Effect of Reticuloendothelial Stimulation on Plasma Acid Hydrolase Activity in Hemorrhagic Shock.* (31977)

PATRICK E. LENTZ† AND JAMES J. SMITH
Department of Physiology, Marquette University School of Medicine, Milwaukee, Wisc.

Cellular adaptation(1) and the state of the reticuloendothelial system (RES)(2) have been proposed as possible factors involved in shock protection. Resistance to tumbling(3,4), hemorrhagic(5) or endotoxin shock can be increased by stimulation of the RES. Extracts of the yeast *Saccharomyces cerevesiae* cause hyperplasia of RE cellular elements(6,7,8), enhance phagocytosis(7), and increase acid phosphatase activity in the Kupffer cells of liver(9). Zymosan, the complete cell wall extract, induces a hyperfunctional RES and affords protection against tumbling trauma, although the mechanism of this protective action is unknown(3,4). Glucan, which also stimulates the RES, apparently does not induce the shock-protective effect(4,10).

Hydrolytic enzymes associated with lysosomes, *e.g.*, acid phosphatase, beta glucuronidase and cathepsins are found in high concentration in the RES(9,11) and are increased in RE hyperfunctional states(9,12). Elevation of circulating acid phosphatase and beta glucuronidase activity in shock is apparently associated with acid hydrolase release from injured tissues(11,12,13,14). Shock tolerance (induced by drum conditioning) and cortisone treatment minimize the release of acid hydrolases during subsequent stress, perhaps as a result of an increased lysosomal stability(13).

The purpose of this investigation was to compare the effects of zymosan and glucan on 1) plasma activity of acid phosphatase and beta glucuronidase during hemorrhagic shock, and 2) free and total activity of lysosomal enzymes in large-granule fractions of liver homogenates after 3 hours of hypotension. A further objective was to attempt correlation of these findings with shock resistance.

* This investigation was supported by Research Grant HE 06588 from Nat. Heart Inst.

† Present address: Division of Med. Sci., Brown University, Providence, R. I.

Methods and procedures. Male Sprague-Dawley rats (250 ± 25 g) were used in these experiments.

Experimental groups. A. Uninjected, unshocked controls had no previous injections but the animals were cannulated and arterial pressure monitored for 3 hours on day of experiment.

B. Uninjected, shocked controls had no previous injections before hemorrhage.

C. Zymosan and *D. Glucan* animals received three 10 mg/kg intravenous injections of zymosan or glucan suspensions respectively on days 5, 3 and 1 before hemorrhage.

E. Saline-injected controls had equivalent volume injections of pyrogen-free saline on days 5, 3 and 1 before hemorrhage.

All groups except Group A were subjected to 3 hours of hypotension at 40 mm Hg.

The zymosan and glucan (Standard Brands, Inc., Stamford, Conn.) were injected via the dorsal vein of the penis at a dose of 10 mg/kg which was previously shown to stimulate the reticuloendothelial system(4). The extracts were suspended in sterile 0.9% sodium chloride (Abbott Laboratories, North Chicago, Ill.) and boiled for 10 minutes in a water bath to achieve uniform particle dispersion.

Hemorrhage and sampling procedure. Under pentobarbital anesthesia, the right carotid artery was cannulated and connected to a mercury manometer and blood reservoir. An anticoagulant, mepesulfate, 2.4 mg/100 g body weight (Hoffmann-La Roche, Nutley, N.J.), was injected intravenously. After a 15-minute control period the animals were bled to a mean arterial pressure of 40 mm Hg and maintained at this level for 3 hours. The blood remaining in the reservoir after 3 hours was reinfused and the animals observed until death or until sacrificed for biochemical analysis. Blood samples (1.0 ml) were taken from the right carotid cannula immediately before and at the first, second and third hour of the hypotensive period.

Preparation of large-granule fractions of rat liver homogenates. Livers were excised, washed in ice cold 0.25 M sucrose and 10% homogenates in 0.25 M sucrose prepared with a Potter-Elvehjem homogenizer equipped with a teflon pestle. The homogenate was separated into fractions according to the method of Weissmann(15). Nuclei and unbroken cells were sedimented at 800 × *g* for 10 minutes in a Sorvall refrigerated automatic centrifuge. The pellet was washed once and the wash combined with the original cytoplasmic extract. Large-granule fractions which contain mitochondria and lysosomes were isolated between 800 × *g* and 15,000 × *g* for 20 minutes. The supernatant from this centrifugation contained enzyme from the original homogenate which was unsedimentable after 20 minutes at 15,000 × *g;* this activity is referred to in the text as unsedimentable activity. After washing once, the pellet from the 15,000 × *g* centrifugation was resuspended in 0.25 M sucrose and homogenized in an ice cooled micro-attachment for the Waring blendor for 5 minutes at full speed. Activity released into the supernatant after mechanical disruption in the blendor represents sedimentable activity. Total activity of large-granule fractions is calculated as the sum of the unsedimentable and sedimentable activity.

Enzyme and protein assay. Beta glucuronidase was assayed by the method of Fishman(16) using phenolphthalein glucuronide (Sigma Chemical Co., St. Louis, Mo.) as substrate. Acid phosphatase was assayed by the Baginski-Zak method(17) using beta glycerophosphate (Sigma Chemical Co., St. Louis, Mo.) as substrate. Protein was determined by the Lowry method(18) with crystalline egg white lysozyme as standard.

Data are expressed as group means ± standard error of the mean (S.E.M.); statistical significance was determined by use of Students' "t" test.

Results. A. Effect of zymosan and glucan on RES organ weights. The organ weight changes (Table I) induced by zymosan and glucan are similar to those previously reported by Filkins *et al*(4). In this study 89% of the saline-injected animals and 27% of the zymosan animals died after 3 hours of hemorrhagic hypotension. Some of the glucan-injected animals died during the first hour of hypotension—primarily due to respiratory arrest—and were excluded from these data.

TABLE I. Effect of Zymosan and Glucan Injection on RES Organ Weights.

Exp groups	No. of rats	Organ wt as % body wt (mean ± S.E.) Liver	Spleen	Lung
Saline	19	3.67 ±.13	.28 ±.02	.63 ±.04
Zymosan	22	4.33 ±.15*	.52 ±.03*	.86 ±.12*
Glucan	15	4.52 ±.33*	.65 ±.03*	2.34 ±.12*

* Probability ≤0.05 as compared to saline controls.

B. Plasma acid phosphatase and beta glucuronidase activity during hypotension. Plasma acid phosphatase activity before and at the first, second and third hour of hypotension is shown in Table II. The increase in activity in the unshocked controls was rather surprising; it may have been due to a nonspecific stress response to the anesthetic, to the cannulation procedures or to the anticoagulant. Before hemorrhage and as a result of previous injections, plasma acid phosphatase activity was significantly elevated in the saline and glucan animals, particularly in the latter. Zymosan-injected animals however did not have an elevated prehemorrhage acid phosphatase activity compared to the shocked controls. After hypotension, the plasma acid phosphatase activity was considerably elevated from prehemorrhage levels in all the experimental groups but the final values in the zymosan group were less than in any of the other groups subjected to shock.

Prehemorrhage plasma beta glucuronidase activity was not much affected by prior injections of saline or zymosan but glucan injections did elevate the levels (Table III). Sizable increases in plasma beta glucuronidase activity also occurred in all the groups during the experimental period. After 3 hours of hypotension, plasma beta glucuronidase had increased about 700% in glucan animals, 400% in saline animals, 350% in uninjected controls and 250% in zymosan rats. At the end of shock, the plasma beta glucuronidase activity in zymosan animals was significantly less than that of the shocked control, saline- or glucan-injected rats.

TABLE II. Plasma Acid Phosphatase Activity During Hemorrhagic Shock in the Rat (Mean ± S.E.).

Hour of hypotension	Unshocked controls	Shocked controls	Zymosan	Glucan	Saline
0	.52 ±.41 (11)*	.87 ±.12 (7)	.79 ±.14 (7)	3.14 ±.15†(5)	1.85 ±.26†(10)
1	.81 ±.52 (4)	1.31 ±.26 (6)	1.01 ±.20 (5)	3.59 ±.69‡(5)	3.57 ±.63‡ (9)
2	1.12 ±.53 (4)	1.94 ±.53‡(5)	1.27 ±.38‡(4)	5.38 ±.27‡(3)	4.25 ±.88‡ (8)
3	1.14 ±.52 (4)	2.65 ±.55‡(3)	1.94 ±.33‡(4)	6.82 ±.67‡(2)	4.51 ±.67‡ (3)

* No. of animals.
† Probability of significant difference from uninjected control prehemorrhage activity ≤.05.
‡ Probability of significant difference from respective prehemorrhage activity ≤.05.
One unit of activity equals 1 mg of inorganic phosphorus per 100 ml plasma per hr at 37°C. Data are expressed as units in the table.

TABLE III. Plasma Beta Glucuronidase Activity During Hemorrhagic Shock in the Rat (Mean ± S.E.).

Hour of hypotension	Unshocked controls	Shocked controls	Zymosan	Glucan	Saline
0	.35 ±.05 (6)*	.47 ±.11 (5)	.42 ±.09 (8)	.82 ±.11 (6)	.46 ±.11 (10)
1	.85 ±.05†(6)	.82 ±.12 (6)	.84 ±.11 (6)	2.02 ±.13†(6)	1.35 ±.29†(10)
2	1.13 ±.16†(4)	1.29 ±.09†(6)	1.31 ±.20†(6)	3.24 ±.85†(4)	2.30 ±.19† (8)
3	1.29 ±.14†(3)	2.13 ±.28†(5)	1.50 ±.20†(3)	6.63 ±.43†(3)	2.27 ±.21† (3)

* No. of animals.
† Probability of significant difference from respective prehemorrhage activity ≤.05.
One unit of activity equals 1 mg of phenolphthalein per 100 ml plasma per hr at 37°C. Data are expressed as units in the table.

Plasma acid phosphatase and beta glucuronidase activity were increased after 3 hours of hypotension compared to the unshocked animals at the same periods; however it is noteworthy that incident to shock the greatest absolute increase in plasma hydrolase activity occurred in the glucan animals and the smallest increase in zymosan-treated animals.

C. Alteration in hepatic lysosomes during shock. Attempts were made to correlate the increase in plasma hydrolase activity during shock with hydrolase release from hepatic lysosomes.

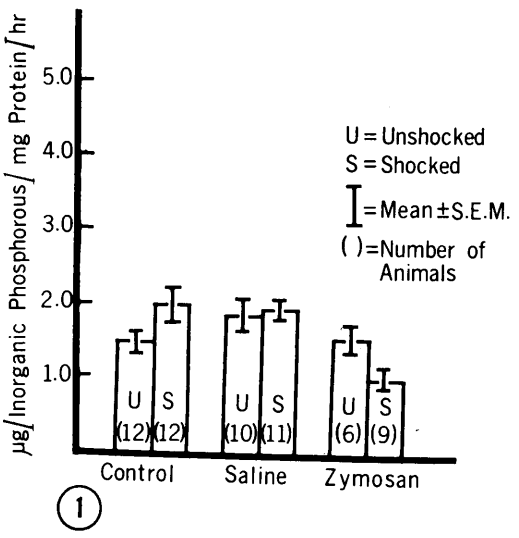
Slight increases in total acid phosphatase and beta glucuronidase were detected in large-granule fractions of liver homogenates prepared from uninjected shocked control and saline-shocked rats (Fig. 1 and 2). However, decreased total activities of acid phosphatase and beta glucuronidase were found in zymosan animals after 3 hours of hypotension.

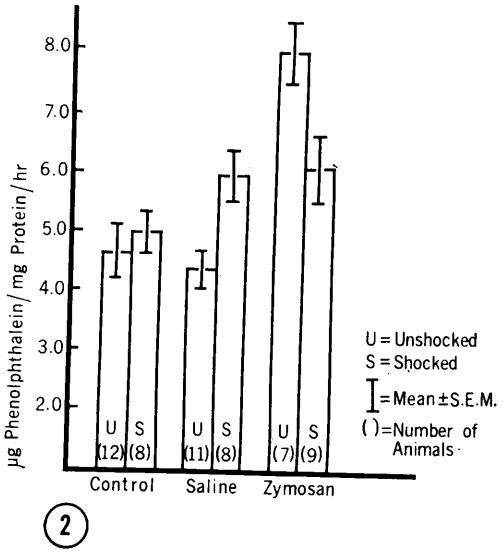
After 3 hours of shock, unsedimentable acid phosphatase (in terms of the proportion of total activity) increased 12% in uninjected shocked controls, 28% in saline animals and 24% in zymosan animals (Fig. 3). Similarly, the unsedimentable beta glucuronidase portion of the total activity increased 19% in control, 16% in saline and 21% in zymosan-shocked animals (Fig. 4). An increase in unsedimentable activity may be comparable to enzyme release *in vivo* although this is only a presumption and must be viewed with reservation.

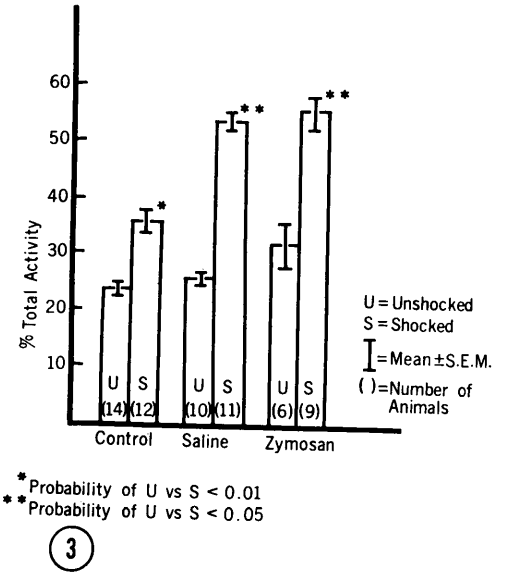
Discussion. The present experiments confirm the protective effect of the complete cell wall extract, zymosan, in hemorrhagic shock (3). It is, furthermore, evident that treatment with RE stimulating agents markedly altered the plasma acid phosphatase and plasma beta glucuronidase activity during hypotension. The results indicate that zymosan-treated rats responded to hemorrhage with a smaller release of acid phosphatase and beta glucuronidase than did glucan-treated rats. It is not clear whether this lesser release of acid hydrolases is the mechanism underlying the protective action of zymosan or is merely a result of it.

The increased plasma hydrolase activity

during shock may be ascribed to one or more of several factors, *i.e.*, 1) possible changes in binding characteristics of enzymes to cellular structures, 2) altered sensitivity of lysosomal membrane to shock with resultant release of its enzymes, 3) altered permeability of the cell membranes or 4) impaired elimination of hydrolases from the blood. The marked differences in hydrolase release observed in glucan and zymosan animals suggest that either the binding of acid phosphatase and beta glucuronidase to cellular supporting structures







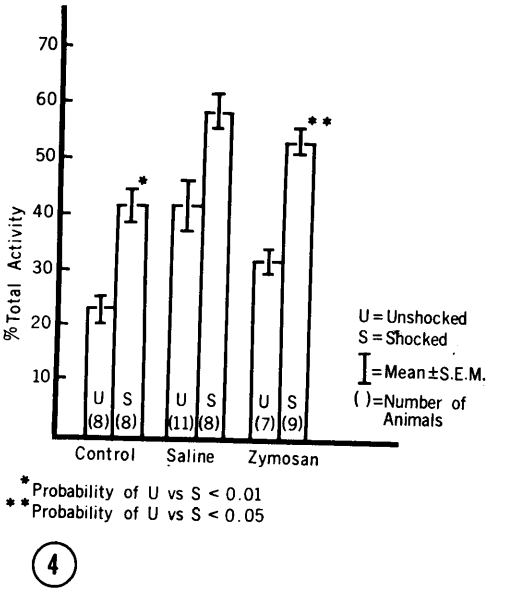


FIG. 1. Total acid phosphatase activity in large-granule fractions of art liver homogenates after hemorrhagic shock.

FIG. 2. Total beta glucuronidase activity in large-granule fractions of rat liver homogenates after hemorrhagic shock.

FIG. 3. Unsedimentable acid phosphatase activity (expressed as % total activity) in large-granule fractions of rat liver homogenates after hemorrhagic shock.

FIG. 4. Unsedimentable beta glucuronidase activity (expressed as % total activity) in large-granule fractions of rat liver homogenates after hemorrhagic shock.

may be different in these two situations or that lysosomes of zymosan rats are somehow less susceptible to the anoxic, toxic and acidotic environment developed during hypotension. Measurement of unsedimentable activity, considered to be an index of lysosomal fragility(13), did not support the concept that lysosomes of zymosan-treated rats are more stable in shock. The variations in total hydrolase content of the large-granule fractions of liver were not large nor statistically significant and it is not possible from the present data to derive any meaningful association between the hydrolase content of the liver fractions and the altered plasma activity in shock.

Previously reported elevations of cytoplasmic enzymes such as transaminases(19, 20) or dehydrogenases(20,21) as well as plasminogen activators(22) suggest that cell membrane permeability to these enzymes may alter in traumatic, endotoxin or hemorrhagic shock, and it is possible that membrane permeability rather than lysosomal stability may be important in the release of the hydrolases. Little information is available on the elimination of enzymes from the circulation although Wakim(23) has shown that zymosan may interfere with the destruction of enzymes by the RES.

It should be emphasized that these data do not provide direct evidence regarding either the nature of the primary injury in shock or to origin of the hydrolases in the blood of shocked animals. Although acid phosphatase and beta glucuronidase are found in high concentration in lysosomal fractions, other cell fractions also contain significant quantities of these enzymes(24). Whether these enzymes are released from lysosomes *per se* or from other cell organelles such as the endoplasmic reticulum requires further investigation.

Although resistance to stress has been ascribed to an altered release(13,25) or production(22) of hydrolytic enzymes, there is little evidence that acid hydrolases play the critical role in the development or progression of cellular injury(26). An alternative to this pathological function of lysosomes in shock is the suggestion of Bitensky *et al*(25) who maintained that intact lysosomes are required for the destruction of toxins liberated from the intestine of shocked animals. The latter hypothesis seems more consistent with our data and it is possible that the number of intact, rather than disrupted, lysosomes may be the important factor in the animals' response to shock.

Summary. Prior stimulation of the reticuloendothelial system (RES) with zymosan and glucan markedly altered plasma acid phosphatase and plasma beta glucuronidase activity during subsequent hemorrhagic shock in the rat. There was an increase in the plasma activity of both hydrolases during the hypotensive period in glucan-injected and saline-injected animals compared to uninjected shocked controls; the increase in zymosan-treated animals was substantially less than that after glucan or saline. Hemorrhagic shock caused small increases in total acid phosphatase and beta glucuronidase activities of large-granule fractions of liver homogenates in control and saline-injected animals but a decrease in zymosan-treated animals. Unsedimentable activity of acid phosphatase and beta glucuronidase in the large-granule fractions of liver was increased in all groups after 3 hours of hypotension. It is suggested that the marked differences in hydrolase activation in zymosan and glucan rats may be due to enzyme binding or membrane permeability properties and that the lesser release of hydrolytic enzymes may be a characteristic feature of the shocked-protective action of zymosan.

The authors are pleased to acknowledge the helpful comments of Dr. Julian Van Lancker of Brown University in preparation of this manuscript.

1. Rosenthal, S. M., Tabor, H., Lillie, R. D., Am. J. Physiol., 1945, v143, 402.

2. Zweifach, B. W., Ann. N. Y. Acad. Sci., 1960, v88, 203.

3. Zweifach, B. W., Benacerraf, B., Thomas, L., J. Exp. Med., 1957, v106, 403.

4. Filkins, J. P., Lubitz, J. M., Smith, J. J., Angiology, 1964, v15, 465.

5. Fine, J., Rutenberg, S., Schweinburg, F. B., J. Exp. Med., 1959, v110, 547.

6. Stiehm, E. R., J. Appl. Physiol., 1962, v17, 293.

7. Benacerraf, B., The Liver, v2, C. Rouiller, ed., 1964, 37.

8. Kampine, J. P., Banaszak, E. F., Smith, J. J., J. RE Soc., 1965, v2, 172.
9. Thorbecke, G. J., Old, L. J., Benacerraf, B., Clarke, D. A., J. Histochem. Cytochem., 1961, v9, 392.
10. Filkins, J. P., Stekiel, W. J., Smith, J. J., Fed. Proc., 1962, v21, 118.
11. Saito K., Suter, E., J. Exp. Med., 1965, v121, 727.
12. ———, ibid., 1965, v121, 739.
13. Janoff, A., Weissmann, G., Zweifach, B. W., Thomas, L., ibid., 1962 v116, 451.
14. Dummont, A. E., Weissmann, G., Nature, 1964, v201, 1231.
15. Weissmann, G., Thomas, L., J. Clin. Invest., 1963, v42, 661.
16. Fishman, W. H., Springer, B., Brunetti, R., J. Biol. Chem., 1948, v173, 449.
17. Baginski, E., Zak, B., Clin. Chim. Acta, 1960, v5, 834.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., J. Biol. Chem., 1951, v193, 265.
19. Sievert, H. W., Finley, R. A., Proc. Soc. Exp. Biol. & Med., 1964, v116, 754.
20. Konttinen, A., Rajasalmi, M., Paloheimo, J., Am. J. Physiol., 1964, v207, 385.
21. Vesell, E. S., Feldman, M. P., Frank, E. D., Proc. Soc. Exp. Biol. & Med., 1959, v101, 644.
22. Beard, E. L., Hampton, J. K., Jr., Am. J. Physiol., 1963, v204, 405.
23. Wakim, K. G., Fleisher, G. A., J. Lab. Clin. Med., 1963, v61, 107.
24. Walkinshaw, C. H., Van Lancker, J. L., Lab. Invest., 1964, v13, 513.
25. Bitensky, L., Chayen, J., Cunningham, G. J., Nature, 1963, v199, 493.
26. Trump B. F., Ericsson, J. L. E., The Inflammatory Process, B. W. Zweifach, L. Grant, R. T. McCluskey, ed., 1965, 35.

Received January 3, 1967. P.S.E.B.M., 1967, v124.

Enhanced Efficiency of Echovirus 6 by a Transferable Factor in Cultured Human Cells.* (31978)

FAY RIGHTHAND AND DAVID T. KARZON†
Departments of Pediatrics and Bacteriology and Immunology, School of Medicine, State University of New York at Buffalo

In previous studies, stable mutant pairs of echovirus 6 strains were isolated in monkey kidney cell cultures(1). The plaque mutants, *m*+ and *m*, could be distinguished by several biological properties. In addition to differences in plaque size, cytopathogenicity, sensitivity to polyanions and neutralizability by antisera, the mutants differed in their host susceptibility(2). Most human cells were shown to have 100- to 1,000-fold greater sensitivity than monkey cells to infection by the large plaque mutant, *m*+. In contrast, human and monkey cells were approximately equally susceptible to the minute plaque mutant, *m*.

The greater sensitivity of human cells for *m*+ mutant was investigated further. The present paper describes a transferable factor in human cells which enhances specifically the efficiency of *m*+ virus for monkey cells. The intracellular location and some of the properties of the enhancing factor are reported.

Materials and methods. Cell cultures. Primary rhesus monkey kidney cells were grown and maintained in Eagle's basal medium (Hanks' salts) containing 2% calf serum and 0.2% SV_5 antiserum. Green monkey kidney cells (GM) were grown serially in Eagle's minimal essential medium (MEM) with 10% calf serum, and maintained in MEM containing 5% calf serum. BSC-1 cells were kindly provided by Dr. H. E. Hopps, Division of Biologics Standards, National Institutes of Health(3), and by Dr. F. Rapp, Baylor University, Houston, Texas. The latter lines were maintained in medium 199 with 2% fetal calf serum, and in MEM with 5% calf serum, respectively. Serially progagated human amnion cells, AV3(4), were grown in 0.45% lactalbumin hydrolyzate supplemented with Hanks' salts and 15% calf serum, and

* Supported in part by research grant AI-02396 and training grant AI-98, from Nat. Inst. of Allergy & Infect. Dis.

† Recipient of Research Career Award AI-1136 from Nat. Inst. of Allergy & Infect. Dis.