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A QUANTITATIVE STUDY OF CORONARY ARTERIAL CALCIFICATION

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Calcification is generally considered to occur at a late stage in the formation of atheromas and may represent an irreversible event in this process. Much remains unknown concerning the manner in which calcium accumulates. It has been difficult to study the structural pattern of calcification in advanced atheromas because these lesions must be decalcified before they can be properly sectioned. In the present study, coronary atheromas with and without gross evidence of calcium deposit and segments of normal coronary artery have been extracted with ethylenediamine tetra-acetic acid (EDTA) by a new method which permits determination of calcium content, determination of calcium removal rate, and microscopic examination of the decalcified specimen. This approach has yielded new information regarding the distribution of atheromatous calcium and has shown calcium deposition to be related to hematoxylin-ringed lacunar spaces, a morphologic feature of atheromas not previously associated with mineral deposits.

MATERIAL AND METHODS

Coronary atheromas and segments of normal coronary arteries, obtained at necropsy, were fixed in 25 volumes of 4 per cent aqueous calcium-free formalin for periods of 3 to 15 days. All solutions used in this experiment were made with water which was distilled once, and then passed through an ion exchange column.‡ Care

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was taken to prevent tissues from drying before fixation. After fixation, specimens were blotted dry on filter paper, weighed, and transferred to individual polyethylene bottles containing known volumes of 0.34 M NH_4-EDTA (100 cc. per gm. of tissue). These were kept in a 5° C. cold room and gently agitated for 14 days. Following this extraction, specimens were dehydrated in graded alcohols, embedded in paraffin, sectioned, and stained with Harris' hematoxylin and eosin.

NH_4-EDTA (0.34 M) was prepared from NA_2-EDTA * as follows: 500 gm. of NA_2EDTA were dissolved in 5 l. of water and concentrated hydrochloric acid (HCl; approximately 300 ml.) was added to bring the solution to pH 1.5. EDTA was allowed to precipitate for 12 hours, separated by decantation, and washed with 2 volumes of water. Next, the precipitate was dissolved in 4 l. of water and the pH adjusted to 7 with saturated ammonium hydroxide. EDTA was again precipitated in its acid form with concentrated HCl, washed, dissolved in water, adjusted to pH 7 with ammonium hydroxide, and stored in polyethylene containers. NH_4-EDTA prepared in this manner leaves no ash when heated to 500° C. and allows quantitative recovery of as little as 50 μg. of calcium from 0.9 gm. of NH_4-EDTA.

At frequent intervals during the period of extraction, aliquots of EDTA solution were withdrawn for calcium assay. One to 5 cc. samples of the solution were transferred to test tubes (Kimble #45048, 19 by 150 mm., Kimax), and dried at 105° C. Test tubes were next heated to 500° C. in a muffle oven for 8 hours. The ash was dissolved in 4 to 6 drops of 15 per cent aqueous perchloric acid and reheated to 200° C. for an hour. Residual ash was dissolved in 1 ml. of 0.1 N HCl, and calcium determined by titration as follows: 2 ml. of 1.25 N potassium hydroxide (stored in a polyethylene container), 1 drop of 1 per cent sodium cyanide, and 0.4 cc. of 1 per cent Cal-Red were added.† Titration was performed with 2.6 M NA_2-EDTA, standardized against calcium carbonate and delivered from a microburette. This determination is specific for calcium. Magnesium is not measured by EDTA titration when Cal-Red is used as the indicator. Interference by copper, aluminum and iron is eliminated by the addition of potassium hydroxide and sodium cyanide.

From these determinations, calcium content of the atheroma at the beginning of extraction and at the time of each sampling was calculated. The rate of calcium extraction from tissue by chelating solution was then determined by solving the equation: $K = \frac{2.303}{t_2 - t_1} \times \log \frac{C_1}{C_2}$. ($K$ = extraction rate; C_1 = calcium content at t_1; C_2 = calcium content at t_2; t = time in hours.)

RESULTS

Table I gives the calcium content of normal coronary artery and coronary atheromas. Complete removal of calcium by NH_4-EDTA was demonstrated in 3 of these plaques by ashing them and finding no residual calcium. Calcium content is given in per cent of wet weight to allow comparison with previous data for aortic and iliac lesions.[1-3] When coronary atheromas show gross evidence of calcification, they may contain as much or more calcium based on percentage of wet weight as advanced aortic or iliac lesions.

Text-figure 1 illustrates the extraction rates of 8 atheromas. The loga-

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† Scientific Service Laboratory, Inc., P.O. Box 175, Dallas 21, Texas.

rithm of the calcium content of lesions during EDTA extraction is plotted against time, and a straight line results in all instances, indicating an essentially constant rate of calcium removal. This finding allows

TABLE I

CALCIUM CONTENT OF NORMAL CORONARY ARTERIES AND CORONARY ATHEROMAS

Case No.	Sex & age (yr.)	Cause of death*	Specimen	Calcium, per cent of wet weight
65302	M 70	Mesenteric thrombosis	1. Atheroma + Ca†	11.3
			2. Atheroma + Ca	14.1
			3. Atheroma — Ca	1.08
65428	M 68	Gastric carcinoma with metastasis	5. Normal coronary artery	< 0.09
			6. Atheroma + Ca	10.1
65390	M 66	Esophageal carcinoma with metastasis	7. Atheroma + Ca	2.42
65421	M 65	Arteriosclerotic heart disease; congestive failure	8. Atheroma + Ca	10.3
			9. Atheroma + Ca	10.3
			12. Atheroma + Ca	8.6
65474	M 80	Arteriosclerotic heart disease; massive intracerebral hemorrhage	10. Atheroma + Ca	14.1
			11. Atheroma + Ca	13.7
			13. Atheroma + Ca	2.08
			14. Atheroma + Ca	13.9
			16. Atheroma + Ca	12.6
65399	F 81	Carcinoma of pancreas with metastasis	17. Atheroma — Ca	0.69
			18. Atheroma + Ca	5.26
65426	F 81	Acute necrotizing pancreatitis	19. Normal cornonary artery	< 0.04
			20. Normal coronary artery	< 0.04
65427	F 85	Arteriosclerotic heart disease; congestive failure	21. Normal coronary artery	< 0.04
			4. Normal coronary artery	< 0.04
			15. Atheroma — Ca	< 0.07

* Determined at necropsy.

† + Ca = gross calcification; — Ca = no gross calcification.

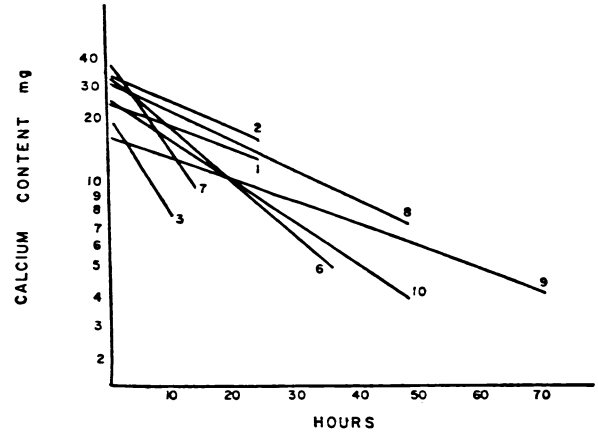
calcium exchange rates to be calculated by the formula given, which describes the rate of a unimolecular chemical reaction.[4]

Heavy, blue hematoxylin staining (Figs. 2 and 3) was frequently encountered in these atheromas although all calcium had been removed. This confirmed the work of Cameron[5] who previously reported that hematoxylin staining might take place in calcific lesions after complete removal of calcium. The extent and intensity of hematoxylin staining could not be correlated with total calcium content or extraction rates. Lack of correlation between hematoxylin staining and calcium content of aortic atheromas has been reported previously.[6]

During a search for morphologic features which might be correlated with calcium contents and extraction rates, the appearance of connective tissue in a plaque with high calcium content and slow extraction rate attracted attention because it was riddled with small, irregular, hematoxylin-ringed lacunas (Figs. 1 and 4). These lacunas had an appear-

ance somewhat suggestive of capillaries or lymphatic spaces and were so numerous that they comprised a considerable portion of the lesion. However, the plaque was not vascular, was quite hard upon gross examination, and contained 10 per cent calcium (wet weight).

Serial sections from all plaques whose extraction rates are illustrated in Text-figure 1 were searched for lacunas. These were easily recognized, and two examiners classifying each plaque independently were in complete agreement as to whether a plaque could be classified as containing lacunas or not. The results are shown in Table II. The rate of calcium removal in plaques containing lacunas was significantly slower than those which showed none.



TEXT-FIGURE 1. The rate of calcium removal from atheromas. Each line demonstrates a change in calcium content in one plaque, identified by the number assigned to it in Table I. The calcium content of plaques 3 and 7 has been multiplied by 10.

DISCUSSION

The procedure employed in this experiment is new and provides a unique method for the study of tissue calcification because both the calcium content and calcium extraction rate of tissues examined microscopically can be known. Previous combined chemical and histologic studies of vascular calcification have employed methods for calcium assay which required destruction of the tissue; thus, microscopic examination of the tissue assayed could not be done. The present technique is made possible by the use of a new salt of EDTA which is readily destroyed by heat, leaving an ash-free residue. EDTA cannot be purchased in the form required, but disodium EDTA is readily converted to the proper salt by a technique described here. The staining characteristics of tissue decalcified by this new method are comparable to tissue decalcified by other techniques, and calcium removal is rapid and complete.

Serial determinations of the calcium content in extraction solutions

have demonstrated that extraction of atheromatous calcium by NH_4-EDTA follows a definite pattern. It has been shown that the calcium content of plaques declines in an exponential manner at a rate which is

TABLE II

CALCIUM EXTRACTION RATE

Case No.	Plaque*	Lacunas		Average extraction rate (μg./hr.)
65302	1	Yes		26.1
65302	2	Yes		29.2
65421	8	Yes		31.7
65421	12	Yes		24.3
			Average	27.8
65302	3	No		78.1
65428	6	No		60.3
65390	7	No		82.2
65474	10	No		48.5
			Average	67.2
				$t = 4.38$†
				$p < .01$†

* The numbers are specimen numbers from Table I.

† The t and p values were determined by standard statistical techniques.[7]

constant and unique for each plaque. Exponential equations have been solved to determine the extraction rate for each plaque shown in Text-figure 1 and these plaques can be divided into two classes: slowly extractable plaques and rapidly extractable plaques. Difference in extraction rate cannot be attributed to factors introduced by the patient's age, sex, or general metabolic state because both rapidly and slowly extractable plaques have been found in the same individual. In addition, because both rapid and slow rates have been found in heavily calcified atheromas, different extraction rates cannot be attributed to differences in the extent of calcification. It seems more probable that extraction rates are determined by the distribution of calcium in atheromas.

It has been shown that extremely hard, heavily calcified lesions may be riddled with lacunar spaces when all calcium has been removed. Clefts are known to occur in lipid-rich atheromas after extraction with fat solvents, and so it seems reasonable to believe that spaces may be left in heavily calcified atheromas when calcium is removed. The possibility that decalcification may leave spaces in tissue does not appear to have received previous consideration. At present, because heavily calcified portions of atheromas cannot be properly sectioned prior to removal of calcium, evidence that the removal of calcium can cause such spaces remains indirect.

The lacunas described in this paper are not unique to atheromas

treated with NH_4-EDTA; they can also be found in plaques decalcified with NA_2-EDTA or with mineral acids. It is possible that their size and number depend upon completeness of calcium removal. They may be more numerous in the present series of atheromas than in material decalcified by other means. Because NH_4-EDTA extraction is the only present method allowing the same tissue to be examined microscopically and assayed for calcium, this question cannot be answered with certainty.

Extraction of calcium is significantly slower from atheromas which are left with lacunas than those which are not. If lacunas are considered to be the sites of previous discrete masses of calcium, this difference in rate can be explained as follows: Calcium contained in granules large enough to leave visible spaces must be assumed to be in larger deposits than calcium in a form too diffuse to leave visible spaces. Geometric considerations indicate that larger masses have a smaller ratio of surface to mass and therefore can be expected to be extracted more slowly. It therefore seems probable that two different forms of calcific deposits occur in advanced atheromas—a diffuse and a discrete form. The diffuse form is rapidly extractable and leaves no visible spaces. The discrete form is slowly extractable and leaves visible spaces. It should be emphasized that although extraction rate data and the occurrence of lacunas are reported together and correlated, they represent two independent lines of evidence, and both suggest that calcification in advanced atheromas can occur in more than one form.

Conclusions

A quantitative method of decalcifying tissues which utilizes NH_4-EDTA is described. Total calcium content of coronary vessels and calcium removal rates have been determined upon specimens left intact for microscopic examination. This technique indicates that coronary atheromatous calcification can be as extensive as that which occurs in aortic atherosclerosis. In addition, the method gives evidence that two different forms of calcification occur in coronary atheromas. Certain calcific atheromas exhibit characteristic hematoxylin-ringed lacunas when calcium is removed. These atheromas are slowly extracted by EDTA and are believed to contain calcium in discrete granules. Other calcific atheromas show no lacunas and are rapidly extracted by EDTA. These atheromas are believed to be more diffusely infiltrated by calcium.

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[*Illustrations follow*]

Legends for Figures

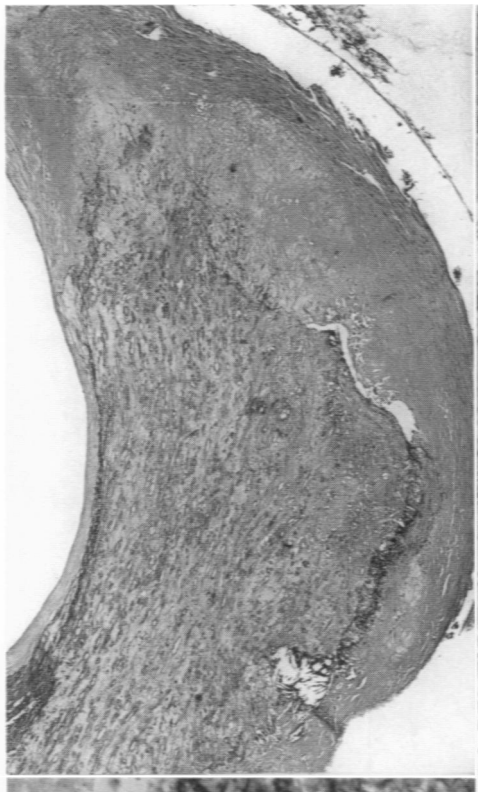
Photomicrographs were prepared from sections stained with hematoxylin and eosin. They show microscopic detail of a very hard, heavily calcified atheroma. Ten per cent of the weight of this plaque was calcium.

Fig. 1. The plaque in eccentric. Heavy hematoxylin staining occurs in two areas, but the major portion of the plaque is filled with lacunar spaces. × 30.

Fig. 2. Detail from the outer margin of the plaque, showing heavy hematoxylin staining adjacent to an area containing lacunar spaces. × 125.

Fig. 3. Detail of the heavy hematoxylin-stained granules shown in Figure 2. × 500.

Fig. 4. Lacunar spaces from the center of the lesion. Hematoxylin stains their margins. × 500.



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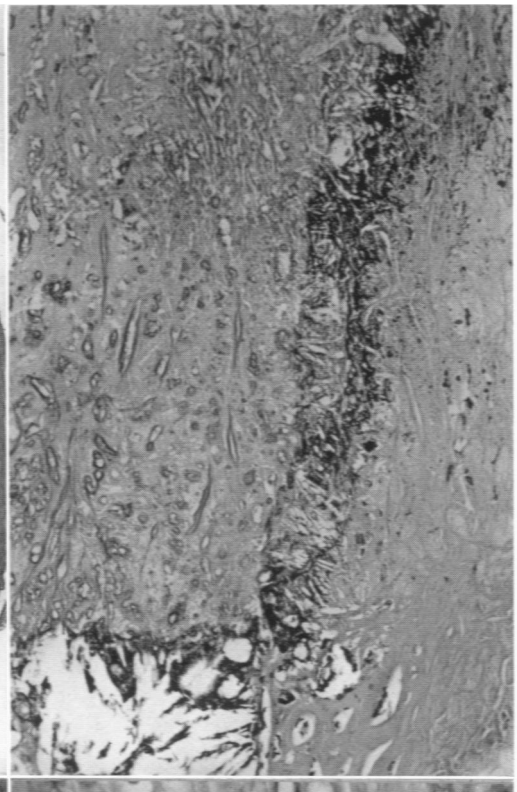

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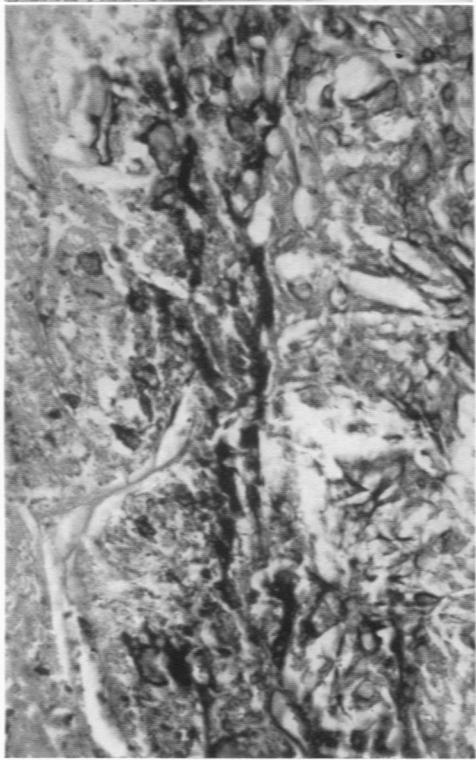

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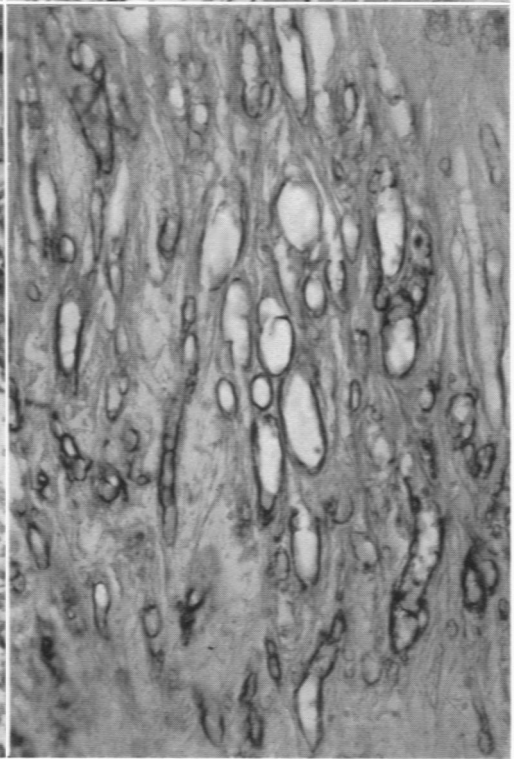

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