Exp. 1, 22% in Exp. 2, and by 9% in the rooster experiment.

Discussion. In theory, saponins could exert their cholesterol-complexing action either inside the intestinal lumen or in the blood after their absorption. However, absorption of significant amounts of saponin would probably cause hemolysis(2,10) which was not apparent in these experiments. Furthermore, if complexing were to take place in the blood stream, cholesterol would be released from its complex with saponin during analysis and therefore would still be measured as plasma cholesterol. Hence, the reactions by which saponins reduce plasma cholesterol must occur not in the blood, but in the intestinal lumen. Peterson(11) suggested that an insoluble sterol-saponin compound is formed in the digestive tract when cholesterol and saponins are fed simultaneously. This could explain the depressing effect of dietary saponins on blood cholesterol, when the diet contains cholesterol. In an endogenous hypercholesterolemia and with a cholesterol-free diet, the cholesterol in the intestinal tract originates from biliary and intestinal secretions. Extending Peterson's theory to endogenous cholesterol, one might explain lowering of blood cholesterol levels, as observed in lots 3 in each experiment, by failure to reabsorb part of this cholesterol due to its unavailability after complexing.

Feed consumption data suggest that age might play a role in degree of depression of feed intake with dietary saponin. Chicks put on saponin diets at the age of one week showed less depression than chicks started on diets at one day, and adult roosters seemed to be least affected.

Summary. A study with growing chicks and adult roosters indicated that dietary saponin will depress blood plasma cholesterol previously elevated by feeding low protein levels in presence and absence of dietary cholesterol. It is suggested that complexing of saponin with cholesterol secreted in the intestinal lumen makes less cholesterol available for reabsorption from the intestinal tract.

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Host Resistance in Hemorrhagic Shock XV. Isolation of Toxic Factor from Hemorrhagic Shock Plasma* (24372)

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Previous reports from this laboratory describe several properties of hemorrhagic shock plasma which distinguish it from normal plasma, *viz.*: 1. Infusion of irreversible shock plasma is lethal to a reversibly shocked animal, whereas infusion of normal plasma restores normal peripheral blood flow and leads to recovery(1). 2. Shock plasma induces the local and general Shwartzman reaction in suit-

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ably prepared animals, while normal plasma does not(2). 3. Shock plasma is pyrogenic; normal plasma is non-pyrogenic(3). 4. Repeated daily injections of small doses of shock plasma in normal animals induce tolerance to the pyrogen and resistance to hemorrhagic shock. Normal plasma is completely inert in these respects.

These biologically significant properties suggest that the toxic factor in the plasma of the shocked animal is an endotoxin. The present report describes the isolation of this toxic factor from shock plasma, demonstrates its lethal potential, and discusses the basis for its identification as a bacterial endotoxin.

Methods. Normal dogs and dogs in irreversible hemorrhagic shock were exsanguinated into heparinized flasks. The plasma was rapidly processed, as described earlier(2), by a modification of the technic of Boivin and Mesrobeanu(4) for the purification of bacterial lipopolysaccharides. The dialyzed polysaccharide fraction was concentrated to approximately 1/100th of the starting volume of plasma by evaporation at 50-55°C under reduced pressure (4-6 mm Hg). The concentrated extract prepared from normal plasma was water clear, but the same fraction from shock plasma exhibited an amber color. The visible absorption spectrum of the shock plasma extract showed only a smooth rise in absorption coefficient from 700 mμ to 410 mμ, with no identifying peaks. No precipitate formed on addition of ethanol to normal plasma extracts. Shock plasma extracts yielded a colloidal white precipitate at 65% (v/v) ethanol concentration, and precipitation was complete at 75-80% (v/v) ethanol. The precipitate dissolved readily in distilled water or saline, and was shown in pilot experiments to contain *all* the toxicity of the crude extract. The solution of ethanol-precipitated toxin was water clear and colorless, indicating that the tan color of the crude extract was due to the presence of some other, non-toxic component of shock plasma. Ethanol fractionation was omitted in preparing the extracts for the studies described below. The toxic properties† of the extracts were investigated in reversibly shocked rabbits prepared as follows: an unanesthetized normal rabbit was bled from a femoral artery, exposed under local anesthesia, into a heparinized reservoir which was elevated to maintain the blood pressure constant at 50 mm Hg. After 90 minutes a volume of extract equivalent to 100 ml of plasma from either a normal or shocked dog was injected into the femoral arterial cannula, and washed into the circulation by returning all the reservoir blood to the animal immediately thereafter. The artery was ligated and the animal observed for 48 hours. In our experience the return of all the shed blood to the animal after this degree and duration of hemorrhagic hypotension is well tolerated by rabbits, and has consistently resulted in survival of 90% or more of the animals in many series. In this study the reversibly shocked rabbits infused with the extracts of normal plasma are taken as controls for the reversibly shocked rabbits infused with extracts of hemorrhagic shock plasma.

Results. Thirteen extracts of shock dog plasma and 6 of normal dog plasma were individually tested for toxicity in reversibly shocked rabbits, each extract being transferred to a single recipient only. All such recipients of the normal plasma extract recovered in response to transfusion of the reservoir blood, and showed no gross abnormalities when killed and examined 48 hours later. All such recipients of the shock plasma extract exhibited the usual initial pressor response to transfusion of the reservoir blood, yet all died within 6 to 24 hours, and showed changes in the intestinal tract characteristic of lethal hemorrhagic shock (Table I, Series I).

A second series of 11 extracts of shock dog plasma and 6 extracts of normal dog plasma was then prepared and tested in the same way. All 6 recipients of normal plasma extract recovered, but of the 11 recipients of shock plasma extract only 7 died. None of the survivors in either group showed any gross abnormalities on post-mortem examination, but

† Normal rabbits are not visibly affected by intravenous injection of extracts of either normal or shock plasma. Closer examination, however, reveals that extracts of shock plasma induce a transient leukopenia and febrile response in normal recipients, whereas extracts of normal plasma are inert in these respects.

TABLE I. Effect of Polysaccharide Fraction of Plasma from Normal Dogs and from Dogs in Prolonged Shock on Recovery of Recipients Exposed to Reversible Shock.

Series	Donor*	Dose (ml)†	Recipient response		
			Survived	Died	% survival
I	Normal (6)	100	6	0	100
	Irreversible shock (13)	"	0	13	0
II	Normal (6)	"	6	0	100
	Irreversible shock (11)	"	4	7	36
III	Normal pool (6)	300	6	0	100
	Shock pool #1 (5)	100	1	2	33
	Shwartzman positive	50	1	2	"
		20	1	2	"
		10	1	2	"
	Shock pool #2 (5)	100	0	3	0
	Shwartzman positive	50	0	3	0
		20	0	3	0
		10	0	3	0
	Shock pool #3 (3)	100	3	0	100
	Shwartzman negative	75	2	0	"

* No. in parentheses represents No. of animals in each donor category or No. of donors to each pool (Series III).

† Dosage of extract is expressed in ml of plasma from which it was obtained. Actual volume of extract administered to the rabbits ranged from 0.30 to 1.0 ml.

all recipients of shock plasma extract which succumbed exhibited the typical pathological changes of irreversible hemorrhagic shock (Table I, Series II).

To account for the difference in response to the shock extract in the 2 series it became necessary to determine whether the 4 survivals in the second series were due to (a) absence of toxin in the donor plasma; to (b) increased resistance to toxin in the recipients; to (c) a combination of these 2 factors; or to (d) technical errors in the preparation of the extracts. For this purpose a third series of 13 extracts of shock plasma and 6 extracts of normal plasma was prepared. The individual extracts in this series were first tested for the presence of endotoxin by their ability to induce the generalized Shwartzman reaction in Thorotrast-primed rabbits, which are far more sensitive to endotoxin than are normal rabbits(2,5). All extracts of normal plasma failed to induce the reaction. Ten of the 13 extracts of shock plasma produced the reaction, but 3 did not, even when retested using larger aliquots. To correlate the response to the Shwartzman test with the response of reversibly shocked rabbits to the plasma extracts, and to test the possibility of variability in resistance of reversibly shocked rabbits to the toxin, the extracts were pooled as follows: The normal plasma extracts were combined to form one pool; the 10 Shwartzman-positive extracts of shock plasma were grouped in 2 pools of 5 each, and the 3 Shwartzman-negative extracts of shock plasma were mixed to form a fourth pool.

A dose of extract equivalent to 300 ml of normal plasma was uniformly non-toxic in each of 6 reversibly shocked rabbits. The first pool of shock plasma extract was administered in doses equivalent to 100, 50, 20 and 10 ml of shock plasma to groups of three animals at each dose level. Two of the 3 rabbits in each group died within 6 to 24 hours, with the typical pathologic changes of irreversible shock. The second pool of shock plasma extract, tested in identical manner, was lethal to *all* recipients. The pool of Shwartzman-negative shock plasma extract proved *non-toxic* to all of 5 recipients in doses equivalent to 100 ml (3 rabbits) or 75 ml (2 rabbits) of shock plasma (Table I, Series III).

It is clear from the observations on the first pool of shock plasma extract that recovery of a test animal may be due to supranormal resistance, since one-third of the recipients of a demonstrably potent extract survived. It is equally clear that resistance may vary by a dose factor of 10 or more, since one recipient survived ten times the dose of extract which

killed 2 other recipients. On the other hand, it is evident from the data on the pool of Shwartzman-negative shock plasma extract that the plasma of the shocked donor animal may, in some instances, be devoid of toxin. It is, therefore, impossible to determine whether the 4 recipients of the shock plasma extract in Series II which survived did so because they were unusually resistant to the toxin or because the extracts were impotent.

It is unlikely that technical errors in preparation account for the non-toxic extracts of shock plasma, for these were prepared in parallel with potent preparations in different runs, and because there is little opportunity for error in the simple method employed for isolation of the active material.

Discussion. There can be little doubt that the extract of shock plasma, prepared as described above, contains most, if not all, of the toxin which appears in the blood during shock. This fraction, and whole shock plasma, can induce (a) fever and leukopenia, (b) resistance to the febrile response and resistance to hemorrhagic shock after repeated daily administration, and (c) the generalized Shwartzman reaction. This is strong presumptive evidence for the very close similarity, if not identity, of the toxic material in shock plasma with bacterial endotoxin. The bacterial origin of this factor is strongly indicated by the absence of toxic activity in the plasma of endotoxin-resistant or antibiotic-pretreated animals in hemorrhagic shock(1). Isolation of the toxic activity of shock plasma in a dialyzed TCA-soluble fraction, and its further purification by precipitation with ethanol within limits of 65% to 75% (v/v) concentration, indicates that the toxin is a macromolecular polysaccharide or lipopolysaccharide. Thus there is good agreement from chemical, biological, and pharmacological observations to substantiate the thesis that the toxic factor present in the plasma of the animal in shock which converts reversible to irreversible hemorrhagic shock is an endotoxin.

Many students are agreed that, irrespective of the experimental methods employed, the same mechanisms are evoked in all forms of shock, and that if the process continues unrelieved, it becomes self-perpetuating and eventually unresponsive to all therapy. There appears to be general agreement also with the thesis that the fundamental lesion in shock is a functional deterioration, and later perhaps also a morphological alteration, of the metarterioles and venules. Finally, it is characteristic of shock that there is a delay, generally varying from a few hours up to about 18 hours, between delivery of the lethal blow and time of death. Obviously, any agent proposed as the toxic factor responsible for the irreversible state of the circulation in shock should be capable of producing these characteristic features of the disorder. Bacterial endotoxin, with which we identify the toxin in the plasma of shocked dogs, is such an agent, because it is capable of (a) inducing shock, (b) sensitizing the metarterioles and venules to adrenalin, and thereby, if one or more MLDs are given, (c) precipitating irreversible injury to the peripheral circulation and death, usually after a lag period varying from a few hours up to 18 to 24 hours.

It does not appear likely that the toxic polysaccharide in shock plasma is a tissue polysaccharide with endotoxin-like properties, a possibility suggested by Landy and Shear(6), because (a) it makes its appearance too early in shock to accord with the degree of extensive cell breakdown implied in their thesis, and because (b) it is not present in animals even after six hours of shock pretreated with non-absorbable antibiotics.

The fact that plasma extracts prepared from 7 of 37 shocked dogs in this series were free of demonstrable toxic activity at a time when the shock was judged to be irreversible may seem inconsistent with the hypothesis that endotoxemia is the cause of irreversibility. The assumption that irreversibility *was* present when the dogs were exsanguinated is based on the observation from hundreds of experiments with dogs and rabbits that 80% of animals in shock die in spite of transfusion if (a) the animal has been kept at maximal hypotension (35 mm Hg in the dog, 50 mm Hg in the rabbit) for 6 hours, or (b) has taken 40% or more of the shed blood back into its circulation from the reservoir. But the *only positive proof* of irreversibility is the death of

the animal. Since the dogs were killed by exsanguination in order to prepare the extracts it is impossible to predict which of them would have died and which would have survived. Since 10% to 20% do survive, one may conclude that the survivors are unusually resistant to shock, just as there are other animals that are unusually vulnerable. Elsewhere we discuss the evidence for the thesis that the degree of resistance to shock depends on the status of the R.E. system, which is conditioned by the prior experience of the animal(7). According to this thesis the R.E. system is progressively depleted as shock persists, so that eventually the endotoxin being continuously absorbed can no longer be inactivated, and is then free to damage or destroy the responsiveness of the peripheral vessels. Those few animals which survive an ordinarily lethal exposure to shock recover because they have "native resistance" or because they have acquired an increased resistance (to endotoxin and to shock) by prior exposure to endotoxin. They possess an R.E. System capable of inactivating far more endotoxin than the R.E. system of the average animal. Hence the absence of demonstrable endotoxemia does not necessarily indicate that the animal in shock was not exposed to endotoxin, but rather that its R.E. system remained equal to the challenge because of its greater initial detoxifying potential.

It does not detract from the significance of the role of bacterial endotoxin in the pathogenesis of irreversible shock to recognize that endotoxin is only one of many factors with which the shocked animal must contend. The fact that germ-free rats appear to respond to shock in the same way as normal animals does not make the endotoxemia of animals in a normal environment either irrelevant or of secondary importance(12,13). For commensalism with bacteria, especially in the gastrointestinal tract, is the normal state of affairs.

Nor is it safe to assume that endotoxemia can be *excluded* as a factor in germ-free rats. The first line of defense against endotoxin is the R.E. system, which removes approximately 70-85% of injected endotoxin within a few minutes(8,9,10). There is ample evidence that R.E. system functions are initially depressed by small doses of endotoxin(1). Germ-free animals of several species regularly exhibit a notable paucity of lymphoid and R.E. tissues. It is not surprising therefore that the germ-free animal is highly susceptible to bacteria, and succumbs following accidental exposure to relatively small inocula of commonly encountered non-pathogenic bacteria. Successful resistance of the germ-free animal to the bacteria (or their products) in the normal environment required a very carefully controlled period of adaptation to a diluted inoculum of a single bacterial species(11a). The germ-free animal, therefore, appears to have a R.E. potential as weak as that encountered in the normal animal subjected to reversible shock or to "blockade" of the R.E. system by Thorotrast. The latter exhibits an astounding increase in vulnerability to endotoxin, and may be killed by as little as 10^{-5} to 10^{-6} part of the normal MLD/100(1). The absence of demonstrable viable micro-organisms in the tissues or environment of the germ-free animal does not exclude the presence of endotoxin, for endotoxin is almost certainly ingested with the sterile food provided these animals. Observations on the specific antibody content of the germ-free animal support the contention that (a) bacterial endotoxin is ingested in the sterile diet, and (b) is absorbed from the gastrointestinal tract in sufficient quantity to ultimately induce a low specific antibody titer to those bacteria present in the diet prior to sterilization(11b). It seems reasonable to suppose, therefore, that the germ-free animal, like the Thorotrast-blockaded animal, becomes even more susceptible to absorbed traces of endotoxin when its already deficient R.E. system is still further burdened by the deficient blood flow of hemorrhagic shock.

Summary. 1) Previous observations on the toxicity of plasma from animals in irreversible hemorrhagic shock were confirmed and extended by the isolation of a toxic polysaccharide fraction from this plasma which cannot be isolated, under identical conditions, from normal plasma. The ability of this polysaccharide fraction to elicit responses characteristic of bacterial endotoxins, and to convert the reversible to the irreversible state of hemorrhagic shock has been demonstrated. The

ability of bacterial endotoxins, in turn, to elicit the lesions characteristic of shock, was reviewed. 2) Although the entire quantity of toxic polysaccharide circulating in the irreversibly shocked animal is innocuous to a normal animal, only a fraction of the total is sufficient to inflict lethal injury to an animal with its defenses already depleted by reversible shock. The mounting evidence for the presence of endotoxemia in shock provides a logical basis for incriminating the loss of functional integrity of the R.E. system in the development of irreversibility to transfusion in hemorrhagic shock, and for an understanding of the phenomenon of variation in resistance to shock under different conditions. The development of irreversibility to transfusion in hemorrhagic shock in the germ-free animal is discussed in the light of these considerations.

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Silicic Acid Chromatography of Lipids of Whole Human Blood.* (24373)

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We examined constituent lipids of 2 samples of human blood by chromatography on columns of silicic acid. One sample (A) was provided by a normal donor (male, age 44 years) and the other by a xanthomatous patient (male, age 11 years). The experiments on both samples were conducted simultaneously, under conditions as nearly identical as possible.

Methods. Blood was collected in evacuated bottles containing citrate anticoagulant solution (62.5 ml), and within 5 min. was poured into a shaken flask containing *n*-propanol (800 ml) at −78°. After 2 hr at −20° the resulting mixture was filtered at 0°; the residue was then extracted successively with eight 100 ml portions of propanol at room temp. The total extract was evaporated in a rotary evaporator at <55°, the residue extracted with ligroin (b.p.<60°), and the lipid weighed after evaporation of this solution. It was then chromatographed on silicic acid (50 g, column dia. 28 mm). The eluate was collected (200 hr) in fractions with a drop-counting collector (originally set to deliver 8.5 ml, but the size of fractions varied with lipid and methanol concentrations). The fractions were evaporated in a vacuum oven at <55°, the residues weighed, and the elution curves A and B (Fig. 1) then plotted. The fractions were grouped for chemical study. Seven paper chromatograms of each group were made, using as solvent propanol: 0.25 N aqueous ammonia: acetone (4:1:1, v/v), which rapidly separates lipids from more polar materials (1). The results of tests applied to these chromatograms are shown in Table I, together with findings after hydrolysis(1). Scherer (inositol) and Pettenkofer (bile acid) tests

* Phospholipids containing amino acids other than serine. Part II (Part I, ref. 1). This work was supported in part by grant from Nat. Sci. Fn.