Assay, Kinetics, and Lysosomal Localization of an Acid Cholesteryl Esterase in Rabbit Aortic Smooth Muscle Cells*

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SUMMARY

A sensitive radioisotope microassay has been developed for the estimation of cholesteryl ester hydrolyase (EC 3.1.1.13) in preparations of isolated rabbit aortic smooth muscle cells. Kinetic studies served to establish optimal assay conditions, which involve incubation in a total volume of 0.2 ml containing 3 μg cholesteryl oleate tritiated in the cholesterol moiety, 0.4 mM egg yolk lecithin, 0.3 mM taurocholate, 25 μg of bovine serum albumin (defatted) mg ml, and 0.05 M sodium acetate buffer, pH 4.25. The labeled cholesterol was separated from the ester by thin layer chromatography with double development. Hydrolysis of as little as 1 pmole of substrate could be detected by this method. Under these conditions no measurable activity was found at neutral or alkaline pH in either phosphate or Tris buffer. The enzyme is several times more active on cholesteryl esters of unsaturated fatty acids than on those of saturated fatty acids in the C₁₈ and C₂₀ series. Among esters of even numbered saturated fatty acids (C₁₈ to C₂₀), cholesteryl myristate (C₁₈) was hydrolyzed fastest. Fractionation studies indicate that cholesteryl esterase is associated with lysosomes in aortic smooth muscle cells.

It is well known that there is a marked accumulation of cholesterol and of cholesteryl esters in the arterial wall during the development of atherosclerosis (1). These lipids accumulate both within the cells and in the extracellular region of the arterial wall (2-4). Earlier investigations from this laboratory have shown that, in the aortic smooth muscle cells of rabbits rendered severely atheromatous by a cholesterol-rich diet, the lysosomes are an important site of intracellular cholesterol and cholesteryl ester accumulation (4-6). Marked elevations in lysosomal acid hydrolyase activities (up to more than 12-fold for certain enzymes) and an increase in lysosomal stability accompany this accumulation. Many workers subscribe to the view, recently reiterated by Kothari et al. (7), that cholesterol enters the artery in free form and is then esterified locally. Such a mechanism does not, however, readily account for the lysosomal localization of the deposits. Lysosomes are digestive organelles, and their overloading is most often the consequence of inadequate breakdown of materials taken in by endocytosis or sequestered by autophagy (8).

In view of these facts, it seemed of importance to measure the ability of aortic smooth muscle cells to hydrolyze cholesteryl esters, and to determine the intracellular localization of the enzyme or enzymes responsible for this ability. Our technique of cell isolation yields only about 1 to 2 mg of cell protein from a single aorta, and this is then further subdivided into some 15 subcellular fractions (9). A highly sensitive assay method was therefore required, especially since the cholesteryl ester hydrolase (EC 3.1.1.13) activity of rabbit aortic cells was found to be low. This paper describes the development of such an assay, together with the results of relevant kinetic studies carried out on homogenates of isolated aortic smooth muscle cells. Data on the subcellular localization of cholesteryl esterase in normal aortic cells will also be presented.

EXPERIMENTAL PROCEDURE

Materials

Labeled cholesterol ([1,2-3H]cholesterol, 50 Ci per mmole) was purchased from New England Nuclear Corporation (Boston, Mass.); unlabeled cholesterol, cholesteryl esters, and fatty acids from Applied Science Laboratories, Inc. (State College, Pa.). Bovine serum albumin (essentially fatty acid free) and commercial fatty acid chlorides were products of the Sigma Chemical Co. (St. Louis, Mo.), except for linoleyl and arachidonoyl chlorides, which were prepared from the corresponding fatty acids by reaction with oxalyl chloride followed by vacuum distillation of the acyl chloride (10). Sodium taurocholate and bovine, plant, and egg yolk lecithins were obtained from Supelco, Inc. (Belleville, N.J.). Dioleyl lecithin was purchased from Applied Science. Lecithins were standardized by total organic phosphorus determination (11, 12). Triton WR-1339 was purchased from Rupel Chemical Co., Inc. (Irvington, N.J.). All other reagents were of analytical grade. Labeled cholesteryl esters were synthesized by allowing labeled

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cholesterol to react with 0.02 ml of fatty acyl chloride as described by Deykin and Goodman (13). Quantitative analysis of the labeled substrates by the thin layer chromatographic method described below indicated a radiochemical purity of the order of 99.9%.

Methods

Preparation of Aortic Cell Homogenates—Rabbit aortic smooth muscle cells were isolated after enzymic digestion of the connective tissue matrix, and homogenized in 0.25 M sucrose containing 1 mM EDTA and 0.1% ethanol, exactly as described before (9). The homogenates, which contained about 200 µg of protein per ml, were used as such or after removal of a crude nuclear fraction by centrifugation at 900 x g for 10 min (postnuclear supernatant fraction). Fractionation of the postnuclear supernatant by isolectric centrifugation in a sucrose gradient was carried out as in our earlier studies (5, 9).

Assay of Cholesteryl Esterase—The following procedure was used to prepare 1 ml of buffered substrate. The appropriate labeled cholesteryl ester (3 x 10⁶ cpm) in 0.1 ml of benzene-10 µmol of carrier cholesteryl ester in 0.1 ml of benzene, and 0.8 amole of lecithin in 0.05 ml of benzene were mixed and taken to dryness under a stream of N₂ (extra dry grade, Matheson Gas Products, East Rutherford, N. J.). Then 0.25 ml of 2.4 mM sodium taurocholate was added and the resulting suspension sonicated twice for 10 s at a setting of 10 amps in a Branson Sonifier (Branson Instruments, Inc., Danbury, Conn.). To minimize overheating during sonication, the probe was pre-chilled in Dry Ice before each run and the tube was kept in an ice bath. Bovine serum albumin, 0.25 ml of 200 µg per ml, and 0.5 ml of 0.2 M sodium acetate-acetic acid buffer, pH 4.25, were added and, after mixing, the substrate was centrifuged for 10 min in a bench top International Clinical Centrifuge (Ace Scientific Co., Linden, N. J.). A suitable ultraviolet light plate was developed by ascending chromatography (Eastman Chromagram Sheets No. 13181 with fluorescent indicator, East Rutherford, N. J.). The chromatogram was viewed under ultraviolet light, and the plate was then cut into five portions: (a) l-cm strip in-}

<table>
<thead>
<tr>
<th>Table I</th>
<th>Effect of lecithin source on cholesteryl oleate hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lecithin</td>
<td>Activity (micromoles/mg protein)</td>
</tr>
<tr>
<td>Egg yolk</td>
<td>12.8</td>
</tr>
<tr>
<td>Plant</td>
<td>7.6</td>
</tr>
<tr>
<td>Bovine</td>
<td>8.1</td>
</tr>
<tr>
<td>Dioleyl (synthetic)</td>
<td>0.6</td>
</tr>
<tr>
<td>Egg yolk lysolcholethin</td>
<td>0.3</td>
</tr>
<tr>
<td>None</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Clinical Centrifuge (Ace Scientific Co., Linden, N. J.). A suitable ultraviolet light plate was developed by ascending chromatography (Eastman Chromagram Sheets No. 13181 with fluorescent indicator, East Rutherford, N. J.). The chromatogram was viewed under ultraviolet light, and the plate was then cut into five portions: (a) l-cm strip including origin, (b) strip extending to within 1 cm of free cholesterol front, (c) strip including free cholesterol spot, (d) 0.1-cm strip between free and esterified cholesterol spots, (e) the remainder up to solvent front, including cholesteryl ester. The strips were transferred to scintillation vials, together with 7.5 ml of scintillation fluid made up of 6 g of 2-(4'-t-butylphenyl)-5-(4'-biphenyl)-1,3,4-oxadiazole (Beckman Instruments, Inc., Fullerton, Calif.) per liter in toluene. After mixing and leaving overnight, the samples were counted in a Packard Tricarb liquid scintillation spectrometer, model 3375 (Packard Instrument Co., Downers Grove, III.). Total recovered radioactivity was estimated, and the fraction of substrate hydrolyzed was calculated and converted to units, 1 unit being defined as the amount of enzyme producing 1 amole of free cholesteryl per min under the conditions of the assay.

Essentially the same procedure was followed in the kinetic experiments, with the appropriate factor modified as indicated in the text. In particular, the substrate was always suspended by sonication with the amounts of lecithin and taurocholate included in the tests, as described above. Effectors were added together with the buffer.

Other Biochemical Assays—As previously described (9), cytochrome oxidase was measured by a sensitive spectrophotometric assay, whereas protein, N-acetyl-p-glucosaminidase, and α-glucosidase were assayed fluorometrically.

RESULTS

Chromatographic Separation—To achieve the necessary sensitivity, it was essential to measure the hydrolysis of as little as 0.1% of the substrate. Complete chromatographic separation of free cholesterol from the ester was necessary for this purpose, especially since cholesterol is the slower moving component and any trailing of ester would result in unacceptably high blanks.

We were unable to achieve adequate resolution with the use of silicic acid column chromatography and different solvent systems (14, 15), especially when starting from a mixture containing bile salts, lecithin and an aortic cell homogonate. Improved separation was obtained with thin layer chromatography on silicic plates, but complete separation of cholesterol from the cholesteryl ester was achieved only when double development of the chromatogram was carried out. Under these conditions the blank was usually reduced to about 0.1% of the original substrate added.

Influence of Emulsifiers—Both lecithin and taurocholate were necessary ingredients of the incubation mixture. As shown in Table 1, the best results for the hydrolysis of cholesteryl oleate were obtained when egg yolk lecithin was used to prepare the micellar substrate. Optimum stimulation of the reaction was achieved when egg yolk lecithin was present at about 0.4 mM final concentration (Fig. 1). Excess lecithin caused an inhibition of esterase activity.

As illustrated in Fig. 2, the activity measured over a 30-min incubation period tended to increase in a pseudo-hyperbolic fashion as a function of increasing taurocholate concentration. However, as shown in Fig. 3, the effect of taurocholate on cholesteryl esterase activity was dependent upon both time and bile salt concentration. The higher initial reaction rate obtained in the presence of 1 mM taurocholate was not maintained, but declined progressively, presumably owing to inactivation of the enzyme. In contrast, with 0.3 mM taurocholate, the initial rate
of cholesteryl oleate hydrolysis was lower but linear kinetics was achieved for at least 4 hours. Assay conditions were chosen on the basis of these results.

Influence of Bovine Serum Albumin—With some preparations, addition of bovine serum albumin had no effect on the assay, with others it increased the observed activity. Its inclusion in the incubation medium was decided as a precautionary measure. Its mechanism of action was not investigated.

Influence of pH—The influence of pH on the enzyme activity is shown in Fig. 4. Under our assay conditions, the highest rate of cholesteryl oleate hydrolysis is observed at pH 4.25, and there is no measurable activity around neutrality, in either phosphate or Tris buffer.

Influence of Enzyme Concentration—As shown in Fig. 5, good proportionality with enzyme concentration was observed up to an activity of about 0.1 microunit, which in the present case corresponded to hydrolysis of 0.6% of the substrate. As little as 0.5 µg of this activity could be detected. These limits define the range of optimal applicability of our assay.

Influence of Substrate Concentration—Hydrolysis of the micellar substrate was found to follow normal Michaelis-Menten kinetics (Fig 6). The apparent $K_m$ for the micellar substrate calculated from the linear double reciprocal plot (Fig. 6, inset) is 40 µM.

Influence of Effectors—Table II shows the effect of various activators and inhibitors on the hydrolysis of cholesteryl oleate. There was slight activation of the enzyme by 10 mM CaCl$_2$, whereas CuCl$_2$ was inhibitory at this concentration. EDTA and eserine had no effect on enzyme activity. The lack of inhibition by eserine is significant since this compound is a potent inhibitor of cholinesterase activity. Among the sulfhydryl group inhibitors, p-chloromercuribenzoate was strongly inhibitory at 0.1 mM concentration, whereas N-ethylmaleimide had little effect on enzyme activity and iodoacetamide became inhibitory only at 10 mM concentration.

Specificity—The fatty acid specificity of cholesteryl esterase on a series of substrates with different fatty acid moieties is illustrated in Table III. All assays were carried out under standard conditions at identical substrate concentration (5 µM).

It is apparent that the shorter chain fatty acid esters of cholesterol were hydrolyzed more rapidly than the longer chain esters.
The most striking differences were observed when fatty acid esters of the same chain length but different degrees of unsaturation were compared. Hydrolytic activity was much greater against the unsaturated than against the corresponding saturated fatty acid esters.

Since the differences shown in Table III could have resulted from differences in the physical state of the substrate, experiments were also performed on identical mixtures of four or five esters with a different ester labeled in each sample. As shown in Table IV, when presented with an equimolecular mixture of cholesteryl esters of even numbered saturated fatty acids from C_{12} to C_{20}, the enzyme acted mainly on the myristate and laurate, much less on the palmitate, and very little on the stearate and arachidate. Out of a mixture of four esters of C_{18} fatty acids with increasing number of double bonds, the enzyme hydrolyzed the three unsaturated esters 4 to 5 times faster than it did stearate. Increasing the number of double bonds from 1 to 3 increased the reaction rate by only 44%.

**Table III**

Fatty acid specificity of cholesteryl ester hydrolysis

<table>
<thead>
<tr>
<th>Cholesteryl ester*</th>
<th>Activity (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitate (16:0)</td>
<td>3.4</td>
</tr>
<tr>
<td>Stearate (18:0)</td>
<td>1.3</td>
</tr>
<tr>
<td>Oleate (18:1)</td>
<td>8.7</td>
</tr>
<tr>
<td>Linoleate (18:2)</td>
<td>9.8</td>
</tr>
<tr>
<td>Linolenate (18:3)</td>
<td>12.9</td>
</tr>
<tr>
<td>Arachidate (20:0)</td>
<td>0.9</td>
</tr>
<tr>
<td>Arachidonate (20:4)</td>
<td>11.0</td>
</tr>
</tbody>
</table>

* Number of carbon atoms to number of double bonds is given in parentheses.

**Table IV**

Fatty acid specificity of cholesteryl ester hydrolysis

<table>
<thead>
<tr>
<th>Labeled cholesteryl ester*</th>
<th>Labeled cholesterol released (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
</tr>
<tr>
<td><strong>Series I</strong></td>
<td></td>
</tr>
<tr>
<td>Laurate (12:0)</td>
<td>10.9</td>
</tr>
<tr>
<td>Myristate (14:0)</td>
<td>12.6</td>
</tr>
<tr>
<td>Palmitate (16:0)</td>
<td>4.8</td>
</tr>
<tr>
<td>Stearate (18:0)</td>
<td>2.4</td>
</tr>
<tr>
<td>Arachidate (20:0)</td>
<td>1.7</td>
</tr>
<tr>
<td>Total</td>
<td>32.4</td>
</tr>
<tr>
<td></td>
<td>100.0</td>
</tr>
<tr>
<td><strong>Series II</strong></td>
<td></td>
</tr>
<tr>
<td>Stearate (18:0)</td>
<td>1.4</td>
</tr>
<tr>
<td>Oleate (18:1)</td>
<td>5.0</td>
</tr>
<tr>
<td>Linoleate (18:2)</td>
<td>5.6</td>
</tr>
<tr>
<td>Linolenate (18:3)</td>
<td>7.3</td>
</tr>
<tr>
<td>Total</td>
<td>19.3</td>
</tr>
<tr>
<td></td>
<td>100.0</td>
</tr>
</tbody>
</table>

* Number of carbon atoms to number of double bonds is given in parentheses.

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**Intracellular Localization**—The subcellular localization of cholesteryl oleate hydrolase was studied in isolated aortic smooth muscle cells from normal rabbits with the "inner layering" technique previously described (5, 9). Fig. 7 shows the distribution profiles of cholesteryl oleate hydrolase, cytochrome oxidase, N-acetyl-β-glucosaminidase, and α-glucosidase in preparations from normal rabbits. The distribution of cholesteryl esterase resembles most closely that of the lysosomal marker N-acetyl-β-glucosaminidase. It differs greatly from that of the mitochondrial cytochrome oxidase, and significantly from that of the presumably microsomal α-glucosidase, which shows distinctly more activity in the region of density higher than 1.20.

Earlier studies have shown that pretreatment of the rabbits with repeated injections of Triton WR-1339 causes a selective decrease in the density of aortic cell lysosomes (9). As shown in Fig. 8, this treatment altered the distributions of N-acetyl-β-
The conditions worked out in the present study seem well adapted to the measurement of the acid cholesteryl esterase. The enzyme has been shown to be of critical importance (7, 16, 26) for the differences between our results and those of other workers. With a substrate concentration equal to \( K_m \) of the enzyme, as

\[ pH \text{ optimum in the neighborhood of neutrality has been demonstrated} \]

In particular, the physical state of the substrate presented to the enzyme, as

\[ pN \text{ optimum of 6.6 when tested against a micellar substrate, and of 7.4 when acting on an emulsion of cholesteryl oleate} \]

There is less activity in the starting layer, which contains both soluble enzyme and low density particles, and correspondingly more associated with the main peak. The modal density of \( \alpha \)-glucosidase is essentially unaltered. These results provide a strong indication that at least the major part of the cholesteryl esterase activity assayed under our conditions is localized in lysosomes.

**DISCUSSION**

**Properties of Enzyme**—Cholesteryl esterase activity with a pH optimum in the neighborhood of neutrality has been demonstrated in the aorta of several animal species (7, 16–22). But this is the first report of the existence of a distinctly acid cholesteryl esterase in aortic tissue. Such an enzyme has, however, been detected in human, rat, and bovine liver (23–25), in mouse peritoneal macrophages (26), and in rabbit ovary (27). For all three tissues, there is evidence of a lysosomal localization of the enzyme, as in the rabbit aorta. The macrophage cholesteryl esterase resembles the aortic enzyme also in having a strong preference for esters of unsaturated fatty acids (26).

It seems likely that differences in assay conditions account for the differences between our results and those of other workers. In particular, the physical state of the substrate presented to the enzyme has been shown to be of critical importance (7, 16, 25). The conditions worked out in the present study seem well adapted to the measurement of the acid cholesteryl esterase. With a substrate concentration equal to \( \frac{1}{2} K_m \) of the enzyme, we find an activity of the order of 12.5 microunits per mg of cell protein, corresponding to about 0.25 milliunits per g (wet weight) of aortic tissue. Almost 10 times as much would be found at saturating substrate concentration.

Closest to our results are those of Kothari and co-workers, who, working with human aorta (16), and later with acetone powders of rat and rabbit aortas (7), have investigated a cholesteryl esterase apparently localized in lysosomes (16), but showing a pH optimum of 6.6 when tested against a micellar substrate, and of 7.4 when acting on an emulsion of cholesteryl oleate stabilized by bovine serum albumin. These results are in striking contrast with our finding of an optimum at pH 4.25 and total lack of activity above pH 8.5, even in phosphate buffer, used by Kothari et al. (Fig. 4). Another difference between the two sets of results lies in the \( K_m \) value, which Kothari et al. (7) estimate at 200 \( \mu M \) for the rabbit enzyme, as against 40 \( \mu M \) determined in the present work. On the other hand, the maximum activities measured in the two laboratories are of the same order of magnitude. For their rabbit aorta preparation, Kothari et al. (7) report values of 8 to 10 milliunits per g of acetone powder, or about 3 milliunits per g (wet weight) of tissue, which is essentially what we would find in our preparations at saturating substrate concentration.

At first sight it would appear that two distinct enzymes are involved, were it not for the difficulty of explaining how each group manages to miss entirely the enzyme detected by the other. It is conceivable that, in the work of Kothari et al. (7), the enzyme may be altered by the procedure used to prepare an acetone powder, but this does not seem very probable since these authors also find a pH optimum of 6.6 for the activity in fresh human aortic homogenates (16). It would seem more plausible that the differences in enzyme properties are due to differences in the composition of the assay medium, which are considerable: Kothari et al. use 120 times more cholesteryl oleate and about

**Fig. 7** (left). Fractionation by isopycnic centrifugation (inner layering) of postnuclear supernatant fraction of aortic cells isolated from normal rabbit aortas. Graph shows frequency-density distributions (±S.D.) for cholesteryl esterase and marker enzymes. Frequency is defined as fraction of total recovered enzyme activity present in subcellular fraction, divided by density span covered. The shaded area represents an arbitrary abscissa interval the enzyme remaining in the sample layer. Results are averaged and shown with S.D., as in our earlier publications (4, 5, 9). The per cent recovered activity (±S.D.) for each enzyme and the number of experiments (in parentheses) are: cholesteryl oleate hydrolase, 72.4 ± 17 (four); cytochrome oxidase, 84.5 ± 19 (four); \( N \)-acetyl-\( \beta \)-glucosaminidase, 81.4 ± 16 (four); \( \alpha \)-glucosidase, 87.5 ± 3.9 (two).

**Fig. 8** (right). Fractionation by isopycnic centrifugation (inner layering) of postnuclear supernatant fraction of aortic cells isolated from aortas of rabbits treated with Triton WR-1339 (1 g intravenously per kg on days 6, 4, and 2 before killing). Results of two experiments, represented as in Fig. 7. Broken lines give distributions in normal preparations (Fig. 7). The per cent recovered activity (±S.D.) for each enzyme is: cholesteryl oleate hydrolase, 95.2 ± 20; cytochrome oxidase 95.5 ± 1.2; \( N \)-acetyl-\( \beta \)-glucosaminidase, 99.0 ± 7.3; \( \alpha \)-glucosidase, 96.7 ± 25.
4 times more taurocholate and lecithin per unit volume than we do. However, the results of Fig. 6 render this explanation also improbable since we can go up to 14 the substrate concentration used by Kothari et al., with substrate to emulsifier ratios very similar to theirs, without detecting any anomalous behavior of the enzyme. In their early work (10) Kothari et al. for some unexplained reason included oleic acid in their incubation mixture at 3 times the concentration of cholesteryl oleate. But they did not do so in their later work (7). Presumably, therefore, this factor is not responsible for the observed differences. These cannot be explained either, as we have verified, by the presence of serum albumin in our test. There remains the possibility that the method followed for preparing the substrate is the critical factor. Kothari et al. add their lipid components in ether solution to the aqueous buffer, homogenize, remove the ether by evaporation at 37°, and then sonicate at 10 to 20 kc per s for 15 to 30 min or longer. What precautions are taken against thermal damage during this treatment is not stated. In our procedure, the mixture of cholesteryl oleate and lecithin is introduced first in benzene solution, taken up to dryness, suspended in a taurocholate solution by two 10-s sonications with rigid precautions against overheating, and finally mixed with the buffer and albumin.

Assay Method—As an analytical tool, the procedure described here appears reasonably optimized, and it obeys satisfactorily the requirements for linearity with respect to time (Fig. 3) and enzyme concentration (Fig. 5). It is highly sensitive and can be applied to subcellular fractions isolated from a single aorta preparation (Figs. 7 and 8). The choice of a very low substrate concentration, not generally advocated in enzyme work, has several advantages in the case of a radioassay of this type. It is economical, not only in terms of cost, but also in terms of time and effort since very pure substrate is more easily prepared on a small than on a large scale.

Biological Significance—The presence of an active acid cholesteryl esterase in the lysosomes of aortic smooth muscle cells is of considerable interest in view of the massive intralysosomal accumulation of cholesteryl esters in the aortic cells of cholesterol-fed rabbits (5, 6). As we have mentioned in a preliminary report (4) and will elaborate in more detail in a subsequent paper in this series, there is evidence that the amount of cholesteryl ester stored in the lysosomes is inversely related to their cholesteryl esterase activity, suggesting that storage may be the consequence of a relative enzyme deficiency, as in many congenital storage diseases (28).

Acknowledgments—We gratefully acknowledge the expert technical assistance of Bogumila Koss, Fred Davidson and Armando Pelaschier. We are also gratefully indebted to Drs. Robert Donaldson and Stanley Fowler for their collaboration in several aspects of this work.

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