

## THE EFFECT OF ASCORBIC ACID ON EXPERIMENTAL ATHEROSCLEROSIS

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Among those factors which affect the development and course of atherosclerosis, ascorbic acid is of particular importance. It is known that ascorbic acid administration decreases cholesterol (1, 4, 6) and lipoprotein (3) levels in the blood of patients with atherosclerosis. In rabbit experiments ascorbic acid prevents the development of alimentary atherosclerosis (5, 11, 12). Ascorbic acid deficiency gives rise to atherosclerosis in guinea pigs even with normal blood cholesterol levels (15). However, some investigators (9, 13) have not observed an inhibitory effect of ascorbic acid on experimental atherosclerosis.

Disagreement in the literature influenced us to study the effect of this substance on the development of experimental atherosclerosis using various methods of estimation the degree of atherosclerosis.

The mechanism of action of ascorbic acid in atherosclerosis has not been adequately studied. It apparently affects various stages of lipid metabolism. Ascorbic acid administration results in an increase in concentration of a number of substances in the blood which suggests accentuation of lipid oxydation (7). Ascorbic acid also decreases the rate of cholesterol synthesis from acetate, whereas with ascorbic acid deficiency there is an increased rate of cholesterol synthesis (14). Vascular wall permeability plays an important role in the development of atherosclerosis. Ascorbic acid decreases capillary permeability (2). Cholesterol secretion from the blood into the bile and gut is increased by ascorbic acid (8). Apparently ascorbic acid administration results in an increase in the liver cholesterol transport (10). We require, therefore, more detailed information on the mechanisms of ascorbic acid action in atherosclerosis. Cholesterol  $^{14}\text{C}_4$  was used to that end.

### METHODS AND MATERIALS

The observations were carried out on 83 rabbits of the same strain, body weight 2.5 to 3 kg. In the first series of experiments 53 animals were used to observe the

effect of ascorbic acid on the development of experimental alimentary atherosclerosis. During 3 months these animals received 0.2 g. cholesterol per kg. body weight/day, in sunflower seed oil. 27 of these also received 0.1 g./kg. body weight ascorbic acid, all per os. 29 animals were experimented on in the spring, 24 in the autumn, in order to exclude seasonal effects. As the activity of the latter gland may influence the development of atherosclerosis, 29 animals of the spring group were equally distributed between controls and experiments according to their latter gland activity measured by <sup>131</sup>I.

Every third week of the experiment total blood cholesterol was estimated by Abel's method and ascorbic acid by titration.

The degree of lipid infiltration of the aorta in all rabbits was determined in two ways: indirect planimetry and extraction of total lipids from tissue by the method of

Tab. I. Total lipids content in the aortic wall of rabbits.

Series I

Controls (cholesterol)			Experimentals (cholesterol + ascorbic acid)		
No.	Total content (mg.)	Content in mg./100 mg. wet wgt.	No.	Total content (mg.)	Content in mg./100 mg. wet wgt.
1.	38.9	5.2	15.	42.9	17.8
2.	52.7	6.5	16.	10.0	2.5
3.	41.7	3.1	17.	70.2	22.4
4.	44.2	4.6	18.	37.5	58.5
5.	37.8	5.5	19.	27.2	7.4
6.	34.9	4.9	20.	26.7	11.2
7.	29.7	4.3	21.	23.1	8.3
8.	35.0	4.2	22.	39.5	18.8
9.	91.4	6.8	23.	32.4	16.0
10.	63.5	8.1	24.	21.3	5.8
11.	45.1	12.4	25.	50.0	49.6
12.	154.6	6.6	26.	34.4	18.6
13.	115.5	5.2	27.	22.2	11.1
14.	61.5	4.8	28.	12.9	3.2
			29.	23.3	18.9

Average: 60.5 ± 5.6 mg.      5.9 ± 0.6 mg.      32.5 ± 3.2 mg.      3.8 ± 0.5 mg.

Series II

30.	48.9	2.6	42.	23.3	3.6
31.	30.5	4.5	43.	40.7	4.3
32.	35.6	4.3	44.	25.2	3.1
33.	46.8	5.6	45.	54.0	7.0
34.	30.4	4.2	46.	21.0	2.7
35.	49.7	3.2	47.	16.5	2.2
36.	27.5	3.2	48.	16.8	2.3
37.	31.4	3.7	49.	23.9	3.7
38.	55.9	6.8	50.	15.3	2.8
39.	57.9	12.0	51.	20.9	2.5
40.	25.5	4.6	52.	22.1	2.8
41.	36.0	4.5	53.	20.2	2.5

Average: 39.6 ± 3.4 mg      5.0 ± 0.5 mg      24.7 ± 3.1 mg      3.2 ± 0.2 mg

Tab. II. Tissue radioactivity after 50  $\mu$ C  $^{14}$ C-cholesterol (in imp./min./mg. dry tissue or mg. serum protein).

Group	Serum	Aorta	Liver
Control	325 $\pm$ 59.2	32 $\pm$ 0.6	642 $\pm$ 86.2
Cholesterol	337 $\pm$ 214.9	44 $\pm$ 1.4	142 $\pm$ 16.4
Cholesterol + ascorbic acid	2177 $\pm$ 215.5	30 $\pm$ 1.4	194 $\pm$ 25.2

Buck & Rossitor. Aortas were taken from the aortic valve to the bifurcation, and the adventitia was stripped off. The tissue was minced and lipids extracted first with ethanol-ether and then for 8 hours in a Soxhlet apparatus. The coronary arteries were investigated histologically.

In the second experimental series we used radioactive cholesterol. 12 of 30 rabbits received 0.2 g. cholesterol per kg. body weight for 45 days, 11 animals received in addition 0.1 g. ascorbic acid/kg. body weight daily. 7 animals received an ordinary diet. On the 45th day all animals received by stomach tube 50  $\mu$ C of cholesterol- $^{14}$ C. 24 hours later blood radioactivity was measured. At post mortem aorta and liver activities were also measured. These organs were cleaned of blood by perfusion and dried in vacuo to a constant weight, then dissolved in HCl. 10 mg. dry solids or 0.025 ml. serum were used for counting. The 4 II gasflow counter was used, and counts were expressed in imp./min. per mg. dry solids or per mg. serum proteins. Organ cholesterol was also determined, and the specific activity of organ cholesterol was calculated.

#### RESULTS

In the spring group, no effect of ascorbic acid on cholesterolaemia was observed: controls at the end of 3 months had an average of 1200  $\pm$  99 mg.%, and experimentals 1400  $\pm$  140 mg.  $\pm$  140 mg.% (the difference is statistically not reliable). Blood ascorbic acid levels in the first group were 0.4  $\pm$  0.09 and in the second 0.7  $\pm$  0.11 mg. %.

In evaluating the degree of atherosclerotic alteration of the aorta planimetrically, there were obvious changes attributable to ascorbic acid (Tab. I). The mean atherosclerotic formula of the aorta (AFA) in cholesterol animals was 35/16 + 13 + 6, in the cholesterol-ascorbic acid group 19/5 + 8 + 6. In these figures is given the % area involved with atherosclerotic elements, and the degree of damage with thick or thin plaques

Table III. Total cholesterol content in mg./mg. dry tissue or mg. serum proteins.

Group	Serum	Aorta	Liver
Control	1 $\cdot$ 10 <sup>-2</sup>	10 $\cdot$ 10 <sup>-4</sup>	20 $\cdot$ 10 <sup>-4</sup>
Cholesterol	14 $\cdot$ 10 <sup>-2</sup>	18 $\cdot$ 10 <sup>-4</sup>	200 $\cdot$ 10 <sup>-4</sup>
Cholesterol + ascorbic acid	17 $\cdot$ 10 <sup>-2</sup>	11 $\cdot$ 10 <sup>-4</sup>	1390 $\cdot$ 10 <sup>-4</sup>

or lipid blotches. Ascorbic acid administration was also associated with a decrease in total lipids in the aorta. The control group showed values of  $60.5 \pm 5.6$  mg., or  $5.9 \pm 0.6$  mg./100 mg. wet weight of aorta. The ascorbic acid group showed  $32.5 \pm 3.2$  mg. or  $3.8 \pm 0.5$  mg./100 mg. wet weight.

In the autumn group, ascorbic acid in addition succeeded in lowering blood cholesterol: control group showed  $1500 \pm 222$  mg.% and experimental  $800 \pm 142$  mg.%, with blood ascorbic acid levels resp. of  $0.5 \pm 0.06$  and  $0.9 \pm 0.05$  mg.%.

In both spring and autumn groups there was a clear inhibitory effect of ascorbic acid on aortic alimentary atherosclerosis. Mean AFA in cholesterol animals was  $30/9 + 15 + 6$ , whereas in the autumn ascorbic acid group it was  $13/0.6 + 1.9 + 10.5$ . In the latter there were practically no thick plaques. Mean aortic lipid content was  $3.2 \pm 0.25$  mg./100 mg. wet weight or  $24.7 \pm 3.1$  mg. with ascorbic acid whereas with only cholesterol feeding the values were  $5.0 \pm 0.5$  mg./100 mg. wet weight or  $39.6 \pm 3.4$  mg.

In most animals on ascorbic acid no marked changes were observed histologically in the coronary arteries, whereas with cholesterol alone there was lipoidosis of the small intramural branches and some "fracture" of small and large branches (Fig. 1 on Plate).

In the second series of experiments the activity of serum, liver and aorta was studied in healthy animals, animals 45 days on cholesterol alone, and animals which received in addition ascorbic acid over the same period (Tab. II).

In the cholesterol group there was a rise in serum activity by 2502 imp./min. — in the ascorbic acid group values were higher than normal, but lower than with cholesterol alone — 1352 imp./min. With cholesterol alone, aortic activity was 12 imp./min. higher than normal, with ascorbic acid there was no difference from the norm. With cholesterol alone, there was a decrease in liver activity. With ascorbic acid, they were also below normal, but 52 imp./min. more than with cholesterol alone.

Tab. III shows total organ cholesterol contents. Cholesterol animals and ascorbic acid animals both showed higher serum levels, but showed no differences between themselves. Aortic cholesterol was above normal only in the cholesterol group, and this applied to liver cholesterol as well.

Tab. IV shows the relative specific activities of tissue cholesterol. This

Table IV. Relative specific activity of tissue cholesterol (least value set at 0.1, all other values as multiples).

Group	Serum	Aorta	Liver
Control	8.2	32.2	321.0
Cholesterol	2.3	25.2	7.0
Cholesterol + ascorbic acid	1.0	30.0	1.3

serves as an indicator of the intensity of cholesterol turnover, and is expressed as imp./min./mg. total cholesterol. For comparative purposes, the lowest specific activity of cholesterol, in the ascorbic acid group serum, was set at 1.0, and all other values were taken as multiples of this value.

Serum specific activity was less than normal in the cholesterol group, and even lower in the ascorbic acid group. Aortic specific activity was lowest in the cholesterol group, highest in the normals, and in between in the ascorbic acid group. Liver specific activity was lowest in the ascorbic acid group, highest in controls, and in between in the cholesterol group. These results show that administration of ascorbic acid decreases cholesterol specific activity in serum and liver, and increases it in the aorta.

#### DISCUSSION

The results suggest that ascorbic acid has a transitory hypocholesterolaemic effect. Inhibition of alimentary hypercholesterolaemia was observed in animals experimented on in the spring, but not in those experimented on in the autumn. Obviously the factor of season plays an important role.

Labelled cholesterol experiments were carried out in the spring. Less cholesterol appears to have gotten into the blood. But the decrease in serum specific activity suggests a decreased turnover.

In all cases ascorbic acid appeared to have decreased the development of atherosclerosis as determined by various methods (planimetric, radiologic and aorta content of lipids and cholesterol). The last data suggest that the principle effect of ascorbic acid is to decrease permeability of the aortic wall, a view confirmed by the radioactive measurements. It should be stressed that ascorbic acid also increases cholesterol turnover in the aorta, as expressed by specific activity calculations.

Ascorbic acid also increased liver take-up of cholesterol, but decreased liver turnover, with a subsequent rise in content.

The favourable effect of ascorbic acid on experimental atherosclerosis, therefore, consists of:

1. decreasing the permeability of the aortic wall — less cholesterol penetrates,
2. increasing cholesterol turnover in the aorta — more cholesterol leaves,
3. increasing cholesterol accumulation in the liver.

#### SUMMARY

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The effect of ascorbic acid on the course of experimental rabbit atherosclerosis has been studied in various seasons. In the spring there was no hypocholesterolaemic

effect of ascorbic acid, but in the autumn there was. In both seasons, however, ascorbic acid administration was associated with less alimentary atherosclerosis in the actual tissue concerned, as estimated by various methods (planimetry, radiology, aortic content of total lipids and cholesterol, etc.). Experiments with  $^{14}\text{C}_4$ -cholesterol explained the effect of ascorbic acid on atherosclerosis: less penetration into the aorta, increased turnover in the aorta, decreased turnover in the liver, accumulation in the liver.

#### ZUSAMMENFASSUNG

Es wurde die Wirkung von Ascorbinsäure auf den Verlauf der experimentellen Cholesterinatherosklerose bei Kaninchen untersucht, die zu verschiedenen Jahreszeiten hervorgerufen worden war. Bei den im Frühjahr durchgeführten Versuchen trat keine hypocholesterinämische Wirkung der Ascorbinsäure in Erscheinung, aber bei einer Wiederholung des Versuches im Herbst machte sich ein leichter hypocholesterinämischer Effekt bemerkbar. Gleichzeitig wurde unter dem Einfluss der Ascorbinsäure in beiden Versuchsgruppen eine deutliche Hemmung des atherosklerotischen Prozesses in der Aorta und den Kranzarterien beobachtet, was durch verschiedene Methoden nachgewiesen wurde (planimetrisch, histologisch, radiologisch und mittels Bestimmung der Gesamtlipide und des Cholesterins in der Aorta).

Versuche mit markiertem  $4\text{-C}^{14}$ -Cholesterin haben den Mechanismus der lipotropen Wirkung der Ascorbinsäure aufgedeckt. Es wurde festgestellt, dass diese das Eindringen von Cholesterin in die Aorta herabsetzt, die Intensität des Cholesterinmetabolismus in der Aorta erhöht und dessen Eindringen in die Leber verstärkt.

#### RÉSUMÉ

Etude de l'influence de l'acide ascorbique sur le développement de l'athérosclérose cholestérolique du lapin, provoquée pendant des saisons différentes. Dans les expériences faites au printemps, l'effet hypocholestérolémique de l'acide ascorbique ne s'est pas manifesté, mais lors de la répétition de l'expérience en automne, on a observé un effet légèrement hypocholestérolémique. En même temps, l'acide ascorbique présente dans les deux groupes un effet inhibiteur marqué sur le développement des altérations athéroscléreuses dans l'aorte et les artères coronaires, démontré par des méthodes différentes (planimétrique, histologique, radiologique et par l'évaluation de la concentration des lipides totaux et du cholestérol dans l'aorte).

Des expériences faites avec du cholestérol marqué  $4\text{-C}^{14}$  ont démontré le mécanisme de l'effet lipotrope de l'acide ascorbique. On a pu constater que celui-ci fait diminuer la pénétration du cholestérol dans l'aorte, tout en augmentant l'intensité du métabolisme du cholestérol dans l'aorte et sa pénétration dans le foie.

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