Lysosomal acid cholesteryl esterase activity in normal and lipid-laden aortic cells

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Abstract We have investigated the kinetic properties of acid cholesteryl esterase (EC 3.1.1.13) in preparations of rabbit aortic cells, with the aim of establishing conditions suitable for the quantitative assay of the enzyme in freshly prepared homogenates and subcellular fractions, whether derived from normal cells or from atheromatous cells heavily laden with cholesterol and cholesteryl esters. As substrate we used cholesteryl [1-^{14}C]oleate incorporated at a 1:100 molar ratio into egg-lecithin liposomes as described by Brecher and co-workers (*J. Lipid Res.* 1976. **17:** 239), and measured the radioactivity remaining in the alkaline buffer phase after organic solvent extraction of unhydrolyzed substrate. When the liposome substrate was used as such, more than 80% of the enzyme activity was latent in fresh homogenates. It could be released quantitatively without inactivation by addition of digitonin, but this created a requirement for taurocholate, presumably because digitonin disrupts liposomes. The following conditions gave satisfactory linearity with both time of incubation and enzyme concentration, with both normal and atheromatous cell preparations, and were adopted for the assay: 12.7 μM cholesteryl oleate dispersed in 1.27 mM egg lecithin; 50 mM acetate buffer, pH 3.9; 2.0 mM Na taurocholate; and 0.005% digitonin. The enzyme has a pH optimum of 3.9, and, under our conditions, has an apparent Km of about 1.5 μM. It is markedly inhibited by salt solutions and is sensitive to freezing, especially at high ionic strength. Subcellular fractionation by sucrose density gradient centrifugation indicates that a considerable part of the enzyme is localized in lysosomes, both in normal and in either moderately or heavily lipid-laden atheromatous aortic cells. The proportion of activity associated with the soluble fraction is however higher for acid cholesteryl esterase than for two acid glycosidases, in all three cell preparations. When assayed under optimal conditions, lipid-laden atheromatous cells displayed up to 3.5 times the acid cholesteryl esterase activity of normal aortic cells. This finding does not support the hypothesis that lipid overloading of lysosomes in atheromatous arterial cells occurs as a consequence of a relative deficiency of acid cholesteryl esterase.—**Haley, N. J., S. Fowler, and C. de Duve.** Lysosomal acid cholesteryl esterase activity in normal and lipid-laden aortic cells. *J. Lipid Res.* 1980. **21:** 961–969.

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In previous papers from this laboratory, the hypothesis was put forward that the intralysosomal accumulation of lipid that occurs in aortic cells of cholesterol-fed rabbits may be the consequence of a relative deficiency of the digestion of the cholesteryl esters that are taken up by the cells in association with pinocytized lipoproteins (1–3). Supporting such a mechanism were some preliminary findings suggestive of an inverse correlation between lysosomal lipid storage and cholesteryl esterase activity (2), and the clinical observation that a congenital deficiency of this enzyme does indeed lead to massive atherosclerosis (4). The possibility could not be excluded, however, that the low cholesteryl esterase activity observed in the lipid-laden lysosomes was a consequence, rather than the cause, of the engorgement of these particles (2).

Implicit in the enzyme deficiency hypothesis is the assumption that the lipid deposits in the lysosomes consist of undigested cholesteryl esters. It has, however, been found recently that much of the intralysosomal lipid of atheromatous aortic cells consists of unesterified cholesterol, whereas the stored cholesteryl esters of these cells occur largely as cytoplasmic droplets (5). This finding does not agree with the enzyme deficiency hypothesis. Consequently we have reinvestigated the acid cholesteryl esterase activity of normal and atheromatous aortic cells. Our results, recorded in the present paper, show that the activity of this enzyme was underestimated in our earlier work. Possible reasons for this are inactivation of the enzyme during isolation and storage of the cell preparations and its incomplete release from the lysosomes in the assay. With optimal assay conditions, the lipid-laden atheromatous cells display more than three-fold the lysosomal acid cholesteryl esterase activity of normal cells.

A preliminary report of this work has been presented previously (6).

Abbreviations: PNS, postnuclear supernatant fraction of aortic cell homogenates; SVE, homogenization medium containing 250 mM sucrose, 1 mM EDTA, and 0.1% ethanol.

MATERIALS AND METHODS

Preparation of tissue samples

Aortas of female New Zealand white rabbits were used in all of the studies presented; atheromatous vascular lesions were induced in the animals by cholesterol feeding as described before (7). For the preparation of whole tissue homogenates, fresh aortas were chilled to 0°C, stripped free of adventitia, and the remaining media chopped into 0.2 mm squares, and homogenized to a uniform suspension in ten volumes of ice-cold medium with a Duall homogenizer (Kontes Glass Co., Vineland, NJ). The composition of the homogenization medium was varied and will be given in the text. Arterial cells were isolated from control or atheromatous aortas by the procedure of Haley, Shio, and Fowler (8). Atheromatous cells were further separated by Metrizamide density gradient centrifugation into low density (ρ = 1.02–1.07 g/ml) and high density (ρ = 1.09–1.14 g/ml) atheromatous cells, as described previously (8, 9). All cell and tissue preparations were kept ice-cold until used for enzyme assay.

Subcellular fractionation

Subcellular fractionation by sucrose density gradient centrifugation was performed on preparations of isolated cells. Each cell preparation was first washed twice by low speed centrifugation in Hanks' medium to remove the Metrizamide and twice in 250 mM sucrose containing 1 mM EDTA and 0.1% ethanol (SVE). As in earlier work (10), the pellet of washed cells was suspended in 2 ml SVE medium and disrupted with 15 strokes of a type B pestle in a small Dounce homogenizer (Kontes Glass Co.). The homogenate was transferred to a conical tube and centrifuged 10 min at 1000 rpm. The supernate was removed with a Pasteur pipette and kept at 4°C. The pellet was resuspended in 2 ml SVE and homogenized and centrifuged as above. The pooled supernate (postnuclear supernate, PNS) was used as enzyme source or for subcellular fractionation. Subcellular fractionation was performed with a Beaufay zonal rotor by the outer-layering technique described by Peters and de Duve (7), except that centrifugation was carried out for 90 min at 35,000 rpm.

Acid cholesteryl esterase assay

Acid cholesteryl esterase activity was determined by a modification of the procedure of Brecher et al. (11). Cholesteryl [1-^{14}C]oleate (sp act 55 mCi/mmol) (New England Nuclear Corp., Boston, MA), was freed of contaminating fatty acids by the extraction procedure of Belfrage and Vaughn (12), and then added to a small volume of benzene containing 1.27 μmol of cold cholesteryl oleate (Applied Science Lab., State College, PA) and 127 μmol of egg lecithin (Grade I from Lipid Products, South Nutfield, Surrey, England). The solvents were removed by evaporation under N_2, followed by lyophilization of the lipid for at least 1 hr to insure that all traces of the solvent had been removed. The lipids were resuspended by thorough vortex mixing into 10 ml of 100 mM KCl containing 10 mM Tris-HCl buffer, pH 7.0, and 3 mM Na azide. This suspension was transferred to a 15 ml water-jacketed glass vessel maintained at 45°C, and sonicated for 12 min with a Branson W-350 sonifier equipped with a standard 0.5 inch horn (Branson Instruments, Danbury, CT), at an output setting of 4 (about 100 watts). The resulting opalescent suspension was centrifuged at 30,000 rpm in a Beckman Ti-50 rotor (Beckman Instruments, Palo Alto, CA) for 15 min to remove metal fragments released from the sonicator horn, and any aggregated lipid. The preparation was stored at 4°C and used within 2 weeks. A free fatty acid standard was prepared similarly, except that [1-^{14}C]oleic acid was substituted for the radioactive cholesteryl oleate. This standard was used with each set of assays to measure the efficiency of extraction of liberated fatty acids.

In the standard assay, a substrate mixture was prepared by the addition of one part lecithin–cholesteryl oleate dispersion to four parts of 125 mM Na acetate buffer, pH 3.9, containing 5.0 mM Na taurocholate. The enzyme preparation, consisting of a tissue homogenate or subcellular fraction, was diluted in SVE containing 0.1 mg digitonin/ml, and incubated for 10 min at 0°C. The reaction was started by the addition of 100 μl of the diluted enzyme to 100 μl of substrate solution. Incubation was carried out at 37°C for 30–90 min. Substrate blanks were run under identical conditions with SVE and digitonin added in place of the enzyme. The final reaction mixture contained enzyme, 1.27 mM lecithin, 12.7 μM cholesteryl oleate, 2.0 mM Na taurocholate, 0.005% digitonin, and 50 mM Na acetate buffer, pH 3.9. Essentially the same incubation conditions were followed in the kinetic experiments with the factor under investigation modified as described in the text. The reaction was stopped, and the unhydrolyzed substrate was removed by addition of chloroform, methanol, heptane, and alkaline buffer, as described by Belfrage and Vaughn (12). The samples, in screw-cap test tubes, were shaken together on a Kraft S-500 shaker (Kraft Apparatus, Inc., Mineola, NY), for 30 min and then centrifuged at room temperature to clear the phases. A 0.5-ml aliquot of the upper aqueous phase was

added to 10 ml of scintillant (Formula 963, New England Nuclear Corp., Boston, MA) and counted. The free fatty acid standard was counted before and after extraction and the efficiency of extraction (about 90%) determined. One unit of enzyme activity corresponds to the hydrolysis of 1 μmol of cholesteryl oleate per min.

Other analytical procedures

DNA, protein, free and esterified cholesterol, and marker enzymes were determined as described previously (8).

RESULTS

Kinetic properties

Influence of surface-active agents on enzyme activity and latency. In an earlier study from this laboratory (3), acid cholesteryl esterase was assayed on aortic cell preparations with a substrate mixture consisting of cholesteryl oleate dispersed by low temperature sonication in the presence of lecithin and Na taurocholate as emulsifiers. Since then, Brecher et al. (11) have described a new procedure using cholesteryl ester incorporated into lecithin vesicles and without added Na taurocholate. The activities measured on this liposome substrate are distinctly higher than those reported by Takano et al. (3).

Unfortunately, the substrate of Brecher et al. (11) cannot be used with intact lysosomes. As shown in **Table 1,** less than 20% of the total enzyme activity of a freshly prepared aortic homogenate was measured with a liposome substrate without added Na taurocholate. Freezing and thawing the homogenate five times released only a small part of the latent activity. Additional freezing cycles were not very effective, owing to the instability of the enzyme (see below). Even 2 mM taurocholate did not elicit the full activity of the enzyme. Apparently, incomplete breakage of the lysosomes is responsible for this observation. We have found in separate experiments that 2 mM Na taurocholate has only a small influence on the degree of latency of N-acetyl-β-glucosaminidase, which was of the order of 60% in whole aortic homogenates, and fell to 46% after addition of 2 mM Na taurocholate. It is possible that membrane disruption by Na taurocholate does not occur below the critical micellar concentration, which has been reported to be 4 mM at 37° C (13).[1] In the early work, it had been assumed as a matter of course that the amount of bile salt added (0.3 mM final concentration) would suffice to break open the lysosomes, but this was never verified experimentally.

TABLE 1. Effects of Na-taurocholate and freezing and thawing on acid cholesteryl esterase activity in homogenates of control aortas

Aortic Homogenate	Acid Cholesteryl Esterase Activity	
	−Na Taurocholate	+Na Taurocholate
	(mU/mg DNA)	
Fresh	0.89	2.95
Frozen and thawed five times	1.65	4.73

Homogenates freshly prepared in 250 mM sucrose were used as such, or after five cycles of freezing in dry ice–acetone followed by thawing at 37°C. Assays were run under standard conditions except for absence of 2 mM taurocholate where indicated.

As shown in **Fig. 1,** Triton X-100, which is very effective in releasing latent N-acetyl-β-glucosaminidase and other lysosomal hydrolases, cannot be used in high concentrations in the assay of acid cholesteryl esterase because it inhibits the enzyme. Digitonin, on the other hand, gives satisfactory results (**Table 2**); it releases the enzyme fully at a concentration a little over 0.001%, and becomes inhibitory only above the 0.01% level.

The optimal concentration of Na taurocholate with a normal aortic cell preparation is 2 mM (**Fig. 2**). Addition of 0.005% digitonin changes the shape of the

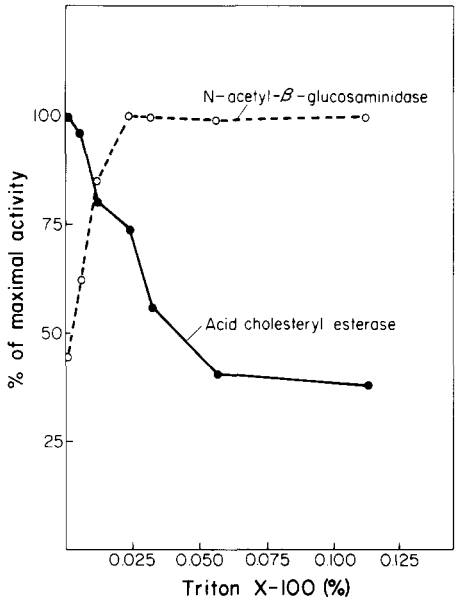

Fig. 1. Effect of Triton X-100 on the activities of N-acetyl-β-glucosaminidase and acid cholesteryl esterase in homogenates of control aortas. The assays for both enzymes were done under standard conditions, except that digitonin was excluded from the enzyme preparation for acid cholesteryl esterase. Incubations run in duplicate, for 15 min for N-acetyl-β-glucosaminidase, and 30 min for acid cholesteryl esterase.

[1] We are indebted to Dr. D. Small for pointing this out.

TABLE 2. Effect of digitonin on activity of N-acetyl-β-glucosaminidase and of acid cholesteryl esterase in aortic cell preparations

	% of Maximal Activity			
	N-Acetyl-β-glucosaminidase		Acid Cholesteryl Esterase	
Digitonin	Control Cells	Atheromatous Cells	Control Cells	Atheromatous Cells
(mg/ml)				
0	16	21	57	40
0.006	49	66	90	70
0.012	85	93	100	95
0.025	98	100	96	100
0.050	100	100	98	98
0.100	100	100	90	86
0.200	100	100	79	74

Assays were performed on freshly prepared homogenates of isolated control cells and of atheromatous cells (not separated by Metrizamide centrifugation) with free activity substrate (containing 250 mM sucrose) for N-acetyl-β-glucosaminidase, and standard substrate minus digitonin for acid cholesteryl esterase. The maximal activities measured were 4.1 and 8.0 mU/mg DNA, for the control cells and atheromatous cells, respectively.

curve, but not the optimum. The optimum in the presence of digitonin is shifted towards higher Na taurocholate concentrations with preparations of high density atheromatous cells, even more so with low density atheromatous cells (**Fig. 3**). Whether this shift is due to the increased lipid load or to the increased enzyme activity of the diseased cells was not investigated. Since the 2 mM concentration, which is optimum for normal cells, gave activities very close to the maximum values with the atheromatous cells, this was adopted in our assays. Takano et al. (3) used a suboptimal Na taurocholate concentration of 0.3 mM because of poor linearity with time at higher bile salt concentration. We did not encounter this problem.

Influence of pH. Under our assay conditions, maximum activity was observed at pH 3.9, irrespective of the lipid load of the cells (**Fig. 4**). This value, which was adopted for our measurements, is lower than

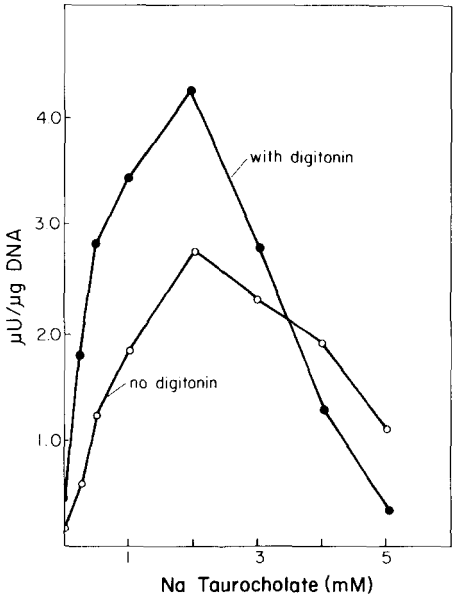

Fig. 2. Effects of Na taurocholate and of digitonin on acid cholesteryl esterase activity in isolated control aortic cells. Assay conditions were standard except for the variables shown. Incubations were run in duplicate for 45 min. Each sample assayed contained 10 μg of protein and 0.4 μg of cholesterol.

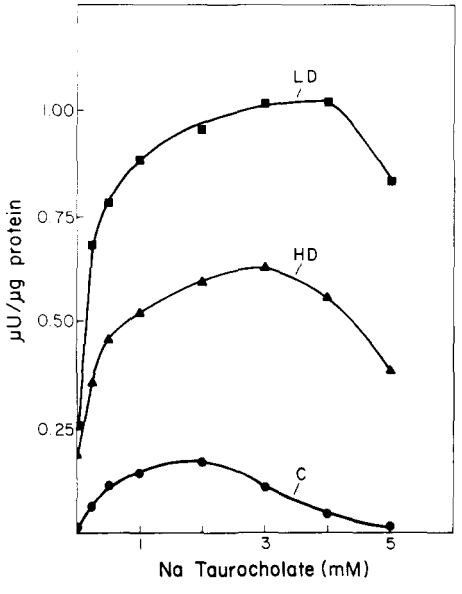

Fig. 3. Influence of Na taurocholate on cholesteryl oleate hydrolysis. Standard assay conditions were used except for Na taurocholate concentration. Incubations were run in duplicate for 45 min for homogenates of control cells (C), and 30 min for high density (HD) and low density (LD) atheromatous cell homogenates. Protein and total cholesterol content per assay were, respectively: control cells, 6.5 and 0.46 μg; high density atheromatous cells 10 and 2.1 μg; low density atheromatous cells, 13.5 and 12 μg.

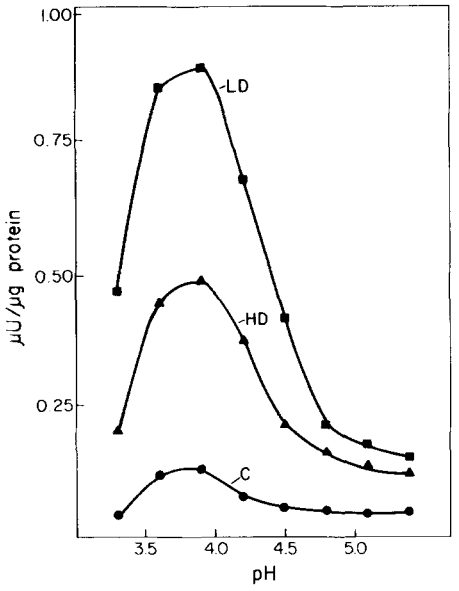

Fig. 4. Effect of pH on cholesteryl oleate hydrolysis. Standard assay conditions were used with the exception of the pH of the acetate buffer. Incubations were run in duplicate for 45 min for homogenates of control aortic cells (C), and 30 min for high density (HD) and low density (LD) atheromatous cell homogenates. The amounts of protein and total cholesterol present in each enzyme sample were the same as in Fig. 3.

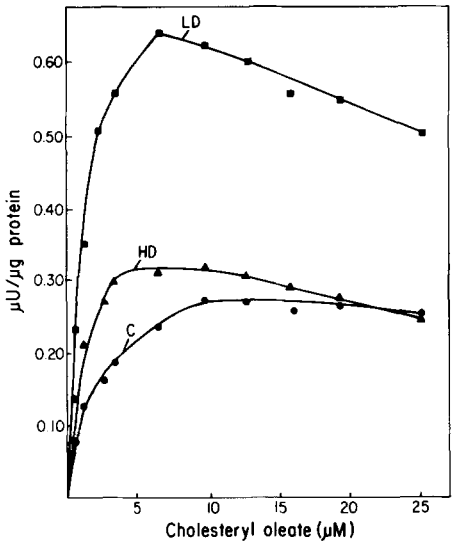

Fig. 5. Effect of substrate concentration on cholesteryl oleate hydrolysis. Standard assay conditions were used except for the amount of cholesteryl oleate-lecithin. The incubations were run in duplicate for 30 min. Protein and total cholesterol content per assay were, respectively: control aortic cells (C), 13 and 0.78 μg; high density atheromatous cells (HD), 13.5 and 2.73 μg; low density atheromatous cells (LD), 10.4 and 7.37 μg.

those reported by Takano et al. (3) and by Brecher et al. (11), but is the same as that found by Fowler, Shio, and Wolinsky (14) in calf aortic cells.

Influence of substrate concentration. Enzyme activity increased with increasing amounts of cholesteryl oleate–lecithin mixture added, up to a substrate concentration of about 10 μM. At higher concentrations, a certain degree of inhibition was observed, more so, apparently, in lipid-laden cells than in control cells (**Fig. 5**). Assuming Michaelis-Menten kinetics, an apparent Km of about 1.5 μM can be calculated for the enzyme in control cells. This value is considerably lower than those reported by Takano *et al.* (3) (40 μM) and by Brecher, Pyun, and Chobanian (15) (28.5 μM). Keeping the lecithin concentration constant at 1.27 mM, while varying only the cholesteryl oleate concentration, did not alter the shape of the curve for control cells or the calculated apparent Km value. The substrate concentration adopted for our assay (12.7 μM) is close to the optimum for both normal and lipid-laden cells.

Influence of incubation time. The enzyme reaction rate remains constant with time for at least 1 hr with normal and moderately lipid-laden cells, but tends to fall off after 30 min with low density atheromatous cells (**Fig. 6**). In practice, incubation times were restricted to 30 min or less in most cases.

Influence of enzyme concentration. As shown by **Fig. 7,** satisfactory linearity as a function of enzyme concentration was observed with both normal and atheromatous cell preparations. Notable is the fact that the cholesterol content of the cells in no way modifies the

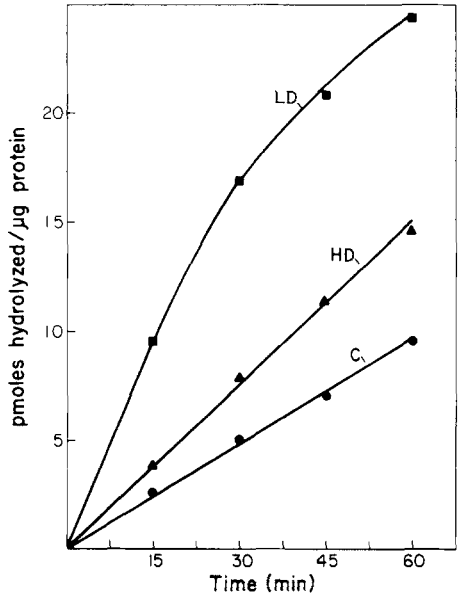

Fig. 6. Time course of hydrolysis of cholesteryl oleate by homogenates of control cells (C), and of high density (HD), or low density (LD) atheromatous cells. Standard assay conditions were used. The amounts of protein and total cholesterol present in each enzyme sample were, respectively: control cells, 9.6 and 0.37 μg; high density atheromatous cells 8.8 and 0.83 μg; low density atheromatous cells, 3.4 and 3.0 μg.

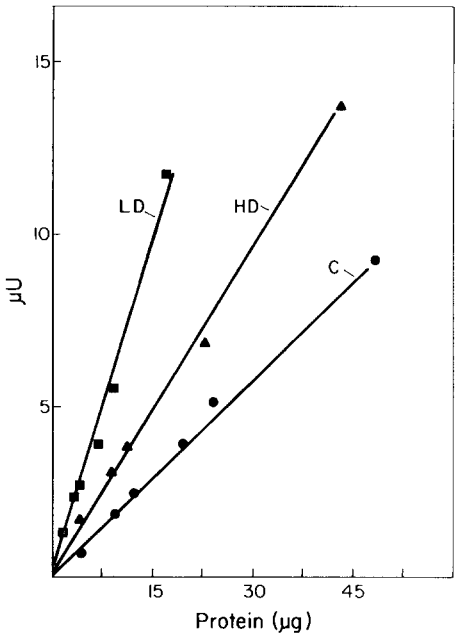

Fig. 7. Effect of enzyme concentration on cholesteryl oleate hydrolysis by homogenates of control cells (C), and of high density (HD), or low density (LD) atheromatous cells. Standard assay conditions were used, with incubations run in duplicate for 45 min for control cells, and 30 min for atheromatous cells. Cholesterol to protein ratios in the three preparations were the same as in Fig. 6.

linearity of the observed relationship. This is good evidence that endogenous cholesteryl esters do not compete with the substrate, nor dilute it isotopically. In the low density atheromatous cell preparation, for instance, the ratio of endogenous cholesterol (much of which is esterified) to substrate cholesterol increases from about 1.5 to 15 over the measured range. The relationship remains perfectly linear.

TABLE 3. Influence of various factors on acid cholesteryl esterase activity in homogenates of whole aortic tissue and of isolated cells

Diluent	Relative Activity	
	Whole Aorta	Isolated Cells
250 mM Sucrose	100	100
250 mM Sucrose, 50 mM dithiothreitol	99	94
250 mM Sucrose, 1 mM EDTA, 0.1% ethanol	131	152
250 mM Sucrose, 1 mM EDTA, 0.1% ethanol, 50 mM dithiothreitol	121	129
Hanks' balanced salt solution	51.4	64.6
Hanks' balanced salt solution, 1 mM EDTA	60.3	83.6
NaCl 125 mM	45.9	55.6
NaCl 500 mM	19.1	15.4
NaCl 1000 mM	6.5	4.7

Homogenates of whole aortic tissue and of isolated aortic cells carefully washed with 250 mM sucrose were prepared in 250 mM sucrose, diluted ten-fold with the various solutions shown, and assayed under standard conditions. The activities measured on the preparations diluted with sucrose alone were 2.8 and 2.1 mU/mg DNA, for the whole aorta and isolated cells, respectively.

Influence of salts. The results presented in **Table 3** show that the aortic acid cholesteryl esterase activity is inhibited by increasing ionic strength. The degree of inhibition is of the order of 50% in Hanks' balanced salt solution, the medium used for isolation of aortic cells. Consequently, all isolated cell preparations were carefully washed with sucrose before homogenization and enzyme assay. The enzyme activity is enhanced by 1 mM EDTA, but not by 50 mM dithiothreitol. Maximum activity was observed in the standard SVE medium used for the preparation of our tissue homogenates.

Influence of frozen storage. Although relatively resistant to rapid freezing and thawing, acid cholesteryl esterase suffers progressive inactivation when stored at −20°C, especially in Hanks' medium (**Table 4**). Accordingly, all our assays were performed on fresh preparations.

Cell content and subcellular distribution

Total enzyme activity. In **Table 5** are summarized the acid cholesteryl esterase activities measured on isolated normal aortic cells, and on high and low density atheromatous cells separated by Metrizamide centrifugation. It is seen that acid cholesteryl esterase activity increases with increasing lipid load of the cells, but less markedly than do the activities of the two lysosomal glycosidases, N-acetyl-β-glucosaminidase and β-galactosidase.

Intracellular distribution. The distributions found for acid cholesteryl esterase in the three aortic cell preparations are shown in **Fig. 8,** where they can be compared with those observed in the same density gradients for two lysosomal markers. The results obtained on normal cells are in agreement with previous findings that have indicated a lysosomal localization of acid cholesteryl esterase in aortic cells (1, 3, 14), except that there seems to be more cholesteryl esterase remaining in the starting layer, presumably in soluble form, than there is of the two glycosidases.

Lipid overloading of the cells was associated with the expected shift of the distributions of the glycosidases towards lower densities, a change typically correlated with intralysosomal accumulation of lipid (7, 8). The distribution of acid cholesteryl esterase was shifted similarly, though perhaps not as markedly. The very low density lysosomes of the atheromatous cells have less demonstrable acid cholesteryl esterase activity, relative to their content in glycosidases, than have the particles, presumably less lipid-laden, that equilibrate at higher densities. This difference

TABLE 4. Effect of storage on acid cholesteryl esterase activity in control aortic homogenates

Storage Conditions	Homogenization and Storage Medium			
	250 mM Sucrose		Hanks' Balanced Salt Solution	
	mU/mg DNA	*% of fresh activity*	*mU/mg DNA*	*% of fresh activity*
Fresh	3.91		1.91	
Fresh, frozen and thawed ten times	3.26	83.4	1.48	77.5
Storage 4°C, 24 hr	2.83	72.4	0.93	48.7
Storage −20°C, 24 hr	2.94	75.2	0.43	22.5
Storage −20°C, 7 days	2.78	71.1	0.08	4.2
Storage −20°C, 18 days	0.69	17.6	0.03	1.6

resembles that observed previously (2), although it is less pronounced.

DISCUSSION

Acid cholesteryl esterase activity of normal and atheromatous vessels has been studied in a number of experimental animals (3, 14–19) and in man (18, 20, 21), and attempts have been made to purify the enzyme (21–23). It is, however, difficult to compare the results of these experiments, because of differences in the method of substrate dispersion, assay conditions, and mode of enzyme extraction from the tissue. Biochemical evidence has been presented that the arterial enzyme may act both on cholesteryl esters and on triacylglycerols (23), as was earlier suggested by the observation that the genetic deficiency of acid lipase (Wolman's disease) leads to tissue deposition of both lipid classes (24). Recent studies have shown that acid cholesteryl esterase activity in vessels is sensitive to various physiological stimuli, including vessel tension, insulin level, and moderate exercise (19, 25, 26).

For a variety of reasons—alteration of the physical state of the substrate, isotope dilution, competitive inhibition of enzyme, binding of enzyme to endogenous lipid—the presence of large amounts of cholesteryl esters and free cholesterol in the arterial cells could conceivably interfere with the accurate measurement of cholesteryl esterase, especially with the enzyme actually being located in a major cellular site of lipid deposition. It was important, therefore, to develop assay conditions close to optimal both for normal cells and for cells heavily laden with lipid. The procedure finally adopted here provides a quantitative assay that satisfactorily obeys the requirements of linearity with time of incubation and enzyme concentration. As already pointed out, the fact that the latter feature is unaffected by the amount of endogenous lipid present indicates clearly that most of the possible causes of error considered above are not operative. There seems to be no alteration of the physical state of the substrate, no isotope dilution, no competitive inhibition of the enzyme. Only noncompetitive inhibition remains as a possible artifact.

Our assay combines key features of the method previously used in our laboratory (3) and of that described more recently by Brecher et al. (11). From the latter, we have adopted the procedure of extensive sonication at high temperature for dispersion of the insoluble cholesteryl ester substrate. As observed by Takano et al. (3), Na taurocholate stimulates the enzyme activity and was also included in our assay mixture. Whether this bile salt acts by altering the physical state of the substrate or by affecting the enzyme directly is unclear. Because of the need to overcome the latency of the enzyme in preparations containing intact lysosomes, we added digitonin, which was not inhibitory to acid cholesteryl esterase, at the concentration needed for full release of the lysosomal enzymes. No doubt use of both digitonin

TABLE 5. Activities of acid cholesteryl esterase and other lysosomal enzymes in aortic cell preparations

Cells	Number of Experiments	Acid Cholesteryl Esterase	N-acetyl-β-glucosaminidase	β-Galactosidase
			(mU/mg DNA)	
Control	(6)	4.07 ± 0.6	52.2 ± 10	23.4 ± 9
High density atheromatous	(7)	6.05 ± 1.7	105 ± 36	77.7 ± 21
Low density atheromatous	(6)	14.4 ± 2.0	391 ± 110	419 ± 107

Values given are means ± standard deviation.

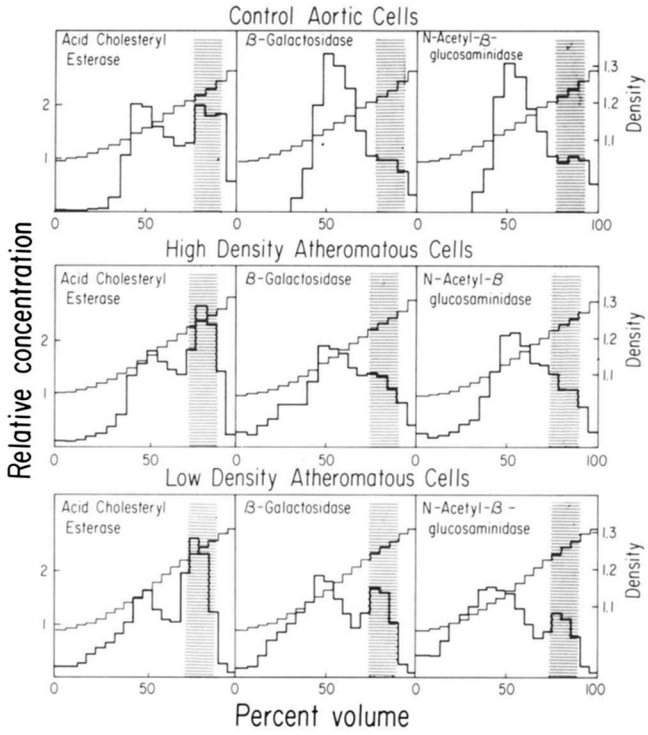

Fig. 8. Sucrose density gradient centrifugation of postnuclear supernates of control cells (C), and of high density (HD), and low density (LD) atheromatous cells. The histograms present the distributions of three acid hydrolases as a function of the volume recovered from the rotor. The shape of the density gradient is given by the "staircase" on top. Shaded areas represent initial position of PNS sample. Percent enzyme recoveries for control cells, and for high density, and low density atheromatous cells, respectively, were: acid cholesteryl esterase, 76,100,66; β-galactosidase, 92,88,85; N-acetyl-β-glucosaminidase, 100,88,76.

and Na taurocholate results in lysis of the unilamellar liposomes, which serve as a vehicle for the substrate in the procedure of Brecher et al. (11).

For reasons that we are unable to explain, the new method yields an apparent K_m value that is more than one order of magnitude lower than the values obtained by the other two techniques. It is known, however, that lipases and related enzymes are very sensitive to the physical form in which their water-insoluble substrate is presented to them. Apparently, our procedure for dispersing cholesteryl oleate is favorably adapted to the particular requirements of the acid cholesteryl esterase occurring in the lysosomes of rabbit aortic cells.

With the new assay, we find that the lysosomal cholesteryl esterase activity of foam cells is increased some 3.5-fold with respect to the enzyme level found in normal smooth muscle cells. It is increased only about 1.5-fold in the less lipid-laden high density cells separated from atheromatous aorta. Brecher, Pyun, and Chobanian (15) observed similarly a 2.5-fold increase in the acid cholesteryl esterase levels of rabbit atheromatous aortas as compared to control aortas, and Peters, Takano, and de Duve (1) and Corey and Zilversmit (27) found elevated levels of acid triacylglycerol lipase in atheromatous vessels. Contrary, therefore, to our earlier surmise, there is a positive correlation, not an inverse one, between lipid load and enzyme activity, rendering unlikely the pathogenic model proposed previously according to which cells become foamy because they do not have enough cholesteryl esterase activity in their lysosomes to satisfy the increased demand induced by cholesterol feeding (1, 2). The fact that a substantial amount of the cholesterol in lysosomes is free, rather than esterified, also argues against this model (5).

The matter should, however, not be considered entirely settled. Compared to several other lysosomal hydrolases (7, 8, also Table 5), acid cholesteryl esterase increases only moderately in the lipid-laden cells. Its level could still be below what is needed for coping efficiently with the increased influx of lipid to which the cells are subjected. Agreeing with this possibility is the fact that part of the intralysosomal cholesterol appears to occur also in esterified form, presumably representing undigested cholesteryl esters. Physical retention of free cholesterol by association with these deposits of undigested esters could conceivably account for its occurrence in the lysosomes of foam cells. It must be noted in this connection that even with our new assay we still find the lysosomes of lowest density to be poorest in acid cholesteryl esterase, relative to their content in glycosidases, as was found previously (2).

According to our morphometric measurements, the lysosomes of the aortic cells in cholesterol-fed rabbits do not become overloaded with lipid until sizable lipid droplets have accumulated in the cytoplasm. If, as shown by Goldstein and Brown (28) on other biological systems, these droplets arise through reesterification of cholesterol released by lysosomal digestion, we may take it that whatever deficiency affects the lysosomes in these cells arises progressively. It seems likely that saturation of the cytoplasmic system involved in clearance of cholesterol from the lysosomes (sterol-carrying protein?) causes a backlog, first of free cholesterol, and later of undigested cholesteryl esters, in the lysosomes. But one cannot entirely exclude a more complex mechanism involving inadequate lysosomal hydrolysis of cholesteryl esters as the primary causal event.

Clearly, more detailed investigation of these processes is required, preferably on an in vitro system in which in vivo conditions can be approximated in a controlled fashion.

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