Radioautographic evidence using $^{45}\text{CaCl}_2$ is presented to demonstrate that the aorta has an inherent ability to bind calcium ion regardless of age, sex or species. The binding occurs primarily in the intima and is not homogeneously distributed. Experiments with boiled aorta suggest that the primary substance responsible is collagen. The binding of calcium by the aorta has a marked effect upon the stability of emulsions. A theory of arterial plaque formation is presented.

The mechanism by which arterial plaques are produced in atherosclerosis has evoked an enormous effort to elicit its nature. The numerous hypotheses and speculations, based upon evidence from diversified avenues of approach, instead of clarifying the situation, merely point up unresolved complexity.

One must be impressed with the physiologic insolubility of the two principal substances comprising the plaque, namely cholesterol and calcium phosphate or carbonate, an insolubility which suggests an irreversible process once either of these substances is precipitated in the artery. Since cholesterol is transported in chylomicrons and lipoproteins, factors influencing the stability of these transporting agents may conceivably influence the deposition of cholesterol. With the knowledge that many proteins effectively bind calcium ion, and that calcium salts themselves markedly effect the stability of emulsions, it appeared profitable to investigate the binding of calcium ion by the aorta and its effect upon emulsions containing cholesterol. This was accomplished by means of radioautography.

**METHODS**

Unless otherwise stated, all experiments were conducted with human aortas. These were stripped of adhering tissue, washed thoroughly with distilled water, placed between filter paper and weighted down with lead bricks. After 48 hours, the aorta emerged dry, brittle and translucent, and could be kept indefinitely. Upon immersion in water, it resumed its original elastic and opaque properties.

Pieces of thoracic aorta up to 30 Gm. were extracted with acetone-petroleum ether (1:1, v/v) for 2 days, rinsed thoroughly in distilled water and decalcified in 10 per cent sodium ethylene diamine tetra-acetate. The tissue was...
washed thoroughly again in distilled water. For calcium binding studies the tissue was shaken for 4 hours at room temperature with 20 μe. Ca^{45}Cl₂ in a volume of 250 ml. distilled water, after which it was incubated overnight at 37 C. Following incubation, the tissue was removed and washed with 10 L. of running, distilled water, dried between lead bricks, and radioautographed using dental film. In experiments designed for comparative purposes, identical conditions were maintained for control and test specimens. This includes the processing of film. Radioactive emulsions were made with 5 mg. stearic acid, 5 mg. sodium desoxycholate, 0.5 Gm. glycerol trioleate, 2 mg. cholesterol, 20 μe. oleic acid-1^131 in 5 ml. distilled water. These were shaken vigorously and added to 250 ml. distilled water containing the aorta. When cholesterol-4-C¹⁴ was used, the radioactive oleic acid was omitted.

RESULTS

Figure 1 shows a sclerotic piece of aorta from a 60-year-old male after the extraction-decalcification procedure. Although the plaques themselves appear untouched, the area around them is perfectly clear. This includes the indented area (arrow) which represents the location of a plaque that had been removed physically prior to the extraction and decalcification. Figure 2 shows a radioautograph of the same tissue following incubation with 20 μe. Ca^{45}Cl₂. It is apparent that the uptake of Ca^{45} is specifically confined to the areas represented by the position of the plaques in figure 1. More important is the observation that the area formerly occupied by a plaque (fig. 2), (arrow), still has the ability to bind calcium ion. The inherent ability of the aorta to bind calcium ion is demonstrated further with an infant’s aorta (23 months, male, fig. 3) and a cat’s aorta (fig. 4). The interesting observation is that the binding is not homogeneous, but appears to be localized in specific positions. Moreover, the localization appears primarily in the intima, for radio-
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FIG. 4. Radioautograph of decalcified-extracted cat's aorta demonstrating Ca$^{45}$Cl$_2$ uptake. Radioautograph exposure, 17 hours.

FIG. 5. Radioautograph of decalcified-extracted sclerotic aorta, 56 year old male demonstrating uptake of Ca$^{45}$Cl$_2$. Radioautograph exposure, 17 hours.

Radioautographs of the adventitia show little uptake of calcium. Information as to the nature of the substance(s) responsible for the binding of calcium ion is obtained by noting the effect of boiling upon the extent of binding. Figure 5 is a radioautograph of an aorta (male 56 years) prior to boiling in water for an hour. Figure 6 is a radioautograph of the same piece of tissue after boiling and reincubation with Ca$^{45}$Cl$_2$. The shrinkage of tissue is characteristic of elastic tissue upon boiling, and it is obvious that the treatment has resulted in a great loss in the original binding ability.
The influence of bound calcium upon the stability of the emulsions was studied in two ways. First, the Ca$^{45}$ binding pattern was recorded. Whereupon the aorta was then incubated with the radioactive emulsion containing oleic acid-$I^{131}$. It was then possible to select a short exposure time without any interference from the soft radiation of Ca$^{45}$.

When the aorta strip shown in figure 1 was incubated with oleic acid-$I^{131}$ emulsion, after it had bound Ca$^{45}$ (fig. 2), the radioactive oleic acid is found deposited in the same sites as those occupied by the calcium (fig. 7).

The other method of studying the effect of bound calcium upon the emulsion stability is demonstrated in figures 8 and 9. Figure 8 is a radioautograph of an aorta strip (female, 20 years) which after extraction and decalcification was incubated with the oleic acid-$I^{131}$ emulsion. Figure 9 is a radioautograph of an adjacent strip of the same aorta which was not decalcified, but was incubated with nonradioactive 0.1 M CaCl$_2$, washed thoroughly, and incubated at the same time and with the same concentration of radioactive oleic emulsion as the previous aorta strip. The influence of bound calcium upon oleic acid deposition is apparent. When cholesterol-C$_{14}$ is used in the emulsions instead of oleic acid-$I^{131}$, the deposition is the same (fig. 10) (female, 20 years).

**DISCUSSION**

The binding of calcium ion appears to be an inherent characteristic of the aorta regardless of age, sex or species. The binding has been shown to be confined to localized areas in the intima instead of being homo-
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Fig. 8. Radioautograph of decalcified-extracted aorta, 20 year old female, decalcified prior to incubation with emulsion containing oleic acid—$^{131}$I. Radioautograph exposure, 7 hours.

generously distributed, which suggests that the substance(s) responsible for the localization is more or less randomly dispersed. One possibly responsible substance is collagen, well known for its calcium binding ability. The experiment with the boiled aorta is evidence in this direction. The ground substance which also can be conceived as binding calcium is too uniformly distributed to account for the heterogenous type of binding observed. The so-called polyuronides described by Faber remain as another possibility. Elastin which also is randomly dispersed cannot account for the large loss of binding capacity following boiling, because of its insolubility.

The influence of calcium ion upon the stability of emulsions has been amply recorded in the literature. Whereas the concentration of serum calcium is never high enough to be influential, it is conceivable that bound calcium may present a local concentration which is capable of producing a phase reversal and precipitation of the emulsifying agent. We have obtained evidence of this by using an ion exchange resin of the carboxylic type, IRC-50. In the acid or hydrogen form, a column of this resin has no effect upon the emulsion stability of the composition used in these experiments, that is, the emulsion passes through unaltered. However, if the resin is converted into the calcium form, the emulsion is retained irreversibly in the column.

One may visualize in the same manner the precipitation of radio-oleic acid and radiocholesterol from the emulsion, caused by the calcium ion bound to the artery wall. That bound calcium is necessary for the precipitation of the emulsion has been demonstrated in figures 8 and 9.

In the light of the present information, we envisage arterial plaque formation to be...
due to a series of events which must follow one another in a definite order. The artery has an inherent ability to bind calcium ion. The present studies suggest that the substance primarily responsible is collagen. The bound calcium is capable of reducing the stability of chylomicrons by a reversal of phase and precipitating the emulsifying agent. Injury to the arterial wall would cause more collagen to be formed in repairation. This enhances the calcium binding capacity which in turn is able to precipitate more emulsion. It would then appear that of the various components of the chylomicrons, only the precipitated cholesterol is not metabolized and the insoluble calcium salts of the fatty acids, which appear as fatty streaks in the vessel, are disposed of slowly, the final deposit being cholesterol and insoluble calcium salts. The present results point to the investigation of plausible sources of arterial injury as being the logical avenue of research in atherogenesis. The influence of diet would appear to be secondary.

Various authors in the past have emphasized one or more of the above factors in atherogenesis. Several localizing factors have been cited: mucoid degeneration of collagen, mucinous material in the ground substance, and degenerate elastic fibers. Moreton in particular has stressed the importance of chylomicrons. That calcification preceded plaque formation was noted by Blumenthal et al. and Lansing et al.

SUMMARY

Radioautographic evidence using Ca\(^{45}\)Cl\(_2\) is presented to demonstrate that the aorta has an inherent ability to bind calcium ion regardless of age, sex or species. The binding of calcium ion occurs primarily in the intima and is not homogeneously distributed. The aorta loses much of its ability to bind calcium ion upon boiling. The primary substance responsible is believed to be collagen.

The binding of calcium by the aorta has a marked effect upon the stability of emulsions, causing precipitation. This is demonstrated with emulsions containing oleic acid and cholesterol-C\(^{14}\).

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SUMMARIO IN INTERLINGUA

Es presentate observationes radioautographiche, obtenite per medio del uso de Ca\(^{45}\)Cl\(_2\), que demonstra que le aorta ha le capacitate inherente de ligar iones de calcium, sin reguardo al etate del subjecto ab que le
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aorta proveni, a su Sexo, o al Specie que illo representa. Le ligation de iones de calcium occurre primarimente in le intima e non es distribuite homogeneamente. Quando un aorta es ebullite, illo perde un grande parte de su capacitate de ligar iones de calcium. Es exprimite le opinion que collageno es le substantia primarimente responsabile pro le ligation de iones de calcium.

Le ligation de calcium per le aorta ha un effecto marcate in le stabilitate de emulsiones. Illo causa precipitation. Isto es demonstrate con emulsiones que contine acido oleic con $^{131}$ e cholesterol con $^{14}$.  

REFERENCES

Radioautographic Study of Aortic Plaque Formation

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