

STUDIES ON THE DERMAL CONNECTIVE TISSUE BARRIER OF
ALLOXAN-DIABETIC RABBITS AND ITS RECONSTITUTION
AFTER ADMINISTRATION OF TESTICULAR AND
BACTERIAL HYALURONIDASE

BY LENNART JUHLIN
UPSALA, SWEDEN

In non-diabetic rabbits the dermal connective tissue barrier and its reconstitution after supramaximal doses of hyaluronidase were studied by measuring the spread of haemoglobin injected intracutaneously immediately after death. A transient hyaluronidase-insensitive barrier to spread was found 6 hours after the administration of bacterial or testicular enzyme. At the same time a hyaluronidase-sensitive barrier had also formed after the testicular enzyme but not after the bacterial enzyme (Juhlin 1956 a, b).

Reports on the connective tissue in diabetic conditions are scanty and no studies on the state of the dermal connective tissue barrier seem to have been published. Altshuler & Angevine (1951) found the metachromatic substances in human connective tissue to be increased in diabetes and took this as evidence for an increased amount of acid mucopolysaccharides. Azerad et al. (1956) have reported what seems to be similar findings in some but not all of a group of diabetic patients. Schiller & Dorfman (1955) were able to isolate less hyaluronic acid and chondroitinsulfuric acid from the skin of alloxandiabetic rats than from controls. In these animals they also injected ^{14}C -carboxyl-labeled sodium acetate and $\text{Na}_2^{35}\text{SO}_4$. They found a decreased turnover rate of hyaluronic acid, while that of chondroitinsulfuric acid was unaffected, and suggested that the synthesis of the connective tissue mucopolysaccharides was inhibited in these diabetic animals.

In untreated diabetics the glucose content of the skin is increased (for ref. see Urbach 1946). The increased amount of glucose in the skin was thought to make it a better bacterial substrate, thus explaining the prevalence of dermal infections in diabetics. The connective tissue barrier, however, is also of importance for spreading of infections (Duran-Reynals 1942). It would therefore be of interest to determine whether the barrier, or its reconstitution after break-down by hyaluronidase, is different in diabetes. With these aims in mind the present experiments were performed.

*From the Department of Pharmacology, University of Upsala, Sweden.
(Chief: Professor Ernst Bárány, M. D.)*

Methods

Test animals

Male albino rabbits weighing 1.8—2.2 kg before the alloxan injection and of a strain inbred for several years were used. They were fed on hay, oats and water ad lib.

Indicator

The same solution of human haemoglobin as in earlier experiments (Juhlin 1956 a) was used throughout. All injections were made by the same person, the rate of injection being about 0.1 ml/sec.

Hyaluronidase

(a) Hyalas[®], (Leo, Hälsingborg, Sweden). Highly purified testicular hyaluronidase. This was injected intracutaneously in a dose of 6 VRU per wheal.

(b) Hyason[®], (N. V. Organon, Oss, Holland). Highly purified staphylococcal hyaluronidase. The dose used was 0.6 VRU per wheal.

The hyaluronidase solutions were prepared immediately before use by addition of sterile saline or haemoglobin solution. The doses used were about 100 times greater than those necessary to produce the maximal effect. For comparison between the properties and doses of the enzymes see Juhlin (1956 a).

Experimental procedure

Alloxan injection

Alloxan (Eastman-Kodak) was administered intravenously during 2—3 minutes in a dose of 150 mg/kg body wt. as a freshly-prepared, 5 per cent solution in distilled water. To counteract early death from hypoglycaemia glucose was given during the first 24 hours as described by Bárány & Brolin (1953). After 5 days and on the day before the spreading experiments urine-sugar was determined with Clinitest Reagent Tablets[®] (Ames Company, London). Animals with less than 0.5 % glucose in the urine on both occasions were discarded.

The alloxan treated animals were weighed again on the day before the spreading experiments. Some animals had now increased in weight and some had decreased. The mean body weight was slightly decreased and the range was now 1.45—2.20 kg. No correlation was seen between the degree of glucosuria and change in body weight.

Nitrogen and Hexosamine determinations

Circular pieces (5.72 cm²) were taken from the middle of the dried skin. The hair was carefully removed by shaving and with the aid of some barium sulphide paste. The paste was washed off under running water. The skin pieces were weighed after being kept at 80° C for 24 hours. The nitrogen was then determined according to a modification of Kjeldahl's macro-method, as described by Hallgren (1953). On similar skin pieces Blix's modification (1948)

of Morgan-Elson's method was used for hexosamine determinations. Control experiments showed that the use of the barium sulphide paste was no source of error.

Depletion experiments

In order to determine whether the skin could be depleted of mucopolysaccharides by repeated injections of hyaluronidase the following experiments were performed. The day before, the fur on the back of the rabbit had been shorn with ordinary scissors. On one side of the backbone a wheal with testicular hyaluronidase (6 VRU/ml) and another with bacterial hyaluronidase (0.6 VRU/ml) were injected. The other side was used as control side where only saline was injected. The limits of the wheals were marked out with an indelible pencil. One ml. hyaluronidase solution, or saline was injected in the same skin area 6, 4, 2 and $\frac{1}{2}$ hours before the animal was killed. Some minutes before death the hyaluronidase wheals were also injected with 1 ml saline. After death the skin was dissected free and fixed with even tension on a cork sheet. The blood vessels were emptied by gentle pressure and the skin dried about 12 hours at 80° C. Circular pieces were then taken from the site of the wheals and treated for hexosamine determinations as described above.

Spreading experiments

The spreading experiments were started 3—4 weeks after the injection of alloxan. The day before, the fur on the back of the rabbit was shorn with ordinary scissors. The general procedure was as described in detail by Juhlin (1956 a).

The rabbits were pretreated by injecting 0.6 ml hyaluronidase or 0.5 ml saline symmetrically at 8 points in the skin of the back. The margins of the wheals were marked out. The animals were then allowed to move about freely in cages.

After 6 hours the animals were killed by a blow on the head. As soon as the heart stopped beating 0.3 ml of haemoglobin solution with or without hyaluronidase was injected into the pretreated areas. One hour later the skins were dissected free, fixed on a cork sheet, and dried. The areas of spread could then be traced onto paper and measured by weighing the cut-out tracing.

The arrangement of the wheals on the skin of the dorsum was the same as described by Juhlin (1956 a). The wheals were classified as follows.

- Wheal A. Injection of haemoglobin into skin pretreated with saline.
- Wheal B. Injection of haemoglobin into skin pretreated with hyaluronidase.
- Wheal C. Injection of haemoglobin + hyaluronidase into skin pretreated with saline.
- Wheal D. Injection of haemoglobin + hyaluronidase into skin pretreated with hyaluronidase.

The following table (I) is reproduced from Juhlin (1956 a) and indicates the information obtainable from the relationship between the wheal areas.

Results

The results are given in table II—V. The following conclusions may be drawn.

Table I.

Expression	100 x	Explanation	Interpretation
I	C/A-1	Increase in spread due to hyaluronidase, per cent of control area (A)	Expression of maximum effect
II	B/A-1	Increase in spread due to pretreatment with hyaluronidase, per cent of control area (A)	Expression of residual effect
III	D/B-1	Increase in spread due to second injection of hyaluronidase into pretreated area, per cent of pretreated area (B)	Expression of the amount of hyaluronidase-sensitive barrier formed
IV	C/B-1	Difference between non-pretreated area injected with hyaluronidase and pretreated area, per cent of pretreated area (B)	Expression of total amount of barrier formed
V	C/D-1	Difference between non-pretreated and pretreated areas injected with hyaluronidase, per cent of hyaluronidase-injected and pretreated area (D)	Expression of the amount of hyaluronidase-insensitive barrier formed

Table II.

	Source of hyaluronidase injected	Mean of the areas in cm ² ± the standard error of the mean			
		A	B	C	D
Control animals	Test.	3.80 ± 0.08 (n = 16)	6.09 ± 0.25 (n = 23)	9.29 ± 0.19 (n = 24)	7.38 ± 0.28 (n = 14)
	Bact.		7.17 ± 0.22 (n = 29)	9.74 ± 0.36 (n = 10)	7.67 ± 0.34 (n = 15)
Diabetic animals	Test.	3.74 ± 0.10 (n = 16)	5.85 ± 0.36 (n = 15)	10.64 ± 0.66 (n = 16)	9.11 ± 0.43** (n = 15)
	Bact.		6.43 ± 0.32 (n = 14)	10.52 ± 0.62 (n = 15)	8.38 ± 0.50 (n = 13)

n = Number of animals

Test. = Testicular hyaluronidase

Bact. = Bacterial hyaluronidase

P. = The probability that the difference between diabetic and nondiabetic animals is caused by random factors

** = P < 0.01

() = P < 0.02

* = P < 0.05

Table III.

	Source of hyaluronidase injected	100x {mean of the ratios - 1 ± the standard error of the mean				
		$\frac{B}{A} - 1$	$\frac{C}{A} - 1$	$\frac{D}{B} - 1$	$\frac{C}{B} - 1$	$\frac{C}{D} - 1$
Control animals	Test.	65 ± 5 (n=23)	143 ± 11 (n=24)	32 ± 5 (n=13)	53 ± 8 (n=13)	17 ± 4 (n=13)
	Bact.	90 ± 6 (n=29)	153 ± 7 (n=45)	5 ± 6 (n=15)	32 ± 8 (n=15)	26 ± 5 (n=10)
Diabetic animals	Test.	62 ± 8 (n=15)	$191 \pm 18^*$ (n=16)	$61 \pm 9^{**}$ (n=15)	$86 \pm 10^{***}$ (n=15)	18 ± 6 (n=15)
	Bact.	$70 \pm 7^*$ (n=14)	182 ± 19 (n=15)	$37 \pm 10^{**}$ (n=13)	$64 \pm 12^*$ (n=13)	25 ± 6 (n=13)

For explanations see table II.

Table IV.

	Mean of the amount of N in g per 100 g dry skin ± the standard error of the mean.	Mean of the amount of hexosamine in mg per 100 g dry skin ± the standard error of the mean.
Control animals	16.2 ± 0.06 (n=14)	419 ± 12 (n=8)
Diabetic animals	16.3 ± 0.09 (n=16)	410 ± 8 (n=8)

Table V.

Enzyme used for depletion	Amount of hexosamine in mg per 100 g dry skin
Testicular hyaluronidase	282, 293, 314, 316, 321. Mean = 305
Bacterial hyaluronidase	266, 318, 324, 334, 350. Mean = 318
Saline controls	393, 396, 409, 410, 435. Mean = 409

1. The spread of haemoglobin alone (A) in intact skin was the same in diabetic and control animals. Nor was the spread of haemoglobin with hyaluronidase (C) significantly altered, though some increase was noted.
2. In areas pretreated with bacterial hyaluronidase a hyaluronidase-sensitive barrier (D/B) had formed after 6 hours in diabetic animals whereas in controls no enzyme-sensitive barrier was formed. The hyaluronidase-sensitive barrier normally formed after testicular enzyme was increased in diabetic

- animals ($P < 0.02$). For bacterial enzyme the effect of the hyaluronidase-sensitive barrier (D/B) was due to some increase in D and some decrease in B. For testicular enzyme it was mainly due to an increase in D.
3. The expression for the hyaluronidase-insensitive barrier (C/D) formed after break-down with hyaluronidase was the same in diabetic and control animals.
 4. In keeping with the results 2) and 3) some increase in diabetic animals of the total amount of barrier formed (C/B) was noticed ($P < 0.02$ resp. 0.05) and the residual effect (B/A) after bacterial enzyme was slightly decreased ($P < 0.05$).
 5. The hexosamine and total nitrogen content per mg dry skin was the same in diabetic and control animals. In areas pretreated with repeated doses of hyaluronidase the hexosamine content was diminished by about 25 %.
 6. No difference in spread was found between animals which had decreased or increased in weight. No correlation could be found between spread and skin thickness or the hexosamine or nitrogen in the skin.

Discussion

Hyaluronic acid and chondroitinsulfuric acid have been isolated from the skin of different species whereby in general similar values have been obtained for the yield of the isolated substances (Meyer & Chaffee 1941; Pearce & Watson 1949; del Conte et al. 1954; Schiller & Dorfman 1955). Schiller et al. (1956) in studies on the half-life times of hyaluronic acid and chondroitinsulfuric acid found comparable analytical data in rabbits and rats. Schiller & Dorfman (1955) also isolated hyaluronic acid and chondroitinsulfuric acid from the skin of diabetic rats where the values found were respectively 30 mg and 26 mg per 100 g dry skin against 60 mg and 38 mg in normal rats. Calculated from the values obtained by Schiller et al. (1954) these would correspond to a total of about 36 mg acid mucopolysaccharide hexosamine in normal and 20 mg per 100 g dry skin in diabetic rats.

In this investigation about 410 mg hexosamine per 100 g dry skin was found, figures which are in rather good agreement with those of Schlamowitz et al. (1950) in rabbit skin, Bowes (1951) in ox hide and Consden et al. (1953) in subcutaneous connective tissue from the human elbow. Part of the hexosamine found might be due to neutral mucopolysaccharides and mucoproteins. The observed discrepancy between the present results and those of Schiller & Dorfman seems, however, chiefly to be due to the fact that isolation of the acid mucopolysaccharides is not a suitable method for quantitative determinations of total content (Schiller et al. 1954). That a large part of the total hexosamines in skin is derived from hyaluronic acid is evident from the hexosamine determinations in hyaluronidase-pretreated skin areas. The hexosamine content decreased by about 100 mg per 100 g dry skin after pretreatment with testicular or bacterial hyaluronidase. The value obtained for lost hexosamine thus are in considerable excess of the total values for acid mucopolysaccharide hexosamine reported by earlier authors even though the enzyme treatment probably did not affect the entire thickness of the skin and the absorption of the breakdown products may have been incomplete.

In the present experiments no difference in hexosamine content was found between diabetic and normal animals. If such a marked diminution of the amount of hyaluronic acid as noted in diabetics by Schiller & Dorfman (1955) had occurred it should have been detectable by the hexosamine determinations. A possible cause of the diminution found by Schiller & Dorfman may be that the mucopolysaccharides in diabetic animals are more easily lost during the isolation procedure, possibly due to different degrees of polymerisation. The discrepancy could also be due to species differences.

Since about 75 per cent of the total nitrogen of skin is due to collagen (Eisle & Eichelberger 1945), nitrogen was determined to get some information on the relation between the amount of collagen and spread. No correlation could be seen between skin thickness or nitrogen content per cm² and spread of haemoglobin with or without hyaluronidase, which would indicate that spread in these cases is independent of the amount of collagen present.

The main difference in spread between non-diabetic and diabetic animals was an increase in the latter of the hyaluronidase-sensitive barrier (D/B) built up 6 hours after break-down by bacterial or testicular hyaluronidase. The same increase in the newly-formed hyaluronidase-sensitive barrier has previously been found in skin pretreated with 48/80 to degranulate the mast cells (Juhlin 1956 b). It is of interest in this connection that Cottenot & Tanret (1953) found an abundance of mainly degranulated mast cells in the epidermis of diabetic patients.

Some alloxan-treated animals had increased in weight and some decreased. In animals which lost weight the skin was usually thinner than in those which had gained. No difference in spread was, however, obtained between these two groups. Hence, it is not likely that the increase in the hyaluronidase-sensitive barrier is due to a decrease in skin or body weight.

An increase in serum of glucoproteins and hexosamine is known to occur in severe diabetes (for ref. see Törnblom 1955 and Andreani & Gray 1956). Increased amounts of mucoproteins, probably chondroitin sulfate (Craddock & Kerby 1955) and of glucuronic acid (Brox 1953) have also been found in the urine of diabetic patients. It is not known whether such substances act as precursors of the hyaluronidase-sensitive material built up after break-down of the tissue mucopolysaccharides with hyaluronidase.¹ The possibility can therefore not be excluded that the increase in newly formed enzyme-sensitive barrier (D/B) is due to an increase in the serum of precursors of the mucopolysaccharides of connective tissue.

Schiller & Dorfman (1955) in a preliminary report suggested a decreased rate of turnover for hyaluronic acid in the skin of diabetic rats. The turnover rate was determined by measuring the amount of C¹⁴-acetate in isolated hyaluronic acid 1 and 5 days after injection of the acetate. Their results are difficult to interpret, since they gave no data for the turnover rate of the total acetate-pool which might influence their results.

The functional importance of the increased hyaluronidase-sensitive barrier in diabetic animals is obscure. It might inhibit the local spread of an inflammatory process, when hyaluronidase is produced in submaximal doses. If it is

¹ A review of the relationship between circulatory seromucoides and mucopolysaccharides of connective tissue is given by Jayle & Boussier (1955).

permissible to draw conclusions from studies on the spread of spherical particles of different sizes (Juhlin 1956 c) it is not probable that hyaluronidase is of importance as a direct-acting spreading factor for bacteria. The spread of bacteria may, however, be influenced by the formation and motion of oedema fluid around an inflammatory focus (Juhlin 1956 d), and movement of oedema might well be influenced by hyaluronidase and thus indirectly affect the invasiveness of bacteria.² A decreased spread of bacteria or toxic substances may result in their concentration within a smaller area of tissue and thus more easily produce a lesion (Duran-Reynals 1942, Sprunt 1954). Such a chain of events might be a factor contributing to the high incidence of localized necrotic and suppurative processes in diabetic skin, although direct experimental evidence is lacking.

SUMMARY

Dermal connective tissue of alloxan-diabetic rabbits has been examined with respect to its content of hexosamine and nitrogen. In control rabbits the amount of hexosamine was also determined in skin areas which had been depleted of hyaluronic acid by repeated injections of bacterial or testicular hyaluronidase. The barrier against spreading of haemoglobin with or without testicular or bacterial hyaluronidase in supra-maximal doses injected immediately after death, was examined in both non-pretreated skins and skins which had been pretreated with hyaluronidase 6 hours earlier. The following results have been obtained:

No differences in the hexosamine and nitrogen content of the skin were found between diabetic and control animals. In the depletion experiments the amount of hexosamine derived from hyaluronic acid in rabbit skin has been shown to be at least 100 mg per 100 g dried tissue.

In skin areas not pretreated with hyaluronidase no difference was found between diabetic and non-diabetic rabbits in the spread of haemoglobin alone or haemoglobin with hyaluronidase. In skin pretreated with bacterial or testicular hyaluronidase the hyaluronidase-sensitive barrier re-formed after 6 hours was increased in the diabetic animals. No correlation was found between the spreading areas and body weight, skin thickness or the amounts of hexosamine and nitrogen in the skin.

RÉSUMÉ

Le tissu connectif du derme dans le diabète alloxanique du lapin a été étudié en vue d'établir les quantités d'héxosamine et d'azote qu'il contient. Chez les animaux de contrôle, la quantité d'héxosamine a été également déterminée, sur des parties de peau ayant subi plusieurs injections d'hyaluronidase d'origine bactérielle ou testiculaire dans le but d'éliminer l'acide hyaluronique.

La barrière limitant la diffusion de l'hémoglobine, avec ou sans hyaluronidase bactérielle ou testiculaire dans des injections à des doses supermaximum fait immédiatement après la mort, a été examinée sur les peaux non traitées ou traitées avec l'hyaluronidase 6 heures auparavant.

Les résultats suivants ont été obtenus:

Il n'a pas été trouvé de différence dans les quantités d'héxosamine et d'azote

² Bøe (1944) found no correlation between the virulence of staphylococci and their hyaluronidase production, whereas Sallman & Birkeland (1950) demonstrated that hyaluronidase is an important factor in the pathogenicity of hemolytic streptococci.

de la peau, entre les animaux diabétiques et ceux de contrôle. Dans les expériences d'élimination de l'acide hyaluronique de la peau du lapin la quantité d'hexosamine issue de cet acide est au moins de 100 mg par 100 g tissu sec.

Dans les parties de peau non traitées préalablement avec l'hyaluronidase, il n'a pas été constaté de différence entre les lapins diabétiques et les non diabétiques dans la diffusion de l'hémoglobine seule ou de l'hémoglobine et hyaluronidase. Dans les parties de peau traitées avec de l'hyaluronidase bactérielle ou testiculaire la barrière de sensibilité à l'hyaluronidase à nouveau présente après 6 heures, est plus importante chez les animaux diabétiques. Il n'a pas été trouvé de relations entre les dimensions des zones de diffusion et le poids du corps, l'épaisseur de la peau ou les quantités d'hexosamine et d'azote contenues dans la peau.

ZUSAMMENFASSUNG

Bindegewebe von Alloxan-diabetischen Kaninchen wurde auf seinen Gehalt an Hexosamin und Stickstoff untersucht. Bei Kontrollkaninchen wurde das Hexosamin auch in Hautbereichen in denen die Hyaluronsäure durch wiederholte Injektionen von Bakterienhyaluronidase oder Hodenhyaluronidase entfernt war, bestimmt. Die Barriere gegen das »spreading« von Haemoglobin, mit oder ohne Hyaluronidase in übermaximalen Dosen gleich nach dem Tode injiziert, wurde in sowohl unvorbehandelter Haut als in Haut welche 6 Stunden früher mit Hyaluronidase vorbehandelt war, untersucht. Folgende Resultate wurden gefunden.

Hexosamin- und Stickstoffgehalt der Haut der diabetischen Tiere sind gleich denen der Kontrolltiere. Die Enzymvorbehandlungsversuche zeigten, dass die Menge der Hyaluronsäure in normaler Kaninchenhaut mindestens 100 mg Hexosamin pro 100 g getrocknetem Gewebe entspricht.

In Hautbereichen die nur mit Kochsalzlösung vorbehandelt waren, ist in »spreading« des Haemoglobins allein oder des Haemoglobins mit Hyaluronidasezusatz zwischen diabetischen und nicht-diabetischen Kaninchen kein Unterschied zu finden. In Haut die mit Bakterien- oder Hodenhyaluronidase vorbehandelt war, die neu aufgebaute hyaluronidase-empfindliche Barriere bei den diabetischen Tieren mehr ausgeprägt. Zwischen der Grösse der Ausbreitungsbereiche und dem Körpergewicht, der Hautdicke oder der Menge von Hexosamin und Stickstoff in der Haut war keine Korrelation festzustellen.

RESUMEN

Ha sido estudiado el tejido conectivo dérmico de conejos con diabetes aloxánica respecto a su contenido en hexosamina y nitrógeno. En el conejo control se determinó también la cantidad de hexosamina en las zonas de piel desprovistas de ácido hialurónico por inyecciones repetidas de hialuronidasa bacteriana o testicular. La barrera que se opone a la diseminación de la hemoglobina con o sin hialuronidasa bacteriana o testicular en dosis supramaximales inyectadas inmediatamente después de la muerte, fué explorada en pieles no tratadas y en pieles que previamente recibieran hialuronidasa 6 horas antes.

Los resultados obtenidos han sido los siguientes:

No se hallaron diferencias de contenido de hexosamina ni de nitrógeno entre la piel de animales diabéticos y la de los control. En las experiencias en zonas de piel inyectada con hialuronidasa, la cantidad de hexosamina derivada del

ácido hialurónico de la piel de conejo ascendía, por lo menos, a 100 mgrs. por 100 grs. de tejido seco.

En las zonas de piel que no habían recibido hialuronidasa no se vió diferencia entre los conejos diabéticos y los no diabéticos en la diseminación de hemoglobina sólo o de hemoglobina con hialuronidasa. En la piel previamente tratada con hialuronidasa bacteriana o testicular, la barrera hialuronidasa-sensible reformada a las 6 horas estaba aumentada en los animales diabéticos. No se halló correlación entre las zonas de diseminación y el peso corporal, espesor de la piel o las cantidades de hexosamina y nitrógeno cutáneo.

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Note added in proof

Boas (Boas, N. F.: Arch. Biochem. 1955, 57, 367.) studying rat subcutaneous tissue found similar values for hexosamine as reported here (0.4 % dry wt.). About 33 per cent of this could be accounted for by the presence of mucopolysaccharides. These results are in good agreement with those obtained in the present experiments.