Phospholipases in Arterial Tissue

IV. THE ROLE OF PHOSPHATIDE ACYL HYDROLASE, LYSOPHOSPHATIDE ACYL HYDROLASE, AND SPHINGOMYELIN CHOLINE PHOSPHOHYDROLASE IN THE REGULATION OF PHOSPHOLIPID COMPOSITION IN THE NORMAL HUMAN AORTA WITH AGE

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ABSTRACT The role of phospholipases in the regulation of the changing phospholipid composition of normal human aortae with age was studied. Portions of grossly and histologically lesion-free ascending aortae from 16 females and 29 males obtained at autopsy, were analyzed for deoxyribonucleic acid (DNA), phospholipid, and cholesterol content and phospholipid composition. Enzymic activity toward four substrates, lecithin (LE), phosphatidyl ethanolamine, lysolecithin, and sphingomyelin (SP), was determined on portions of the same homogenate. By regression analysis for correlation between all determinations and age the following results were obtained: (*a*) total phospholipids and choleserol increased linearly with age; (*b*) the increase in sphingomyelin accounted for about 70% of the phospholipid increment; (*c*) hydrolysis of lecithin and phosphatidyl ethanolamine increased markedly with age, that of lysolecithin only moderately; (*d*) hydrolysis of sphingomyelin decreased with age; and (*e*) an inverse relation between the SP/LE ratio and age and sphingomyelinase/lecithinase activity and age was obtained. These results were interpreted to indicate that a causal relation exists between the fall in sphingomyelinase activity, both absolute and relative to lecithinase activity, and the accumulation of sphingomyelin with age.

INTRODUCTION

The origin, role, and fate of cholesterol in the pathogenesis of atherosclerosis has been the focus of interest and the subject of many investigations during the past decades. The bulk of the information obtained tended to overshadow the well known fact that apart from cholesterol the diseased artery accumulates considerable amounts of phospholipids, especially sphingomyelin. The increase in sphingomyelin has been shown to be a function of age (1–5) and its accumulation in atherosclerosis might be regarded as an exaggeration of the normal process of aging. It seems well established that a part of aortic phospholipids does originate *in situ* (6–12) and thus an imbalance in local regulation of synthesis and degradation might contribute to an increase in the phospholipid content of the vessel wall with age. Recently the role of aortic phospholipases in the regulation of the concentration and composition of aortic phospholipids has been studied. The presence of enzymes active in degradation of lecithin (LE), phosphatidyl ethanolamine (PE), lysolecithin (LL), and sphingomyelin (SP) in aortae of various mammals was described (13, 14). Conditions for the quantitative determination of these different phospholipases in samples of the same artery have been determined and the best reproducibility was obtained when the enzymic activity was related to the deoxyribonucleic acid (DNA) content and thus to the cell population of the artery. Since the method of assay was based on the hydrolysis of added radioactive substrate, the latter's complete miscibility with the endogenous substrate of the artery was ascertained. More recently the activity of the aortic phospholipases was studied in rats and rabbits and an unequivocal rise in lecithinase with age was found, whereas sphingomyelinase did not show a significant change (15). The present com-

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munication deals with the study of phospholipases in the normal human aorta, and their role in the regulation of phospholipid composition and content during maturation and aging.

METHODS

Radioactive substrates. The preparation of labeled substrates has been previously described in detail (13, 14). Sphingomyelin, labeled with tritiated choline, was prepared biosynthetically. Pregnant mice were kept on a choline-deficient diet during the last 5 days of pregnancy and for 5 subsequent days of lactation. From the 2nd day postpartum, the mice were injected daily with 0.5 mCi of choline (methyl-^{3}H) chloride (SA 100 mCi/mmole), and were killed 24 hr after the fourth injection. The maternal liver was removed, extracted in 20 vol of chloroform: methanol (2:1 v/v), and lipids were washed according to Folch, Lees, and Sloane Stanley (16). The lipids were fractionated on silicic acid columns (17) and the sphingomyelin recovered in the chloroform: methanol fraction 2:3 v/v, was further purified from traces of labeled lecithin and lysolecithin by mild alkaline hydrolysis (0.4 M KOH in 70% methanol for 2 hr at 37°C) in the presence of unlabeled lecithin. The products of hydrolysis of lecithin were eluted from a silicic acid column with chloroform: methanol 3:2 (v/v) and the labeled sphingomyelin was eluted with chloroform: methanol 2:3 (v/v) and it proved to be more than 99% pure on thin-layer chromatography in two different solvent systems, chloroform: methanol: water (70:25:4) and chloroform: methanol: acetic acid: water (50:25:8:4).

Lecithin, labeled specifically in the 2 position with oleic acid-9,10-$^3H_2$ was prepared from lysolecithin (obtained after hydrolysis of liver lecithin with *Crotalus adamanteus* venom) and tritiated oleic acid, using rat liver microsomes as the source of enzyme according to Robertson and Lands (18). When the labeled lecithin was subjected to hydrolysis with *Crotalus adamanteus* venom (19) more than 95% of the labeled oleic acid was released from the 2 position of the molecule.

Phosphatidyl ethanolamine labeled biosynthetically with palmitic acid-9,10-$^3H_2$ was isolated from rat liver 1 hr after intravenous injection of palmitic acid-9,10-$^3H_2$. The lipids were separated on silicic acid columns (17) and the neutral lipids and free fatty acids were eluted with 5% methanol in chloroform and PE with 15% methanol in chloroform. The distribution of the tritiated palmitic acid in PE was found to be 95% in the 1 position and 5% in the 2 position of the PE. Lysolecithin, labeled specifically in the 1 position, with palmitic acid-9,10-$^3H_2$ was prepared after hydrolysis of biosynthetically labeled palmitoyl-^{3}H lecithin with *Crotalus adamanteus* venom (19).

Aqueous suspensions of lecithin, sphingomyelin, and PE were prepared as follows: aliquots of the labeled substrate were dried under N_2 in a conical all-glass homogenizer, homogenized in ice-cold glass-distilled water, and subjected to ultrasonic irradiation while kept in ice for 3 min at 10 kilocycles/min (Branson Instruments, Inc., Stamford, Conn.). Aqueous suspensions of lysolecithin were prepared similarly but for the use of ultrasonic irradiation.

Preparation of aortic homogenates. Aortae were obtained at autopsy performed within 20 hr of death, during which time the body was kept under refrigeration. Enzymic determinations were carried out within 24 hr of death and the preservation of enzymic activity during this interval has been ascertained previously (13, 14). The principal cause of death encountered in the different age groups is presented in Table I. At autopsy the aortae were examined for gross changes and samples from the arch and ascending aorta were fixed in buffered glutaraldehyde for microscopic examination whereas adjacent portions were taken for chemical and enzymic studies. Sections prepared from paraffin blocks were stained with hematoxylin and eosin. Only those samples which were microscopically free of any atheromatous changes or fatty streaks were included in the present report. For the preparation of homogenates the adventitia and outer part of the media were removed and the intima and most of the media were used. The tissue was minced well with sharp scissors and samples of 0.3–0.5 g were weighed and processed. Samples of the aorta used for enzyme studies were homogenized in 9 volumes of ice-cold glass-distilled water, using an all-glass conical homogenizer (size B, Kontes Glass Co., Vineland, N. J.) packed in ice. Portions of this homogenate were rehomogenized in a buffer appropriate for the determination of each particular enzyme. Portions of the minced aorta taken for chemical studies were homogenized in 20 volumes of chloroform: methanol (2:1 v/v).

TABLE I

Main Causes of Death in the Different Age Groups

Age	No. of males	No. of females	Cause of death
yr			
0–10	9	2	Pneumonia (7), hyaline membrane disease (1), astrocytoma (1), trauma (1), congenital heart disease (1)
11–20	4	3	Trauma (4), pneumonia (1), myotomia (1), muscular dystrophy (1)
21–50	9	4	Trauma (10), rheumatic heart disease (2), cancer (1)
51–70	5	2	Cancer (3), coronary artery disease (3), pyelonephritis (1)
71–97	2	5	Cancer (5), coronary artery disease (2)

Determination of enzymic activities. Enzymic activities were determined according to procedures described previously (13, 14). To 0.2 ml of 7.5% aortic homogenate (by weight) in an appropriate buffer, labeled substrate and cofactors were added to a final volume of 0.3 ml.

Sphingomyelin choline phosphohydrolase (EC 3.1.4.3) activity was determined in 0.05 M acetate buffer, pH 5.1 in the presence of 0.17% Triton X-100. Sphingomyelin choline-^{3}H was used as the labeled substrate at a concentration of 0.215 mmole/liter and the concentration of endogenous unlabeled sphingomyelin in the incubation mixture ranged from 0.030 mmole/liter to 0.145 mmole/liter in the young and old age groups respectively; thus the final concentration ranged from 0.245 to 0.360 mmole/liter. Since in the presence of Triton X-100 complete mixing of exogenous and endogenous sphingomyelin had been demonstrated (13), it was necessary to ascertain that no substrate inhibition of enzyme takes place in the samples with the higher concentration of sphingomyelin. To this end, hydrolysis of sphingomyelin by aortic homogenates was measured in the presence of various concentrations of exogenous sphingomyelin and no

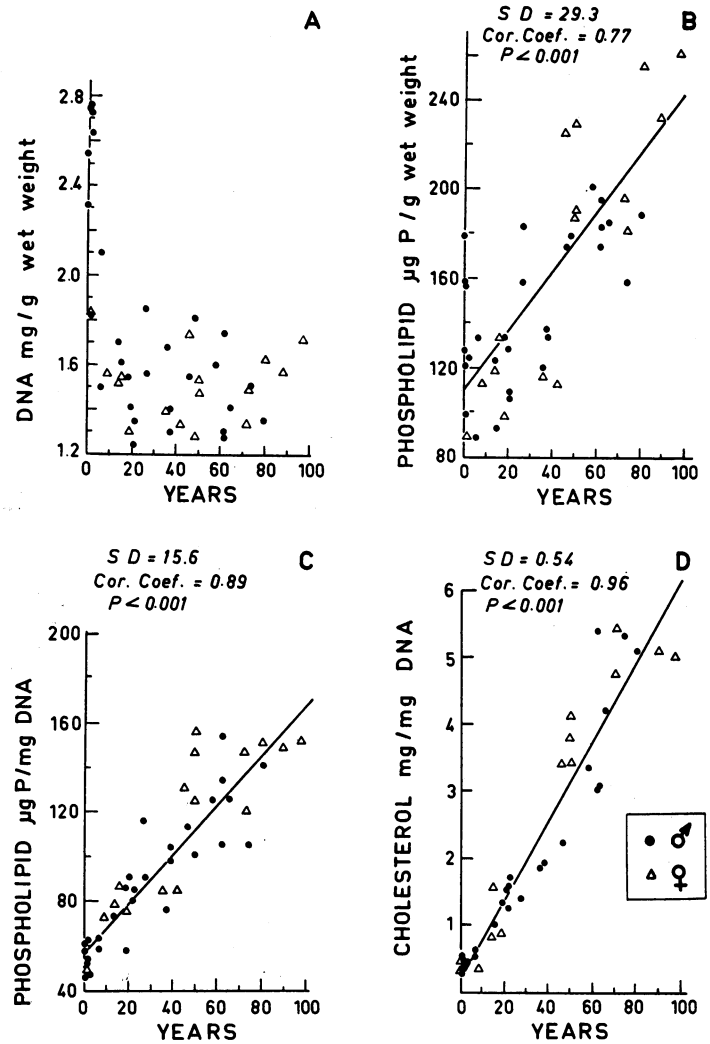

FIGURE 1 A–D Regression analyses of relation between DNA, phospholipids, and cholesterol and age in normal human aorta. In this and all subsequent figures the correlation coefficient is for fit between the experimental data and a linear function and the significance of its difference from zero is given by *P*. The standard deviation (SD) is for the linear function presented in the figure. Data for males and females were plotted together as no difference between values obtained from aortae of male and female subjects has been found.

substrate inhibition of enzymic activity occurred even when the concentration of sphingomyelin was raised to 0.460 mmole/liter, which was higher than in any incubation used in the present study. Incubations were carried out for 60 and 120 min and the rate of hydrolysis was determined by the appearance of water-soluble radioactivity, which consisted mainly of phosphorylcholine (13).

Phosphatidyl acyl hydrolase (EC 3.1.1.4) activity using lecithin and PE as substrate was determined in 0.015 M Tris buffer, pH 8.6, in the presence of 0.15% sodium deoxycholate and 0.01 M $CaCl_2$. The concentration of labeled substrate in the incubation mixture was 0.215 mmole/liter, and the concentration of endogenous unlabeled substrate varied with age from 0.08 to 0.1 mmole/liter for lecithin and from 0.05 to 0.07 mmole/liter for PE. Incubations were for 30, 60, and 120 min and in each experiment at least two assays at two time intervals were performed. The rate of lecithin hydrolysis was measured by the appearance of labeled free fatty acid (14) and that of PE by the appearance of labeled lysophosphatidyl ethanolamine using thin-layer chromatography as described previously (14).

Aortic lysolecithin acyl hydrolase (EC 3.1.1.5) activity was determined in 0.026 M KCl: 0.08 M Tris buffer (9:1 v/v) pH 7.45 with 0.215 mM 1-palmitoyl-^{3}H lysolecithin as substrate. The amount of endogenous lysolecithin in the aorta was negligible in all age groups. The rate of hydroly-

TABLE II

Change of Phospholipid Content and Activity of Phospholipases in Aortae of Different Age Groups

Age	Yr 0*–19 (A)	20–44 (B)	45–70 (C)	71–97 (D)	P of differences A vs. B	B vs. C	C vs. D
Phospholipid, *μg P/mg DNA*	63.2 ±3.2‡ (17)	91.0 ±3.8 (10)	129.2 ±5.6 (11)	138.0 ±7.4 (7)	<0.001	<0.001	>0.1
Sphingomyelin, *μg P/mg DNA*	12.5 ±1.0 (17)	27.0 ±1.7 (10)	55.3 ±3.2 (11)	62.7 ±3.3 (7)	<0.001	<0.001	>0.1
Lecithin, *μg P/mg DNA*	26.7 ±1.6 (17)	30.7 ±1.4 (10)	33.3 ±1.9 (11)	35.1 ±2.6 (7)	<0.1	>0.1	>0.1
PE, *μg P/mg DNA*	17.2 ±1.0 (17)	19.7 ±1.3 (10)	25.1 ±1.3 (11)	24.5 ±1.6 (7)	>0.1	<0.01	>0.1
PI + PS, *μg P/mg DNA*	3.2 ±0.4 (17)	6.0 ±0.4 (10)	9.1 ±0.6 (11)	10.1 ±0.7 (7)	<0.001	<0.001	>0.1
Cholesterol, total, *mg/mg DNA*	0.64 ±0.09 (16)	1.59 ±0.10 (7)	3.60 ±0.27 (10)	5.12 ±0.10 (6)	<0.001	<0.001	<0.001
Free cholesterol, *mg/mg DNA*	0.61 ±0.10 (12)	1.15 ±0.08 (6)	2.40 ±0.17 (10)	3.07 ±0.06 (5)	<0.001	<0.001	<0.01
Lecithinase, *μmoles hydrolyzed/mg DNA* sec hr	0.34 ±0.04 (16)	0.79 ±0.14 (9)	1.71 ±0.15 (10)	1.77 ±0.22 (7)	<0.01	<0.001	>0.1
Sphingomyelinase, *μmoles hydrolyzed/mg DNA* sec hr	0.13 ±0.01 (16)	0.09 ±0.01 (7)	0.06 ±0.01 (9)	0.05 ±0.01 (7)	<0.05	<0.05	<0.1
Lysolecithinase, *μmoles hydrolyzed/mg DNA sec hr*	0.35 ±0.06 (10)	0.83 ±0.08 (7)	0.65 ±0.05 (7)	0.66 ±0.11 (5)	<0.001	<0.1	>0.1

P = Phosphorus.
* From 1st day of life.
‡ The numbers are mean ±SE; the figures in parentheses are number of determinations.

sis was determined by the appearance of labeled free fatty acid, as determined by thin-layer chromatography (14).

All incubations were carried out in 15-ml stoppered test tubes, air being the gas phase, in a shaking incubator at 37°C, and the enzymic reaction was terminated by the addition of 20 vol of chloroform: methanol (2:1 v/v).

Analytical procedures. Determination of lipid composition was performed on aortic homogenates, prepared in chloroform: methanol, and purified following the procedure of Folch et al. (16). DNA was determined on the chloroform-insoluble residue (20), and cholesterol (21), phospholipid phosphorus (22), and phospholipid composition were determined on aliquots of the purified chloroform phase. Cholesterol ester and free cholesterol were separated on alumina columns (23). Phospholipid composition of human aortae was determined according to Parker and Peterson (24). A sample containing 10–20 μg of lipid phosphorus (lipid-P) was streaked on a HR silica gel plate (Merck, Darmstadt, Germany), using the Radin-Pellick applicator (Applied Science Laboratories, Inc., State College, Pa.). The plates were developed in chloroform: methanol: acetic acid: water (50:25:8:4) as described by Skipski, Peterson, and Barclay (25). The areas corresponding to lysolecithin, sphingomyelin, lecithin, phosphatidyl inositol + phosphatidyl serine (PI + PS), and PE, identified with the help of purified reference standards, as well as the origin and front, were scraped off the plate and lipid phosphorus was determined. The five identified fractions accounted for more than 95% of the lipid-P recovered from the plate. The products of hydrolysis of lecithin, PE, and lysolecithin were separated by thin-layer chromatography by use of the solvent system of chloroform: methanol: water (70:25:4). The areas corresponding to the

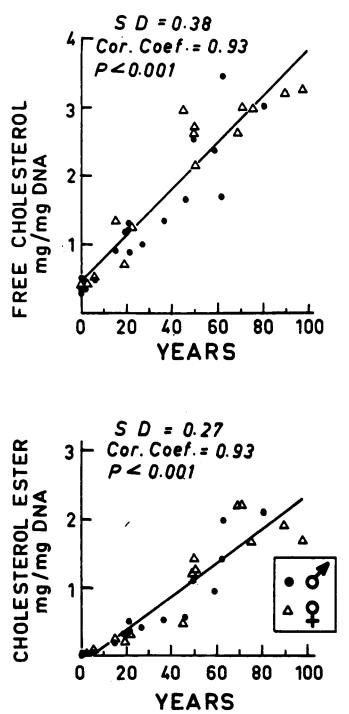

FIGURE 2 Regression analyses of relation between free cholesterol and esterified cholesterol of normal human aortae and age.

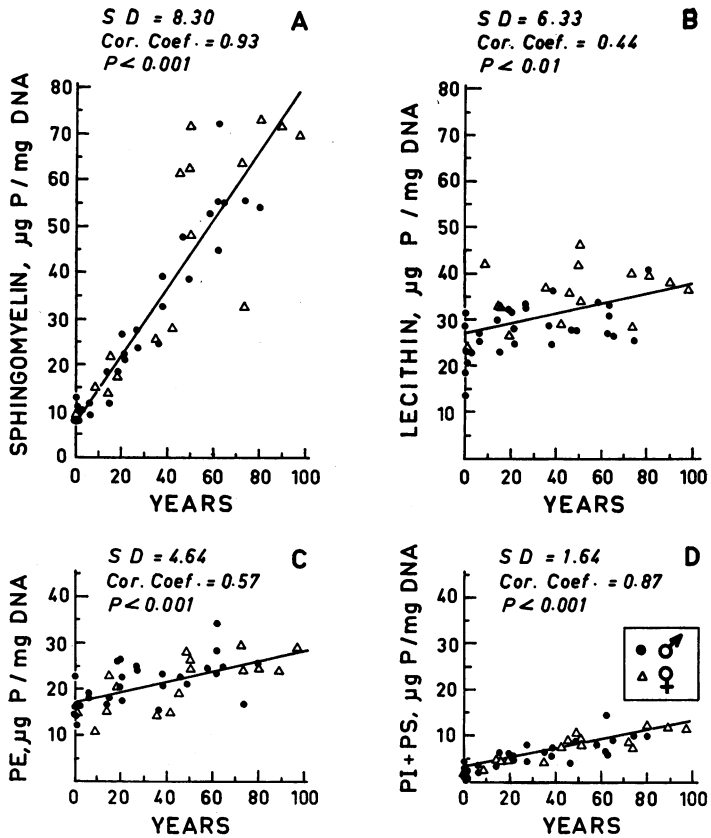

FIGURE 3 A–D Regression analyses of the relation between sphingomyelin, lecithin, phosphatidyl ethanolamine (PE), and phosphatidyl inositol + phosphatidyl serine (PI + PS) concentration of normal human aortae and age. All the values are drawn to the same scale to allow comparison of absolute amounts of the various components and their respective contribution to the over-all increase in aortic phospholipids.

individual compounds were scraped off the plates into counting vials containing the scintillation fluid and the radioactivity was determined with a β-liquid scintillation spectrometer, Packard model 4332. The scintillation fluid consisted of dioxane: water 5:1 (v/v) containing 8.33% naphtalene, 0.58% 2,5-diphenyloxazole and 0.025% 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene. All radioactive materials were obtained from The Radiochemical Centre, Amersham, England. Triton X-100 was obtained from Rohm and Haas Co., Philadelphia, Pa. All solvents were reagent grade.

Statistical analysis. The data for male and female subjects were analyzed separately as to their fit to linear, parabolic, and logarithmic functions by regression analysis with the help of a CDC 6400 computer. A simple linear correlation between the values measured and age was chosen and, as the difference between the data obtained from males and females was not significant, when determined according to Brownlee (26) they were pooled together for final analysis and presentation. The coefficient of correlation was determined and its significance was evaluated with the help of tables (27). The significance of differences between means was detemined by Student's *t* test.

RESULTS

In a comparative study of enzymic activities the choice of a reference parameter presents some problems; the most commonly used are wet weight, protein content, or DNA content. Owing to the increase in aortic extracellular collagen and elastin with age (28) both protein content and wet weight did not seem suitable as a basis for comparison of phospholipid content and phospholipase activity. Since the latter are controlled by the function of aortic cells, the DNA content, i.e. the cell population, was chosen as the common denominator. Fig. 1 summarizes the changes in DNA, phospholipid, and cholesterol content of aortae of various age groups. There was a marked fall in the DNA content of the aortae during the first decade and later the values showed some variability but did not continue to decrease. Since the ratio of DNA to wet weight remained constant between 10 and 97 yr, one could have used wet

weight as a parameter for comparison of the various determinations. As seen in B and C of Fig. 1, there was a rise in aortic phospholipid content with age, irrespective of whether it was related to DNA or wet weight. However, a better correlation with age was obtained when the phospholipid of the artery was related to DNA. When the increments in phospholipids between four age groups were analyzed, a highly significant increase between the first three age groups was observed, but there was no further rise in phospholipid content in the last age group, from 70 yr up (Table II). The correlation between the rise in total cholesterol and age was highly significant (Fig. 1 D), and the increase continued also in the oldest age group examined (Table II). The increase in free cholesterol with age was linear throughout the whole range of age groups. Cholesterol ester was found in trace amounts only during the first decade, and increased with age thereafter (Fig. 2).

The graphs in Fig. 3 A–D were drawn to the same scale and thus allow a true comparison between the various phospholipids. The rise in sphingomyelin with age is quite prominent, whereas lecithin and PE show only a slight increase. Of the minor components PI + PS

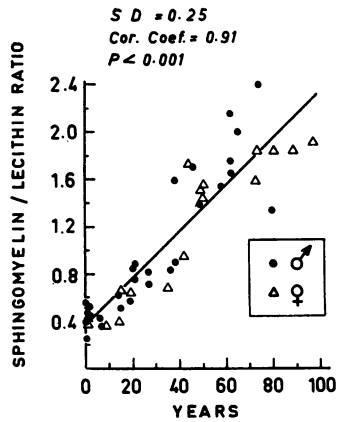

FIGURE 4 Regression analysis of relation between the sphingomyelin to lecithin ratio and age.

show a marked increase. Lysolecithin content (not shown) did not vary markedly and was less than 5% of total phospholipids in all age groups.

As seen in Table II, the mean increment in total phospholipid, as determined by the difference between the means of the youngest and oldest age groups ex-

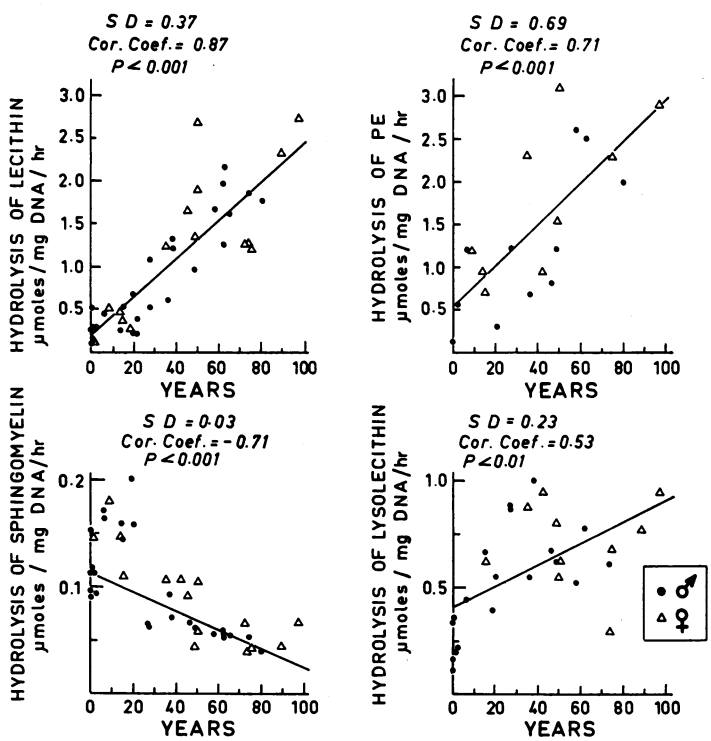

FIGURE 5 A–D Regression analyses of relation between the activity of aortic phospholipases and age. All enzymic activities of each aorta were determined at the same time on different portions of the same homogenate. The details of the determination of enzymic activity are given in Methods.

TABLE III

Comparison of Phospholipid and Cholesterol of Normal Human Aortae of Various Ages

Reference	Site	Layer	Age	Choles-terol	Free choles-terol	Phospho-lipids	SP	LE	PE	SP/LE
			yr							
(1)	Entire aorta	Media	12–40	5.4	3.2	7.1	0.7*			
			41–60	6.7	4.1	8.4	4.6*			
			61–84	11.6	5.7	8.7	4.8*			
(2)‡	Random	Intima + media	1	1.0	0.9	3.5	0.6	1.5	2.4	0.4
			40	4.0	3.2	6.1	2.4	1.5	2.4	2.2
			60	5.7	4.1	7.5	3.3	1.5	2.4	2.2
(3)	Entire aorta	Intima + media	3–12	1.0	0.7	5.5	1.9	2.4	1.0	0.8
(5)§	Entire aorta	Intima	15	3.2	2.2	6.1				1.0
		Media	15	1.4	1.2	4.7				1.4
		Intima	53	13.5	4.5	8.0				1.2
		Media	53	6.3	3.0	5.7				2.7
(29)	Thoracic aorta	Intima	25–44	2.8	1.4	3.4				
		Media	25–44	3.1	2.2	4.9				
Present study‡	Ascending aorta	Intima + media	1	0.4	0.4	2.7	0.4	1.3	0.8	0.3
			15	1.2	0.9	3.2	0.8	1.2	0.8	0.7
			40	2.7	1.8	4.0	1.5	1.2	0.8	1.2
			60	3.8	2.4	4.6	1.9	1.2	0.9	1.5

SP = sphingomyelin; LE = lecithin; PE = phosphatidyl ethanolamine. All data were normalized to mg/g of wet weight; dry weight was converted to wet weight using the factor of 70% moisture (1).

* Ether-insoluble fraction.

‡ Data obtained from regression lines.

§ The age is a mean of six boys and four adults respectively. The sphingomyelin fraction contained lysolecithin.

amined, was 74.8 μg of lipid-P/mg DNA. The mean increment in sphingomyelin was 50.2, that of lecithin 8.4, PE 7.3, and PI + PS 6.7 μg lipid-P/mg DNA. Thus the increase in sphingomyelin accounted for about 70% of the rise in aortic phospholipids. When the increment is examined in relation to the initial value it can be seen that a rise in lecithin of 8.4 μg lipid-P amounts to not more than a 30% change, whereas the rise of PI + PS of 6.7 μg lipid-P indicates an increase of 300%. Similar to the change in total phospholipid, the increments of sphingomyelin and PI + PS between the two last age groups examined were statistically not significant. The data plotted in Fig. 4 express the comparative change in phospholipid composition with age. As each value for lecithin and sphingomyelin was obtained from the same artery the rise in their ratio from 0.3 to 2.0 with age becomes even more significant.

Enzymic activity of aortic homogenates towards four substrates was determined, using aliquots of the same preparation. As shown previously (14) hydrolysis of lecithin and PE in aortic homogenates occurs predominantly at the 2 position; lysolecithinase hydrolyses the fatty acid in the 1 and 2 positions while sphingomyelinase cleaves phosphorylcholine (13), leaving the ceramide portion of the molecule.

As shown in Fig. 5 the activities of aortic homogenates towards the substrates investigated differed with age. There was a pronounced (up to 5-fold) increase in the rate of hydrolysis of lecithin and PE with age, and again as in the analysis of phospholipid composition no significant difference was obtained between males and females. The change in lysolecithinase activity was more difficult to evaluate. By regression analysis a fit for a linear correlation was tested and even though the coefficient of correlation was relatively low when compared to those obtained for the other data, it was still statistically significant. In contradistinction to the above, a significant decrease of sphingomyelinase activity with age was seen. The change in enzymic activity was also evaluated in four age groups. Data for hydrolysis of PE were not included, as this enzyme activity was determined only on a part of the homogenates. As seen in Table II the increase in lecithinase activity was highly significant for the first three age groups, but the increment in the last group was not significant. Lysolecithinase activity increased only initially, and the increment

of its activity between the second and third age groups was significant at the 10% level only. The decrease in the sphingomyelinase activity was progressive and tended to level off in the last age group examined. The fall in sphingomyelinase activity becomes even more prominent when related to lecithinase activity as their ratio falls from 0.65 at age 0 to 0.0018 at age 90.

DISCUSSION

Lipid accumulation in normal aging aorta has been reported by several investigators and in order to compare our data with those of others it seemed helpful to normalize all results to a common denominator (Table III). Whenever the original data were related to dry weight, they were converted to wet weight by using the factor of 70% moisture (1). Some discrepancies became apparent between the results of the various studies; according to Smith (4, 5) the phospholipid content of the intima in young and aging male aortae is higher than that of the media and an opposite result is shown by Insull and Bartsch (29). The other divergency concerns the rise of free and esterified cholesterol with age. Whereas both were shown to increase in all the investigations, the bulk of the increase up to the age of 60 was accounted for by free cholesterol (1, 2, and present authors), but in the study of Smith (5) more than 50% of the cholesterol was in the form of ester in the media at the mean age of 53 yr.

When the sample examined contained both the intima and media (2 and present study), or the media alone (1, 5), there was a marked rise in the sphingomyelin to lecithin ratio with age, whereas no such change was observed in the intima (5). Our phospholipid and cholesterol values for all age groups are somewhat lower than those described by other investigators and agree most closely with those of Insull and Bartsch (29) for the medial lipids. This is probably so since in both studies samples were derived from the same region, i.e. the thoracic aorta, and Smith (4) has shown that both cholesterol and phospholipid is higher in the abdominal than in the thoracic region of the aorta.

The preferential accumulation of sphingomyelin could result from an imbalance of the usual regulatory mechanisms, i.e., the rate of influx which comprises both synthesis *in situ* and entry from the circulation and the rate of removal which comprises the rate of egress and degradation.

Following the report of Zilversmit and coworkers (9) that ^{32}P is incorporated into phospholipids by preparation of human aorta, other investigators have shown incorporation of labeled fatty acids into phospholipids, including sphingomyelin (30–32). The present investigation did not include the determination of the rate of synthesis of phospholipids as it was uncertain whether this enzymic activity would be adequately preserved in the postmortem material to allow a comparative study. Hence the main effort was focused on the quantitation of the degradative mechanisms, as preservation of the enzymic activity of the phosphohydrolases had been ascertained previously. In order to reduce some of the variables expected in a study of a heterogeneous human population the samples were taken from the ascending portion of the thoracic aorta as it has been shown that the normally occurring increase in intimal width with age is far less pronounced in the thoracic than in the abdominal aorta (33). The adventitia and outer part of the media were always excluded and thus the cellular population of the sample examined consisted almost solely of smooth muscle cells and endothelial cells as a minor component.

The changes with age in activities of phospholipases seen in the human aorta showed some similarities to those encountered in the aortae of rats and rabbits (15). In the three species examined there was an increase in lecithinase activity with age, which was more pronounced in the rat and human than in the rabbit. In the rat and human the increment of aortic lecithin with age averaged 10.7 and 8.4, whereas in the rabbit it was 22.2 μg P/mg DNA. An inverse relationship was found between the increment of aortic sphingomyelin and change in sphingomyelinase activity with age. In the rat and rabbit the increment in sphingomyelin was 8.8 and 18.0 μg P/mg DNA, respectively, and there was little or no change in sphingomyelinase activity; in the human, the increment in sphingomyelin was 50.2 μ P/mg DNA and there was a fall in sphingomyelinase activity. Thus it seems that, in addition to changes in local phospholipid synthesis and (or) influx from the circulation, aortic phospholipases may particpate in the regulation of the phospholipid concentration.

If one assumes that aortic cholesterol is derived entirely or mainly from the serum in the form of β-lipoproteins, its accumulation with age could be taken as an indirect measurement of the influx of phospholipids. The ratio of cholesterol to phospholipid (w/w) in the human serum β-lipoprotein in normal adult males is 1.4–1.5 (34, 35) and since lecithin accounts for 65% and sphingomyelin for 25% of the β-lipoprotein phospholipids (36) the ratio of cholesterol to lecithin in the lipoprotein would be about 2.1 and of cholesterol to sphingomyelin 5.6–6.0. When one determines the *increment* of these lipids in the aortic wall with age (by taking the difference between the mean values of group 0–19 yr and 71–97 yr, Table II) and obtains their ratio, it becomes apparent that the cholesterol : lecithin ratio is 20 and cholesterol : sphingomyelin ratio is 3.4. As no degradation of cholesterol in the aorta has been demonstrated so far, the change in the cholesterol to lecithin ratio

is most probably the result of increased activity of lecithinase with age occurring also in vivo, like in the system examined in vitro. One could speculate also that the increase in the cholesterol-bearing lipoprotein in the aortic wall might contribute towards the rise in lecithinase activity with age. A different chain of events appears to evolve with regard to sphingomyelin. In contradistinction to the high cholesterol: lecithin ratio, the cholesterol: sphingomyelin ratio in the artery is lower than that expected from the influx of serum β-lipoprotein. Since a preferential efflux of cholesterol from the arterial wall is not a very likely explanation, the low cholesterol: sphingomyelin ratio might be due to an increased rate of formation of this phospholipid, accompanied by a fall in the catabolism of sphingomyelin, and the latter was observed indeed in the present study.

The localization of the sphingomyelin accumulating in the normal aging aorta has not been elucidated so far. Adams and Bayliss (37), on the basis of a histochemical stain (osmium tetroxide-α-naphtylamine), postulated that phospholipids and especially sphingomyelin are present mainly in elastic fibers. The specificity of that histochemical method has been questioned by Lojda and Elleder (38). The most serious objection was raised by the fact that brief extraction of sections in cold acetone, which removes hardly any sphingomyelin, prevented the development of the color indicative of the presence of phospholipids. Another attempt at the localization of sphingomyelin in human aorta was made with the help of the *cis*-aconitic anhydride reaction, which stains choline-containing phospholipids (39). In very young individuals the histochemical reaction of the elastic lamellae was negative, while the positive reaction in the older age groups was prevented by prior saponification (40). As the detection of phospholipids after saponification has been regarded as indicative of the presence of sphingomyelin, the question of the localization of sphingomyelin in normal aorta remains not solved so far. We have attempted to study this problem in experimental animals in which a 3-fold rise in sphingomyelin with age was observed. Electron microscopic studies of aortae of rats and rabbits disclosed an impressive change in the shape of the smooth muscle cells and the cellular population of membranes with age (41). This change consisted of an increase in the cell outer membrane and its derivatives and a decrease in rough endoplasmic reticulum. Since of all the cellular membranes the plasma membrane was found to be the richest in sphingomyelin (42, 43), it seems that it might be the site of accumulation of sphingomyelin with age. A similar interpretation was proposed also by Portman, Alexander, and Maruffo (44), who were able to isolate from monkey aortic homogenates a vesicular fraction, rich in sphingomyelin, which was probably derived from the plasma membrane. One could speculate then that in the human aorta as well some of the increase in sphingomyelin with age is related to the formation of a cellular membrane which might participate in the assimilation of surplus cholesterol entering from the circulation.

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