

## THE INFLUENCE OF ANTICOAGULANTS ON THE FORMATION AND REGRESSION OF EXPERIMENTAL ATHEROSCLEROSIS

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Recent studies by Graham *et al.* (1) and Constantinides *et al.* (2) have provided evidence that anticoagulants, particularly heparin, are effective in retarding the appearance of experimental atherosclerosis in the rabbit. Studies on the effects of anticoagulants on the regression of experimental atherosclerosis however have not been reported in the literature to this date. Studies on the effects of these agents on the genesis and the regression of experimental atherosclerosis in the chick are reported in this paper.

Heparin was selected as one of the anticoagulants in these experiments, primarily because of its potent antilipemic action. It was reasoned that if heparin were effective in clearing lipemic turbidity it might also be effective in preventing or retarding the formation and possibly hastening the regression of cholesterol-induced atherosclerosis. Dicoumerol was used in the study because it has no antilipemic action and it represents an anticoagulant with a mechanism of action different from that of heparin. By the use of the two types of anticoagulants some information might be obtained regarding the significance of antilipemic action as well as anticoagulant action on experimental atherosclerosis.

Chicks were employed as the experimental animals because of their consistent response to atherogenic stimuli. The many advantages of the chick in experimental atherosclerosis have been thoroughly reviewed by Katz and Stamler (3).

### *Methods and Procedures*

Two series of experiments were undertaken using in all 86 chicks. In both, 4 and 5 week old Rhode Island Red cockerels were employed. They were obtained from a local hatchery and reared on commercial mash and tap water until the age of 7 weeks. The birds of the first series were then divided into four dietary groups: Group I was fed mash supplemented with 5 per cent cottonseed oil (Wesson oil), and groups II, III, and IV were fed mash supplemented with 2 per cent cholesterol plus 5 per cent cottonseed oil. Six birds from each group were sacrificed after 8 weeks and 8 birds from each group after 12 weeks on the experimental diet. During this period group III was treated with daily subcutaneous injections of 10 mg.

per bird of heparin repository<sup>1</sup> while birds of group IV received orally an aqueous suspension of dicoumerol in a dosage of 2 to 3 mg. per kilogram on alternating days.

Birds in the second series were fed *ad libitum* a 2 per cent cholesterol-5 per cent cottonseed oil-mash diet for a period of 12 weeks. The experimental diet was discontinued and a straight mash diet was substituted. The birds were divided into three groups at this time and the following treatment pursued: Group I, no treatment; group II, daily subcutaneous administrations of mg. per bird of heparin repository; and group III, oral administration of 2 or 3 mg. per kilogram of an aqueous suspension of dicoumerol on alternating days. The diet and treatments in the two series of experiments are summarized on Table I.

Changes in body weight, serum cholesterol, and serum lipid phosphorus were determined for each bird at 2 week intervals throughout the entire experiment. 2 to 3 ml. of blood was

TABLE I  
*Summary of Diets and Anticoagulant Treatments Given to Chicks in These Experiments*

Series	Group	Diet		Treatment	
		Mash and oil	Mash and oil and cholesterol	Heparin	Dicoumerol
I	I	+	-	-	-
	II	-	+	-	-
	III	-	+	+	-
	IV	-	+	-	+
II	I	-	+	-	-
	II	-	+	+	-
	III	-	+	-	+

withdrawn from the wing vein and clotting and prothrombin times were performed in order to determine the presence and effectiveness of the anticoagulants. The cholesterol was determined according to the direct method of Zlatkis *et al.* (4). Lipid phosphorus was determined by King's method (5).

After the designated periods the birds were sacrificed and the brachiocephalics, aorta, and the iliac arteries were inspected for atheromatous lesions. Severity of the involvement was graded on a scale of 0 to 4 according to the method followed by Duff and McMillan (6). The gross observations and evaluations of the vessels were confirmed microscopically. Vessels with questionable areas as well as obvious lesion were inspected for lipid material.

Serum turbidity was also observed in each of the serum samples by gross inspection. This was also arbitrarily graded on a scale from 0 to 4 as described by Constantides *et al.* (2).

#### RESULTS

*Series I.*—The incidence and severity of atherosclerosis in the four groups showed considerable differences at the end of the 8 week period. The means of the grades of atheroma of the various groups are listed in Table II. Group I, as expected, was completely free from atheroma, but groups II, III and IV exhibited lesions ranging from minimal to severe. The results indicate a much

<sup>1</sup> The heparin repository was generously supplied for these experiments by the Lederle Laboratories, Division, American Cyanamid Company.

greater incidence of severe lesions in group II, the untreated cholesterol-fed animals, than in those treated with either heparin or dicoumerol. The protection provided by the anticoagulants was evidenced by fewer plaques, smaller areas of involvement, and less advanced lesions in the aorta and other great vessels. Only one specimen in the dicoumerol group exhibited lesions which compared in severity with those in group II.

Microscopic sections of the vessels tended to support the gross observations. Chickens of group I showed no evidence of atherosclerosis. In sections taken from the anticoagulant-treated groups atheromatous involvement was evident,

TABLE II  
*Mean Values and Ranges of Weight and Serum Lipids and the Grade of Atheromata of Chicks after 8 Weeks*

Group	Weight	Cholesterol	Lipid P	C/P ratio	Grade
	<i>gm.</i>	<i>mg. per cent</i>	<i>mg. per cent</i>		
I Control	1807 (1610-2085)*	205 (172-266)	8.8 (6.7-13.4)	23.2	0.0
II Cholesterol	1687 (1310-2040)	2152 (1750-2560)	27.3 (23.7-32.9)	78.8	2.7
III Cholesterol and heparin	1582 (1195-1850)	1528 (605-2960)	25.6 (8.6-43.5)	59.9	1.5
IV Cholesterol and dicoumerol	1760 (1540-2050)	1789 (1035-2470)	24.6 (8.9-36.0)	73.0	1.5

\* Range.

although none in group III showed as extensive changes as seen in group II. Only one bird in the dicoumerol-treated group showed the thick cholesterol-laden plaques seen in the untreated cholesterol fed group.

The serum lipid picture of the animals after 8 weeks on the experimental diet showed a marked rise in both cholesterol and phospholipid values. As indicated in Table II and in Fig. 1 the untreated animals exhibited the highest serum cholesterol in the cholesterol-fed groups. The serum lipid phosphorus rose in a manner paralleling the cholesterol as can be seen in Fig. 2. Although the anticoagulant-treated groups showed a lower lipid phosphorus level at the end of 8 weeks the difference from the group II values was not nearly as marked as in the case of the cholesterol figures.

When the serum cholesterol and lipid phosphorus values of the anticoagulant-treated birds were compared statistically with the untreated group the following results were obtained:—

(a) A highly significant difference was found between the serum cholesterol levels of the untreated and the heparin-treated animals ( $p < 0.01$ ); (b) A barely significant difference was found between the cholesterol levels of the untreated and the dicoumerol-treated groups ( $p > 0.1$ ); (c) The lipid phosphorus levels of

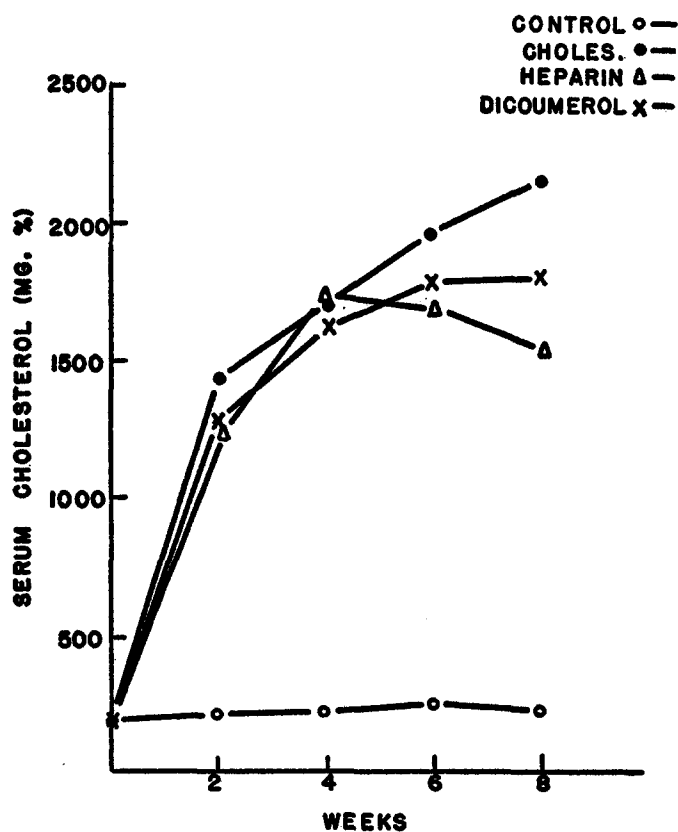


FIG. 1. The influence of anticoagulants on the serum cholesterol levels in chicks fed a cholesterol diet over an 8 week period.

both the heparin and dicoumerol-treated animals were not significantly different from that of the untreated groups ( $p > 0.7$  and  $> 0.4$ , respectively).

Serum turbidity was also observed at the 8 week period. The sera of groups II, III, and IV showed similar opacities at this time. Thus heparin was ineffective in clearing the turbidity produced by the feeding of a high cholesterol diet in the chick. In all cases however, the anticoagulant-treated birds showed markedly prolonged clotting or prothrombin times.

After 12 weeks on the experimental diet the cholesterol-fed groups showed a

marked advancement in the gross lesions. The vessels again revealed a wide range of involvement, but compared to the earlier period the lesions were much more severe. The clear cut differences in degree of atherosclerosis which had existed between the groups in the earlier series of experiments was no longer

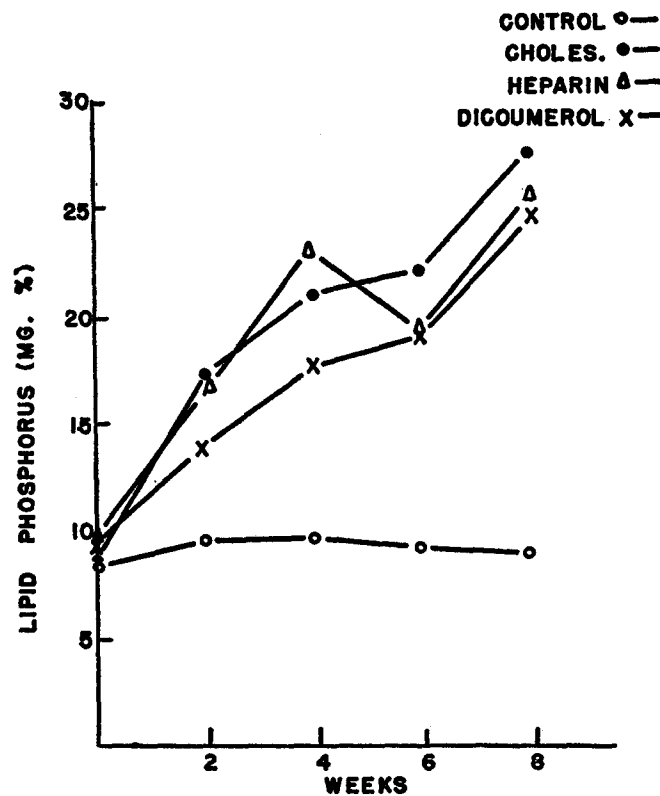


FIG. 2. The influence of anticoagulants on the serum lipid phosphorus levels in chicks fed a cholesterol diet over an 8 week period.

apparent at this time. The means of the gradings of the four groups at the 12 week period are listed on Table III.

These results indicate that the protective property of the anticoagulants diminished greatly as the cholesterol feedings progressed. Careful inspection of the individual vessels however gave some evidence of protection in treated birds, particularly in the heparin group. The serum lipid picture also demonstrated the decrease of effectiveness of the anticoagulants against hypercholesteremia and phospholipemia. Although the mean values of the serum lipids of the treated groups showed were lower than the untreated group the difference be-

tween the two was decreased considerably. Analysis of the data given in Table 3 revealed the following changes:—

(a) Although the means of the serum cholesterols of the heparin-treated groups differed by some 500 mg. per cent they were only barely significant ( $p > 0.02$ ).

TABLE III  
*Mean Values and Ranges of Weight and Serum Lipids and the Grade of Atheromata of Chicks after 12 Weeks*

Group	Weight	Cholesterol	Lipid P	C/P ratio	Grade
	<i>gm.</i>	<i>mg. per cent</i>	<i>mg. per cent</i>		
I Control	2441 (1960-2730)	150 (132-175)	7.4 (6.5-13.3)	20.3	0.0
II Cholesterol	1966 (1395-2615)	2414 (900-3740)	28.0 (16.2-43.4)	86.2	3.3
III Cholesterol and heparin	1940 (1475-2440)	1905 (1550-2570)	25.3 (16.1-33.4)	75.3	2.5
IV Cholesterol and dicoumerol	2025 (1885-2225)	2240 (1000-3280)	23.7 (14.3-33.3)	94.5	3.0

TABLE IV  
*Mean Values and Range of Weight and Serum Lipids and the Grade of Atheromata after 10 Weeks of Regression*

Group	Weight	Cholesterol	Lipid P	C/P Ratio	Grade
	<i>gm.</i>	<i>mg. per cent</i>	<i>mg. per cent</i>		
I Control	2856 (2620-3515)	166 (131-244)	7.7 (7.2-8.6)	21.6	2.8
II Heparin	2711 (2325-3375)	181 (144-256)	8.6 (7.2-9.3)	27.8	2.8
III Dicoumerol	2626 (2350-2990)	166 (147-238)	8.3 (6.5-15.7)	20.0	2.9

(b) The difference between the dicoumerol-treated and the untreated groups was not significant ( $p > 0.6$ ). (c) The difference in serum lipid phosphorus between the heparin and dicoumerol-treated groups and the untreated group were as before not significant ( $p > 0.4$  and  $> 0.2$ , respectively).

As before the serum samples revealed that heparin was ineffective in preventing or abolishing the turbidity produced by the experimental diet.

*Series. II*—The influence of anticoagulants on the regression of experimental

atherosclerosis was determined in the second series of experiments. Birds made atherosclerotic by placing them on high cholesterol diets for a period of 12 weeks were divided into three groups and two of these were treated with anticoagulants for another 10 weeks. During the latter period of anticoagulant therapy the animals were maintained on a plain mash and tap water diet.

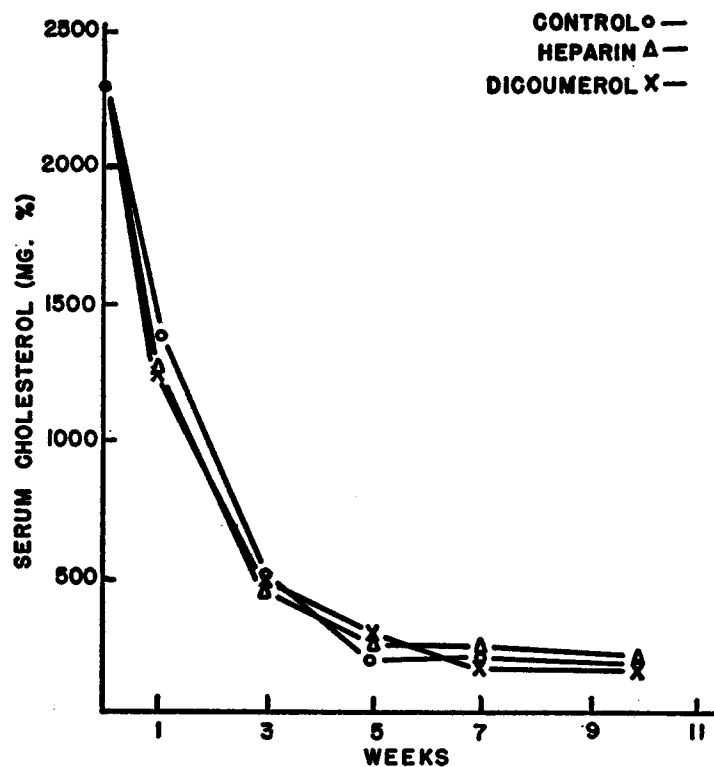


FIG. 3. The influence of anticoagulants on the regression of serum cholesterol levels in chicks after withdrawing the cholesterol diet.

The degree of atherosclerosis in these groups was definitely less than in those birds sacrificed immediately after the prolonged cholesterol feeding. The mean grades of the birds in this series of experiments are listed in Table IV. Comparison of these values with those for the three cholesterol-fed groups in the previous experiments indicate that there was a gradual regression of the atheroma in the absence of the atherogenic stimulus. The average grades of 2.8, 2.8, and 2.9 obtained in these experiments also demonstrate that neither heparin nor dicoumerol in the doses employed were effective in hastening the regression of experimentally induced atherosclerosis in the chick.

The serum lipid concentration showed a rapid decline after cessation of the high cholesterol diet. As early as 5 weeks after the cessation of the high cholesterol diet the serum lipid values were essentially back to normal control levels. The most outstanding feature of the serum lipid picture was the remarkable similarity of the regression of the untreated and anticoagulant treated animals.

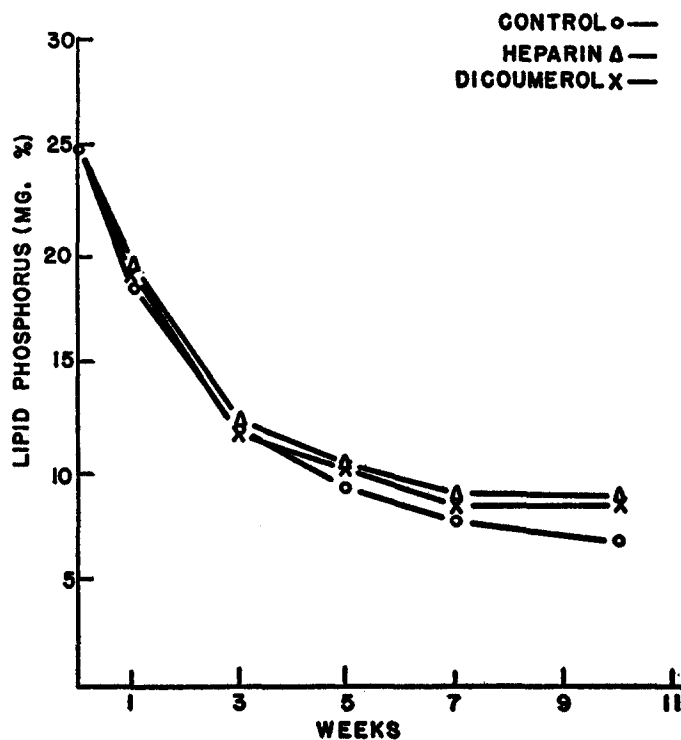


FIG. 4. The influence of anticoagulants on the regression of serum lipid phosphorus levels in chicks after withdrawing the cholesterol diet.

Both the serum cholesterol and the lipid phosphorus curves showed almost identical slopes (see Figs. 3 and 4). The regression of atheroma appears to coincide with the rate of decrease in the serum lipid levels; that is, regression appears to have proceeded at the same rate in the three groups. Serum turbidity was also determined at the end of the 10 week period. As expected there was no turbidity in any of the serum samples.

#### DISCUSSION

The results of the experiments reported have demonstrated that both heparin and dicoumerol are effective in retarding the formation of atheroma in the cholesterol-fed chick. Complete protection however was not observed at 8 or 12



weeks with either heparin or dicoumerol therapy. This is in contrast to the effects of heparin in the formation of atheroma in the cholesterol-fed rabbit. Constantinides (2) reported that after  $5\frac{1}{2}$  and  $7\frac{1}{2}$  weeks heparin-treated rabbits were completely free of atheroma, while at  $9\frac{1}{2}$  weeks his animals still showed considerable protection, although a few lesions had appeared. The chicks in our experiments on the other hand showed considerable atherosclerosis after only 8 weeks on the cholesterol diet. This difference in the ability of heparin to influence the genesis of atherosclerosis can be partially explained by the difference in susceptibility of the chicks and rabbits to experimentally induced atheroma. Katz and Stamler (3) cite from earlier experiments that rabbits develop gross lesions much later than the chick.

The protection against atheroma formation is most likely attributable to the ability of heparin to inhibit the marked hypercholesteremia seen in the untreated cholesterol-fed animals. This effect was also reported in rabbits fed a high cholesterol diet by Constantinides *et al.* (2). Jones *et al.* (7) and Graham *et al.* (1) found that heparin was effective in preventing the development of high concentrations of  $S_2$  10 to 50 lipoproteins and retarding the development of atherosclerosis. However, they also noted little or no effect on the total plasma cholesterol. This is in contrast to the results obtained by both Constantinides and this laboratory.

Whether an anticholesteremic effect is evident or not it is quite possible that the beneficial effect of heparin in these conditions is related to its antilipemic property. This lipemia-clearing property of heparin was first described by Hahn (8) and is easily demonstrated in man, dogs, and rats, but not in rabbits and chicks. The difficulty lies in the fact that a visible chylomicronemia cannot be produced in the chicken by the usual methods. Feeding of cream, corn oil, or cottonseed oil in large amounts did not result in a cloudy serum. This phenomenon has also been observed in the rabbit (9). When the animals were fed a high cholesterol diet however the resulting sera were turbid. Furthermore the serum turbidity caused by the cholesterol could not be cleared by heparin. The inability of heparin to decrease the turbidity of cholesterol-induced lipemia has been noted by Anfinsen *et al.* (10).

The ability of dicoumerol to retard the formation of atherosclerosis was first noted by Dedichen *et al.* (11) in the stilbesterol-implanted chick. The protection provided by this anticoagulant is also probably the result of its ability to prevent the usual marked rise in serum cholesterol. Although in the present experiments the differences in serum cholesterol between the untreated and dicoumerol-treated groups were not highly significant at the 8 week period, the majority of the treated animals showed a considerably lower level than the untreated group. At 12 weeks there was no difference in the serum cholesterol; likewise the protective ability of dicoumerol at this time was almost totally absent.

The second series of experiments have demonstrated the ineffectiveness of

heparin and dicoumerol in influencing the regression of cholesterol-induced atherosclerosis in the chick. After the development of the lesions the anticholesteremic actions of these agents appear to be of no value in hastening the regression. The fact that in the treated and untreated groups the blood lipids declined in an almost identical manner is also indicative of the absence of any influence of the anticoagulants on the regressive processes. This can be compared to the effects observed in the first series of experiments in which the serum cholesterol were much lower in the treated groups, especially in the earlier parts of the experiment.

#### SUMMARY AND CONCLUSIONS

The prolonged administration of either heparin or dicoumerol has been demonstrated to be effective in retarding the formation of atheromata in the cholesterol-fed chick. This beneficial action is possibly produced as a result of a demonstrated antihypercholesteremic action exerted by these anticoagulants.

Heparin is ineffective in preventing the marked turbidity of sera from cholesterol-fed chickens.

After the formation of atherosclerosis in the chick neither heparin nor dicoumerol is effective in hastening the regression of this condition. The serum lipid picture as well as the degree of atheroma of the various groups in the regression experiments is unaffected by the anticoagulants.

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