groups of final injury. In other words, our material underwent a change and we do not know whether this change was selective or not.

Furthermore, we cannot give a numerical representation for the groups "no gangrene", "partial gangrene", "total gangrene". Our experimental results do not enable us to assume that the extent of necrosis is a continuous function of increasing severity of injury since all types of tissue do not show the same sensitivity to cold.

The statistical data given in the paper serve only the purpose of showing that the constellation of figures on which our conclusions are based are not due to random variation.

Summary. A total of 153 rabbits had their legs exposed to temperatures of −25°C or −15°C for 30 minutes and subsequently treated with heparin administered intravenously for a period of 6 days. Twelve animals received 20 mg of heparin every 4 hours, 39 received 30 mg every 12 hours, and 102 received 30 mg every 6 hours.

Eighty animals were frostbitten at the same temperatures and for the same exposure times and used as untreated controls.

A statistical analysis of the results showed that tissue necrosis from frostbite was not significantly less in the heparin-treated animals.

Of 153 animals that were treated with heparin 74 died whereas 17 of 80 control animals succumbed during the experiment. The fatalities in the heparin-treated series were due to fatal internal hemorrhages in 53 cases; in 17 instances to a shock-like condition especially observed when the initial injection of heparin was delayed 4 or more hours after injury, and 4 animals died from unknown causes.

Conclusions. 1. The excellent results reported by Lange and his co-workers on the use of heparin in frostbite were not reproducible.

2. The results of heparin therapy by the regime recommended by Lange were not more satisfactory in our experiments than those in untreated control animals.

3. The death rate was significantly increased in animals that received heparin in doses proposed by Lange *et al.*

We wish to give credit to Mr. Allyn Kimball of the Department of Biometrics, School of Aviation Medicine, Randolph Field, Texas, for making the statistical analyses of the experimental data.

Received August 9, 1949. P.S.E.B.M., 1949, **72**.

Alteration in Permeability of Some Membranes by Hyaluronidase and Inhibition of this Effect by Steroids. (17355)

JOSEPH SEIFTER, DAVID H. BAEDER, AND ALPHONSE DERVINIS. (Introduced by W. Ehrich.) (With the technical assistance of Leonard Dann.)

From the Wyeth Institute of Applied Biochemistry, Wyeth, Inc., Philadelphia, Pa.

It is well established that hyaluronidase increases the "permeability" of the ground substance and can thereby markedly facilitate the spread of India ink, dyes, bacteria, drugs, etc. through the cutaneous tissues.[1,2] Hyaluronidase may also facilitate the penetration of some drugs into the cornea by attacking the monosulfuric ester of hyaluronic acid of this tissue.[3] It was of interest to investigate the effects of hyaluronidase on the permeability of membranes both *in vitro* and *in vivo* since this has not been done heretofore. Such a study is of timely interest in light of the possible relationship of hyaluronidase as a causative factor in arthritis and the alleviation of arthritis by steroids such as Compound E.[4,5]

Most of the *in vitro* tests were made using the urinary bladder of the rabbit as a semi-

[1] Duran-Reynals, F., *Bact. Rev.*, 1942, **6**, 197.

[2] Meyer, K., *Physiol. Rev.*, 1947, **27**, 335.

[3] Seifter, J., *Ann. N. Y. Acad. Sci.*, in press.

permeable membrane. The *in vivo* studies were made by injecting a dye into the synovial cavity of rabbits and using the speed of appearance of dye in the urine and the rate of excretion into the urine as an index of permeability of the synovial membrane. These studies have been extended to human subjects under the influence of various drugs and diseases. In this paper we are presenting the data obtained *in vitro*. Those obtained *in vivo* correspond in nearly every detail to these and will be presented in a separate paper.

Materials and methods. The hyaluronidase* was prepared from bull testes and assayed 7000 to 9400 turbidity reducing units per mg of nitrogen. Membranes used in this study were the lens capsule, urinary bladder membrane processed according to the method of Barnes,[6] and freshly prepared urinary bladder of the rabbit. We also studied the effects of hyaluronidase on water imbibition by skeletal muscle, penetration of drugs into excised frog hearts, and filtration through isolated frog skin, because membrane permeability may be involved in these phenomena.

Crystalline lens membrane. Freshly excised, intact lens of frogs and rabbits were used. Series of test tubes were set up, four in each series, with one pair containing distilled water and the other pair physiological salt solution. One lens was placed in each solution, the tube agitated, and an aliquot of the supernatant removed. To one tube of each pair hyaluronidase was added to make a 0.05% solution, the tubes again agitated, and samples removed. The tubes were maintained constantly at 38°C. The degree of turbidity of each solution was read every five minutes, using the B scale of the Fischer Electrophotometer. A water clear solution gave a light transmission of 100%; an increase in turbidity decreased the amount of light transmitted (Fig. 1).

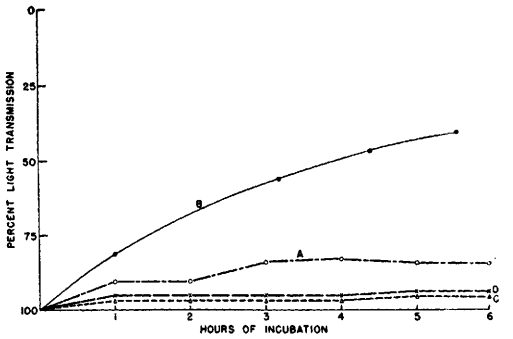

FIG. 1.

Turbidity of incubating medium containing whole rabbit lens (38°C). Fischer electrophotometer used for readings).

Curve A—Distilled water.

Curve B—0.05% hyaluronidase (assaying 9400 T.R.U. per mg N) in distilled water.

Curve C—Physiological salt sol.

Curve D—0.05% hyaluronidase (assaying 9400 T.R.U. per mg N) in physiological salt sol.

Urinary bladder membrane. The urinary bladder was removed surgically from rabbits by excision at the neck. Each membrane was prepared by washing and storing overnight at 36°C in 0.85% NaCl. The following morning a membrane was stretched and mounted over the open end of a thistle tube, the adventitia being toward the inside of the tube. After drying for 30 minutes in the hot air oven at 100°C, the membrane was waterproofed at the edge with paraffin and stored in the refrigerator until used. The preparation served as an osmometer with a semipermeable membrane separating M/1 sucrose on the inside from distilled water against the mucosal surface on the outside. One membrane could be used several times if after each experiment it were reprocessed by repeating the above procedure. When reprocessing was repeated on 5 separate membranes for 3 successive days the curves of the rate of osmosis obtained on the first day could be superimposed on those obtained on the third day, thus ruling out the possibility that reprocessing altered permeability. A total of 58 permeability experiments were done on 30 processed membranes.

Urinary bladder membranes maintained in the fresh state were also studied. After killing the rabbits by a sharp blow across the

[4] Hench, P. S., Kendall, E. C., Slocumb, C. H., and Polley, H. F., *Proc. Staff Meet., Mayo Clin.*, 1949, **24**, 181.

[5] Hench, P. S., Slocumb, C. H., Barnes, A. R., Smith, H. L., Polley, H. F., and Kendall, E. C., *Proc. Staff Meet., Mayo Clin.*, 1949, **24**, 277.

* Hydase, Wyeth.

[6] Barnes, T. C., Textbook of General Physiology, 1937, Blakiston Co., Philadelphia, Pa.

cervical vertebrae, the urinary bladder was immediately removed, washed in Tyrode's solution at 37 ± 1°C, and by means of a purse string suture of surgical silk secured as quickly as possible to the open end of a thistle tube. The adventitia was inside and the mucosa outside. Tyrode's solution containing M/1 sucrose was placed within the osmometer which was immersed in a beaker containing Tyrode's solution. Oxygen was bubbled into the solution on each side of the membrane. A freshly excised bladder was used for each of 21 experiments performed at 37°C in a hot air incubator.

The following were tested for their effect on permeability of urinary bladder membranes: (1) hyaluronidase; (2) adrenal cortical extract (10% alcoholic, Upjohn Co.); alcoholic solutions of cholesterol, testosterone, estrone, desoxycorticosterone acetate,† Equilenin,† and Equilin;† (3) alarm reaction induced by colchicine. The final concentration of alcohol in the steroid tests did not exceed 5 γ of ethanol per ml. This concentration was found to have no effect on membrane permeability. All substances tested for effect on permeability were added to the solution in contact with the mucosal surface.

Skeletal muscle membrane. The gastrocnemius muscle of frogs of near weight was immersed in a bath of 50 ml distilled water or 50 ml distilled water-drug solution, immediately removed from the solution and weighed after the water was shaken off. The muscle was then returned to its constant temperature bath maintained at 0 to 2°C. Thirty minutes after immersion and each one-half hour thereafter for 5 hours, the muscles were weighed. In several instances a weighing was also taken at 24 to 48 hours. Seventy-five muscles were used in this study and the following compounds were tested: hyaluronidase; an antihistaminic, Neohetramine; and histamine diphosphate.

Results. Lens membrane. Incubation of frog and rabbit lens in distilled water at 38°C resulted in progressive turbidity of the supernatant (Fig. 1, curve A). The lens capsule usually blistered in one hour and ruptured up to 6 hours later. Incubation in physiological salt solution, on the other hand, did not produce turbidity and did not injure the capsule (Fig. 1, curve C). Addition of 0.05% hyaluronidase produced a marked increase in the turbidity of the distilled water (Fig. 1, curve B) but had no effect on the appearance of the physiological salt solution (Fig. 1, curve D). Tests with anthrone reagent[7] revealed that the turbidity was not due to release of carbohydrate material. This reagent, however, does not detect hyaluronic acid or its breakdown products.

It could not be assumed from the turbidity that hyaluronidase affected the permeability

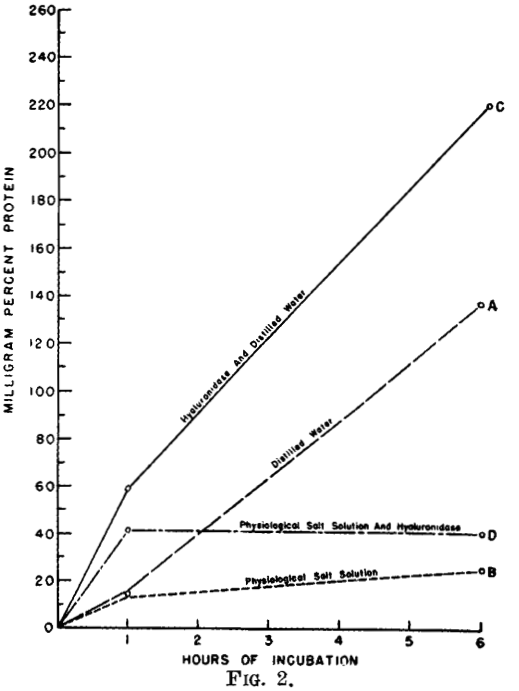

FIG. 2.

Leakage of proteins from rabbit lens exposed to hyaluronidase.

Curve A—Lens incubated at 37°C in distilled water. Capsule blistered in 1 hr.

Curve B—Lens incubated at 37°C in physiological salt sol.

Curve C—Lens incubated at 37°C in 0.05% hyaluronidase (assaying 9400 T.R.U. per mg N) in distilled water. Capsule blistered in 1 hr.

Curve D—Lens incubated at 37°C in 0.05% hyaluronidase (assaying 9400 T.R.U. per mg N) in physiological salt sol.

† We wish to thank the Schering Corp., Bloomfield, N. J., for the desoxycorticosterone acetate, and Ayerst, McKenna & Harrison Limited, Montreal, Canada, for the Equilenin and Equilin.

[7] Morris, D. L., *Science*, 1948, **107**, 254.

TABLE I.
Effect of Hyaluronidase and Steroids on the Permeability of Processed Membranes.

	No. of exp.	Avg rise at 360 min. (mm)	Range (mm)	Median (mm)
Distilled water	24	101	67-189	105
Hyaluronidase	10	245	130-360	240
Adrenal cortical extract*	3	91	62-124	87
Adrenal cortical extract + hyaluronidase	5	125	100-146	125
Cholesterol*	2	122.5	120-125	
Cholesterol + hyaluronidase	1	133		
Estrone*	2	110.5	110-111	
Estrone + hyaluronidase	2	50	29- 71	
Testosterone*	1	93		
Testosterone + hyaluronidase	1	176		

* These membranes had much greater permeability in the control period when only distilled water was used.

only in distilled water, since the released material might be soluble in physiological salt solution. Protein determinations of the supernatants were made by the sulfosalicylic acid method, and some of the data are presented in Fig. 2. Each curve was obtained by averaging the milligrams percent of protein for the supernatants of 6 to 8 lenses. The data for distilled water and distilled water-hyaluronidase were taken only from lenses showing blistering or rupture in one hour in order to make a comparison with physiological salt solution-hyaluronidase which for some as yet unexplainable reason became inactivated in one hour. Leakage of protein through the membrane immersed in distilled water or physiological salt solution did not occur to a significant degree during the period when the lens was intact (curves A and B). Hyaluronidase caused leakage of protein through the membrane during this period (curves C and D). Individual capsules differed markedly in their resistance to injury by the hypotonicity of distilled water and to alteration of permeability by hyaluronidase.

Urinary bladder membrane. The results on the processed membranes are listed in Table I and typified in Fig. 3. The rate of osmosis through a semi-permeable bladder membrane is shown by Curve A. The distilled water at the end of the experiment was sugar free by the anthrone method. Two γ of hyaluronidase per ml markedly increased the rate (Curve B). At the end of this experiment the concentration of sugar outside the membrane was 0.30 mg per ml as compared with 0.34 mg per ml inside; therefore the semi-permeable nature was abolished. The permeability effect was reversible since removing the hyaluronidase

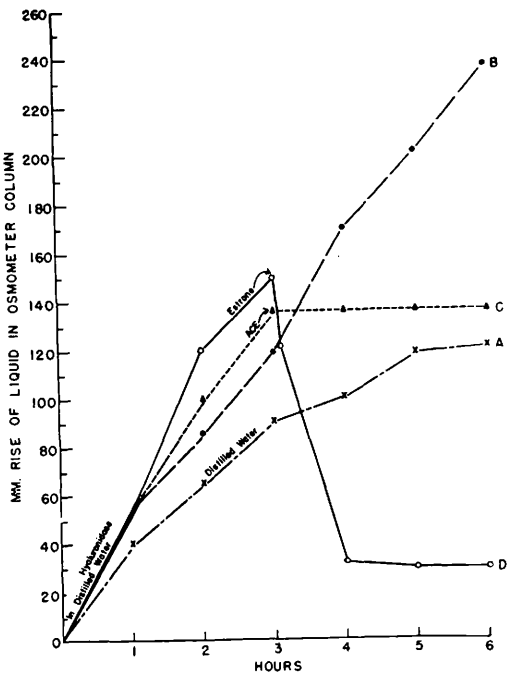

FIG. 3.

Rate of osmosis. (Urinary bladder membrane semi-permeable to M/1 sucrose).

Curve A—Distilled water outside osmometer.

Curve B—Hyaluronidase 1:10^6 (assaying 7000 T.R.U. per mg N) in distilled water (2 γ per ml).

Curve C—Same concentration of hyaluronidase in distilled water as in curve B, but aqueous adrenal cortical extract, 10 γ per ml, added at the arrow.

Curve D—Same concentration of hyaluronidase in distilled water as in curve B, but aqueous estrone, U.S.P., 10 γ per ml, added at the arrow.

TABLE II.
Effect of Hyaluronidase, Steroids, and an Alarming Stimulus on the Permeability of Surviving Membranes.

	No. of expts.	Avg rise at 360 min. (mm)	Range (mm)	Median (mm)
Tyrode's sol.	5	39	38- 40	39
Hyaluronidase	5	160	159-161	160
Adrenal cortical extract + hyaluronidase	2	74	74- 75	
Estrone	1	20		
Estrone + hyaluronidase	1	13		
Desoxycorticosterone acetate	1	104		
Desoxycorticosterone acetate + hyaluronidase	1	150		
Equilenin + hyaluronidase	1	3		
Equilin + hyaluronidase	1	5		
Colchicine *in vivo*	1	0		
Colchicine *in vivo* + hyaluronidase *in vitro*	1	0		
Plasma from rabbit in alarm reaction	1	13		

after the first hour decreased the rate of osmosis, and adding it again at the third hour reestablished it. This alternation could be demonstrated for the first 6 hours.

Ten γ of either adrenal cortical etxract, testosterone, or cholesterol per ml inhibited the passage of water through the membrane immersed in hyaluronidase solution. This observation resembles the *in vivo* inhibiting effect of adrenal cortical extract on the spreading activity of hyaluronidase[8] and decreased absorption through the synovial membrane.[9] Ten γ of estrone per ml not only stopped osmosis through the hyaluronidase treated membrane, but reversed the flow (Curve D). Injection of estrone *in vivo* increased the resistance of the ground substance[10] and of synovial membranes[9] to hyaluronidase.

The results obtained with fresh membranes in oxygenated Tyrode's solution were identical with those just described and are listed in Table II. Further studies were carried out using an estrogenic type steroid. Twenty-four γ of Equilenin or 30 γ of Equilin per ml reversed the flow of water through the hyaluronidase treated membrane in a manner similar to that of estrone. When the compounds were removed and replaced with a fresh solution of hyaluronidase and Tyrode's solution, osmosis proceeded as it had before the steroids had been added indicating that the membranes were not injured.

Ten γ of desoxycorticosterone acetate per ml enhanced considerably the effect of hyaluronidase on osmosis. To further study the action of this steroid, a rabbit was given 35 mg desoxycorticosterone acetate in oil intramuscularly once a week for two weeks. Os-

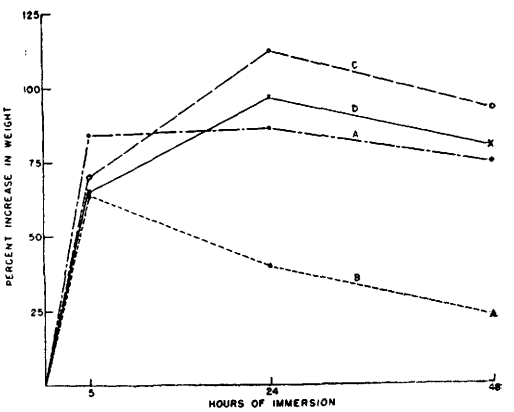

FIG. 4.

Influence of antihistaminics and histamine on lyophilia of frog gastrocnemius muscle (immersed in distilled water at 2°C).

Curve A—Distilled water.

Curve B—0.1% neohetramine hydrochloride in distilled water. (1% neohetramine hydrochloride causes the muscle to decrease below the original weight).

Curve C—0.1% to 0.2% histamine diphosphate in distilled water.

Curve D—0.1% neohetramine hydrochloride plus 0.1% to 0.2% histamine diphosphate in distilled water. (1% neohetramine counteracts 0.1% histamine diphosphate).

[8] Opsahl, J., White, A., and Duran-Reynals, F., *Ann. N. Y. Acad. Sci.*, in press.

[9] Seifter, J., Baeder, D. H., and Begany, A. J., PROC. SOC. EXP. BIOL. AND MED., 1949, **72**, in press.

[10] Sprunt, D. H., and McDearman, S., *Endocrinology*, 1940, **27**, 893.

mosis through this bladder was more rapid in Tyrode's solution alone than through normal bladder membranes treated with hyaluronidase.

In order to determine whether an alarm reaction with the concomitant release of adrenal steroids would affect the permeability of membranes, 3 mg colchicine per kg were administered subcutaneously to rabbits. Two such membranes were prepared. Osmosis was completely inhibited through them even in the presence of hyaluronidase.‡ One ml of plasma from a rabbit in colchicine alarm reaction immediately inhibited osmosis and reversed the flow through a normal membrane.

Striated muscle membrane. Thirty gastrocnemius muscles immersed in distilled water gained an average of 82% of their original weight in 5 hours, 87% in 24 hours, and 78% at 48 hours (Fig. 4, curve A). Neohetramine inhibited the uptake of water so that at 5 hours there was an average increase to 62%, at 24 hours 39%, and at 48 hours only 31% (typical curve, Fig. 4, curve B), thus confirming the observations of Halpern[11] that antihistaminic drugs decrease the permeability of skeletal muscle immersed in distilled water. The uptake of water in gastrocnemius muscles immersed in a solution containing 0.1 to 0.2% histamine diphosphate was 73% of the original muscle weight at 5 hours, 103% at 24 hours, indicating a slight "edema" effect, and 98% at 48 hours (typical curve, Fig. 4, curve C). This concentration of histamine was sufficient to counteract the "anti-edema" effect of the lower concentrations of the anti-histaminic compound (Fig. 4, curve D). Neohetramine in a concentration of 1%, however, antagonized the effect of 0.1% histamine diphosphate.

Addition of hyaluronidase in amounts up to 1% in the distilled water solution alone or in the distilled water containing histamine or Neohetramine did not result in deviations from the usual behavior of the compound tested. The curves obtained in each instance could be superimposed on those shown in Fig. 4. Similar results were obtained with the rabbit soleus muscle incubated at 37°C.

The experiments with skeletal muscle are illustrative of our finding that hyaluronidase does not enhance penetration of drugs in isolated frog hearts and does not affect ultrafiltration through frog skin.

Summary. 1. Purified testicular hyaluronidase had no effect on the normal or altered permeability of skeletal muscle, cardiac muscle, or of isolated frog skin.

2. Hyaluronidase strikingly increased the permeability of the lens capsule and urinary bladder membranes, and abolished their semipermeable character.

3. The enhancing effect of hyaluronidase on osmosis through the bladder membrane was increased by desoxycorticosterone acetate.

4. Adrenal cortical extract, testosterone, and cholesterol abolished the enhancing effect of hyaluronidase. Estrone, Equilin, and Equilenin reversed the direction of flow through hyaluronidase treated membranes.

5. Hyaluronidase did not alter the complete inhibition of osmosis through the bladder membrane of rabbits which had been exposed to an alarming stimulus.

6. The products released *in vivo* during the alarm reaction rendered bladder membranes impermeable even in the presence of hyaluronidase.

Received August 12, 1949. P.S.E.B.M., 1949, **72**.

[11] Halpern, B., personal communication. Published reference unavailable.

‡ Preliminary *in vitro* and *in vivo* experiments indicate that cortisone acetate is as effective as the alarm reaction.