STUDIES ON BACILLUS TYPHOSUS TOXIC SUBSTANCES.

I. Phenomenon of Local Skin Reactivity to B. typhosus Culture Filtrate.*

By GREGORY SHWARTZMAN, M.D.

(From the Laboratories of the Mount Sinai Hospital, New York.)

Plates 6 and 7.

(Received for publication, April 21, 1928.)

INTRODUCTION.

Brieger (1) initiated the studies on toxic substances derived from *B. typhosus* cultures. Schütze (2) claimed that these substances possessed no antigenic properties. Further work on the nature of the toxic factors was done by Sirotinin (3), and Beumer and Peiper (4). Bitter (5), Chantamesse (6), Besredka (7), Rodet and Lagriffoul (8), Kraus and Stenitzer (9), Arima (10) and others obtained immune sera which were supposed to neutralize the toxic substances derived from *B. typhosus* cultures grown in fluid and solid media. Pfeiffer and Bessau (11) showed that immune sera had no superior neutralizing properties over normal sera and that there was no neutralization according to the law of multiple proportions. The toxic substances of *B. typhosus* have been claimed to be heat resistant by Sirotinin (3), Chantamesse (6) and Besredka (7). According to Kraus and Stenitzer (9) these substances are not influenced by light or by exposure to room temperature. The majority of the authors considered the toxic substances as endotoxins (Pfeiffer (11), Besredka (7) and others). Kraus and Stenitzer (9) and later Arima (10) were of the opinion that the typhoid cultures contain both endotoxins and exotoxins. The symptoms produced by the toxic substances are devoid of any specific features. In acutely poisoned rabbits they consist of convulsions, Cheyne-Stokes respiration, diarrhea, paralysis and, possibly, swelling of Peyer's patches (Arima (10)). Similar symptoms have been demonstrated by injection of anaphylatoxins (Friedberger (12)).

On the basis of the work thus far mentioned, a general belief arose that no true toxins exist in *B. typhosus* cultures. In fact, the classical requirements for demonstration of a true toxin, namely, heat lability, serum neutralization in multiple proportions and specific pathological effect upon animals were not fulfilled. The subject has been revived by Zinsser (13) in recent years. The toxic substances obtained from fluid cultures of various microorganisms, including *B. typhosus*,

*Read before the Society for Experimental Biology and Medicine, April 18, 1928.

were called by him x-substances. They produced no specific pathological changes when injected into animals. Antigenic properties could not be determined because of fluctuations in the response of the animals and inability to continue immunization over a prolonged period of time. The x-substances were also heat resistant, were more toxic for rabbits than for guinea pigs and their effect upon animals was always accompanied by a short but definite incubation period. Zinsser thought that they could not be dismissed merely as split products and considered them somewhat similar to exotoxins as reported by Kraus and Stenitzer, and by Arima.

The present author has carried out experiments as preliminary to the work here to be reported, which can be summarized as follows:

Whole cultures and filtrates of cultures of *B. typhosus* grown under various conditions presumably favorable to the production of powerful toxins were injected intravenously into rabbits. The symptoms produced by the intravenous injections were similar to those described by previous authors. The susceptible animals became sick shortly after the injection. Before death there was very profuse diarrhea, increased respiration, paralysis and, frequently involuntary muscular contractions. Analysis of the highly inconstant results of titration of the toxic effect in a large group of rabbits showed the impossibility of establishing any relation between the dose of the toxic substances injected and the reaction of the animals. The inconstancy of the effect was most likely due to individual variations in the susceptibility of the animals to the effect of these substances.

The effect of *B. typhosus* culture filtrate upon the skin of normal rabbits was also studied. About 50 per cent of all the normal rabbits tested by skin injections of the filtrate showed no reactions whatsoever. In the remaining rabbits the skin injections produced erythemas. Only a small percentage of the positively reacting animals (12 per cent) gave well pronounced erythemas. A majority of positive rabbits reacted weakly. Moreover, different areas of the skin of the abdomen of the same rabbits presented considerable variations in the intensity of reactions to *B. typhosus* culture filtrate.

As can be seen from this summary, the general response and the skin reactions of rabbits to *B. typhosus* culture filtrate could not be used as criteria for studies on the nature of the toxic factors of these filtrates. Further experiments now to be described demonstrated a phenomenon of local skin reactivity to *B. typhosus* culture filtrates.

Phenomenon of Local Skin Reactivity to B. typhosus Culture Filtrate.

Methods.—The *strains* employed for this work were T_L and T_{240}. They were obtained from the stool of convalescent typhoid fever patients 1 and 4 years ago, respectively. Both strains appeared smooth and were easily agglutinated by *B. typhosus* antisera to a high titer. The floccules were large. The strain T_{240}, however, was inagglutinable during the 1st month of cultivation.

The *toxic* substances were prepared as follows:

200 cc. of tryptic digest broth of initial pH 7.8 were added to 2000 cc. Erlenmeyer flasks to give a large surface area. The entire growth of one 24 hour old agar slant culture of *B. typhosus* was suspended in 10 cc. of salt solution. 10 cc. of the suspension were added to each Erlenmeyer flask. The period of incubation was 6 days. The cultures were then filtered through paper and cotton and finally through Berkefeld V candles. The filtrates were tested for sterility, stored in the refrigerator and used for a period of approximately 2 to 3 weeks following their preparation. No preservative was added.

The entire skin of the abdomen of rabbits was epilated with barium sulfate. The animals were injected not less than 24 hours after shaving if no signs of irritation were seen. For the local injections described in this paper, the needle was inserted subcutaneously and then pushed upwards until the point was just seen through the skin. 4 areas, near the upper and lower right and left corners of the epilated areas, about 2 inches apart from each other, were used for the injections. The amount of fluid injected into each area was usually 0.25 cc.

The type of the reaction to be described in the following part of the paper was termed *hemorrhagic.* The severity was expressed in pluses. The size of the reaction was recorded. Distinction was made between the hemorrhagic reaction and the erythema described further on which varied from light pink to a distinctly red color.

Experimental: Protocol 1.—Upper and lower right and left areas of the skin of the abdomen of Rabbits 14-1 and 14-2 were injected each with 0.5 cc. of T. D. T_L filtrate. 24 hours later Rabbit 14-1 showed no skin reaction, Rabbit 14-2 a slightly pink erythema (2+) in the injected areas. The same morning intravenous injections of 3 cc. per kilo of body weight of the filtrate were given to both rabbits. The skin of the animals was examined every hour afterwards. Approximately, 2 hours after the intravenous injections blue discoloration appeared at the site of previous skin injections in Rabbit 14-1. The skin of Rabbit 14-2 remained unchanged. The discoloration observed in Rabbit 14-1 rapidly increased until the reaction became extremely pronounced in about 4 hours after the intravenous injection. At this time, all 4 areas were dark blue in the center with a deep red zone at the periphery. The skin over the hematomas was glossy and swollen. The size of these areas was considerable, the reaction in the upper right corner measuring 2 × 2 cm., the lower right 3 × 2 cm., the upper left 4 × 2 cm. and the lower left corner 3 × 2½ cm. The reaction described resembled a severe bruise. The animal was killed 5 hours after the intravenous injection and sections of the

skin were made. Rabbit 14-2 showed no reaction in the course of 24 hours following the intravenous injection.

Protocol 2.—4 areas of the skin of the abdomen of Rabbit 7-6 were injected each with 0.25 cc. of T. D. T_{240} filtrate. 20 hours later there were "2+" erythemas at the site of skin injections. 24 hours after the skin injections this rabbit was injected intravenously with the filtrate in a dose of 1 cc. per kilo of body weight. The previously injected skin areas showed discoloration in about 2 hours after the intravenous injection. The reactions again, progressively, increased and 4½ hours after the intravenous injection they were extremely severe. The areas were hemorrhagic. They appeared dark blue, glossy and swollen. The upper right corner reaction measured 3½ × 3 cm., the lower right 2 × 2½ cm., the upper left 2 × 2 cm. and the lower left 1½ × 3 cm. Rabbit 7-6 was reexamined 24 hours later. The areas were then black with a dark red zone at the periphery. The size remained unchanged. It appeared that the reaction reached its maximum size in about 5 hours after the intravenous injection. The healing of the hemorrhagic areas was slow. Sloughs formed in about 48 hours after the intravenous injection. The sloughs were followed by scab formation, their gradual separation and scarring. The complete process of healing took about 8 days.

Histological examinations of the hemorrhagic areas were made.

Sections of these areas from Rabbit 14-1 obtained 5 hours after the intravenous injection can be described briefly, as follows:

The general impression was that of the severest type of hemorrhage and necrosis. The skin was edematous in places. Some of the blood vessels were ruptured. The subcutaneous tissue was engorged with blood. There was also an extensive migration of polymorphonuclear neutrophil leucocytes. There was observed pronounced necrobiosis of these cells located inside and outside the blood vessels. Some of the blood vessels contained small parietal thrombi. While it was clear that the process affected the veins, it remained unsettled whether there was any primary injury to the arteries. Some of the arteries were found normal. Others were almost entirely obliterated. The obliteration, however, was probably secondary to the hemorrhagic infiltration outside the arteries. Occasionally, hyalinization was seen in the blood vessels. The hemorrhage and necrosis extended to the corium of the skin, which was thin and broken in places.

The sections of the hemorrhagic areas of Rabbit 7-6 made 24 hours after the intravenous injection were almost identical with the sections of Rabbit 14-1.

More extensive histological studies of the phenomenon are under progress.

As is seen from these protocols, a phenomenon of local skin reactivity to *B. typhosus* culture filtrate was observed. The reactivity was due to skin injection of the filtrate 24 hours prior to the intravenous injection of the same filtrate. The local response was that of severest hemorrhage and necrosis and was fully developed 4 to 5 hours

after the intravenous injection.[1] This phenomenon was reproduced in many rabbits.

In order to determine the characteristic features of the phenomenon the following points were investigated:

1. Susceptibility of Normal Rabbits to the Phenomenon of Local Skin Reactivity to B. typhosus Culture Filtrates.

In the course of the work it was observed that certain animals did not respond to the above described treatment. Statistical data were accumulated in order to determine the percentage of normal rabbits which were susceptible to the phenomenon of local reactivity to *B. typhosus* culture filtrates.

In this group of experiments filtrates of Strain T_L or T_{240} cultures in tryptic digest broth were employed. Each area of the skin was injected with 0.25 cc. of undiluted or diluted 1:2 culture filtrate. The dose for intravenous injection varied from 1 to 3 cc. per kilo of body weight. The interval between the skin and intravenous injections was from 20 to 24 hours. Some of the animals died shortly after the intravenous injection (1 to 3 hours) or in the course of the following 48 hours. A considerable percentage of rabbits survived 48 hours. Although in many cases it was already possible to make readings of the reactions 2 hours after the intravenous injection the rabbits which died earlier than 3 hours after the intravenous injection were not taken into consideration in this part of the work.

In the animals which were considered resistant to the phenomenon there was no local hemorrhagic necrosis following the intravenous injection. When, prior to the intravenous injection, there was erythema at the site of skin injections, it became more pronounced 4 to 5 hours after the intravenous injection. Frequently swellings appeared. 24 hours later the skin appeared normal again.

The positively reacting animals showed very severe hemorrhagic necrosis at the site of preliminary skin injections. No mild reactions which would constitute an intermediate group between the negative and positive animals were obtained under the conditions of this part of the work. Of the 212 animals tested in this manner, there were 45 negatively reacting rabbits and 167 rabbits which showed severe reactions (approximately, 78 to 79 per cent positive animals).

[1] The factors which induced the local skin reactivity are termed "*skin preparatory factors*" and those involved in production of local hemorrhagic reactions following the intravenous injection "*reacting factors*."

TABLE I.

The Uniformity of the Hemorrhagic Reaction in Various Areas of the Skin of the Abdomen.

No. of rabbits	Dose of each skin injection	Dose of intravenous injection per kilo of body weight	Filtrates used for skin and intravenous injections	Size, intensity and type of reaction in various areas of the skin of the abdomen			
				The upper right area of the abdomen	The lower right area of the abdomen	The upper left area of the abdomen	The lower left area of the abdomen
	cc.	*cc.*					
I	0.5	2.8	$T.D.T_L$	Hem. 4+ $2 \times 2\frac{1}{2}$ cm.	Hem. 4+ $1\frac{1}{2} \times 2$ cm.	Hem. 4+ 3×2 cm.	Hem. 4+ 3×2 cm.
II	"	2	$T.D.T_{240}$	—	—	—	—
III	"	3	$T.D.T_L$	Hem. 4+ 2×3 cm.	Hem. 4+ 3×2 cm.	Hem. 4+ 4×2 cm.	Hem. 4+ $3 \times 2\frac{1}{2}$ cm.
IV	"	"	"	—	—	—	—
V	0.25	0.5	"	Hem. 4+ $2 \times 1\frac{1}{2}$ cm.	Hem. 4+ 2×2 cm.	Hem. 4+ 2×2 cm.	Hem. 4+ 3×1 cm.
VI*	"	3	"	—	—	—	—
VII	"	"	"	Hem. 4+ 2×1 cm.	Hem. 4+ $1\frac{1}{2} \times 2$ cm.	Hem. 4+ 3×2 cm.	Hem. 4+ 2×2 cm.
VIII	"	0.2	$T.D.T_{240}$	Hem. 4+ 2×2 cm.	Hem. 4+ 3×1 cm.	Hem. 4+ 2×2 cm.	Hem. 4+ $1\frac{1}{2} \times 1\frac{1}{2}$ cm.
IX	"	3	$T.D.T_L$	Hem. 4+ 2×2 cm.	Hem. 4+ 1×1 cm.	Hem. 4+ 2×3 cm.	Hem. 4+ 3×1 cm.
X	"	"	"	Hem. 4+ 2×2 cm.	Hem. 4+ 1×2 cm.	Hem. 4+ 4×4 cm.	Hem. 4+ $2\frac{1}{2} \times 2$ cm.
XI	"	"	"	Hem. 4+ 1×2 cm.	Hem. 4+ 1×1 cm.	Hem. 4+ 2×2 cm.	Hem. 4+ $1\frac{1}{2} \times 2$ cm.
XII**	"	"	"	Hem. 4+	Hem. 4+	Hem. 4+	Hem. 4+
XIII	"	"	"	Hem. 4+ $1\frac{1}{2} \times 1\frac{1}{2}$ cm.	Hem. 4+ $1\frac{1}{2} \times 2$ cm.	Hem. 4+ $2\frac{1}{2} \times \frac{1}{2}$ cm.	Hem. 4+ $1\frac{1}{2} \times 2$ cm.

XIV	0.5	0.5	"	−	−	−	−
XV	"	0.8	"	Hem. 4+ $3\frac{1}{2} \times 3$ cm.	Hem. 4+ 2×2 cm.	Hem. 4+ 2×2 cm.	Hem. 4+ $1\frac{1}{2} \times 3$ cm.
XVI***	0.25	2	"	Hem. 4+	Hem. 4+	Hem. 4+	Hem. 4+
XVII*	"	"	"	−	−	−	−
XVIII***	"	"	"	Hem. 4+	Hem. 4+	Hem. 4+	Hem. 4+
XIX	"	"	"	Hem. 4+ 1×1 cm.	Hem. 4+ 2×1 cm.	Hem. 4+ $1\frac{1}{2} \times 2$ cm.	Hem. 4+ $1\frac{1}{2} \times 1\frac{1}{2}$ cm.
XX***	"	"	"	Hem. 4+	Hem. 4+	Hem. 4+	Hem. 4+
XXI***	"	"	"	Hem. 4+	Hem. 4+	Hem. 4+	Hem. 4+
XXII****	"	"	"	.....	.....	.....	.....
XXIII****	"	"	"	.....	.....	.....	.....
XXIV****	"	"	"	.....	.....	.....	.....

....., not protocolled.
−, no reaction.
Hem., hemorrhagic.
4+, very severe.
* Died in ½ hour after intravenous injection. No reaction was seen yet.
** Confluent reactions.
*** Not measured. Approximately 2×2 cm. in size each.
**** Died in 1 to 2 hours after intravenous injection. Beginning of reaction.

2. *The Uniformity of the Hemorrhagic Reaction in Various Areas of the Skin of the Abdomen.*

Experiments were carried out in order to determine whether there was a uniform response of different areas of the skin of the abdomen to the intravenous injection of *B. typhosus* culture filtrates.

Protocol 1.—Rabbit 43-4 received injections of T. D. T_L filtrate into 5 areas, namely, upper and lower right and left corners and the center of the skin of the abdomen. 0.1 cc. was injected into each area. The next day there was a slight erythema (1+) in each injected area. 24 hours after the skin injections the rabbit received 3 cc. per kilo of body weight of the same filtrate intravenously. 4 hours later the previously injected skin areas were severely hemorrhagic. There was no appreciable difference in the *severity* of the reaction in different areas. The upper right area measured $1\frac{1}{2} \times 1$ cm., the other areas were 1×1 cm. in size. 24 hours following the intravenous injection no change in the size and type of reaction was observed.

As is seen from this experiment, 5 different areas of the skin of the abdomen responded to intravenous injection of the filtrate with an equal degree of severity and showed no appreciable difference in size of the reactions.

In view of the importance attached to these findings it was deemed necessary to extend the work to a large group of animals, in which the doses for both skin and intravenous injections varied to a certain extent.

Protocol 2.—A group of 24 rabbits was used. The conditions of the experiments and the results are summarized in Table I. It will be noted from this table that the positively reacting animals showed uniformly extremely severe hemorrhagic reactions in prepared skin areas following intravenous injections of *B. typhosus* culture filtrates in various doses. The only variations observed were in the size of the reaction. The smallest reaction was 1×1 cm. and the largest 4×4 cm. The size of the reaction did not definitely depend on the amount of filtrate used for the skin preparation. Injection of 0.25 cc. into the skin, frequently led to reactions as large and larger than the injection of 0.5 cc. of the filtrates. For the same animals variations in the size of different areas lay between 1 and 3 cm. in diameter, as is seen from Animal X, in which the widest variations were obtained. Ordinarily, the difference was 1 to 2 cm. Occasionally confluent reactions were obtained. No well marked relationship between the intravenous dose and the size of the reaction of the skin was observed under the conditions of these experiments.

3. Titration of the Skin Preparatory Factors of B. typhosus Culture Filtrates.

Protocol 1.—In order to titrate the skin preparatory factors of *B. typhosus* culture filtrates various dilutions were injected into the skin of the abdomen. 24 hours later the undiluted filtrate was injected intravenously in a dose of 3 cc. per kilo of weight. Readings of the reactions were made 5 and 24 hours after the intravenous injections. In this group of experiments T. D. T_L filtrate was employed.

The results obtained on 12 rabbits susceptible to the phenomenon can be summarized as follows:

The filtrate diluted up to 1:4 was consistently able to induce the local skin reactivity. Reactions following the intravenous injections were severely hemorrhagic, necrotic and varied in size from 1 × 1 cm. to 3 × 2 cm.

There were observed fluctuations in the preparatory effect of higher dilutions. Filtrate dilutions from 1:8 to 1:64 were able to induce local skin reactivity in some animals. The severity of the reactions following the intravenous injections varied from slight blue (1+) to deep blue with an angry red zone at the periphery (4+). It would be of interest to determine whether the fluctuations observed would serve as an indicator of the degree of susceptibility of the rabbits to the phenomenon of local skin reactivity to *B. typhosus* culture filtrates.

Dilutions higher than 1:64 failed to induce the local skin reactivity to *B. typhosus* culture filtrate.

Similar results were obtained with a mixture of equal parts of filtrates derived from different strains (Mt. Sinai, 240, 215_A, 215_B, T_L)

4. Relation of Intensity of Erythema Following Preparatory Skin Injection to the Local Hemorrhagic Reaction Produced by Intravenous Injection of B. typhosus Filtrate.

The purpose of the observations reported here was to determine whether the skin reaction following the preparatory skin injection had any influence on the size and severity of the local hemorrhagic response produced by intravenous injection of *B. typhosus* culture filtrate.

Rabbits were injected into 4 areas of the skin of the abdomen with 0.25 cc. of T. D. T_L filtrate. The intensity of the erythema obtained 20 to 24 hours later

TABLE II.

The Relation of the Erythema Following Skin Injection of B. typhosus Filtrate to the Hemorrhagic Reaction Following Intravenous Injection of the Same Filtrate.

No. of rabbits	*Upper right corner*		*Lower right corner*		*Upper left corner*		*Lower left corner*	
	Intensity of erythema after skin injection	Size and intensity of reaction after intravenous injection	Intensity of erythema after skin injection	Size and intensity of reaction after intravenous injection	Intensity of erythema after skin injection	Size and intensity of reaction after intravenous injection	Intensity of erythema after skin injection	Size and intensity of reaction after intravenous injection
XXVI	Negative	3.5 × 4.5 cm. Hem. 4+	Negative	5 × 3 cm. Hem. 4+	–	–	–	–
XXVII	2+	2 × 2 cm. Hem. 4+	"	1 × 1½ cm. Hem. 4+	2+	3 × 1 cm. Hem. 4+	2+	1½ × 1 cm. Hem. 4+
XXVIII	Doubtful	1½ × 1 cm. Hem. 4+	"	1 × 1 cm. Hem. 4+	Doubtful	3 × 2 cm. Hem. 4+	Doubtful	2 × 2 cm. Hem. 4+
XXIX	3+	1 × 1½ cm. Hem. 4+	3+	1 × 1½ cm. Hem. 4+	3+	1 × 1 cm. Hem. 4+	–	–
XXX	3+	1 × 1½ cm. Hem. 4+	2+	1 × 1 cm. Hem. 4+	1+	1 × 1½ cm. Hem. 4+	Negative	1 × 1 cm. Hem. 4+
XXXI	2+	2 × 3½ cm. Hem. 4+	2+	4 × 1½ cm. Hem. 4+	2+	4 × 3 cm. Hem. 4+	"	1½ × 2 cm. Hem. 4+
XXXII	Negative	2 × 2 cm. Hem. 4+	Negative	2 × 2 cm. Hem. 4+	Negative	2½ × 1½ cm. Hem. 4+	–	–
XXXIII	"	1½ × 1 cm. Hem. 4+	–	–	1+	1 × 1 cm. Hem. 4+	Negative	1½ × 1½ cm. Hem. 4+
XXXIV	"	1½ × 1½ cm. Hem. 4+	Negative	1½ × 1 cm. Hem. 4+	–	–	"	1½ × 2 cm. Hem. 4+
XXXV	2+	1× 2 cm. Hem. 4+	"	1 × 1½ cm. Hem. 4+	4+	1½ × 1 cm. Hem. 4+	2+	2 × 2 cm. Hem. 4+

XXXVI	3+	1½ × 1½ cm. Hem. 4+	3+	1½ × 2 cm. Hem. 4+	Doubtful	1½ × 1 cm. Hem. 4+	Doubtful	1½ × 2 cm. Hem. 4+
XXXVII	1+	1½ × 1 cm. Hem. 4+	Negative	1 × 1 cm. Hem. 4+	2+	1 × 2 cm. Hem. 4+	Negative	1 × 1 cm. Hem. 4+
XXXVIII	Negative	3 × 3 cm. Hem. 4+	"	3 × 3 cm. Hem. 4+	Negative	2 × 2 cm. Hem. 4+	"	1 × 1 cm. Hem. 4+
XXXIX	2+	2½ × 2 cm. Hem. 4+	"	3 × 1½ cm. Hem. 4+	2+	1 × 3½ cm. Hem. 4+	"	1 × 1 cm. Hem. 4+
XL	1+	1 × 2 cm. Hem. 4+	–	–	2+	1 × 1 cm. Hem. 4+	"	1 × 1½ cm. Hem. 4+
XLI	Negative	2½ × 2 cm. Hem. 4+	–	–	Negative	2 × 2 cm. Hem. 4+	"	2 × 2 cm. Hem. 4+
XLII	"	1½ × 1½ cm. Hem. 4+	Negative	1½ × 1½ cm. Hem. 4+	3+	1½ × 2 cm. Hem. 4+	"	2 × 1 cm. Hem. 4+
XLIII	"	1 × 1 cm. Hem. 4+	2+	1 × 1 cm. Hem. 4+	–	–	–	–
XLIV	3+	1½ × 1½ cm. Hem. 4+	2+	1½ × 1½ cm. Hem. 4+	Negative	1½ × 1 cm. Hem. 4+	–	–
XLV	Negative	3 × 2½ cm. Hem. 4+	Negative	2 × 1 cm. Hem. 4+	–	–	Negative	2½ × 2½ cm. Hem. 4+
XLVI	1+	1½ × ½ cm. Hem. 4+	1+	1 × 1 cm. Hem. 4+	1+	1 × 1 cm. Hem. 4+	1+	1 × 1 cm. Hem. 4+
XLVII	2+	1 × 1 cm. Hem. 4+	1+	1 × 1 cm. Hem. 4+	–	–	–	–
XLVIII	4+	2 × 2 cm. Hem. 4+	2+	2 × 2 cm. Hem. 4+	4+	2 × 2 cm. Hem. 4+	–	–

–, not tested.

was recorded. 24 hours after the preparatory injections the same filtrate was injected intravenously into these animals. The dose varied from 2 to 3 cc. per kilo of body weight. Readings recorded here were made 24 hours after the intravenous injections.

Some of the animals died 1 to 2 hours after the intravenous injection. The readings of these animals were not included in the protocols.

In Table II, animals susceptible to the phenomenon of local skin reactivity to *B. typhosus* culture filtrate are recorded. It is seen from this table that a considerable number of areas which did not react to the local injection responded severely locally to the intravenous injection of the filtrate. In addition, areas which had erythemas of various intensity showed an approximately equally active response to the intravenous injections.

In this experiment there were also 5 rabbits which were not included in Table II. 2 animals showed no erythema following the preparatory skin injections. 3 rabbits had erythema of various intensity from 1+ to 4+. All these animals were entirely resistant to the phenomenon of local skin reactivity to *B. typhosus* culture filtrate.

As is seen from the observations reported above, there was no relationship between the intensity of the erythema following the preparatory skin injections and the size and intensity of the local hemorrhagic response to intravenous injections of *B. typhosus* culture filtrate. Evidently, the local trauma produced by the preparatory skin injections was not responsible for the skin localization of the toxic factors introduced by the intravenous route. This finding was confirmed by the observation that some animals resistant to the phenomenon of local hemorrhagic response showed erythemas of various intensity after the preparatory skin injections.

5. Attempts to Produce Local Skin Reactivity to B. typhosus Culture Filtrate by Skin Injections of Various Substances.

A series of experiments was performed in order to determine whether the local skin reactivity to *B. typhosus* culture filtrate could be produced by skin injections of various substances.

A: The purpose of the following experiment was to determine whether uninoculated culture medium by itself was able to induce the local skin reactivity to *B. typhosus* culture filtrates.

Protocol 1.—5 rabbits were used for this experiment. The upper and lower right and upper left areas of the skin of the abdomen were injected each with 0.5 cc. of sterile tryptic digest broth. The lower left areas were injected with 0.5 cc. of *B. typhosus* culture filtrate. 24 hours later these rabbits were injected intravenously each with *B. typhosus* culture filtrate. The dose was 3 cc. per kilo of weight. 1½ hours after the intravenous injections 1 rabbit died. No readings were made. 5 hours after the intravenous injections the remaining rabbits showed no reactions in the upper and lower right and upper left areas. The lower left areas of the skin of these rabbits showed very severe hemorrhagic necrosis. The size of the reactions varied from 2½ × 2½ cm. to 3½ × 4 cm.

As is seen from this experiment, sterile tryptic digest broth failed to produce local skin reactivity to *B. typhosus* culture filtrate.

B: Experiments were performed in order to determine whether culture filtrates of various strains of streptococci were able to induce local skin reactivity to *B. typhosus* culture filtrate:

Protocol 1.—In addition to the *B. typhosus* culture filtrate, 2 strains of green producing streptococci (530 and 941) were employed in this experiment. Both strains of streptococcus were isolated from the blood of cases of subacute bacterial endocarditis. The culture filtrates were made in a manner identical with that employed for the preparation of *B. typhosus* culture filtrates.

Rabbits 14-3 and 14-4 received into the upper and lower right areas of the skin injections of streptococcus filtrates 530 and 941, respectively. The upper and lower left areas were injected with T. D. T_L filtrate. 24 hours after the skin injections no reactions were seen. The rabbits were immediately injected intravenously with T. D. T_L filtrate in a dose of 3 cc. per kilo of weight. 5 hours after the intravenous injections no reactions were seen in the upper and lower right areas. The upper and lower left areas, however, showed very severe hemorrhagic reactions. The size of the reactions varied from 1 × 1 cm. to 3 × 2 cm.

Protocol 2.—For this experiment filtrates of 2 strains of *pyogenes Streptococcus hæmolyticus* were prepared in the same manner as *B. typhosus* culture filtrates. In addition toxin of *Streptococcus erysipelatis*, kindly sent to me by Dr. K. Birkhaug under the name E_1—E_5, was employed. A group of 10 rabbits was prepared by skin injections of these filtrates into the upper and lower right and upper left areas. The lower left areas were injected with T. D. T_L filtrate. 24 hours later the rabbits were each injected intravenously with T. D. T_L filtrate. The dose was 3 cc. per kilo of weight. In 1 to 2 hours after the intravenous injections 3 rabbits died. Readings were made 5 hours after the intravenous injections. In 6 rabbits the lower left areas showed very severe hemorrhagic reactions (4+) which varied in size from 1 × 1 cm. to 3 × 2 cm. The other prepared areas were entirely negative. 1 rabbit reacted negatively in all the 4 prepared areas.

It is evident from the experiments of Protocols 1 and 2 that no local skin reactivity to *B. typhosus* culture filtrate was induced by skin injections of 5 different streptococcus culture filtrates. The susceptibility of the rabbits employed to the phenomenon of local skin reactivity to *B. typhosus* culture filtrate was controlled.

C: In this group of experiments the skin of rabbits was prepared by injections of turpentine and 24 hours later *B. typhosus* culture filtrate was injected intravenously.

Protocol 1.—Rabbits 49-4, 49-5, 49-6 were injected with turpentine into 4 areas of the skin of the abdomen. The upper and lower right areas were injected with dilution 1:10 and the upper and lower left areas with dilution 1:5. 24 hours later there were no abscesses in turpentine injected areas, but they were distinctly red. The intravenous injections of potent *B. typhosus* culture filtrate (T. D. T_L) in a dose of 3 cc. per kilo of weight produced no reactions in the prepared areas.

Protocol 2.—The upper and lower right and left areas of the skin of the abdomen of Rabbit 6-3 and 6-9 were injected with 0.2 cc. of undiluted turpentine and turpentine diluted 1:10, 1:20 and 1:40, respectively. 24 hours later the upper right corners of the skin of the abdomen of both animals showed well formed abscesses, the remaining areas showed reddening, but no pus was seen. 24 hours after the skin injections, T. D. T_{240} filtrate, which proved to be potent on the day of the experiment, was injected intravenously into these animals. The dose was 3 cc. per kilo of weight. No local skin reactions followed the intravenous injections.

From the observations reported in this part of the work it was concluded that non-specific irritating substances such as sterile tryptic digest broth and turpentine, and culture filtrates of certain microorganisms biologically unrelated to *B. typhosus* could not substitute the skin preparatory factors of *B. typhosus* culture filtrate. Additional studies are under way in order to determine whether the local skin reactivity to *B. typhosus* culture filtrate can be produced by microorganisms biologically related to *B. typhosus*.

6. The Effect of Heat upon the B. typhosus Skin Preparatory Factors.

To determine the effect of heat upon the skin preparatory factors a number of experiments was made:

Protocol 1.—Rabbits 28-0, 28-1 and 28-2 were injected into the upper and lower right and left areas of the skin of the abdomen respectively with unheated T. D. T_L diluted 1:2, with the same dilutions of T. D. T_L heated to 60°C. for 1 hour with 1:2 T. D. T_L heated in the Arnold steam sterilizer for 1 hour and with 1:2 T. D.

T_L heated in the autoclave for 45 minutes. 24 hours after the skin injections Rabbits 28-0 and 28-1 showed no reactions and Rabbit 28-2 had a diffuse redness extending over the entire skin of the abdomen (2+). 24 hours after the skin injections these rabbits received intravenously unheated T. D. T_L filtrate in a dose of 3 cc. per kilo of body weight. 5 hours after the intravenous injections the upper and lower right and upper left areas of all 3 rabbits showed severe hemorrhagic reactions which varied in size. The lower left areas were entirely negative. Rabbit 28-2 died 6 hours after the intravenous injection. Rabbits 28-0 and 28-1 showed no change in reactions 24 hours after the intravenous injection.

These experiments were repeated on 10 more rabbits with identical results. The slight variations in the size of severe hemorrhagic reactions could be disregarded.

As is seen from these observations, the skin preparatory factors of this strain of *B. typhosus* culture filtrate could be inactivated by heating in the autoclave for 45 minutes. Lower temperatures had no appreciable effect on the ability of the filtrate to induce the local skin reactivity.

Protocol 2.—Similar experiments were performed with a filtrate derived from a culture of a different strain of *B. typhosus* (T_{240}). The culture was made in tryptic digest broth in the usual manner. Rabbits 26-5 and 26-6 were injected into the upper and lower right and left areas of the skin of the abdomen respectively with unheated filtrate T_{240}, with T_{240} filtrate heated to 60°C. for 1 hour, with T_{240} filtrate heated in the Arnold steam sterilizer for 1 hour and the T_{240} filtrate heated in the autoclave for 45 minutes. 24 hours later the rabbits were injected intravenously with the unheated filtrate T. D. T_{240}. The dose was 2 cc. per kilo of body weight. 4½ hours after the intravenous injections all the 4 prepared areas showed extremely severe hemorrhagic reactions which varied in size from 2 × 2 cm. to 4 × 3 cm.

Further attempts were made to inactivate the skin preparatory factors of T. D. T_{240} filtrates by autoclaving:

The filtrates were first diluted 1:2 and then autoclaved for 45 minutes. In other experiments the autoclaving of diluted and undiluted T. D. T_{240} filtrate was prolonged to 1 hour. In all the experiments the skin preparatory factors remained unaffected by the process.

Protocol 3.—A mixture of equal amounts of filtrates derived from cultures of 4 different strains of *B. typhosus* (Mt. Sinai, L, 215_A and 215_B) was employed for these experiments. 6 rabbits were used. The experiment was performed in a manner identical with that described in Protocol 1. 1 rabbit died 1 hour after the injection. No reading was made. 4 rabbits showed very severe hemorrhagic

reactions in the upper and lower right and upper left areas. The lower left areas were entirely negative. 1 rabbit was resistant to the phenomenon of local skin reactivity to *B. typhosus* culture filtrate. The same readings were obtained 24 hours after the intravenous injection.

As is seen from the experiments of this part of the work, the skin preparatory factors of *B. typhosus* possessed considerable resistance to heating. Filtrates derived from certain strains lost these factors when autoclaved for 45 minutes. There was encountered, however, a filtrate of one strain (T_{240}) which resisted autoclaving for 1 hour.

7. *The Effect of Different Hydrogen Ion Concentrations upon the B. typhosus Skin Preparatory Factors.*

It was desirable to determine the effect of acid and alkali upon the skin preparatory factors of *B. typhosus* and, incidentally, to determine whether there is a difference in the heat resistance of filtrates adjusted to various pH.

The experiments were performed as follows:

The *B. typhosus* culture filtrate T. D. T_L was adjusted under sterile precautions to pH 9.0, 8.6, 7.6, 7.0, 6.6, 5.4 and 4.0.[2] The final dilution of the filtrates of various pH was 1:2. The filtrates of pH 9.0 and 4.0 were used on the day of adjustment; those of pH 8.6 to 6.0 were injected 25 hours after the adjustments. Before use, given amounts of filtrates of various pH were heated for 1 hour at 60°C., in the Arnold sterilizer and in the autoclave. A group of 4 rabbits was employed for experiments with heated and non-heated filtrates of each pH. The upper right areas of the skin of the abdomen were injected with the unheated filtrates. Filtrates heated for 1 hour at 60°C., filtrates heated for 1 hour in the Arnold sterilizer and filtrates heated for 1 hour in the autoclave were injected into the lower right and upper and lower left areas, respectively. The center of the skin of the abdomen was injected with non-adjusted and unheated T. D. T_L filtrate diluted 1:2.[3] 24 hours after the preparatory skin injections the rabbits received intravenously the unheated and non-adjusted T. D. T_L filtrate. The dose was 3 cc. per kilo of weight. The susceptible animals reacted severely 5 hours after the intravenous injections. Reactions were obtained in upper and lower right and upper left areas. The lower left areas were entirely negative. The same readings were made 24 hours after the intravenous injection.

[2] No buffers were used for adjustments.

[3] The pH of the filtrate was 7.9 to 8.0.

As is seen from these experiments, there was no inactivation of the skin preparatory factors at a hydrogen ion concentration range of from 9.0 to 4.0 and there was no change in the heat resistance at the various pH. The factors derived from the strain used (T. D. T_L) were invariably inactivated by autoclaving for 1 hour.

8. Incubation Period in Preparation of the Skin to the Phenomenon of Local Skin Reactivity to B. typhosus Culture Filtrate.

In the experiments thus far reported there was allowed an interval of 24 hours between the skin preparatory injections and the intravenous injection of *B. typhosus* culture filtrate. The following experiments were made in order to determine the optimum interval between the injections necessary to elicit the reactivity:

Protocol 1.—6 rabbits (Nos. 48-2 to 48-6) were injected into 4 areas of the skin with T. D. T_L filtrate. Rabbits 48-2, 48-3 and 48-4 received intravenous injections of the filtrate 2 hours after the skin injections. The dose was 3 cc. per kilo of weight. No reactions were seen in these rabbits in the course of the following 48 hours.

Rabbits 48-5, 48-6 and 48-7 were injected intravenously with 3 cc. per kilo of body weight 48 hours after the skin injections. The skin remained unchanged for 48 hours later.

No definite conclusions could be drawn from this experiment, since as shown on page 251 a certain percentage of animals was spontaneously resistant to the phenomenon of local reactivity to *B. typhosus* filtrate. In order to eliminate this objection, the following experiment was performed:

Protocol 2.—The upper right areas of the skin of the abdomen of Rabbits 1-7, 1-8, 2-0, 2-6, 2-7 and 43-9 were injected with 0.25 cc. of T. D. T_L filtrate. 24, 48 and 55 hours later 0.25 cc. of the same filtrate was injected into the lower right, upper left and lower left areas of skin of the abdomen respectively. 56 hours after the first skin injections filtrate T. D. T_L was injected intravenously into these rabbits. The dose was 3 cc. per kilo of body weight. The morning following the intravenous injections namely 15 hours after the intravenous injections the following results were obtained: Rabbit 1-7 was found dead and no reading was possible. Rabbits 1-8, 2-0, 2-6, 2-7 and 43-9 showed no reactions in the upper right and lower left areas. Both lower right and upper left areas showed very pronounced hemorrhagic reactions which varied in size from 1×1 to 2×2 cm.

From these experiments the following could be concluded: For the reproduction of the described phenomenon a definite interval of time was required between the preparatory skin injection and the intravenous injection of the filtrate.

No definite data were available as yet in reference to the exact number of hours required to allow for the preparation of the skin. It could only be stated that 2 hours were insufficient and that the state of reactivity did not last longer than 32 hours. The phenomenon could be invariably reproduced if an interval of 24 hours was allowed between the skin and intravenous injections.

In Protocol 2 it will be noticed that skin reactivity appeared 8 hours after the skin preparatory injection. In these animals, however, the reading of the skin reaction was made 15 hours following the intravenous injections, instead of after the customary 5 hours. The significance of this observation will be considered in a subsequent report.

9. Local Reaction to Repeated Skin Injections of B. typhosus Culture Filtrates.

In the work described up to this point severe hemorrhagic and necrotic local reactions were obtained by skin-intravenous injections. Although no attempts were made as yet to study the mechanism of the phenomenon, it was of interest to determine whether the intravenous route was essential for the reaction. The following protocol illustrates these attempts:

Protocol 1.—Rabbits 16-0, 16-1 and 16-2 received each 4 skin injections of T. D. T_L filtrate into the usual areas. 0.5 cc. was injected into each area. No reactions were seen in these rabbits 24 hours after the injections. The same areas were then reinjected with the filtrate. The amount of filtrate was again 0.5 cc. for each area. The readings were, then, made every hour for the following 6 hours and again 20 hours later. About 1 to 2 hours after the reinjections there appeared reddening and swellings in the 4 areas. The reddening became very pronounced towards the end of 5 hours (4+ erythema). Rabbit 16-1 was killed and sections of the inflamed skin were made. The following morning Rabbits 16-0 and 16-2 showed reactions of the same type as the day before. They were then given intravenous injections of T. D. T_L filtrate in a dose of 3 cc. per kilo of body weight. 4 hours after the intravenous injections both rabbits showed very extensive hemorrhagic necrotic reactions which varied from 2 × 3 to 3 × 4 cm. in size.

The skin section of Rabbit 16-1 showed inflammatory changes. There was an

infiltration of polymorphonuclear neutrophil leucocytes. The leucocytes did not appear necrobiotic. There was no rupture of the blood vessels. No thrombi were found. Some dilatation of the blood vessels was seen in places. The experiment was repeated on 8 more animals with identical results.

As is seen from this experiment repeated skin injections of the filtrate with a 24 hour interval between the injections did not result in the hemorrhagic and necrotic type of reaction described in this paper. The type of the reaction produced by repeated skin injections consisted of a pronounced cellular infiltration, but in contrast to the phenomenon under consideration, there was no breaking up of the cells and no severe damage to the blood vessels. Moreover, the susceptibility of the animals to the hemorrhagic type of reactions was demonstrated, for, 24 hours after the second skin injections, intravenous injections of the *B. typhosus* filtrate were given and there developed severe hemorrhagic and necrotic reactions at the site of previous skin injections. It seems, therefore, that the preparatory skin injections have to be followed by injection of the filtrate through the intravenous route for the reproduction of the phenomenon described.

Further work on the mechanism of the reaction is under progress. It is intended also to amplify the experiments on repeated skin injections with toxic filtrates of different strength.

CONCLUSIONS AND SUMMARY.

A phenomenon of local skin reactivity to *B. typhosus* culture filtrates is described in this report. The reactivity was induced by skin injections of the filtrate followed 24 hours later by an intravenous injection of the same filtrate. The local response consisted of severe hemorrhagic necrosis and was fully developed 4 to 5 hours after the second injection.

About 78 to 79 per cent of the rabbits employed were susceptible to this phenomenon.

Different areas of the skin of the abdomen, when similarly treated, responded with equal severity to the intravenous injection. There were variations in the size of different areas in the same animals.

The intensity and size of the local hemorrhagic reactions were not related to the intensity of the erythema produced by the preparatory skin injections. Following intravenous injection very severe hemor-

rhagic reactions were obtained, in those areas which reacted negatively in this respect to the preparatory skin injections. Evidently, the local trauma produced by the preparatory skin injections was not responsible for the localization of the toxic factors introduced by the intravenous route.

It was necessary to allow a short interval of time between the skin preparatory injections and the intravenous injection, for the reproduction of the phenomenon. An incubation period of 2 hours was insufficient. An interval of 24 hours was invariably sufficient. The ability to react disappeared in 48 hours after the preliminary skin injections.

Repeated direct injections of the filtrate into the same areas of the skin, with an interval of 24 hours between the injections, did not result in reactions similar to the above described hemorrhagic necrosis. The second skin injection was followed by reddening, some swelling and a local accumulation of polymorphonuclear neutrophil leucocytes which showed no signs of necrobiosis. There was no rupture of blood vessels. Skin injections followed after a suitable interval by intravenous injection, were necessary for the reproduction of the severe local hemorrhagic response.

Skin reactivity to *B. typhosus* culture filtrate injected intravenously was not induced by turpentine in various dilutions, sterile tryptic digest broth, culture filtrates of 4 strains of streptococci or by the *Streptococcus erysipelatis* toxin.

It was possible to titrate the skin preparatory factors. Dilutions of the filtrate up to 1:64 were able to induce the local skin reactivity. But, whereas dilutions up to 1:4 invariably prepared the skin so that very severe hemorrhagic reactions followed the second injection in susceptible animals, dilutions from 1:8 to 1:64 were uncertain and their preparatory effect varied in different animals.

The skin preparatory factors showed considerable heat resistance. The heat resistance varied with the strains employed. One strain produced factors totally resistant to heating in the autoclave for 1 hour. However, there was definite and unquestionable inactivation of these factors as derived from other strains when the filtrate was diluted 1:2 and heated in the autoclave.

Various hydrogen ion concentrations in the range from 9.0 to 4.0 had

no effect upon the skin preparatory factors. Heat resistance was not modified by the various pH within this range.

The mechanism of the phenomenon described has not been fully studied as yet. An experimental comparison of it with the manifestations of bacterial allergy of the skin is necessary. There are certain features, however, which *considered together*, distinguish this phenomenon from the known phenomena of bacterial hypersusceptibility. These features are: local reactivity; the short incubation period necessary to induce the local reactivity; the short duration of the state of reactivity; the ability to induce local reactivity by a single skin injection; the severity of the reaction; and the necessity to make the second injection of the toxic agent by the intravenous route.

Studies on the relation of specific antisera to the phenomenon described are under way.

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EXPLANATION OF PLATES.

PLATE 6.

FIG. 1. 5 areas of the skin were injected each with 0.3 cc. of T. D. T_L filtrate. 24 hours later the rabbit received intravenous injection of the same filtrate. The dose was 2.5 cc. per kilo of body weight. Appearance of reaction at the site of prior skin injections 5 hours after the intravenous injection.

FIG. 2. This rabbit was treated in a manner identical with that represented by Fig. 1. In this rabbit there was a confluent reaction extending from the upper right and lower right areas to the center of the skin of the abdomen.

PLATE 7.

FIG. 3. Hematoxylin-eosin. × 230. Section of skin from Rabbit 14-1. Microscopic appearance of hemorrhagic reaction 5 hours after intravenous injection of T. D. T_L. Note necrobiosis of white blood cells.

FIG. 4. Hematoxylin-eosin. × 270. Section of skin from Rabbit 16-1. Microscopic appearance of reaction produced by repeated skin injections of T. D. T_L. No necrobiosis of white blood cells.

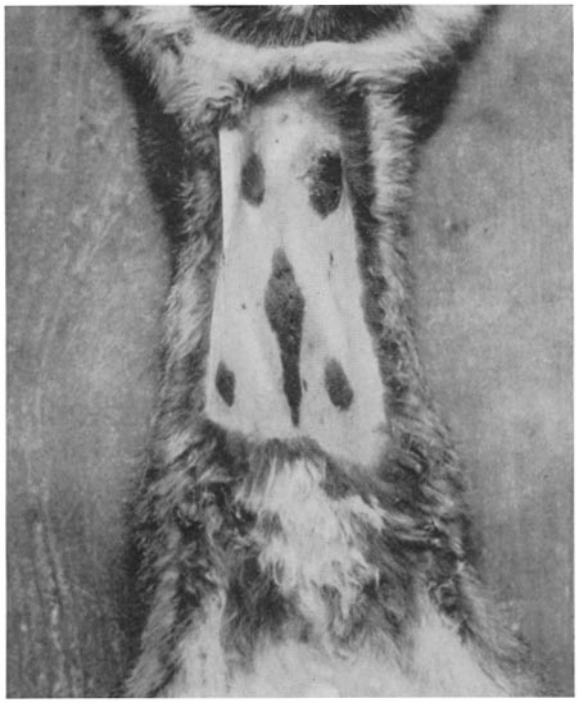

FIG. 1.

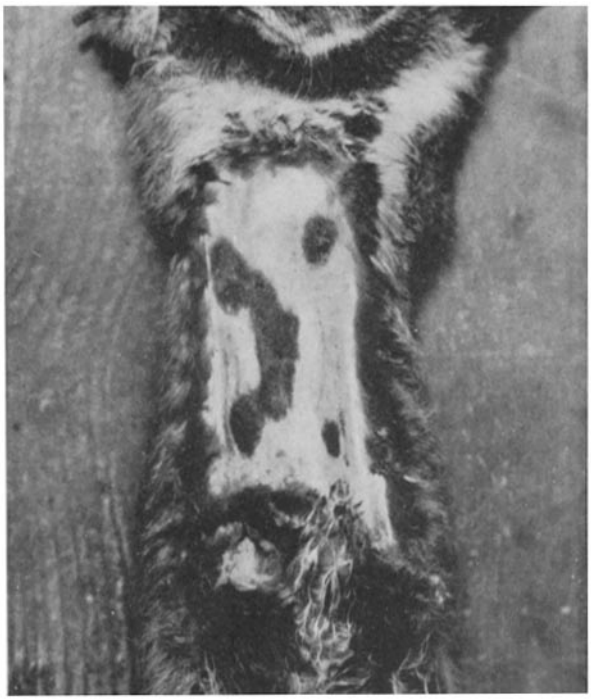

FIG. 2.

(Shwartzman: *B. typhosus* toxic substances. I.)

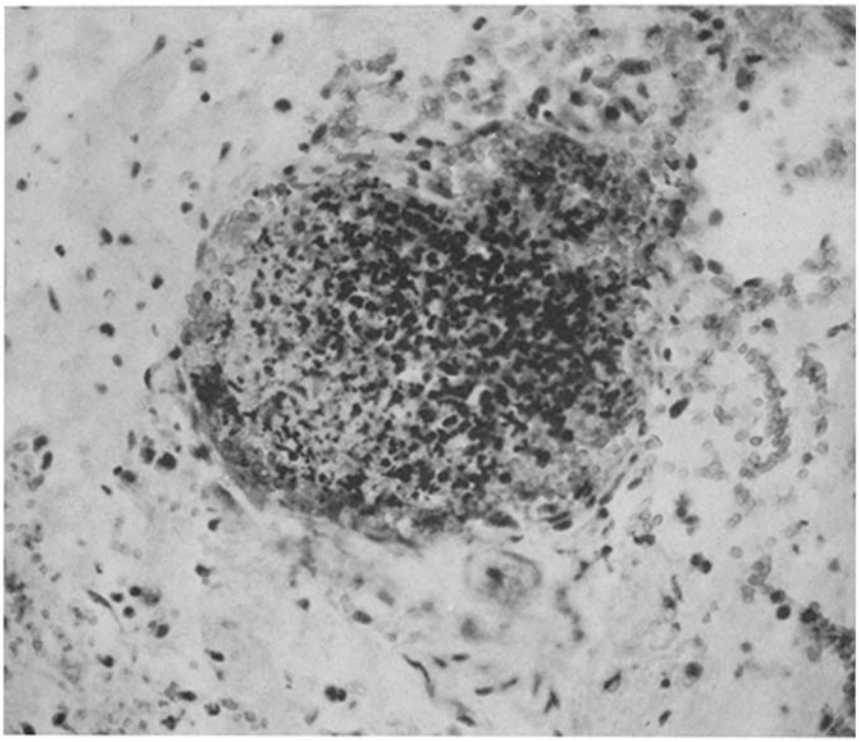

FIG. 3.

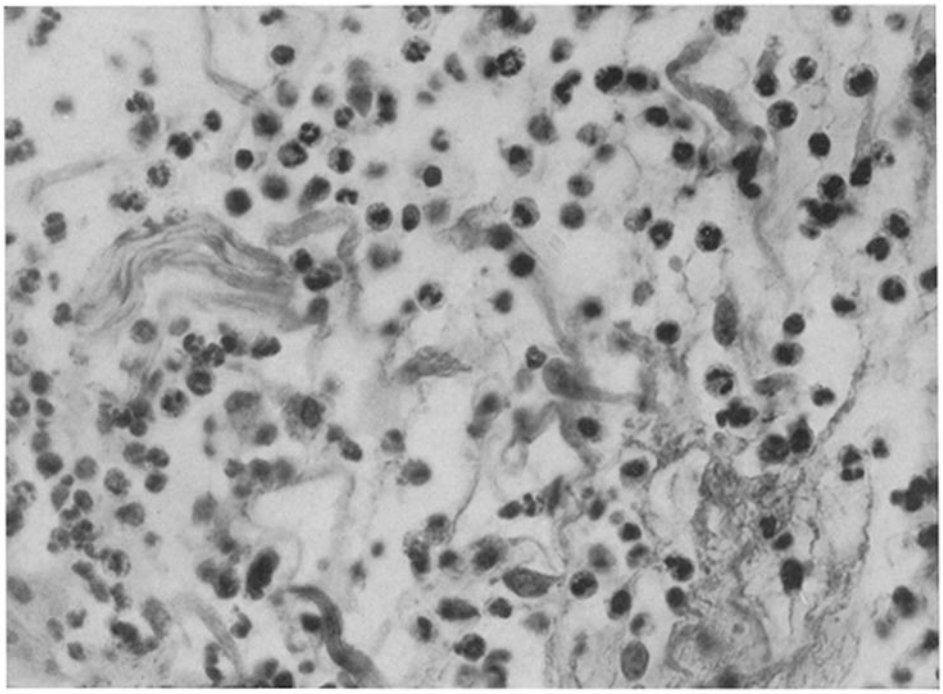

FIG. 4.