

APPROVAL SHEET

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Studies on Tests for the Detection of the Endotoxic Substance Produced by a Strain of Aerobacter cloacae Causing Acute Illness Among Workers Using Low-grade Stained Cotton.

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STUDIES ON TESTS FOR THE DETECTION OF THE ENDOTOXIC  
SUBSTANCE PRODUCED BY A STRAIN OF ARBOBACTER GLOABER  
CAUSING ACUTE ILLNESS AMONG WORKERS USING LOW-GRADE  
STAINED COTTON

By

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## I. INTRODUCTION

During the period from April 1941 to August 1942, widespread outbreaks of acute illness were reported among workers in rural mattress making centers, cotton mills, cotton seed processing plant, and upholstering plants where a very low grade of dusty stained cotton was used. This illness occurred among workers exposed to high dust concentrations, and its severity varied with the degree of exposure.

Investigations, which have already been reported (14, 21), demonstrated conclusively that the etiological agent responsible for this acute illness was an endotoxin-like substance produced by a mucoid, Gram-negative, motile, encapsulated, rod-shaped microorganism previously referred to as the "cotton bacterium." Subsequent taxonomic studies indicate that this bacterium is a strain of Aerobacter cloacae (3).

The disease is characterized by its sudden onset (1-6 hours after exposure), the short duration of the acute phase (usually 24-48 hours after exposure), and by the development of a marked leucocytosis. The principal subjective symptoms are conjunctival irritation, substernal oppression, dryness of throat, generalized aches, fatigue, headache, cough, chills, fever, anorexia, nausea and vomiting.

All the samples of cotton incriminated in outbreaks of illness were dusty, low-grade and stained, and contained varying amounts of plant debris. The majority of these samples were classified within official grades for tinged and stained cotton by graders of the U. S. Department of Agriculture, and many of them were described as "bally (26) in character." The mucoid bacterium occurred to the exclusion of



other types of microorganisms in these samples and its incidence ranged from 3,000,000 to more than 10,000,000,000 per gram. It was not isolated from high grade cotton. Seventy of eighty-one samples of materials reported to have caused illness contained the same strain of this bacterium. Typical cultures of this strain of Aerobacter cloacae have also been isolated from hemp and grain dust.

A heat-stable, endotoxin-like substance was demonstrated in filtrates from saline extracts of stained cotton, in filtrates from broth cultures and in killed culture suspensions. It is believed that this toxic substance is in the nature of an endotoxin because of its heat stability, lower antigenic capacity, and its increase with age of culture or destruction of cells.

The same type of disabling illness, which resembles an acute intoxication, could be produced experimentally in humans by inhalation of (a) dust from normal cotton contaminated with this organism and its culture filtrates, (b) dust from stained cotton containing large numbers of the organism and (c) a fine mist of sterile filtrates from cultures of this bacterium. In experimental cases, the severity of symptoms and physical findings were dependent upon the presence and concentration of this bacterium or its products in the cotton dust inhaled, and upon the duration of exposure (14, 21).

Reports of similar diseases among workers exposed to organic dusts have appeared in the literature for many years.

One of the earlier references to mill fever among cotton mill workers was made by Leach (12) in 1863.

Arlidge (1) described illness among flax workers as characterized by chills, nausea, vomiting, headache, thirst, and fever.

Light (13) described a case of "thresher's fever" in which the symptoms were headache, substernal oppression, generalized aches, chills, fever and anorexia. He also reported the development of marked leucocytosis.

Oliver (15) described mill fever among jute workers, resulting from exposure to high concentrations of jute dust.

Respiratory ailments, among which were the so-called mill fever, or Monday fever, have constituted important occupational diseases among cotton mill workers in Great Britain for many years (6, 29). These diseases follow the inhalation of cotton dust. According to the literature, cotton mill workers, while engaged in the initial stages of cotton processing, are subject to frequent illness due to a special and complex cause, which has not been completely explained, but which has been considered to be both allergic and mechanical (16).

The Departmental Committee on Dust in Cardrooms in the Cotton Industry 1932 (18) reported that the symptoms of the illness among cotton cardroom operatives were due to the action of the dust on the mucous membranes of the respiratory passages. According to this report there was no evidence of aspergillosis or other similar disease of the lung due to inhalation of fungus mycelia, conidia or spores. In an appendix to this report (18), Pittland and his associates reported the presence of histamine in cotton dust and suggested the possibility of histamine being a causative factor in cotton mill illness.

The desirability of following up this clue led the Medical Research Council of Great Britain to institute a study of illness in cotton mills. The results of this investigation are given in the report of Frausnitz (16). He concluded that histamine, although present in cotton dust, was

not the cause of the illness. On the basis of particle size determinations and animal experiments with cotton dust, however, Fraenitz formulated the following conception of the pathogenesis of the cotton mill illness:

1. Penetration of irritating soluble proteins of the cotton dust into the alveolar tissue, gradually producing a thickening of the alveolar walls.
2. The development of supersensitiveness to the cotton dust.
3. Superficial irritation of the bronchial mucous membrane by the dust resulting in bronchitis, cough, and ultimately in emphysema.

In connection with this illness among cotton cardroom operatives, the British Parliament in 1940 (29) passed an act to provide for compensation for workmen who died or became permanently and totally incapacitated for work as the result of the respiratory disease known as byssinosis.

Mill fever has never been attributed to bacteria or their toxic products. However, the British Departmental Committee on Dust in Cardrooms in the Cotton Industry in 1932 (18) reported the presence of large numbers of Gram-negative bacilli in cotton dust. No importance was attributed to these bacteria since no factor common to the bacteriology of the dust and the patients' sputum was found.

The acute illness occurring among workers using low grade stained cotton, except for its greater severity, closely resembles mill fever, Monday fever, or gin fever in cotton mill workers. Similar diseases called heckling fever, mill fever, grain fever, and hemp fever have been

reported in workers inhaling flax, jute, grain and hemp dust, respectively, and, as mentioned above, toxigenic strains of A. cloacae have been isolated from hemp dust and grain dust. Therefore, it is suggested that toxic products liberated by these strains of A. cloacae may cause these diseases.

This acute illness, to which only humans are susceptible, may be considered as a potential occupational health hazard for employees in industries utilizing low-grade stained cotton, or other dusty plant materials. It is therefore readily apparent that practical methods for the detection of the endotoxin responsible for this illness should be developed in order to facilitate the investigation and prevention of future outbreaks of this illness. Studies on methods for the treatment of cotton for the destruction of this endotoxin are also limited until tests for its detection are developed.

During the earlier phases of the investigation into the etiology of this acute illness a heat-stable, endotoxin-like substance was demonstrated in filtrates from saline extracts of stained cotton and in filtrates of broth cultures of the organism isolated from it by means of the Shwartzman reaction and the Dolman and Hammon tests for enterotoxin (21). Since positive Shwartzman reactions and Dolman and Hammon enterotoxin tests may be elicited by other toxic substances, it was necessary to resort to the inhalation exposure of human subjects to contaminated cotton dust, and culture filtrates of the organism or to the intradermal injection of such subjects with filtrates from saline extracts of incriminated cotton, and of culture filtrates in order to demonstrate conclusively the etiological agent responsible for this disease.

Since it would be impossible to resort to the use of human subjects

as a routine procedure, the following studies were undertaken in an attempt to develop practical and specific methods for the detection of this endotoxin.

II. METHODS FOR DETERMINING THE INCIDENCE IN AND  
THE ISOLATION OF TOXIC STRAINS OF ANEROBACTER  
GLOABAE FROM COTTON OR OTHER MATERIALS

A. Bacteriological Methods

In the investigation of outbreaks of this illness, it is obviously necessary to ascertain the incidence of this toxic microorganism in suspected materials. Studies which were reported previously (14, 21) demonstrated that the following procedure is practical for this purpose. The sampling and analytical procedures are carried out essentially as follows. Samples should be collected in sterile containers such as one-gallon Mason jars, friction top cans, or in clean, tight shipping cartons in order to avoid extraneous contamination. A 1:100 dilution of each sample is prepared by aseptically weighing 1 gram of material into 100 ml of sterile physiological saline in wide-mouth, screw-cap bottles. The bottles are agitated mechanically for 30 minutes. Serial dilutions from 1:1000 to 1:100,000,000 are made from the resulting suspensions. Aliquot portions of these dilutions are plated on potato-carrot-dextrose agar\*. Inoculated plates are incubated 48 hours at 37° C. A microscopic examination is made of stained preparations from representative colonies in order to ascertain their morphological type, Gram-stain reaction and the presence or absence of capsules and flagella. Representative colonies are selected for additional studies and purified by repeated replating.

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\*Formula: Potatoes, 2000 gm; carrots, 500 gm; dextrose, 200 gm; magnesium sulfate, 3.0 gm; calcium carbonate, 2.0 gm; agar, 150 gm; and water, 10 liters. pH adjusted to 6.8.

### B. Biochemical Studies

The strains of A. gloabae isolated from materials incriminated in outbreaks of acute illness attack the usual carbohydrate test substances except adonitol, inositol and inulin. Four different types of A. gloabae were arbitrarily distinguished on the basis of these fermentations (3). Strains of this organism, which are known to produce endotoxin, have been placed in Type I. Type I A. gloabae was found to predominate in samples of materials incriminated in outbreaks of illness (3). The limited number of characteristics or reactions which serve to identify these microorganisms as A. gloabae and to differentiate the four types are given in Table I.

Purified cultures, isolated from suspected materials, may therefore be subjected to a limited number of morphological and biochemical studies, which will definitely indicate whether these cultures are identical biochemically with toxigenic types of A. gloabae.

### C. Serological Studies

It has been demonstrated (21) that homologous antisera can be produced in rabbits through intravenous, immunizing injections with saline suspensions of a killed culture of toxigenic strains of A. gloabae, with Berkefeld filtrates from 7-day tryptose broth cultures of the organism, and with filtrates from vacuum distillation concentrates of saline extracts of the stained cotton from which toxigenic cultures were isolated. The highest titer that it has been possible to attain consistently shows positive agglutination in a serum dilution of 1:5120. All attempts to produce an immune serum of higher titer have been unsuccessful. Recent studies

TABLE I.

Differentiation Characteristics of  
Four Types of Acrobacter slonans

	Type I	Type II	Type III	Type IV
Gram stain	-	-	-	-
Capsule formation	+	+	+	+
Motility	+	+	+	+
Lactose fermentation	AG	AG	AG	AG
Reaction on E.M.B. agar	atypical	atypical	atypical	atypical
Invis reaction	--++	--++	--++	--++
Gelatin liquefaction	+	+	+	+
Glycerol	A	A	A	A
Adonitol	-	-	-	-
Inulin	-	-	-	-
* Saccharides	AG	AG	AG	±
Dulcitol	AG	-	±	±
Sorbitol	AG	AG	±	±
Inositol	-	-	AG	±

\* Monosaccharides: Arabinose, galactose, glucose, levulose, mannose, rhamnose and xylose.

Disaccharides: Cellobiose, maltose, sucrose, trehalose and lactose.

Trisaccharides: Raffinose.

Polysaccharides: Dextrin and starch.



have demonstrated that the following immunization schedule is the most satisfactory for the routine preparation of immune serum. The dosages employed and the injection intervals are shown in Table II.

TABLE II  
Immunization Schedule

Injection Interval	Quantity Injected ml
1st day	0.35 ml of a 1:10 dilution
3rd day	0.50 ml do.
5th day	0.75 ml do.
7th day	1.00 ml do.
9th day	0.25 ml Undiluted material
11th day	0.80 ml do.
13th day	0.75 ml do.
15th day	1.00 ml do.

This schedule is applicable to the immunization of rabbits with killed culture suspensions, culture filtrates, and concentrates from extracts of stained cotton. Immunized rabbits are permitted to rest for 2 days after the final injection. Blood is then obtained by cardiac puncture, and the immune serum separated from the clot by centrifugation.

The antigens employed for agglutination tests throughout these studies consisted of filtered, standardized, uniform suspensions of 18 to 24 hour potato-carrot-dextrose agar slant cultures of typical cultures (11b, 56b) of toxigenic strains of *A. glaucus*.

The method consisted of diluting the serum in the usual manner with sterile physiological saline throughout a range from 1:10 to 1:5,120; adding 0.5 ml of antigen to each dilution and after thorough mixing incubating all agglutination tubes in a constant temperature bath at 37° C for 2 hours, followed by refrigeration at 5° C overnight.

All of the immune sera employed throughout these studies were pre-

pared and their agglutination titer determined by the methods outlined above.

The results of agglutination tests for determining the titer of immune sera are given in Table III.

Agglutination tests have been found very useful for ascertaining whether suspected cultures belong to the same serological group as known toxigenic strains of A. glaucus.

Agglutination Tests for Filter of Immune Serum

TABLE III

Immune Serum	Serum Dilutions									
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
Serum Antigen	+	+	+	+	+	+	+	+	+	+
Control Antigen	-	-	-	-	-	-	-	-	-	-

Antigen = culture 11b.

On 56K 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ -

do. 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ -

On 56F 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ -

On 56G 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ -

do. 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ -

Antigen = culture 56b.

On 56K 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ -

do. 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ -

On 56F 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ -

On 56G 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ -

do. 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ -

On 56K = Serum from rabbits immunized with killed culture.  
 On 56F = Serum from rabbits immunized with Berkeley filtrate from 7-day typhoid growth culture.  
 On 56G = Serum from rabbits immunized with filtrate from warm conen-  
 trate of saline extract of contaminated stained cotton.

### III. EXPERIMENTAL

#### A. Methods Investigated for the Detection of Endotoxin

As previously reported (14, 21) toxigenic strains of A. cloacae have a very low pathogenicity for experimental animals.

Kittens, hamsters, guinea pigs, monkeys, baboons, and rabbits exposed to contaminated dust from low-grade stained cotton for one or more seven-hour periods showed no symptoms of illness. Massive doses of viable organisms, killed cultures, and culture filtrates were required to kill mice and guinea pigs when injected intravenously or intraperitoneally. However, intradermal injections of the filtrates of stained cotton extracts and of a toxigenic culture of A. cloacae in man resulted in the production of severe inflammatory lesions characteristic of an intoxication rather than a hypersensitivity.

It was therefore considered advisable to investigate other species of animals and fowls as test subjects for the detection of endotoxin. Preliminary studies (14, 21) had also indicated that certain serological methods such as the Shwartzman test and precipitation tests, might be developed as practical, specific methods for the detection of the endotoxin. Therefore, all methods investigated for the detection of the endotoxin were confined to animal and serological tests.

1. Materials Employed. Seven types of materials were employed during these studies.

a. Viable Culture Suspensions. Filtered saline suspensions from 18 to 24 hour potato-dextrose agar slant cultures of toxigenic strains of A. cloacae containing approximately one billion cells per ml of suspension.

b. Killed Culture Suspensions. These suspensions were prepared by heating viable culture suspensions in a boiling water bath for 30 minutes.

- c. Culture Filtrates. During investigations previously reported (14, 21) it was found that toxigenic strains of *A. glaucus* autolyse rapidly in Macto tryptose phosphate broth; therefore, the following procedure was adopted for the preparation of culture filtrates. Two hundred fifty ml of sterile tryptose broth was inoculated from an 18 to 24 hour potato-carrot-dextrose agar slant culture of each microorganism studied, and incubated at 37° C for 7 days. At the end of the incubation period, each broth culture was centrifuged to remove the cells and sterilized by filtration through a Berkefeld K-candle. Each culture filtrate was tested for sterility before use by incubation for 48 hours at 37° C.
- d. Cotton Extract. It was found (14, 21) that the endotoxin in stained cotton, which was liberated by toxigenic strains of *A. glaucus*, could be removed by prolonged extraction with isotonic salt solution. The procedure adopted for the preparation of extracts from contaminated stained cotton, as well as from uncontaminated normal cotton, was as follows. One hundred grams of cotton were weighed aseptically into a 2-liter Erlensmeyer flask containing 1000 ml of sterile isotonic salt solution. This saline suspension of cotton was extracted for 3 to 4 days in a refrigerator at 5° C. Each suspension was mixed or agitated twice each day. At the end of the extraction period the extract was removed from the cotton suspension by vacuum filtration through a Buchner funnel containing two circles of No. 1 filter paper. Compression was applied to the cotton in order to remove all of the extract. Each extract was centrifuged to remove sediment, then sterilized by filtration through a Berkefeld K-candle. All filtrates were tested for sterility before use.
- e. Cotton Extract Concentrates. All cotton extract concentrates were prepared by the concentration of the filtrate from saline extract of cotton

20 to 30 times at 25 to 33° C in a vacuum distillation apparatus (Figure

1). All concentrates were sterilized by filtration through a Berkefeld N-candle and tested for sterility by incubation at 37° C for 48 hours.

f. Sterile Isotonic Salt Solution. Sodium chloride, G. P., 0.55 gm and distilled water, 1000 ml.

g. Tryptose Phosphate Broth. Lacto tryptose, 20.0 gm, Kato dextrose, 2.0 gm, sodium chloride, G. P., 5.0 gm, disodium phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ), 2.5 gm, distilled water, 1000 ml. Final pH was 7.3.

All of the viable and killed culture suspensions, culture filtrates, cotton extract filtrates and cotton extract concentrates employed in these studies are listed in Tables IV and V. These tables show the culture or cotton sample number, the symbol employed for reference to each material throughout the studies, and the source of each culture or cotton sample. Each time that a substance is employed throughout the following studies it will be referred to by its standard symbol as listed in Tables IV or V.

3. Animal Tests. Since marked local and systemic reactions are produced in humans through the intradermal administration of materials containing the endotoxin, it was decided to try to find an experimental animal or fowl which would respond in the same manner to the intradermal or other administration of this toxic material. In order to properly evaluate skin reactions, it is obviously necessary to use animals or fowls with a light skin. Accordingly, white rabbits, white mice, and young chickens were chosen for these studies.

a. Tests with White Rabbits. Twenty-eight white rabbits were employed in these tests. Each animal was carefully observed for 7 days prior to the test. The hair was clipped over the injection site on each animal before test.

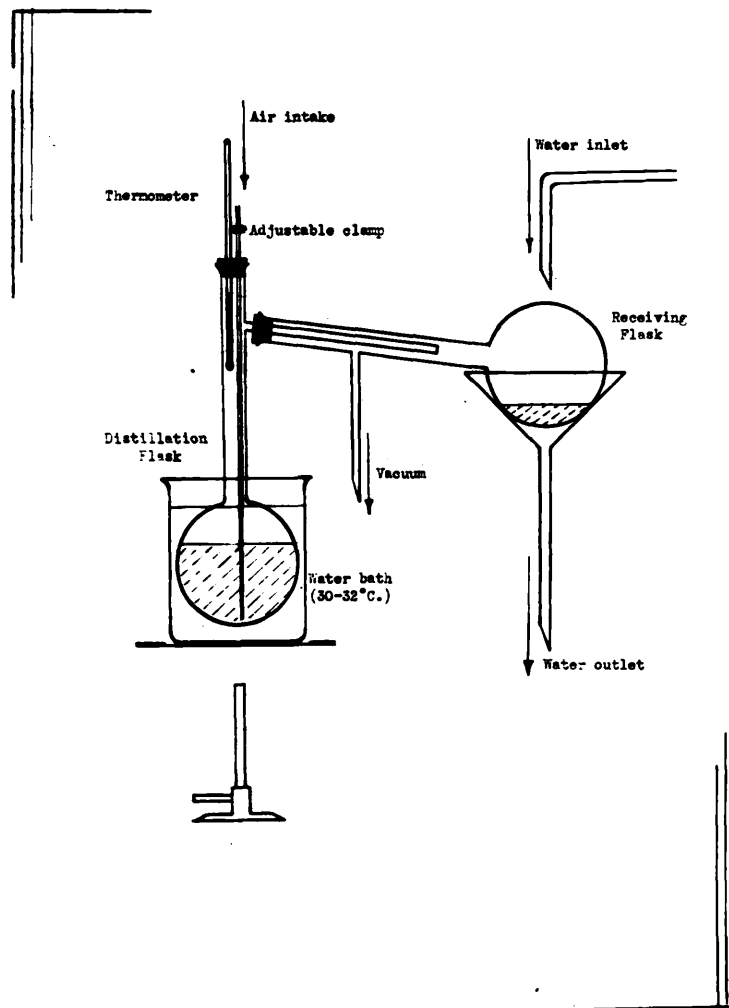


Figure 1: Vacuum Distillation Apparatus

TABLE IV  
Cultures Employed

Culture No.	Viable Culture Symbol	Killed Culture Symbol	Culture Filtrate Symbol	Species	Source
4			Cu 4F	<u>Escherichia coli</u>	Frozen whole egg after 1½ years' storage.
7			Cu 7F	Atypical <u>E. coli</u>	Frozen egg yolk after 1½ years' storage.
10			Cu 10F	Citrobacter	Frozen egg yolk after 5 years' storage.
11	Cu 11L	Cu 11R	Cu 11F	<u>A. gloabae</u> (toxigenic Type I)	Stained cotton sample No. 11, West Virginia.
14			Cu 14F	Atypical Citrobacter	Frozen whole egg after 6 years' storage.
19			Cu 19F	<u>A. aerogenes</u>	Putrid frozen whole egg after 5 years' storage.
26			Cu 26F	Atypical <u>A. aerogenes</u>	Putrid frozen whole egg after 6 years' storage.
H-35		Cu H-35X	Cu H-35F	<u>A. aerogenes</u>	From Dr. Francis E. Clark, U. S. Dept. Agriculture.
49			Cu 49F	<u>A. gloabae</u> (Type III)	Stained cotton sample No. 49, Georgia.
56	Cu 56L	Cu 56R	Cu 56F	<u>A. gloabae</u> (toxigenic Type I)	Stained cotton sample No. 56, West Virginia
63			Cu 63F	<u>A. gloabae</u> (Type II)	Cotton mill dust sample No. 63, North Carolina.
222			Cu 222F	<u>A. gloabae</u>	American Type Culture Collection.
539			Cu 539F	do.	do.
961			Cu 961F	do.	do.
962			Cu 962F	do.	do.



TABLE V  
Cotton Extracts and Extract Concentrates Employed

Sample No.	Cotton Extract Symbols	Cotton Extract Concentrate Symbols	Type of Cotton	Source	Incidence <i>A. cloacae</i> Numbers per gram
5	Co 5E	Co 5E <sub>c</sub>	Stained, toxigenic	U.S. Dept. Agr.	30,000,000
7	Co 7E	Co 7E <sub>c</sub>	do.	Tennessee	750,000,000
8	Co 8E	Co 8E <sub>c</sub>	do.	Kentucky	2,500,000,000
11	Co 11E	Co 11E <sub>c</sub>	do.	West Virginia	2,050,000,000
43	Co 43E	Co 43E <sub>c</sub>	do.	Arkansas	200,000,000
44-45	Co 44-5E	Co 44-5E <sub>c</sub>	White M. and G. M.*	U.S. Dept. Agr.	None
46	Co 46E	Co 46E <sub>c</sub>	White, dirty	Georgia	1,870,000,000
56 <sub>1</sub>	Co 56E <sub>1</sub>	Co 56E <sub>c1</sub>	Stained, toxigenic	West Virginia	3,700,000 to 70,000,000
56 <sub>2</sub>	Co 56E <sub>2</sub>	Co 56E <sub>c2</sub>	do.	do.	190,000,000
56P <sub>1</sub>	Co 56PE <sub>1</sub>	Co 56PE <sub>1c</sub>	Stained, processed	do.	Before processing 3,700,000 After processing None
56P <sub>2</sub>	Co 56PE <sub>2</sub>	Co 56PE <sub>2c</sub>	do.	do.	Before processing 3,700,000 After processing None
56P <sub>3</sub>	Co 56PE <sub>3</sub>	Co 56PE <sub>3c</sub>	do.	do.	Before processing 20,000,000 After processing 1,900
56P <sub>4</sub>	Co 56PE <sub>4</sub>	Co 56PE <sub>4c</sub>	do.	do.	Before processing 70,000,000 After processing 42,000
56P <sub>5</sub>	Co 56PE <sub>5</sub>	Co 56PE <sub>5c</sub>	do.	do.	Before processing 190,000,000 After processing (Sporulates) 100
70	Co 70E	Co 70E <sub>c</sub>	White, G. M.	U.S. Dept. Agr.	Type III (3) 12,000
71	Co 71E	Co 71E <sub>c</sub>	White, S. M.	do.	None
72	Co 72E	Co 72E <sub>c</sub>	White M.*	do.	do.
73	Co 73E	Co 73E <sub>c</sub>	White, S. L. M.*	do.	do.
74	Co 74E	Co 74E <sub>c</sub>	White, L. M.	do.	do.

\* U. S. Department of Agriculture official cotton grades (27).

(1) Method. Five rabbits were injected with toxigenic culture filtrate Cu 11F and two rabbits with culture filtrate Cu 56F. Five rabbits were injected with the toxigenic cotton extract filtrate Co 11% and two rabbits with the extract filtrate Co 56%. Seven control rabbits were injected with sterile tryptose broth, and seven rabbits were retained as non-injected controls. Twenty-five hundredths ml of each material was injected intradermally.

The test animals were injected over the lymph nodes in the inguinal and axillary regions. Each animal was observed for skin reactions and other symptoms for a 30-hour period following injection. Rectal temperatures were taken on some animals in each group twice during a 24-hour period before injection and three times during a thirty-hour period following injection. Blood specimens were taken for total leucocyte and differential counts each time that the temperature was determined. The temperature records and the total and differential leucocyte counts are given in Table VI.

(2) Results. None of the animals developed any marked skin reactions at the site of injection, nor did they show any evidence of lymphangitis or lymphadenitis.

The animals injected with toxigenic culture filtrates showed a 1.1 to 3.1° F rise in temperature within 4 to 5 hours after injection. The animals receiving injections of toxigenic cotton extract filtrates showed a 0.0 to 1.0° F rise in temperature 4 to 5 hours following injection. The non-injected control animals and the control animals receiving sterile tryptose broth showed changes in temperature from -0.1 to 1.0° F during the same periods in which the injected animals were observed.

The variations in total leucocyte and differential counts were so

TABLE VI

Effect of the Intradermal Injection of Toxic Material in Rabbits  
Temperature Changes and Leucocyte Counts

Rabbit No.	Material Injected	Temperature		Total Leucocyte Count per mm <sup>3</sup>	Differential Leucocyte Count					Skin Reaction
		Before or After Injection	Rectal °F		Seg- Bands	Lympho- monere cytes	Mono- cytes	Eosino- philes	Baso- philes	
1	Cu 11F	After	106.8	-	-	-	-	-	-	None
		do.	105.2	-	-	-	-	-	-	
2	do.	do.	105.9	-	-	-	-	-	-	do.
		do.	104.0	-	-	-	-	-	-	
3	do.	Before	102.6	22,800	9	43	39	9	0	Edema
		do.	102.6	10,800	4	23	68	4	0	
		After	103.7	43,600	21	42	22	4	4	
		do.	102.2	15,800	1	27	68	2	2	
		do.	102.5	26,700	2	44	41	7	0	
4	do.	Before	101.1	8,000	3	43	41	4	4	None
		do.	102.3	10,600	3	28	53	6	2	
		After	104.7	8,800	3	45	38	4	2	
		do.	102.6	13,400	1	45	50	2	0	
		do.	103.3	11,600	1	30	66	2	1	
5	do.	Before	103.6	11,400	0	39	48	6	1	Slight Edema
		do.	103.3	14,400	1	36	51	6	2	
		After	106.4	10,400	9	69	19	1	0	
		do.	103.0	14,200	3	16	74	2	3	
		do.	103.2	20,200	2	31	63	3	1	
6	Cu 86F	After	103.0	-	-	-	-	-	-	None
		do.	103.8	-	-	-	-	-	-	
7	do.	Before	104.8	7,400	0	51	65	0	0	do.
		do.	104.6	9,800	0	24	72	2	0	
		After	105.8	14,600	3	78	16	2	0	
		do.	104.7	8,400	0	39	54	7	0	
		do.	103.3	9,600	0	43	52	5	0	
8	Co 11E	After	106.8	-	-	-	-	-	-	do.
		do.	105.2	-	-	-	-	-	-	
9	do.	do.	105.9	-	-	-	-	-	-	do.
		do.	104.9	-	-	-	-	-	-	

TABLE VI (Cont.)

Rabbit No.	Material Injected	Temperature		Total Leucocyte Count per mm <sup>3</sup>	Differential Leucocyte Count					Skin Reaction	
		Before or After Injection	Rectal °F		Seg- Bands	Lympho- cytes	Mono- cytes	Baso- philes	Eosino- philes		
10	Co 11B	Before	103.2	12,400	2	20	72	5	1	0	Slight Edema
		do.	103.2	11,400	2	31	60	2	1	4	
		After	103.6	20,600	6	31	56	3	1	3	
		do.	102.8	11,800	0	25	69	6	0	0	
		do.	103.5	14,400	6	26	58	7	0	3	
11	do.	Before	103.2	12,400	5	28	64	6	0	0	Slight Edema
		do.	102.9	13,800	0	27	56	9	5	4	
		After	103.5	13,000	6	44	37	3	2	8	
		do.	103.1	13,400	0	25	72	3	0	0	
		do.	102.9	11,000	4	30	61	6	0	0	
12	do.	Before	103.4	10,400	3	30	52	9	0	6	Slight Edema
		do.	103.8	11,000	3	31	54	9	1	2	
		After	103.0	17,800	4	34	50	6	1	5	
		do.	103.1	9,400	2	53	42	3	0	1	
		do.	103.8	10,400	6	62	28	3	1	0	
13	Co 56E	do.	108.0	-	-	-	-	-	-	-	None
		do.	103.8	-	-	-	-	-	-	-	
14	do.	Before	104.0	16,000	0	54	40	2	0	4	Slight Edema
		do.	103.4	13,800	0	50	48	2	0	0	
		After	104.4	16,000	0	58	32	7	2	1	
		do.	103.5	11,800	0	36	57	7	0	0	
		do.	103.1	16,400	0	50	44	6	0	0	
15	T	do.	-	-	-	-	-	-	-	-	None
		do.	-	-	-	-	-	-	-	-	
16	T	do.	-	-	-	-	-	-	-	-	do.
		do.	-	-	-	-	-	-	-	-	
17	T	do.	-	-	-	-	-	-	-	-	do.
		do.	-	-	-	-	-	-	-	-	
18	T	Before	103.4	11,400	0	40	56	2	0	2	
		do.	103.2	12,000	0	34	59	5	0	2	

TABLE VI (Cont.)

No. of material injected	Temp- Before infection	Total leuco- cyte count	Differential Leucocyte Count			Seg- -lymph- -mono- -baso- -Histo- -Skin	Reaction
			bands	neutrophils	lymphocytes		
18	After 104.2	12,000	34	64	2	0	None
	do. 104.0	15,600	37	61	1	1	
	do. 104.0	12,400	62	35	3	0	
19	Before 104.0	12,800	42	45	5	2	0
	do. 103.4	21,400	30	53	7	5	0
	After 104.3	22,800	38	49	8	6	0
	do. 103.4	29,600	10	76	8	2	0
	do. 102.9	60,200	27	45	24	2	0
20	Before 104.0	10,800	39	43	3	2	10
	do. 104.0	18,400	31	50	11	0	5
	After 102.9	13,000	34	45	11	0	5
	do. 102.6	10,200	14	75	5	1	3
	do. 102.9	20,800	36	62	1	0	1
21	Before 103.6	10,600	36	52	4	1	2
	do. 102.2	12,600	40	44	10	1	4
	After 102.4	20,200	45	43	10	0	1
	do. 103.6	13,100	23	69	5	0	2
	do. 102.8	8,200	30	66	2	1	0
22	Control 4:25 PM 104.2	-	-	-	-	-	Not injected
	10:40 AM 102.3	-	-	-	-	-	
	4:30 PM 104.8	-	-	-	-	-	
	10:25 AM 106.0	-	-	-	-	-	
	4:25 PM 103.8	-	-	-	-	-	
	10:25 AM 103.7	-	-	-	-	-	
23	10:30 AM 102.0	12,600	33	61	3	0	1
	4:30 PM 102.1	10,600	36	60	4	0	0
	10:15 AM 102.9	29,600	54	39	5	0	1
	4:35 PM 102.9	14,800	52	48	0	0	0
	1:30 PM 103.2	14,000	35	51	6	3	4
	9:50 AM 103.0	11,800	31	49	5	1	8
	2:05 PM 103.4	14,200	30	47	6	1	12
	9:50 AM 102.8	15,900	35	56	2	0	2
	2:40 PM 102.8	10,200	4	51	0	0	0

TABLE VI (Cont.)

Rabbit No.	Material Injected	Temperature		Total Leucocyte Count per mm <sup>3</sup>	Differential Leucocyte Count					Skin Reaction	
		Before	Rectal		Seg- Bands	Lympho- monera	Mono- cytes	Baso- philes	Eosino- philes		
		or After Injection	°F								
27	Control	1:50 PM	103.4	14,800	2	25	63	10	0	0	Not Injected
		9:30 AM	103.4	12,800	3	20	69	7	0	1	
		1:50 PM	103.4	14,800	4	21	66	3	0	6	
		9:45 AM	102.8	11,800	0	29	62	7	0	2	
		2:50 PM	103.4	12,600	0	30	68	2	0	0	
28	do.	1:40 PM	103.2	12,800	4	45	35	10	2	4	do.
		9:40 AM	103.3	20,800	2	23	62	9	0	6	
		2:00 PM	103.5	16,400	5	43	38	5	2	7	
		9:40 AM	103.5	18,600	7	37	48	5	0	3	
		2:45 PM	103.6	17,000	1	46	49	1	0	3	

marked in the control as well as in the test animals that no definite conclusions could be drawn.

While the intradermal injections of culture filtrates and cotton extract filtrates resulted in definite temperature increases in the test animals, skin reactions, physical signs, and white blood cell counts were too variable to be of diagnostic value. It was, therefore, concluded that rabbits are not suitable animals for intradermal skin tests for the detection of the endotoxin produced by toxigenic strains of A. glaucus.

b. Tests with Chickens. Bunyee and MacDonald (2) reported that certain strains of A. aerogenes and E. coli var. acidilactici are capable of causing mortality in turkeys. Their pathogenicity tests showed that both organisms when recently isolated from turkeys were capable, by intravenous or intraperitoneal inoculation, of producing disease and death in young or adult turkeys. Other workers have reported that young chickens are also susceptible to infection with certain members of the coliform group of bacteria. In view of these reports, young chickens were studied as test subjects for the detection of endotoxin in cultures of A. glaucus and in toxigenic filtrates from extracts of stained cotton.

(1) Materials Tested. Viable culture suspensions of toxigenic A. glaucus (Cu 56L); killed culture suspensions of toxigenic A. glaucus (Cu 56K); culture filtrates from toxigenic A. glaucus (Cu 56F and Cu 11F); toxigenic cotton extract filtrate (Co 56E); normal cotton extract filtrate (Co 44-5E); sterile tryptose broth (T); and isotonic salt solution (S).

(2) Methods of Administration and Dosage. Gavage, 0.50 ml and 1.0 ml; intradermal injection, 0.25 ml; intravenous injection, 0.50 ml; intraperitoneal injection, 1.00 ml; and inhalation exposure to contaminated cotton dust.

Test No. 1. Fifty-six chickens were employed in this test series. These chickens were obtained at the age of one day and held under observation in an electric brooder for 7 days prior to test. They were fed a balanced starter mash, watered, and their environment was maintained in a sanitary condition. All chicks were strong and healthy at the time of test.

Five-tenths ml of each test substance (Cu 56F, Cu 56L, Cu 56K, Co 56X, Co 44-5X, T, and B) was administered to six 7-day-old chicks by gavage. Fourteen uninoculated chicks were retained as controls. All birds were observed throughout a two-week period. None of the chicks developed any abnormal signs or symptoms during the observation period. At the end of the first two-week period, 1.0 ml of each test material was administered to the same birds as in the initial experiment. All birds were observed for an additional 10-day period following the second series of injections. None of the birds developed any abnormal signs or symptoms of infection or intoxication following the second series of injections with larger doses of the test materials.

At the termination of the experiment all of the birds receiving viable culture suspensions, representative birds receiving the other test materials, and control birds were killed by ether anesthesia and autopsied. All gross autopsy findings were negative. It was concluded that young chicks are not susceptible to infection or intoxication with toxigenic strains of A. gloaceus or their products when the test materials are administered by gavage.

Test No. 2. These tests were undertaken in order to ascertain whether young chicks were susceptible to infection or intoxication with A. gloaceus or its products following other avenues of administration.



Forty-nine chickens were employed in this test series. These chicks were obtained at the age of one day and held in an electric brooder for 24 hours without food. They were kept on a balanced starter mash and water, and their environment was maintained in a sanitary condition. The chicks were kept under observation for 16 days before test and all were strong and healthy at the time of test.

Each test substance (Cu 56L, Cu 56K, Cu 11F, Co 56E, Co 44-5K, and T) was administered to three chicks by intradermal injection and to three birds by intraperitoneal injection, except in the case of the six birds receiving Cu 56F. This filtrate was administered to three birds by intradermal injection, to two birds by intravenous injection, and to one bird by intraperitoneal injection. Intravenous injections were found to be impractical, hence this method of administration was not employed for any of the other test materials. Seven uninoculated birds were retained as controls.

The birds receiving a killed culture suspension, culture filtrates, cotton extract filtrates and sterile tryptose broth were kept under observation for a period of two weeks while the chicks receiving live culture suspensions were kept under observation for 32 days. None of the birds developed any symptoms or reactions indicative of intoxication or infection following the intradermal, intravenous, or intraperitoneal administration of the test substances. Representative birds from each group were autopsied at the end of the final observation period. The gross autopsy findings were negative.

It was concluded that 7 to 38-day-old chicks are not susceptible to infection or intoxication with toxigenic A. gleucosus or its products.

Test No. 3. These studies were undertaken in order to ascertain

whether immature chickens were susceptible to acute respiratory illness either during or following exposure to dust from stained cotton heavily contaminated with a toxigenic strain of A. slouzei and its toxic products.

Eight of the control birds employed in the previous tests were used for these studies. These chickens were 40 days old at the time of test. They were exposed, by inhalation, to a heavy concentration of dust from stained cotton for a continuous period of 7 hours. The incidence of mold microorganisms in the stained cotton employed was 78,000,000 per gm.

The temperature in the exposure chamber ranged from 89 to 92° F during the 7-hour exposure period. Water was provided for the birds after the first  $3\frac{1}{2}$  hours' exposure.

The chickens did not exhibit any evidence of illness during the exposure period, except symptoms of discomfort such as restlessness, ruffled feathers, panting, etc. These minor symptoms were attributed to the high temperatures prevailing in the exposure compartment throughout the exposure period. The chickens were normal and ready for food when removed from the exposure compartment; they remained in a normal condition throughout a 48-hour period following exposure. It was concluded that immature chickens are not susceptible to acute respiratory illness following inhalation exposure to contaminated stained cotton dust.

c. Tests with White Mice. These tests were carried out in order to determine whether white mice would exhibit a skin reaction or other symptoms of intoxication following the intradermal injection of the toxic products obtained from cultures of A. slouzei or from extracts of stained cotton.

Eight healthy, mature white mice were employed in these studies. The abdomen of each animal was carefully shaved 24 hours before the beginning of the test.

(1) Materials tested. Killed culture suspension of A. gloabae (Cu 11E); culture filtrates from toxigenic A. gloabae (Cu 11F and Cu 56F); toxigenic cotton extract filtrates (Co 11E and Co 56E); normal cotton extract filtrate (Co 44-EE); sterile tryptose broth (T); and sterile isotonic salt solution (S).

Each mouse was injected intradermally with 0.125 ml of one of the test materials. None of the mice developed any skin reactions or other symptoms following these intradermal injections. It was concluded that white mice are unsatisfactory for skin tests, since they do not respond to the intradermal administration of materials containing the endotoxin.

### 3. Serological Methods.

a. Shwartzman Reaction. Shwartzman (22, 23, 24) first demonstrated a new phenomenon of local skin reactivity to Bacillus typhosus culture filtrates. The reactivity was induced in rabbits by intradermal injections of the filtrate followed, after a 24-hour interval, by the intravenous injection of the same filtrate. The local reactions appeared at the site of the skin injections; they were fully developed within 4 to 5 hours after the intravenous injection; they were extremely severe and showed pronounced necrosis of tissue with rupture of blood vessels and extensive local hemorrhage. The reactions in different rabbits varied in size from 1x1 cm to 4x4 cm. About 78 percent of the animals tested developed severe local hemorrhagic reactions. The remaining animals were spontaneously resistant to the phenomenon. The toxic factor inducing the reaction was thermostable. Hydrogen ion concentration did not inactivate the skin preparatory factors throughout a pH range of 4.0 to 9.0. There was no change in the heat resistance of the preparatory factors at any pH within this range. Shwartzman (23) reported that the mechanism of this

hemorrhagic reaction differs from bacterial hypersusceptibility in (a) local reactivity, (b) short incubation period necessary to induce the local reactivity, (c) the short duration of the state of reactivity, (d) the ability to induce local reactivity by a single skin injection, (e) the severity of the reaction, and (f) the necessity to make the second injection of the toxic agent by the intravenous route.

In preliminary studies previously reported (14, 21) it was revealed that extracts of stained cotton, incriminated in outbreaks of acute illness, killed culture suspensions, and culture filtrates of A. cloacae isolated from this cotton contained a thermostable, endotoxin-like substance capable of eliciting the Shwartzman reaction in rabbits. The results of these preliminary studies were sufficiently encouraging to warrant additional investigation of the Shwartzman phenomenon as a specific routine test for the detection of this endotoxin in culture filtrates and in extracts from stained cotton or other materials incriminated in future outbreaks of illness.

(1) **Animals employed.** White mice and rabbits were employed in these tests.

(2) **Materials tested.** The materials tested included killed culture suspensions and culture autolysates of toxigenic strains of A. cloacae and other closely related microorganisms, filtrates from saline extracts of stained and of normal cotton, vacuum distillation concentrates from saline extracts of cotton, sterile tryptose broth and isotonic salt solution. The identifying symbols and the description of these materials are given in Tables IV and V. Each test substance will be referred to by symbol throughout these tests in order to simplify the tabular data.

(3) **Technique.** In demonstrating the Shwartzman phenomenon, sensi-

tivity is induced by intradermal injections (preparatory injections) of 0.25 to 0.5 ml of the substances to be tested. Reactivity is induced by intravenous injections (reacting injections) of 1.0 to 3.0 ml of the test materials per kg of body weight, 24 hours after the preparatory injections. A positive Shwartzman reaction is evidenced by a severe hemorrhagic necrosis, usually beginning at the site of the intradermal injection within 4 hours after the reacting injection. The sites become swollen and dark blue with a red border. Typical positive Shwartzman reactions are shown in Figures 2, 3, and 4.

Tests with Mice. Since mice are cheap and readily available in the majority of laboratories, tests were made to ascertain whether the Shwartzman phenomenon could be induced in these animals. Eight healthy mature mice were prepared for injection by shaving the skin over the entire abdomen with a safety razor. One mouse was used for each material tested. Each mouse was given an intradermal injection in the skin of the abdomen with 0.125 ml of one of the materials tested, followed 24 hours later by an intravenous injection of 0.25 ml of the same materials. The materials used and the results of the tests are given in Table VII.

All of the toxic materials tested failed to elicit any response in mice following either the intradermal or intravenous injections. Therefore, it was concluded that mice are not suitable test subjects for the Shwartzman reaction. This is in accord with the results reported by Stolywe (25).

Tests with Rabbits. Two hundred and twenty-one healthy mature rabbits were employed in these studies. Each animal was prepared for test by clipping or by epilating the hair from the skin over an area 3"x5" on its side 24 hours prior to the initial injection.



Figure 2: Shurtman Reaction (ccc)  
Preparatory Injection = 0.25 ml. On 567.  
Reacting Injection = 1.00 ml. On 567.



**Figure 3: Shwartzman Reaction (ccc)**  
**Preparatory Injection = 0.25 ml Co 56K.**  
**Reacting Injection = 5.75 ml Co 56K.**



**Figure 4: Shartsman Reaction (+)**  
**Preparatory Injection = 0.25 ml Ca 119.**  
**Reacting Injection = 0.50 ml Ca 567.**



TABLE VII

Shwartzman Reaction in Mice for the Detection of Endotoxin

Mouse No.	Sensitizing Injections		Reacting Injections		Reaction
	Materials	Quantity ml	Materials	Quantity ml	
1	Cu 11F	0.125	Cu 11F	0.25	None
2	Co 11B	do.	Co 11B	do.	do.
3	Cu 56F	do.	Cu 56F	do.	do.
4	Co 56B	do.	Co 56B	do.	do.
5	Co 44-5E	do.	Co 44-5E	do.	do.
6	Cu 11K	do.	Cu 11K	do.	do.
7	F	do.	F	do.	do.
8	S	do.	S	do.	do.

Test 1: Only materials in which there was a reasonable certainty of toxicity were employed in this test. These materials consisted of killed culture suspensions, culture autolyates, extracts of stained cotton, and vacuum concentrates from saline extracts of stained cotton. All tests were carried out according to the technique previously described. The materials injected for direct and cross reactions and the results of this series of tests are given in Table VIII.

These studies demonstrated that positive Shwartzman reactions could be obtained with killed culture suspensions and culture autolyates of toxigenic strains of *A. glaucus* or with extracts and extract concentrates of the stained cotton from which these microorganisms were isolated. Positive reactions were obtained regardless of whether each substance was used separately as sensitizing and reacting injections or when cross injected with each other. It is obvious from these results that a similar toxigenic substance is present in the cultures and in the stained cotton from which the cultures were isolated. More consistent results were obtained when killed culture suspensions or culture autolyates were employed for the reacting injections. However, it was found that a reacting dosage of less than 0.5 ml per kg should be employed in order to avoid killing the animal before a positive reaction could be obtained. The dosage required when stained cotton extracts or extract concentrates were employed for the reacting injections ranged from 1.0 to 3.0 ml per kg. It is evident that the potency of the endotoxin-like substances is much higher in killed cultures and culture autolyates than in cotton extracts or extract concentrates.

Ninety rabbits were employed in this test series. Positive Shwartzman reactions were obtained with 53 (58.9 percent) of these rabbits. A

TABLE VIII

Positive Shwartzman Reactions Produced with Killed Cultures and Culture Antolysates of *A. glaucus* and Extracts of Stained Cotton

Rabbit No.	Weight kg	Sensitizing Injections		Reacting Injections			Reaction	
		Materials	Quantity ml	Materials	Quantity ml per kg	Quantity ml	Degree	Type
29	3.17	Cu 11K	0.25	Cu 11K	0.94	3.00	4+	HN* (died)
34	3.45	Cu 11F	do.	do.	0.43	1.50	4+	HN
41	2.61	Cu 11K	do.	Cu 11F	0.59	1.50	3+	HN
37	3.01	do.	do.	Co 11E	1.99	6.00	-	No reaction
38	2.91	Co 11E	do.	Cu 11K	0.51	1.50	2+	HN (died)
37	3.60	Cu 11K	do.	Co 11E	2.22	8.00	-	No reaction
32	2.91	do.	do.	Cu 56K	0.34	1.00	-	do.
45	2.88	Cu 56K	do.	Cu 11K	0.34	1.00	-	do.
40	3.64	Cu 11K	do.	Cu 56K	0.42	1.50	2+	HN
42	3.60	Cu 56K	do.	Cu 11K	0.42	1.50	+	HN
21	2.54	Cu 11K	do.	Cu 56F	0.39	1.00	2+	HN
26	3.42	Cu 56F	do.	Cu 11K	0.43	1.50	2+	HN
48	3.54	Cu 11K	do.	Cu 56F	0.35	1.25	2+	HN
50	2.92	Cu 56F	do.	Cu 11K	0.50	1.50	3+	HN
48	2.73	Cu 11K	do.	Cu 56F	1.83	5.00	-	No reaction
49	3.52	Cu 56F	do.	Cu 11K	0.49	1.75	3+	HN
49	4.10	do.	do.	do.	0.43	1.75	2+	HN
65	1.48	Cu 11K	do.	Cu 56F	2.70	4.00	4+	HN

\*H = Hemorrhagic; N = Necrosis.

TABLE VIII (Cont.)

Rabbit No.	Weight kg	Sensitizing Injections		Reacting Injections			Reaction	
		Materials	Quantity ml	Materials	Quantity ml per kg	Quantity ml	Degree	Type
43	2.43	Cu 56K	0.25	Cu 56K	0.41	1.00	4+	HN (died)
66	2.30	do.	do.	do.	0.43	1.00	2+	HN
31	3.04	do.	do.	Cu 56F	0.49	1.50	4+	HN (died)
39	2.88	Cu 56F	do.	Cu 56K	0.52	1.50	2+	HN (died)
46	2.44	Cu 56K	do.	Co 56E	2.04	5.00	4+	HN
47	2.52	Co 56E	do.	Cu 56K	0.59	1.50	3+	HN (died)
25	2.34	Cu 11F	do.	do.	0.29	1.00	-	No reaction
27	3.70	Cu 11K	do.	Cu 11F	0.47	1.75	4+	HN
26	3.54	Cu 11F	do.	Cu 56K	0.43	1.50	+	HN
40	3.28	Co 11E	do.	do.	0.33	1.00	-	No reaction
50	2.75	Cu 56K	do.	Co 11E	2.54	7.00	-	do.
56	3.12	do.	do.	do.	2.56	8.00	-	do.
62	1.94	Co 11E	do.	Cu 56K	0.51	1.00	3+	HN (died)
20	2.64	Cu 56F	do.	Cu 56F	1.13	3.00	4+	do.
49	3.81	Cu 56K	do.	do.	0.33	1.25	4+	HN
34	3.88	Cu 56F	do.	do.	0.39	1.50	3+	HN
101	2.85	do.	do.	do.	0.49	1.40	4+	HN (died)
143	3.13	do.	do.	do.	0.30	0.94	4+	do.
119	2.64	do.	do.	do.	0.05	0.125	4+	HN
120	2.60	do.	do.	do.	0.10	0.25	4+	HN (died)
121	2.88	do.	do.	do.	0.20	0.60	4+	do.

TABLE VIII (Cont.)

Rabbit No.	Weight kg	Sensitizing Injections		Reacting Injections			Reaction	
		Materials	Quantity ml	Materials	Quantity ml per kg	Quantity ml	Degree	Type
122	2.90	Cu 56F	0.25	Cu 56F	0.30	0.90	4+	HN (died)
124	3.64	do.	do.	do.	0.40	1.50	4+	do.
123	3.70	do.	do.	do.	0.50	1.90	3+	HN
140	2.75	do.	do.	(1:10 dilution) Cu 56F	1.10	0.275	4+	HN
139	2.87	do.	do.	Cu 56F	0.05	0.143	4+	HN
136	3.01	do.	do.	do.	0.10	0.30	4+	HN
132	2.98	do.	do.	do.	0.20	0.60	4+	HN (died)
143	3.13	do.	do.	do.	0.30	0.94	4+	do.
164	2.42	do.	do.	(1:10 dilution) Cu 56F	0.20	0.50	-	No reaction
201	2.37	do.	do.	do.	0.20	0.50	-	do.
33	3.19	Cu 11F	do.	Cu 11F	0.47	1.50	4+	HN
18	3.22	Co 11E	do.	Co 11E	1.39	4.50	-	No reaction
19	3.02	do.	do.	do.	2.98	9.00	-	do.
51	4.34	do.	do.	do.	2.20	10.00	-	do.
59	4.10	do.	do.	do.	1.92	8.00	-	do.
21	2.94	Co 56E	do.	Co 56E	1.53	4.50	-	do.
30	3.42	do.	do.	do.	2.33	8.00	3+	HN (died)
63	1.94	do.	do.	do.	2.06	4.00	-	No reaction
102	2.82	do.	do.	do.	2.03	5.75	4+	HN

TABLE VIII (Cont.)

Rabbit No.	Weight kg	Sensitizing Injections		Reacting Injections			Reaction	
		Materials	Quantity ml	Materials	Quantity ml per kg	Quantity ml	Degree	Type
89	2.04	Co 56E <sub>c</sub>	0.25	Co 56E <sub>c</sub>	0.98	2.00	4+	HN (died)
18	3.08	Cu 11F	do.	Cu 56F	0.32	1.00	+	H (died)
19	3.11	Cu 56F	do.	Cu 11F	0.48	1.50	-	No reaction
53	3.48	do.	do.	do.	0.50	1.75	3+	HN
54	3.54	Cu 11F	do.	Cu 56F	0.35	1.25	3+	HN
163	1.96	do.	do.	(1:10 dilution) Cu 56F	0.20	0.40	+	HN
197	2.46	do.	do.	do.	0.20	0.50	2+	HN
15	4.73	do.	do.	Co 11E	1.88	9.00	2+	HN (died)
16	4.33	Co 11E	do.	Cu 11F	0.36	1.50	3+	do.
35	2.72	Cu 11F	do.	Co 56E	2.20	6.00	4+	HN
36	3.11	Co 56E	do.	Cu 11F	0.48	1.50	4+	HN
26	3.24	Cu 56F	do.	Co 56E	1.38	4.50	-	No reaction
24	2.92	Co 56E	do.	Cu 56F	1.02	3.00	4+	HN (died)
17	4.44	Cu 56F	do.	Co 56E	2.02	9.00	4+	do.
40	3.03	do.	do.	Co 11E	1.98	6.00	-	No reaction
44	2.97	Co 11E	do.	Cu 56F	0.50	1.50	4+	HN (died)
63	1.83	Cu 56F	do.	Co 11E	2.73	5.00	-	No reaction
65	1.58	Co 56E <sub>c</sub>	do.	Cu 56F	0.47	0.75	3+	HN (died)
87	2.02	Cu 56F	do.	Co 56E <sub>c</sub>	0.99	2.00	3+	HN
103	2.93	Co 56E	do.	Cu 56F	0.51	1.50	3+	HN (died)

TABLE VIII (Cont.)

Rabbit No.	Weight kg	Sensitising Injections		Reacting Injections			Reaction	
		Materials	Quantity ml	Materials	Quantity ml per kg	Quantity ml	Degree	Type
91	3.52	Co 56K	0.25	Cu 56F	0.30	1.05	-	No reaction (died)
205	2.31	do.	do.	(1:10 dilution) Cu 56F	0.30	0.45	-	No reaction
168	2.46	Co 11E	do.	do.	0.20	0.50	-	do.
172	2.58	Co 56K	do.	do.	0.20	0.50	-	do.
71	4.00	Co 11E <sub>g</sub>	do.	do.	0.20	0.80	-	do.
140	3.40	Co 56K <sub>g</sub>	do.	do.	0.20	0.70	-	do.
159	2.60	do.	do.	do.	0.20	0.50	+	HN
42	2.90	Co 11E	do.	Co 56E	2.06	6.00	-	No reaction
45	3.20	Co 56E	do.	Co 11E	2.03	6.50	2+	HN
66	2.24	Co 11E	do.	Co 56E	2.67	6.00	2+	HN
70	2.02	Co 56K	do.	Co 56K <sub>g</sub>	0.99	3.00	4+	HN (died)
71	2.04	Co 56K <sub>g</sub>	do.	Co 56E	1.96	4.00	+	HN

positive response could not be elicited from 28 (31.1 percent) of the animals. These figures confirm the results reported by Schwartzman (22, 23) who found that approximately 21 to 22 percent of rabbits are spontaneously resistant to the phenomenon. Of the 28 rabbits, from which a positive reaction could not be obtained, 14 (50 percent received pre-paratory or reacting injections of cotton extract filtrate (Co 11X). This extract was known to possess an endotoxin of low potency. The age or size of the rabbits did not appear to influence the reaction.

Test 2: These studies were carried out in order to demonstrate that the control materials used in these studies would not elicit a positive Schwartzman reaction. Physiological saline was used throughout this investigation for the extraction of the toxin from the cotton and tryptose phosphate broth was used in the preparation of culture antolyzates. A saline extract of normal cotton was also included in order to provide evidence that extractable fractions of this cotton were nontoxic. The control substances were injected separately as both pre-paratory and reacting factors. They were also injected for gross reactions with each other and with known toxicogenic substances.

The materials injected for both direct and gross reactions and the results obtained are recorded in Table IX.

Forty-five rabbits were employed for these tests. Positive Schwartzman reactions could not be obtained with any of the control materials, regardless of whether they were employed singly as both sensitizing and reacting factors, or in gross reactions with each other or with known toxic materials. It was concluded that sterile tryptose phosphate broth, isotonic salt solution, and saline extracts of normal noncontaminated cotton are incapable of eliciting a positive response whether employed alone or in conjunction with known toxicogenic materials.



TABLE IX

Shwartzman Reaction. Tests with Control Materials.

Rabbit No.	Weight kg	Sensitizing Injections		Reacting Injections			Reaction	
		Materials	Quantity ml	Materials	Quantity ml per kg	Quantity ml	Degree	Type
23	2.81	T	0.25	T	1.77	5.00	-	No reaction
27	3.65	Cu 56F	do.	T	2.46	9.00	-	do.
23	2.86	T	do.	Cu 56F	0.52	1.50	-	No reaction (died)
19	3.48	T	do.	Co 56K	1.72	6.00	-	No reaction
21	2.95	Co 56K	do.	T	8.71	8.00	-	do.
27	3.84	T	do.	Co 44-5E	2.08	8.00	-	do.
32	2.64	Co 44-5K	do.	T	3.03	8.00	-	do.
35	3.22	S	do.	T	2.86	8.00	-	do.
36	2.44	T	do.	S	2.32	8.00	-	do.
37	3.36	T	do.	Cu 11F	0.60	2.00	-	do.
40	3.00	Cu 11F	do.	T	2.35	8.00	-	do.
41	3.18	T	do.	Co 11E	2.51	8.00	-	do.
3	4.32	Cu 11K	do.	T	1.85	8.00	-	do.
12	4.74	Cu 56K	do.	T	1.68	8.00	-	do.
21	3.10	T	do.	Cu 11K	0.57	1.75	-	do.
26	3.60	T	do.	Cu 56K	0.42	1.50	-	do.
34	4.10	Co 11E	do.	T	1.92	8.00	-	do.
22	2.83	S	do.	S	1.76	5.00	-	do.
25	2.09	Cu 56F	do.	S	4.30	9.00	-	do.

TABLE IX (Cont.)

Rabbit No.	Weight kg	Sensitizing Injections		Reacting Injections			Reaction	
		Materials	Quantity ml	Materials	Quantity ml per kg	Quantity ml	Degree	Type
22	3.09	S	0.25	Cu 56F	0.48	1.50	-	No reaction (died)
25	3.52	Co 56E	do.	S	2.27	8.00	-	No reaction
26	3.54	S	do.	Co 56E	1.69	6.00	-	do.
33	3.66	Co 44-5E	do.	S	2.18	8.00	-	do.
24	3.34	S	do.	Co 44-5E	3.29	8.00	-	do.
42	3.36	Cu 11F	do.	S	2.38	8.00	-	do.
46	2.82	S	do.	Cu 11F	0.71	2.00	-	No reaction (died)
48	3.24	Co 11E	do.	S	2.46	8.00	-	No reaction
11	5.30	Cu 11K	do.	S	1.50	8.00	-	do.
13	5.60	Cu 56K	do.	S	1.42	8.00	-	do.
25	3.42	S	do.	Cu 11K	0.49	1.75	-	do.
32	3.24	S	do.	Cu 56K	0.46	1.50	-	do.
33	4.10	S	do.	Co 11E	1.95	8.00	-	do.
27	3.52	Co 44-5E	do.	Co 44-5E	1.42	5.00	-	do.
32	2.81	Cu 56F	do.	do.	3.20	9.00	-	do.
28	2.86	Co 44-5E	do.	Cu 56F	0.52	1.50	-	No reaction (died)
51	3.56	Co 11E	do.	Co 44-5E	2.81	10.00	-	No reaction
53	2.94	Co 44-5E	do.	Cu 11F	0.51	1.50	-	do.
54	3.02	Cu 11K	do.	Co 44-5E	2.98	9.00	-	do.

TABLE IX (Cont.)

Rabbit No.	Weight kg	Sensitizing Injections		Reacting Injections			Reaction	
		Materials	Quantity ml	Materials	Quantity ml per kg	Quantity ml	Degree	Type
55	3.85	Co 44-5E	0.25	Cu 11K	0.51	2.00	-	No reaction (died)
56	2.52	Cu 56K	do.	Co 44-5E	3.14	8.00	-	No reaction
57	3.60	Co 44-5E	do.	Cu 56K	0.41	1.50	-	No reaction (died)
61	3.60	Cu 56K	do.	Co 44-5E	2.22	8.00	-	No reaction
50	2.90	Co 11E	do.	do.	2.75	8.00	-	do.
59	3.74	Co 44-5E	do.	Co 11E	2.13	8.00	-	do.
35	3.44	Co 44-5E	do.	Co 56E	2.32	8.00	-	do.

Test 3: This series of tests was undertaken in order to ascertain whether immune serum prepared for toxigenic strains of A. cloacae would neutralize the toxic skin preparatory factors present in killed culture suspensions, and culture filtrates of the organism and in extracts and extract concentrates from stained cotton. It seemed reasonable to assume that the Shwartzman reaction could be made a more specific test if the immune sera for known toxigenic materials from stained cotton or culture autolysates would neutralize or prevent a reaction with these materials, while permitting a reaction with toxic substances from other microorganisms. Immune serum was mixed with killed culture suspensions, culture autolysates, stained cotton extracts, or stained cotton extract concentrates in the ratio of 1 part toxigenic material to 3 or 4 parts of serum. In some cases the serum filtrate mixtures were incubated for one hour at room temperature and in other cases at 37° C for 18 hours. The temperature and the length of the incubation did not appear to influence the results. Both culture materials from known toxigenic strains of A. cloacae and from other closely related strains of bacteria known to give a positive Shwartzman test were employed in these tests. The serum-filtrate mixtures were used as preparatory or sensitizing injections. Eighteen rabbits were given the preparatory injections with serum-filtrate mixtures. Fourteen of the eighteen rabbits failed to show a positive Shwartzman reaction following the intravenous injection. A positive reaction was elicited in four rabbits in spite of the serum neutralisation of the toxigenic materials employed. In order to ascertain the effect of the dilution factor, eight rabbits were given preparatory injections with toxic filtrates which had been diluted with sterile isotonic saline in the same ratios as with serum and incubated as in the case of serum. Four

of the rabbits receiving the toxic filtrate-saline mixtures gave the positive Shwartzman reaction, while the results obtained with the remaining four rabbits were negative. The materials employed, the quantities injected, and the results obtained in these tests are summarized in Table X.

Shwartzman (23) reported that toxic filtrates from B. typhosus cultures diluted 1:4 were consistently able to induce local skin reactivity. Since the results of the studies reported here were somewhat variable, it is believed that the dilution factor may have influenced the results.

It is also apparent that toxic skin preparatory factors may be neutralized with immune serum. Additional studies on the effect of dilution and serum neutralization on skin preparatory factors should be carried out at some future time when rabbits are more readily available.

Test 4: From the results obtained in Test 1, it was evident that more consistent results could be obtained when autolysates from toxigenic cultures of A. cloacae were employed for the intravenous or reacting injections in the Shwartzman test, and cotton extracts or extract concentrates were employed as the sensitizing or preparatory injections. The culture autolysates were found to be of more constant potency, thus making it possible to adopt a standardized reacting dosage for the test.

Shwartzman (22, 23) recommended the use of 1.0 to 3.0 ml per kg of body weight of the toxic substance liberated by typhoid bacilli for reacting injections. This dosage is too high for A. cloacae endotoxin. The following experiments were carried out in an attempt to standardize the Shwartzman reaction for the detection of endotoxin in culture filtrates or extracts from suspected cotton or other similar materials.

TABLE X

**Shwartzman Reaction. Prevention of Reaction  
by Homologous Leams Sera**

Rab- bit No.	Weight kg	Sensitizing Injections		Reacting Injections			Reaction	
		Materials	Quan- tity ml	Materials	Quan- tity ml per kg	Quan- tity ml	De- gree	Type
61	3.74	Cu 56F+ Serum (1:3)	0.25	Cu 56F	0.33	1.25	-	No reaction (died)
8	4.54	do.	do.	do.	0.33	1.50	+	H N* (died)
125	3.20	Cu 56F + Serum (1:4)	do.	do.	0.40	1.50	-	No reaction (died)
202	2.79	do.	do.	(1:10 dilution) Cu 56F	0.20	0.55	-	No reaction
198	2.86	Cu 11F+ Serum (1:4)	do.	do.	0.20	0.60	-	do.
72	4.80	Cu 56F + Serum (1:3)	do.	Cu H-55K	0.62	3.00	-	No reaction (died)
74	3.94	Cu H-55K+ Serum (1:3)	do.	Cu 56F	0.51	1.25	-	do.
75	4.67	do.	do.	Cu H-55K	0.64	3.00	-	No reaction
7	4.40	Cu 56K + Serum (1:5)	do.	Cu 56K	0.45	2.00	3+	H N (died)
67	2.24	Co 56K+ Serum (1:3)	do.	Co 56K	2.23	5.00	2+	H N
80	1.98	Co 56K+ Serum (1:3)	do.	do.	2.02	4.00	-	No reaction
84	1.60	Co 56K+ Serum (1:3)	do.	Co 56K <sub>0</sub>	1.25	2.00	-	do.
118	2.78	Cu 56F+ Serum (1:3)	do.	Cu 56F	0.50	1.40	-	No reaction (died)
135	4.09	do.	do.	do.	0.50	1.25	-	do.
186	2.78	Cu 4F+ Serum (1:4)	do.	(1:10 dilution) Cu 56F	0.20	0.55	-	No reaction
188	3.18	Cu 7F+ Serum (1:4)	do.	do.	0.20	0.55	3+	H
192	2.63	Cu 14F+ Serum (1:4)	do.	do.	0.20	0.50	-	No reaction
195	2.82	Cu 19F+ Serum (1:4)	do.	do.	0.20	0.55	-	do.
51	3.96	Cu 56F+ Saline (1:3)	do.	Cu 56F	0.32	1.25	3+	H N

\*H -- Hemorrhagic; N -- Necrosis

TABLE X (Cont.)

Rab- bit No.	Weight kg	Sensitizing Injections		Reacting Injections			Reaction	
		Materials	quan- tity ml	Materials	quan- tity ml per kg	quan- tity ml	Re- gree	Type
126	3.02	Cu 56F <sup>+</sup> Saline (1:4)	0.25	Cu 56F	0.40	1.20	+	H (died)
187	2.89	Cu 4F <sup>+</sup> Saline (1:4)	do.	(1:10 dilution) Cu 56F	0.20	0.60	2+	H
189	3.20	Cu 7F <sup>+</sup> Saline (1:3)	do.	do.	0.20	0.65	2+	H
193	3.04	Cu 14F <sup>+</sup> Saline (1:4)	do.	do.	0.20	0.60	-	No reaction
196	3.16	Cu 19F <sup>+</sup> Saline (1:4)	do.	do.	0.20	0.65	-	do.
199	2.40	Cu 11F <sup>+</sup> Saline (1:4)	do.	do.	0.20	0.50	-	do.
204	2.45	Cu 56F <sup>+</sup> Saline (1:4)	do.	do.	0.20	0.50	-	do.

In order to accomplish standardization of the test, it was necessary to ascertain (a) the minimum reacting dosage which would elicit a strongly positive Shwartzman reaction in rabbits without causing the death of the animals and, (b) whether the preparatory or sensitizing injection has any influence on the toxicity of the reacting injection. All rabbits were selected for uniformity of weight in these studies. Six rabbits (Nos. 119 to 124, Table XI) were given preparatory injections with 0.25 ml quantities of culture autolysate Cu 56F. Twenty-four hours later each animal received one of a gradually increasing series of intravenous dosages. These dosages ranged from 0.05 to 0.50 ml per kg. The results of these studies are summarized in Table XI. It was found that 0.05 ml of Cu 56F per kg, when employed as a reacting injection, elicited a 4+ Shwartzman reaction without resulting in the death of the animal. An intravenous dosage of 0.1 ml per kg, while producing a strongly positive reaction, also resulted in the death of the animal.

The following experiment was designed to ascertain whether the sensitizing injection has any influence on the toxicity or potency of the reacting injection. Two series of animals were employed for the tests. The corresponding animals in each series were selected for uniformity of weight. One series of animals received preparatory and reacting injections for the Shwartzman tests, while the animals in the second received only the same intravenous injections as the corresponding animals in the first series. The materials and quantities injected and the results of the test are shown in Table XI. The last ten animals in Table XI were included in this test. The minimum intravenous dosage producing death in this series of animals receiving a prior sensitizing injection was 0.2 ml per kg of body weight. The minimum fatal dosage for the animals



TABLE XI

## Shwartzman Reaction

## I. Minimum Reacting and Toxic Dosages

## II. Effect of Sensitising Injection on Toxicity of Intravenous Injection.

Rabbit No.	Weight kg	Sensitising Injections		Reacting Injections			Reaction	
		Materials	Quantity ml	Materials	Quantity per kg	Quantity ml	Degree	Type
119	2.64	Cu 56F	0.25	Cu 56F	0.05	0.125	4+	H N* (died)
120	2.60	do.	do.	do.	0.10	0.025	4+	do.
121	2.33	do.	do.	do.	0.20	0.60	4+	do.
122	2.90	do.	do.	do.	0.30	0.90	4+	do.
124	3.64	do.	do.	do.	0.40	1.50	4+	do.
125	3.70	do.	do.	do.	0.50	1.90	3+	do.
140	2.75	do.	do.	(1:10 dilution) Cu 56F	0.10	0.275	4+	H N.
139	2.87	do.	do.	Cu 56F	0.05	0.143	4+	H N
136	3.01	do.	do.	do.	0.10	0.30	4+	H N
132	2.98	do.	do.	do.	0.20	0.60	4+	H N (died)
143	3.13	do.	do.	do.	0.30	0.94	4+	do.
141	2.68	None	None	(1:10 dilution) Cu 56F	0.10	0.275	-	No reaction
139	2.92	do.	do.	Cu 56F	0.05	0.150	-	died
137	2.96	do.	do.	do.	0.10	0.30	-	do.
145	3.03	do.	do.	do.	0.20	0.60	-	do.
104	3.33	do.	do.	do.	0.30	1.00	-	do.

\*H -- Hemorrhagic; N -- Necrosis

not receiving sensitizing injections was 0.06 ml. per kg. of body weight. There is an indication, from the limited data available, that the preparatory injection may afford the animal some degree of protection to the toxicity of the intravenous injection. As a result of these studies the following standardized procedure was adopted for future studies with the Shwartzman reaction: Preparatory (sensitizing) injections = 0.25 ml. of the unknown; intravenous (reacting) injections = 0.2 ml. of a 1:10 dilution of Ou 567 per kg. of body weight.

**Test 5:** The following tests were carried out in order to evaluate the specificity of the standardized procedure proposed for the Shwartzman test. In order to accomplish this purpose, it seemed desirable to ascertain (a) the specificity of the standardized test for the detection of the endotoxin in culture autolysates from strains of toxigenic A. slovacae, and in filtrates from saline extracts of stained cotton and in vacuum concentrates from stained cotton extracts obtained from lots of cotton incriminated in outbreaks of acute illness; and (b) whether closely related species or strains of bacteria were capable of producing toxic factors, which would elicit a positive Shwartzman when used in combination with filtrates from toxigenic strains of A. slovacae.

The materials employed in these studies included culture filtrates from all types of coliform bacteria and miscellaneous strains of A. slovacae; extracts from numerous samples of stained cotton incriminated in outbreaks of acute illness; vacuum distillation concentrates from these stained cotton extracts and with filtrates from extracts of normal cotton, which was not contaminated with strains of A. slovacae. The culture filtrates employed, their identifying symbols, and the source of the cultures are given in Table IV. The cotton extracts and extract concentrates tested, the

source and type of cotton and the incidence of mucoid bacteria in the cotton are given in Table V.

All test materials were used as preparatory injections, and the toxigenic culture filtrate Cu 56F was employed for all reacting injections during these studies.

The results of these tests are shown in Tables XII, XIII, and XIV.

Shwartzman (24) and Freund and Mosner (8) have reported that culture filtrates from other microorganisms possess preparatory factors which will induce local skin reactivity in rabbits. This is confirmed by the data reported in Table XII, which shows that filtrates from coliform cultures Cu 4F, Cu 7F, Cu 10F, Cu 14F, Cu 19F, and Cu H-35K possess preparatory factors which will induce local skin reactivity in rabbits, which gave a positive Shwartzman reaction when the sensitizing injection with these filtrates was followed by an intravenous (reacting) injection with a toxigenic culture filtrate of A. cloacae. While these results are indicative of test non-specificity, it is believed that they do not detract from the general usefulness of the test. The test should only be employed for the detection of the endotoxin responsible for this acute respiratory illness, after the incidence and identity of the toxigenic strain of A. cloacae has been determined.

In summary, it may be stated that these studies on the application of the Shwartzman phenomenon revealed that (a) a heat-stable, endotoxin-like substance is liberated by toxigenic strains of A. cloacae; (b) a similar toxic substance is present in filtrates from saline extracts of stained cotton incriminated in outbreaks of acute illness; (c) rabbits may be cross sensitized with filtrates from either source; (d) the toxic preparatory factors present in culture filtrates, and cotton extract fil-

TABLE XII

Shwartzman Reactions to Check Specificity of Toxicogenic A. cloacae  
with Autolysates of Closely Related Bacteria

Rabbit No.	Weight kg	Sensitising Injections		Reacting Injections		Reaction		
		Materials	Quantity ml	Materials	Quantity ml per kg	Degree	Type	
147	3.90	Cu 4F	0.25	(1:20 dilution) Cu 56F	0.20	0.60	3+	H N* (died)
185	3.80	do.	do.	do.	do.	0.65	-	No reaction
148	2.68	Cu 7F	do.	do.	do.	0.55	3+	H N
190	2.46	do.	do.	do.	do.	0.50	-	No reaction
149	3.52	Cu 10F	do.	do.	do.	0.70	-	No reaction (died)
150	3.06	Cu 14F	do.	do.	do.	0.80	4+	H N
191	2.78	do.	do.	do.	do.	0.55	-	No reaction
151	3.24	Cu 19F	do.	do.	do.	0.65	2+	H N (died)
194	2.74	do.	do.	do.	do.	0.55	+	H
182	2.34	Cu 26F	do.	do.	do.	0.45	-	No reaction
88	3.32	Cu H-35K	do.	Cu H-35K	0.60	2.00	-	do.
54	3.20	do.	do.	do.	0.78	2.80	-	do.
59	3.42	Cu 56K	do.	do.	0.58	2.00	-	do.
60	2.92	Cu H-35K	do.	Cu 56K	0.34	1.00	3+	H N (died)
158	2.34	Cu H-35F	do.	(1:10 dilution) Cu 56F	0.20	0.45	-	do.
53	3.10	Cu 56F	do.	Cu H-35K	0.60	2.50	-	do.
56	2.91	Cu H-35K	do.	Cu 56F	0.42	1.25	-	do.
159	2.48	Cu 222F	do.	(1:10 dilution) Cu 56F	0.20	0.50	-	do.
160	2.40	Cu 529F	do.	(1:10 dilution) Cu 56F	0.20	0.50	-	No reaction

\*H -- Hemorrhagic; N -- Necrosis

TABLE XII (cont.)

Sub- bit No.	Weight kg	Sensitizing Injections		Nonetizing Injections		Reaction Do- type	
		Bacteria ml	Quant- ity ml	Bacteria ml	Quant- ity ml per kg		
181	2.19	Cu 961W	0.29	(1:10 dilution) Cu 56P	0.20	0.45	- No reaction
182	2.41	Cu 962P	do.	do.	do.	0.50	- do.
183	1.96	Cu 11P	do.	do.	do.	0.40	• M
187	2.46	do.	do.	do.	do.	0.50	2• M M
184	2.42	Cu 86W	do.	do.	do.	0.50	3• M M
201	2.37	do.	do.	do.	do.	0.50	2• M M
185	3.18	Cu 63P	do.	do.	do.	0.65	• M M
194	2.24	Cu 49P	do.	do.	do.	0.65	- No reaction (died)

Exp- No.	Weight kg	Material (quant- ity ml)	Material (quant- ity ml per 100 ml sol- vent)	Reaction
165	2.22	CO 5N	0.25 (1:10 dilution) 0.20 0.45	No reaction
166	2.20	CO 7N	do.	No reaction
167	2.53	CO 8N	do.	No reaction (died)
168	2.46	CO 11N	do.	No reaction
169	1.76	CO 43N	do.	do.
170	2.63	CO 44-5N	do.	do.
172	2.58	CO 66N	do.	+
177	2.33	CO 70N	do.	No reaction
178	2.67	CO 71N	do.	do.
179	2.64	CO 72N	do.	do.
180	2.26	CO 73N	do.	do.
181	2.20	CO 74N	do.	do.

\*H -- Hemorrhagic; H -- Hemorrhagic

Shewan reactions: specificity of reaction with cotton extracts

TAB. XIII

TABLE XIV

Shwartzman Reaction: Specificity of Reaction with Vacuum Concentrates of Cotton Extracts

Rabbit No.	Weight kg	Sensitizing Injections		Reacting Injections			Reaction	
		Materials	Quantity ml	Materials	Quantity ml per kg	Quantity ml	Degree	Type
65	4.50	Co 5%	0.25	(1:10 dilution) Cu 56%	0.20	0.90	+	H*
66	3.30	Co 7%	do.	do.	do.	0.75	+	H
67	4.60	Co 8%	do.	do.	do.	0.90	+	H
71	4.00	Co 11%	do.	do.	do.	0.80	-	No reaction
84	3.77	Co 45%	do.	do.	do.	0.75	+	H
87	4.28	Co 46%	do.	do.	do.	0.85	+	H
140	3.40	Co 56% <sub>01</sub>	do.	do.	do.	0.70	-	No reaction
159	2.60	Co 56% <sub>02</sub>	do.	do.	do.	0.50	+	H N

\*H -- Hemorrhagic; N -- Necrosis

trates may be neutralized with homologous immune serum; (e) this endotoxic substance is not present in saline extracts of normal cotton, sterile tryptose broth or isotonic saline.

While the Shartzman reaction is non-specific in that culture filtrates from heterologous types of bacteria contain toxic factors capable of inducing the reaction, it is believed that the test is useful when employed in conjunction with other tests for the detection of the endotoxin responsible for acute illness among cotton workers.

b. Phagocytic Tests for Endotoxin. The general term phagocytosis refers to the phenomenon of the ingestion of particles of foreign materials by living cells. However, in the restricted sense considered here the term refers to the power of leucocytes to ingest or engulf bacteria. Phagocytosis can be readily demonstrated "in vitro," where measured amounts of bacterial suspensions can be added to known quantities of phagocytes, along with any material the effect of which in promoting or inhibiting phagocytosis is to be studied. Many studies relative to the practical application of the phenomenon of phagocytosis have been reported.

Huddleson, Johnson, and Hermann (9) proposed a method for determining the opsono-cytophagic power of blood for Brucella. They use the term "opsono-cytophagic" to indicate the phagocytic activity of blood in the presence of serum opsonins and homologous leucocytes. These authors recommend this test as one index to the susceptibility of immunity to Brucella infection.

Rabstein and Cotton (17) employed a modification of Huddleson's opsono-cytophagic test in their studies on Bang's disease immunity tests.

Welch and Hunter (28) studied the effect of chemical antiseptics on phagocytosis. They proposed a method for testing the toxicity of antisept-



tics based on the inhibition of the phagocytosis of artificially opsonized staphylococci.

Many studies have been directed toward analyzing the effect on phagocytic systems of various chemical compounds and of purely physical entities such as heat, osmotic pressure, etc. (29). Although conflicting results have been obtained, it is known that certain chemical substances exhibit a marked inhibitory effect on phagocytosis.

A marked leucocytosis has been demonstrated in experimentally induced cases of acute illness resulting from exposure to contaminated stained cotton dust or a fine mist of a filtrate from a toxigenic culture of A. glaucus. It was considered possible that the endotoxin liberated by this microorganism might exert an inhibitory effect on the phagocytic activity of leucocytes, in which case a phagocytic method could be developed for the detection of the endotoxin.

The method developed by Welch and Hunter (29) for determining the effect of chemical antisepsis on phagocytosis and the method employed by Rabstein and Cotton (17) for determining immunity to Bang's disease were employed in these studies.

**Welch and Hunter method.** The method of Welch and Hunter (29) depends upon the ability of the normal guinea pig or human leucocyte to engulf artificially opsonized staphylococci in the presence of increasing concentrations of the germicidal substance under test. All tests take place in the presence of 40 percent fresh guinea pig or human blood. The method is as follows:

**Whole Blood:** Fresh human blood drawn by venipuncture was immediately mixed with sodium citrate in the proportion of 5.0 ml of blood to 0.2 ml of 20 percent sodium citrate in isotonic salt solution resulting in a

final concentration of 0.78 percent citrate.

Antigen: Staphylococcus aureus (F. D. A. strain 209). Saline suspensions from 48-hour nutrient agar slant cultures artificially opsonized with fresh sterile one percent chrome alum. The antigen is diluted to give a corrected Gates reading of 1.04 before use. This reading corresponds to a bacterial count of 500 million staphylococci per ml.

Materials tested: Filtrates from tryptose broth cultures of A. cloacae (Cu 11F, Cu 56F); filtrates from saline suspensions of killed cultures (Cu 11K, Cu 56K); extract filtrates (Co 44-5E, Co 56E) from normal and stained cotton; vacuum concentrate (Co 56K<sub>0</sub>) from extract of stained cotton, sterile tryptose broth (T) and sterile isotonic salt solution (S). The materials tested are described in Tables IV and V. Serial dilutions from 1:5 to 1:160 were prepared from each filtrate.

Technique of test: One-tenth ml of filtrate dilution, 0.2 ml of fresh citrated (0.78 percent) human blood, and 0.2 ml of standardized antigen were mixed and rotated on a shaking machine at 4 r.p.m. in a 37° C incubator for 30 minutes. Smears were prepared in the same manner as ordinary blood smears and stained by flooding the slide with 0.5 ml of 1.0 percent methylene blue in absolute alcohol for one minute. One ml of buffered distilled water (pH 7.2) was then added and the staining continued for four minutes. The slides were carefully washed in running water. The smears were examined microscopically to determine the number of staphylococci ingested by each of 25 leucocytes. The degree of phagocytosis was estimated as follows:

No phagocytosis = no organisms engulfed.

Slight phagocytosis = 1 to 20 organisms engulfed.

Moderate phagocytosis = 20 to 40 organisms engulfed.

Marked phagocytosis = over 40 organisms engulfed.

The results of a representative series of tests are given in Table XV. Since no inhibition of phagocytosis occurred with any filtrate dilution above 1:5, the results are recorded only for this dilution.

TABLE XV  
Results of Phagocytic Tests for Endotoxin  
(Welch and Hunter Method)

Filtrates Tested 1:5 dilution	Degree of Phagocytosis (percent)			
	Negative	Slight	Moderate	Marked
Cu 11F	16	52	16	16
Cu 11K	28	52	20	0
Cu 56F	40	44	12	4
Cu 56K	16	52	20	12
Co 56E	8	60	16	16
Co 56E <sub>g</sub>	88	12	0	0
Co 44-5E	16	56	20	8
T	8	60	20	12
S	0	48	28	24

It is evident from the results given that the vacuum distillation concentrate from an extract of stained cotton (Co 56E<sub>g</sub>) was the only substance tested which caused marked inhibition of phagocytosis. The culture filtrates, stained cotton extract filtrates, and the control materials, e.g., normal cotton extract, sterile tryptose broth and sterile isotonic salt solution did not exhibit any marked degree of inhibition of phagocytosis.

**Habstein and Cotton method.** The method of Habstein and Cotton (17) differs from the Welch and Hunter method (28) as follows: A standardized saline suspension of a homologous microorganism instead of artificially opsonized staphylococci is employed as antigen; equal quantities of anti-

gen, citrated blood, and test substance are employed in the test, and the test mixtures are not agitated during incubation. Details of this method are as follows:

Blood preparation: Fresh human blood (0.78 percent sodium citrate)..

Antigen: Saline suspension from 24-hour potato-carrot-dextrose agar slant culture No. 56 of toxigenic A. glaucus, standardized to turbidity of Tube No. 16 of McFarland scale.

Test materials: Culture filtrates, cotton extract filtrates, vacuum concentrates of cotton extracts, sterile tryptose broth and sterile isotonic salt solution.

A description of the materials tested is given in Tables IV and V.

Technique of test: One-tenth ml of whole blood, 0.1 ml of antigen suspension, and 0.1 ml of the material to be tested were placed in clean Wassermann tubes. The contents of each tube were thoroughly mixed and the tubes placed in a water bath at 37° C for 30 minutes. Sedimentation of the blood cells takes place during the incubation period; hence, the cells were resuspended by shaking after the period of incubation. A drop of each cell suspension mixture was placed at one end of a thoroughly cleaned and polished glass slide. The smears were made by placing the end of another glass slide at an angle in contact with the drop of cell suspension and pushing across the surface of the slide so that a thin blood cell suspension film terminates near the opposite end of the slide. The slides were dried rapidly in a current of warm air. The blood film smears were dipped in chloroform for 2 to 3 seconds and dried. The smears were then stained in toluidine blue\* for 1 to 2 minutes and washed in running water. The stained smears were dried on a flat surface without blotting.

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\*Toluidine Blue, 0.5 gm; Ethyl Alcohol (95%), 10.0 ml; Phenol, 3.0 ml; and Distilled water to make 100.0 ml.

The degree of phagocytosis was ascertained by the microscopic examination of 25 leucocytes in each blood film and determining the number of microorganisms engulfed by each leucocyte.

A protocol of a representative series of tests is shown in Table XVI.

TABLE XVI

Results of Phagocytic Tests for Endotoxin  
(Rabstein and Cotton Method)

Materials Tested 1:3 dilution	Degree of Phagocytosis (percent)			
	Negative	Slight	Moderate	Marked
Cu 11F	0	60	32	8
Cu 56F	0	56	28	16
Co 56E	0	48	40	12
Co 56% <sub>c</sub>	36	44	20	0
Co 5E	8	72	20	0
Co 5% <sub>c</sub>	4	44	28	24
Co 44-5E	0	72	28	0
Co 70E	0	64	36	0
T	0	60	28	12
S	0	60	28	12

As in the previous test, the only substance which exhibited a marked inhibition of phagocytosis was a vacuum distillation concentrate from an extract of toxigenic stained cotton (Co 56%<sub>c</sub>).

The vacuum distillation concentrates from saline extracts of stained cotton are hypertonic, since they are prepared by concentrating isotonic saline extracts of stained cotton 20 to 30 times. The most probable explanation for the inhibition of phagocytosis by this material is that it was due to the hypertonicity of the concentrate rather than the endotoxin present in the material. The validity of this explanation is further enhanced by the statement of Zinsser et al (30) that hypertonic sodium

chloride tends to inhibit phagocytosis.

Since the potency of the endotoxin is greater in filtrates from broth cultures and killed culture suspensions than in stained cotton extract filtrates or concentrates from extracts of stained cotton, it must be concluded that the endotoxin exerts little effect upon the phagocytic activity of leucocytes.

e. **Precipitation Tests.** It is a well-known fact that precipitation occurs when any antigen in solution is allowed to react with its corresponding (homologous) antiserum in the presence of electrolytes, provided that the concentration of each of these reagents and the experimental conditions, such as temperature of incubation and time of incubation, are suitably arranged. All antigenic substances produce precipitating antibodies and the precipitation reaction is another manifestation of the union of an antigen with its antibody.

Precipitation reactions are carried out with undiluted or slightly diluted antiserum against increasing dilutions of the antigen. This is necessary because larger amounts of antibody-carrying globulin of the serum are required for covering the surface of the precipitating solution than would be required for coating a suspension of bacterial cells as in the case of agglutination.

The phenomenon of precipitation has been employed for many purposes. Lancefield (11) described methods for the classification of streptococci on the basis of precipitin reactions with antigenic substances extracted from the organisms. Lancefield found that hot H91 extracts of streptococci, when cooled and neutralized, contained precipitable substances which were both group specific and type specific, i.e., "H" substance is type specific, "O" substance is group specific, and "P" fraction nucleoprotein is group specific.

The "C" substance, which is a carbohydrate haptene, precipitates both with homologous and heterologous sera and is used in the classification of streptococci.

Earlier limited studies (14, 21) demonstrated that immune rabbit serum contained antibodies capable of precipitating precipitinsogens prepared from cultures of toxigenic A. glaucus and that contaminated stained cotton contained an extractable substance which is precipitated by this immune serum. The results of these studies were sufficiently encouraging to warrant a re-evaluation of precipitation methods for the detection of the endotoxin-like substance liberated by toxigenic strains of A. glaucus.

Precipitinsogens employed: Four types of precipitinsogens were used in these studies.

(1) Lancefield type -- Crude extracts obtained from toxigenic strains of A. glaucus and other closely related microorganisms. The precipitinsogen for each microorganism was prepared as follows: Bacterial sediment from 250 ml of an 18-hour broth culture was suspended in 3.0 ml of physiological salt solution containing sufficient normal HCl to make a final concentration of 1/20 HCl. The reaction of the suspension was tested with Congo red paper and, if necessary, enough HCl was added to turn the paper blue. The tube containing the acidified cell suspension was then immersed in boiling water for 10 minutes, cooled under running water, and centrifuged. The supernatant fluid was removed and neutralised, and the resulting inactive precipitant discarded. The water-clear supernatant fluid was used as the precipitinsogen. Obviously, such a crude extract contains a mixture of substances. However, these apparently do not interfere with the reaction under consideration. The species of microorganisms employed and their sources are shown in Table IV.

(2) Vacuum-distillation-concentrates from saline extracts of stained, processed stained, and normal cotton -- These concentrates were prepared as previously described in this paper. The number, type and source of each concentrate are given in Table V.

(3) Filtrates from 7-day tryptose broth cultures of strains of A. cloacae and other closely related microorganisms -- The culture filtrate number, species of organism and source are given in Table IV.

(4) Filtrates from saline extracts of stained, processed stained, and normal cotton (Table V).

Serum employed: The serum employed for these studies was obtained through the immunization of rabbits with Berkefeld filtrates from a toxigenic strain of A. cloacae (Cu 56F). The serum was aseptically obtained and prepared for use without preservative.

Method: The procedure followed in these tests was essentially as follows: 0.1 ml was placed in the bottom of each of nine 3.0 mm precipitation tubes. Two-tenths ml of the precipitinogen, both undiluted and that diluted with sterile physiological (isotonic) saline throughout a range from 1:5 to 1:320, was then laid over the serum with a capillary pipette. One tube containing 0.2 ml of undiluted precipitinogen and 0.1 ml of physiological saline and a second tube containing 0.1 ml of serum and 0.2 ml of physiological saline were prepared for controls. The tubes were incubated for 30 minutes at 37° C and then observed for ring formation. The tubes were then thoroughly agitated to insure mixing of the precipitinogen and the serum, incubated for an additional 3 hours at 37° C and stored in the refrigerator at 5° C over night. Final examination for precipitates was then made.

Tests with Lancefield's precipitinogens: These tests were carried



out in order to ascertain whether tests employing immune serum homologous for known toxigenic strains of A. gloaceus were specific for these microorganisms or whether positive tests could also be obtained with culture extracts from other closely related species of bacteria. Precipitinogens were prepared from two toxigenic strains of A. gloaceus, Nos. 11 and 56, six strains of A. gloaceus, Nos. 49, 63, 222, 529, 961, and 962, not known to be toxigenic, and seven strains of other coliform bacteria (Table IV).

The data recorded in Table XVII are characteristic of the results obtained in a series of repeated tests. The only positive precipitation tests were obtained with precipitinogens prepared from the two known toxigenic strains of A. gloaceus, Nos. 11 and 56, and one strain of A. gloaceus, No. 961, obtained from the American Type Culture Collection. The tests with all other cultures were negative. It is believed that this test, when carried out as described, is specific for the detection of toxigenic strains of A. gloaceus.

#### Tests with vacuum distillation concentrates from cotton extracts:

Vacuum distillation concentrates from saline extracts of 7 samples of contaminated stained cotton, incriminated in outbreaks of illness, 5 samples of stained cotton processed for destruction of the endotoxin, and from 6 samples of normal cotton were employed in these studies (Table V).

The results of one series of studies with this type of precipitinogen are shown in Table XVIII. They are characteristic of the data obtained in a series of repeated tests. Positive precipitation tests were obtained with vacuum concentrate precipitinogens from all samples of cotton incriminated in outbreaks of acute illness. Reactions occurred in precipitinogen dilutions as high as 1:30 and 1:160. Positive tests were also obtained with precipitinogens derived from extracts of processed contaminat-

RESULTS OF PRECIPITATION TESTS WITH LAMPROLID'S CULTURE EXTRACT PRECIPITIN

TABLE XVII

No.	In- di- cator	Precipitation Dilutions							
		1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640
1	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-
11	4+	4+	4+	3+	2+	-	-	-	-
12	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	-
27	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-
30	1+	1+	1+	1+	2+	+	-	-	-
31	-	-	-	-	-	-	-	-	-
32	-	-	-	-	-	-	-	-	-
33	-	-	-	-	-	-	-	-	-
34	-	-	-	-	-	-	-	-	-
35	-	-	-	-	-	-	-	-	-
36	-	-	-	-	-	-	-	-	-
37	-	-	-	-	-	-	-	-	-
38	-	-	-	-	-	-	-	-	-
39	-	-	-	-	-	-	-	-	-
40	-	-	-	-	-	-	-	-	-
41	-	-	-	-	-	-	-	-	-
42	-	-	-	-	-	-	-	-	-
43	-	-	-	-	-	-	-	-	-
44	-	-	-	-	-	-	-	-	-
45	-	-	-	-	-	-	-	-	-
46	-	-	-	-	-	-	-	-	-
47	-	-	-	-	-	-	-	-	-
48	-	-	-	-	-	-	-	-	-
49	-	-	-	-	-	-	-	-	-
50	-	-	-	-	-	-	-	-	-
51	-	-	-	-	-	-	-	-	-
52	-	-	-	-	-	-	-	-	-
53	-	-	-	-	-	-	-	-	-
54	-	-	-	-	-	-	-	-	-
55	-	-	-	-	-	-	-	-	-
56	-	-	-	-	-	-	-	-	-
57	-	-	-	-	-	-	-	-	-
58	-	-	-	-	-	-	-	-	-
59	-	-	-	-	-	-	-	-	-
60	-	-	-	-	-	-	-	-	-
61	4+	3+	2+	2+	+	-	-	-	-
62	-	-	-	-	-	-	-	-	-

TABLE XVIII

Results of Precipitation Tests. Precipitinogens:  
Vacuum Distillation Concentrates From  
Saline Extracts of Cotton

Cotton Extract Concen- trate Precipitin- ogen	Precipitinogen Dilutions								Anti- gen Con- trol	Serum Con- trol
	Un- di- lu- ted	1:5	1:10	1:20	1:40	1:80	1:160	1:320		
Co 5E <sub>0</sub>	4+	4+	3+	3+	2+	+	+	-	-	-
Co 7E <sub>0</sub>	3+	3+	3+	2+	2+	+	+	-	-	-
Co 9E <sub>0</sub>	4+	3+	3+	2+	2+	+	+	-	-	-
Co 11E <sub>0</sub>	4+	4+	4+	3+	2+	+	+	+	-	-
Co 45E <sub>0</sub>	3+	3+	3+	2+	2+	+	+	-	-	-
*Co 44-5E <sub>0</sub>	-	-	-	-	-	-	-	-	-	-
*Co 44-5E <sub>0</sub>	-	-	-	-	-	-	-	-	-	-
*Co 46E <sub>0</sub>	4+	4+	4+	3+	2+	+	-	-	-	-
Co 56E <sub>01</sub>	4+	4+	4+	3+	2+	+	-	-	-	-
*Co 56E <sub>01</sub>	4+	4+	3+	3+	2+	2+	+	-	-	-
*Co 56E <sub>02</sub>	4+	4+	4+	4+	3+	2+	+	+	-	-
Co 56PE <sub>1c</sub>	4+	2+	+	-	-	-	-	-	-	-
Co 56PE <sub>2a</sub>	4+	3+	2+	+	-	-	-	-	-	-
Co 56PE <sub>3a</sub>	4+	3+	2+	+	-	-	-	-	-	-
Co 56PE <sub>4a</sub>	4+	2+	+	-	-	-	-	-	-	-
Co 56PE <sub>5a</sub>	4+	3+	2+	+	-	-	-	-	-	-
Co 70E <sub>0</sub>	-	-	-	-	-	-	-	-	-	-
Co 71E <sub>0</sub>	-	-	-	-	-	-	-	-	-	-
Co 72E <sub>0</sub>	-	-	-	-	-	-	-	-	-	-
Co 73E <sub>0</sub>	-	-	-	-	-	-	-	-	-	-
Co 74E <sub>0</sub>	-	-	-	-	-	-	-	-	-	-

\*Additional extracts or concentrates of the same samples.

ed cotton. However, the highest dilutions in which reactions occurred were 1:10 and 1:20. Positive precipitation tests were never obtained with vacuum concentrate precipitinogens prepared from saline extracts of normal cotton, which was not contaminated with toxigenic strains of A. glaucus or its toxic products.

The results of this series of tests indicate (a) that contaminated stained cotton contains an extractable substance which is precipitated with immune serum prepared from known toxigenic strains of A. glaucus, and (b) that a precipitation test employing a vacuum concentrate precipitinogen affords a convenient and specific method for the detection of toxin-containing cotton.

Tests with culture filtrate and cotton extract precipitinogens: It was decided to investigate the possibilities of using filtrates from 7-day tryptose broth cultures and filtrates from saline extracts of cotton as precipitinogens in tests for the detection of toxigenic microorganisms and the presence of endotoxin in cotton. The former are more easily prepared than Lancefield's culture extracts and the latter filtrates do not involve vacuum distillation. Hence, it was hoped that a simplified precipitation test could be developed. Fifteen culture filtrate and 19 cotton extract filtrate precipitinogens were used in these tests. The type and source of these precipitinogens are given in Tables IV and V. Positive precipitation tests could not be obtained with these materials. It was concluded that the endotoxin, when present in these materials, is not sufficiently concentrated for precipitation. The results of these tests are summarized in Table XIX.

d. Agglutination-Precipitation tests for Endotoxin. Previous studies have demonstrated that toxigenic strains of A. glaucus and stained cotton

TABLE XIA

Results of Precipitation Tests  
 Precipitinogens: (a) Filtrates from 7-day tryptose  
 broth cultures. (b) Filtrates from cotton extracts.  
 Serum: Homologous for toxigenic A. cloacae.

Culture Filtrate Precipi- tinogens	Precipitation	Cotton-Extract Filtrate Precipitinogens	Precipitation
Cu 4F	-	Co 5B	-
Cu 7F	-	Co 7B	-
Cu 10F	-	Co 8B	-
Cu 11F	-	Co 11B	-
Cu 14F	-	Co 43B	-
Cu 19F	-	Co 44-5B	-
Cu 26F	-	Co 46B	-
Cu H-55F	-	*Co 56B	-
Cu 49F	-	*Co 56B	-
Cu 56F	-	Co 56PB <sub>1</sub>	-
Cu 63F	-	Co 56PB <sub>2</sub>	-
Cu 222F	-	Co 56PB <sub>3</sub>	-
Cu 529F	-	Co 56PB <sub>4</sub>	-
Cu 961F	-	Co 56PB <sub>5</sub>	-
Cu 962F	-	Co 70B	-
		Co 71B	-
		Co 72B	-
		Co 73B	-
		Co 74B	-

\*Two extracts from same samples.

incriminated in outbreaks of acute illness contain a similar extractable substance (endotoxin), which is precipitated by sera from rabbits immunized with culture filtrates, with killed culture suspensions of the organism, or with vacuum distillation concentrates from extracts of the stained cotton.

Data have been presented which indicate that the regular precipitation test is a practical, sensitive test for the detection of the endotoxin. However, numerous workers have proposed modifications of the precipitation test by which it is claimed that the test may be simplified and its sensitivity increased. These modifications involve the coating of the precipitinogen or extractable substance on foreign bacterial cells or on colloidal particles, which in turn are employed as antigens for a routine agglutination test.

Cannon and Marshall (4) proposed a method for determining the precipitative titer of antisera, which involves the coating of the precipitinogen on uniform colloidal particles contained in a standardized suspension. The antiserum, rather than the antigen, is diluted and the test is carried out as an agglutination test. The authors employed this test in the study of soluble proteins and protein antisera. It is claimed that the method is sharply specific and delicate enough to demonstrate the presence of precipitins in weak sera.

Eisler (7) employed the methods of Cannon and Marshall in studies on the adsorption of bacterial suspensions and autolysates of pneumococci, meningococci, and gonococci on colloidal particles and a demonstration of their reactivity with specific antisera. He reported that colloidal particles combined with autolysates of pneumococci, meningococci, and gonococci were agglutinated when the suspensions were mixed with specific

antisera. The phenomenon of increased titer effected by use of colloidal particle-antibodyate antigens was observed with both low and high titered antisera. Hinler found by experiment that the most satisfactory titers were obtained by allowing colloidal particles to remain suspended in autolyates. He suggested that the particles should not be washed free of excess antigen since antigenic substances other than protein might be present.

Jones (10) reported that bacteria sensitized with bovine serum and subsequently washed until bovine serum no longer remained in the washing solution, agglutinated when low concentrations of bovine antisera were added.

Roberts and Jones (19) developed a method which involves coating the cells of a suspension of ERRHALLIA MERTENSIS with serum antigen and observing the agglutination of such cells upon the addition of the animal serum being tested. It was called the "B. A." method. It is claimed that antibodies can be demonstrated with this method within 24 hours after the injection of a single dose of antigen. The authors report that this method is more convenient and the bacteria employed exhibit greater suspension stability than the colloidal particles recommended in the methods employed by Cannon and Marshall (4) and Bistler (7).

Roberts and Jones, in a subsequent paper (20), reported on the application of their bacterial agglutination (B. A.) method for the detection of antibodies for the St. Louis type of encephalitic virus in the blood of man and animals.

After a review of these methods, it was concluded that the procedures recommended for the preparation of suspensions of colloidal particles to be used for agglutination-precipitation tests were too involved to be practical for routine purposes. Therefore, it was decided to investigate the

"B. A." bacterial agglutination method of Roberts and Jones (19) for the detection of the endotoxin in toxigenic filtrates from cultures of A. gloabae and from extracts of stained cotton.

Roberts and Jones "B. A." method. Details of this method are as follows:

Preparation of cell suspension: The cultures of S. marcescens were grown on meat infusion agar slants incubated at 37° C for 24 hours. The cells were scraped from the agar slants, suspended in sterile isotonic saline, filtered, and washed twice with additional saline by centrifugation. The cells were then suspended in distilled water and killed by heating in flowing steam for 10 minutes.

Coating of cells with precipitinogen: The distilled water suspension of killed cells was added to each material to be tested in the ratio of 1 part of cell suspension to 5 parts of the filtrate to be tested. The cell-filtrate mixture was incubated for 18 hours at 37° C and then centrifuged to remove the cells. The cells were washed twice with sterile saline and then diluted with the same material to an opacity equivalent to a reference standard containing 0.35 mg of ground pyrex glass per ml. This standardized cell suspension was centrifuged at 300 r.p.m. for 5 minutes to throw down the larger clumps of cells.

Technique of tests: One ml quantities of serial dilutions of serum from 1:10 to 1:10,240 were placed in agglutination tubes. Five-tenths ml of the supernatant cell suspension was added to each serum dilution tube for the agglutination test. Each test series of tubes and antigen and serum controls were incubated for 2 hours at 40° C followed by storage in the refrigerator at 5° C over night.

Materials tested: Culture autolyate filtrates (Cu 11F and C u 56F);



filtrates from extracts of contaminated stained cotton (Co 56W and Co 5B); vacuum concentrate from saline extract of stained cotton (Co 56Kc); and filtrates from extracts of normal cotton (Co 44-5B and Co 70E). The description and sources of the test materials are given in Tables IV and V.

Serum: Pooled sera from rabbits immunised with Berkeleyfield filtrates from a 7-day tryptose broth culture of No. 56, toxigenic *A. glaucus*, were employed in all of the agglutination-precipitation tests.

Test series I: Two sets of tests were prepared as outlined. One set of tubes was incubated at 40° C and the second set at 37° C for 2 hours. Both sets were stored in the refrigerator at 5° C over night.

The test set-up which was incubated at 40° C failed to show any significant agglutination in any dilution. The test set-up which was incubated at 37° C gave low titer agglutination (1:40 dilution) of cells adsorbed with culture autolysates Cu 11F and Cu 56F and with filtrates from saline extracts of stained cotton. However, cells adsorbed with filtrates from saline extracts of normal cotton, Co 44-5B and Co 70E, did not show any agglutination. The results of this test series were inconclusive.

Test series II: The procedure varied as follows. All series of tubes were prepared in duplicate. To 0.05 ml of packed dead cells was added 2.0 ml of each filtrate or material to be adsorbed, and the mixture was incubated for eighteen hours at 37° C. After incubation the cell-filtrate suspension in one set of tubes was centrifuged to remove the excess filtrate. The packed adsorbed cells thus obtained were diluted to the proper turbidity without washing. The cell-filtrate mixtures in the second set of tubes were diluted to the proper turbidity for antigen without centrifuging to remove the excess filtrate from the cells. These procedures were suggested by the work of Eisler (7) who suggested that no

washing of the particles be made to free them of excess antigen, in view of the fact that antigenic substances other than adsorbable proteins might be present. A series of 0.5 ml serum dilutions to which 0.5 ml of a saline suspension of killed cells of *S. marcescens* had been added was prepared as a control.

All test set-ups in this series were incubated in a water bath at 37° C for 2 hours followed by overnight storage in the refrigerator at 8°C.

Low titer agglutination (1:40 to 1:80) was obtained with all filtrate adsorbed cells, whether the cells were adsorbed with culture autolyates, filtrates from saline extracts of stained or normal cotton, or with vacuum concentrates from extracts of stained cotton. The same results were obtained regardless of whether the adsorbed cells were centrifuged free from the excess filtrates before dilution, or were allowed to remain suspended in the filtrates and diluted.

Test series III: The procedure employed in this test series was essentially the same as that employed in test series II, except for the following minor variations: (a) 0.05 ml of packed dead cells were adsorbed with 3.0 ml of each filtrate or autolyate to be tested rather than with 2.0 ml of each material, and (b) a lower range of serum dilutions (halved dilutions from 1:2 to 1:512). The same incubation procedure was followed in each experiment. The results of this test series are given in Table XX. The results obtained in this experiment are in agreement with those obtained in test series II. Low titer agglutination was obtained with all of the materials tested. These materials included known non-toxic filtrates as well as toxic substances.

Test series IV: The procedure followed in this test series was iden-

TABLE XX

Results of "B. A." Agglutination Tests for Endotoxin  
Test Series III.

Antigen: Coated Cells of <u>S. marcescens</u>	Serum Dilutions										Anti- gen Con- trol	Serum Con- trol
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512			

0.06 ml packed cells centrifuged from excess filtrate and diluted to proper turbidity without washing.

Cells Coated with Cu 11F	3+	3+	3+	+	-	-	-	-	-	-	-
Cu 56F	3+	3+	2+	+	-	-	-	-	-	-	-
Co 56E	3+	3+	2+	2+	+	-	-	-	-	-	-
Co 56E <sub>o</sub>	4+	4+	3+	3+	+	-	-	-	-	-	-
Co 44-5E	4+	3+	2+	2+	+	-	-	-	-	-	-

0.05 ml packed cells + 0.0 ml of filtrate diluted to proper turbidity for antigen.

Cells plus Cu 11F	4+	3+	2+	+	1	-	-	-	-	-	-
Cu 56F	3+	3+	3+	2+	2+	+	-	-	-	-	-
Co 56E	3+	3+	2+	2+	+	-	-	-	-	-	-
Co 56E <sub>o</sub>	4+	3+	3+	2+	+	1	-	-	-	-	-
Co 44-5E	3+	3+	2+	+	-	-	-	-	-	-	-

tical with those employed in test series II and III , except for the fact that 0.5 ml of antigen (coated cell suspension) was added to 0.5 ml of each serum dilution rather than 1.0 ml as outlined in the original "B. A." agglutination method.

The results of this test series are in agreement with the results obtained in test series II and III; hence, they are not incorporated in this report. Positive agglutination tests were obtained with both toxigenic and non-toxigenic materials.

Test series V: In this test series killed cells of S. marcescens were coated with immune serum and tested with dilutions of the filtrates. This test series was carried out as follows: A saline suspension of killed cells of S. marcescens was prepared as previously outlined. One-tenth ml of packed cells, obtained by centrifugation, was placed in each of two centrifuge tubes. Four ml of immune rabbit serum were added to the packed cells in each tube. After thorough agitation of the serum-cell suspension the tubes were incubated for 18 hours at 37° C. Two types of antigen were prepared from serum-coated cells as follows: (a) One tube of the cell-serum suspension was centrifuged to remove the excess serum. The packed cells thus obtained were diluted to the proper turbidity for antigen without washing the cells; and (b) the cell-serum suspension in the remaining tube was diluted to the proper turbidity for antigen without removal of the excess serum.

Serial dilutions were prepared with each of the culture autolysates and cotton extract filtrates to be tested. Five-tenths ml of each cell-serum antigen was added to a like quantity of each dilution of each filtrate or autolysate. All tubes were incubated for 2 hours at 37° C, and stored in the refrigerator at 5° C over night.

Agglutination of each type of antigen occurred in all serial dilutions of each filtrate or autolyate as well as in the antigen control tubes.

In all of the five series of tests with the "B. A." agglutination method, the test set-ups were incubated for an additional 2 hours at 56° C before they were discarded. While the higher incubation temperature increased the degree or intensity of the reactions, it did not change the character of the results in any of the five tests.

It must be concluded from the extremely variable results obtained in these studies that the "B. A." agglutination test recommended by Roberts and Jones (19) for "Detection of minute amounts of serum antibody by agglutination of antigen-coated bacterial cells" is apparently not applicable for the detection of the endotoxin produced by toxigenic strains of A. glaucus. The variable results obtained are certainly not comparable to the excellent results obtained with the ordinary precipitation tests.

#### B. Investigation of Methods for the Treatment of Stained Cotton to Destroy Toxigenic Strains of A. glaucus and their Endotoxin

Previous studies have indicated that the exposure of stained cotton on two successive days for 4 hours at 175° C in dry heat did not destroy these microorganisms and their toxic products. Also this sterilization procedure was apparently too drastic, since it resulted in the deterioration of the cotton fibers. A sterilization process involving the application of moist heat for destruction of these bacteria and their toxic products might be employed without injury to the cotton fibers.

A limited number of studies was carried out on the utilization of superheated steam under vacuum for the treatment of contaminated stained cotton. Details of the process will be reported elsewhere.

Four 10-gm samples and one 100-gm sample were obtained from a bale of stained cotton, which had been incriminated in an outbreak of acute illness, for these processing studies. Duplicate samples were also withdrawn at the same time for extraction with physiological saline, so that the cotton could be tested for the presence of endotoxin before processing. A quantitative bacteriological examination was made on each of the 5 samples prior to and after processing. Each of the 5 cotton samples was processed for at least 30 minutes with superheated steam under vacuum. After removal of a one-gram portion from each processed sample for the bacteriological examination, the remainder of each sample was extracted with saline in order to obtain filtrates for toxin tests.

The incidence of mucoid bacteria in each of the 5 cotton samples both before and after processing is given in Table XXI.

TABLE XXI

Incidence of Mucoid Bacteria in Processed  
and Unprocessed Stained Cotton

Cotton Sample No.	Type Cotton	Incidence Mucoid Bacteria	
		Nos. per gm Before Processing	After Processing
56P <sub>1</sub>	Stained Cotton	3,700,000	None
56P <sub>2</sub>	do.	do.	do.
56P <sub>3</sub>	do.	20,000,000	1,900
56P <sub>4</sub>	do.	70,000,000	43,000
56P <sub>5</sub>	do.	190,000,000	None

It is obvious from these results that the processes employed destroyed all of the viable microorganisms in samples 56P<sub>1</sub>, 56P<sub>2</sub>, and 56P<sub>5</sub>. While the organisms were not completely destroyed in samples 56P<sub>3</sub> and 56P<sub>4</sub>, their numbers were greatly diminished.

The extracts obtained from the processed and unprocessed cotton samples were tested for the presence of endotoxin by means of the Shwartzman reaction and precipitation tests. The procedures followed were the same as those outlined in previous sections of this thesis. Descriptions of the cotton extracts, extract concentrates, and culture filtrates employed for these tests are given in Tables IV and V.

The results of these tests are contained in Tables XXII and XXIII.

The results of the Shwartzman test (Table XXII) show that the toxic products present in the stained cotton (rabbits Nos. 102 and 103) were not entirely destroyed during processing. The only processed cotton extract which gave a negative test for toxin was Co 56PE<sub>1</sub> (rabbit No. 116).

The results of the precipitation tests (Table XXIII) indicate that while the toxin in the processed cotton samples was greatly diminished, it was not entirely destroyed. The precipitation titer for the processed cotton extract concentrates (Co 56PE<sub>1c</sub>, 56PE<sub>5c</sub>) ranged from 1:10 to 1:20, while precipitation occurred with unprocessed cotton concentrates (Co 56E<sub>01</sub>, 56E<sub>02</sub>) in dilutions from 1:80 to 1:160. The concentrate Co 70E<sub>0c</sub> from normal contaminated cotton did not precipitate.

TABLE XXII

Results of Smartman Tests for Yersin in Processed  
and Unprocessed Stained Cotton

Rab- bit No.	Weight kg	Sensitizing Injections		Resisting Injections		Reaction		
		Materials ml	Quan- tity ml	Materials	Quan- tity ml per kg		De- gree Type	
101	2.85	Cu 56P	0.25	Cu 56P	0.49	1.40	4+	H H *
102	2.82	Cc 56S <sub>1</sub>	do.	Cc 56S <sub>1</sub>	2.83	5.75	4+	H H
103	2.95	do.	do.	Cu 56P	0.51	1.50	3+	H H
116	2.82	Cc 56P <sub>1</sub>	do.	do.	0.49	1.40	"	"
117	3.46	Cc 56P <sub>2</sub>	do.	do.	0.50	1.75	"	H
144	2.64	Cc 56P <sub>3</sub>	do.	do.	0.30	0.80	3+	H
146	3.60	Cc 56P <sub>4</sub>	do.	do.	0.30	1.08	"	H
157	2.80	Cc 56P <sub>5</sub>	do.	(1:10 dilution) Cu 56P	0.20	0.55	2+	H

\*H --- Hemorrhagic; H --- Necrotic



TABLE XXIII

Precipitation Tests for Detection of Endotoxin  
in Processed and Non-processed Stained Cotton

Cotton Extract Concen- trate	Precipitinogen Dilutions								Antigen Control	Serum Control
	Undi- luted	1:5	1:10	1:20	1:40	1:80	1:160	1:320		
Co 56% <sub>01</sub>	4+	4+	3+	3+	2+	2+	+	-	-	-
Co 56% <sub>02</sub>	4+	4+	4+	4+	3+	2+	+	±	-	-
Co 56% <sub>10</sub>	4+	2+	±	-	-	-	-	-	-	-
Co 56% <sub>20</sub>	4+	3+	2+	+	-	-	-	-	-	-
Co 56% <sub>30</sub>	4+	3+	2+	+	-	-	-	-	-	-
Co 56% <sub>40</sub>	4+	2+	+	-	-	-	-	-	-	-
Co 56% <sub>50</sub>	4+	3+	2+	+	-	-	-	-	-	-
Co 70% <sub>0</sub>	-	-	-	-	-	-	-	-	-	-

It is concluded from the results of these studies that processing procedures employing the application of superheated steam under pressure, while not destroying all of the viable microorganisms and their products in contaminated stained cotton, do bring about a diminution of these bacteria and their products.

There was no apparent injury to the cotton fibers. It is believed that this method for processing contaminated cotton offers sufficient promise to warrant further investigation.

#### IV. DISCUSSION

While outbreaks of food poisoning have been attributed to the ingestion of toxic substances contained in products contaminated with A. cloacae (3), the illness among workers handling low-grade, stained cotton appears to be the first reported instance of respiratory disease due to the inhalation of such toxic products. Illness similar to that observed in workers handling stained cotton has been reported in hemp, flax, jute and grain workers. Since A. cloacae is commonly known to be widely distributed in nature, it might be expected as a major contaminant of organic plant materials offering suitable conditions for its growth.

The acute illness caused by this endotoxin may be considered as a potential occupational health hazard for employees in industries utilizing low-grade, stained cotton or other dusty plant materials. Therefore, practical methods for the detection of this endotoxin are essential in order to facilitate the investigation and prevention of future outbreaks of this illness.

Topley and Wilson (26) state that toxic bacterial components (toxic antigens) are widely distributed among the Gram-negative bacilli of the coliform group, and perhaps among other more distantly related species. They suggest that these endotoxins may all act in much the same way, irrespective of the particular bacterium from which they have been derived. According to these authors, endotoxins do not, in general, give rise to diagnostic symptoms, so that it is usually not possible to recognize any particular endotoxin by its pharmacological action as in the case of bacterial exotoxins.

These studies have demonstrated that the toxic product of the strain of A. cloacae causing acute illness among workers with low-grade, stained

cotton has the following characteristics which are similar to those of an endotoxin:

1. It is thermostable -- it will resist boiling for 30 minutes and when present in stained cotton it has been found to resist heating with super-heated steam under vacuum at 121° to 137.6° C. for 30 minutes.
2. It increases with age or autolysis of cells in broth cultures (14).
3. It stimulates the formation of agglutinins and precipitins, when injected into rabbits.
4. Its toxic skin preparatory factors (Shwartzman reaction) may be neutralized with low dilutions of immune serum.
5. Although inducing severe inflammatory lesions and systemic symptoms in humans following intradermal injection, it does not affect mice, rabbits or chickens when administered by the same route.
6. It induces a marked leucocytosis and acute illness in humans following inhalation (14).
7. The minimum lethal dose is much greater than the m.l.d. of an exotoxin.
8. The toxin does not affect the phagocytic activity of leucocytes.
9. It was demonstrated by means of the Shwartzman reaction that similar toxic preparatory factors were also present in strains of Escherichia coli, atypical E. coli, Citrobacter, atypical Citrobacter, A. aerogenes and other strains of A. cloacae.

None of the characteristics or combination of characteristics outlined is sufficiently specific for this endotoxin to be of use for its detection in culture extracts, culture autolysates, cotton extracts or other suspected materials. However, the precipitation test, although time-consuming and requiring large quantities of immune serum, is apparently a specific

and reliable test for the detection of this endotoxin in suspected materials.

Since this endotoxin does not give rise to diagnostic symptoms or lesions in experimental animals, it cannot be detected through its pharmacological action, except through administration to human subjects. However, it is believed that biochemical studies on the nature and composition of this endotoxin might provide additional means for its detection and identification.

## V. SUMMARY

The results of studies on tests for the detection of the endotoxic substance produced by a strain of A. cloacae causing acute illness among workers using low-grade stained cotton are presented.

Bacteriological and biochemical methods for the isolation and identification of the responsible organism in suspected materials are outlined.

Serological methods for the preparation of immune sera, and a determination of the serological grouping of the bacterium are presented.

Both animal and serological methods for the detection of the endotoxin were investigated.

A wide variety of materials were employed during this investigation. These included viable and killed culture suspensions of toxigenic strains of A. cloacae; culture filtrates (autolysates) of this bacterium and other closely related species; filtrates from saline extracts of stained cotton inactivated in outbreaks of acute illness; filtrates from extracts of normal cotton; sterile tryptose phosphate broth; and, sterile isotonic salt solution.

Since marked local and systemic reactions are produced in humans through the intradermal administration of materials containing the endotoxin, an attempt was made to find an experimental animal or fowl which would respond in the same manner to intradermal or other administration. Twenty-five white rabbits, 8 white mice, and 106 1 to 3-week old chickens were employed for these studies. It was found (a) that rabbits and white mice do not respond to the intradermal administration of materials containing the endotoxin, and (b) that 1 to 3-week old chickens are not susceptible to infection or intoxication with the microorganism or its toxic products regardless of whether the materials are administered by

gavage, intradermal, intravenous, and intraperitoneal injections or by inhalation of contaminated cotton dust.

An extensive series of studies was carried out on the evaluation of the Shwartzman reaction for the detection of the endotoxin. White mice were found unsuitable for the test. Two hundred and twenty-one white rabbits were employed in these studies. It was found that approximately 30 per cent of normal white rabbits are not susceptible to the phenomenon of local skin reactivity to toxic materials. The results of the studies with the Shwartzman reaction indicate (a) that an endotoxin-like substance is present in filtrates from broth cultures and saline suspensions of killed cultures of strains of A. cloacae isolated from stained cotton, (b) that a similar toxic substance is present in filtrates from saline extracts of stained cotton incriminated in outbreaks of acute illness, (c) that rabbits may be cross-sensitized with the toxic products in killed culture suspensions or culture filtrates of the organism or with filtrates from saline extracts of stained cotton, (d) that sterile tryptose broth, sterile physiological saline, and saline extracts from normal non-contaminated cotton do not contain this toxic substance and are incapable of eliciting the Shwartzman phenomenon, (e) that the Shwartzman reaction is non-specific, since culture filtrates from closely related species of bacteria are capable of inducing local skin reactivity to intravenous injections of filtrates from toxigenic cultures of A. cloacae, and (f) that the specificity of the test may be increased through the use of serum-neutralized toxic materials as controls. A standardized test procedure is proposed.

It was considered possible that the endotoxin liberated by this strain of A. cloacae might exert an inhibitory effect on the phagocytic

activity of leucocytes, in view of the fact that a marked leucocytosis has been demonstrated in experimentally induced cases of acute illness resulting from exposure to contaminated stained cotton dust or a fine mist of a culture filtrate of the organism. Although two different procedures were employed for studying the effect of the endotoxin on phagocytosis by leucocytes, the theory could not be substantiated.

Precipitation tests were found to be specific and reliable for the detection of the endotoxin in cultures and in extracts from stained cotton incriminated in outbreaks of acute illness.

Agglutination-precipitation methods involving the coating of killed cells of S. marcescens with the precipitinogen (toxic culture filtrates or cotton extracts) followed by agglutination of the coated cells with immune serum are unsatisfactory for the detection of the endotoxin.

A method, involving the use of superheated steam under vacuum, for the treatment of stained cotton to destroy toxigenic strains of A. elcaas and their endotoxin was investigated.

## VI. CONCLUSIONS

It is concluded that the Shwartzman test, while non-specific, is a useful method for the rapid detection of toxic factors in cultures isolated from stained cotton, or in extracts from stained cotton incriminated in outbreaks of acute respiratory illness. Furthermore, precipitation tests afford accurate specific methods for the detection of the endotoxin.



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PUBLICATIONS:

1. Schmeiter, R. Report on Microbiological Methods for Examination of Frozen Egg Products. Jour. of the Assoc. of Off. Agr. Chem., 22(5): 625-628. 1939.
2. Schmeiter, R. Report on Frozen Egg Products. Jour. of the Assoc. of Off. Agr. Chem., 23(3): 615-617. 1940.
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