Effect of Hyaluronidase on Experimental Hypercholesterolemia in Rabbits and Rats and Atheromatosis in Rabbits. (20388)

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Both Anitchkow(1) and Aschoff(2) in their studies on atheromatosis suggested that changes in permeability of the arterial wall preceded deposition of cholesterol. Aschoff further suggested that physico-chemical alterations in the ground substance rather than simple permeability changes might be the initial lesion. Both authors agreed that the deposits were secondary. These ideas have recently been revived by Pollack(3) and Reinhardt(4) who utilized more modern methods of observing the ground substance. Since hyaluronidase affects permeability of the ground substance by altering mucopolysaccharides it was of interest to investigate the effects of the enzyme on experimental hypercholesterolemia in rabbits and rats and cholesterol-induced atheromatosis in rabbits.

Materials and methods. The regimen for producing atheromatosis was that of Bauman (5) who showed that 100% of rabbits developed the lesion after ingesting 0.43% cholesterol and 5% cottonseed oil for 14 weeks. Fifteen male and 15 female albino rabbits weighing between 1.3 and 2.4 kg were housed in individual cages and fed a basic diet of Purina Rabbit Checkers (PRC)* and tap water *ad libitum*. The animals were weighed twice weekly and their food consumption was recorded daily. For the purposes of this experiment they were divided into 5 treatment groups of 3 males and 3 females as follows:

Group A—PRC
B—PRC + 5% cottonseed oil (CSO)†
C—PRC + 5% CSO + 0.43% cholesterol (Ch)
D—PRC + 5% CSO + daily 1000 TRU hyaluronidase (H)‡ subcut.
E—PRC + 5% CSO + 0.43% Ch + daily 1000 TRU H subcut.

Total blood cholesterol was determined weekly on Manuronate®§-treated blood by the method of Bloor(6) with the following modifications. The blood was extracted twice with the 3:1 alcohol-ether mixture in glass-stoppered centrifuge tubes. The supernatant was removed after centrifuging and evaporated to the point of dryness. The residue was dissolved in redistilled reagent-grade chloroform and clarified where necessary with iron-free Norite A and recentrifuged. The clear supernatant was treated in accordance with the Lieberman-Burchard reaction. In 100 tests involving cholesterol ranges of 20-1000 mg % the results were statistically indistinguishable from those obtainable by the Sperry-Schoenheimer method(7). The *anti-hyaluronidase activity* of serum was determined by a turbidimetric assay procedure. Several 2-fold dilutions of serum were prepared in volumes of 0.4 ml in 0.1 M acetate buffer at pH 6.0. A concentration of hyaluronidase (8.4 TRU-1000 TRU/mg) that will completely reduce the turbidity of a standardized preparation of vitreous humor potassium hyaluronate was added in a volume of 0.2 ml to each tube. To each tube was also added 0.2 ml of physiological salt solution and 0.2 ml of a solution of purified hyaluronate. After 30 minutes of incubation at room temperature to allow for neutralization of the enzyme by antibodies, the residual unneutralized hyaluronidase was measured by the usual turbidimetric procedure and expressed as per cent inhibition. Normal rabbit serum and an anti-hyaluronidase serum of known potency served as controls. All

* No cholesterol detectable by our method of analysis.

† Puritan Brand (Procter & Gamble, Inc.) contained 0.44% Ch = 0.022% Ch in diet.

‡ Wydase®, Wyeth Laboratories—7000 Turbidity Reducing Units per mg N.

§ A synthetic heparinoid anticoagulant.

sera were treated for 30 minutes at 56°C to eliminate non-specific inhibitors. The rabbits were *killed* by electro-shock and *exsanguination*. The experiment was terminated at the end of 7 weeks because the hyaluronidase treatment resulted in high antibody titers at this time. Heart, lungs, liver, kidneys, spleen, pancreas, adrenals, thyroid, pituitary, gonads, uterus, salivary glands, cervical lymph node, thymus, skeletal muscle, and marrow from the femur were removed. The organs were weighed and specimens fixed and prepared for histological examination. The aorta was dissected free to the bifurcation below the level of the adrenals and sections taken of the descending thoracic aorta and the abdominal aorta at the renal artery level. These were mounted on corks and the intima was stained with oil red 0 according to the method of Lilli(8). The sections were graded without prior knowledge of the treatment each animal had received.

The definition of grades was decided before examination of the specimens and was based on previous studies of the aortas of chickens.

0—No visible oil red 0 stain in the aorta.
1—Particles stained by oil red 0 visible with 10x hand lens.
2—Particles easily visible with naked eye after staining but few in number and discrete.
3—Large particles or plaques, no ulceration.
4—Large and ulcerated plaques.

Microscopic examination. Frozen sections stained with oil red 0 and hematoxylin from representative sections of each specimen were taken at random (preventing pathologist's view of identifying labels) and again graded on a 1 to 4 basis. Grades 1 and 2 represent small and moderate amounts of fat-stained material without displacement of the endothelium, Grade 3 larger plaques with irregular elevations of the endothelium and Grade 4 ulcerated plaques. An attempt was made to provoke the *systemic* Shwartzman reaction in 12 intact and 17 adrenalectomized rabbits. The meningococcus endotoxin which we used was kindly supplied by Dr. Gregory Shwartzman. We found that it contained no measurable cholesterol. It was administered in the doses suggested by him. Cholesterol levels were determined on blood obtained by cardiac puncture on the unanesthetized animal at appropriate intervals in the experiment as indicated in Table II. The rabbits were divided into 4 treatment groups:

Group F—6 intact rabbits received 2 intrav. inj. of meningococcus endotoxin.
G—6 intact rabbits received 5000 TRU hyaluronidase twice daily for 6 days. First dose of toxin administered intrav. on third day of hyaluronidase treatment and second dose on fourth day. All animals sacrificed 48 hr after second inj. of toxin.
H—9 adrenalectomized rabbits—same treatment as Group F.
J—8 adrenalectomized rabbits—same treatment as Group G.

The rabbits were adrenalectomized by the method of Baeder and Seifter(9) and maintained on 0.9% sodium chloride drinking water. No rabbits were used sooner than 1 week after removal of the second adrenal. The *nephrotic syndrome* was produced in 36 Long-Evans rats by injecting anti-rat kidney rabbit serum globulin free of cholesterol. The rats were divided into 4 treatment groups:

Group K—6 rats: Physiological salt sol. intraper. twice daily.
L—12 rats: 1 cc anti-kidney serum/100 g intrav. Eleven rats survived.
M—12 rats: 10000 TRU hyaluronidase daily for 4 days subcut.; on second day of hyaluronidase medication challenging dose of anti-kidney serum intrav. Eight rats survived.
N—12 rats: 100 mg streptococcal hyaluronic acid intraper. twice daily for 4 days; on second day of hyaluronate medication challenging dose of anti-kidney serum intravenously. Eight rats survived.

Seventy-two hours after administration of the anti-kidney serum to Groups L, M, and N, all the rats were anesthetized with ether, the thorax opened, and blood was obtained by

TABLE I. Data of Atheromatosis Experiment.

Sex	Group	Wt (kg) Initial	Wt (kg) Final		Total blood cholesterol levels (mg %) Wk of exp. 0	1	2	3	4	5	6	7	Degree of athermatosis in aorta Thoracic G	Thoracic M	Abdominal G	Abdominal M	Anti-hyaluronidase titer*
♂	A	2.1	3.0		97	72	51	104	92	88	107	74	0	0	1	0	—
♀		2.2	3.6		35	73	84	86	96	80	100	68	0	0	0	0	—
♀		2.0	2.9		96	94	80	99	95	86	103	71	1	0	1	0	—
♀		2.3	2.8		98	85	67	90	88	100	83	85	0	0	0	0	—
				Mean	82	81	70	95	93	89	98	75					
				± S.D.	±28.4	±30.6	±17.3	± 8.3	± 2.7	± 8.5	±10.4	± 7.0					
♂	B	2.1	3.1		109	35	30	92	69	64	151	87	1	0	0	0	—
♂		1.3	3.5		108	102	86	85	91	97	84	76	2	1	1	0	—
♂		1.5	3.9		34	69	105	113	84	87	99	99	1	0	1	0	—
♀		2.3	3.7		60	94	94	108	82	95	82	88	0	0	0	0	—
♀		1.6	3.1		39	108	99	92	91	77	98	91	2	0	1	0	—
♀		1.8	3.2		70	75	107	97	85	91	102	84	1	0	1	0	—
				Mean	70	81	87	98	84	85	103	88					
				± S.D.	±36.7	±28.5	±29.1	±10.8	±10.4	±12.6	±25.1	± 7.9					
♂	C	2.2	3.4		91	96	131	180	228	217	655	177	0	0	0	1	—
♂		2.2	3.6		103	123	156	169	197	146	526	335	1	0	1	2	—
♀		1.7	3.7		95	332	158	199	—	330	428	585	3	3	3	2	—
♀		2.1	3.6		120	357	124	147	217	282	413	533	1	2	1	2	—
♀		2.0	3.0		72	306	200	194	192	—	459	257	2	3	2	2	—
				Mean	96	303	155	178	209	244	496	377					
				± S.D.	±19.0	±13.5	±26.9	±20.9	±21.3	±25.3	±98.8	±175.7					
♂	D	2.2	3.2		70	112	100	90	65	64	100	120	0	0	1	1	1:80
♂		2.4	3.3		62	74	86	67	79	71	66	75	1	0	3	1	1:20
♂		2.2	3.2		71	54	104	109	64	81	98	105	1	0	2	0	1:160
♀		1.8	3.2		66	97	94	110	79	80	101	119	0	0	1	0	1:80
♀		1.5	3.3		89	88	100	91	91	82	105	95	1	1	1	1	1:160
♀		2.0	3.8		74	91	91	—	76	77	112	111	0	2	0	1	1:40
				Mean	72	86	96	93	76	76	97	104					
				± S.D.	± 9.7	±22.0	± 6.8	±17.9	±17.8	± 6.9	±14.1	±17.1					
♂	E	1.7	3.5		70	124	80	122	122	108	275	712	3	3	3	3	1:20
♂		2.1	3.2		113	165	74	126	109	119	302	591	2	2	2	3	1:40
♂		1.6	2.6		34	82	84	98	123	123	282	600	3	1	2	3	1:80
♀		1.9	3.8		71	309	97	145	114	125	244	660	3	3	3	2	"
♀		2.1	3.7		49	113	103	85	137	165	255	643	3	3	3	2	"
♀		1.6	2.5		92	72	138	107	121	207	244	625	3	3	3	3	1:40
				Mean	72	144	96	114	121	141	267	641					
				± S.D.	±28.4	±87.0	±23.1	±21.3	± 9.9	±12.1	±22.9	±12.9					

Two rabbits in Group A were sacrificed 3rd wk of exp. One rabbit in Group C died 4th wk of exp. Cause of death not determined.
* Highest dilution of serum showing at least 10% inhibition. G = Gross grading; M = Microscopic grading.

TABLE II. Total Blood Cholesterol Levels in Rabbits Treated with 2 Intravenous Injections of Meningococcus Endotoxin.

Treatment group		Total blood cholesterol levels (mg %) Control period	24 hr Hyaluronidase treatment	48 hr Hyaluronidase treatment	24 hr after 2nd toxin inj.	48 hr after 2nd toxin inj.
F		72			248	236
		84			203	209
		91			179	196
		65			198	222
		70			196	184
		81			184	192
	Mean	77			201	207
G		61	74	81	135	132
		70	68	82	162	148
		69	82	71	151	129
		91	102	84	140	150
		101	96	96	178	162
		101	90	88	149	159
	Mean	82	85	84	153	147
H		104			195	202
		97			199	224
		72			245	232
		84			239	201
		81			186	172
		78			201	165
		84			180	180
		71			184	175
		86			193	182
	Mean	84			202	192
J		52	68	72	68	71
		101	84	86	109	98
		96	91	92	94	92
		82	84	86	88	89
		80	105	88	85	94
		85	101	100	87	88
		92	89	70	92	95
		87	84	88	94	90
	Mean	84	88	85	90	90

puncture from the beating heart. The *hyaluronidase solution* was prepared fresh each day by dissolving the freeze-dried enzyme in physiological salt solution. The dose of hyaluronidase was much larger than that used clinically, and was selected to be in excess of the amount necessary to neutralize the non-specific inhibitors. The hyaluronic acid was prepared from *Streptococcus pyogenes*. It contained 3.67% N and was free from S, P, and halogen. The solutions were prepared fresh on the day of injection.

Results. The pertinent data for individual animals are found in Tables I, II, and III. For the purposes of our experiment we arbitrarily assigned a total blood cholesterol value of 150 mg % as hypercholesterolemia.

Effect of hyaluronidase and hyaluronic acid on hypercholesterolemia. Fig. 1 shows the prevention of the hypercholesterolemia due to cholesterol feeding in Group E as compared to Group C during the first 5 weeks of the experiment and the rapid rise in blood cholesterol from this time until the termination of the experiment. During the first 2 weeks the lower limit of the standard deviation of the mean total blood cholesterol levels of Group C coincided with the upper limit of the standard deviation of Group E. The standard deviation of Group C did not overlap the other 3 groups at any time during the experiment. Group E did not show definite hypercholesterolemia until the sixth week. After that it was marked and exceeded that of Group C. The abrupt rise coincided with the appearance of high anti-hyaluronidase titers (Table I).

TABLE III. Total Blood Cholesterol Levels of Nephrotic Syndrome Rats.

Treatment group	Total blood cholesterol (mg %)	Treatment group	Total blood cholesterol (mg %)
K	91	M	99
	115		96
	140		73
	112		110
	165		101
	126		90
			98
			100
Mean	125		96
L	171	N	120
	168		96
	236		104
	215		81
	248		92
	181		87
	248		90
	301		101
	241		
	331		
	301		
Mean	240		96

Hyaluronidase had a similar effect on endogenously produced hypercholesterolemia in the rabbits treated for the Shwartzman reaction (Table II) and in the nephrotic rats (Table III). In the toxin-treated rabbits the hypercholesterolemia did not depend on the presence of kidney lesions or on the adrenals. Hyaluronic acid also prevented hypercholesterolemia in the nephrotic rats. The failure to develop hypercholesterolemia did not appear to alter the course of these experimental diseases.

Effect of hyaluronidase on the degree of atheromatosis. Table I shows the results of gross and microscopic grading of aortic lipid content. The minimal change observed microscopically in aortas (Grade 1) consisted only of lipid droplets in the endothelial cells. In larger deposits (Grades 2 and 3) many large foam cells loaded with fat-stained droplets lay beneath the endothelium and elevated it. In the fat stains some material appeared to lie outside the cells but most of the lipid was intracellular. In areas with the greatest deposition, the fat-stained material was seen extending into the wall of the vessel to a depth which indicated penetration into the inner portion of the media. Groups C, D, and E showed increased deposition of lipids in the intima when compared to Groups A and B. Isolated droplets of fat-staining material also were scattered through the media in some animals in Groups C, D, and E. Foam cells were noted even in smaller deposits deep in the media.

Other than the fatty deposits it was difficult to be certain of any differences in the aortas of these animals. The general appearance was similar in the hematoxylin and eosin, Ritter-Oleson polysaccharide and trichrome stains in the several groups. Similar differences were observed in liver, spleen, and kidneys. While in liver and spleen the lipids were deposited in the Kupffer cells, in the kidney they were seen chiefly in the endothelial cells of the capillaries both in the glomeruli and in the parenchyma, but in the more severe cases many large macrophages laden with lipid were found in the connective tissue spaces between capillaries and tubules.

Discussion. The antihypercholesterolemic effect of subcutaneously injected hyaluronidase may be accounted for by 2 hypotheses. The first supposes that hyaluronidase is absorbed from the site of injection into the general circulation and is then capable of increasing the permeability of the ground substance at various sites. The greater rate of deposition of cholesterol would prevent hypercholesterolemia. The antihypercholesterolemic action of intravenously injected hyaluronidase lends support to this view. Further evidence for this hypothesis is the development

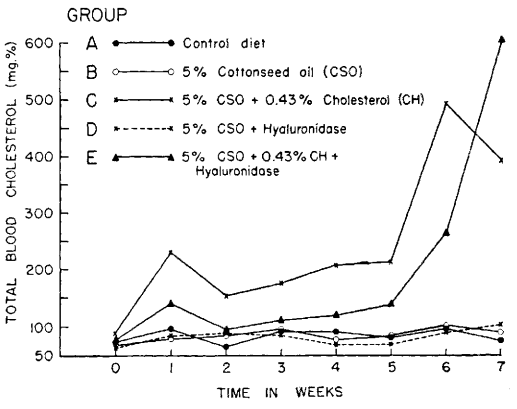

Fig. 1

of the specific anti-hyaluronidase in the circulation following prolonged administration of the enzyme. The alternative hypothesis is that hyaluronidase released hyaluronic acid from the site of injection and possibly from other sites. Hyaluronic acid has been shown to have protective colloid and dispersing action(10). It can be assumed that the hyaluronic acid released in this experiment dispersed the cholesterol in a form favoring rapid deposition. This hypothesis is supported by the fact that hyaluronic acid injected intraperitoneally suppressed the hypercholesterolemia in nephrotic rats.

The finding that the reduction of hypercholesterolemia by hyaluronidase resulted in more extensive arterial involvement casts some doubt on the treatment of human atherosclerosis by agents that lower the blood cholesterol without determining its subsequent distribution and fate. We should like to point out, however, that the morphological alterations observed in this experimental disease resemble those in human lipidoses (Xanthomatoses) rather than those of atherosclerosis. No significance can be attached at the present time to the finding of minimal intimal lesions in rabbits which received cottonseed oil and hyaluronidase without added cholesterol.

Our experiments do not reveal whether hypercholesterolemia developed following the first or second injection of endotoxin. The failure of hyaluronidase to lower the blood cholesterol level as significantly in intact rabbits as in adrenalectomized ones may be due to an anti-hyaluronate effect of the adrenal cortical hormones present in the former group.

It is of interest that a specific antibody was capable of abolishing the antihypercholesterolemic action of hyaluronidase. The circulating antibody probably neutralized the absorbed hyaluronidase before it could react at the various ground substance sites.

Facilities were not available for making ultracentrifuge examinations. If the Gofman theory(11) applies to our experiments the decrease in the blood cholesterol which is accompanied by deposition should be correlated with a decrease in the macromolecular bound cholesterol of the blood.

Summary. 1. Subcutaneous or intravenous injection of hyaluronidase depressed hypercholesterolemia in rabbits and rats. 2. Intraperitoneal injection of hyaluronic acid prevented hypercholesterolemia in nephrotic rats. 3. Hyaluronidase facilitated general lipidoses and aggravated atheromatosis in rabbits. 4. The advisability of indiscriminately lowering the blood cholesterol level in hypercholesterolemics is questioned. 5. The antihypercholesterolemic effect of hyaluronidase was abolished when specific antibodies to the enzyme developed. 6. Endogenous experimental hypercholesterolemia in rabbits does not depend on the presence of the adrenals.

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1. Anitchkow, N., *Deut. med. Wochenschr.,* 1913, v39, 741.

2. Aschoff, L., *Z. Ges. Neurol. Psychiat.,* 1939, v167, 214.

3. Pollack, O. J., *J. Gerontol.,* 1951, v6, 175.

4. Reinhardt, J., *Cal. Med.,* 1951, v75, 335.

5. Bauman, C. A., and Rusch, H. P., PROC. SOC. EXP. BIOL. AND MED., 1938, v38, 647.

6. Bloor, W., Pelkam, H. P., and Allan, D. M., *J. Biol. Chem.,* 1922, v52, 191.

7. Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.,* 1934, v106, 745.

8. Lilli, R. D., *Stain Techno.,* 1944, v19, 55.

9. Baeder, D. H., and Seifter, J., *J. Pharmacol. Exp. Therap.,* 1952, v106, 372.

10. Butt, A. J., Hauser, E. W., and Seifter, J., *Cal. Med.,* 1952, v76, 123.

11. Gofman, J. W., Lindgreen, F. T., Ellent, H. A., Mantz, W., Hewitt, J., Strisomer, B., and Herring, V., *Science,* 1950, v3, 166.

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