1. Treadwell, C. R., Flick, D. F., Vahouny, G. V., *J. Nutrition*, 1957, v63, 611.

2. Best, C. H., Ridout, J. H., *Am. Rev. Biochem.*, 1939, v8, 349.

3. Best, C. H., Huntsman, M. E., *J. Physiol.*, 1935, v83, 255.

4. Dury, A., Treadwell, C. R., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 719.

5. Hubbell, R. B., Mendel, L. B., Wakeman, A. J., *J. Nutrition*, 1937, v14, 273.

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Host Resistance in Hemorrhagic Shock. XIV. Induction of Shwartzman Reaction by Shock Plasma and Tissues.* (23767)

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The plasma of an animal in hemorrhagic shock contains a toxin which is not present in normal plasma(1). Certain properties of this toxin, shared with bacterial endotoxins, are: 1. Ability to reduce the phagocytic activity of normal phagocytes and macrophages *in vitro*(2). 2. Induction of "flight of leukocytes," *i.e.* granulocytopenia with segregation of granulocytes, primarily in capillaries of the lung, liver, and spleen(3). 3. Production of a characteristic febrile reaction in trained rabbits, with development of resistance to the pyrogen following repeated daily administration(4). 4. Development in such resistant animals of tolerance of prolonged hemorrhagic shock, with recovery following a transfusion which is not curative in non-resistant animals (4). The presence of this toxin in shock plasma is not simply an incidental abnormality, for it has been shown to be responsible for the failure of animals in prolonged shock (6 hours) to respond to transfusion; and it is capable of converting reversible shock, in which transfusion of normal plasma leads to full recovery, into a state of shock which is not responsive to any therapy(1). The thesis that this toxin is of bacterial origin stems from two observations: 1. Demonstration, already referred to, that resistance to hemorrhagic shock, induced by making animal resistant to a known bacterial endotoxin, is also induced by the toxin in shock plasma(4). 2. Finding that broad-spectrum non-absorbable antibiotics, administered orally so as to nearly sterilize the gastro-intestinal tract, prevent prolonged shock from becoming irreversible to transfusion, and render blood in prolonged shock free of the toxin(1,5). Two further lines of investigation into the properties of this toxin are presented in this paper to provide additional evidence that the circulating toxin in shock is similar to, or identical with, bacterial endotoxin. The first is the demonstration that the plasma and tissues of the shocked animal elicit a generalized Shwartzman reaction under conditions that may be considered virtually specific for endotoxin(6), while normal plasma and tissues, under identical test conditions, fail to do so. The second is the observation that this activity of shock plasma and tissues resides within a polysaccharide fraction extractable with trichloroacetic acid, in the manner employed by Boivin and Mesrobeanu(7) for the isolation and purification of bacterial endotoxins.

Method. Hemorrhagic shock was induced in dogs and rabbits, which were exsanguinated after the shock was shown to be irreversible to transfusion(5).

The blood from rabbits was drawn with sterile precautions into a heparinized syringe, and centrifuged in a refrigerated sterile container. Ten ml of the plasma was then injected intravenously into young (1 kg) rab-

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bits prepared for the Schwartzman reaction by an intravenous injection of 0.03-0.06 mg of *E. coli* endotoxin (Difco #1027-B8) given 18 hours earlier. Plasma from normal rabbits was prepared and tested in the same way.

The blood from dogs was drawn into clean glassware, quickly cooled, and the plasma separated in a refrigerated centrifuge. The cooled heparinized dog plasma was diluted with an equal volume of 0.85% sterile saline or distilled water, and deproteinized by the addition of 16 ml of 30% trichloroacetic acid (w/v) per 100 ml of diluted plasma (0.25 N TCA). The suspension of denatured protein was allowed to stand at 4°C for 4 to 16 hours, centrifuged in the cold, and the supernatant fluid dialyzed at 4°C against frequent changes of large volumes of distilled water for 3 to 4 days. The dialyzed extract was then concentrated to approximately 1/100th the starting volume by evaporation at 50-55°C under reduced pressure (4-6 mm Hg).† One- to three-tenths of an ml of the concentrated extract was injected intradermally into adult rabbits (2-3 kg) prepared for the Shwartzman reaction by an intravenous injection of 3 ml/kg of 25% sterile colloidal thorium dioxide suspension given 6 hours earlier.‡ Extracts which failed to produce a Shwartzman reaction when given intradermally were retested by intravenous injection. Tissues from normal and shocked rabbits were homogenized in sterile saline, diluted to a concentration of 20 mg/ml, and centrifuged to obtain a nearly clear saline extract. Three-tenths of an ml of the saline tissue extract was given intravenously to young rabbits (1 kg) prepared for the Shwartzman reaction by an injection of 0.03-0.06 mg of the *E. coli* endotoxin 18 hours earlier. Trichloroacetic acid extracts were prepared from aliquots of whole tissue homogenate suspensions from normal and shocked rabbits, in the manner described for plasma. The concentrated extracts were adjusted to represent the TCA-soluble fraction of 200 mg of fresh wet tissue per ml. These extracts were tested in adult rabbits primed for the Shwartzman reaction with Thorotrast as described above. All recipient animals were killed and examined 48 hours after the challenge or at death, whichever occurred sooner, for gross and microscopic evidence of the Shwartzman reaction. The tissues were fixed in 10% neutral formalin and stained with phloxin and hematoxylin for histologic examination.

TABLE I. Generalized Shwartzman Reaction in Endotoxin-Primed Rabbits Challenged with Plasma.

No. of animals	Priming dose,* intrav.	Challenge dose, intrav.	Response	
			Pos.	Neg.
4	.03 mg/kg	20 ml normal plasma	0	4
6	.03 "	20 ml shock plasma	5	1
4	.06 "	*Idem*	3	1
3	2 ml shock plasma	10 ml shock plasma	2	1

* *E. coli* endotoxin (Difco #1027-8B) admin. 18 hr before challenge.

Results. None of four rabbits primed with endotoxin and challenged with normal rabbit plasma (Table I) showed any of the gross or microscopic features of the generalized reaction described by Shwartzman(8). In contrast, 8 of 10 rabbits primed with endotoxin and challenged with shock plasma (Table I) developed gross and/or microscopic lesions of the generalized Shwartzman reaction. In addition, 2 of 3 rabbits given a small priming dose of shock plasma (2 ml) in place of endotoxin exhibited the generalized Shwartzman reaction following a challenge dose of shock plasma (Table I).

The saline extracts of liver, lung and spleen

† Use of concentrated extract of dialyzed trichloroacetic acid soluble fraction, in place of whole plasma or saline tissue extracts, in addition to providing evidence on the chemical nature of the toxic agent, permits testing of material from differing species without concern for protein-protein incompatibilities between species, and provides much greater flexibility of dosage and sensitivity of detection of the toxin.

‡ Thorotrast was substituted for *E. coli* endotoxin in preparation of these animals for the Shwartzman reaction because it appears to be capable of inducing a greater degree of sensitivity and regularity of response to known Shwartzman-inciting reagents(9). Thorotrast provides the additional advantage of uniform potency, in contrast to endotoxins, which require biological standardization of each preparation, and which occasionally lose potency on storage(10).

TABLE II. Generalized Shwartzman Reaction in Endotoxin-Primed Rabbits Challenged with Saline Extracts of Rabbit Tissue.*

Challenge tissue	Response: Normal tissue extr. Pos.	Normal tissue extr. Neg.	Shock tissue extr. Pos.	Shock tissue extr. Neg.
Lung	1?	5	4	2
Liver	0	6	5	1
Spleen	0	6	2	4

* 0.06 mg *E. coli* endotoxin (Difco #1027-8B) admin. 18 hr before challenge. Two of the 6 animals challenged with saline extract of shock lung died, one of them too soon to develop the Shwartzman reaction.

of 6 normal and 6 shocked rabbits were each tested in endotoxin-primed rabbits. All recipients of normal tissue extract survived and, with the exception of one animal, showed no gross or microscopic signs of the Shwartzman reaction. The one exception, which had received a lung extract, appeared normal on gross examination, but revealed moderate pulmonary congestion and a few petechial hemorrhages in the kidney on microscopic examination. Two of 18 recipients of saline extracts of shocked tissue died, one within 4 hours of the challenge dose, without the development of gross or microscopic changes of the Shwartzman reaction. Ten of the 16 survivors, and the second non-survivor, demonstrated the generalized Shwartzman reaction (Table II).

Typical *positive* findings at post-mortem examination were (1) heavy, edematous, lungs with numerous discrete areas of hemorrhagic extravasation; (2) suffusion of the liver with blood; (3) petechiae and/or interstitial hemorrhages in the kidneys; (4) focal necrosis on the capsular and cut surface of the kidneys; and, in a few instances, (5) intramural hemorrhages in the small and large intestine. In some instances organs appeared to be within normal limits on gross inspection, but revealed typical changes on microscopic examination.

In general, the microscopic examination simply confirmed the gross findings of congestion, interstitial hemorrhage, and focal necrosis. In addition, however, there was diffuse infiltration of tissues with granulocytes, particularly in the lungs; and some of the renal glomeruli were occluded with a homogenous acidophilic substance resembling fibrin.

The concentrated polysaccharide fraction of the plasma of 13 shocked dogs and of 6 normal dogs was tested by intradermal injection in Thorotrast-primed rabbits. Four of 13 recipients of the shock plasma extract died, while none of the 6 recipients of normal plasma extract succumbed. Ten of the 13 animals tested with shock plasma extract showed gross and microscopic signs of the Shwartzman reaction (Table III). All 6 animals tested with normal plasma extract were free of the stigmata of the Shwartzman reaction. All extracts giving a negative response on intradermal injection were retested *via* intravenous administration, with the result that one of the 3 negative shock plasma extracts gave a positive response, and all 6 normal plasma extracts remained totally inactive.

The 4 rabbits killed by the shock plasma extract exhibited hemorrhagic peritoneal fluid and a large retro-peritoneal and/or peri-renal hemorrhage, in addition to the typical gross lesions of the Shwartzman reaction. The same occurred to a lesser degree in 5 of the 9 Shwartzman-positive survivors.

The concentrated polysaccharide extract of tissues (liver, lung, kidney, and skeletal muscle) of irreversibly shocked rabbits and of normal rabbits was injected intravenously into Thorotrast-primed rabbits in doses equivalent to 3 to 10 times the amount of whole tissue represented by the saline extracts (Table IV). Extracts prepared from normal rabbit tissues were uniformly non-toxic, and failed to induce the Shwartzman reaction. Extracts prepared from irreversibly shocked rab-

TABLE III. Generalized Shwartzman Reaction in Thorotrast-Primed Rabbits Challenged with 0.3 ml Trichloroacetic Acid Extract Concentrate* of Plasma.

No. of animals	Challenge material		Response: Pos.	Neg.
6	Ext. normal plasma	intraderm.	0	6
6	*Idem*	intrav.	0	6
13	Ext. shock plasma	intraderm.	10	3
3	*Idem*	intrav.	1	2

* 0.3 ml concentrate equivalent to 20 ml of plasma.

TABLE IV. Generalized Shwartzman Reaction in Thorotrast-Primed Rabbits Challenged with 0.3 ml Trichloroacetic Acid Extract Concentrate* of Tissue.

Challenge tissue	Response	
	Normal	Shock
2 lung	0	+
2 liver	0	+
2 kidney	0	+
2 muscle	0	?

* 0.3 ml concentrate equivalent to extractable polysaccharide from 60 mg fresh tissue. The data in this table are from 2 normal and 2 shocked rabbits.

bits, with the exception of skeletal muscle extract, produced the generalized Shwartzman reaction in each instance, and 2 of 6 animals died. In the case of the skeletal muscle extract, the results are still inconclusive.

Discussion. The induction of the generalized Shwartzman reaction by a challenge dose of plasma, saline tissue extract, or a partially purified polysaccharide fraction of the plasma or tissues of shocked animals, demonstrates that the toxin present in these preparations is bacterial endotoxin. This statement is based on the prevailing view that, whereas a number of high molecular weight substances other than bacterial endotoxin are capable of *preparing* an animal for the Shwartzman reaction, and of *inducing* a *localized* Shwartzman reaction, only *bacterial* endotoxin has, to date, been shown to be capable of inducing the generalized Shwartzman reaction(6,8).

Recently Landy and Shear stated that the biological properties of endotoxin are not specific for bacterial polysaccharides, since polysaccharide fractions from the tissues of normal mice, isolated by tryptic digestion and trichloroacetic acid-phenol extraction, exhibit the same properties as bacterial endotoxin, except that they are less potent(10). Such tissue polysaccharides are said to "manifest endotoxic activities only when they are released from their native state of firm combination with other cellular components"(10). The endotoxic properties consisted of a lethal effect, a pyrogenic effect, a tumor necrotizing effect, and a preparative activity for the local Shwartzman reaction. But a generalized Shwartzman reaction has been difficult to elicit. Several facts are in conflict with the suggestion that these polysaccharides rather than bacterial endotoxins may be the source of the toxin in the shocked animal. First, the oral administration of non-absorbable antibiotics prior to the induction of shock renders an animal tolerant to hemorrhagic shock, and prevents the appearance of the toxin in the plasma(1). It is unreasonable, in our view, to postulate that non-absorbable antibiotics might have an action upon the host cells so as to prevent the release of tissue polysaccharides that Landy and Shear imply may be released in shock(10). Moreover, the lack of effect of antibiotics upon the course of hemorrhagic shock when the intestinal flora are resistant to the antibiotics points again to the bacteria, not to the host tissue, as the source of the toxin. Second, the fact that endotoxic activity is present in the plasma long before shock becomes irreversible, and can be demonstrated after as little as 5 minutes of hemorrhagic shock(11), is equally difficult to reconcile with the thesis of a toxic tissue polysaccharide. The conventional procedures for the isolation of polysaccharides, which are less drastic than that of Landy and Shear, fail to demonstrate toxic polysaccharides in normal tissue, but do demonstrate toxic polysaccharide in shock tissue. If the thesis of Landy and Shear is correct, the degree of tissue injury equivalent to homogenization, tryptic digestion, and trichloroacetic acid-phenol extraction, used by them to demonstrate toxic polysaccharide, would have to result from an exposure to as little as 5 minutes of hemorrhagic shock. This is far more severe tissue damage than other observations would indicate for such a short exposure to hemorrhagic shock. On the other hand, relatively minor differences in the methods of isolation and purification of endotoxin may alter the antigenic and/or toxic properties of polysaccharides.

Zweifach and Thomas state that the development of tolerance to shock following induced tolerance to endotoxin may represent a non-specific enhancement of R.E. function, and does not necessarily imply that the cross-tolerance to shock is specifically related to an increased capacity to destroy endotoxin *per se*

(11). It is, of course, possible that stress, in some undefined way, may induce a non-specific enhancement of R.E. function; but such a hypothesis will not explain the observation that a circulating toxin in the shocked animal, which can convert reversible to irreversible shock, and which can induce tolerance to shock, is absent in shocked animals rendered tolerant to shock by pre-treatment with antibiotics, or by repeated prior exposure to shock plasma or endotoxin. In support of their hypothesis that non-specific resistance is involved in shock they report data purporting to show that while endotoxin can induce cross tolerance to shock from drum trauma, the reverse is not the case; for they state that sublethal drum trauma fails to induce cross-tolerance to endotoxin(11). A statistical examination of their data, however, indicates that sub-lethal drum trauma *does* induce significant cross-tolerance to endotoxin. Since the cross-tolerance so demonstrated does not proceed via classic immunochemical mechanisms, one cannot regard it as constituting adequate proof of the *chemical* identity of the shock toxin and endotoxin. Nevertheless, the demonstration of cross-tolerance in both directions (*i.e.* trauma/endotoxin and endotoxin/trauma) is best explained on the theory that endotoxemia is a concomitant of shock.

Summary. It has been demonstrated that whole plasma and saline extracts of lung, liver, spleen, and kidney of the shocked animal, but not the normal animal, are capable of inducing the generalized Shwartzman reaction in endotoxin or Thorotrast-primed recipients with the same severity and frequency as purified bacterial endotoxin. The active material may be isolated by extraction with trichloroacetic acid and dialysis in the manner employed for bacterial polysaccharides. A number of similarities are noted in the biologic effects of shock toxin and bacterial endotoxin. The appearance of the toxin in the circulation within minutes of the onset of shock and the prevention of its appearance by antibiotic pretreatment are discussed with respect to the endogenous or exogenous source of the toxin.

1. Schweinburg, F. B., Shapiro, P. B., Frank, E. D., Fine, J., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 646.

2. Rutenburg, S. H., Fine, J., *ibid.*, 1956, v93, 484.

3. Schweinburg, F. B., Smiddy, F. G., Fine, J., *ibid.*, in press.

3a. Weiss, L., Bach, F., Sebestyen, K., Shapiro, P., in preparation.

4. Smiddy, F. G., Fine, J., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 558.

5. Schweinburg, F. B., Frank, H. A., Fine, J. *Am. J. Phys.*, 1954, v179, 532.

6. Thomas, L., Good, R. A., *J. Exp. Med.*, 1952, v96, 605.

7. Boivin, A., Mesrobeanu, I., Mesrobeanu, L., *Compte rend. Soc. Biol.*, 1933, v114, 307.

8. Shwartzman, G., *Phenomenon of Local Tissue Reactivity*, Paul Hoeber, N. Y., 1937.

9. Good, R. A., Thomas, L., *J. Exp. Med.*, 1952, v96, 625.

10. Landy, M., Shear, M. J., *ibid.*, 1957, v106, 77.

11. Zweifach, B. W., Thomas, L., *J. Exp. Med.*, 1957, v106, 385.

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A Vulnerable and Rate-Limiting Step in Urea Synthesis in Patients with Hyperammoniaemia.* (23768)

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The role of portal-systemic shunts, either spontaneously developed or surgically constructed, in the production of hyperammoniaemia and ammonia intoxication is well established(1,2,3) but there has been no direct evidence regarding vulnerability in man of the mechanism of synthesis of accumulated ammonia to urea. Animal experiments, how-

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