is a marked loss of potassium during the first 4 days following irradiation, with a gradual recovery to a positive value by 8-10 days following irradiation. The changes in sodium balance are roughly parallel with those in the potassium balance data. Quite a different picture is seen in the chloride data, however. The chloride balance remains close to zero for the first 4 days of the post-irradiation period, and only after the 4th day is there a significantly negative balance. Again, there is evidence of recovery in the later periods.

The data of Table I represent the means of over 800 independent determinations carried out in triplicate. The control day-to-day standard deviations, representing the over-all analytic and physiologic variations, are only 5-7% of the intake. This degree of uncertainty is even further decreased by the large amount of control data available for comparison. The changes observed under these conditions are statistically significant ($P<.05$ for chloride and $P<.01$ for sodium and potassium), and clearly define the nature of the post-irradiation electrolyte balance changes in the rat exposed to an LD_{50} dose of total body x-irradiation.

Discussion. From previous reports in the literature, it can be inferred that following irradiation the body electrolyte balances become positive(1-3), negative(4-7), or are uninfluenced(8). Unfortunately, none of these reports presents balance results which are statistically significant.

The main features of the post-irradiation electrolyte balances, as defined in Table I, are: (A) a prompt (0-4 day) loss of K^+ and Na^+, (B) a delayed (4-6 day) loss of Cl^-, and (C) a recovery of electrolyte beginning at the 8-10 day period.

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Studies on the O Antigen of *Salmonella typhosa* IV. Endotoxic Properties of the Purified Antigen. (21937)

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Toxic manifestations induced in man and experimental animals by Gram-negative bacteria have been established as referable to their production of a distinctive component, variously described as somatic antigen, endotoxin, pyrogen, and tumor hemorrhagic agent. These specific attributes of endotoxins have been described, particularly by Burrows(1), Bennett and Beeson(2), and Thomas(3). Endotoxins are generally referred to as complexes of polysaccharide, lipid, and protein; however, the precise chemical composition of the complex in relation to endotoxic activity has not been established. Biological properties of endotoxins have been studied for the most part with relatively crude preparations, frequently culture filtrates or extracts(4). Those purified products in recent investigations(5-9), contained significant quantities of nitrogen, which probably represented appre-

ciable protein or polypeptide components. Thus, the essentiality of the protein moiety for biological activity remains obscure.

Our papers described isolation of the somatic antigen of *Salmonella typhosa* as a lipopolysaccharide containing only 0.6% nitrogen, and showed that despite the essentially complete removal of protein, its activity as an antigen was not diminished, but increased (10,11). This report shows that this product possesses the properties characteristic of the classical endotoxins. Inasmuch as the biological effects of endotoxins are well-documented, this report is restricted to a summary of data on endotoxic activities of the lipopolysaccharide derived from *S. typhosa*.

Materials and methods. Endotoxin: The product was isolated by Dr. M. E. Webster, from viable *S. typhosa*, 0-901, by an ethanol-ammonium sulfate fractionation procedure. The purified endotoxin is a phosphorylated lipopolysaccharide containing only 0.6% nitrogen. Its chemical and immunological properties have been described(10,11). *Pyrogenicity tests*: Female New Zealand white rabbits weighing approximately 3 kg were employed. Animals free of demonstrable typhoid O agglutinins were utilized. They were quartered in individual cages in animal room maintained at 75°F. Rectal temperatures were obtained with clinical mercury thermometers. Normal or baseline temperatures were obtained several days prior to use. Following administration of test material, temperatures were taken hourly for 5-7 hours. Rabbits were not used for more than a single test.

Results. General toxic properties: The purified lipopolysaccharide derived from *Salmonella typhosa* possessed, to a high degree, the toxic attributes of colonially smooth Gram-negative bacilli. When administered intravenously to susceptible host, such as the rabbit, exceedingly small amounts were capable of inducing fever, leucopenia, diarrhea, prostration, and death. The activity of the purified endotoxin in eliciting this toxic syndrome is as follows:

Lethal Activity: The lethal action of intravenously administered endotoxin for rabbits is shown in Table I. Despite control of strain, sex, weight, and age of rabbits, considerable variation in susceptibility of individual animals to the lethal action of the endotoxin exists. It is significant that administration of endotoxin over a wide range of dosage in no instance resulted in 100% mortality. On several occasions, the percentage of rabbits succumbing to large doses of endotoxin (100 to 500 μg) was less than that obtained with 20-50 μg. At present there is no explanation for this paradox. Despite this apparent lack of dose-response relationship, a large series of individual tests in this and in related studies have shown that the lipopolysaccharide generally, but not invariably, is lethal for rabbits in dose range of 20-50 μg.

TABLE I. Lethality of Typhosa Endotoxin for Rabbits.*

μg endotoxin (i.v.)	No. of animals dead / Total animals inj.
10	2/10
20	7/10
50	6/10
100	3/10
500	8/15

* New Zealand white females weighing 2.1-2.4 kg.

As observed by other workers(1), we found that in contrast to the rabbit, the mouse is relatively refractory to endotoxin. While much larger doses (on the basis of body weight) are required, the lethal action of intraperitoneally injected endotoxin in this species was more reproducible and consistent than in the rabbit. Thus, the LD_{50} for 15 g Bagg albino mice was approximately 250 μg. Like the mouse, the guinea pig tolerated relatively large doses of endotoxin. The LD_{50} for this species was somewhat more variable and lay in the range of 500-1000 μg.

The lethal activity of the lipopolysacchar-

TABLE II. Lethality of Typhosa Endotoxin for Mice. Effect of alkali treatment.

μg endotoxin (i.p.)	Endotoxin	Alkali-degraded endotoxin
200	7/10*	0/10
400	8/10	0/10
600	6/10	0/10
1000	10/10	0/10
3000	—	3/10

* Fractions equal No. of animals dead of total animals injected.

TABLE III. Pyrogenic Activity of Typhosa Endotoxin. Comparison with intact typhoid bacilli.

Preparation	Dose (μg)	Pyrogenic response of individual rabbits (°F)				Mean temp. rise (°F)	M.P.D.* (μg)
S. typhosa 0-901	.3	2.6	1.4	1.7		1.9	.015
	.03	2.0	1.1	.8		1.3	
	.003	.4	.7	.1		.4	
Purified typhosa endotoxin	.008	2.2	2.9	3.0	3.1	2.8	.0002
	.0016	1.3	2.1	1.6	1.9	1.7	
	.00032	1.5	1.3	1.2	1.1	1.2	
	.00006	.4	.6	.5	.3	.4	

* Minimum pyrogenic dose: Quantity giving rise to 1°F elevation in temperature.

ide was considerably decreased following autoclaving, boiling for 30 minutes, or exposure to weak alkaline hydrolysis (*i.e.* 0.02 N NaOH for 18 hours at room temperature). A comparison of the lethal action for mice of alkali-degraded endotoxin with undegraded endotoxin is illustrated in Table II. On a weight basis, the product subjected to alkaline hydrolysis exhibited less than 10% of the lethal activity of the untreated product.

Pyrogenicity. During investigations on the chemical and immunological properties of the typhosa endotoxin, it was observed that intravenous injection of rabbits with 0.01 μg or less of this lipopolysaccharide resulted in rapid production of fever, associated with leucopenia and subsequent leucocytosis. Generally, temperatures rose within an hour after administration of the product, reached a maximum at approximately 3 hours, and gradually declined to normal in 6 to 8 hours. A marked neutrophilic leucopenia was observed one hour after injection, and the total leucocyte count gradually rose to normal at 4-6 hours, followed by a leucocytosis. These alterations in temperature and leucocyte counts were primarily dependent on the dose of lipopolysaccharide administered. The remarkable pyrogenic activity of this lipopolysaccharide is shown in Table III where the response of groups of rabbits to graded doses of this product are compared with those of animals receiving acetone-killed and dried typhoid bacilli. When the mean temperature response is plotted against dose of lipopolysaccharide administered, the response is rather well related to dose over the range shown in this Table. It is apparent that the purified product is a far more potent pyrogen than the whole organism. The quantity of this component present in intact typhoid bacilli of the 0-901 strain has been reported as 3.3% by Staub and Combes(12), and 6.2% by Webster *et al.*(10). These data indicate that the isolated component should be approximately 15-30 times as active as the bacterial cell. The finding that it is in fact somewhat more active suggests that greater availability may favorably influence pyrogenic activity.

A state of insusceptibility to the pyrogenic action of this endotoxin could be developed in rabibts by a series of injections of the lipopolysaccharide. A group of 10 rabbits treated with 10 daily injections ranging from 1-20 μg (initial dosage 1 μg was progressively increased at 2-day intervals), exhibited little or no thermal response to 1 μg of endotoxin 24 hours after the last injection (mean temperature rise of 0.6°F). This is in contrast to the mean temperature rise of 4°F. developed following injection of 1 μg on the first day of the experiment. On the other hand, a constant daily dose of 1 μg administered to a second group of 6 rabbits for a period of 15 days, did not result in as significant a depression of the fever peak obtained after the initial injection. After 1, 2, 3, 10, and 15 injections, mean temperature rises were 4.6°, 3.5°, 2.9°, 2.6° and 2.4° respectively. In addition, this peak was attained relatively earlier as the injection series progressed.

Animal species were found to vary greatly in their pyrogenic response to this product. Thus, the administration of 10 μg of lipopolysaccharide intravenously brought about a marked thermal response in 2 horses and 2 dogs. On the other hand, during an 8-hour

period, no rise in temperature was observed to occur in groups of mice, rats, guinea pigs, or chicks injected intravenously with 10 μg of this product. Three 20-25 lb chimpanzees were given a single intravenous injection of 1 μg of lipopolysaccharide. A mean elevation of 2.7°F ensued and the leucocyte count fell to approximately one-half the normal values shortly after injection, followed by a 3-4 fold increase in the number of these cells during a period of 24 hours. The pyrogenic response of man to intravenous injection of the purified endotoxin has not yet been determined.† However, in a recent study of its antigenicity in man(13), it was observed that a subcutaneous injection of 20 μg frequently, but not invariably, resulted in erythema, induration, fever, and malaise, while edema and lymphadenitis were encountered occasionally. On the whole, the reactions exhibited by the purified endotoxin were somewhat more marked than those induced by the conventional typhoid vaccine.* These more pronounced reactions might be expected since the quantity of purified endotoxin administered was greater than that calculated to be contained in the standard dose of 0.5 ml of typhoid vaccine (7.5 μg).

Activity in Eliciting the Shwartzman Reaction. Local Reaction: A quantitative study was made of the activity of purified endotoxin as a preparing and provoking agent for the phenomenon of local skin reactivity in the rabbit. Groups of animals were prepared for this reaction with amounts of endotoxin varying from 100 to 0.5 μg. Dilutions of 0.2 ml of this product were injected intradermally at 4 sites on the clean-shaven abdominal skin of each animal. Rabbits, so prepared, were divided into groups of 5-12 animals, and 20 hours later each group was provoked with an intravenous injection of lipopolysaccharide ranging from 100 to 0.2 μg. The ability of individual animals of the same sex, size and strain to respond to this component with development of the characteristic local reaction varied; however, in those rabbits capable of responding the incidence of reactivity followed a decreasing gradient as either the preparatory or provocative dose was reduced (Table IV). As would be expected, the extent and severity of the lesions tended to be minimal when the area had been prepared or provoked by the smallest quantities of endotoxin. Nonetheless, it is apparent that as little as 1 μg, or even less, was sufficient to prepare and provoke this reaction.

TABLE IV. Quantitative Assessment of Activity of Typhosa Endotoxin in Production of Local Shwartzman Reaction.

Provocative dose (μg) admin. i.v. 20 hr after preparatory dose	Preparatory dose (μg) inj. intraderm.					
	100	25	10	5	1	.5
100		80*	60	40	40	
25	83	100	57	57	44	10
20	67	50	50		29	
15	67	33	0		0	
10	40	14	14	0	0	
5	40	40	40		20	
1	60				10	10
.2	10				0	0

* Figures indicate % of rabbits exhibiting positive reaction sites as indicated by visible hemorrhage and purpura. Groups of 5-12 New Zealand white rabbits (3 kg) were used.

The generalized Shwartzman reaction was induced with purified endotoxin from *S. typhosa* in young (2-3 lb) New Zealand white rabbits. Gross and histopathologic examination† of the characteristic lesion, bilateral renal cortical necrosis, showed that the pathology evoked by this purified component was in all respects similar to the classical changes induced by crude bacterial products (*e.g.* occlusion of small blood vessels of kidney with an amorphous fibrinoid-like material. The resultant punctate hemorrhagic and necrotic areas of the cortex occasionally extended into the medullary area) as described by Thomas and Good(7,8). The results of tests in which it was attempted to determine quantitatively

* Adsorption of the lipopolysaccharide on aluminum hydroxide resulted in complete suppression of these toxic manifestations. However, groups of 60 subjects each, inoculated with 20 μg of adsorbed endotoxin showed relatively poor antibody response (mean O agglutinin titer 1:26) in contrast to those of individuals receiving endotoxin as a saline solution (mean O agglutinin titer 1:90).

† The authors are indebted to Lt. Col. Helmuth Sprinz, Walter Reed Army Hospital, who prepared and examined sections of kidneys obtained from rabbits in this study.

TABLE V. Activity of Typhosa Endotoxin in Production of Generalized Shwartzman Reaction.

Preparatory dose of endotoxin (μg)	Hr elapsed between doses	Provocative dose of endotoxin (μg)	No. of animals exhibiting bilateral renal cortical necrosis of total inj.*
20	18	50	0/6
20	12	50	4/6
20	8	50	1/2
20	6	50	1/2
10	12	50	1/3
5	12	50	0/4
20	12	100	2/2
20	12	20	3/5
10	12	20	3/4†
10	12	10	2/4†
5	12	10	0/4

* Reaction evident in the gross 18 hr after provocation.

† Degree of reaction was considerably less in these animals.

the amount of endotoxin necessary to prepare and provoke this pathology in the rabbit are given in Table V. The principal variables in these experiments were preparatory dose, provocative dose, and time interval between the 2 injections. The data obtained indicated that production of this reaction does not follow a clear-cut dose-response relationship, but appears to be associated primarily with quantities of endotoxin not far removed from dosage shown to be lethal for the rabbit. The optimal time interval between the two injections, required for production of the characteristic kidney lesion, appeared to be somewhat less than that previously reported by Thomas and Good(7), who employed meningococcal washings as endotoxin.

Discussion. General toxic manifestations associated with administration of typhoid bacilli have been reproduced by a protein-free lipopolysaccharide isolated from an O variant strain of *S. typhosa*. For the most part, these studies have been conducted in the rabbit, a species highly susceptible to endotoxin. However, various animal species differ in their response to the endotoxic action of this product. Thus, man, horse, dog, and rabbit reacted strongly to the pyrogenic and antigenic stimulus of this product. On the other hand, it may be significant that species such as the mouse, rat, guinea pig, and chick, which did not exhibit a thermal response to injection of 10 μg of lipopolysaccharide, were the species previously shown to be refractory to this product as an antigen(11). Examination of sera from the latter group by the highly sensitive bacterial agglutination test showed no evidence of production of O antibody following this injection. However, it is not known (and it is difficult to obtain acceptable experimental evidence) whether a causal relationship exists between these two properties. In this connection, it is noteworthy that alterations in the lipopolysaccharide which resulted in decreased antigenic activity were accompanied by reduction in pyrogenicity(11). This lipopolysaccharide possessed antigenicity of a high order in the rabbit, *e.g.* a single intravenous injection of 0.001 μg elicited production of significant levels of agglutinins(11). The present study shows that in terms of one of the characteristic properties of endotoxin, namely pyrogenic action, this product exhibits extraordinary potency. It is difficult directly to compare and evaluate the pyrogenic potency of this product with that of the various fever-producing agents reported in the literature; nonetheless, the finding that the minimum pyrogenic dose for a 3 kg rabbit is approximately 0.0002 μg affords evidence that this typhosa lipopolysaccharide is one of the most potent pyrogens known. It has been shown that these attributes of pyrogenicity and antigenicity in the rabbit are manifested in essentially the same range of concentrations and exhibit similar extinction values(11). Depending on the species involved, lipopolysaccharide evokes either both effects, or neither. These findings may therefore be indicative of a common functional mechanism. Experimental evidence tending to support the thesis that these physiological activities of endotoxin may be responsible for marked antigenic activity, subsequently expressed by the host, was obtained in our studies on enhancement of antibody response to proteins by typhosa lipopolysaccharide(14).

The remarkable activity of this purified bacterial component in eliciting these experimental host reactions may provide further insight into the classical reactions encountered clinically in those diseases of man caused by

endotoxin-bearing organisms. For example, calculations based on rhamnose content and on actual isolation, indicated that 1 billion organisms (*S. typhosa,* 0-901) can contribute approximately 15 μg of lipopolysaccharide. That the minimum pyrogenic dose is approximately 0.0002 μg/3 kg rabbit, makes it apparent that endotoxic material released from relatively few bacteria (1×10^4) can produce fever in the rabbit. Man is considered to be more susceptible to endotoxin than the rabbit (15), and this study offers further evidence for the hypothesis advanced by Favorite and Morgan that under conditions of natural infection, extremely small quantities of endotoxin may elicit the pyrexial response in the typhoid fever patient.

A similar situation may apply in the Shwartzman reaction. It is apparent that rabbits vary in ability to react to the endotoxin used by us. The dose-response relationship of an individual rabbit is such that if susceptible to this reaction, it will respond over a wide range of concentrations of endotoxin. That nonsusceptible rabbits exhibit no reduction in their refractory state even when doses of endotoxin are greatly increased further attests to the "all or none" nature of this phenomenon. A comparable individual hyperreactive state has been postulated by Black-Schaffer *et al.* as being a prerequisite for the occurrence in man of the pathology characteristic of the Shwartzman reaction(16).

Summary. A study was made of the endotoxic properties of a protein-free lipopolysaccharide isolated from the 0-901 strain of *S. typhosa.* The product was lethal for rabbits in a dose range of 20-50 μg while 300-500 μg were required to kill mice. The minimum pyrogenic dose in rabbits was 0.0002 μg, characterizing it as a pyrogen of extremely high potency. In a quantitative study of the local Shwartzman reaction, 1 μg or less, was effective as a preparatory or provocative dose. Its activity in eliciting bilateral renal cortical necrosis, the lesion characteristic of the generalized Shwartzman reaction, was in the range of 5-20 μg. Thus, very small quantities of a lipopolysaccharide, freed of protein, are capable of producing the various biological alterations generally attributed to endotoxins.

Addendum. Since this report was submitted for publication preliminary tests of the effects of the typhosa lipopolysaccharide in man were undertaken(17). These indicate that when injected i.v. the minimum pyrogenic dose for a 70 kg subject lies in the range of .01-.04 μg.

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