XIV. THE INFLUENCE OF CHANGES INDUCED BY CHOLESTEROL UPON THE CALCIFICATION *IN VITRO* OF RABBIT AORTA.

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(Received November 30th, 1935.)

It was previously shown [Rosenheim and Robison, 1934] that the aortae of normal rats and rabbits can be calcified *in vitro* by immersion in solutions of similar composition to those used for calcification of hypertrophic cartilage. The course of the process is not the same in these different tissues. In cartilage and osteoid tissue, which normally become calcified *in vivo*, calcification *in vitro*, in suitable solutions, begins rapidly and may be complete in 12 to 24 hours; after such periods of immersion a marked diminution in the power of the calcifying mechanism becomes evident [Robison and Rosenheim, 1934]. Calcification of the aorta *in vivo* is an abnormal phenomenon, and *in vitro* the process is delayed and erratic. Deposition rarely begins until the tissue has been immersed for two days and may not occur within 4 or 5 days, even in solutions which are much more highly supersaturated with calcium carbonatophosphate than those required for deposition of this salt in hypertrophic cartilage. From a consideration of these and other facts it was suggested that conditions or properties favourable to calcification may be developed in the aorta as the result of injury or of degenerative changes. Some attempts were made to obtain experimental evidence in support of this view by subjecting the aorta to various treatments after removal from the animal; these attempts were unsuccessful.

It was decided to continue these experiments by producing in the living animal changes in the aorta which are commonly associated with eventual calcification. The choice of cholesterol sclerosis for our first experiments was taken with the helpful advice of Prof. J. B. Duguid to whom we express our thanks. Experimental cholesterol arteriosclerosis in rabbits simulates in many respects human atherosclerosis [Anitschkow, 1933]. It is characterised by deposition of lipid substances, probably consisting of cholesterol and cholesteryl esters, in the intima, which becomes greatly thickened with formation of large, fat-laden "foam" cells. Fatty plaques are formed on the inner surface, beginning in the arch and extending into the thoracic aorta and abdominal aorta, ultimately covering large areas of the vessel wall. At later stages, calcification of the degenerate tissues often occurs. The earliest microscopical changes may be observed in aortae of rabbits which have received cholesterol over periods of 20 days and upwards. This type of arteriosclerosis is associated with disturbance of cholesterol metabolism, but some form of injury or alteration of the aortic tissues is commonly supposed to play a part in predisposing these tissues to the infiltration of lipoids.

The problem which we set out to study was whether such changes as are induced in the rabbit aorta by diets rich in cholesterol provide conditions more favourable for the deposition of calcium salts than are found in the normal tissues. The plan of our experiments was to compare the rapidity, extent and

character of the calcification *in vitro* produced by supersaturated solutions of the bone salt in aortae of normal rabbits and of those suffering from experimental cholesterol arteriosclerosis in varying degrees of severity.

EXPERIMENTAL

Twelve rabbits, each about 1 year old, were divided into pairs of approximately the same weight and were given a diet consisting of oats, bran, hay, cabbage and carrot. One of each pair received in addition 1 g. cholesterol daily, this being thoroughly mixed with minced carrot and eaten by the animals before they received the rest of the food. Five pairs of rabbits were killed after periods of 35, 43, 50, 81 and 91 days respectively. One rabbit of the sixth pair received cholesterol during 112 days; thereafter it received the normal diet without cholesterol for a further 41 days and was then killed along with the rabbit which had received the normal diet for the whole period. Immediately after death the aortae were excised with aseptic precautions and were divided transversely into segments some of which were placed in the calcifying solution, whilst the remainder were fixed at once as controls. The whole of the ascending aorta and arch, the thoracic aorta and a small part of the abdominal aorta were used, the segments being numbered consecutively, beginning from the heart.

The calcifying solution (8 : 5 : 0) used in all experiments contained 8 mg. Ca and 5 mg. P, as inorganic phosphate, per 100 ml., together with other inorganic salts in the concentrations in which they occur in plasma [Robison and Rosenheim, 1934]. Each segment was placed in a tube containing 18 ml. of this solution at p_H 7.4 and 37°. In the previous investigation [Rosenheim and Robison, 1934] solution 8 : 5 : 0 produced calcified deposits in 5 days in 3 out of 6 rabbit aortae.

In the first three experiments of the present series each aorta was divided into four segments; the first and third were immersed in solution 8 : 5 : 0 for 6 days, whilst the second and fourth served as controls. In Exps. 4–6 the aortae were divided into ten smaller segments and the period of immersion was varied from 1 to 5 days in order that any increased tendency to calcification might be more readily detected. In order to minimise the risk of spontaneous precipitation of calcium salts the experimental solution was renewed at intervals of 1 or 2 days.

The segments were fixed in 10 % neutral formalin, dehydrated and embedded in paraffin. Serial sections were cut and stained with silver nitrate and eosin; alternatively, some sections were stained with purpurin and orange G or with haemalum and eosin. In Exps. 4–6 some control segments were cut with the freezing microtome and stained with Sudan III and haematoxylin for the detection of lipoids.

The results of these experiments are summarised in Table I.

Changes induced by cholesterol.

Extensive fatty plaques were found in all segments of the aortae of rabbits which had received cholesterol for periods of 81, 91 and 112 days (Exps. 4–6). Considerable deposits of lipoids were present in the intima and in the inner layers of the media. Definite, though less extensive, intimal lesions were observed in the aortae of the rabbits which had received cholesterol for 35, 43 and 50 days (Exps. 1–3). The calcification which often accompanies the later stages of cholesterol arteriosclerosis did not appear in any of these experiments; no calcified deposits were seen in any segments which had not been immersed in calcifying solution.

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Table I. *Calcification in vitro of rabbit aorta.*

Exp.	Sex	Initial wt. g.	Final wt. g.	Diet	Period on diet days	Control segments			Segments immersed in solution 8 : 5 : 0			
						Nos. of segments	Calcifi- cation	Chol- esterol lesions	Nos. of seg- ments	Period of immer- sion days	Calcification	
1	♂	2530	2890	N	35	2, 4	-	-	1, 3	6	++	i, m, a
	♂	2550	2730	C	35	2, 4	-	+	1, 3	6	++	i, m, a
2	♂	3470	3050	N	43	2, 4	-	-	1, 3	6	++	i, m, a
	♂	3620	3020	C	43	2, 4	-	+	1, 3	6	++	i, m, a
3	♀	2050	2520	N	50	2, 4	-	-	1, 3	6	++	i, m, a
	♀	2525	3200	C	50	2, 4	-	+	1, 3	6	++	i, m, a
4	♂	3080	2835	N	81	3, 6, 9 2, 5, 8	-	-	1	1	-	
									4	2	-	
									7	4	-	
									10	5	-	
	♀	3300	2850	C	81	3, 6, 9 2, 5, 8	-	+	1	1	-	
									4	2	-	
									7	4	-	
									10	5	++	i, m, a, p
5	♂	2845	2990	N	91	3, 6, 9 2 5, 8	s	-	1	1	s	m
									4	2	-	
									7	3	-	
									10	4	+	m
	♂	2970	2575	C	91	3, 6, 9 2, 5, 8	-	+	1	1	s	m
									4	2	+	p
											(Few granules)	
									7	3	-	
									10	4	+	m
6	♂	2795	3570	N	153	3, 6, 9 2, 5, 8	-	-	1	1	-	
									4	2	++	m
									7	3	++	m
									10	5	-	
	♂	2685	2820	{ C N	112 41	3, 6, 9 2, 5, 8	-	+	1	1	s	m
									4	2	-	
									7	3	++	m
									10	5	++	i, m, a

Diet: N, normal; C, normal + 1 g. cholesterol daily.

Calcification: -, no deposit; +, very light deposit; ++, heavy deposit; s, very small calcified area occurring *in vivo*.

The letters indicate the location of the deposit: i, intima; m, media; a, adventitia; p, fatty plaque.

The most extensive deposition was almost always found in the media.

In Exps. 4-6, segments 3, 6, 9 were cut with the freezing microtome and examined for lipid deposits.

In the aorta of the normal rabbit of Exp. 5, a very small area of calcification, involving one or two elastic laminae in the media, was found in segment 2 (arch) which had been fixed as a control (Plate I, Fig. 1). Very similar calcified areas were seen also in segment 1 of the same aorta and in the first segments of the aortae of two rabbits receiving cholesterol (Exps. 5 and 6). Although the last three segments had been immersed in solution 8 : 5 : 0 for one day, it was considered that the deposits were most probably formed *in vivo* but were not necessarily induced by the cholesterol diet. This type of calcification is indicated in Table I by the letter "s". The presence of such deposits in the aortae of apparently normal rabbits has been frequently noted. In a recent study, Kesten [1935] observed spontaneous medial degeneration in the aortae of 68 out of 125 apparently normal young rabbits aged from 1 day to 8 months. Histologically, the

earliest changes were swelling, fragmentation and fraying of elastic fibres in the inner media, and at later stages calcium salts were laid down along and closely apposed to these swollen fibres.

Calcification in vitro.

In Exps. 1-3, in which alternate segments were immersed for 6 days in solution 8:5:0, heavy calcification was obtained *in vitro* in the aortae of the normal rabbits and of those which had received cholesterol. As in the earlier experiments [Rosenheim and Robison, 1934] the deposits were found chiefly in the media but occurred also in the intima and adventitia, sometimes extending throughout the whole width of the wall. In lightly calcified areas the appearance of the stained deposit was that of scattered granules formed on or around the elastic fibres. This was shown particularly well in sections stained with purpurin in which fibres could be seen through the translucent pink granules (Plate I, Fig. 2). In the more densely calcified areas the deposit extended between the elastic laminae and frequently appeared to lie chiefly in the muscle tissue. Under high magnification deposits of the latter type showed particles attached to fine off-shoots joining the elastic laminae, but whether deposition always began in this way could not be determined. The impression that elastic fibres play an important part in the initiation of calcification in the aorta was strengthened by the examination of these deposits.

In these three experiments the aortae of the normal rabbits and of those which had received cholesterol did not show any significant differences in their *in vitro* calcification.

In Exps. 4-6, in which different segments were immersed in calcifying solution for periods of 1-5 days, calcification *in vitro* occurred erratically. In Exp. 4 no calcification occurred in any segment of the normal rabbit aorta, whilst in that of the rabbit receiving cholesterol a heavy deposit was formed in one segment only, immersed for 5 days; in this segment, calcification took place in some parts throughout the vessel wall and in the extensive fatty plaque (Plate I, Fig. 3). The presence of numerous bacteria was, however, observed in the peripheral layers of this segment and these organisms may conceivably have played some part in determining calcification in these regions; no bacteria were seen in the middle zone where a heavy deposit was also found.

In Exp. 5 a light deposit was observed in the media of one segment of the normal aorta, immersed for 4 days; the corresponding segment of the aorta from the rabbit receiving cholesterol also showed slight calcification after 4 days' immersion, the deposit being formed on the innermost elastic laminae. In another segment of the same aorta, immersed for 2 days only, a few granules of calcium salt were found in the fatty plaque (Plate I, Fig. 4); but no calcification occurred in segment 7 after 3 days' immersion. In Exp. 6 segments of the normal aorta were calcified after 2 and 3 days' immersion (Plate I, Fig. 5) but not after 1 or 5 days, whilst the aorta of the rabbit which had received cholesterol became calcified to about the same extent after 3 and 5 days' immersion but not after 2 days. A section of segment 7, which was immersed for 3 days, is shown in Plate I, Fig. 6. The fat has been removed during dehydration but one end of an extensive intimal plaque is seen. There is no calcification in this plaque or in the sub-intimal layers; but a heavy deposit is present in the middle region of the media and resembles that found in segment 7 of the normal aorta after immersion for a similar period (Plate I, Fig. 5).

Most of the deposits observed in Exps. 4-6 occurred in the media, and granules formed on or around elastic fibres were very conspicuous.

The staining of the deposits.

The staining properties of the deposits of calcium salts (probably calcium carbonatophosphate) formed in these experiments were investigated in some detail. With haemalum the staining was very slight, only the heaviest deposits retaining the colour around their edges; a deeper, though rather patchy, stain was obtained if the sections were previously immersed for some time in a 1% solution of ferric chloride. Purpurin and silver nitrate stained the deposits in approximately the same degree, the purpurin giving a deep clear pink colour, characteristic of calcium in absence of iron. The greatest reliance was placed on silver nitrate but the method has certain defects. With heavy deposits, there is a tendency to spreading of the brown stain into surrounding tissues; whilst, occasionally, the reduced silver may form relatively large and apparently crystalline particles, which probably do not show the exact distribution of the original calcium salt. As pointed out by Cameron [1930] it is not the calcium ion but the anion, carbonatophosphate, which reacts with silver nitrate.

Through the kindness of Dr C. V. Harrison, we were able to compare these reactions with those given under precisely similar conditions by the deposits formed *in vivo* in his experiments on arterial disease produced by cholesterol and vitamin D [Harrison, 1933]. In sections cut from his material, all deposits stained by silver nitrate were also stained by haemalum and purpurin, the latter giving a reddish purple colour indicating the presence of iron. In addition, certain areas not stained by silver nitrate were stained by haemalum and showed also a faint purple stain with purpurin. Cameron [1930] concluded that "haematoxylin does not stain calcium salts though it often identifies areas in which changes favourable to the deposition of calcium salts are taking place. The reaction obtained in these areas depends partly on the presence of iron and mostly on a peculiar ground substance which is normally present in bone and cartilage and which also appears at the site of pathological calcification and, if mordanted with aluminium or chromium, stains deeply with haematoxylin." In accordance with this view we may consider either that this peculiar ground substance was not present in the aortae used for our experiments and did not develop *in vitro* as a preliminary to calcification, or that the absence of iron from our experimental solution was the sole cause of the failure to stain with haemalum. These alternatives will be investigated in further experiments.

The possible influence of bacteria in the tissues upon calcification.

The precautions taken to avoid bacterial contamination of the excised aortae were not so successful as in similar experiments with bone slices. Many of the calcifying solutions when tested at the end of the experimental period produced bacterial growths on agar slopes at 37°. Since the solutions were sterilised before use, the bacteria must have been introduced with the tissue segments. The contamination was not sufficient to produce visible turbidity in the solutions or to affect their p_H .

Microscopical examination of these segments revealed the presence of bacteria in the surface layers of intima and adventitia; occasionally the contamination extended for some little distance into the media. Rarely could any association be traced between the areas so contaminated and those in which calcium deposition took place. The chief instance of such possible relationship was seen in Exp. 4, segment 10 (Plate I, Fig. 3); but even here, dense calcification also occurred in the middle region where no bacteria were seen.

The effect of bacterial invasion on the calcification of tissues *in vitro* is a question of sufficient interest to warrant further study. It is, however, reasonably certain that the formation of most of the deposits observed in our experiments was not due to this cause.

DISCUSSION.

From the results of these experiments it appears that the specific changes induced in the rabbit aorta *in vivo* by diets rich in cholesterol do not provide conditions more favourable for the deposition of calcium carbonatophosphate *in vitro* than are present, or may develop *in vitro*, in aortae excised from normal rabbits.

Neither the lipid deposits characteristic of cholesterol arteriosclerosis nor the tissues in which these lipoids occur have proved specially prone to calcification in our experimental solutions, although deposits of calcium salts were occasionally produced in these areas. This conclusion does not stand in contradiction to the fact that calcification often occurs *in vivo* in atheromatous lesions of long standing which have undergone further necrotic changes. Our experiments were probably of too short duration for such secondary degenerative changes to take place.

It is believed [Duff, 1935] that some alteration of the tissues precedes and favours the deposition of lipoids in this type of arteriosclerosis. We consider that some alteration of the tissues may also precede and favour deposition of calcium salts in the aorta *in vivo* and *in vitro*; but our present results do not justify the assumption that changes which lead to or accompany the accumulation of lipoids in the aorta have any immediate relationship to its subsequent calcification.

The histological interpretation of calcification produced *in vitro* is made difficult by the cell degeneration which accompanies the prolonged immersion of the tissues in the experimental solutions; after 5 or 6 days few stainable nuclei remain. It is permissible, however, again to emphasise that the phenomenon has certain aspects in common with calcification occurring in vitamin D sclerosis as described by Duguid [1930] and others. Both processes are associated with high values of the product $\text{Ca} \times \text{P}$ in the fluids bathing the tissues; in both, calcification affects, primarily, the media, picking out first the elastic laminae and later extending into the tissue between them. Harrison induced cholesterol sclerosis and vitamin D sclerosis successively in the same rabbit and found that the respective lesions were confined to different parts of the vessel. In some of his experiments the two lesions, intimal and medial, alternated in an almost unbroken chain around the circumference of the aorta, a result attributed by him to the movements of the vessel and the relative immobility of those areas affected by the preliminary lesion, rendering them less susceptible to the subsequent lesion. Factors of this nature were not operative in our experiments. In Harrison's experiments, calcification was sometimes found in the fatty debris in the deeper parts of the cholesterol plaques and was more marked in those rabbits which had subsequently received irradiated ergosterol. With these observations, apart from the differences inherent in the two types of experiments, our own results are in agreement.

From information kindly given to us by Prof. I. M. Heilbron it appears that the "pure cholesterol" of commerce is not entirely homogeneous but contains about 0.1% of another sterol which shows selective absorption similar to ergosterol but is probably not identical with the latter. The quantity of cholesterol which must be administered to rabbits in order to produce athero-

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

matous lesions in the aorta is so large in comparison with the quantity of calciferol which produces medial calcification that the possibility that the second sterol, although present in such small proportion, may play some significant part in the development of experimental cholesterol sclerosis would seem to be worth investigation.

SUMMARY.

1. The influence of the changes induced in the rabbit aorta *in vivo* by diets rich in cholesterol on subsequent calcification has been investigated by the method of calcification *in vitro* previously described.

2. The development of typical cholesterol arteriosclerosis was not found to be associated with any increased proneness to calcification of the aorta *in vitro* nor did the deposits so obtained specially favour those portions of the vessel in which the cholesterol lesions occurred.

We wish to thank Dr C. V. Harrison and Dr E. Weston Hurst to whom we are indebted for much helpful advice on the histology of this investigation. Our thanks are also due to Messrs Glaxo Laboratories Ltd., who kindly provided the cholesterol used in these experiments.

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DESCRIPTION OF FIGURES IN PLATE I.

ca, calcification; e.l., elastic lamina; p, cholesterol plaque.

- Fig. 1. Exp. 5. Ascending aorta of normal rabbit, segment 1, showing calcification in the media on elastic laminae (occurring *in vivo*). AgNO₃ and eosin. × 280.
 Fig. 2. Exp. 2. Ascending aorta of rabbit which had received 1 g. cholesterol daily for 43 days; segment 1, 6 days in solution 8 : 5 : 0. Note typical appearance of granules of Ca salt deposited on the elastic laminae in the media. Purpurin and orange G. × 280.
 Fig. 3. Exp. 4. Abdominal aorta of rabbit which had received 1 g. cholesterol daily for 81 days; segment 10, 5 days in solution 8 : 5 : 0. Note extensive calcification in intima, media, adventitia and in the large intimal plaque. Numerous bacteria were present in the surface layers of the segment. AgNO₃ and eosin. × 30.
 Fig. 4. Exp. 5. Intimal plaque, in side artery at junction with aortic arch, of rabbit which had received 1 g. cholesterol daily for 91 days; segment 4, 2 days in solution 8 : 5 : 0. Note granules of calcium salt deposited in plaque. AgNO₃ and eosin. × 520.
 Fig. 5. Exp. 6. Thoracic aorta of normal rabbit, segment 7, 3 days in solution 8 : 5 : 0. Note calcification in the media, on and between the elastic laminae. AgNO₃ and eosin. × 120.
 Fig. 6. Exp. 6. Thoracic aorta of rabbit which had received 1 g. cholesterol daily for 112 days, then normal diet for 41 days; segment 7, 3 days in solution 8 : 5 : 0. Note calcification in the media, but none in the intima or plaque. AgNO₃ and eosin. × 120.