8. Gillespie, J. M., and Lennox, F. G., *Biochim. Biophys. Acta,* 1953, v12, 481.
9. Einsele, W., *J. Gent.,* 1937, v34, 1.
10. Heidenthal, G., *Genetics,* 1940, v25, 197.
11. Russell, E. S., *ibid.,* 1939, v24, 332.
12. Hanna, B. L., *Am. J. Human Genetics,* 1953, v5, 293.

Received May 20, 1954. P.S.E.B.M., 1954, v86.

Anticoagulant and Other Pharmacologic Effects of Sulfated Chitin in Animals. (21084)

L. W. ROTH, I. M. SHEPPERD, AND R. K. RICHARDS.
From the Department of Pharmacology, Abbott Laboratories, North Chicago, Illinois.

The sulfation of various polysaccharides to produce compounds having anticoagulation properties was first described by Bergstrom (1). He reported on the characteristics of the sulfated products of cellulose, starch, glycogen, pectin, chitin and chondroitin sulfuric acid. Most of his investigations were conducted *in vitro,* but later workers, including Piper(2) and others, extended much of the study to *in vivo* testing. A side reaction frequently observed was the agglutination of the thrombocytes. This, coupled with other toxic symptoms, led to the conclusion that the compounds were not suitable for clinical application. Sorenson and Wright(3) described a polysulfuric acid ester of polyanhydromannuronic acid which was tried clinically. Field *et al.* (4) tested the sodium salt or sulfated polygalacturonic acid methyl ester methyl glycoside, and Walton(5) reported on the biological behavior of sulfated dextran. In all instances there was evidence of potential side reactions or toxic phenomena.

Preliminary tests with the sulfuric acid ester of chitin were sufficiently promising to justify extensive studies on the biological behavior of this material. The properties and toxicity of various preparations are herein reported.

Materials and methods. Samples with various average molecular weights were produced by Cushing *et al.*(6), who also described the material as follows: "The product can be defined as the sodium salt of the sulfuric acid ester of poly (1-4) N acetylglucosamine. The sulfur assays (13.5 to 14.5%) showed the degree of sulfation to be somewhat below the maximum theoretical sulfur content of 15.7% for a product having two sulfate groups on each acetylglucosamine residue. The nitrogen assays ranged from 2.3 to 3.2%, the variation being due chiefly to small amounts of residual triethylamine (as a salt on sulfate) used for neutralization of the sulfation mixture. Curiously, the elimination of the residual triethylamine did not significantly decrease the toxicity of the product. All molecular sizes are represented according to the usual frequency distribution curve except that molecules below 5000 mol wt are mostly eliminated during dialysis or repeated alcohol precipitation. In a product having an average molecular weight of 14000, the amount of high molecular weight material (above 17000) is less than 0.5% of the total. In the selected material, relative viscosities (R.V.) in 1% aqueous solution were remarkably uniform, with 1.3 R.V. as the preferred product, and a maximal permissible range of 1.25 to 1.35. The *in vitro* potency varied between 27 and 35 I.U./mg(7)."

Injections were almost exclusively intravenous. Unless high doses were required, sulfated chitin and heparin were used as 1% aqueous solutions. Blood samples for all tests were withdrawn through silicone-treated needles, either into siliconed syringes for transfer to siliconed, chilled glassware or directly into capillary tubes thrust into the needle hub. Coagulation times were determined by the capillary tube method(8). Prothrombin determinations were made by the Quick one-step method. Platelets were counted directly from samples diluted with 3.8% sodium citrate, as described by Quick *et al.*(9). Acute intravenous toxicity was determined in mice. Subacute intravenous toxicity was observed

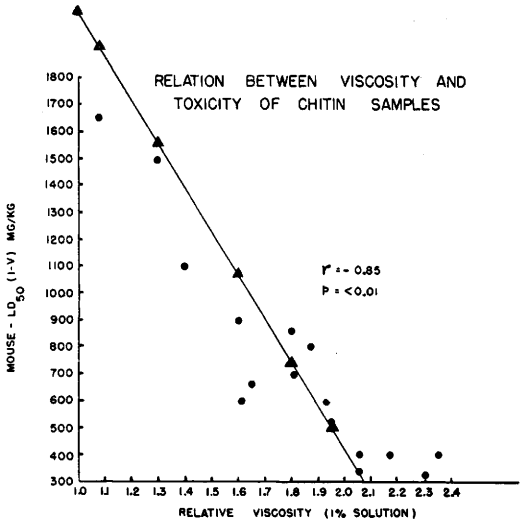

FIG. 1. Triangles = calculated points for regression line. Circles = actual values.

in rabbits, dogs and monkeys with selected preparations.

Results. The *in vitro* potencies of samples having different average molecular weights were assayed by the U.S.P. heparin technic. The acute toxicity in mice was proportional to the average molecular weight (estimated by the R.V. of a 1% aqueous solution.) Fig. 1 shows the LD_{50} of each sample plotted against its relative viscosity. The values closely approximate the calculated regression line, and the correlation is statistically significant (P 0.01). The duration of action was roughly proportional to the R.V.

In contrast, there was no relationship between *in vitro* anticoagulant potency and viscosity, nor did the *in vivo* results show good correlation with *in vitro* values, or provide an indication of duration of action. Therefore, assays in rabbits were chosen as a more reliable criterion of possible usefulness.

Low molecular weight samples had much shorter duration for a given dose; for example, a sample of R.V. 1.1 had a duration of action of less than 30 minutes at a dose of 10 mg kg (see below and Fig. 2 for data on 1.3 R.V. material). Higher molecular weights were more toxic, as shown in Fig. 1. Finally, material having a R.V. of 1.3 (as described under Materials) was chosen for more extensive study. This represented a compromise between toxicity and duration of action. By osmometric and light scattering methods the average molecular weight of this sample was determined to be between 13000 and 15000. The LD_{50}, in mice calculated by the method of Miller and Tainter(10), was found to be 1.5 g/kg.

Blood samples were taken from dogs and rabbits before, and 5, 15, 30 and 60 minutes after the injection of the compound, or until the coagulation time had returned to or below 3 times the normal coagulation time, which was arbitrarily defined as a limit of "significant drug effect". In rabbits, 10 mg/kg of sulfated chitin had a potency approximately equal to 2.0 mg/kg of heparin, while 5 mg/kg of sulfated chitin was appreciably more potent than 1.0 mg/kg of heparin. With equipotent doses, the duration of "significant drug effect" was 2 to 3 times longer with sulfated chitin. In dogs, 1.5 mg/kg of heparin and 5 mg/kg of sulfated chitin were approximately equal in potency and duration. Platelet counts were performed on the same blood samples. The results in dogs and rabbits were in agreement with the reports of earlier workers. There was a moderate reduction (40-60%) in the number of circulating platelets, but the magnitude of change was no greater, though the duration was somewhat longer than that obtained with equipotent doses of heparin. However, as also reported by others for heparin, the platelet count again reached control levels with or before the return of normal coagulation time. No significant influence of sulfated chitin, in doses up to 10 mg/kg, upon the blood prothrombin level was noted. The anticoagulant effect can be neutralized by protamine sulfate.

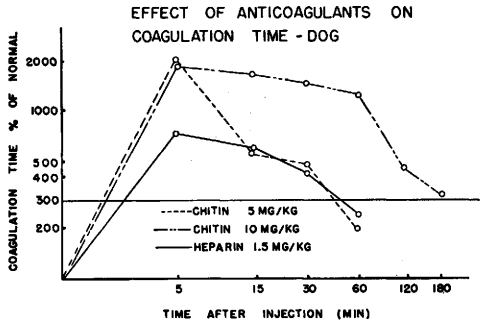

FIG. 2. Horizontal line at 300% of normal coagulation time represents arbitrary lower limit of "significant drug effect."

These findings strengthen the assumption that its physiological action is similar to that of heparin.

Rapid intravenous injection into anesthetized cats or dogs of as much as 50 mg/kg produced no change in blood pressure other than the effect of volume of fluid given. Attempts to sensitize animals with sulfated chitin by methods which detect weak antigens [Roth *et al.*(11)] failed to demonstrate any antigenic potentialities.

Four dogs were given 10 mg/kg daily of a higher molecular weight sample having a R.V. of 1.95. In all 4 animals, bloody diarrhea and vomiting occurred after 6 to 8 days of treatment. In those sacrificed for histological study, acute hemorrhagic changes were confined to the intestine. All other tissues showed no significant pathological changes.

Daily intravenous administration to dogs of 10 mg/kg of 1.3 R.V. sulfated chitin produced no significant symptoms for the first week. After about 10 days some lethargy and slight ataxia appeared but no other symptoms were noted, and animals sacrificed showed no gross or microscopic abnormalities. At 20 mg/kg per day, the animals showed some loss of weight, and after 8 days ataxia and weakness appeared. Death occurred at 9 and 10 days, but the animals were not seen in time to justify autopsy. No diarrhea occurred with either dose level. These untoward effects were entirely absent in rabbits and monkeys. Daily doses of 20 mg/kg for 3 weeks produced no detectable symptoms in either species. Some rabbits received 50 mg/kg/day for 2 weeks without untoward effects, while others tolerated 100 mg/kg/day for a few days. Death ultimately resulted from direct hemorrhage from injection sites, reopened after return to their cages. Hematological studies of animals during chronic administration showed no significant departure from normal values for red or white cell count, differential count or hemoglobin.

Attempts were made to eliminate unknown toxic factors and to improve purity by modifying the chemical process. The mortality data obtained with numerous samples on a total of 4044 mice (at least 30 per sample) provided LD_{50} values ranging between 1.25 and 1.8 g/kg which represented no encouraging improvement over earlier material.

Discussion. Survey of recent literature disclosed several reports which express doubts as to the safety of various sulfated polysaccharides. Astrup(12) observed that citration of blood appears to stabilize human platelets. Therefore, if citrate is used for any observations on the effects of anticoagulants on platelets, negative results may be misinterpreted. He also implied that any synthetic anticoagulants of the sulfated polysaccharide type might be expected to agglutinate thrombocytes and would be considered undesirable for clinical use. Other reports (Pratt(13); Fischer *et al.*(14); Hirschboeck *et al.*(15)) described the occurrence of alopecia following the use of synthetic anticoagulants, and Wright(16,17) reported many other side effects. In our results on dogs, it was first thought that the effects were possibly a species idiosyncrasy similar to that observed with polyvinylpyrollidone. However, Hirschboeck (15) describes the occurrence of a bloody diarrhea in human patients treated with another sulfated polysaccharide. This information indicates the need for more information on the basic mechanisms involved, and for improvement in the synthetic approach, before a completely safe heparin substitute can be assured.

Summary. The anticoagulant effects of a sulfated polysaccharide, the sodium salt of the sulfuric acid ester of chitin, have been described. It has an *in vivo* potency ¼ to ⅕ that of heparin activity. Duration of action and toxicity are both directly related to average molecular weight. The occurrence of toxic side reactions in the dog are similar to some of those reported in the human for another sulfated polysaccharide. Limitations of potential clinical usefulness are discussed.

The authors express appreciation to R. A. Hendricks and B. B. Morphis for valuable technical assistance and to R. T. Olsen and R. A. Rieker for the *in vitro* potency assays.

1. Bergstrom, S., *Z. f. Physiol. Chem.*, 1936, v238, 163.

2. Piper, J., *Acta Physiol. Skand.*, 1945, v9, 28.

3. Sorenson, C. W., and Wright, I. S., *Circulation* 1950, v2, 658.

4. Field, J. B., Ramsay, G. D., Attyah, A. M., and Starr, P., *J. Lab. & Clin. Med.*, 1953, v41, 208.
5. Walton, K. W., *Brit. J. Pharmacol.* 1952, v7, 370.
6. Cushing, I. B., Davis, R. V., Kratovil, E. J., and MacCorquodale, D. W., *J. Am. Chem. Soc.*, 1954, in press.
7. Cushing, I. B., personal communication.
8. Hepler, O. E., *Manual of Clinical Laboratory Methods.* 4th Ed. p83, C. C. Thomas, Springfield, Ill., 1949.
9. Quick, A. J., Shanberge, J. N., and Stefanini, M., *J. Lab. and Clin. Med.*, 1948, v33, 1424.
10. Miller, L. C., and Tainter, M. L., PROC. SOC. EXP. BIOL. AND MED., 1944, v57, 261.
11. Roth, L. W., Richards, R. K., and Shepperd, I. M., *J. Lab. and Clin. Med.*, 1949, v34, 531.
12. Astrup, T., *Skand. J. Clin. and Lab. Invest.*, 1953, v5, 137.
13. Pratt, G. H., *Surg. Clin. N. Am.*, 1952, v32, 369.
14. Fischer, R., Bircher, J., and Reich, T., *Schw. Med. Wchnschr.*, 1953, v83, 509.
15. Hirschboeck, J. S., Madison, F. W., and Pisciotta, A. V., *Am. J. Med. Sci.*, 1954, v227, 279.
16. Wright, I. S., *Cardiologia,* 1952, v21, 709.
17. ———, *Am. J. Med.*, 1953, v14, 720.

Received April 2, 1954. P.S.E.B.M., 1954, v86.

Intracellular Pattern of Nucleic Acid in Virus Infection.* (21085)

R. BERNAL JOHNSON AND W. WILBUR ACKERMANN.
(Introduced by Thomas Francis, Jr.)

From the Department of Epidemiology and Virus Laboratory, School of Public Health, University of Michigan, Ann Arbor

Upon infection with herpes virus, the heart and liver of chick embryos reveal an enlargement which results from an increased rate of cellular proliferation. This is paralleled by a markedly increased rate of nucleic acid synthesis such that the total nucleic acid of the liver may be 70% greater than normal(1). While these first observations were concerned with differences in the whole organ which were a result of the increased mass of the liver, the present paper deals with observations at the cellular level.

The amount of nucleic acid per cell has been estimated in normal and viral infected tissues. Nuclei isolated from herpetic liver have been analyzed for the nucleic acids, and data concerning the intracellular distribution of deoxyribonucleic acid and ribosenucleic acid have been obtained. Parallel observations have been made of the nucleic acid pattern of the Ehrlich ascites tumor infected with influenza virus.

Methods and materials. Virus. The herpes virus used was the Armstrong 1166 strain. It had undergone 54 passages in mouse brain and was adapted to the embryonate egg by 40 passages on the chorionic membrane(2). The WS strain of Type A influenza virus was used in the studies with the Ehrlich mouse carcinoma. This virus was maintained for several years by continuous passage in tissue culture and since has undergone 102 passages in mouse brain. It will multiply both in the brain and lungs of mice. After adaptation to the Ehrlich ascites tumor, it has been passed serially in that tissue 40 times(3). *Tissue.* The Ehrlich mouse carcinoma was adapted to ascitic form by Dr. G. A. LePage and in this laboratory has undergone 60 passages in the ascitic form in Swiss white mice (Webster strain). *Fractionation.* A 20% homogenate of liver was prepared in 0.25 M sucrose containing .0018 M $CaCl_2$. The disruption of the tissue was achieved with the Potter-Elvehjem glass homogenizer. The resulting preparation was diluted to 4% and fractionated essentially by the procedure of Hogeboom and Schneider(4). A nuclear and a cytoplasmic fraction were obtained. The latter contained the soluble proteins as well as the mitochondria and microsomes. All operations were performed at temperatures

* These studies were aided by a grant from the National Foundation for Infantile Paralysis.