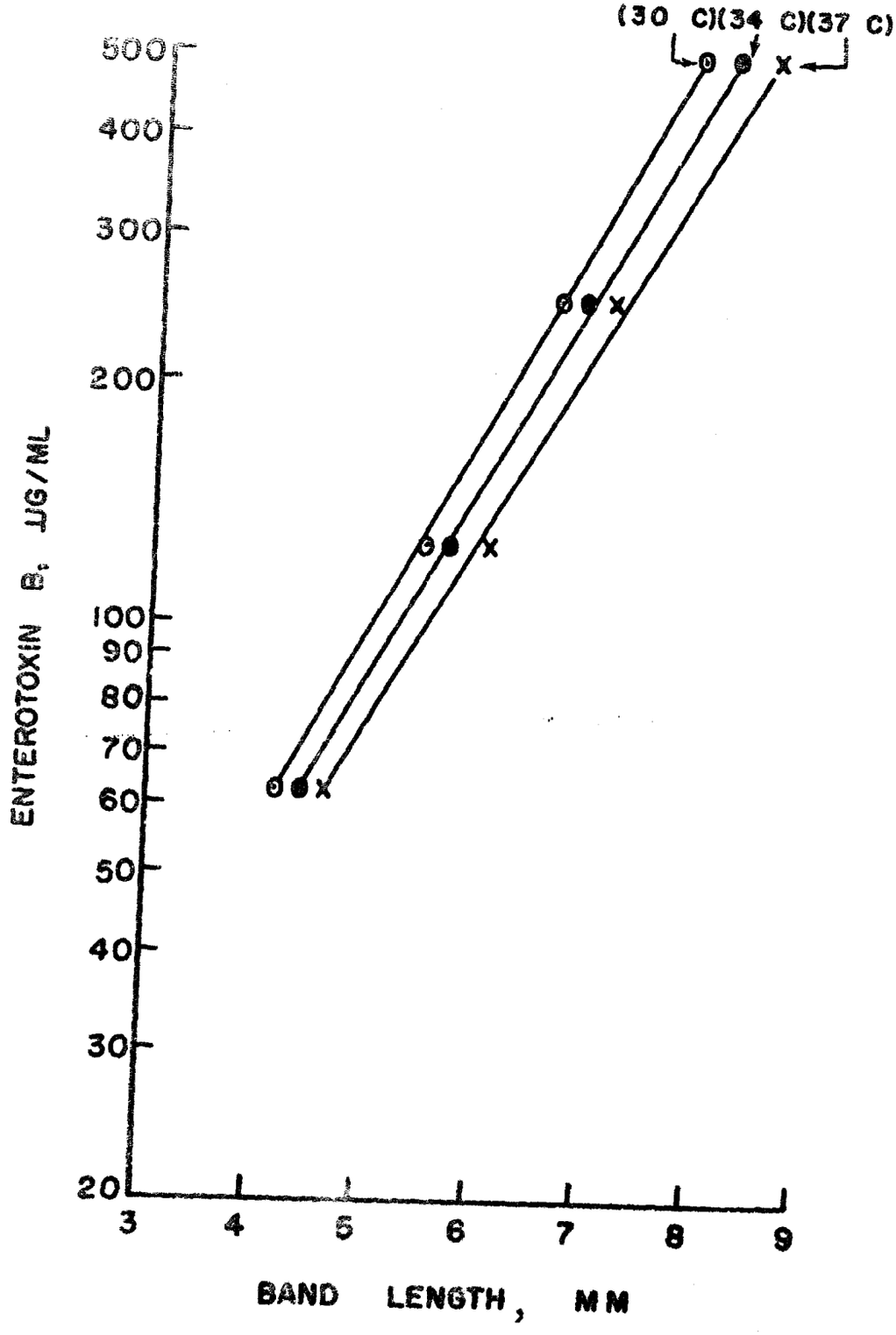

Figure 2. Temperature Response Curves (24-hr reading).

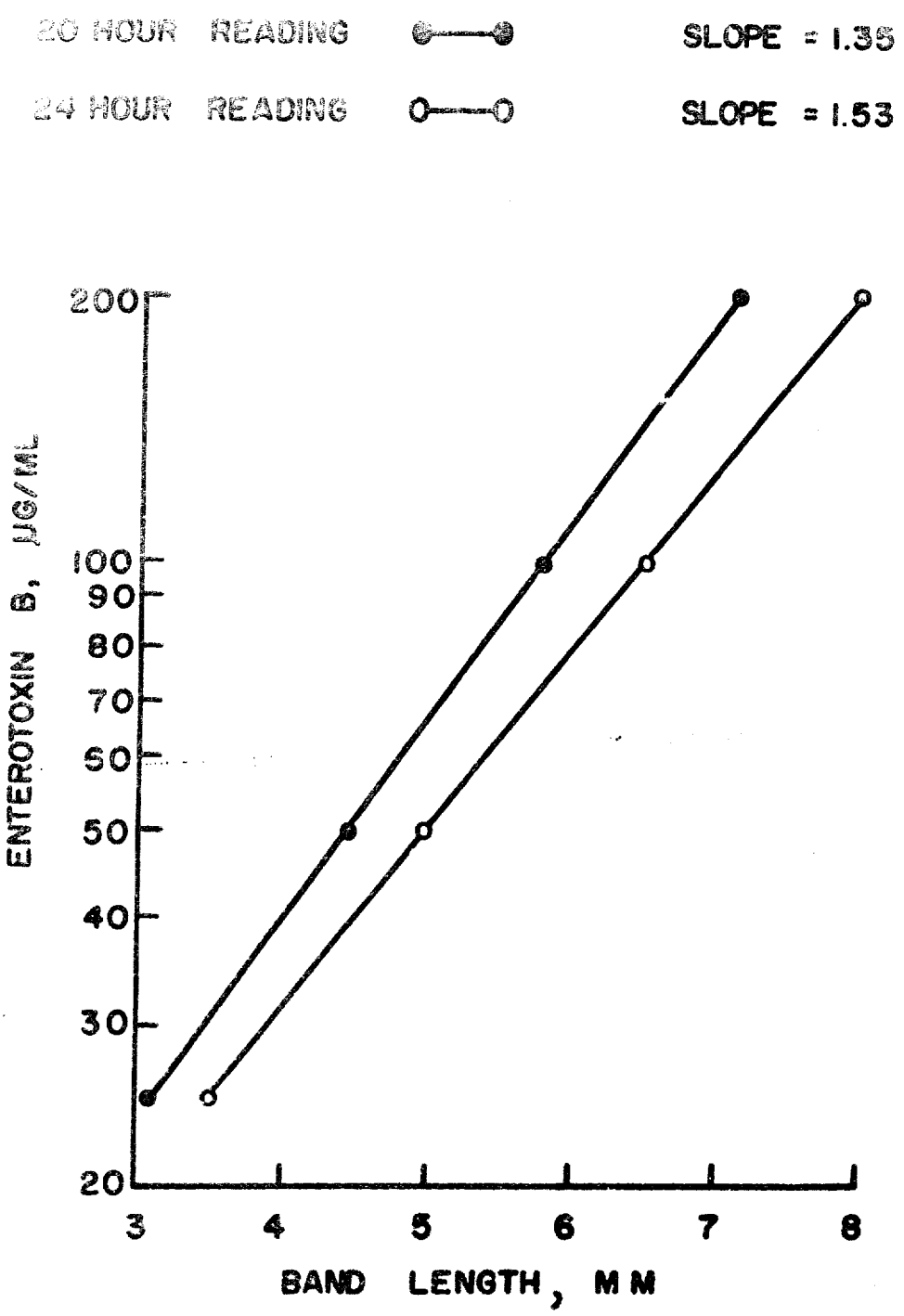

Figure 3. Changes in Band Lengths Between 20- and 24-hr Reading.

3. Diluents

The effect of salt concentration in phosphate-buffered saline on assay results was investigated. We found that correct assay values are obtained only when the salt (NaCl) concentration is 0.5 M (or 2.9%) (Fig. 4) with approximately correct readings being obtained through the range 0.2 to 1.0 M (or 1.2 to 5.8%). Hall et al.[4,8] reported that it was necessary to employ organic diluents in preparing reference standards when assaying organic material; however, we found a variation between organic diluents that was dependent upon their concentration and composition. Therefore, to obtain correct assay values the same culture medium was used both to prepare reference standards and to dilute samples of culture supernatants. Large errors were obtained if the ionic strength of the system was not standardized. Figure 5 shows the variation in band movement when purified enterotoxin B was diluted with the indicated diluents. Assay values of undiluted, 3% N-Z Amine, Type A (NZAA), broth samples would be expected to be about 10% low when calculated from a reference standard curve prepared with salt buffer. Table 1 shows an average error of -11.2% between diluted and undiluted samples due to different diluents in standard solutions and in the sample, i.e., culture supernatant enterotoxin level calculated from salt-buffer standard curve. When samples and standards were prepared in phosphate-buffered saline plus 2% added salt (salt buffer) or in solutions of the same ionic strength, good agreement was obtained between replicates within and between sample dilutions. Table 1 indicates reproducibility of assay values for undiluted culture supernatant and supernatants diluted 1:2 and 1:4; the averages for 17 samples were 174.9, 194.3, and 195.0 μg/ml of toxin, respectively. The average value of the undiluted cultures was 11.2% lower than that of the diluted culture.

4. Filtration

Filtration of assay samples resulted in adsorption of enterotoxin B on UF glass, Seitz pads, and other filter media. When sterile broths were desired, they were obtained by treatment with merthiolate to a final concentration of 1:10,000. Accurate readings were obtained after correction for dilution. Centrifugation of assay samples, the method of choice, did not cause a detectable error.

5. pH

The test was most accurate at a pH of 7.4, with an acceptable range of 6.8 to 7.8 (Table 2). At pH 6.3 and 8.8 band migration was slower, resulting in a low assay value.

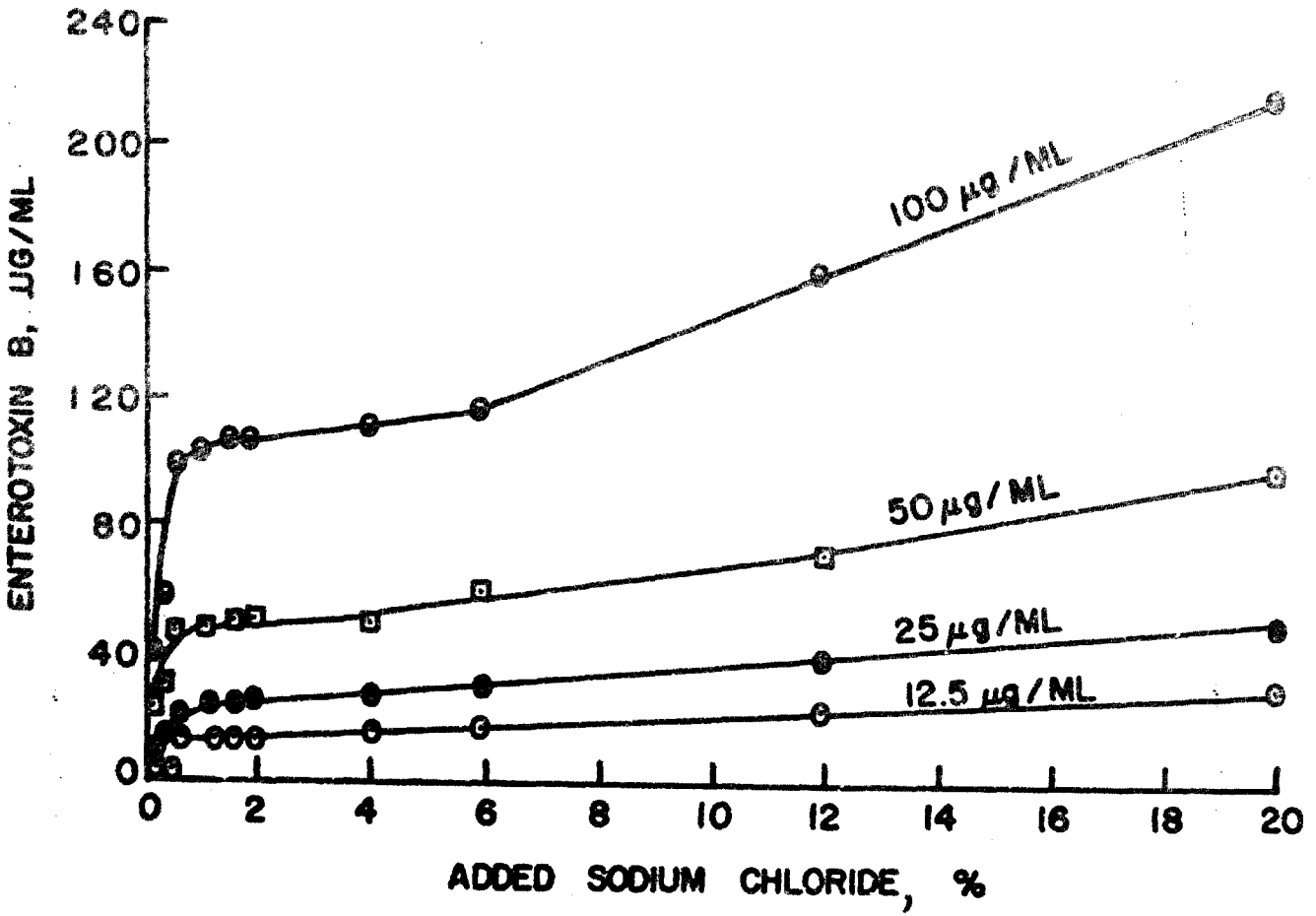

Figure 4. Effect of Added Salt on Enterotoxin B Assay. Increasing amounts of sodium chloride were added to phosphate-buffered saline. Reference standard curve solutions were prepared in phosphate-buffered saline diluent containing 2% added NaCl.

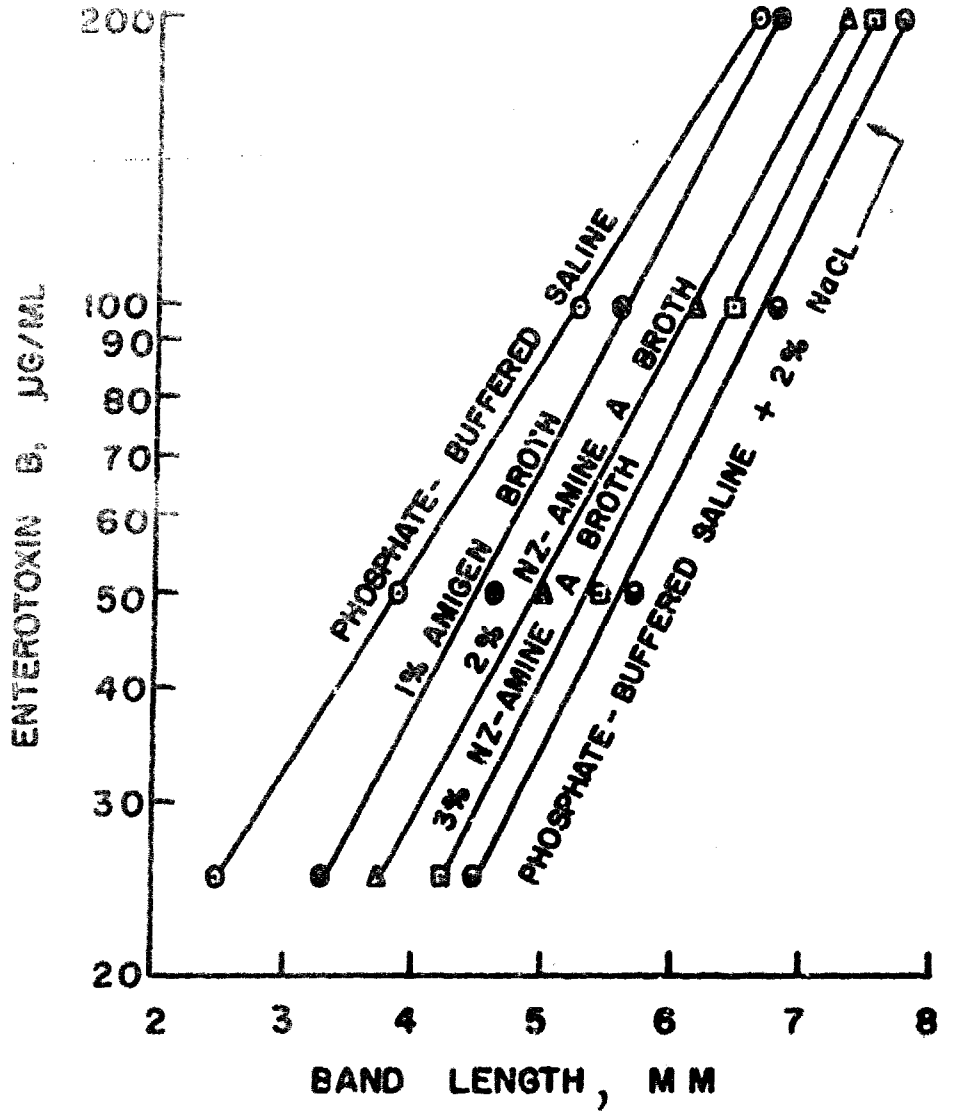

Figure 5. Effect of Diluents on Enterotoxin B Assay (24-hr reading).

TABLE 1. ENTEROTOXIN B ASSAY VALUES AS AFFECTED BY DILUTION

Culture Supernatant No.	Undiluted Culture, µg/ml	Culture Diluted in Salt Buffer, µg/ml	
		1:2	1:4
437	168	192	160
439	171	192	185
441	150	183	175
442	171	180	165
443	203	231	225
447	179	213	220
448	150	192	190
449	231	243	265
450	222	258	280
452	147	162	170
453	150	165	140
456	141	171	160
457	157	180	180
458	164	159	180
461	168	186	205
462	199	213	190
463	203	183	225
Avg	174.9	194.3	195.0

TABLE 2. ASSAY VALUES, µg/ml, AT DIFFERENT pH LEVELS (24-HR READINGS)[a]

Reference Standard Enterotoxin B, pH 7.4	pH[b]					
	6.3	6.9	7.4	7.8	8.3	8.8
205	187	207	207	204		
107	93	101	108	106	105	107
29	21	29	29	29	26	21
10	8	9	10	11	11	8
5	<2	5	5	2	<2	<2

a. Standards and test solutions were diluted in phosphate-buffered saline.

b. The pH of the test solutions was modified by adjusting phosphate-buffer ratio.

6. Agar

Tests were performed to determine the optimum concentration of agar in the antiserum agar gel. Final concentrations from 0.1 to 0.5% agar gels were prepared. There was no difference between the precipitin band-lengths for 0.3 to 0.5% agar gels. Gel tubes containing 0.1 or 0.2% agar had shorter precipitin bands and were erratic in replicates.

7. Sodium Salts

The effect of varying salt concentration (0.1 to 1.0 N) and various sodium salts (SO_4^{2-}, Cl^-, NO_3^-, Br^-, and I^-) in aqueous solutions on precipitin band migration was studied at two levels of enterotoxin (10 and 100 μg/ml). The results are shown in Figure 6. Band migration increased sharply with salt concentration. Constant diffusion rate occurred with concentrations of 0.4 to 1.0 N of NaCl. A gradual increase in band lengths occurred for toxins diluted in 0.2 to 1.0 N Na_2SO_4 solutions. The results followed a reverse-order Hofmeister series, $I^- > Br^- > NO_3^- > Cl^- > SO_4^{2-}$, indicating that the ability of these anions to bind protein is the reverse of their salting-out ability. The results indicated that NaI stabilizes the diffusion of enterotoxin B over a wider concentration range and may be a better choice of sodium salt in precipitin systems than the conventional sodium chloride-phosphate diluent.

8. Comparison of Conventional Three-Day with Rapid One-Day Assay

The rapid one-day assay was compared with the three-day assay for accuracy and variability. Pure toxin diluted in phosphate-buffered saline was compared with reference standards prepared with the same buffer. Enterotoxin containing culture supernatant was compared with reference standards prepared in sterile NZAA broth. Table 3 reports duplicate values made a week apart for undiluted and diluted samples in inorganic and organic systems. The only difference between the three-day and rapid assay results shown in Table 3 was the method used to calculate the potencies of the samples. Rapid assay values reported were calculated for each day that measurements were made, and show that the calculations of assay results based on measurements made at 24, 48, or 72 hr were in close agreement among themselves and with the calculated potencies.

The results indicate approximately the same variation for inorganic and organic assay systems. The rapid assay method yielded comparable results to the three-day assay method, the advantage being that the results are obtained in one day.

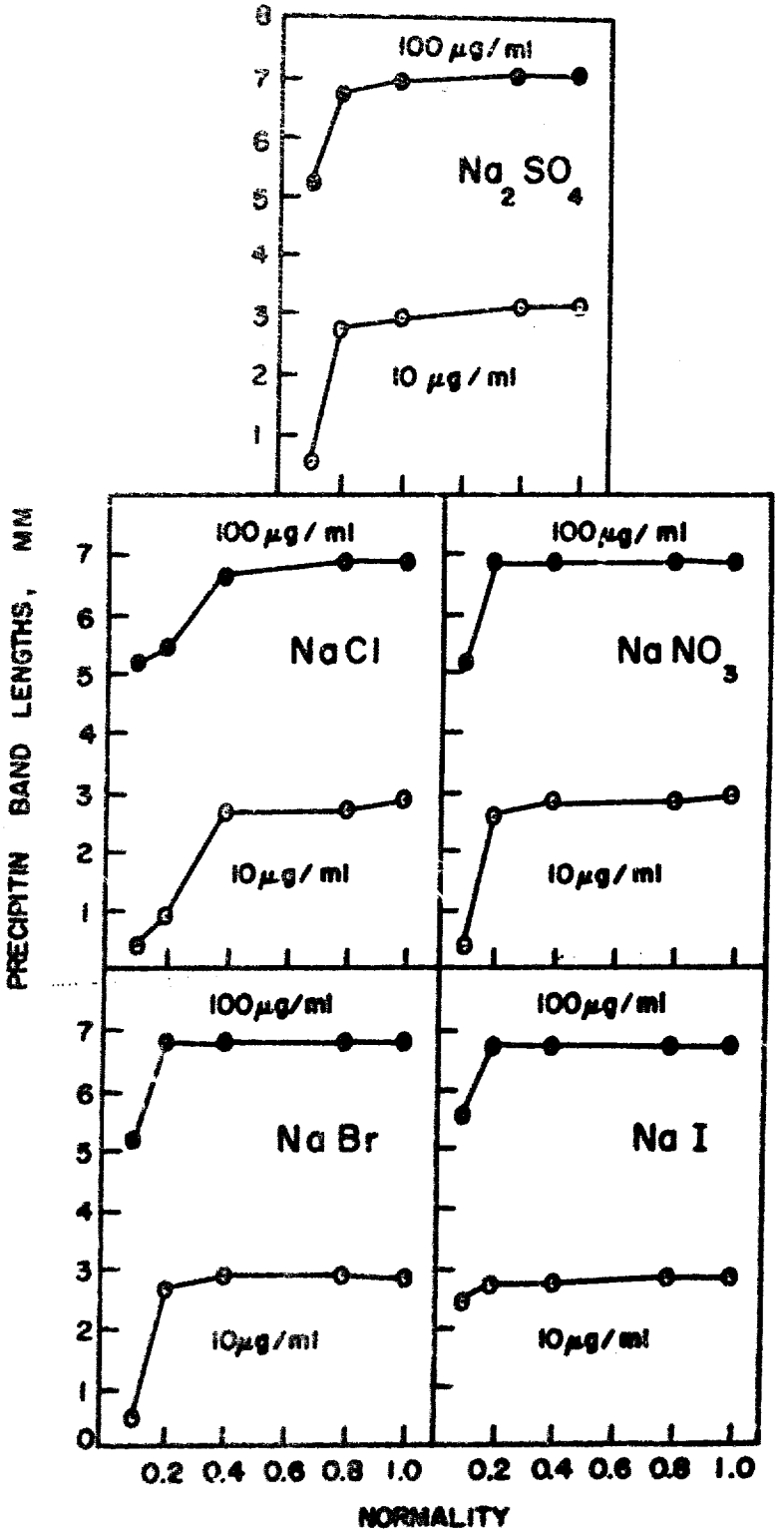

Figure 6. Effect of Varying Salt Concentration and Various Sodium Salts in Aqueous Solution on Precipitin Band Migration (22-hr) at Two Levels of Enterotoxin B. All solutions adjusted to pH 7.4 with 0.1 N NaOH.

TABLE 3. COMPARISON OF OUDIN THREE-DAY ASSAY WITH RAPID ASSAY. EACH ASSAY VALUE, µg/ml, IS AN ARITHMETIC MEAN OF FOUR ASSAY TUBES

Sample Description	Subsample or Dilution	Oudin 3-Day Assay Replicate[a] 1[b]	Oudin 3-Day Assay Replicate[a] 2	Rapid Assay Values Calculated When Read at Indicated Time: 24 hr Replicate 1	24 hr Replicate 2	48 hr Replicate 1	48 hr Replicate 2	72 hr Replicate 1	72 hr Replicate 2	Avg. of 3 Readings Replicate 1	Avg. of 3 Readings Replicate 2
Undiluted											
Pure enterotoxin B, potency 208.3 µg/ml. Sample and standard curve solutions prepared in phosphate-buffered saline diluent.	1	255	245	205	205	205	205	210	230	207	213
	2	241	195	210	212	210	240	209	220	210	224
	3	282	197	205	212	225	215	220	220	217	216
	Avg.	259.3	212.3	206.7	209.7	213.3	220.0	213.0	223.3	211.3	217.7
	Assay/Standard, %	124.5	101.9	99.2	100.7	102.4	105.6	102.3	107.2	101.4	104.5
N-Z Amine A culture supernatant. Estimated potency 97.9 µg/ml. Sample and standard curve solutions prepared in N-Z Amine A broth.	1	122	116	98	82	109	90	100	105	102	92
	2	113	98	98	90	100	100	94	104	97	98
	3	130	-	98	93	105	90	99	90	101	91
	Avg.	121.7	107.0	98.0	88.3	104.7	93.3	97.7	99.7	100.0	93.7
	Assay/Standard, %	124.3	109.3	99.9	90.2	106.9	95.3	99.8	101.8	102.1	95.7
Diluted											
Pure enterotoxin B, potency 208.3 µg/ml. Sample dilutions and standard curve solutions prepared in phosphate-buffered saline diluent.	1:2	245	265	210	192	220	210	210	230	213	216
	1:4	245	228	224	216	208	220	200	234	211	223
	1:8	250	238	232	216	216	216	224	240	226	226
	Avg.	246.7	243.7	222.0	208.0	214.7	215.3	211.3	234.7	216.7	221.7
	Assay/Standard, %	118.4	117.0	106.6	99.9	103.1	103.4	101.4	112.7	104.0	106.4
N-Z Amine A culture supernatant. Estimated potency 97.9 µg/ml. Sample dilutions and standard curve solutions prepared in N-Z Amine A broth.	1:2	124	108	100	84	109	90	100	105	102	92
	1:4	127	110	100	80	104	104	104	108	103	99
	1:8	145	116	88	77	100	102	108	110	103	97
	Avg.	132.0	111.3	96.0	80.3	104.3	98.7	104.0	107.7	102.7	96.0
	Assay/Standard, %	134.8	113.7	98.1	82.0	106.5	101.8	106.2	110.0	104.9	98.1

a. Samples were stored at -15 C between replications made one week apart.
b. First replicate antiserum was diluted 1:30 resulting in very light precipitin bands. High variation in first replicate three-day assay was due to decreased band migration by the third day. The same lot of antiserum was used in the second replicate but diluted 1:20.

IV. DISCUSSION

The present study was conducted to devise a practical rapid assay method for enterotoxin B based on the Oudin single gel diffusion assay system. Rubinstein[7] quantitated Oudin's[2] method by plotting the relationship between the length of the precipitate band (in mm) and the square root of time (in min) and then plotted the resultant slopes against the logarithms of the respective antigen concentrations. The latter plot is the calibration curve for determining the concentrations of unknown antigen solutions. Several readings of an unknown antigen solution are taken and plotted against log $t^{\frac{1}{2}}$ to determine the slope, which is then compared with the calibration curve to determine the concentration of the unknown solution. The rapid quantitation of the Oudin method described here is based on the linear relationship between the length of the band of precipitate and the logarithm of the concentration of the antigen (enterotoxin B) when the antibody concentration and the time of reading are held constant. This method simplified and shortened the time of calculation of the concentration of the unknown antigen.

Several factors affected the assay results. Large errors resulted when there were differences of ionic strength between the enterotoxin standard and the sample assayed. Both organic and inorganic systems are capable of yielding reliable assays if the ionic strengths are nearly equivalent in the antigen-antibody preparations. Because culture medium constituents are not standardized, a reliable assay system based on an organic system[4,8] is difficult to achieve. Changes in culture medium ingredients result in erroneous assay values (Fig. 5). However, both inorganic and organic systems can be stabilized to yield a uniform band migration per unit of toxin, within a relatively wide range of salt concentration, 1.2% (0.2 M) to 5.8% (1.0 M), Figures 4 and 6. Below 0.2 M salt concentration, assay results varied widely with small changes in concentration; above 1.0 M the assay values increase gradually and the precipitin bands become lighter. The assay system was stabilized by increasing the salt to concentrations greater than that used by Silverman.[3] In a no-salt buffer system, 25 µg/ml of toxin is the lowest toxin level that will form a precipitin band. In 0.02 M salt buffer, 12.5 µg/ml form bands and 2 µg/ml of toxin can be determined if salt buffer is increased to 0.2 M. Thus, if it is desired to detect very small amounts of enterotoxin, 0.2 M buffer should be used. Sodium salts other than those with Cl^- should be considered for precipitin reactions; the data indicated I^-, Br^-, and NO_3^- to be more effective than Cl^- or SO_4^{2-}.

Band migration continues in linear proportion to the square root of time during the period of measurements. Ideally, it would be advantageous if the band migration could be halted at the time of reading; however, since no practical method is known that would stop the band migration the time between readings of the standards and unknowns should be kept as short as possible and always less than 30 minutes.

Dense secondary bands will form if the environmental temperature is suddenly lowered by a few degrees or if the temperature fluctuates a few degrees during incubation. The dense secondary band is fixed.[8] A sudden rise in temperature results in a clear gap. The problem of secondary band or gap formation can be prevented by incubating the tubes at the same temperature as that at which the bands are read.

The rapid quantitative assay procedure described is not subject to the limitation of the quantitative method as reported by Rubinstein[7] and Silverman.[8] Unknown antigen concentration can be determined by precipitin band measurements in 16 hr and good estimates of toxin concentration can be obtained in as short a time as 4 hr. Involved titration and plottings of each lot of antiserum are not required, although somewhat more toxin is required than for the three-day assay. Rigid control of reagents and conditions of the assay are not necessary. Involved and time-consuming plotting and/or calculation of assay values are unnecessary because standard enterotoxin B solutions and unknown toxin samples are both subjected to the same manipulations and environmental conditions. The method is also applicable for other precipitating antigen-antibody systems. A specific single component toxin of high purity is desirable; however, crude toxins can also be used as a reference point and for preparing antiserum during purification procedures.

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RAPID QUANTITATIVE SEROLOGICAL ASSAY OF STAPHYLOCOCCAL ENTEROTOXIN B

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13. ABSTRACT

A simple, rapid method, based on the Oudin single gel diffusion technique, is described for the quantitative assay of staphylococcal enterotoxin B. The method yields reproducible results without close control of such assay variables as temperature, antiserum dilution, and assay time, provided that the ionic strength is maintained above 0.2 N sodium chloride equivalent.

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