A METHOD FOR TITRATING ENDOTOXIC ACTIVITY IN THE SKIN OF RABBITS

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Plates 1 and 2

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Several well established properties of bacterial endotoxins (1, 2) may be employed to assay the potency of experimental materials. During the course of studies directed toward the isolation of active fractions from certain species of *Salmonella* (3), the median lethal dose in mice, injected intraperitoneally, has been employed routinely as a measure of toxicity. While this method yields reproducible results, the native resistance of mice is such that large quantities of material are required to establish end points. Assays based on pyrogenicity for rabbits require only small amounts of preparations but are expensive in terms of animals, and the necessary numbers of suitable rabbits were not readily available. Furthermore, the test is so delicate that guarding against pyrogenic effects from extraneous causes becomes difficult. Ability to prepare normal rabbit skin for the local Shwartzman reaction is another property of endotoxins which may be adapted to an assay requiring only modest amounts of toxin; however, a varying proportion of rabbits fails to show the reaction and, as the test is ordinarily performed, relatively few inoculations may be made into any one animal.

In the past, we have successfully employed the reaction of normal rabbits to intradermal injection of *Bacterium tularense* for estimating the toxicity of the whole organisms (4) and so were prompted to determine whether the fractions from *Salmonella* would have a similar effect. The primary inflammatory response to intracutaneous injections of culture filtrates or toxic extracts from Gram-negative bacilli has been recognized for many years. Ecker and Welch (5) cited numerous investigations, beginning at least as early as 1914, of the cutaneous response to products of enteric bacteria. Many of these were confused through preoccupation with attempts to make a diagnostic use of bacterins by analogy with the tuberculin test. In 1927, Ecker and Rimington (6) reported that concentrated and refined culture filtrates (principally carbohydrate in nature) from paratyphoid bacilli invariably produced in the skin of normal rabbits "a raised edematous and reddened area," often 2 to 3 cm. in diameter. This finding was later reaffirmed and extended to products of several related bacteria

(5). In 1932, Hanks and Rettger (7), in the interest of conserving animals, explored the use of rabbit skin tests as a means of comparing the endotoxic activity of various preparations from *Salmonella pullorum* and other Gram-negative bacteria. They reported that both crude and refined materials, when injected into the skin of normal albino rabbits, produced local tissue damage which was evidenced by edema, erythema, and occasionally necrosis, and that the severity of response was proportional to the dose of toxin injected. They stated that reactions to the same filtrate in different rabbits were "fairly uniform," and demonstrated a correlation between the abilities of their materials to produce skin reactions in rabbits and to kill mice; but their method did not lead to a statistic which could be tested and compared.

Contrariwise, Shwartzman (8, 9) has asserted that only a small proportion of rabbits responded with lesions visible in the gross to most substances active in the phenomenon of local tissue reactivity, and that his phenomenon could regularly be produced with doses which gave rise to no primary lesion whatever. Others who have worked with the local Shwartzman reaction tend to support this judgment, so far as lesions detectable in the gross are concerned (10–13), but the consensus is that local inflammatory changes may be seen microscopically. Thomas and Good (14) concluded that an intradermal injection of bacterial endotoxin capable of preparing normal rabbit skin for the Shwartzman reaction always produced edema and erythema at the injected site, with infiltration of the tissue by many inflammatory cells. Digeon and Raynaud (15) found that intradermal injections of an endotoxin produced congestive zones that varied in size with the dose, but reported that variability of response from animal to animal made the reaction unsuitable as a method of titration.

We have found that intradermal injection of rabbits with endotoxins, even in high dilutions, isolated from *Salmonella typhosa, Salmonella enteritidis, Salmonella oranienburg, Salmonella typhimurium, Escherichia coli, Citrobacter freundii,* and *Serratia marcescens* resulted in production of characteristic lesions and that the quantity of endotoxin which produced a lesion was directly related to the amount which was lethal for mice. As will appear below, this relationship was established for injections made into the skin of the lateral surfaces of thorax and abdomen, but was not valid for intradermal injections on the ventral surface where the Shwartzman reaction is commonly elicited. The present report describes a relatively simple method of titrating endotoxins in rabbits. Amounts of material comparable to those used by Smith and Thomas (16) in the chick embryo are required.

Materials and Methods

Rabbits were obtained from a number of sources including local rabbitries in Hamilton, Montana, commercial rabbitries in Spokane, Washington, and the animal colony maintained at the National Institutes of Health, Bethesda, Maryland.

Preparations.—1. Soluble endotoxins isolated from washed cells of *S. enteritidis, S. typhosa* (strains 0-901 W and Ty 2), *S. oranienburg,* and *Citrobacter freundii* by treatment with aqueous ether and clarification of the extracts by centrifugation (3); or by treatment with trichloracetic acid (TCA) according to the method of Boivin and Mesrobeanu (17).

(*a*) Dialyzed aqueous ether and TCA extracts, and 68 per cent alcohol precipitates of these, composed of lipid, polysaccharide, and protein (L-Ps-Pr).

(*b*) 68 per cent alcohol precipitates of dialyzed aqueous ether extracts of *S. enteritidis* and *S. typhosa*, strain 0-901 W., from which the protein moiety had been removed by fractional precipitation with alcohol in saturated saline solutions and fractional precipitation with ammonium sulfate according to the method of Webster *et al.* (18) (L-Ps).

(*c*) 68 per cent alcohol precipitates of dialyzed aqueous ether extracts of *S. enteritidis* with content of lipid reduced by reflux in a mixture of chloroform and methanol or in a mixture of monochlorobenzene and ethanol followed by extraction with ether and chloroform (19).

2. Washed cells of *S. enteritidis*, strain S-795, and *S. typhosa*, strain Ty 2, killed with formalin.

3. Fractions consisting of protoplasm and purified cell walls of *S. enteritidis* and *S. typhosa* Ty 2 prepared by mechanical disruption of washed cells in the Mickle apparatus and separated by centrifugation.

4. Samples of endotoxins supplied by Dr. A. G. Johnson, University of Michigan; Dr. A. L. Lane, Difco Laboratories; and Dr. M. Landy, National Cancer Institute.

Intraperitoneal Injection of Mice.—Mouse toxicity tests were performed in 21-day-old male white mice of the Rocky Mountain Laboratory strain. Groups of 5 mice were inoculated intraperitoneally with 0.5 and 1.0 ml. aliquots of serial twofold dilutions of preparations in saline. Animals were observed for 6 days (although most deaths occurred within 48 hours) and mice dying later than the 3rd day were examined for evidence of infection. The median lethal dose (LD_{50}) was calculated by the method of Kärber (20); standard errors and 95 per cent confidence limits were estimated by the method of Irwin and Cheeseman (21), except when otherwise noted.

Intradermal Injection of Rabbits.—Albino or Himalayan rabbits in apparent good health weighing approximately 2.5 kg. were selected. The materials were dissolved or suspended in 0.85 per cent NaCl solution, and serial twofold dilutions were made in this diluent. Doses of 0.2 ml. of each serial dilution were injected into closely clipped skin of at least 4 rabbits. The serial dilutions were injected consecutively, from greatest to least dilution, in horizontal rows. In the usual test, each rabbit received all (8 to 12) dilutions of 4 different preparations, 2 preparations on each side, with the bands spaced above and below the lateral median lines. Some care was taken to avoid using lethal amounts of toxin, although this was rarely a problem because rabbits exhibit much tolerance to intradermal injections of endotoxin. Preliminary readings were made 24 hours after inoculation and final readings at the end of 48 hours. At this time the lesions appeared to be at their height and the end points were most consistent from animal to animal. In order to visualize the lesions adequately, hair was reclipped when necessary and the skin was soaked with alcohol while the lesions were being studied. The latter procedure greatly facilitates reading of the tests and is considered essential.

The designations "skin lesion dose" (SLD_{50}) and "Shwartzman preparing dose" (SPD_{50}) were introduced to give numerical expression to the results of skin tests. The method for obtaining these statistics is given below.

RESULTS

The lesions observed are depicted in Fig. 1. Their size was determined primarily by the concentration and activity of the material injected and only secondarily by the volume of inoculum. Infrequently, inflamed areas as large as 35 x 40 mm. were produced by injection of highly potent materials. In most instances, an area of reaction approximately 20 mm. in diameter was produced by injection of 0.2 ml. of soluble endotoxin. As the end point was approached,

size of lesions decreased considerably, and areas less than 5 mm. in diameter were not recorded as positive. Typical lesions were raised and had smooth, firm, erythematous edges. In many cases the center was higher than the edges and the surface was smoothly convex. Usually, however, the central portion appeared to be slightly depressed, and in those lesions appearing as a result of injection of low concentrations of endotoxin the center was paler than the periphery. In those resulting from injections of large concentrations of endotoxin the central portion was hemorrhagic but not to the degree noted in a Shwartzman reaction.

If lesions were rubbed firmly with a gauze pledget soaked in alcohol, marked hemorrhages often developed in the center, most conspicuously in those areas which had received the greatest amount of toxic substance. Lesions in sites which received only small amounts of toxin regressed rather rapidly and might not be detectable 72 to 96 hours after initiation of the experiment. In most instances the lesions were apparent within 24 hours (Fig. 1 *a*), fully developed at 48 hours (Fig. 1 *b*), and no longer visible by the end of a week; but on occasion sufficient damage was done by the initial amount of toxin to produce necrosis followed by sloughing of a considerable area of skin.

A protocol of one experiment is presented in order to demonstrate the method of evaluating results and the variability of response to intradermal injection into rabbits of samples of material from bacterial cells. The materials selected for testing were all derived from *S. enteritidis* and included two samples of protoplasm, one of cell walls, and one of endotoxin. The pertinent data concerning them are as follows:—

No.	Designation	Derivation	Mouse LD_{50}
			mg.
1	Se 123 II B	Cell walls	0.40
2	Se 76 B	Protoplasm	>8.0
3	Se 132-133 B	Endotoxin (heated at 100°C. for 30 minutes)	0.18
4	Se 123 II A	Protoplasm	>4.0

The starting sample of each of these preparations contained 100 μg. of solids per 0.2 ml. of saline. Eleven serial twofold dilutions of each material were made and 0.2 ml. amounts of all dilutions of all 4 materials were injected into each rabbit. Six rabbits were used for the test. Readings, in this case, were made at intervals of 24, 48, and 72 hours, and the presence or absence of lesions was recorded. It was convenient to record the smallest quantity of substance producing a lesion in terms of the serial twofold dilution rather than the actual content in micrograms. In this test, dilution 1 equals 100

μg., 2 = 50, 3 = 25, 4 = 12.5, 5 = 6.25, 6 = 3.12, 7 = 1.56, 8 = 0.78, 9 = 0.39, 10 = 0.195, 11 = 0.098, and 12 = 0.049. The results were as follows:—

Preparation No.	Highest dilution producing a lesion (6 rabbits)			
	24 hrs.	48 hrs.	72 hrs.	SLD_{50} (μg).
1	3, 5, 6, 5, 7, 5	5, 7, 7, 8, 6, 8	5, 5, 7, 5, 6, 5	1.30
2	–, –, –, –, –, –*	–, –, –, –, –, –	–, –, –, –, –, –	>100
3	7, 9, 9, 8, 9, 8	9, 10, 10, 10, 9, 10	9, 10, 9, 9, 8, 9	0.18
4	–, –, –, –, –, –	–, –, –, –, –, –	–, –, –, –, –, –	>100

* = No lesion produced by any dilution tested.

It was desirable to calculate from such results a statistic which would be reasonably comparable to the LD_{50} for mice. Because more than one preparation was tested on each rabbit without randomization of the individual sites, the usual calculations for median effective dose were inappropriate. Instead, the geometric mean of the responses in rabbits was determined. For this purpose a "determining dose," halfway between the doses contained in the highest dilution producing a lesion at least 5 mm. in diameter and the next greater dilution was assumed. Since the serial dilution numbers are functions of the log doses, the determining doses for preparation 1 (Se 123 II B) may be represented as 5.5, 7.5, 7.5, 8.5, 6.5, and 8.5. The average is 7⅓. The geometric mean, (mean threshold dose or SLD_{50}) therefore lies ⅓ of the way between 1.56 and 0.78 and is found to be 1.30 μg. The 95 per cent confidence limits (mean ± t_{05} S.E.) are 0.58 to 2.86 μg. The corresponding figures for preparation 3 are 0.18 μg (0.13 to 0.26).

Although the test reported here was designed to measure the toxicity of soluble endotoxins, it was also used to study preparations consisting of whole cells, cell walls, and protoplasm from species of *Salmonella*. Results of rabbit skin and mouse toxicity tests performed on these preparations are presented in Table I. They indicate that nearly all reactive material was located in the cell wall (3, 22). The 4 protoplasmic fractions listed were not lethal for mice at the highest doses tested (2.7 to 8 mg.). However, the skin test provided an estimation of the endotoxic potency (or, more likely, of the amount of contaminating cell wall endotoxin) of 3 of these preparations.

Comparison of different preparations of endotoxin is probably vitiated to some extent by great differences in solubility which exist between them. Table II summarizes toxicity data (LD_{50} for mice and SLD_{50} in rabbit skin) obtained with preparations which could be loosely termed "soluble" although differences in clarity and stability of solutions were still apparent. Preparations in roughly the upper half of the table were toxic in a range expected for

potent endotoxins from Gram-negative bacteria. Materials with LD_{50} for mice of less than 0.4 mg. yielded SLD_{50} values in rabbit skin of less than 0.6 μg., approximately a thousandfold difference in sensitivity. Also included in Table II are data from additional preparations of varying toxicity in a lower range. The correlation between results of mouse toxicity and rabbit skin tests is treated statistically below.

Certain of the results in Table II are of special interest. Reproducibility of results may be estimated from data on preparations which have been tested more than once; *e.g.*, Se 132-133 B and Se 143-148 III. Since these were tested

TABLE I

Results of Mouse Toxicity and Rabbit Skin Tests Performed on Whole Cells, Cell Walls, and Protoplasm of Salmonella enteritidis and S. typhosa

Preparation		Remarks	Mouse LD_{50}	Rabbit SLD_{50}
			mg.	*μg.*
Se 123 IV	Whole cells *S. enteritidis*	Sterilized with 0.4 per cent formalin	1.2	3.5, 8.8
Sty 7 Kp	" " *S. typhosa* (Ty-2)	Residual cells after dioxane extraction	1.3	4.4
Se 140 A	" " *S. enteritidis*	Sterilized with 0.4 per cent formalin	1.5	6.2
Se 123 III	" " "	" " ether	1.6	3.5
Sty 7 G	" " *S. typhosa* (Ty-2)	" " 0.4 per cent formalin	1.8	8.8
Se 123 II B	Cell walls *S. enteritidis*	Sterilized with 0.4 per cent formalin	0.40	1.1, 1.2, 2.9
Sty 6 C II Cb	" " *S. typhosa* (Ty-2)	" " " " " "	0.47	2.8
Sty 2 Bb	" " "	" " " " " "	0.54	1.8
Sty 14 I C	" " "	" " " " " "	0.71	6.2
Se 76 B	Protoplasm *S. enteritidis*	Sterilized with ether	>8	71, 50, >100
Se 123 II A	" " "	" " "	>4	28, >100
Sty 6 C II-III AC	" *S. typhosa* (Ty-2)	" " "	>5	35
Sty 14 I B	" " " "	" " "	>2.7	>135

in small numbers of animals, differences between values are not considered extreme. Fraction Se 108 Q was a lipid-protein-polysaccharide complex. Removal of the protein moiety did not significantly alter the toxicity of the resulting fractions (Se 108 Qff and Se 108 Qkf) for mice or their inflammatory effect in normal rabbit skin. Endotoxins isolated with aqueous ether from live cells contain less so called "bound" lipid (*i.e.* lipid not extractable with common fat solvents) than TCA extracts. An aqueous ether extract from *S. enteritidis* whose lipid content was reduced from 7 to 3 per cent (Se 143-148 III and IV) had approximately the same toxicity as the original extract (Se 143-148 I) and was somewhat more active in these tests than a TCA extract of *S. typhosa* (Lot B 12) reported to contain 20 to 30 per cent lipid (18). This is of interest since some investigators (23, 24) are of the opinion that the lipid component of an endotoxin plays an important role in eliciting toxic manifestations.

TABLE II

Results of Mouse Toxicity and Rabbit Skin Tests Performed on Endotoxins Isolated from Enterobacteriaciae

Preparation	Source	Remarks	LD_{50}	SLD_{50}
			mg.	*μg.*
Se 132-133 B	*Salmonella enteritidis*	Aqueous ether extract, 68 per cent ETOH precipitated (L-Pr-Ps)	0.18, 0.18, 0.38	0.23, 0.29
Se 132-133 B	" "	Same, heated 100°C. for 30 min.	0.18	0.19, 0.23
Se 136 I	" "	TCA extract, dialyzed (L-Pr-Ps)	0.15	0.20
Se 137 G	" "	" " " "	0.23	0.36
Se 108 Da	" "	Aqueous ether extract, dialyzed (L-Pr-Ps)	0.25	0.12
Se 108 Q	" "	Aqueous ether extract, dialyzed 68 ETOH precipitated (L-Pr-Ps)	0.20	0.12
Se 108 Qff	" "	Aqueous ether extract, dialyzed deproteinized (L-Ps)	0.27	0.25
Se 108 Qkf	" "	Aqueous ether extract, dialyzed deproteinized (L-Ps)	0.19	0.15
Se 143-148 I	" "	Aqueous ether extract, dialyzed 68 per cent ETOH precipitated (L7 per cent-Pr Ps)	0.27	0.34
Se 143-148 II	" "	Aqueous ether extract, dialyzed 68 per cent ETOH precipitated $CHCl_3$-MEOH (L4 per cent-Pr-Ps)	0.20	0.29
Se 143-148 III	" "	Aqueous ether extract, dialyzed 68 per cent ETOH precipitated C_6H_5Cl-ETOH (L3 per cent-Pr-Ps)	0.31	0.12, 0.20, 0.29
Se 143-148 IV	" "	Aqueous ether extract, dialyzed 68 per cent ETOH precipitated C_6H_5Cl-ETOH (L3 per cent-Pr-Ps)	0.31	0.25
St 1 DFM	*S. typhosa* (0-901W)	Aqueous ether extract, deproteinized (L-Ps)	0.25	0.15
Se 140 ID-IF	*S. enteritidis*	TCA extract, dialyzed (L-Pr-Ps)	0.36	0.49
Se 108 K	" "	Aqueous ether extract, 0.1 mole ETOH precipitated in saturated saline (L-Pr-Ps)	0.38	0.59
St 5-03	*S. typhosa* (0-901W)	TCA extract, 0.2 mole ETOH precipitated in saturated saline (L-Pr-Ps)	0.47	0.69
St 4-03	" " "	Aqueous ether extract, 0.3 mole ETOH precipitated in saturated saline (L-Pr-Ps)	0.54	0.98
RX B25624	*S. typhimurium*	Difco	0.41	1.2
No. 902203	*Escherichia coli*	"	0.47	1.2
Sty 14 IV D	*S. typhosa* (Ty-2)	Aqueous ether extract, dialyzed (Vi + 0 antigen)	0.46	1.4
Sty 8 Cc	" " "	Aqueous ether extract, dialyzed (Vi + 0 antigen)	0.60	1.4
Lot B 12	" " (0-901W)	TCA extract, deproteinized (L-Ps) Dr. M. Landy	0.71	2.7
RX B115513	*Serratia marcescens*	Difco	0.71	4.1
RX B12555	" "	"	0.76	2.0
Se 129 I	*S. enteritidis*	Aqueous extract after treatment with dioxane	0.76	2.2
Sty 3 N	*S. typhosa* (Ty-2)	Aqueous ether extract, dialyzed (Vi + 0 antigen)	1.1	3.7
So 2 K	*S. oranienburg*	Aqueous ether extract, 0.1 mole ETOH precipitated in saturated saline (L-Pr-Ps)	1.5	5.5
St 4 Bb	*S. typhosa* (0-901W)	Aqueous ether extract, dialyzed (L-Pr-Ps)	1.7	6.2

Abbreviations: ETOH = ethanol; L = lipid; MEOH = methanol; Pr = protein; Ps = polysaccharide; TCA = trichloracetic acid.

Removal of lipid with retention of toxic and antigenic activities will be reported in detail elsewhere (19). Dermal activity was retained when a water solution of endotoxin (Se 132-133 B) had been treated at 100°C. for 30 minutes. As was also to be expected, an O-haptenic polysaccharide, prepared from a potent endotoxin (Se 143-148 II) by treatment with 0.1 M acetic acid (19, 25) was not lethal for mice in a dose of 2 mg., and 400 μg. failed to produce typical endotoxic skin lesions on rabbits (see also preparation 4 in Table III).

TABLE III

*Results of Mouse Toxicity and Rabbit Skin Tests of Endotoxins from Another Laboratory**

Preparations		Mouse LD_{50}	Rabbit SLD_{50}
No.	Description		
		mg.	*μg.*
1	TCA extract, deproteinized (L-Ps), treated with normal serum and reprecipitated.	>2	2.6
2	Control preparation for No. 1 (recovered following incubation in saline).	>2	2.6
3	TCA extract, acetone-precipitated, dialyzed (L-Pr-Ps).	0.47	0.37
4	Polysaccharide haptene (Ps), prepared according to Freeman (25)	>2	>100
5	TCA extract, deproteinized (L-Ps), Lot 5L10.	0.53	0.55
6	TCA extract of *S. marcescens* (Difco)	1.1	2.6

* These preparations were supplied by Dr. A. G. Johnson, University of Michigan Medical School. All except No. 6 derived from *S. typhosa*. Nos. 1, 2, and 5 were prepared originally by the method of Webster *et al.* (18). Solubility and activity of Nos. 1 and 2 had been altered by manipulation; skin reactions were atypical.

The rabbit skin test also was employed during attempts to separate O and Vi antigens of *S. typhosa*. The toxic mixture containing both antigens was released in water-soluble form by treatment of cell wall fractions or intact cells with aqueous ether (22). These antigens were then quantitatively separated by means of curtain electrophoresis, a technique employed in this laboratory by Dr. F. G. Jarvis for the purification of Vi antigen from *Citrobacter freundii* (26). The Vi-O mixture had a mouse LD_{50} of 1.1 mg. and a rabbit SLD_{50} of 3.5 μg. After separation, the O fraction had an SLD_{50} of 2.2 μg. The Vi fraction did not kill mice or produce lesions in rabbits at the highest levels tested (0.5 mg. and 25 μg., respectively). Another preparation of Vi antigen, similarly obtained, produced an inflammatory lesion when 400 μg. was injected but not at a dose of 200 μg.; mice were not killed by 1 mg. The corresponding O fraction was not tested in rabbits, but had a mouse LD_{50} of 0.33 mg.

To examine further the usefulness of the method, additional endotoxins prepared in another laboratory were tested. Results of a comparative study

of 6 samples supplied by Dr. A. G. Johnson are presented in Table III. Preparation 4 did not yield end points in either test, and so was least active. Preparations 1 and 2 produced atypical lesions in rabbit skin (probably because they had been manipulated in unusual ways in the course of experiments on the effects of serum on endotoxin), and their SLD_{50}'s did not bear the expected relationship to the results of mouse toxicity tests. Nevertheless, the toxicity data in Table III agree well with Dr. Johnson's gradation (13) of the preparations for ability to prepare for the local Shwartzman reaction and to elicit fever in rabbits ($4 < 1$ and $2 < 6 < 5$ and 3).

TABLE IV

Comparison of Sensitivity of Skin Test Method and Local Shwartzman Reaction

Rabbit No.	Weight	Intravenous toxin (20 μg.)	Shwartzman reaction	Least skin test dose*	
				24 hrs.	48 hrs.
	gm.			μg.	μg.
1	1980	No	—	0.39	0.39
2	1980	"	—	0.39	0.19
3	1870	Yes	Neg.	0.78	0.39
4	2160	No	—	0.39	0.39
5	2200	"	—	0.19	0.19
6	1880	Yes	Positive (3.12 μg.)	0.39	0.39
7	1730	"	" "	0.39	0.78
8	1760	"	" "	0.19	0.39

* Minimal dose producing primary skin lesion.

The observed skin reactions to the materials under study are produced by a technique similar to that used for preparatory doses in the local Shwartzman reaction. In order to determine the relative sensitivity of the skin test method and the local Shwartzman reaction, preparation Se 132-133 B was tested both ways in a group of 8 rabbits. All were injected intracutaneously with 0.2 ml. amounts of each of 12 serial twofold dilutions of endotoxin in saline. The initial dose contained 100 μg. and the final dose about 0.05 μg. After 20 hours, 4 of the rabbits were given 20 μg. of the same endotoxin intravenously. Within 4 to 7 hours, hemorrhages developed in the preparatory lesions of 3 of the 4 rabbits given the provoking dose. The results are summarized in Table IV.

The 3 animals which responded to the second injection of endotoxin developed hemorrhages at the sites of preparatory doses of 3.12 μg. or above, but failed to show a change at sites injected with lesser amounts of toxin. At the time of administration of the provoking dose, all the animals had visible lesions in areas treated with 0.78 μg. or less of the fraction used. A similar range was noted at the end of 48 hours. In this instance, then, the rabbit skin test was

sufficiently more sensitive than the Shwartzman reaction to detect at least a fourfold greater dilution of toxin. The lesions of rabbits with and without an intravenous provoking dose of toxin are compared in Fig. 2. Only lesions resulting from the 6 higher concentrations are shown; succeeding dilutions were injected into opposite sides of the rabbits, but resulting lesions are not clearly visible in photographs.

The foregoing results are, of course, in conflict with those of Shwartzman and others. It was suggested (13) that differences in sites of injection might be chiefly responsible: all intradermal injections in these studies had been made on the rabbits' sides whereas the Shwartzman reaction is commonly elicited on the ventral surface of the abdomen. (For simplicity, "side" and "lateral surface" are hereafter used interchangeably, as are "belly" and "ventral surface.") An additional experiment was designed to compare the development of primary lesions with the local Shwartzman reaction following injection of endotoxin in the skin of the sides as well as the belly of white rabbits. An aqueous ether extract from *Citrobacter freundii* was adjusted to an initial concentration of 160 μg./ml. (32 μg./0.2 ml. dose) in 0.85 per cent NaCl and further diluted through 8 serial doubling dilutions. Each of 10 rabbits was then given intradermal injections of each dilution on the right side, left side, and belly. The injections were staggered so as to occupy both bands ordinarily employed on each side and to alternate right and left of the ventral median line. The appearance of all sites of injection was recorded 24 hours later, at which time 5 of the rabbits (1, 3, 4, 8, 10), chosen by a statistical randomization procedure, were given intravenous doses of 30 μg. of the same endotoxin. The appearance of lesions in these animals was recorded 5 hours after the provoking dose, and all animals were observed again 48 and 72 hours after the intradermal injections. Any degree of gross hemorrhage appearing after the provoking dose was considered positive. Table V presents the 48 hour reading of the test. At the initial reading of the Shwartzman reaction, rabbit 4 was exhibiting signs of endotoxin shock. At that time no hemorrhages had developed at any of the injection sites (negative Shwartzman reaction). The animal died during the night.

At the 24 hour reading, primary lesions, extending 4 to 9 places, were visible on the sides of all rabbits. On only 3 rabbits, however, was it possible to count discrete lesions on the belly (1 on No. 10, 4 on No. 3, and 5 on No. 4, the animal that died), although a diffuse erythema of varying extent and intensity was visible on most of the animals. Small hemorrhages were noted in the primary lesions of rabbit 10, extending to the sites of 4 dilutions on the left, 2 on the right, and 1 on the ventral surface.

The reading of the Shwartzman reaction at 5 hours after the provoking dose and that given in Table V, while not identical, did not differ significantly since changes were confined to 1 dilution with about the same number in each

TABLE V

Comparison of Primary Response to Endotoxin with Local Shwartzman Reaction in Skin of Lateral and Ventral Surfaces

Rabbit No.	Weight	Intravenous provoking dose (30 μg.)	Location‡	Reaction* at site injected with indicated dose, μg.								
				32	16	8	4	2	1	0.5	0.25	0.12
	gm.											
1	2290	Yes	R	+	+	+	+	+	+	0	0	0
			L	H	H	+	+	+	+	0	0	0
			B	H	H	0	0	0	0	0	0	0
2	2300	No	R	+	+	+	+	+	0	0	0	0
			L	+	+	+	+	+	0	0	0	0
			B	+	+	+	0	0	0	0	0	0
3	2150	Yes	R	H	H	H	H	+	+	+	+	0
			L	H	H	H	H	+	+	+	0	0
			B	H	H	H	H	+	0	0	0	0
4	2240	Yes	R									
			L	(Died from provoking dose; negative Shwartzman)								
			B									
5	2140	No	R	+	+	+	+	+	0	0	0	0
			L	+	+	+	+	+	0	0	0	0
			B	0	0	0	0	0	0	0	0	0
6	1920	No	R	+	+	+	+	+	0	0	0	0
			L	+	+	+	+	+	0	0	0	0
			B	0 (slight diffuse erythema; no discrete lesions)								
7	1770	No	R	+	+	+	+	+	0	0	0	0
			L	+	+	+	+	+	0	0	0	0
			B	+	+	+	?	+ (edges not defined)				0
8	1890	Yes	R	H	H	H	H	+	+	0	0	0
			L	H	H	H	H	H	+	0	0	0
			B	H	H	H	H	0	0	0	0	0
9	1810	No	R	+	+	+	+	+	+	0	0	0
			L	+	+	+	+	+	+	0	0	0
			B	(confluent edema)			?	+	0	0	0	0
10	1900	Yes	R	H	H	H	H	H	+	0	0	0
			L	H	H	H	H	H	+	+	0	0
			B	H	H	H	0	0	0	0	0	0

* 0 = no visible change; + = edema and/or erythema; H = any degree of visible hemorrhage. Readings made 48 hours after intradermal injections (24 hours after intravenous provoking doses).

‡ R = right side; L = left side; B = ventral surface of abdomen.

direction. It is evident that primary responses to intradermal injection of endotoxin were much more uniform and distinct on the sides than on the belly. Also, minimal preparatory doses for the Shwartzman reaction were about the same on the sides and on the belly and, for purposes of titration, the tests were somewhat easier to read in the former site because adjacent reactions had less tendency to coalesce. It will be noticed that in those rabbits which received an intravenous dose of endotoxin, skin lesions of edema and/or erythema (but no hemorrhages) were observed at sites prepared with less material than that which led to typical Shwartzman reactions. This was especially true on the sides. A comparison of the readings at 24, 29, and 48 hours after injections into the skin has left the impression that the appearance of non-hemorrhagic lesions was wholly independent of the intravenous dose of endotoxin.

Obviously, the Shwartzman reaction could be evaluated with the aid of end points similar to those calculated for the primary reactions. When mean "Shwartzman-preparing doses" (SPD_{50}'s) were calculated from the data of Table V, the sensitivity of both tests in the 3 areas of rabbit skin could be compared as follows:

	Right side	Mean threshold dose (μg.) on	
		Ventral surface	Left side
Primary reaction (SLD_{50})	1.3	10.4 (?)	1.3
Shwartzman reaction (SPD_{50})	5.0	4.0	3.0

On the lateral surfaces, then, the primary cutaneous reaction to endotoxin was probably more sensitive than the Shwartzman reaction in any site. The difference in sensitivity could not be established statistically from these limited data, however. On the ventral surface, discrete primary lesions appeared irregularly and could not be regarded as useful for purposes of assay.

Since the rabbit skin test is proposed as a method of assay, the data have been examined statistically for degree of precision and correlation with another standard assay for endotoxin. The mouse LD_{50}'s and rabbit SLD_{50}'s of the more active preparations (upper portion of Table II), together with their 95 per cent confidence limits, are presented in Table VI. When adequate numbers of rabbits were used, the calculated limits of error were similar in both tests. Values based on only 3 or 4 animals must be expected to show a considerable variability. The fact that the actual variation in SLD_{50} is not extreme among these essentially similar preparations with similar lethality for mice is persuasive evidence that useful estimates of toxicity may be obtained with only a few rabbits.

Because the rabbit skin test was standardized in terms of its relationship

to the mouse toxicity test routinely employed in this laboratory, a somewhat more detailed inquiry into the precision of the latter test may be pertinent. Although the estimates of error in Table VI indicate a probably satisfactory reproducibility of results, a more elaborate version of the test has been employed to check the validity of these estimates. In one experiment, an aqueous ether extract which had already been evaluated for toxicity 3 times in the routine

TABLE VI

*Precision of Measurement of LD_{50} and SLD_{50} Values of Soluble Endotoxins from Salmonella**

Preparations	LD_{50}	Limits of error P = 0.95	No. of rabbits	SLD_{50}	Limits of error P = 0.95
	mg.	*per cent*		*μg.*	*per cent*
Se 132-133 B	0.18	56–178	3	0.23	39–283
Se 132-133 B	0.18	56–178	3	0.29	17–538
Se 132-133 B	0.38	54–184	6	0.19	68–142
Se 132-133 B	0.18	56–178	3	0.23	39–283
Se 136 I	0.15	55–180	4	0.20	15–690
Se 137 G	0.23	55–181	4	0.36	53–169
Se 108 Da	0.25	53–187	4	0.12	58–195
Se 108 Q	0.20	56–177	4	0.12	33–292
Se 108 Qff	0.27	53–187	5	0.25	68–144
Se 108 Qkf	0.19	54–186	4	0.15	40–233
Se 143–148 I	0.27	54–185	4	0.34	56–179
Se 143-148 II	0.20	55–182	4	0.29	41–241
Se 143-148 III	0.31	54–187	‡ 4	0.29	§
			‡ 4	0.20	15–690
			‡ 4	0.12	58–175
Se 143-148 IV	0.31	57–174	5	0.25	68–144
St 1 DFM	0.25	60–167	4	0.15	§

* The preparations from the upper portion of Table II. All mouse tests performed with 5 mice per dose and at least 4 dose levels.

‡ Combining results of all three tests of Se 143-148 III gives an SLD_{50} of 0.19 μg. with confidence limits of 58 to 163 per cent.

§ Same reading in all rabbits, hence the standard error is zero.

test was retested in groups of 25 mice per dose. All doses were injected intraperitoneally in 0.5 ml. volumes. The mice were housed individually and assigned to treatments by randomization procedures. When the experiment was completed, each group of 25 was randomized into 5 subgroups, thus providing, in effect, 5 replicate determinations of the LD_{50} in groups of 5 mice per dose. Table VII presents the protocols for the random subgroups and for the entire experiment as well as the maximum likelihood estimates of the LD_{50}'s, their confidence limits, the estimates of the slopes of the dose-response lines, and their standard errors. The actual variability of the estimated LD_{50}'s of the replicate determinations was within acceptable limits. The standard errors

(and, hence, the confidence limits) of the LD_{50}'s are strikingly similar to those obtained with the test as routinely conducted (see Table VI). The routine assay with 5 mice per dose yields satisfactory results in terms of the reproducibility of the LD_{50}'s and their confidence limits. However, the estimates of the slopes show considerable variation, and unless a preparation is sufficiently well known, it might be advisable to determine the slope with larger groups of animals per dose.

TABLE VII

Precision of Measurement of Toxin LD_{50} Values in Groups of Five or Twenty-Five Mice per Dose

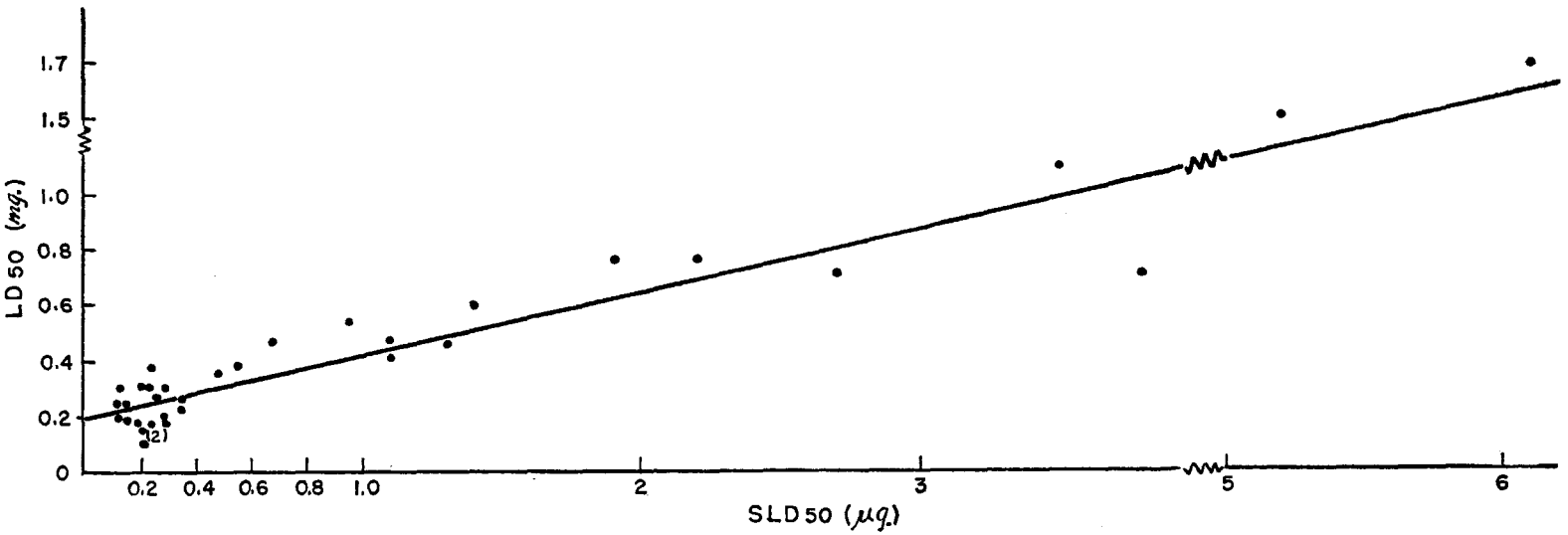
Random subgroup	Deaths/total injected at dose, *mg.*				LD_{50}*	95 per cent confidence limits	Slope (*b*)	S.E. of slope
	0.8	0.4	0.2	0.1				
					mg.	*per cent*		
A	3/5	4/5	1/5	0/5	0.40	55–180	2.54	1.07
B	5/5	5/5	4/5	0/5	0.17	76–129	7.46	2.24
C	4/5	4/5	2/5	0/5	0.29	62–169	2.95	1.10
D	5/5	3/5	2/5	1/5	0.24	58–171	2.74	1.10
E	5/5	4/5	2/5	0/5	0.25	68–144	4.84	1.57
Combined	22/25	20/25	11/25	1/25	0.26	81–123	3.11	0.50

* Maximum likelihood estimates.

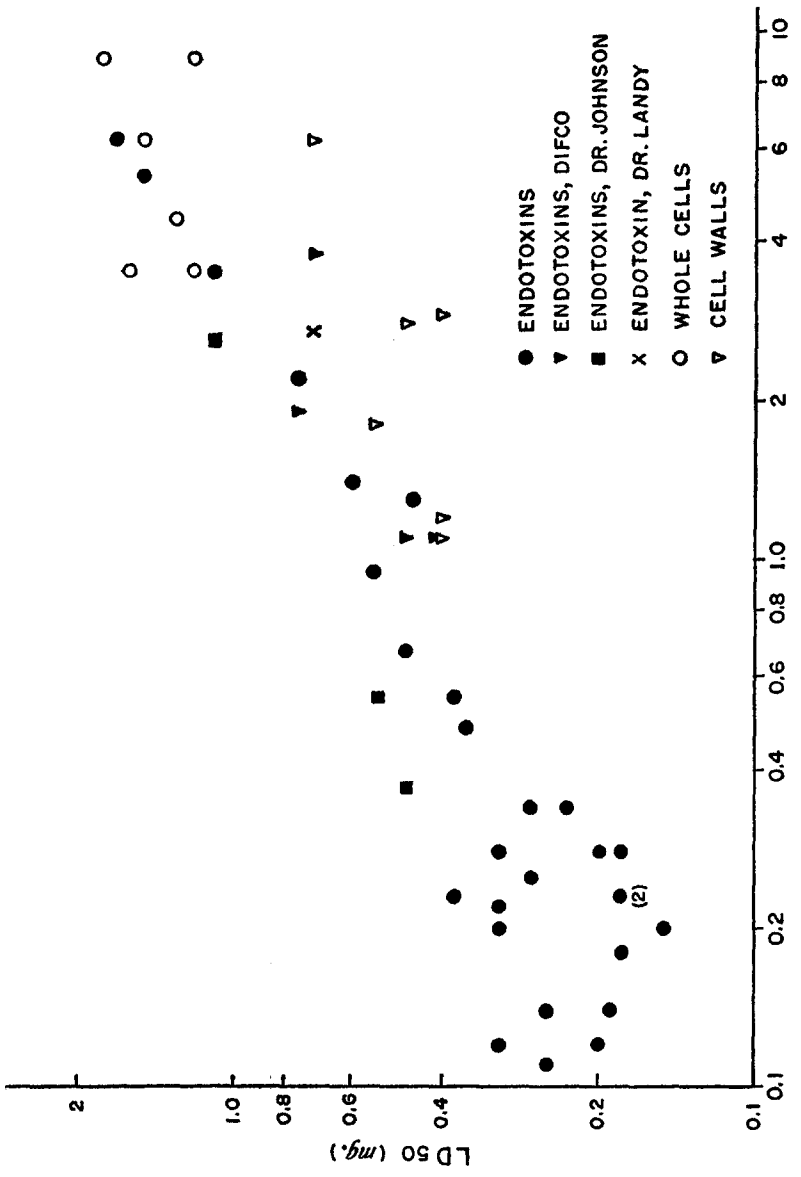
In Text- fig. 1, the LD_{50}'s and SLD_{50}'s for the endotoxins listed in Table II are plotted on arithmetic paper. Each point is determined by the value of the SLD_{50} as abscissa and the value of the LD_{50} as ordinate for each endotoxin. The regression line of the LD_{50} on the SLD_{50} was calculated by the method of least squares. The resulting equation, which may be considered as a formula for converting the rabbit SLD_{50} to the mouse LD_{50} is: $LD_{50} = 0.11 + 0.28\ SLD_{50}$. The slope, 0.28, is significantly different from zero at $p < 0.01$ and therefore a unit (μg.) increase in the SLD_{50} is accompanied by an increase of 0.28 units (μg.) in the LD_{50}. This linear relationship between the two end points is probably the best approximation that can be made from the present data in the sense that the statistical test for departure from linearity is not significant and the postulate of a better fit by a curve rather than a straight line is not tenable. Data from all preparations yielding end points in both tests, including whole cells and cell walls, are presented in Text- fig. 2, which is a scatter diagram obtained by plotting LD_{50} against SLD_{50} on logarithmic paper (in order to include the extended range of values).

DISCUSSION

The foregoing data support the conclusions that primary dermal activity in rabbits may be used as a quantitative assay for endotoxins and that the



TEXT-FIG. 1. Scatter diagram of relation between LD_{50} and SLD_{50} values of endotoxins with calculated linear regression line (plotted on arithmetic paper).



TEXT-FIG. 2. Scatter diagram of relation between LD_{50} and SLD_{50} doses of endotoxins, whole cells, and cell walls (plotted on logarithmic paper).

rabbit skin test is considerably more sensitive than the mouse toxicity test. For potent endotoxins (mouse LD_{50} 0.2 to 0.3 mg.) the SLD_{50} in rabbits is about one-thousandth of the LD_{50} in mice. In our hands, other sensitive tests (for pyrogenicity, for ability to prepare for the local Shwartzman reaction, and for the effect on embryonated eggs described by Smith and Thomas (16)) have not shown a reliability comparable to that of either the mouse toxicity or the rabbit skin test, although the important role of personal experience and preference in such evaluations is recognized.

Conflicting reports were at least partially reconciled by a comparison of primary cutaneous responses to endotoxins with the local Shwartzman reaction elicited by the same materials. With respect to intradermal inoculations on the rabbit's ventral surface, our findings are in agreement with those of Shwartzman: the development of primary lesions was too irregular to be of value for titrations, and hemorrhages appeared, after the intravenous reacting dose, in sites which had not been obviously inflamed from the preparatory dose. But when inoculations were made into the skin of the lateral surfaces, visible edema and/or erythema usually resulted from doses which were too small to prepare for the Shwartzman phenomenon on any of the areas of rabbit skin tested.

Evidence that ability of the described substances to produce inflammatory lesions in the skin of rabbits is attributable to their content of "endotoxin" may be summarized as follows: (*a*) toxic preparations obtained by several methods from a variety of Gram-negative enteric bacteria exhibited this property; (*b*) control preparations, such as polysaccharide haptens, purified Vi antigen, or bacterial protoplasm, were inert in this respect; (*c*) heating of preparations at 100°C. for 30 minutes did not lessen dermal activity; (*d*) statistical analysis of data revealed an excellent correlation between the SLD_{50} in rabbits and the LD_{50} in mice; and (*e*) in one series, the SLD_{50} values served to rank preparations in the same order of reactivity as tests for pyrogenicity and activity in the local Shwartzman reaction performed in another laboratory (13).

These considerations raise the question of the extent to which lethal and dermal activities, as well as other properties, are due to identical chemical groupings in the endotoxin complex. Previous work from this laboratory (3) has demonstrated that by appropriate treatment of preparations of certain species of *Salmonella* with aqueous dioxane two fractions may be obtained: The residual portion is lethal for mice, while the extract has lower toxicity for mice (LD_{50} 1.5 to 2.0 mg.) but maximum serologic reactivity and protective potency. This observation may indicate that there is an alteration of part of an endotoxin molecule, or a selective extraction (from a polymolecular complex) of portions responsible for particular activities. We have discussed certain aspects of the relationship between toxic and antigenic properties of endotoxins

elsewhere (19). Neter *et al.* (27) and Skarnes *et al.* (28) have called attention to the possibility that even some of the various toxic or irritating properties of endotoxins may be functions of different groups and that these may be altered selectively. Therefore, evidence linking dermal reactivity in rabbits to lethality for mice serves to characterize dermal activity as a property of the complexes ordinarily referred to as endotoxins, but does not necessarily imply that these properties are inseparable.

The above-mentioned dioxane extracts and one "lipopolysaccharide" (Difco) from *Brucella abortus* gave results which did not fit the usual pattern, and undoubtedly other endotoxins will be found to produce lesions which differ in morphology or rate of development. The test was devised for typical endotoxins from *S. enteritidis* and *S. typhosa* and has been found to be directly applicable to preparations from several other species of Enterobacteriaceae. Suitable modifications of technique, however, should permit the titration of any materials capable of producing inflammatory lesions in the skin of normal rabbits. It is, of course, essential to work with sterile, readily dispersible preparations which have been carefully separated from solvents or other irritating chemicals.

An important consideration with regard to the mechanics of the test is the possible influence of the site of injection on results, since it is likely that, even within the described bands, some areas of rabbit skin are more reactive than others. We have attempted to compensate for this source of error in two ways: (*a*) reactions at individual sites have been scored as positive or negative, with no attempt at gradation of response; and (*b*) preparations were titrated in each of several rabbits and, as far as possible, distributed equally among the chosen locations (upper or lower, right or left sides).

The advisability of putting more than one preparation on each rabbit may also be questioned. The advantage of economy of animals is obvious, but unknown effects of interference or enhancement may conceivably influence results. This possibility was examined early in the investigation and, for the endotoxins from enteric bacteria then under test, the end points were the same whether 1, 2, or 4 preparations were put on each rabbit. The only contrary behavior was observed with the toxin from *B. abortus* referred to above. When tested in combination with materials from *Salmonella*, it produced lesions which did not develop for several days, whereas, when injected alone, the lesions were mature before 48 hours. Results with other preparations tested at the same time did not appear to be affected.

SUMMARY

The primary inflammatory response of rabbits to intradermal injection of bacterial products has been used as the basis of a sensitive assay for endotoxins. Injection of materials into the skin of the lateral surfaces, rather than the ventral surface of the abdomen, was essential. The method was evaluated by

comparison with the mouse toxicity test. Statistical examination revealed an excellent correlation between the mean threshold dose for producing skin lesions in rabbits (SLD_{50}) and the median lethal dose for mice (LD_{50}). For potent endotoxins (LD_{50} = 0.2–0.3 mg.) the skin test dose was about one-thousandth of the lethal dose for mice. Thereafter the LD_{50} increased in the proportion of about 0.3 mg. per 1 μg. increase in SLD_{50}. The method of testing was devised for endotoxins prepared in various ways from *Salmonella enteritidis* and *S. typhosa* but was directly applicable to endotoxins from several other species of enteric Gram-negative bacteria.

BIBLIOGRAPHY

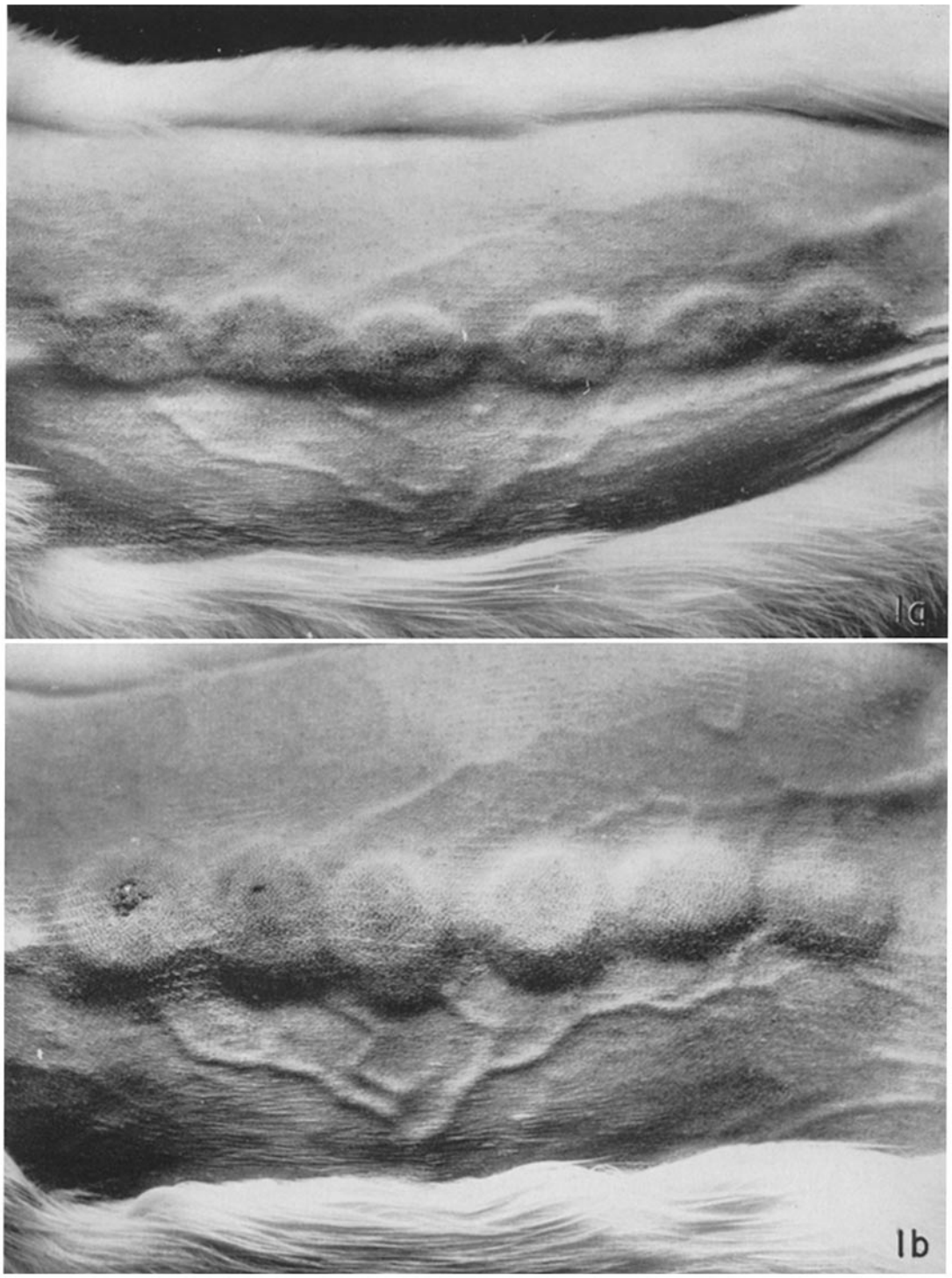
1. Burrows, W., Endotoxins, *Ann. Rev. Microbiol.*, 1951, **5,** 181.
2. Thomas, L., The physiological disturbances produced by endotoxins, *Ann. Rev. Physiol.*, 1954, **16,** 467.
3. Ribi, E., Milner, K. C., and Perrine, T. D., Endotoxic and antigenic fractions from the cell wall of *Salmonella enteritidis.* Methods for separation and some biological activities, *J. Immunol.*, 1959, **82,** 75.
4. Larson, C. L., A skin reaction in rabbits produced by intradermal inoculation of suspensions of killed *Pasteurella tularensis*, *Pub. Health Rep.*, 1946, **61,** 1797.
5. Ecker, E. E., and Welch, H., Cutaneous reactions with culture filtrates of the colon typhoid type, *J. Exp. Med.*, 1930, **51,** 409.
6. Ecker, E. E., and Rimington, C., A preliminary study of the chemical nature of the toxic substances produced by the Salmonella group of organisms, *J. Hyg.*, 1927, **27,** 44.
7. Hanks, J. H., and Rettger, L. F., Bacterial endotoxin. Search for a specific intracellular toxin in *S. pullorum*, *J. Immunol.*, 1932, **22,** 283.
8. Shwartzman, G., Studies on *Bacillus typhosus* toxic substances. I. Phenomenon of local skin reactivity to *B. typhosus* culture filtrate, *J. Exp. Med.*, 1928, **48,** 247.
9. Shwartzman, G., Phenomenon of Local Tissue Reactivity and Its Immunological, Pathological and Clinical Significance, New York, Paul B. Hoeber, Inc., 1937.
10. Gerber, I. E., Morphologic aspects of the local Shwartzman phenomenon, *Arch. Path.*, 1936, **21,** 331.
11. Koplik, L. H., Experimental production of hemorrhage and vascular lesions in lymph nodes: an extension of the Shwartzman phenomenon, *J. Exp. Med.*, 1937, **65,** 287.
12. Moritz, A. R., The relation of altered local tissue reactivity (Shwartzman phenomenon) to infection and inflammation, *J. Exp. Med.*, 1937, **66,** 603.
13. Johnson, A. G., personal communication.
14. Thomas, L., and Good, R. A., The effect of cortisone on the Shwartzman reaction. The production of lesions resembling the dermal and generalized Shwartzman reactions by a single injection of bacterial toxin in cortisone-treated rabbits, *J. Exp. Med.*, 1952, **95,** 409.
15. Digeon, M., and Raynaud, M., Etudes sur la toxine R2 du bacille typhique. VI. Action toxique expérimentale, *Ann. Inst. Pasteur*, 1957, **92,** 642.

16. Smith, R. T., and Thomas, L., The lethal effect of endotoxins on the chick embryo, *J. Exp. Med.*, 1956, **104,** 217.
17. Boivin, A., and Mesrobeanu, L., Recherches sur les antigènes somatiques du bacille typhique. Sur la nature chimique des antigènes "O" et "Vi", *Compt. rend. Soc. biol.*, 1938, **128,** 5.
18. Webster, M. E., Sagin, J. F., Landy, M., and Johnson, A. G., Studies on the O antigen of *Salmonella typhosa.* I. Purification of the antigen, *J. Immunol.*, 1955, **74,** 455.
19. Ribi, E., Hoyer, B. H., Milner, K. C., Perrine, T. D., Larson, C. L., and Goode, G., Physical and chemical analysis of endotoxin from *Salmonella enteritidis*, *J. Immunol.*, 1959, in press.
20. Kärber, G., Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche, *Arch. exp. Path. u. Pharmakol.*, 1931, **162,** 480.
21. Irwin, J. O., and Cheeseman, E. A., On an approximate method of determining the median effective dose and its error, in the case of a quantal response, *J. Hyg.*, 1939, **39,** 574.
22. Ribi, E., Milner, K. C., and Larson, C. L., Antigens from the cell wall of *Salmonella typhosa*, *Bact. Proc.*, 1958, 74.
23. Westphal, O., and Lüderitz, O., Chemische Erforschung von lipopolysacchariden gramnegativer Bakterien, *Angew. Chem.*, 1954, **66,** 407.
24. Westphal, O., Pyrogens, *Tr. 2nd Conf. Polysaccharides Biol.*, Josiah Macy, Jr. Foundation, New York, 1958, 115.
25. Freeman, G. G., The preparation and properties of a specific polysaccharide from *Bact. typhosum* Ty_2, *Biochem. J.*, 1942, **36,** 340.
26. Jarvis, F. G., Ion exchange chromatography of the Vi antigen, *Bact. Proc.*, 1958, 75.
27. Neter, E., Westphal, O., Lüderitz, O., Gorzynski, E. A., and Eichenberger, E., Studies of enterobacterial lipopolysaccharides. Effects of heat and chemicals on erythrocyte-modifying, antigenic, toxic and pyrogenic properties, *J. Immunol.*, 1956, **76,** 377.
28. Skarnes, R. C., Rosen, F. S., Shear, M. J., and Landy, M., Inactivation of endotoxin by a humoral component. II. Interaction of endotoxin with serum and plasma, *J. Exp. Med.*, 1958, **108,** 685.

EXPLANATION OF PLATES

PLATE 1

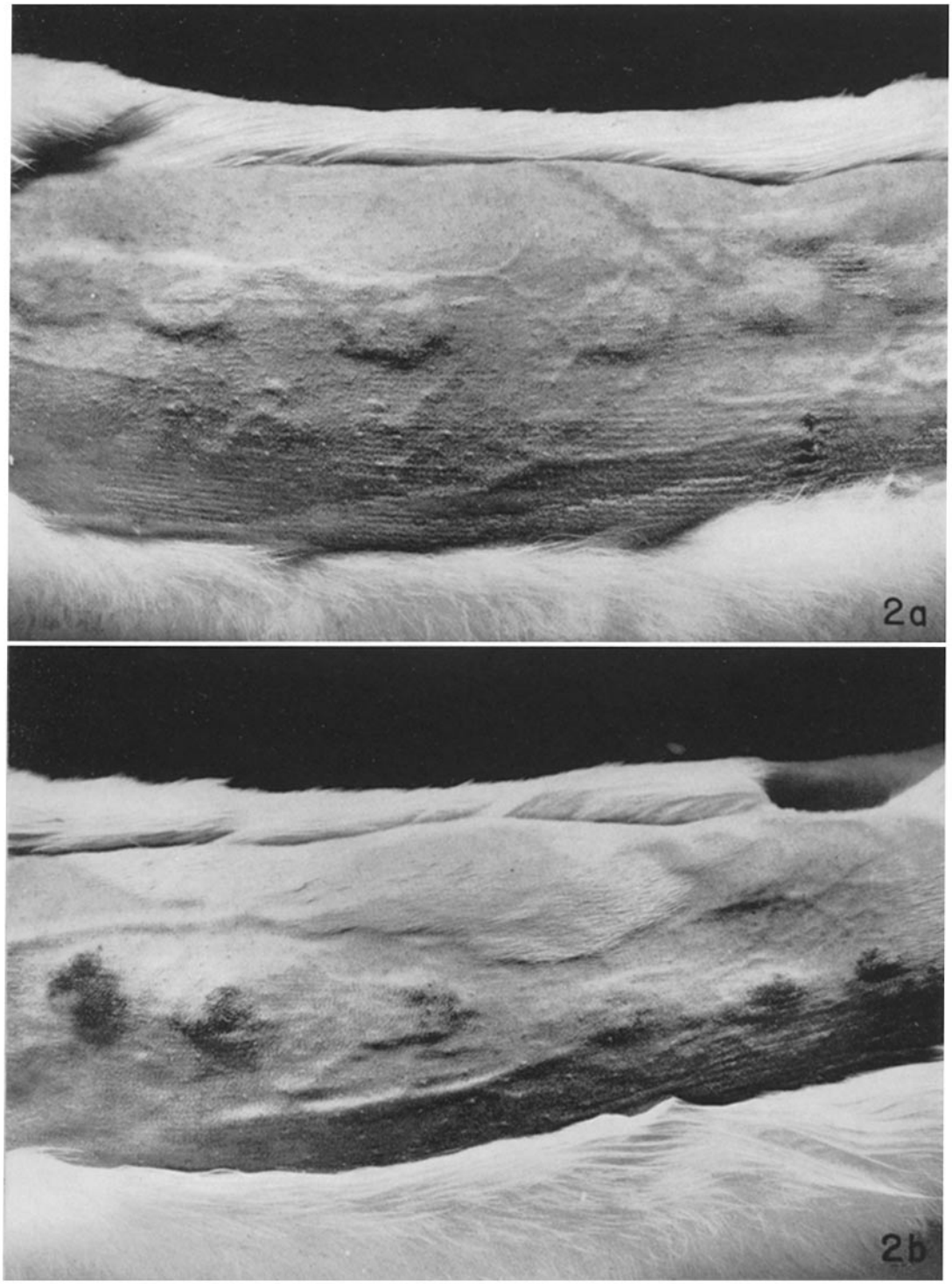
FIG. 1. Skin lesions in rabbits injected with 0.2 ml. of 6 serial twofold dilutions of preparation Se 132-133 B. Responses to doses ranging from 3 to 100 μg. are depicted. Fig. 1 *a*. Lesions after 24 hours. Fig. 1 *b*. Lesions after 48 hours.



(Larson *et al.*: Rabbit skin assay for endotoxin)

PLATE 2

FIG. 2. Comparison of dermal lesions in rabbits (Fig. 2 *a*) (24 hours) with preparatory dose only and, (Fig. 2 *b*) after administration of provoking dose of preparation Se 132-133 B.



(Larson *et al.*: Rabbit skin assay for endotoxin)