Effect of atherosclerosis on lysosomal cholesterol esterase activity in rabbit aorta

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Abstract Radiolabeled cholesteryl oleate, when incorporated into phospholipid vesicles, was hydrolyzed at acid pH by an enzyme present in rabbit aortic homogenates. In contrast, cholesteryl oleate presented as an acetone dispersion was not effectively hydrolyzed at acid pH under identical conditions. Using the vesicle preparation as substrate, a sensitive assay system for the acid hydrolase was developed in which hydrolysis was proportional to protein concentration and incubation time, and was independent of substrate concentration. The physical state of the vesicles was apparently not altered by the assay conditions, and no hydrolysis of the vesicle-associated phospholipid was detected.

Acid cholesterol esterase activity in atherosclerotic aortic tissue was 2.5-fold greater than that of control tissue, and even greater increases were observed in the activities of other lysosomal enzymes (*N*-acetyl-β-*d*-glucosaminidase and β-glucuronidase). Glucose-6-phosphatase activity was also increased in aortas from cholesterol-fed animals while 5′ nucleotidase activity remained unchanged.

Labeled triolein also was incorporated into phospholipid vesicles and was hydrolyzed by an acid lipase in aortic tissue. Similarities between triolein and cholesteryl oleate hydrolysis existed with respect to pH optimum and the effect of cholesterol feeding on activity, suggesting that a single enzyme may hydrolyze both lipids.

Supplementary key words acid lipase · lysosomal enzymes · cholesteryl oleate

The accumulation of cholesteryl esters in rabbit aortic tissue is a characteristic response to sustained ingestion of a cholesterol-rich diet (1). Recent studies have shown that part of the accumulated lipid present in rabbit aortic smooth muscle cells was localized in lysosomes (2–4). Using homogenates obtained from rabbit aortic cell suspensions, it was demonstrated convincingly that the activities of several lysosomal hydrolases increased in response to cholesterol feeding, while the density of the lysosomes decreased as a result of increased lipid accumulation (2). These biochemical findings were supported by morphological and histochemical studies, which also indicated that lysosomes contained intracellular lipid (3). In separate studies, increased aortic lysosomal activity was observed in cholesterol-fed rhesus monkeys (5), and morphological evidence for the intralysosomal accumulation of cholesteryl esters was described (6). These recent studies were in basic agreement with earlier studies that also suggested increased lysosomal activity in atherosclerotic lesions from several species, including man (7–9).

De Duve (10) has proposed that the accumulation of cholesteryl esters in atherosclerosis is due to a relative deficiency in a lysosomal cholesterol esterase, resulting in an inability of the smooth muscle cell to metabolize the increased influx of lipoprotein-associated cholesteryl esters. Takano et al. (11) have described the presence and properties of such a cholesterol esterase in rabbit aortic tissue and have demonstrated the lysosomal origin of this enzymatic activity.

In this study, we have developed an assay for aortic cholesterol esterase activity in which the substrate, radiolabeled cholesteryl oleate, was rendered water soluble by inclusion into phospholipid vesicles (12). We have studied some properties of this assay system and have used it to measure the effect of cholesterol feeding on enzyme activity. The results have been compared with the activities of several other lysosomal enzymes and of other marker enzymes as well.

EXPERIMENTAL PROCEDURE

Cholesteryl [1-^{14}C]oleate (sp act 55 mCi/mmole) and tri-[1-^{14}C]oleoyl glycerol (sp act 44 mCi/mmole) were obtained from New England Nuclear Corporation (Boston, MA), and 1,2-di[1-^{14}C]oleoyl-*sn*-glycero-3-phosphorylcholine (sp act 30.0 mCi/mmole) was obtained from Applied Science Laboratories, Inc. (State College, PA). Egg yolk lecithin was obtained from Lipid Products (Surrey, U. K.). All other nonradioactive lipids were obtained from Applied Science Laboratories, Inc. The reagents used for the assay of

Abbreviation: NAGA, *N*-acetyl-β-*d*-glucosaminidase.

5′ nucleotidase, glucose-6-phosphatase, *N*-acetyl-β-glucosaminidase (NAGA) and β-glucuronidase were obtained from Sigma Chemical Company (St. Louis, MO), as were most other chemical reagents used for these studies.

Preparation of vesicles

All vesicle preparations were made so the final mixture would contain 1% lecithin. A 60 mg portion of egg yolk lecithin dissolved in chloroform–methanol 2:1 was mixed with 8.0 μCi of labeled cholesteryl oleate or triolein. Appropriate amounts of unlabeled cholesteryl oleate or triolein were then added so the molar ratio of lecithin to the corresponding lipid was 66:1. The organic solvents were evaporated under nitrogen, the samples were lyophilized, and the lipids were resuspended in 6.0 ml of 0.1 M NaCl, 0.02% sodium azide, and 0.01 M Tris pH 7.4. A suspension was made by stirring the preparation on a vortex-type mixer, and the suspension was sonicated within a temperature range of 40–50°C for 10 min using a Branson W-350 sonifier fitted with a standard 0.5 inch horn at a power setting of about 85–100 watts (Branson Instruments, Stamford, CT). The resulting suspension was centrifuged at 150,000 *g* for 30 min to sediment small fragments of titanium from the disruptor horn as well as trace amounts of aggregated lipid. Recovery of all lipids throughout the procedure exceeded 95%. To prepare vesicles containing labeled phospholipid, 8.0 μCi of 1,2-di-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphorylcholine was added to the original mixture and the labeled cholesteryl oleate or triolein was omitted. The final concentrations of lipids in the vesicle preparations were: 12.1 mM egg yolk lecithin, 0.18 mM cholesteryl oleate, or 0.18 mM triolein.

The vesicle preparations were stored under nitrogen at 4°C and used for enzymatic studies within 5 days. The preparations were free of lysolecithin as determined by thin-layer chromatography using chloroform–methanol–water 65:25:4 (v/v), with sulfuric acid charring as the visualization technique.

Tissue preparation

Female New Zealand white rabbits were used throughout the study. Rabbits fed a cholesterol-containing diet were given 100 g per day of a standard rabbit chow to which 1.5% cholesterol and 5% peanut oil were added. The animals were killed after 10–15 weeks on the cholesterol diet. Aortas from control or cholesterol-fed animals were removed from the heart to the iliac bifurcation, and the adventitia was carefully stripped away from each. The tissue was divided into two or three transverse segments, wet weights were obtained, and the segments were minced and homogenized in 10 volumes of 0.25 M sucrose–0.01 M Tris pH 7.4. Homogenization was performed at 2°C using a motor-driven glass–glass apparatus. Homogenization was continued until the homogenate contained no visible particles of tissue; this routinely required a total of 2 min homogenization time, spaced over 20 sec intervals. Aliquots of the homogenates were removed for chemical or enzymatic analysis; the homogenate was centrifuged at 10,000 *g* for 20 min, and the resulting supernatant was centrifuged at 150,000 *g* for 45 min to obtain the high speed supernatant. Aliquots of this fraction were used for most enzymatic studies.

Enzyme assays

Lipolytic activities. All experiments were performed in disposable 15 × 100 mm test tubes. Incubation tubes routinely contained 100 μl of 0.15 M acetate buffer pH 4.5, 100 μl of the vesicle preparation, and 50 μl of tissue fraction. The final volume was adjusted to 300 μl with 0.01 M Tris buffer. Incubations were routinely performed at 37°C for 20 min. Following incubation, the labeled free fatty acid formed as a result of hydrolysis was measured essentially as described by Pittman, Khoo, and Steinberg (13). The reaction was terminated by the addition of 3.0 ml of benzene–chloroform–methanol 1.0:0.5:1.2 containing unlabeled oleic acid (0.1 mM) as carrier. A 0.6 ml portion of 0.3 M NaOH was then added; the mixture was stirred for 15 sec, and then was centrifuged at 1000 *g* for 10 min. A 0.5 ml portion of the upper phase was added to a Triton–toluene scintillation cocktail and the radioactivity was determined (^{14}C-efficiency 80%). Ninety-three percent of [1-^{14}C]oleic acid was partitioned into the upper phase using the conditions described. Blanks incubated in the absence of tissue fraction were routinely assayed, and less than 0.2% of the total radioactivity present in the incubation (ca. 2.4 × 10^5 cpm) was found in the upper phase. All measurements were made in duplicate.

β-Glucuronidase. Assays were performed in a total volume of 1.5 ml containing 0.1 M acetate buffer pH 4.5, 0.1% Triton X-100, 1.0 mM phenolphthalein glucuronic acid, and 100 μl of tissue fraction. Following incubation at 37°C for 120 min, the reaction was terminated by adding 3.0 ml of a solution containing glycine (0.133 M), NaCl (0.067 M) and Na_2CO_3 (0.083 M). The tubes were centrifuged at 1000 *g* for 10 min, and the OD was measured at 555 nm. Blanks were incubated both in the absence of substrate and in the absence of tissue fraction.

NAGA. Incubations were made in a total volume

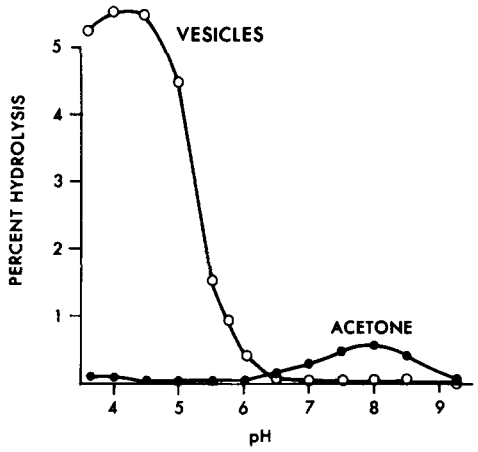

Fig. 1. The effect of pH on the hydrolysis of cholesteryl [1-^{14}C]oleate introduced in vesicles or directly from an acetone solution. Incubation tubes contained 0.05 M acetate buffer pH 3.5–5.0, 0.05 M Tris–maleate pH 5.5–8.5, and 0.05 M glycine at pH 9.2. Where vesicles were added, each tube contained 100 μl of the cholesteryl-[1-^{14}C]oleate-containing vesicles and 50 μl of aortic high speed supernatant fraction (80 μg of protein). For the acetone addition, 40 μl of acetone solution containing 320 μg of cholesteryl oleate (3.94×10^6 cpm) was added to 3.0 ml of high-speed supernatant and 100 μl aliquots (160 μg of protein) were added to each incubation tube. All tubes contained about 17.7 nmoles of cholesteryl oleate in a total volume of 0.3 ml and were incubated at 37°C for 20 min. Hydrolysis was measured as described in the experimental section. Blank values were 0.1–0.15% of the total radioactivity added for both substrate preparations and did not vary with pH.

of 1.5 ml containing 0.1 M sodium citrate buffer pH 4.5, 0.1% Triton X-100, 5.0 mM *p*-nitrophenyl-*N*-acetyl-β-*d*-glucosaminide, and 20 μl of tissue fraction. Following incubation at 37°C for 30 min, the reaction was terminated by adding 1.5 ml of 0.5 M sodium carbonate–0.5 M sodium bicarbonate pH 9.8. The tubes were centrifuged at 1000 *g* for 10 min and the OD was measured at 430 nm. For latency studies, incubations were performed in both the presence and the absence of Triton X-100, and the incubation time was shortened to 10 min at 37°C.

5′ Nucleotidase. The assay was performed according to a published procedure (14). Incubations were at 37°C for 20 min using 20 μl of tissue homgenate.

Glucose-6-phosphatase. The assay was carried out according to a published procedure (14). Incubations were at 37°C for 120 min using 100 μl of tissue homogenate.

Chemical determinations. Protein was measured either by a micro-Kjeldahl method (15) or according to the method of Lowry et al. (16). DNA was measured on aortic homogenate using the procedure of Burton (17). Total cholesterol in the homogenates was determined by adding 0.1 ml of aortic homogenate to 4.0 ml of chloroform–methanol 2:1 and obtaining a lipid extract according to the procedure of Folch, Lees, and Sloan Stanley (18). The lipid extract was saponified at 80°C for 2 hr, and the saponifiable fraction was extracted with hexane and stored at −20°C for cholesterol analysis by gas–liquid chromatography (GLC). GLC was performed using a 2 foot SE-30 column (3.8% on Diatoport S) in a Packard Gas Chromatograph with injection port, oven, and flame ionization detector temperature settings of 260, 250, and 260°C, respectively.

RESULTS

The effect of pH on cholesteryl oleate hydrolysis by the aortic high-speed supernatant fraction differed depending on the mode of substrate presentation **(Fig. 1).** Cholesteryl [1-^{14}C]oleate, incorporated into lecithin vesicles, was effectively hydrolyzed at acid pH, whereas no activity was seen at pH values above 7. In contrast, if an equivalent amount (17.7 nmoles) of cholesteryl oleate was introduced into the incubation mixture directly from an acetone solution, only slight activity was observed at acid pH values; however, hydrolysis was observed at pH values above 7, with a broad optimum between pH 7 and 8. The data in Fig. 1 suggest the presence of two separate enzymatic activities capable of hydrolyzing cholesteryl oleate and show that the vesicle preparation is the more effective substrate for assying the acid hydrolase activity.

Subcellular distribution of the acid hydrolase is shown in **Table 1**. Following homogenization and subcellular fractionation, 50-μl aliquots were taken from each subcellular fraction and incubated under stand-

TABLE 1. Subcellular distribution of cholesterol esterase activity

	Protein Content		Activity (pmoles/min)	
	mg/ml	*Total (mg)*	*ml*	*mg protein*
Homogenate	21.02	222.8	327	15.5
1×10^3 *g* Pellet	17.65	187.1	30	1.7
1×10^4 *g* Pellet	1.38	14.6	0	0
1.5×10^5 *g* Pellet	0.23	2.4	0	0
1×10^3 *g* Supernatant	3.27	27.8	488	149.2
1×10^4 *g* Supernatant	1.88	15.6	509	270.7
1.5×10^4 *g* Supernatant	1.72	13.9	557	323.8

The original homogenate, containing 100 mg of tissue (wet weight) per ml, was centrifuged at 1,000 *g*, 10,000 *g*, and 150,000 *g* for 10, 35, and 45 min, respectively. The 1,000 *g* and 10,000 *g* pellets were resuspended in 10 ml of buffer and washed once by centrifugation at 10,000 *g*. All pellets were resuspended in buffer to the original volume of homogenate (10.6 ml). Assays were performed under standard conditions using cholesteryl oleate-containing vesicles and 50 μl of each subcellular fraction, which represented the extract from 5 mg of tissue wet weight. Activity was expressed as the pmoles of cholesteryl oleate hydrolyzed per min per ml of the respective fraction or per mg protein of each fraction.

ardized conditions (see Experimental Procedure) with vesicles containing labeled cholesteryl oleate. Conditions were adjusted so that each 50-μl aliquot was derived from an equivalent amount of tissue, based on wet weight (5.0 mg wet weight). Hydrolysis was observed in the original homogenate and in all resulting supernatant fractions obtained during the fractionation procedure. Very little activity was measured in the 1000 *g*, 10,000 *g*, or 150,000 *g* particulate fraction. More activity consistently was observed in the supernatant fractions obtained after centrifugation of the original homogenate, perhaps due to removal of the relatively large amount of particulate matter, which may interfere in the assay system. Since aortic tissue contains a relatively high content of collagen and elastin, which are relatively insoluble, the protein contents of the homogenate and particulate fractions were determined using a micro-Kjeldahl method rather than the method of Lowry et al. (16). Although most of the connective tissue protein becomes associated with the particulate fractions, the rigorous homogenization conditions used for aortic tissue may result in some extracellular proteins being distributed in the supernatant fractions. Thus, the expression of enzymatic activity of an intracellular lysosomal enzyme on the basis of protein concentration may be less meaningful in aortic subcellular fractions than in other tissues.

The absence of measurable cholesterol esterase activity in the particulate fraction was surprising, since intact lysosomes would be expected to be associated with such fractions, particularly with the 10,000 *g* pellet. Resuspended particulate fractions were subjected to freeze-thawing using a dry ice–acetone bath and a 37°C water bath for 10 successive cycles to determine whether latent activity was present. However, increased activity was not detected in these fractions following such treatment. The original homogenate also was subjected to freeze-thawing, but no significant increase in activity was observed when compared to the identical untreated homogenate. High-speed supernatant fractions obtained from freeze-thawed or untreated homogenates also contained identical cholesterol esterase activity.

Another technique for uncovering latent lysosomal activity requires the addition of Triton X-100 to the incubation mixture to disrupt lysosomes and enhance the accessibility of enzyme to substrate. Such studies could not be performed with the cholesterol esterase assay since 0.1% Triton X-100 eliminated all activity, presumably by affecting the physical state of the vesicles.

The high-speed supernatant fraction was used in the assay system because *1*) it was relatively free of connective tissue elements, cell organelles, or membrane fragments that could interfere with the accessibility of enzyme and substrate; *2*) it contained most of the enzyme activity measured in the total homogenate; *3*) the specific activity (relative to protein concentration) was highest in this fraction; and *4*) in atherosclerotic tissue, this fraction was the only one that could be obtained relatively free of endogenous cholesteryl esters.

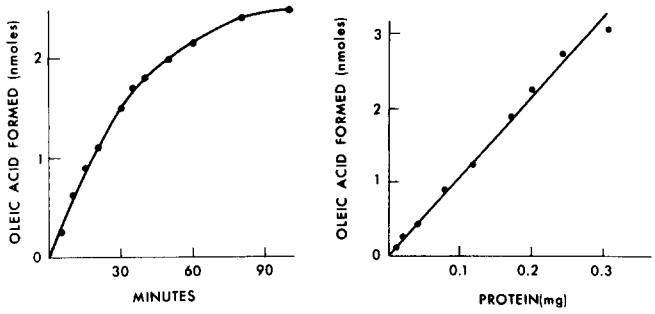

Fig. 2. The effect of incubation time and protein concentration on cholesteryl ester hydrolysis. All incubations contained 100 μl cholesteryl [1-^{14}C]oleate-labeled vesicles in a total volume of 0.3 ml, and were buffered at pH 4.5. Left, each incubation contained 50 μl of high-speed supernatant fraction (85 μg of protein) and was performed under standard conditions (see Experimental Procedure), except that the reactions were terminated at different times. Right, incubation tubes contained 5–150 μl of high-speed supernatant fraction and were incubated at 37°C for 20 min.

Fig. 2 shows the effect of incubation time and protein concentration on the hydrolysis of cholesteryl oleate at acid pH. Hydrolysis proceeded at an almost linear rate for time periods up to 30 min when the cholesteryl ester concentration was 60 μM and 60 μg of tissue protein was present in the incubation medium (Fig. 2, left). Using a 20 min incubation time, hydrolysis was proportional to protein concentration in the range of 10–300 μg of protein, corresponding to 5–150 μl of high-speed supernatant fraction (Fig. 2, right).

The effect of substrate concentration of hydrolysis, using a short incubation time (15 min) and a relatively low protein concentration, is shown in **Fig. 3**. Hydrolysis was proportional to substrate concentration at levels below 30 μM; at higher substrate concentrations, the activity gradually leveled off. Activity was expressed on the basis of a mg of tissue wet weight, which corresponds to 10 μl of high-speed supernatant fraction. The insert shows the data expressed as a double reciprocal plot, which reveals an apparent K_m of 26.8 μM. Similar data obtained from six separate experiments gave an average K_m of 28.5 $\pm$ 5.2 (mean $\pm$ SE). The data obtained in Figs. 1, 2, and 3 indicated that appropriate assay conditions for

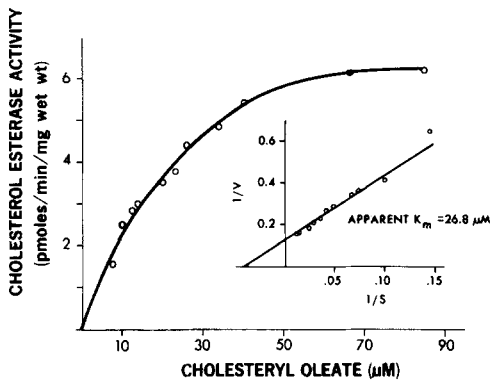

Fig. 3. The effect of vesicle concentration on cholesteryl oleate hydrolysis in high-speed supernatant fraction of a representative control aorta. Tubes contained varying amounts of the vesicle preparation and 50 μl of supernatant fraction, and they were buffered at pH 4.5 in a total volume of 0.3 ml. Incubations were at 37°C for 15 min. The insert contains the data expressed as a double reciprocal plot using cholesteryl oleate concentration as substrate.

cholesterol esterase could be achieved by incubating 20–50 μl of aortic high-speed supernatant (about 40–100 μg of protein) with 100 μl of the vesicle preparation in the presence of 0.05 M acetate buffer pH 4.5 at 37°C for 20 min and in a total volume of 0.30 ml.

To determine whether the substrate preparation was altered during incubation with the aortic high-speed supernatant, an incubated mixture obtained using standard conditions was chromatographed on a Sepharose 4B column, and the radioactive elution pattern was compared with that of the original vesicle preparation **(Fig. 4).** As described previously (12), the cholesteryl oleate-containing vesicles eluted at a region corresponding to the size expected for unilamellar vesicles (about 250 Å diameter), with only a slight peak at the void volume. Radioactivity derived from the incubation mixture eluted in a similar pattern; however, more radioactivity was present at the void volume, suggesting some aggregation of the vesicles. Over 95% of the radioactivity applied to the column was recovered in the eluted fractions. Radioactivity in both peaks was predominantly cholesteryl oleate. The small peak seen at the void volume was probably due to aggregation related to the presence of tissue extract, since it was observed even when incubations were performed at 0°C, when no hydrolysis occurred. Thus, incubation of vesicles with aortic tissue under standard conditions appeared to produce no appreciable effect on the vesicle size.

It was of interest to determine whether cholesterol feeding produced changes in the cholesterol esterase activity **(Table 2).** For comparative purposes, changes in activity of several other aortic enzymes characteristic of different organelles also were assessed. Aliquots of aortic homogenates were used to determine the activity of 5′ nucleotidase and glucose-6-phosphatase, which are considered marker enzymes for plasma membrane and endoplasmic reticulum, respectively. Following subcellular fractionation, the high-speed supernatant fraction was used to assay for the lysosomal enzymes, NAGA and β-glucuronidase, as well as for cholesterol esterase.

Aortic tissue segments from control and atherosclerotic animals were homogenized and the homogenates were analyzed for total cholesterol content. DNA was determined in all aortic homogenates and the concentrations in control tissue (0.11 ± 0.01 mg DNA per ml of homogenate) were not significantly different from those in atherosclerotic segments (0.10 ± 0.01 mg DNA per ml homogenate). Total DNA content was about 0.1% of the tissue wet weight for all segments studied. The comparative data in Table 2 were expressed on the basis of tissue wet weight, since this appeared to be a reliable indicator of the cellularity of the tissue.

Cholesterol feeding resulted in an increased cholesterol content of aortic tissue. The arch and thoracic regions, where the more severe lesions characteristically are produced, contained far more cholesterol than the lower thoracic and abdominal regions, which contain fewer areas of foam cell formation.

Cholesterol feeding produced increased enzymatic activity of all the lysosomal enzymes measured. The aortic arch segments contained 11-fold more

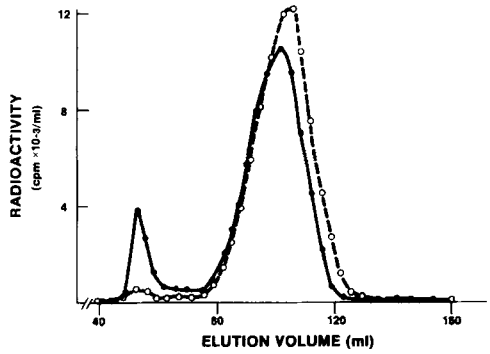

Fig. 4. The effect of incubation on the elution pattern of cholesteryl [1-^{14}C]oleate-containing vesicles. An incubation mixture containing vesicles (200 μl), aortic high-speed supernatant (100 μl), and buffer (300 μl) was incubated at 37°C for 20 min and a 400 μl aliquot was applied to a Sepharose 4B column (2.6 × 32 cm), pre-equilibrated with 0.1 M NaCl, 0.01 M Tris pH 7.4, and 0.02% sodium azide. The elution pattern of the vesicles incubated with high-speed supernatant (solid line, closed circles) was compared to the elution pattern of vesicles incubated under similar conditions but in the absence of aortic supernatant (dotted line, open circles). Samples were eluted at a flow rate of 24 ml/hr and 0.5 ml aliquots of the fractions were analyzed for radioactivity. The void volume and total volume of the column were 49 ml and 159 ml, respectively.

TABLE 2. Effect of cholesterol feeding on aortic enzymes

	Total Cholesterol	5′ Nucleotidase	Glucose-6-phosphatase	NAGA	Beta Glucuronidase	Cholesterol Esterase
	μg/mg wet wt	*nmoles/min/mg wet wt*	*pmoles/min/mg wet wt*	*pmoles/min/mg wet wt*	*pmoles/min/mg wet wt*	*pmoles/min/mg wet wt*
Control Aorta	0.87 ± 0.22	26.61 ± 2.49	58.8 ± 5.3	176.6 ± 13.8	3.55 ± 0.29	4.59 ± 0.26
Cholesterol Fed						
a) Arch and upper thoracic aorta	23.67 ± 1.96[a]	27.45 ± 2.48	162 ± 30.5[b]	1925 ± 298[a]	29.33 ± 6.13[b]	11.81 ± 2.05[b]
b) Lower thoracic and abdominal aorta	10.07 ± 1.72[a]	22.22 ± 1.08	113.1 ± 18.2[c]	580 ± 88.1[b]	5.20 ± 0.49[c]	6.18 ± 0.63[c]

Cholesterol, 5′ nucleotidase and glucose-6-phosphatase were assayed using the total homogenate. NAGA, β-glucuronidase, and cholesterol esterase were assayed using the high speed supernatant fraction. All values are mean ± SE for six determinations.
[a] $P < 0.001$, compared to control value.
[b] $P < 0.01$.
[c] $P < 0.05$.

NAGA activity and over 8-fold more β-glucuronidase activity per unit wet weight than aortic tissue from control animals. Cholesterol esterase activity was also significantly increased in the atherosclerotic arch segments but to a lesser extent (2.5-fold) than with NAGA or β-glucuronidase. The lower thoracic and abdominal segments, which were less atherosclerotic than the arch segments, had correspondingly less activity for all lysosomal enzymes measured, including cholesterol esterase; yet these levels were significantly greater than those measured in aortas from control animals. Increased activity of glucose-6-phosphatase also was seen with cholesterol feeding, the levels again being related to the degree of atherosclerosis. No significant differences were observed in 5′ nucleotidase activity between the groups. Experiments involving only control animals revealed no significant differences between arch and abdominal sections in any of the parameters listed in Table 2. The data for control tissue in Table 2 were obtained using the whole aorta.

Because the assay for cholesterol esterase was performed using aortic high-speed supernatant fractions, this fraction also was used to measure the activities of the lysosomal marker enzymes NAGA and β-glucuronidase. To determine whether the activities in the high-speed supernatant fraction reflected total aortic activity, comparisons were made of the total activity of NAGA and β-glucuronidase in the original homogenate and in the high-speed supernatant fraction in a series of control and atherosclerotic aortic segments **(Table 3).** About half the total NAGA activity (obtained by assaying the original homogenate) was present in the supernatant fraction from both control and atherosclerotic tissues, and no significant difference between the two groups was observed. The partial recovery of NAGA activity in the high-speed supernatant could have resulted from sedimentation of intact lysosomes during the subcellular fractionation. If this were the case, latency of NAGA should be observed in the original homogenate. Separate studies were performed on the latency of NAGA in homogenate and high speed supernatant fractions from control and atherosclerotic tissues.

Latency is the difference between activity measured in the presence of Triton X-100 (total activity) and in the absence of Triton X-100 (free activity) expressed as a percentage of the total activity. Latency in the homogenate fractions averaged 17.8% and 21.5% for control and atherosclerotic segments, respectively, with no significant differences between the two groups. No latency could be detected in high-speed supernatant fractions. The low latency values for NAGA activity in the homogenates suggested that considerable disruption of the lysosomes occurred during tissue preparation and homogenization, and the incomplete recovery of NAGA activity in the supernatant fraction most probably resulted from adsorption of the enzyme to the particulate matter in the cell homogenate.

For β-glucuronidase, the percentage of total activity in the supernatant fraction of atherosclerotic

TABLE 3. Percentage of total aortic NAGA and β-glucuronidase activity in high speed supernatant fractions from control and cholesterol-fed rabbits

Enzyme	Control (N = 7)	Cholesterol-Fed (N = 8)
	% of total activity	
NAGA	48.7 ± 4.4	54.2 ± 3.6
β-Glucuronidase	38.5 ± 4.6	23.4 ± 2.1

Activities were determined in equivalent aliquots (20 μl) of aortic homogenates (total activity) and high speed supernatant fractions from control or cholesterol-fed animals. Activity in the high-speed supernatant was expressed as a percentage of the activity found in the original homogenates. Triton X-100 (0.1%) was present in all incubation mixtures.

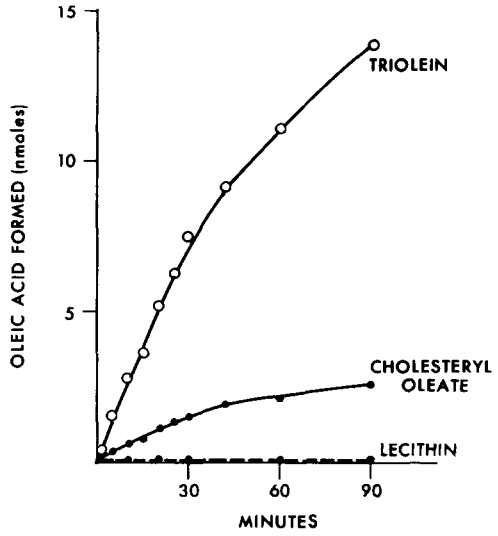

Fig. 5. Comparison of the hydrolysis of cholesteryl oleate, triolein, and lecithin contained in vesicles by aortic high-speed supernatant. Incubations were performed under standard conditions except that tubes contained 100 μl of vesicles containing either labeled cholesteryl oleate, triolein, or lecithin.

preparations (23.4%) was significantly less than that in controls (38.5%). This difference would tend to further exaggerate the increased activity produced by cholesterol feeding shown in Table 2. β-Glucuronidase activity has been shown to be present in microsomal as well as lysosomal fractions from liver tissue (19). Thus, the low percentage recovery of total activity in the supernatant fraction may have been due to sedimentation of non-lysosomal enzyme activity in the microsomal fraction and possibly to adsorption of the enzyme to particulate fractions.

It was of interest to determine whether phospholipid vesicles could be used to measure lipolytic activity other than cholesterol esterase. The specificity of the acid hydrolase was assessed by preparing vesicles containing labeled cholesteryl oleate, triolein, or lecithin (see Experimental Procedure). Under standard incubation conditions, we compared the hydrolysis of the labeled lipids in all three vesicle preparations **(Fig. 5).** No detectable hydrolysis of the fatty acid-labeled lecithin was observed. This lack of hydrolysis was observed using labeled lecithin vesicles prepared in both the presence and the absence of unlabeled cholesteryl oleate. Hydrolysis of the [1-^{14}C]triolein occurred readily and to a greater extent than that of the cholesteryl oleate.

Hydrolysis of triolein contained in vesicles was further studied with respect to pH and to vesicle concentration **(Fig. 6).** With respect to pH, acid hydrolase activity was clearly demonstrated with a pH optimum similar to that of cholesteryl oleate hydrolysis, and essentially no hydrolysis was observed at pH values above neutrality. An identical pH activity curve was observed when the aortic high speed supernatant from a cholesterol-fed rabbit was used, although a greater hydrolytic rate was seen at acid pH values. As with cholesteryl oleate, the rate of hydrolysis changed from first order to zero order kinetics as the vesicle concentration was increased (Fig. 6, right). The apparent K_m for triolein, as determined using a double reciprocal plot (insert), was 18.6 μM, a value somewhat less than the 28.5 μM value obtained using cholesteryl oleate-containing vesicles.

Using identical conditions, the hydrolysis of triolein and cholesteryl oleate was compared in a group of control and atherosclerotic aortic preparations. **Table 4** lists the ratio of triolein to cholesteryl oleate hydrolysis when expressed as nmoles of fatty acid hydrolyzed per unit of time. The ratio was about 6 for both groups, with no significant difference between them. Thus, the relative increases in triglyceride lipase activity in atherosclerotic as compared to control high-speed supernatant fractions resembled those observed with cholesterol esterase activity (Table 2), suggesting that one enzyme may be responsible for both hydrolytic activities.

DISCUSSION

In a previous study, we have shown that phospholipid vesicles, containing small amounts of cholesteryl oleate, were a suitable substrate for a lysosomal cholesterol esterase obtained from rat liver tissue (12). In the present study, we have used the vesicle preparations to devise a sensitive assay system for

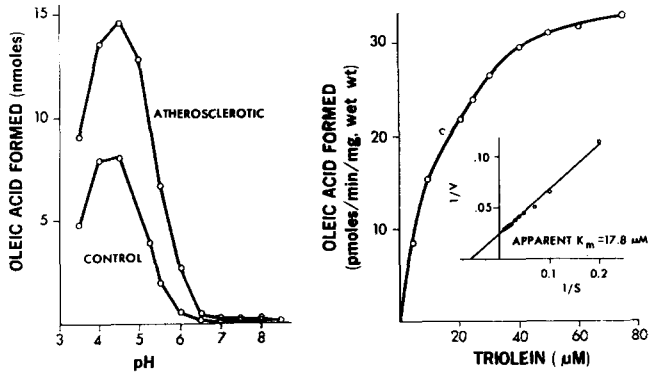

Fig. 6. Effect of pH and substrate concentration on triolein hydrolysis. Incubations were performed using vesicles containing labeled triolein as substrate. Left, 50 μl of high speed supernatant fraction from a representative control and an atherosclerotic aorta containing 96 and 120 μg of protein, respectively, were incubated for 30 min under standard conditions. Right, different amounts of vesicles were incubated with 50 μl of supernatant fraction from a control aorta under standard conditions. The insert is a double reciprocal plot of this data.

aortic cholesterol esterase and have also demonstrated that triglyceride lipase activity could be measured under similar conditions. Using the vesicle preparation as a substrate, an acid hydrolase was indicated that appeared to be similar to the aortic lysosomal cholesterol esterase recently described by Takano et al. (11). These workers used a substrate preparation containing cholesteryl oleate, lecithin, sodium taurocholate, and albumin, and observed an absolute dependency on the presence of the bile salt. In our studies using phospholipid vesicles as a "carrier" for cholesteryl oleate, hydrolysis could readily be measured in the absence of bile salts or albumin. The vesicle preparation would seem to offer an effective means of studying the enzyme–substrate interaction once the enzyme is sufficiently purified. It also can be characterized by physical-chemical techniques, and can be prepared in a reproducible manner.

A striking example of the importance of the substrate's physical state on hydrolysis was shown by the comparison between hydrolysis of cholesteryl oleate when it was incorporated into vesicles and when it was introduced directly in acetone. The far greater effectiveness of the vesicle preparation as a substrate for the acid hydrolase activity was consistent with our previous finding on the lysosomal esterase from rat liver (12). However, unlike the liver studies, where some acid hydrolase activity was observed, practically no activity was seen in the aortic preparation with the acetone dispersion at acid pH. It is possible that the acetone dispersion is effective as a substrate only when the enzyme concentration is high, as in liver tissue, but is not a sensitive substrate for more dilute preparations of cholesterol esterase, such as those present in the aortic fractions used in these studies. Recently, cholesterol esterase from rabbit aortic tissue was partially purified and shown to have both acid and neutral pH optima with acetone dispersion as substrate (20).

Using the vesicle preparation, our assay conditions for the acid hydrolase were linear with respect to time and protein concentration, and were zero order with respect to the amount of vesicles added. A 11.6 μg portion of cholesteryl oleate was added to each assay tube, an amount in excess of the cholesteryl ester content present in 50 μl of aortic high-speed supernatant, even from severely lesioned areas.[1] Thus, the contribution of endogenous cholesteryl esters was minimized in the assay system. The apparent K_m obtained in our assay system averaged 28 μM, a value slightly less than the value of 40 μM obtained by Takano et al. (11), but differences in the substrate preparation could easily account for this discrepancy.

TABLE 4. Ratio of triolein to cholesteryl oleate hydrolysis in control and atherosclerotic aortic high speed supernatant fractions

	Triolein Hydrolysis / Cholesteryl Oleate Hydrolysis (mean ± SE)
Control (N = 6)	6.16 ± 0.21
Atherosclerotic (N = 6)	5.95 ± 0.39

The hydrolysis of triolein and cholesteryl oleate was determined under identical conditions in a series of control and atherosclerotic aortic high-speed supernatant fractions. Incubations were performed under standard conditions using 100 μl of cholesteryl oleate-containing vesicles or triolein-containing vesicles. Hydrolysis was calculated as pmoles of fatty acid hydrolyzed per min per mg wet wt and the ratio of triolein to cholesteryl oleate hydrolysis was determined.

It is important to emphasize that it is premature to attach any physiological significance to the K_m values obtained in our experimental system. Variations in the physical state of the substrate certainly produce wide variations in kinetics of cholesterol esterase activity, and the actual state of the substrate within aortic tissue remains to be determined. Since more than one cholesterol esterase may be present in aortic tissue, it is reasonable to assume that the substrate for each of these enzymes may differ.

The advantages of our assay system over those used by other workers include an easily prepared, physically defined substrate preparation, conditions approximating zero order kinetics, and a sensitivity permitting the assay to be performed readily on a tissue fraction obtained from the equivalent of 5 mg of tissue (wet weight). Furthermore, the close association of the cholesteryl oleate with the phospholipid in our substrate preparation may prove to be a reasonable model for what occurs within the lysosome, where lipoprotein-associated cholesteryl esters are apparently hydrolyzed (21, 22).

The hydrolysis of triolein or cholesteryl oleate when incorporated into vesicles was similar with respect to pH optimum and vesicle concentration, suggesting the possibility that a single enzyme may be responsible for hydrolyzing both lipids. Such a finding is consistent with studies summarized by Patrick and Lake (23) in which biopsy samples of liver from patients with Wolman's disease showed a concomitant deficiency in hydrolytic activity toward both triglycerides and cholesteryl esters. As with most studies dealing with hydrolysis of water insoluble substrates,

[1] Brecher, P., H. Y. Pyun, and A. V. Chobanian. Unpublished data.

differences in substrate presentation make comparative studies difficult. In our studies it is reasonable to assume that triolein or cholesteryl oleate incorporated into the vesicles are in a similar state, that is, the lipid molecules are dispersed and mobile throughout the phospholipid bilayer. Since no detectable phospholipid hydrolysis occurred, it may be postulated that the lipolytic enzyme attacks the fatty acid ester bonds when they are exposed to the outside of the bilayer, resulting in hydrolysis. Further studies using purified enzyme preparations would be required to describe the actual reaction mechanisms. Enzyme purification procedures also are required to determine definitively whether the hydrolysis of triglycerides and cholesteryl esters is due to one or several lysosomal enzymes.

Our data indicate that the cholesterol esterase activity measured in the high speed supernatant fraction provides a reasonable estimate of the relative activity in the original tissue. Cholesterol esterase activity was not observed in particulate fractions, and activity in the original homogenate was less than that seen in supernatant fractions when equivalent volumes of each fraction were used. It is unlikely that appreciable loss of activity occurs during subcellular fractionation due to sedimentation of intact lysosomes, since low latency values for NAGA were observed in the homogenate, and freeze-thawing procedures did not alter cholesterol esterase activity. Furthermore, recent studies by Kwak, Kim, and Lee (24) have shown that lecithin and other phospholipids have a labilizing effect on aortic and hepatic lysosomes, and it is possible that the phospholipid derived from the vesicle preparation, acting in a manner analogous to Triton X-100, eliminated latency effects in the original homogenate.

The high content of particulate matter in aortic homogenates could interfere directly with the enzyme assay and prevent determination of total activity. It also could act as a surface onto which cholesterol esterase would be adsorbed, thereby decreasing recovery in the high speed supernatant. The association of NAGA with membranes from disrupted cell organelles has been observed in swine liver subcellular fractions (25) and similar effects for other lysosomal enzymes have also been reported (26). A tight association of a rat liver lysosomal lipase with lysosomal membranes also has been reported (27). Further studies on the potential solubilization of lipase activity from aortic subfractions may resolve this problem.

Assuming that the lipase activity observed in the high-speed supernatant fraction is a reliable reflection of cellular activity present in the original tissue, the increased aortic cholesterol esterase activity seen as a result of cholesterol feeding suggests that an absolute deficiency of this enzyme is not the major reason for cholesteryl ester accumulation. However, the activities of NAGA and β-glucuronidase, the other lysosomal enzymes measured, showed greater increases than cholesterol esterase. Thus, the recent suggestion by De Duve (10) that a relative decrease in cholesteryl esterase occurs and may be a causative factor in the development of the atherosclerotic lesion would not be in conflict with our data.

An alternate possibility is that lysosomal cholesteryl ester hydrolysis is not a limiting factor. Our studies suggest that the amount of lipid hydrolyzed within lysosomes may be greater in atherosclerotic tissue, producing a greater efflux of free cholesterol into the extralysosomal regions of the cell. According to the model of cholesterol metabolism developed from studies on skin fibroblasts in tissue culture (21), this free cholesterol would become esterified through the action of acyl CoA:cholesterol acyl transferase (ACAT), resulting in cholesteryl ester accumulation. Other studies, including our own, have shown that ACAT activity was increased in atherosclerotic rabbit aortic tissue (28, 29). Thus, by this mechanism, the intracellular cholesteryl esters present in aortic foam cells would arise from intracellular synthesis rather than from a lack of lysosomal hydrolysis. Indeed, it is well known that the fatty acid profile of cholesteryl esters within foam cells differs from the fatty acid pattern of plasma cholesteryl esters (30), a finding that suggests active intracellular metabolism. The mechanism for removal of cholesteryl esters from both normal smooth muscle cells and aortic foam cells is not known. Recent studies on cultured fibroblasts suggested that the lysosomal cholesterol esterase was not involved in the degradation of intracellularly formed cholesterol esters (31). It is possible that the cholesterol esterase activity measured in our studies using acetone dispersions and having a pH optimum above neutrality may be involved in the removal of the intracellularly formed esters, but evidence for such a role is not yet available.

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