Metabolism of Glycosaminoglycans in Alloxan Diabetic Rats

I. Changes in Tissue Glycosaminoglycans

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SUMMARY

The changes in glycosaminoglycans and lipids in the aorta, kidney, skin and retina in alloxan diabetic rats have been studied. Maximum increase in the total cholesterol occurred in the aorta and retina; the skin and kidney showed moderate increase. The increase in phospholipid was moderate in the skin, but less in the kidney, aorta and retina. Glycosaminoglycan concentration was highest in the aorta, while the retina contained moderate amounts. The kidney and skin showed much lower amounts. The changes in the different fractions of glycosaminoglycans in these tissues, fifteen days and two months after alloxan administration, have been studied. In the aorta and retina, the ratio of sulfated glycosaminoglycans to hyaluronic acid is normally high and decreases considerably in the alloxan diabetic rat. In the kidney also there is significant decrease in this ratio, but in the skin there is a decrease of both sulfated glycosaminoglycans as well as hyaluronic acid. The possible role of glycosaminoglycans in the development of cardiovascular complications of diabetes has been discussed. DIABETES *21*:1162-67, December, 1972.

The glycosaminoglycans (gg) form important structural carbohydrates of the vascular system and there is considerable evidence that changes in vascular gg may play a role in the development of atherosclerosis.

The predisposition of the diabetic for arteriosclerosis suggests that in diabetes also, the metabolism of gg is deranged. Changes in the gg in the aorta of diabetic patients as well as experimental diabetic animals have been the subject of investigation by several workers recently. Apart from the aorta, the other tissues that have been investigated from this aspect are the kidney, skin and, to a lesser extent, the retina.

Paterson and Heath[1] have reported a significant decrease in the chondroitin sulfate and heparin content of the aorta of the alloxan diabetic rat. Dorfman[2] found a decrease in hyaluronic acid, chondroitin sulfate and heparin content of rat connective tissue. Schiller and Dorfman[3] observed reduced rates of about one-third of the normal in the gg biosynthesis in the skin of the alloxan diabetic rat on the basis of C^{14} glucose and So_4^{-35} uptake. Paterson and Heath[4] further reported that the incorporation of sulfate^{-35} into chondroitin sulfate fraction of the aorta was reduced very significantly in the diabetic rat aorta. On the other hand, Ichida and Kalant[5] found increased concentrations of hyaluronic acid and chondroitin sulfate-A, while chondroitin sulfate-B was somewhat diminished in the aorta of the alloxan diabetic rabbit. They found changes in the diabetic rat aorta which were less striking, but included increased hyaluronic acid and decreased heparin sulfate, while chondroitin sulfate-A concentration as compared to normal varied with age.

Cohen et al.[6] studied the concentration of specific gg fractions in the aorta of rats in which diabetes was produced by 95 per cent pancreatectomy. It was found that at three months, when the blood sugar levels were only minimally elevated, the concentrations of chondroitin sulfate-A, chondroitin sulfate-C and chondroitin sulfate-B markedly increased, whereas six months after pancreatectomy, the concentrations of chondroitin sulfate-A and chondroitin sulfate-C were lower than normal. They attributed the increase in these fractions at three months to both increased synthesis and decreased degradation, while at six months, concomitant with the rise in blood sugar, there was decreased synthesis and increased degradation. Kofoed[7] reported a 36 per cent reduction in the total gg concentration in the skin in alloxan diabetic rats, while hyaluronic acid decreased by 59 per cent and chondroitin sulfate -A by 40 per cent. Heparin concentration was increased 51 per cent. The other sulfated fractions did not seem to be affected. Berenson et al.[8] studied the changes in the gg fractions in human diabetic kidney. The heparin

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sulfate was increased fairly consistently in most of the diabetic samples, particularly in the severe diabetic group, while hyaluronic acid was increased in some groups.

These divergent results have been reported by different workers for the aortic gg in diabetes, while the pattern varied in the skin and kidney. In view of this, the gg have been studied in the aorta, kidney, retina and skin in alloxan diabetic rats, and in addition the lipid levels are measured in these tissues.

MATERIALS AND METHODS

Young male albino rats of average weight 120 gm. were used for the experiment. The rats were divided into two groups and fed the normal laboratory diet (Hind Lever rat feed). One group (twenty rats) served as the control group.

Diabetes was induced in the rats of the other group by a single subcutaneous injection of 180 mg. of alloxan monohydrate per kilogram of body weight. The rats were fasted for forty-eight hours before alloxan administration. Blood glucose and urine sugar were checked at regular intervals from the seventh day. The animals with marked hyperglycemia were selected for the experiment and grouped into two subgroups (ten rats each). The animals of one subgroup were sacrificed fifteen days after alloxan administration, while those of the second subgroup were sacrificed after two months.

The animals in all groups were fasted overnight and stunned by a blow on the head. The following tissues were immediately removed and temporarily stored in ice-cold containers: the aorta (from its origin up to its bifurcation), kidney, skin and retina. The aortas from the rats in each group were pooled.

The tissue (approximately 500 mg. wet weight) in each case was defatted by successive extraction at 60° C. with ethanol:ether (3:1 v/v) followed by chloroform: methanol (1:1 v/v), twice with each solvent for two hours. The extract was used for the estimation of the lipids. Total cholesterol was estimated by the method of Carr-Drekter,[9] phospholipids by the method described in Gradwohl's textbook.[10]

The dry, defatted tissue was subjected to papain digestion at 65° to 70° C. in 0.1 M phosphate buffer, pH 6.5, containing 0.005 M EDTA and 0.005 M cysteine hydrochloride according to the procedure of Laurent.[11] The digestion was carried out for forty-eight hours, with the addition of fresh papain at the end of every sixteen hours. The digest was centrifuged and the clear supernatant was passed through a column of cellulose. The cellulose column (10 cm. x 1 cm., micro crystalline, chromatography grade, E. Merck, Germany) was washed first with distilled water and then with 15 ml. of 1 per cent cetyl pyridinium chloride (CPC). The digest was then passed through the column and washed again with 15 ml. of 1 per cent CPC. Elution was carried out successively with 15 ml. of each of the following solvents in the order given, and each eluate (15 ml.) collected separately: (1) 0.3 M NaCl in 0.05 per cent CPC, (2) 0.28 M $MgCl_2$ in 0.05 per cent CPC, (3) washed with 15 ml. of 0.05 per cent CPC, (4) n-propanol-methanol-glacial acetic acid-water (40:20:15:38.5 by volume) containing 0.4 per cent CPC, (5) washed with 15 ml. of 0.05 per cent CPC, (6) 0.75 M $MgCl_2$ in 0.1 M acetic acid containing 0.05 per cent CPC, (7) 0.75 M $MgCl_2$ containing 0.05 per cent CPC, (8) washed with 15 ml. of 0.05 per cent CPC and (9) 1.25 M $MgCl_2$ containing 0.05 per cent CPC.

These fractions were considered to contain 1—hyaluronic acid, 2—heparin sulfate, 4—chondroitin sulfate -A, 6—chondroitin sulfate -C and 7—chondroitin sulfate -B. Fraction 9 eluted by 1.25 M $MgCl_2$ may possibly contain heparin (H). The identity of the major gg in each fraction was established by several previous workers[12,13] as well as by this laboratory.[14] To each fraction (15 ml.) was added 10 ml. of 1 per cent CPC, then diluted with 45 ml. of water and allowed to stand at room temperature for twenty-four hours. It was then centrifuged for one hour at 3,000 x g and the supernatant decanted off. The precipitate of CPC-gg complex was decomposed by treating with 5 ml. of 95 per cent ethanol saturated with NaCl, three times; each time the suspension was centrifuged for one hour. The residue (free gg) was dissolved in 1.5 ml. of 0.1 M NaOH and a 1 ml. aliquot was used for determining uronic acid by the modified carbazole reaction of Bitter and Muir.[15]

Blood glucose was estimated according to the method of Asatoor and King[16] except that a weakly alkaline copper reagent was used.[17]

RESULTS

1. *Total cholesterol and phospholipid levels of the aorta, kidney, retina and skin*

The total cholesterol and phospholipid levels of these tissues in the alloxan diabetic rats as compared with the normal rats are given in table 1. Maximum increase in the total cholesterol occurs in the aorta and retina, while moderate increase occurs in the skin and kidney.

TABLE 1

Total cholesterol and phospholipid levels of the aorta, kidney, retina and skin in alloxan diabetic rats*

Tissue	Total cholesterol mg./100 gm. wet tissue			Phospholipid mg./100 gm. wet tissue		
	a†	b‡	c§	a	b	c
1. Aorta	502 ± 10.8	848 ± 12.6	1,122 ± 14.6	2,083 ± 16.4	2,559 ± 12.7	3,100 ± 15.8
t's		85.0 (between a & b)	108.4 (between a & c)		82.5 (between a & b)	141.3 (between a & c)
2. Retina	114.5 ± 10.2	540.9 ± 10.1	1,683 ± 15.1	1,960 ± 12.8	2,317 ± 17.8	3,500 ± 13.9
t's		93.9 (between a & b)	272.3 (between a & c)		51.5 (between a & b)	257.5 (between a & c)
3. Kidney	310 ± 14.0	377.8 ± 11.5	528 ± 12.1	2,670 ± 13.8	3,011 ± 9.8	3,475 ± 10.7
t's		11.8 (between a & b)	37.3 (between a & c)		63.7 (between a & b)	148.5 (between a & c)
4. Skin	180.0 ± 12.6	434 ± 10.6	461 ± 13.1	390 ± 12.8	517 ± 10.6	692 ± 11.6
t's		49.7 (between a & b)	48.9 (between a & c)		24.1 (between a & b)	55.3 (between a & c)

* In every case P is less than 0.001.
† a—Normal rats—blood sugar 72.0 ± 3.5 mg./100 ml.
‡ b—Alloxan diabetic; fifteen days after alloxan administration—blood sugar 330 ± 16.5 mg./100 ml.
§ c—Alloxan diabetic; two months after alloxan administration—blood sugar 420 ± 12.8 mg./100 ml.

The increase in every case was much more two months after alloxan administration than after fifteen days (when there was marked hyperglycemia), except in the case of the skin where the value did not appreciably increase after fifteen days. The increase in the phospholipids was moderate in the skin, but less in the kidney, aorta and retina.

2. *Tissue gg levels in alloxan diabetic and normal rats*

The concentration of the different gg fractions in the aorta, kidney, retina and skin in the alloxan diabetic rats as compared to the normal rats is given in table 2 and figure 1. The aorta shows the maximum concentration of gg, while the retina contains moderate amounts. The kidney and skin contain much lower amounts.

The following changes were found in the alloxan diabetic rat tissue. In the aorta, which has the maximum concentration of gg and where the increase in the lipids is also high, hyaluronic acid (HA) increases considerably fifteen days after alloxan administration, and shows a small decrease from this level two months afterward. Heparin sulfate (HS) first increases and then decreases below the level in normal rats. Chondroitin sulfate-A (Ch S-A) and chondroitin sulfate-C (Ch S-C) show a slight initial decrease, which becomes more marked two months afterward. Chondroitin sulfate-B (Ch S-B) and heparin (H) show a small initial increase but decrease afterward, the decrease being much more in the case of Ch S-B. In the retina, HA shows a similar change, heparin sulfate (HS), Ch S-A and Ch S-C decrease progressively, and Ch S-B and heparin first increase and then decrease. In the kidney, HA and HS show slight increase, Ch S-A after an initial increase shows a decrease, while Ch S-C, Ch S-B and H decrease. In the skin, HA and HS show a decrease after a small initial rise, Ch S-A, Ch S-C, Ch S-B and H decrease.

DISCUSSION

The organs that have been most frequently investigated as sites of possible complications in diabetes are the aorta, which is involved in the vascular complications, retina, kidney, and also the skin. Maximum increase in the lipids, particularly cholesterol, occurs in the aorta and retina. Moderate increase in lipids occurs in the kidney and skin. Regarding the concentration of gg in these four tissues, aorta shows the maximum concentration, followed by the retina. The kidney and skin also contain appreciable amounts of the gg.

The following significant fact emerges from the analysis of the ratio of sulfated gg/HA in these four tissues in normal and alloxan diabetic rats. In the normal rat aorta, this ratio is quite high (9:1) and decreases to 6:1 fifteen days after alloxan administration and to 4:1 two months afterward. This decrease in the ratio of sulfated gg/HA correlates with the increase in the lipid in this tissue and also of the blood glucose. A similar picture emerges for the retina, where these values are 8:1, 6:1 and 5:1 for the normal, fifteen

TABLE 2

Tissue gg levels in alloxan diabetic rats

		Micrograms of uronic acid/gm. dry tissue ± S.E.					
		HA	HS	Ch S-A	Ch S-C	Ch S-B	H
1. Aorta*	a†	820	2,432	1,042	1,623	1,075	1,104
	b‡	1,302	2,696	948	1,605	1,106	1,284
	c§	1,240	2,036	720	1,080	741	1,068
2. Retina	a	235±5.2	388±4.6	880±5.8	304±3.9	210±5.6	146±6.7
	b	282±3.9	328±4.1	550±6.2	240±4.0	250±4.9	186±4.8
t's between a & b		22.8	30.8	122.7	36.2	17.0	15.3
	c	268±4.8	215±3.8	392±3.1	206±4.1	161±6.1	137±5.1
t's between a & c		14.7	91.5	234.6	54.7	18.7	3.4**
3. Kidney	a	230±6.2	430±6.4	138±5.1	246±8.6	184±4.2	160±3.9
	b	280±8.6	440±4.1	156±8.4	198±6.5	127±6.2	150±5.1
t's between a & b		14.0	4.2	5.8	14.1	24.1	9.9
	c	264±4.8	454±5.9	103±7.2	113±6.2	106±7.3	104±6.4
t's between a & c		13.7	8.7	12.5	39.7	29.3	23.6
4. Skin	a	326±7.2	204±3.9	272±8.2	144±6.2	235±7.3	140±5.8
	b	336±5.7	215±8.1	251±4.2	135±5.8	161±4.2	127±4.8
t's between a & b		3.4**	3.9‖	7.2	3.3**	27.8	5.5
	c	211±6.8	201±4.7	201±3.7	106±7.1	130±3.9	107±6.9
t's between a & c		36.7	1.6††	25.0	12.8	40.1	11.6

* The samples from the animals in each group were pooled and used. The results reported are the mean of three estimations using three aliquot portions from the aortic digest.
† a—Normal rats.
‡ b—Fifteen days after alloxan administration.
§ c—Two months after alloxan administration.
‖ P, $0.001 < 0.002$
** P, $0.002 < 0.01$
†† P, $0.05 < 0.1$
In all other cases P is less than 0.001.

days diabetic and two months diabetic animals respectively. In the kidney these values are 5:1, 4:1 and 3:1 respectively. In these tissues, along with the over-all decrease in sulfated gg, the HA increases. In the skin, however, the ratio of sulfated gg/HA does not seem to be affected, because there is a decrease in both the sulfated gg as well as HA.

The aorta is especially significant in view of the high

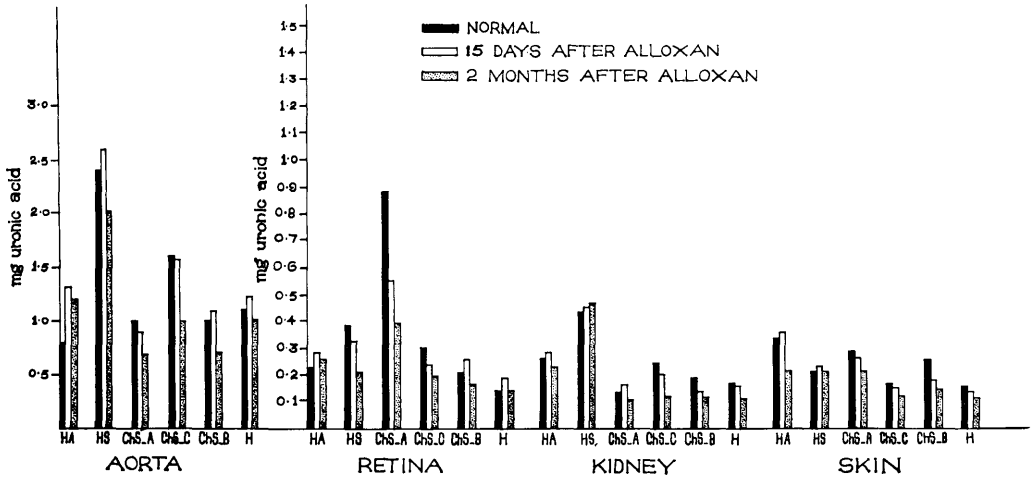

FIG. 1. Tissue glycosaminoglycan levels in alloxan diabetic rats.

concentration of gg present and the marked increase in lipids that occurs both in atherosclerosis and advanced diabetes. Compared with the normal aorta, the gg composition of the aortic wall is considerably altered in alloxan diabetic rat. It is possible that gg may regulate the transport of lipoproteins across the arterial wall. The high concentration of sulfated gg normally present may probably limit the entry of lipoproteins by complex formation at the arterial wall surface. The gg are known to form insoluble complexes with lipoproteins; this complexing ability is much more in the case of sulfated gg than for HA.[18] The concentration of sulfated gg decreases considerably in the diabetic rat aorta, while the serum lipoproteins increase. Hence not sufficient sulfated gg are available at the arterial wall to complex with all the lipoproteins, with the result that free lipoproteins enter the aorta. This might explain the limited entry of lipoproteins into the normal aortic wall from the lumen which is augmented in the atherosclerotic aorta.[19-21]

The decrease in sulfated gg now obtained by us in the alloxan diabetic rat aorta is in agreement with the result reported by Paterson and Heath[1] for chondroitin sulfate and heparin. Dorfman,[2] though he observed decreased heparin sulfate and chondroitin sulfate in alloxan diabetic rat connective tissue, reported decrease in hyaluronic acid, while we have obtained an increase in the hyaluronic acid except in the case of the skin. On the other hand, Ichida and Kalant,[5] even though they reported an increase in hyaluronic acid concentration, found an increase in the concentration of Ch S-A also, while Ch S-B was somewhat diminished in the aorta of alloxan diabetic rabbit. They found changes in the diabetic rat aorta which were less striking, but included increased HA and decreased HS, while Ch S-A concentration varied with age. In our experiments, though, HS showed an initial increase (fifteen days postalloxan), decreased after two months, while Ch S-A showed a definite, progressive decrease.

Cohen et al.[6] reported elevation in the concentration of Ch S-A, Ch S-B and Ch S-C in the aorta three months after 95 per cent pancreatectomy, when the blood sugar was minimally elevated, whereas six months afterward, the concentrations of the Ch S-A and Ch S-C were lower than normal. In our experiments in the fifteen days postalloxan animals, Ch S-A showed a small decrease, Ch S-C was not much affected, but Ch S-B showed a distinct increase, while two months afterward all three showed a definite decrease. The decrease in HA and Ch S-A in the alloxan diabetic rat skin reported by Kofoed[7] is in agreement with our results in the two months postalloxan animals. On the other hand, we could not obtain any increase in the H concentration as reported by that author. Kofoed has also reported that the other sulfated gg fractions did not seem to be affected. This appears to agree with our result for HS, but Ch S-C and Ch S-B showed a definite decrease in our experiments.

Berenson[8] and co-workers observed a fairly consistent increase in the HS in human diabetic kidney, particularly in the severely diabetic group, while HA increased in some groups. Although comparison with the pattern of changes in the human kidney and rat kidney may not be very appropriate, the pattern of changes obtained by us for these two fractions seems to be in agreement with their result.

It is rather difficult to compare data obtained from different investigators in view of several variable factors, such as the animal species used for the study, the method used to induce diabetes, the degree of severity of diabetes obtained, and most important, the methods used in the quantitation of the gg fraction.

The decrease in the sulfated gg in the tissues of alloxan diabetic rats may be due either to their decreased formation or accelerated breakdown. The aspect is being studied in detail.

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