3. Zierler, K. L., Levy, R. I., Lilienthal, J. C., Jr., *Bull. Johns Hopkins Hosp.*, 1953, v92, 41.
4. Rosenkrantz, H., *J. Biol. Chem.*, 1955, v214, 789.
5. Nelson, N., in Colowick, and Kaplan's *Methods in Enzymology*, 1957, v111, 85.
6. Somogyi, M., *J. Biol. Chem.*, 1952, v195, 19.
7. Bodansky, O., *ibid.*, 1958, v232, 859.
8. Dreyfus, J. C., Schapira, G., *Compt. rend. Soc. biol.*, 1954, v147, 1145.
9. Hazzard, W. R., Leonard, S. L., PROC. SOC. EXP. BIOL. AND MED., 1959, v102, 720.
10. Carpenter, M., McCoy, P., Caputto, R., *ibid.*, 1958, v97, 205.

Received November 17, 1960. P.S.E.B.M., 1961, v106.

Effect of Hemorrhagic Shock on Viability of Invading Bacteria.* (26349)

E. D. FRANK, J. B. MACDONALD, C. PALMERIO, F. B. SCHWEINBURG AND J. FINE
Yamins and Kirstein Laboratories for Surgical Research, Beth Israel Hospital, Dept. of Surgery, Harvard Medical School, and Forsyth Dental Infirmary, Harvard School of Dental Medicine, Boston, Mass.

There is now substantial evidence that bacterial endotoxins play a deciding role in development of irreversibility to transfusion in various types of traumatic shock(1,2). Much of this evidence incriminates the intraintestinal bacteria or their endotoxins, which are absorbed from the intestinal lumen into the circulating blood in the normal as well as the shocked animal(3). In the normal animal the endotoxin is taken up and detoxified by the R.E. system, so that there is no detectable amount in the circulation. The R. E. system of the shocked animal can take up very little, and of that it cannot detoxify more than a small fraction(4). The endotoxin is, therefore, free in the circulation to inflict damage on the peripheral vessels, and so to prevent recovery from shock.

It is of considerable importance to know to what extent the gram-negative bacteria in the tissues, whether they are invaders from the gut or contaminants of wounds, might contribute to the amount of circulating endotoxin. The data to be presented show (1), that bacteria regularly traverse the intestinal barrier; and (2) that even though they multiply when the antibacterial defenses are down, their number in the first filtering depots (liver and mesenteric lymph nodes) is too small to contribute significantly to the endotoxemia.

* Aided by a grant from Nat. Heart Inst. Bethesda, Md., and by a contract with Office of Surgeon General, U. S. Army.

Methods. With the use of aseptic technic and sterile equipment, healthy adult white rabbits, with a normal intestinal flora, were given heparin and then bled from a femoral artery, cannulated under local anesthesia, into an elevated reservoir, so as to produce a continuous arterial pressure of 50 mm Hg(5). Two groups (B & C) were maintained at this pressure for 1½ hours, and a third group (D) for 6 hours, after which all the shed blood was returned. Four or 8 hours after this replacement transfusion, a sterile laparotomy was performed under ether anesthesia. The abdomen was prepared for laparotomy by burning the alcohol-soaked skin. Separate sets of instruments were used to incise the skin, to open the peritoneum, to biopsy the ileal lymph nodes, and the liver.

The biopsies were placed in sealed vials of brain heart infusion broth for transfer to the laboratory. Before culturing the samples were removed from the containers, placed in Petri dishes, and exposed to ultraviolet light for 15 minutes to destroy any surface contaminants. They were then ground in a tissue grinder with either brain heart infusion broth or trypticase soy broth. The resultant suspension was inoculated on heart infusion blood agar plates containing 10% defibrinated horse blood, and into brain heart infusion broth and Brewer thioglycollate broth. One set of cultures was incubated aerobically and another anaerobically in Brewer jars under 95% N_2 and 5% CO_2. In most in-

TABLE I. Incidence of Positive Cultures of Mesenteric Nodes and Livers of Normal and Shocked Animals.

Group	Type of exp.	No. of animals	No. of livers with		No. of mesenteric nodes with		No. of animals with	
			Gram-neg. rods*	Gram-pos. rods and cocci	Gram-neg. rods*	Gram-pos. rods and cocci	Gram-neg. rods*	Gram-pos. rods and cocci
Rabbits								
A.	Control	19	0	2	0	7	0	9
B.	1½ hr shock 4 hr after transfusion	7	1	4	3	7	3	7
C.	1½ hr shock 8 hr after transfusion	5	5	3	3	3	5	5
D.	6 hr shock 8 hr after transfusion	3	3	1	2	2	3	2
Dogs								
	Control	10	4	5	7	8	8	8
	2 hr shock 8 hr after transfusion	5	2	4	4	3	4	4

* Gram-negative cocci were found only once in the entire study and so were omitted from the table.

stances, the broth from the transporting container was incubated aerobically. All cultures were incubated at 37°C for 7 days. All tubes and plates showing growth were studied further. Bacteria were examined for morphology and gram reaction. Broth cultures and representative colonies from plates usually were subcultured aerobically and anaerobically on blood agar.

Cultures were considered positive only when organisms were isolated from nodes or liver which differed from any bacteria grown in the broth of the transporting container. Growth in the container was considered to be attributable to surface contamination of the biopsy.

The mesenteric nodes and livers of a control group (A) of rabbits were studied by the same methods as those of the rabbits in Groups B, C and D. Several of the rabbits were subjected beforehand to arterial cannulation, and to 6 hours of restraint in the supine position, but without producing shock, to determine to what extent the manipulation attending induction and maintenance of the shock, rather than the shock itself, may have contributed to bacterial invasion of the nodes and liver.

Similar studies were made on dogs, as indicated in Table I.

Results. (Table I). *A. Rabbits.* Control Group A—The mesenteric lymph nodes and livers of 19 normal rabbits yielded not a single positive culture for gram-negative organisms. Clostridia and gram-positive enterococci were present occasionally. Of the 12 rabbits transfused after 1½ hours of hemorrhagic shock, 7 (Group B) were studied 4 hours afterwards. Three showed gram-negative rods. There was also an increase in incidence of gram-positive organisms. The remaining 5 (Group C) were studied 8 hours afterwards. All 5 showed gram-negative rods, as well as a moderate increase in incidence of gram-positive bacteria. Group D consisted of 3 rabbits in hemorrhagic shock for 6 hours. All 3 showed gram-negative rods in the liver 8 hours after transfusion. Thus, whereas gram-negative bacteria were not detected in any normal rabbit's tissues, 11 of 15 (73%) of shocked rabbit's tissues were positive for these bacteria. Their incidence was higher the longer the interval between transfusion and cultures. The percent of shocked animals with gram-positive organisms was considerably greater than that of normal rabbits with these organisms.

B. Dogs. Contrary to the findings in

rabbits, the incidence of gram-negative bacteria in 10 normal dogs was very high, as was also the case for gram-positive bacteria (80%). The same incidence was found in 2 hour shocked dogs 8 hours after transfusion. Organisms did not grow on the primary plates in the cultures from control dogs but did grow on subculture from broth. Bacteria grew on the primary plates from the shocked dogs, suggesting the possibility that the numbers of organisms in the tissues of these animals was greater than in normal animals.

Discussion. The presence of gram-negative bacteria in the liver and other tissues of the dog was investigated by Jacob *et al.* (6), who reported that these bacteria were rarely found in normal dogs examined immediately after death, whereas gram-positive rods and cocci were found frequently. But when the tissues of dogs subjected to 6 hours of shock were examined immediately after death, there was a sharp increase in incidence of gram-negative as well as gram-positive bacteria. Jacob *et al.* suggested that the bacteria found immediately after death in the tissues were not simply "agonal invaders," but were bacteria that normally and regularly invade from the gut, and that if they could be found only immediatley post-mortem it was because the anti-bacterial potential was adequate until death. The higher incidence of such bacteria in the shocked as compared to the normal dog was taken to signify *in vivo* decline in defense potential. This decline had been demonstrated by showing that the shocked animal cannot destroy a number of intravenously injected gram-negative or gram-positive bacteria, which the normal animal can kill with ease, and that this disability persists beyond 24 hours in the animal recovering from shock(7).

When endotoxemia was shown to be a primary factor in development of irreversibility to transfusion, attention was again focussed on the gram-negative bacteria from which the endotoxin is derived. Even though the evidence pointed to those in the intestine as the major, if not the sole, source of the circulating endotoxin(3), the possibility that those which had invaded and were alive in the tissues might be contributing to the endotoxemia needed reinvestigation. From the results of the present study with the use of more meticulous technics than were employed in the earlier study, the previous report(8) that the gram-negative bacteria cannot be cultured from the living tissues of the dog must be revised.

Their presence in most normal and shocked dogs signifies that they, like the gram-positive bacteria, are continuous invaders from the gut. The fact that they were recovered from the tissues of the normal dog only on subculture, but were recovered from the tissues of the shocked dog without subculturing, suggests the possibility of increased numbers of bacteria in shocked dogs attributable to the decrease in defense potential, which has been repeatedly demonstrated in other ways.

The same general principle applies in the rabbit: Bacteria which cannot be grown out of the normal rabbit's tissues can be found in shocked rabbits with increasing incidence the longer the interval after shock during which they are allowed to multiply. This increasing incidence cannot be attributed to greater bacterial invasiveness during shock, for this would require the finding of a peak incidence during shock, rather than during the recovery period.

In spite of the evidence that gram-negative as well as gram-positive bacteria continuously invade from the gut *via* the lymphatics and portal vein, and that these bacteria persist or multiply during and for some time after shock because of the decline in anti-bacterial potential, the number of colonies growing are so few that it is not possible to account for any significant fraction of the endotoxin in the circulating blood of shocked animals from this source. This conclusion is based on the following data: One gram (wet wt.) of *E. coli,* containing approximately 2×10^{10} to 1×10^{11} bacteria, grown under near optimal cultural conditions, yields 10-20 mg of partially purified endotoxin by the TCA extraction technic of Boivin and Mesrobeanu. Thus a single bacterium can make up to 1 to

2×10^{-7} μg of endotoxin. A rabbit weighing 2 kg and fed 2-3 g (wet wt.) of an identifiable (tagged) strain of *E. coli* will yield some 200 μg of labelled endotoxin from its liver and blood alone(3). If all this were derived from bacteria in the tissues there would have to be approximately 200,000 gram-negative bacteria per gram of tissue in all organs. These bacteria have been found only in the mesenteric nodes and liver, and in only an insignificant fraction of this number.

Studies of the bacteria which may contaminate the groin wound made for cannulating the femoral vessels to induce and maintain shock by the reservoir technic have been made repeatedly. When the experiment is performed with clean though not sterile technic (*i.e.* with use of autoclaved instruments, shaved skin, thoroughly clean glassware, tubing, etc.) bacteria in the wound at the end of experiment are the usual skin contaminants, *i.e.*, staphylococci, *B. subtilis*, diphtheroids and an occasional Clostridium. Gram-negative bacteria are not found. The lack of relevance of these bacteria to the course of events in the rabbit exposed to severe and prolonged hemorrhagic shock is shown by the fact that endotoxemia, irreversible shock and death occur in spite of the use of aseptic technic, or of locally applied antibiotics (Polymyxin and Bacitracin), which prevent bacterial proliferation, as demonstrated by wound cultures at the time of death(9).†

Summary and conclusions. Gram-positive bacteria can be grown from the mesenteric nodes and liver of the normal rabbit and dog. Gram-negative bacteria can be grown from these tissues of the normal dog, but not of the normal rabbit. Gram-negative bacteria are found in the nodes and liver of the rabbit some hours after exposure of the rabbit to 2 hours of hemorrhagic shock. The incidence is higher 8 hours afterward than it is 4 hours afterward. These data confirm the view that bacteria are continuously invading from the intestine. Exposure for 2 hours to hemorrhagic shock so weakens the antibacterial defense that gram-negative as well as gram-positive bacteria can be grown out more readily than in the normal animal. However, the number of bacteria that can be recovered is judged too small to be a significant contribution to the endotoxemia of advanced hemorrhagic shock. Reasons are given for also excluding the bacteria normally found in the groin wound, made for cannulating the femoral vessels, from any significant role in the endotoxemia.

† Schweinburg and Fine(10) state that when such animals are transfused after 2 hours in shock they are hypersensitive to endotoxin because of damage to the R. E. system by the shock. Greisman confirms the occurrence of hypersensitivity to endotoxin, but he attributes it to the combined effects of the hemorrhagic shock and infection in the groin wound (11). This conclusion is based on his view that the groin wound is unavoidably infected with Clostridia, even when aseptic technic is used, because the dissection done to cannulate the vessels activates the Clostridia in the adjacent tissues. Since the Clostridia he finds in these wounds are Perfringens, a strain not found in rabbit tissues, it is clear that his experimental model consists of overt induced sepsis as well as hemorrhagic shock. The mortality in his experiments may be due to the complicating infection rather than the endotoxin. Greisman's experimental model is not comparable to ours, which, as stated, does not result in overt infection. His failure to find hypersensitivity in the rabbits put into hemorrhagic shock by cardiac puncture may be accounted for by the considerably lesser degree of shock induced by this method than by the reservoir technic, for the hypotension part of the time is as high as 60 mmHg, and the intervals allowed between successive bleedings to produce the shock allows a considerably greater mobilization of fluid than is likely with the reservoir technic.

1. Fine, J., Rutenburg, S., Schweinburg, F. B., *J. Exp. Med.*, 1959, v110, 547.

2. Schweinburg, F. B., Fine, J., in press.

3. Ravin, H. A., Rowley, D., Jenkins, C., Fine, J., in press.

4. Wiznitzer, T., Better, N., Rachlin, W., Atkin, N., Frank, E. D., Fine, J., in press.

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

6. Jacob, S., Weizel, H., Gordon, E., Korman, H., Schweinburg, F. B., Frank, H., Fine, J., *ibid.*, 1954, v179, 523.

7. Schweinburg, F. B., Frank, H., Fine, J., *ibid.*, 1954, v170, 532.

8. Schweinburg, F. B., Sylvester, E. M., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 527.

9. Schweinburg, F. B., unpublished observations.
10. Schweinburg, F. B., Fine, J., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 589.
11. Greisman, S. E., *J. Exp. Med.*, 1960, v112, 257.

Received November 18, 1960. P.S.E.B.M., 1961, v106.

Utilization of Serum a_1-Globulin and Glycoprotein by *Diplococcus pneumoniae*.* (26350)

YU-TEH LI AND M. R. SHETLAR
Department of Biochemistry, University of Oklahoma School of Medicine, Oklahoma City

Changes of the serum protein fractions and of the bound carbohydrate (glycoproteins) of these fractions are known to occur in many pathological conditions. However, little is known about the metabolic alterations which lead to these changes, about the normal synthesis and destruction of glycoproteins in the animal body. Information is also lacking concerning destruction of the blood glycoproteins by micro organisms. A preliminary survey in this laboratory involving a number of micro organisms revealed a surprising resistance of serum glycoproteins to bacterial destruction. Data reported by Friedemann and Sutliff(1), however, indicate that the pneumococcus may utilize the protein bound carbohydrate of sera from individuals with certain pathological conditions.

In recent experiments, a strain of *Diplococcus pneumoniae* was utilized which had been isolated from a patient with pulmonary pneumonia at University Hospital, Oklahoma City, and maintained in frozen stock.† Five ml of sterile serum was transferred to a 50 ml sterile Erlenmeyer flask and inoculated with one loopful of 6-hour, rapidly growing culture of the organism. The inoculated serum sample was incubated at 37°C for 20 hours. A control serum sample, similar in all respects except uninoculated, was incubated for the same time in the same way. After incubation, the bacterial cells were removed by centrifugation. Determinations of protein were made on the supernatant by the biuret method; glycoprotein hexose was determined by the method of Shetlar *et al.*(2). Two samples of bovine sera, 2 rat samples, and 8 human samples (4 normal, 2 cancer, 2 rheumatoid arthritis) were investigated. The inoculated bovine samples decreased from 151 to 130 mg per 100 ml in glycoprotein content, and from 7.13% to 6.46% in protein content. Similar changes were noted in rat sera. Results of the human studies are summarized in Table I. In every instance, samples incubated with the organism decreased in both protein and glycoprotein content. When the normal human samples were considered, the difference between serum glycoprotein contents of inoculated and control groups was statistically significant at the 1% level; the decrease of the protein content was also significant at the 1% level.

These data indicate the preferential destruction of carbohydrate-rich protein by the micro organism. This is confirmed by paper electrophoretic studies of protein and glycoprotein of the sera by methods previously described(3). Results of a typical study of serum from a cancer patient are shown in Fig. 1. Results of a_1-glycoprotein determined by paper electrophoresis are given in Table I. It appears from the electrophoretic pattern and from the results in Table I, that the micro organism preferentially utilizes a_1-glycoprotein. Similar results were found in paper electrophoretic investigations of all human samples and of rat and bovine sera. The decrease of a_1-globulin averaged 85% (ranging from 50-100%);

* This study supported by a grant from U.S.P.H.S. (Nat. Cancer Inst.).

† The authors are greatly indebted to Dr. Florene Kelly and Mr. Billy West, Dept of Microbiology, for the culture and for advice on bacteriological technics.