to *Pseudomonas aeruginosa* in experimental frostbite by daily local application of sulfamylon.

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**.

Use of Heparin in Treatment of Experimental Frostbite. (17354)

JOSEF PICHOTKA AND R. B. LEWIS (Introduced by A. W. Hetherington.)

From the Department of Pathology, USAF School of Aviation Medicine, Randolph Air Force Base, Randolph Field, Texas.

During World War II casualties from local cold injury were numerous. In some units they exceeded the number of casualties from every other cause. This was especially true of Air Force personnel on high altitude bombing missions. It was therefore quite natural that much interest was focused on the investigation of the mechanism and therapeusis of cold injury.

Among the publications on the treatment of local frostbite, the reports of Lange and his collaborators were the most remarkable.[1-5] These authors described the results of heparin in the prevention of gangrene in animals and human volunteers after local cold injury. The control cases in their experiments always developed extensive or complete necrosis of the frostbitten area whereas the heparin treated cases almost always escaped gangrene. The details of the experimental procedures and of heparin administration as used in their investigations will be discussed later.

Publications subsequent to those of Lange *et al.* did not confirm their results.[6,7,8] Because of the importance of the subject to the Air Force we deemed it advisable to repeat these experiments.

Material and Method. With few exceptions in the early series we used white male albino rabbits of more than 2,000 g body weight. Female animals were excluded since in one series they seemed to be more susceptible than males to internal hemorrhages after heparin administration. The animals were kept in the open air throughout the year, but at least one week before the experiment they were brought into the air conditioned animal house and kept at 25°C (77°F). The food consisted of Purina rabbit pellets and occasionally carrots; water was unlimited. In the course of the investigation during which more than 300 animals passed through our animal room only 2 animals died spontaneously.

During the first part of the experiment we followed the procedure of Lange *et al.*[2,5] as closely as possible, except that the animals were not anesthetized during freezing. The right hind leg was clipped and depilated with Zip Depilatory Cream from the ankle upwards and loosely covered with a rubber condom boot to about 2 cm to 3 cm above the knee joint. The prepared hind leg was immersed to the knee joint in a bath at −25°C (−13°F) for 30 minutes. The freezing bath consisted of 95% alcohol cooled with dry ice. After some experience it was possible to keep the bath within 0.5°C of the desired temperature. Immediately after freezing, the legs were

1 Friedman, N. B., Lange, K., and Weiner, D., *Am. J. M. Sc.*, 1947, **213**, 61.

2 Lange, K., and Boyd, L. J., *Surg., Gynec., and Obst.*, 1945, **80**, 346.

3 Lange, K., and Loewe, L., *Surg., Gynec., and Obst.*, 1946, **82**, 256.

4 Lange, K., Boyd, L. J., and Loewe, L., *Science*, 1945, **102**, 151.

5 Lange, K., Weiner, D., and Boyd, L. J., *New England J. Med.*, 1947, **237**, 383.

6 Fuhrman, F. A., and Crismon, J. M., *J. Clin. Invest.*, 1948, **27**, 364.

7 Quintanilla, R., Krusen, F. H., and Essex, H. E., *Am. J. Physiol.*, 1947, **149**, 149.

8 Schumacker, H. B., Jr., White, B. H., Wrenn, E. L., Cordell, A. R., and Sanford, T. F., *Surgery*, 1947, **22**, 900.

TABLE I.
Results of Heparin Treatment in Very Severe Frostbite.

No. of animals	Treatment	No necrosis	Partial necrosis	Complete necrosis	Died	Survived
12	2 cc heparin every 4 hr	0	3	2	7	5
30	3 cc heparin every 6 hr	0	4	23 (1)*	4	26
39	3 cc heparin every 12 hr	0	1	12	17	22
Total—81		0	8	46 (1)*	28	53
Controls—42		0	7	28 (2)*	9	33

* Number in parentheses indicates animals which died after fate of the leg was decided and which therefore appear twice in the table.

123 animals frozen in an alcohol bath at —25°C with rubber boot loosely attached to leg.

81 animals were treated with heparin for 6 days.

42 animals were controls.

The distribution of "partial necrosis" and "complete necrosis" is essentially the same in both groups. ($\chi^2 = 1.2$ and $P = 0.27$).

The death rate is not significantly different. ($\chi^2 = 1.7$ and $P = 0.18$).

covered with sterile dressings and the animals put into individual cages. Generally the dressings were changed every day. The animals which showed severe swelling had the first dressing changed after 12 hours to prevent obstruction of the circulation by a tight bandage. At the end of the first day the swelling was at its peak, and thereafter the danger from constrictive dressings was negligible. We originally used a thin layer of wool fat to prevent adherence of the bandages due to the oozing of plasma. After some infections occurred, we added penicillin to the wool fat, but without success. Subsequently sulfamylon was used which eliminated further infection in the course of the investigation. The prevention of infection following experimental frostbite is the subject of a separate paper.[9]

In the second part of the investigation the conditions of exposure were changed since we felt it necessary to use a less severe cold injury to evaluate heparin therapy.

The experiments were performed from February 1948 to March 1949 on 315 animals. Of these, 153 animals received heparin* for 6 days, 80 served as controls, and 82 were used for the determination of a degree of injury suitable for the evaluation of the treatment.

The statistical method used is a test of significance using χ^2 (chi-square) in two-way contingency tables.

Repetition of the Experiments of Lange and Collaborators. Lange and his co-workers used different temperatures and exposure times in their animal experiments.[2,5] The largest group with homogeneous conditions consisted of 41 animals of which one hind leg was exposed to a bath of –30°C (–22°F) for 30 minutes.[5] Twenty-one of these animals received 3 cc of heparin intravenously every 12 hours for 6 days. In a personal communication Lange advised us to give 3 cc of heparin every 6 hours.

The corresponding group in our experiments consisted of 123 animals. One hind leg was depilated, loosely covered with a rubber boot, and immersed to the knee joint in a bath of –25°C (–13°F) for 30 minutes and then allowed to thaw at room temperature. Eighty-one of these animals received heparin and 42 served as controls. The heparin was administered by 3 different regimes as shown in Table I. The initial injection of heparin was made from 1 to 4 hours after freezing in 51 animals, 4 to 8 hours in 14, and 8 to 12 hours in 16.

Blood coagulation times were determined

9 Pichotka, J., and Lewis, R. B., to be published.

* The heparin used was liquaemin (Roche-Organon, Inc.) and heparin (Abbot).

in 28 experiments following the injection of 2 cc of heparin intravenously. The capillary method was used; blood samples were obtained from the ear veins by means of nicking with a sharp needle. Intervals between determinations were 10 minutes in one series and 30 minutes in another.

The results of this experiment do not show any benefit of heparin treatment. In both groups all animals developed tissue necrosis. The statistical evaluation of the observed frequencies of *partial and total necrosis* in *treated and untreated animals* shows a value for χ^2 of 1.2 and for P of 0.27. Hence the results in the two groups are not significantly different. The death rate for the heparin treated animals is slightly greater than for the controls but the difference is not significant ($\chi^2 = 1.7$ and $P = 0.18$). Table I.

Use of Heparin in Less Severe Frostbite. Since our results were not in agreement with those of Lange *et al.* we sought reasons for the discrepancy. The most probable explanation for the differences seemed to be that we did not produce the same degree of injury in our experiment that Lange had in his. Therefore, we first had to standardize our method of producing cold injury. The use of the rubber boot as described by Lange is a possible source of error. Fixation by longitudinal strips allows a considerable amount of air to remain between the leg and rubber boot, which might vary considerably in amount with different sizes, shapes, or positions of the legs. The heat conductivity is considerably altered by the layer of air around the leg and the degree of this change depends on the quantity of air in the boot.

For practical reasons experiments on frostbite with short exposure times must be performed in a fluid with a low freezing point, such as alcohol. Alcohol has an injurious effect on the skin as we could observe in our experiments. Therefore, it is not possible to escape the use of a protective covering which would have been the simplest way to avoid difficulties from altered conductivity.

We overcame this difficulty by attaching the rubber boot as closely to the leg as possible. After the boot was pulled over the foot and fixed loosely with circular adhesive strips at the ankle, the air was pressed out of the boot. The entire depilated limb was then covered with a thin layer of wool fat and the rubber boot pulled over the leg thus making it adherent to the skin. Finally the boot was fixed above the knee by a broad circular strip of tape and at the ankle with a narrow adhesive band. Folds in the rubber boot which sometimes could not be avoided were moved to the posterior aspect of the leg.

The legs of animals prepared in this way were immersed for 30 minutes in a bath of from 0°C to −25°C (+32°F to −13°F) with intervals of 5°C. The actual temperatures in different depths of the exposed leg were recorded simultaneously throughout the exposure and period of thawing.[10]

Judging from the clinical results and from the recorded temperatures inside the leg, the experiments with the rubber boot closely adherent were reproducible to a high degree.

For an exposure time of 30 minutes the temperature range of the freezing bath between −10°C and −15°C (+14°F to +5°F) was of greatest interest (Table II). At −10°C (+14°F) 25 of 26 animals did not develop necrosis and one showed superficial gangrene of the frozen limb; at −15°C (+5°F) 4 animals of 38 showed no necrosis, 16 partial necrosis, and 12 complete loss of the frozen leg. Six died before the fate of the frozen leg was decided. This distribution indicates that the injury of the group exposed at −15°C (+5°F) was just past the borderline from which spontaneous recovery was not possible for the overwhelming majority of our animals. We therefore assumed that any beneficial effect of heparin in the prevention of gangrene after frostbite would become evident under these conditions.

The actual temperatures in the deep muscle recorded in this group during exposure are much higher than in those immersed for 30 minutes at −25°C. On the average they barely passed the range of −6° to −7°C considered by Lake[11] to be decisive for the occurrence of

[10] Pichotka, J., and Lewis, R. B., to be published.

[11] Lake, N. C., *Lancet*, 1917, **2**, 557.

TABLE II.
Results from Exposure for 30 Minutes at Different Temperatures without Treatment.

Bath temp.	Avg deep muscle temp.	No necrosis	Partial necrosis	Complete necrosis	Died	No. animals
—10°C	—3.3°C	25	1	0	0	26
—15°C	—7.4°C	4	16 (1)	12 (1)	8	38

TABLE III.
Results of Heparin Treatment in Less Severe Frostbite.

No. animals	No necrosis	Partial necrosis	Complete necrosis	Died	Survived
Treated—72	3 (2)	21 (6)	22 (12)	46	26
Controls—38	4	16 (1)	12 (1)	8	30

110 animals exposed at —15°C for 30 minutes with rubber boot adherent to the leg.
72 animals received 3 cc of heparin every 6 hours for 6 days.
38 animals were controls.
The incidence of necrosis (partial and complete) is the same in both groups. ($\chi^2 = 0.26$. $P = 0.62$).
The distribution of the results of "no necrosis," "partial necrosis," and "complete necrosis," is essentially the same. ($\chi^2 = 0.46$ and $P = 0.95$).
The increase in the death rate of the treated animals is highly significant. ($\chi^2 = 16.6$ and $P = <0.0001$).

frostbite necrosis.

One hundred and ten animals were subjected to freezing at −15°C for 30 minutes; 72 of these were treated with 3 cc of heparin every 6 hours. The results are given in Table III.

The statistical evaluation of the results of the injury as determined by the chi-square test, including those cases in which the fate of the leg was determined at the time of death, shows that there is no significant difference between treated and untreated animals insofar as the incidence of necrosis is concerned ($\chi^2 = 0.256$ and $P = 0.62$). There is also no difference in the degree of final injury. If the results are computed with regard to the distribution of cases with "no necrosis," "partial necrosis," and "complete necrosis," $\chi^2 = 0.46$ and $P = 0.95$. On the other hand the death rate of the treated animals is significantly increased. ($\chi^2 = 16.6$ and $P = <0.0001$.)

Fatalities During Heparin Treatment. As already mentioned, the fatalities in the heparin-treated groups were much more numerous than in the corresponding controls. Of 153 heparin-treated animals 74 were lost during the 6 days of treatment whereas of 80 controls only 17 animals died. The difference in the death rate between these two groups is highly significant ($\chi^2 = 15.2$ and $P = 0.0001$). The fatalities in the heparin-treated series can be divided into two main groups. In one group, the animals exhibited a shock-like condition after the first or second injection of heparin without hemorrhages. The second group consisted of fatal hemorrhages which occurred in the course of the treatment.

It was soon apparent that fatalities without hemorrhages after the first injection of heparin were directly related to the time which elapsed between injury and heparin injection. This is shown for 72 cases under homogeneous experimental conditions and with intervals between injury and first injection of from 0-12 hours (Table IV). The increase in death rate occurring with increasing intervals between

TABLE IV.
Death Rate of Animals in Relation to the Time Interval Between Cold Injury and the First Injection of Heparin.

Interval between injury and first injection	Survived	Died	Total
0– 2 hr	22	0	22
2– 4 "	16	1	17
4– 8 "	10	7	17
8–12 "	8	8	16

The statistical evaluation shows that the increased death rate with increasing interval between injury and first heparin injection is significant. ($\chi^2 = 20.6$ and $P = 0.00015$).

injury and first injection is significant ($\chi^2 = 20.6$ and $P = 0.00015$).

The deaths from internal hemorrhages were far more numerous than those of the first type. Fifty-three of 153 heparin treated animals died from fatal hemorrhages into the peritoneal cavity, the retroperitoneal space, or into the lungs. The amount of heparin given before death occurred from hemorrhages differed greatly. Some animals developed hemorrhages after the first injection of 30 mg of heparin, others after more than 20 injections. On the average the animals which died from acute hemorrhages received 300 mg of heparin in a period of 60 hours by a regime of either 2 cc every 4 hours or 3 cc every 6 hours.

There was a distinct seasonal difference in the occurrence of hemorrhages during heparin treatment. Fatal hemorrhages occurred more often and with much smaller doses during the winter months. The reason for this observed seasonal difference is not apparent at this time.

Discussion. A comparison of our results with those of Lange *et al.* is possible only if the cold injury is determined by the physical conditions under which it occurs. We have shown in another investigation that under conditions which produce gangrene as a result of acute local cold injury, the immersed hind leg of a rabbit behaves practically like a dead body.[10] The time course of the temperatures in the exposed limb and the clinical results are very much the same in all animals investigated under the same standardized conditions. We should therefore be able to reproduce a comparable degree of cold injury by applying the same experimental conditions.

The cold injury in Lange's experiment was produced by two different procedures. One group of animals was exposed for 45 to 90 minutes to an alcohol dry ice bath which varied in temperature between −12°C and −20°C;[2] the other group was frozen for 30 minutes at −30°C.[5]

The data concerning the first group are not exact enough to permit reproduction. The extreme possibilities for these conditions include almost 100% probability for complete preservation of the immersed legs as well as 100% probability for complete loss. The second regime, *i.e.*, exposure for 30 minutes at −30°C, invariably produces very severe frostbite. But since Lange in a personal communication informed us that the experiments were conducted between −20°C and −30°C, and since in a control series at −25°C very severe frostbite always occurred, we used this latter temperature for the first part of our experiments. During this part of the experimental work the rubber boot was loosely attached by longitudinal strips, as described in the papers of Lange *et al.*[2] We failed completely to find any beneficial influence of heparin. Treated and untreated animals showed the same results. The number of animals was large enough to exclude the possibility that the results were due to random variation. (Table I.) We cannot give a reasonable explanation for the differences between Lange's and our results.

The maintenance of the blood coagulation time between 30 and 60 minutes is not possible when heparin is given intravenously at 12 or 6 hour intervals as described in Lange's publications. In 28 experiments we administered 2 cc of heparin intravenously to rabbits weighing 2,000-3,000 g. Blood clotting times were prolonged to 8 or more hours immediately, but within a few minutes they dropped sharply to reach the normal range within 4 hours or less. More exact investigations on the action of heparin will be published in another paper.

A more accurate evaluation of heparin in the prevention of frostbite gangrene was possible when we were able to perform the experiments with a degree of injury which did not preclude recovery. Systematic investigations showed that a feasible degree of injury could be obtained by an exposure to an alcohol bath of −15°C for 30 minutes with the protective rubber boot closely adherent to the depilated leg.

The relation of occurrence of frostbite to degree of injury is shown in Table V. The progression of the severity of injury from a bath temperature of −12°C to a bath temperature of −15°C with deep tissue temperatures of −3.3°C and −7.4°C, respectively, is rather

TABLE V.
Incidence of Necrosis in Relation to Degree of Injury.*

°C for 30 min.	Temp. in deep tissue (No. of measurements in parentheses) °C	No necrosis	Partial necrosis	Complete necrosis	Total
0	+6.8 (2)	12	0	0	12
—5	+2.2 (2)	12	0	0	12
—10	—0.3 (7)	25	1	0	26
—12†	—3.3 (6)	27	5	0	32
—15	—7.4 (14)	4	16	12	32

* Animals which died before the fate of the tissue was decided are not considered.
† These animals were exposed to a bath temperature of —15°C with different conditions of conductivity (rubber boot not adherent). By measurement of the temperatures in the leg and by interpolation we determined this temperature to correspond to —12°C with the higher conductivity (rubber boot adherent).

striking. While at −12°C only 5 of 32 showed tissue necrosis, at −15°C 28 of 32 suffered gangrene. The distribution of the degree of injury in the group exposed to −15°C, *i.e.*, 12 cases of total gangrene, 16 cases of partial gangrene, and 4 cases without necrosis, should furnish an ideal basis for the evaluation of treatment of acute cold injury. The beneficial effect would become apparent in a shift to the groups of lesser injury.

As shown in Table III, the statistical computation of treated and untreated animals with this degree of injury does not show such a shift. There is significantly no difference in the distribution of the degree of injury between the two groups ($P = 0.95$).

Several other investigators who attempted to repeat Lange's experiments came to the same conclusions as we did. Quintanilla *et al.*[7] were not able to demonstrate any beneficial effect of heparin after various degrees of frostbite. Fuhrman and Crismon[6] in their experiments also did not obtain favorable results from heparin treatment.

It is difficult to explain the failure of several investigators to reproduce Lange's results. The only major point of Lange's experimental procedure which we did not follow was in the use of anesthesia. But Fuhrman and Crismon,[6] Schumacker *et al.*,[8] Quintanilla *et al.*,[7] anesthetized the animals before freezing and did not obtain better results.

The assumption that Lange *et al.* actually produced less severe injuries in their experiments than we did in reproducing them does not abolish this discrepancy. In the second phase of our experiments heparin proved to be ineffective even in the least degree of cold injury that is followed by gangrene. There remains the possibility that some of the factors not sufficiently controlled, such as species of animals used, food, acclimatization, temperatures at which the experiments were performed or at which the animals were kept during the time of observation, might prove to be of major importance.

The incidence of fatalities due to heparin treatment was of secondary interest in our investigations. However, it must be considered in the evaluation of heparin as a therapeutic agent. Furthermore, the death rate determines very much the value of the statistical analysis of the therapeutic results.

The statistical computation applied to our data is not correct in a strict sense. The results were tabulated under the headings of "no gangrene", "partial gangrene", "total gangrene", and "death". These groups are not comparable from a statistical point of view. We can reasonably assume that "no gangrene", "partial gangrene", and "total gangrene" constitute a sequence of increasing severity. Death of the animals either with or without treatment is not comparable to the other groups. Death occurred when, according to the clinical appearance, the injured leg was completely lost, or partially lost, or even completely saved. In 2 large groups we observed that the fatal outcome was due to the delayed first injection of heparin or to seasonal changes in sensitivity to heparin. We do not see how it is possible to determine whether the probability of dying or surviving under these conditions is or is not the same for the three

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.