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# A Study of the Lipids of the Rat Aorta During Induced Calcification (33511)

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Gilman *et al.* (1) produced calcification in the aortas of rats by administering massive oral dosages of vitamin $D_2$. This model of induced ectopic calcification has been used by Fleisch *et al.* (2), Irving *et al.* (3) and others to study factors controlling the calcification process. As had been consistently observed before in normal calciflying tissue (4–7), Irving *et al.* (3) found that calcified areas of the rat aortas, so produced, were strongly Sudan black positive, whereas normal aortas were only weakly stained. This model system seemed useful for the study of lipids in calcifying tissue because calcification could be induced under carefully controlled conditions. Any obvious changes in the lipid composition accompanying the aortic calcification should be of significance in elucidating the role of lipids in the calcification process.

*Materials and Methods.* Two experiments were performed in which 150–200 g female rats of the Wistar strain were given large oral dosages of vitamin $D_3$ for 5 consecutive days, the control groups receiving none. The animals were sacrificed on the fourth day after the final dosage of vitamin $D_3$. In the first experiment 75,000 IU of vitamin $D_3$/kg of body weight per day were given; in the second experiment 75,000 IU/rat per day were administered. The aortas (from the aortic arch to the bifurcation into the iliac arteries) were dissected free, collected in a solution of 0.9% NaCl at 4°, carefully cleaned of adhering tissue, split lengthwise, rinsed three times with fresh cold saline, blotted free of excess moisture, weighed. Lipids were extracted from the pooled fresh tissues with choloroform–methanol (8) using five consecutive extractions, the first of which lasted overnight; the rest allowing 1–2 hr contact between tissue and solvent. The aortas were then dried under $N_2$, weighed, and decalcified with 100 ml of 0.5 *M* EDTA pH 8.0, at 4° for 24 hr. The decalcified aortas were rinsed repeatedly with cold (4°) distilled water to remove the EDTA, blotted free of excess moisture and reextracted with chloroform–methanol five times as before. To insure complete removal of lipids, the tissues were reextracted five more times with chloroform–methanol–conc. HCl, 200:100:1 (v/v) (9). Lipid extracts were purified by previously published methods (10). Phospholipids were analyzed using paper chromatography (11); nonpolar lipids being analyzed by the method of Amenta (12) using silica gel–loaded glass–fiber paper (Gelman Instrument Co., Ann Arbor, Michigan, chromatography media I.T.L.C., type SA, 20 × 20 cm) with solvent systems adapted from the method of Freeman and West (13).

For chemical analysis, cleaned aortas from six rats of each experimental group were blotted free of excess moisture, weighed, freeze dried, and reweighed, moisture content being determined by difference. Calcium and inorganic phosphorus were analyzed from

TABLE I. Composition of Rat Aortas.[a]

| Constituent | | Expt. A | | Expt. B | |
|---|---|---|---|---|---|
| | | Control | Vitamin $D_3$ treated | Control | Vitamin $D_3$ treated |
| Wet wt. | (mg/aorta) | 50.71 ± 0.76 | 52.89 ± 1.15 NS[d] | 44.80 ± 0.63 | 55.74 ± 0.99[c] |
| Dry wt. | (mg/aorta) | 13.47 ± 0.19 | 15.88 ± 0.47[c] | 13.31 ± 0.16 | 18.95 ± 0.40[c] |
| Ash | (mg/aorta) | 0.04 ± 0.001 | 1.01 ± 0.03[c] | 0.04 ± 0.002 | 3.77 ± 0.30[c] |
| Organic matter | (mg/aorta) | 13.34 ± 0.19 | 14.87 ± 0.50[b] | 13.27 ± 0.16 | 15.18 ± 0.70[b] |
| Moisture | (% wet wt.) | 71.44 ± 0.12 | 68.07 ± 0.36[c] | 70.52 ± 0.10 | 66.81 ± 0.31[c] |
| Ash | (% dry wt.) | 0.30 ± 0.02 | 6.34 ± 0.94[c] | 0.30 ± 0.02 | 19.90 ± 2.25[c] |
| Lipid | (mg/aorta) | 1.07 | 1.68 | 1.03 | 1.39 |
| | (% organic) | 8.02 | 11.30 | 7.76 | 9.16 |

[a] For Expt. A, the vitamin D-treated rats were given orally 75,000 IU of vitamin $D_3$/kg of body wt./day for 5 consecutive days, sacrifice being on the 4th day following termination of the vitamin dosage. For Expt. B, the vitamin D-treated rats were given 75,000 IU of the vitamin/animal (i.e., about 5 times that given in A) following the same experimental protocol. Values are the mean ± SEM of six analyses. For lipid analysis in Expt. A, pooled samples of 50 and 52 aortas, respectively, for control and vitamin $D_3$-treated rats were used. In Expt. B, pooled samples of 66 and 65 aortas, respectively, for control and vitamin $D_3$-treated rats were used.

[b] Difference from control value significant at $p < 0.05$.

[c] Difference from control value significant at $p < 0.01$.

[d] NS = difference from control value not significant.

10% trichloroacetic acid digests of the dry aortas, calcium being determined by atomic absorption spectrophotometry using similarly prepared standards; inorganic P being analyzed by the method of Martin and Doty (14). Ash content was calculated from the calcium and phosphorus content (assuming the mineral deposit was of an apatitic nature) using the Ca/P ratio to determine the member of the apatitic series. Organic matter was determined from the dry and ash weights by difference.

*Results.* The general composition of the normal and vitamin $D_3$-treated rat aortas are given in Table I. The wet and dry weights of the calcifying rat aortas were increased, this being due to the larger ash, lipid, and general organic content. The moisture content of the calcifying aortas was decreased in direct proportion to the ash content. The percentage ash (based on Ca and P analysis) in the first and second experiments were 6.3 and 20% of the dry weight, respectively, values markedly greater than that of the controls. Lipid content was increased, whether expressed as a percentage of wet or organic weight, or as mg per aorta. Expressed as a percentage of dry weight, inconsistent differences were obtained which appeared to be caused by the large increase in ash. Analysis of calcium and inorganic phosphate in the mineral deposits of the aortic calcification revealed a progressive increase in the Ca/P ratio with increasing ash content. Although there was a generally heavier calcification in Expt. B, in both experiments the aortas with the least calcification had Ca/P molar ratios of approximately 1.50, whereas those with the highest mineral content had molar ratios of 1.89–1.98, these latter ratios being well above that of pure hydroxyapatite, i.e., 1.67.

Table II presents the composition of rat aorta phospholipids. As in most tissues, phosphatidyl choline was the major phospholipid fraction, followed by phosphatidyl ethanolamine, sphingomyelin, phosphatidyl serine and then the other acidic phospholipids in smaller proportions. In all, over twenty lipid components were observed on chromatograms of aortic phospholipids, however, many represented only a minor fraction of the lipid P and have not been identified. Nevertheless, some of these minor phospholipids are actively labeled with $^{32}P$ in other tissues (11). In all, phospholipids accounted for 20.1–25.9% of the total aortic lipid.

TABLE II. Composition of Rat Aorta Phospholipids.[a]

| Phospholipid | Expt. A | | Expt. B | |
|---|---|---|---|---|
| | Control | Vitamin $D_3$ treated | Control | Vitamin $D_3$ treated |
| | (% of total lipid P[b]) | | | |
| Sphingomyelin | 13.45 ± 0.40 | 14.15 ± 0.14 | 15.14 ± 0.27 | 14.80 ± 0.51 |
| Phosphatidyl choline | 37.93 ± 0.69 | 41.18 ± 0.45[c] | 37.29 ± 0.30 | 44.97 ± 0.08[c] |
| Lyso-phosphatidyl choline | 2.03 ± 0.13 | 2.27 ± 0.22 | 1.85 ± 0.03 | 2.13 ± 0.06 |
| Phosphatidyl ethanolamine | 24.62 ± 0.32 | 24.88 ± 0.20 | 23.76 ± 0.16 | 21.15 ± 0.21 |
| Total neutral phospholipids | *78.03 ± 1.19* | *82.55 ± 0.49[c]* | *78.09 ± 0.68* | *83.05 ± 0.37[c]* |
| Phosphatidyl serine | 9.85 ± 0.52 | 7.94 ± 0.17[d] | 13.01 ± 0.56 | 7.83 ± 0.20[d] |
| Lyso-phosphatidyl serine | 0.06 ± 0.01 | 0.29 ± 0.07[d] | 0.00 ± 0.00 | 0.60 ± 0.05[e] |
| Phosphatidyl inositol | 4.45 ± 0.14 | 3.61 ± 0.23 | 3.48 ± 0.09 | 4.46 ± 0.37 |
| Diphosphoinositide | 0.54 ± 0.17 | 0.46 ± 0.24 | 0.28 ± 0.06 | 0.08 ± 0.04 |
| Phosphatidic acid | 0.91 ± 0.16 | 0.35 ± 0.07 | 0.61 ± 0.11 | 0.87 ± 0.09 |
| Phosphatidyl glycerol | 0.76 ± 0.33 | 0.50 ± 0.18 | 0.25 ± 0.06 | 0.55 ± 0.11 |
| Diphosphatidyl glycerol | 2.92 ± 0.31 | 2.33 ± 0.15 | 2.49 ± 0.03 | 1.29 ± 0.04 |
| Unidentified acidic | 1.91 ± 0.40 | 1.76 ± 0.51 | 1.70 ± 0.30 | 0.86 ± 0.16 |
| Total acidic phospholipids | *21.41 ± 1.10* | *17.23 ± 0.53[d]* | *21.82 ± 0.64* | *16.54 ± 0.48[d]* |

[a] Each phospholipid includes both the diacyl and alkenyl-acyl forms. Percentage of the total lipid P was obtained by summation of P from each component on silica gel-loaded paper chromatograms, the recovery of total lipid P applied to chromatograms being 93.5–95.5%. Italicized and offset values are subtotals of the various lipid classes. Phospholipids accounted for 25.91, 21.81, 20.10, and 23.43% of the total aortic lipid in the normal (A and B) and vitamin $D_3$ treated (A and B) groups, respectively.

[b] Total lipid P is the summation of amounts from the 3 separate extracts. Experimental values are the means ± SEM of 4 analysis (A) and 2 analysis (B).

[c] Difference from the control value in the same experiment significant at $p < 0.05$.

[d] Difference from the control value in the same experiment significant at $p < 0.02$.

[e] Difference from the control value in the same experiment significant at $p < 0.01$.

When the composition of the phospholipids from the aortas of normal and calcifying aortas were compared, certain consistent alterations were observed. The proportion of the phosphatidyl choline and total neutral phospholipids, as well as the lyso derivative of phosphatidyl serine were significantly increased; whereas the proportion of phosphatidyl serine itself was distinctly decreased. The proportion of the other phospholipids were generally quite similar; nevertheless, the proportion of total acidic phospholipids was significantly reduced in the calcifying aortas.

Table III presents the composition of rat aortic nonpolar lipids, which accounted for 74.1–79.9% of the total aortic lipid. In all 16 separate components were detected on chromatograms of the nonpolar lipids; however, many of the minor fractions were not identified. Triglycerides were by far the major fraction, followed by free cholesterol, free fatty acids, diglycerides, monoglycerides, and cholesterol esters. Comparison of the composition of the nonpolar lipid fraction from the control and calcifying aortas revealed no consistent differences in the major lipid classes. There was however, a somewhat higher proportion of monoglycerides, free fatty acids and 1,2-diglycerides, and a significant reduction in the proportion of waxes and unidentified nonpolar lipids in the calcifying aortas. The significance of these findings will be discussed later.

Comparison of the extractability of the polar lipids from the normal and calcifying aortas revealed some distinct differences which have direct bearing on the identify of the Sudan black staining material. Figure 1 shows that in normal aortas virtually all the neu-

TABLE III. Composition of Nonpolar Rat Aortic Lipids.[a]

| Lipid | Expt. A | | Expt. B | |
|---|---|---|---|---|
| | Normal | Vitamin $D_3$ treated | Normal | Vitamin $D_3$ treated |
| | (% total nonpolar lipid[b]) | | | |
| Cholesterol esters | 1.64 ± 0.09 | 1.04 ± 0.05 | 2.25 ± 0.26 | 2.98 ± 0.24 |
| Free cholesterol | 8.49 ± 0.18 | 6.54 ± 0.38 | 6.17 ± 0.18 | 7.15 ± 0.32 |
| *Total sterols* | *10.13 ± 0.27* | *7.58 ± 0.43* | *8.42 ± 0.44* | *10.13 ± 0.56* |
| Triglycerides | 77.50 ± 0.29 | 80.37 ± 0.59 | 71.10 ± 1.50 | 71.98 ± 0.74 |
| 1,3-Diglycerides | 1.47 ± 0.15 | 1.72 ± 0.17 | 2.64 ± 0.68 | 1.31 ± 0.32 |
| 1,2-Diglycerides | 1.19 ± 0.05 | 1.28 ± 0.07 | 2.19 ± 0.30 | 3.18 ± 0.74 |
| Monoglycerides | 1.00 ± 0.01 | 1.27 ± 0.08 | 2.72 ± 0.38 | 3.35 ± 0.49 |
| *Total glycerides* | *81.16 ± 0.50* | *84.64 ± 0.91* | *78.65 ± 2.86* | *79.82 ± 2.29* |
| Free fatty acids | 2.58 ± 0.12 | 2.96 ± 0.12 | 4.27 ± 0.62 | 5.33 ± 0.62 |
| Waxes (tentative) | 2.10 ± 0.09 | 1.78 ± 0.03[c] | 1.74 ± 0.06 | 1.31 ± 0.00[c] |
| Unidentified | 4.03 ± 0.21 | 3.03 ± 0.13[c] | 6.92 ± 0.90 | 3.40 ± 0.33[c] |

[a] Percentage of the nonpolar lipid was obtained by summation of amounts from each component on silica–gel loaded glass–fiber paper chromatograms. Italicized and offset values are subtotals of the various lipid classes. Nonpolar lipids accounted for 74.09, 78.19, 79.90, and 76.57% of the total aortic lipid in the normal (A and B) and vitamin $D_3$ treated (A and B) groups, respectively.

[b] Total nonpolar lipid is the summation of amounts for the 3 separate extracts. Values are the mean ± SEM of 3 analyses.

[c] Difference from the control value in the same experiment significant at $p < 0.05$.

tral phospholipids (phosphatidyl choline, phosphatidyl ethanolamine, and sphingomyelin) and the two major acidic phospholipids (phosphatidyl serine and phosphatidyl inositol) were extracted prior to treatment with EDTA. Significant amounts (5–9%) of certain of the minor acidic phospholipids were not extracted until after the EDTA treatment. However, in the calcifying aortas marked retention of phosphatidyl serine, phosphatidyl inositol, and the minor acidic phospholipids occurred, the amount being directly related to the calcium content of the aortas. The neutral phospholipids were almost completely extracted from calcifying aortas before demineralization just as they were in the normal aortas.

Examination of the extractability of the nonpolar lipids (Fig. 2) revealed a complete lack of relationship to the degree of calcification in the aortas. Although significant amounts of these lipids (for some unidentified lipids, the major fraction) were not removed before treatment with EDTA, no meaningful differences between normal and calcifying aortas within each experiment could be detected.

In Table IV the data are presented as lipid content per aorta. It is shown that the observed reduction in the proportion of certain lipids shown in Tables II and III would apparently be primarily due to preferential synthesis or accumulation of the neutral lipids rather than the destruction of acidic phospholipids. Neutral glycerides, neutral phospholipids, sterols, and free fatty acids were all significantly increased in the calcifying aortas, whereas no increases occurred in the acidic phospholipids, the waxes, or the unidentified nonpolar lipid fraction. Nevertheless, the increased amount of lysophosphatidyl serine, and to a lesser extent, free fatty acids, monoglycerides and 1,-2-diglycerides indicate that lipid degradation may also have been a factor.

*Discussion.* A number of important facts emerge from this study which have bearing on the possible role of lipids in the calcification process. For example, the primary effect of the vitamin D treatment on the rat aorta

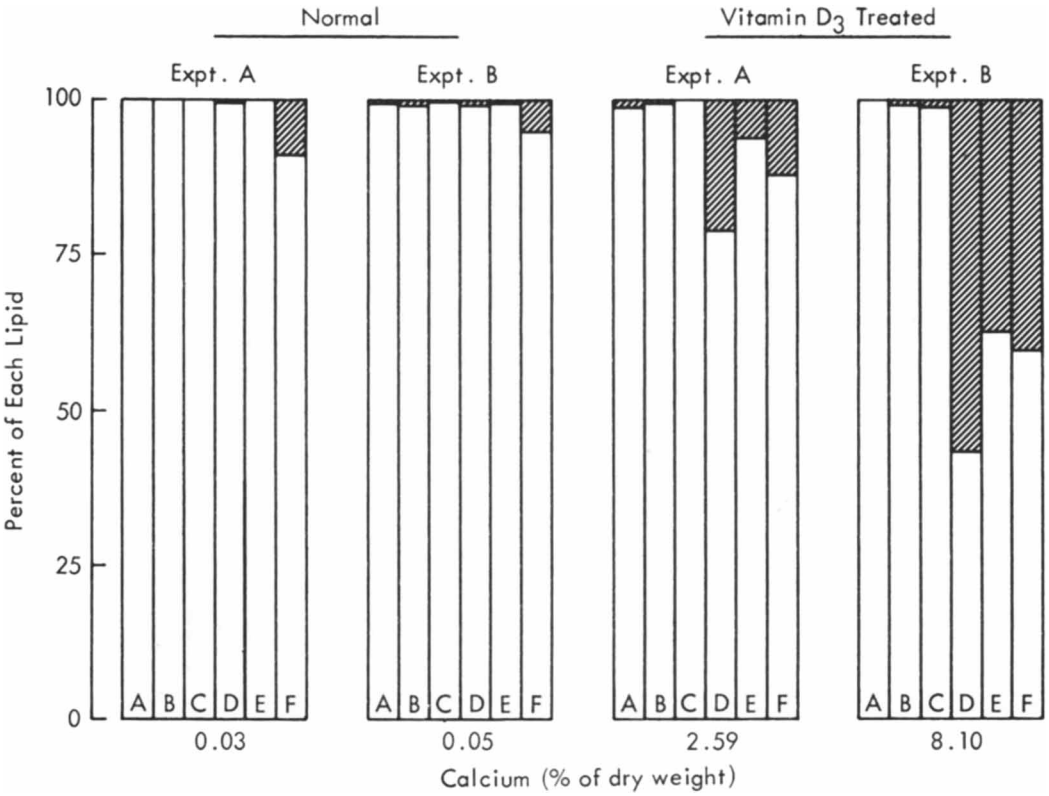


FIG. 1. Relationship between extractability of neutral and acidic phospholipids and the degree of aortic calcification. A, sphingomyelin; B, choline phosphoglycerides; C, ethanolamine phospholipids; D, serine phospholipids; E, inositol phospholipids; F, all other acidic phospholipids. Within each column the clear area represents lipid removed before demineralization; the hatched area, lipid removed after demineralization. Note the decrease in extractibility of acidic phospholipids before demineralization (columns D, E, and F) from the aortas of the vitamin $D_3$-treated rats, the extent of which was related to the calcium content.

lipids appeared to be a stimulation of the synthesis of neutral lipids. While it is possible that these lipids might have accumulated from circulating lipids (16), most studies have indicated that the majority of these lipids are synthesized in situ (15–17). Regardless, the increase in neutral lipids resulted in a significant reduction in the proportion of acidic phospholipids, the lipids which possess the capacity to bind calcium. This raises the possibility that acidic phospholipids may function, in part, to stabilize initial benign deposits of amorphous calcium phosphate (18), preventing their conversion to crystalline apatite. The data on extractability of the acidic phospholipids (Fig. 2) clearly demonstrate that these lipids do indeed bind to calcium deposits, as our previous studies indicated (19, 20).

The data presented in Table IV suggest that increased synthesis of neutral lipids was primarily responsible for the decreased proportion of acidic phospholipids. However, it is interesting to note that while the proportion of phosphatidyl serine was decreased (Table II), the proportion of lysophosphatidyl serine was clearly increased, as were to a lesser extent the proportions of lysophosphatidyl choline, free fatty acids and monoglycerides (Table II). These data suggest that increased hydrolysis of certain lipids may have resulted from the excessive vitamin $D_3$ treatment.

The data on lipid extraction corroborate

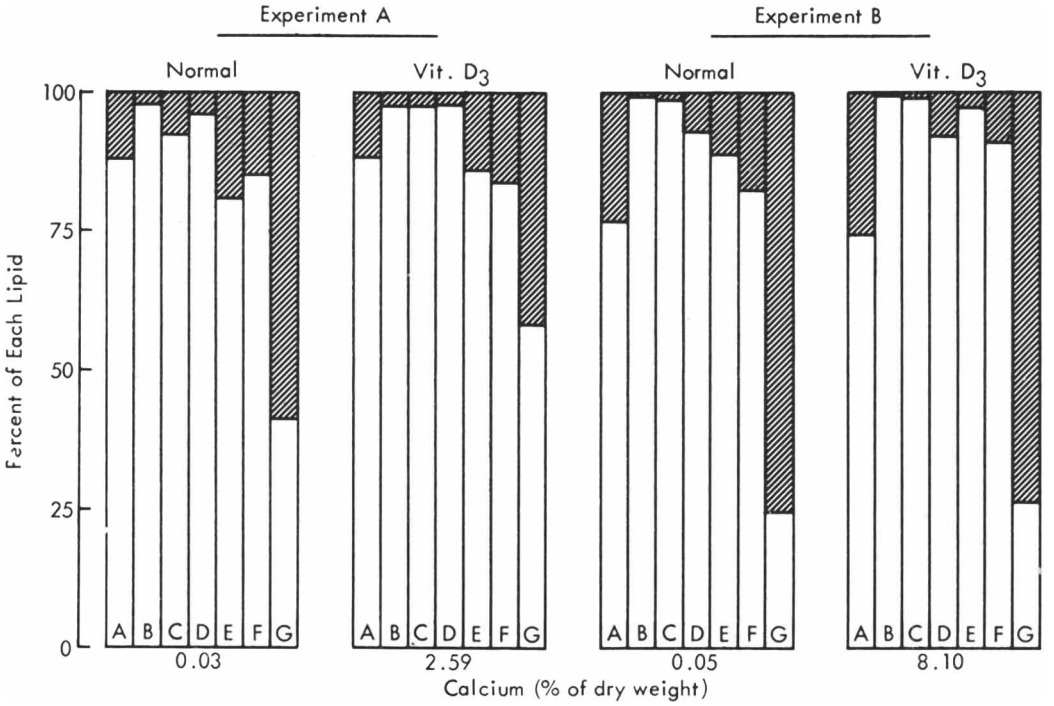


FIG. 2. Lack of relationship between extractability of nonplolar lipids and the degree of aortic calcification. A, cholesterol esters; B, free cholesterol; C, triglycerides; D, diglycerides; E, monoglycerides; F, free fatty acids; G. unidentified nonpolar lipids. Within each column the clear area represents lipid removed before demineralization; the hatched area, lipid removed after demineralization. Although significant amounts of lipid were removed after treatment with EDTA, note that no obvious relationship exists between the extractability of any class of nonpolar lipid and the calcium content of the aortas.

TABLE IV. Lipid Content of Rat Aortas.

| Lipid | Expt. A | | Expt. B | |
|---|---|---|---|---|
| | Control | Vitamin $D_3$ treated | Control | Vitamin $D_3$ treated |
| | (mg/aorta[a]) | | | |
| Waxes (tentative) | 0.0163 ± 0.0007 | 0.0238 ± 0.0004 | 0.0138 ± 0.0005 | 0.0139 ± 0.0001 |
| Sterols | 0.0788 ± 0.0021 | 0.1015 ± 0.0058[c] | 0.0669 ± 0.0035 | 0.1074 ± 0.0059[d] |
| Neutral glycerides | 0.6314 ± 0.0039 | 1.1333 ± 0.0122[d] | 0.6253 ± 0.0227 | 0.8461 ± 0.0243[d] |
| Free fatty acids | 0.0201 ± 0.0009 | 0.0396 ± 0.0016[d] | 0.0339 ± 0.0049 | 0.0565 ± 0.0066[b] |
| Unidentified nonpolar | 0.0314 ± 0.0016 | 0.0406 ± 0.0017 | 0.0550 ± 0.0072 | 0.0360 ± 0.0035 |
| *Total nonpolar* | *0.778 ± 0.009* | *1.339 ± 0.022[d]* | *0.795 ± 0.017* | *1.060 ± 0.028[d]* |
| Neutral phospholipids | 0.215 ± 0.003 | 0.279 ± 0.002[d] | 0.174 ± 0.002 | 0.270 ± 0.001[d] |
| Acidic phospholipids | 0.059 ± 0.003 | 0.059 ± 0.002 | 0.049 ± 0.001 | 0.054 ± 0.002 |
| *Total phospholipids* | *0.274 ± 0.004* | *0.338 ± 0.007[d]* | *0.223 ± 0.002* | *0.325 ± 0.003[d]* |

[a] Amounts of individual lipid classes were calculated from the total yields from pooled aortic samples divided by the number of aortas. Values are the means ± SEM of 3 analyses. Italicized and offset values are subtotals of the various lipid classes. Statistically significant differences from the control values in the same experiment are indicated as: [b] $p \leq 0.05$; [c] $p \leq 0.02$; [d] $p \leq 0.01$. Note that in both experiments the amount of sterols, neutral glycerides, free fatty acids, total nonpolar lipid, neutral phospholipids, and total phospholipids are significantly increased.

our earlier postulation that acidic phospholipids are responsible for the strong sudanophilia seen at sites of new calcification. Although significant amounts of certain nonpolar lipids were not extracted until after treatment with EDTA, there was no significant difference between the normal and the calcifying aortas nor any correlation to calcium content. These findings suggest that these nonpolar lipids must not have contributed to the Sudan black staining seen at sites of calcification, although they may have been responsible for the weak background staining which was observed in noncalcifying areas. In contrast, the extraction of the acidic phospholipids (especially phosphatidyl serine) was markedly different between the normal and calcifying aortas; and this was directly related to the mineral content of the aortas.

The data on the calcium/phosphorus ratios of the ectopic mineral deposits suggest that the calcification pattern in the aorta was not exactly the same as that in normal calcifying tissues. The very high calcium/inorganic phosphorus ratios indicate that the mineral deposit probably contained considerable amounts of carbonate; unfortunately, analyses for this ion were not done. However, despite the differences between normal and ectopic calcification, a close association between acidic phospholipids and mineral deposition was observed once again. The significant reduction in the proportion of phosphatidyl serine and total acidic phospholipids in the calcifying aorta and their association with the early mineral phase suggests that they may function in a regulating role by stabilizing amorphous calcium phosphate and controlling its conversion to crystalline hydroxyapatite.

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