Lipase activity in the human aorta

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ABSTRACT The hydrolysis of triglycerides by grossly normal male human aortas has been studied in vitro. The tissue contains an acid lipase (pH optimum, 5.4) and an alkaline lipase (pH optimum, 8.8). Both lipases catalyze the hydrolysis of saturated triglycerides; the rate decreases with increasing fatty acyl chain from C_{10} to C_{18}. Glycerol trioleate, trilinoleate, and trilinolenate are hydrolyzed at similar rates. Alkaline lipase is inhibited about 50% at 7.2 mM glycerol trioleate, while acid lipase is unaffected at this concentration. Both lipases are activated by Ca^{++} ions. The acid lipase is easily inactivated by deionized water used either as a homogenizing or dialyzing medium. Acid lipase is strongly inhibited by BSA, sodium deoxycholate, and sodium taurocholate; alkaline lipase is unaffected by BSA and is activated about twofold by bile salts. The products of hydrolysis of glycerol trioleate by aortic lipases are predominantly oleic acid and glycerol 1,2-dioleate with a small accumulation of glycerol monooleate.

The aortic preparations appear to contain inhibitors for both the acid and alkaline lipase. The substance which inhibits alkaline lipase also inhibits pancreatic lipase; it is heat-stable and dialyzable. The inhibitor of the acid lipase is also heat-stable but is nondialyzable.

SUPPLEMENTARY KEY WORDS glycerol trioleate · acid lipase · alkaline lipase · pancreatic lipase · lipase inhibitors

THE LIPID COMPOSITION OF human atheromata are reported to be 57% cholesterol (free and esterified), 24% neutral fat, and 19% phospholipid (1). Consequently a considerable number of investigators have focused their attention on the catabolic as well as anabolic processes of lipids in the aortic wall (2). Lipase (glycerol ester hydrolase; EC 3.1.1.3) has been shown to exist in aortas of man (3, 4), pig (5), rabbit (5), and rat (5). However, the limited data available from various animal species do not permit direct comparison because of the differences in the substrates as well as in the assays employed by various investigators. Adams, Bayliss, Abdulla, Mahler, and Root (4) used tributyrin at pH 7.4 for estimation of human aortic lipase, whereas Patelski et al. (5) employed glycerol trioleate in ethanol–water dispersions and reported the pH optimum to be 8.3. Maier and Haimovici (6, 7), however, could not demonstrate lipase activity in man, rabbit, and dog using β-naphthyl laurate at pH 7.4.

In this paper the properties of the lipases in human aortas are described.

Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate (tetrasodium salt); BSA, bovine serum albumin.

MATERIALS

Glycerol trioleate and cholesteryl oleate were purchased from the Hormel Institute, Austin, Minn. Glycerol trilinoleate, trilinolenate, and triarachidonate were purchased from Sigma Chemical Co., St. Louis, Mo. The purity of these substrates was checked by thin-layer chromatography and was found to be greater than 99% (see Fig. 7). Other triglycerides were obtained from Eastman Organic Chemicals, Rochester, N.Y., and were purified by thin-layer chromatography before use. Sodium diethyldithiocarbamate was obtained from J. T. Baker Chemical Co., North Phillipsburg, N.J. Silica Gel F-254 precoated glass plates (20 × 20 cm) were from Brinkman Instruments Inc., Westbury, N.Y. Gum arabic, pancreatic lipase, sodium heparin, sodium deoxycholate, sodium taurocholate, and bovine serum albumin were obtained from Sigma Chemical Co. All the other chemicals employed were of reagent grade.

METHODS

Preparation of Tissue Extract

Portions of the descending thoracic and abdominal aortas of human male subjects (6–32 yr of age) were ob-

tained at autopsy within 12–24 hr of death. The subjects were victims of automobile accidents or homicides. The aortas were cut open longitudinally and cleaned of blood with blotting papers. Only those normal aortas, which did not contain fatty streaks or plaques, were collected, and stored at −20°C until sufficient amounts were obtained. The aortas were thawed, the adhering tissues were removed by forceps, and 10–20 g of the cleaned aortas, randomally selected, were combined. The aortas were minced and homogenized for 2 min in ice-cold 0.01 M phosphate buffer, pH 7.0 (1:8, w/v) in a Waring Blendor at top speed. The homogenate was centrifuged at 34,800 *g* for 120 min in the Sorvall RC2-B centrifuge (SS-34 rotor). The supernatant (I) was filtered through a layer of glass wool in order to remove most of the fatty materials floating on top of the tube. The sediment was resuspended and homogenized as previously described, in half of the original volume of the same buffer; the homogenate was then centrifuged as above to yield supernatant (II). Unless it was necessary to assay the two supernatant fractions (I and II) individually, they were combined and were used as the enzyme source. Normally, approximately 80% of alkaline lipase activity found in the homogenate was recovered in supernatant (I), while most of the remainder of the activity was in supernatant (II). No significant activity was found in a third extraction. The recovery of the activity in the two supernatants was always 90–120% of the activity found in the whole homogenate. Less than 50% of the acid lipase activity in the whole homogenate was recovered in the two supernatants (I and II); therefore, it should be noted that almost all of our experiments were performed with supernatant fractions which contained most of the alkaline lipase and less than 50% of the acid lipase found in the homogenate. Because of the solubility of the enzyme, its specific activity in the supernatant fraction was 3–4 times greater than that in the homogenate; only 20–30% of the proteins in the homogenate was extractable into the two supernatants (Table 1). Further centrifugation of the combined supernatants at 100,000 *g* for 60 min resulted in 4–15% of the activity in the sedimentable fraction (8). The tissue homogenate, as well as the subsequent supernatant fractions thus prepared, could be stored at −20°C for at least 1 month without loss of both lipase activities. Since samples were collected and pooled in such a way, the effect of freezing and thawing was not determined.

Measurement of Lipolytic Activity

The degree of lipolysis was measured by estimation of the amount of released fatty acid according to the method of Duncombe (9). The incubation mixture contained 0.1 ml of triglyceride dispersed in 5% gum arabic, 0.3 ml of either 0.1 M acetate buffer (pH 5.4) or 0.1 M glycine–NaOH buffer (pH 8.8), 0.1 ml of aortic supernatant, and 0.5 ml of deionized water or 0.01 M phosphate buffer. After 30 min of incubation at 37°C, the reaction was stopped by the addition of 0.5 ml of 1 N HCl, and the fatty acid was extracted with 10 ml of chloroform–methanol 1:1. A chloroform phase which separated after the addition of 4 ml of deionized water was then reacted with 2.5 ml of copper reagent (9). 2 ml of chloroform phase was mixed with 1.0 ml of 0.1% sodium diethyldithiocarbamate in *n*-butanol, and the absorbance was measured at 440 nm with a Hitachi Perkin-Elmer UV-VIS spectrophotometer, Coleman Model III. Blank values were obtained by incubating the enzyme preparation and substrate separately and were subtracted from the sample values. Throughout the experiments, duplicate assays were carried out for all samples and were zero-order reactions. The values reported are the average of such duplicate assays.

TABLE 1 EFFECT OF HOMOGENIZING MEDIA ON RELEASE OF ALKALINE LIPASE INTO SUPERNATANT

Medium	Weight of Aortas	Fraction	Total Volume	Total Protein	% of Homogenate Protein in Supernatant	Total Lipase Activity†	Recovery of Lipase in Supernatant	Specific Activity
	g		*ml*	*mg*			%	
0.25 M Sucrose with 1 mM EDTA (pH 7.0)	15.0	Homogenate	128	1050		76800		2.4
		Supernatant (I)	96	156	15	17800	23	3.8
		Supernatant (II)	48	56	5.3	9600	13	5.7
		Supernatant* (III)	110	116	11	50050	65	14.4
0.01 M Phosphate buffer (pH 7.0)	14.9	Homogenate	128	1020		85700		2.8
		Supernatant (I)	94	252	25	70500	82	9.3
		Supernatant (II)	52	50	4.8	11180	13	7.5

Human aortic homogenates were prepared in 0.25 M sucrose solution with 1 mM EDTA (pH 7.0), and in 0.01 M phosphate buffer (pH 7.0). Preparation of each supernatant and assay conditions are described in the text.

* The sediment, after supernatant (II) was removed, was rehomogenized in 8 vol of 0.01 M phosphate buffer (pH 7.0). The homogenate was centrifuged at 34,800 *g* for 120 min to yield supernatant (III).

† mμmole of oleic acid released per 30 min.

While sodium taurocholate did not affect the blank value up to 1 mg in the incubation mixture and under the conditions employed, sodium deoxycholate was found to give a linear response in the range of 0–1 mg in the incubation mixture. This represented a response of approximately one-third of that of fatty acid at equimolar concentration. If, however, after stopping the reaction, the precipitated deoxycholate was removed by centrifugation prior to the fatty acid estimations, its effect became negligible.

When thin-layer chromatography was performed to determine the products of hydrolysis, the chloroform phase was separated and evaporated to dryness at 50°C under a stream of nitrogen. 0.1 ml of chloroform was added to each sample, and 0.02 ml (0.01 ml when pancreatic lipase was used) was spotted on a Silica Gel F-254-coated glass plate. The plate was developed in a solvent system consisting of petroleum ether–diethyl ether–formic acid 75:25:1.5 (v/v), and spots were visualized by exposing the plate to iodine vapor. When commercial pancreatic lipase was used as enzyme source, a solution containing 0.5 mg/ml of 0.01 M phosphate buffer (pH 7.0) was made, and an appropriate volume was used in the same incubation mixture as described for assay of aortic lipase. The reaction was carried out for 5 min at 37°C.

Protein was measured by the method of Miller (10). Specific activity is expressed as mμmole of fatty acid released per mg of protein per min.

Substrate Dispersion

All the substrates were dispersed in a 5% solution of gum arabic according to the following method. A weighed amount of substrate was dissolved in a small volume of chloroform, and this solution was dispersed in ice-cold 5% gum arabic solution with approximately 1 g of crushed ice (11) by a microblender (Eberbach Corp., Ann Arbor, Mich.) at top speed for 3 min. Chloroform was completely removed under a stream of nitrogen while warming the dispersion to 50°C. Usually a final substrate concentration of 36 mM was made. The dispersion, thus prepared, was opaque and could be stored for a few days without spontaneous hydrolysis. Whenever necessary, the pH of the dispersion was adjusted to the desired pH with either 1 N HCl or 1 N NaOH.

RESULTS

Effect of pH

Two distinctly different substrates, glycerol tridecanoate and glycerol trioleate, were used to compare the pattern of lipolysis by aortic supernatant (Fig. 1). With both substrates, pH optima of 5.2–5.4 and 8.8–9.0 were consistently observed when the aortas were homogenized in

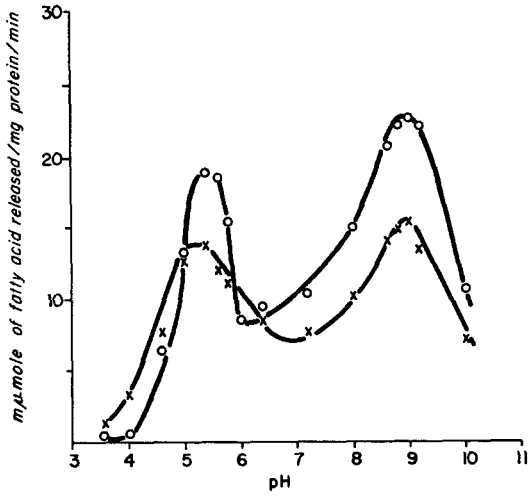

FIG. 1. Effect of pH on the hydrolysis of glycerol tridecanoate and trioleate by aortic supernatant. Incubations were for 30 min at 37°C with 0.01 ml of aortic supernatant (0.244 mg of protein). Buffers used: 0.1 M acetate, pH 3.6–5.6; 0.1 M phosphate, pH 5.6–8.0; 0.1 M tris–HCl, pH 7.2–9.0; 0.1 M glycine–NaOH, pH 8.6–10.0; O—O, glycerol tridecanoate (7.2 mM); ×—×, glycerol trioleate (3.6 mM).

0.25 M sucrose (pH 7.0) or in 0.01 M phosphate buffer (pH 7.0). However, if the same samples were homogenized in deionized water, only the alkaline peak was fully active, and the acid activity was not observed. Other data which will be discussed later, also strongly support the presence of two different lipases in aortic tissue. Thus, the subsequent studies with human aortic preparations were made at pH 5.4 and pH 8.8.

Comparison of Homogenizing Media

Aortas from each individual were cut longitudinally into two identical portions. One portion was suspended in 8 volumes of 0.25 M sucrose medium containing 1 mM EDTA (pH 7.0), and the other was suspended in 0.01 M phosphate buffer (pH 7.0). The homogenate and subsequent supernatant fractions were prepared as described under Methods. Table 1 illustrates a typical distribution of alkaline lipase activity in each preparation. While only 36% of the activity of the homogenate was extracted into two supernatants (I and II) when the sucrose medium was used, more than 95% of the activity was recovered in supernatants (I and II) prepared with hypotonic buffer medium. The amounts of protein in the sucrose and phosphate buffer supernatants were approximately 20 and 30%, respectively. However, as can be seen, the rest of the activity (65%) and an additional 11% of the protein, which had not been extracted by two successive homogenizations in sucrose medium, were recovered in supernatant (III) prepared as described in Table 1. This fraction (supernatant III) had the highest specific activity. It is noted that the percentage of total protein extracted into the supernatant fractions with sucrose (I,

II, and III) and buffer (I and II) media were very close, 31.3, and 29.8%, respectively. In two other experiments, 89 and 116% of the enzyme activity, and 27 and 30% of the proteins were extracted into the two supernatants prepared with buffer medium. Because of the ease of preparation and because there were less contaminants, later studies were made with those supernatants from homogenates prepared in 0.01 M phosphate buffer (pH 7.0). It should be pointed out, however, that the recovery of acid lipase activity in supernatants (I and II) from homogenates prepared in the hypotonic buffer medium varied from 9 to 47% and was never more than 50%. It has not been ruled out that the variation might have been due in part to the freezing and thawing of the samples which had to be stored frozen until sufficient amounts were obtained. For this reason, no attempt was made to compare directly the absolute differences between the acid and alkaline lipase activities in the supernatants.

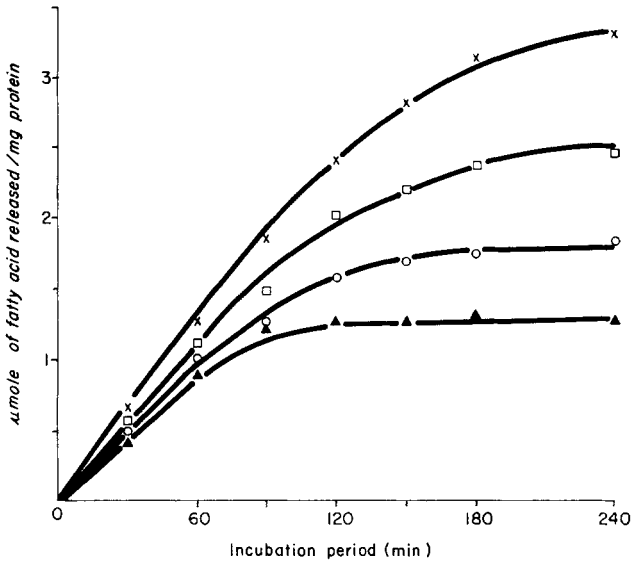

Fig. 2. Effect of time on the hydrolysis of glycerol tridecanoate and glycerol trioleate by aortic supernatant. Incubations were carried out with 0.8 ml of supernatant, 2.4 ml of 0.1 M acetate buffer (pH 5.4) or 0.1 M glycine–NaOH buffer (pH 8.8), 1.6 ml of glycerol tridecanoate (7.2 mM) or 0.8 ml of glycerol trioleate (3.6 mM) in 5% gum arabic solution, and deionized water to bring the total volume to 8.0 ml. At the indicated time intervals, 1.0 ml of the reaction mixture was withdrawn, and the activity was measured as described in the text. Glycerol tridecanoate: □—□, pH 5.4; ×—×, pH 8.8. Glycerol trioleate: ▲—▲, pH 5.4; ○—○, pH 8.8.

Effect of Incubation Time

The hydrolysis of both glycerol tridecanoate and glycerol trioleate was linear up to 60 min under the experimental conditions employed (Fig. 2). No significant difference was found between acid and alkaline lipases.

Effect of Substrate Concentration

Glycerol tridecanoate, trioleate, trilinoleate, and trilinolenate were used as substrates. Since it was found that glycerol trilinoleate and trilinolenate were hydrolyzed in a manner similar to that of glycerol trioleate, Fig. 3 shows only the results obtained with glycerol tridecanoate and glycerol trioleate. Fig. 3 demonstrates that maximum hydrolysis of medium-chain triglyceride (glycerol tridecanoate) occurred at a substrate concentration approximately twice that of long-chain triglyceride (glycerol trioleate). It is also noted that alkaline lipase was saturated with a much lower concentration of glycerol trioelate than was acid lipase. With both

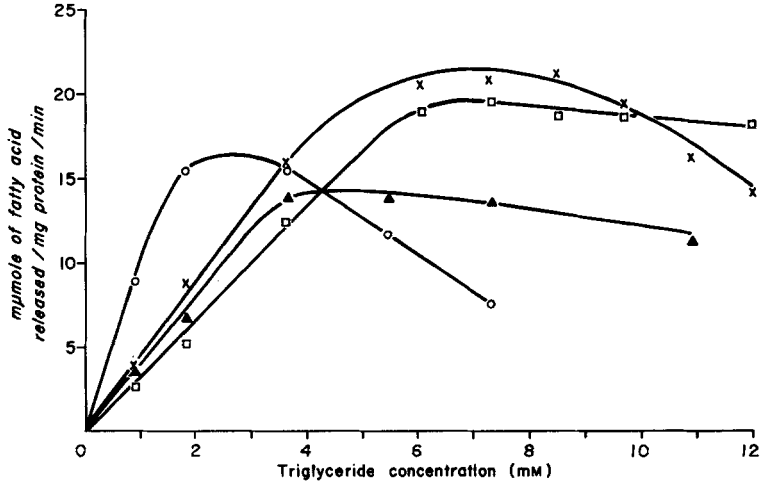

Fig. 3. Effect of substrate concentration on the hydrolysis of triglycerides. Stock solutions of glycerol tridecanoate and glycerol trioleate were made to 60 mM in 5% gum arabic solution. Different amounts of these solutions were used. The amount of total gum arabic solution was kept constant in each tube by the addition of aqueous 5% gum arabic. Activity was measured with 0.1 ml of aortic supernate for 30 min at 37°C under the assay conditions described in the text. Symbols are the same as in Fig. 2.

substrates any concentration beyond saturation began to inhibit alkaline lipase significantly, without much effect on acid lipase. The inhibition of maximal alkaline lipase acitvity was found to be nearly 40% at 12 mM glycerol tridecanoate and 50% at 7.2 mM glycerol trioleate.

Effect of Concentration of Aortic Supernatant

Under optimal conditions of pH, time, and substrate concentration the effect of various concentrations of aortic supernatant was studied with glycerol tridecanoate and glycerol trioleate (Fig. 4). Hydrolysis of glycerol tridecanoate by acid lipase was linear up to about 0.15 ml (or 0.366 mg of protein), and up to 0.25 ml (or 0.61 mg of protein) for alkaline lipase. With glycerol trioleate, linearity was observed over a greater range for both lipases. Unexpectedly, beyond these linear ranges both lipases were strongly inhibited with increasing concentration of supernatant. With 0.5 ml of supernatant, the alkaline lipase activity decreased to nearly one-half of the maximal activity, and the acid lipase activity decreased to one-fourth of its maximal activity; this was found with both substrates. It should be noted that inhibition of acid lipase began with a smaller volume of supernatant solution than did the alkaline lipase. Although the amounts of supernatant which started to inhibit both enzymes varied from preparation to preparation, this finding was consistent. Thus, these observations made us suspect the presence of inhibitors in the tissue preparations.

Effect of Boiled Aortic Supernatant

To demonstrate more clearly the presence of inhibitory substances, the aortic supernatant was boiled for 30 min, which resulted in a complete inactivation of both lipases. Various amounts of the boiled supernatant were then added to the incubation mixtures which contained the same untreated aortic supernatant or pancreatic lipase as did the enzyme source (Table 2). As can be seen, the patterns of inhibition are similar to those observed with various amounts of untreated aortic supernatant (Fig. 4), except that a slightly greater inhibition was observed with boiled supernatant than with an equal volume of the untreated supernatant. These results indicate that a certain concentration of inhibitors was necessary to produce the effect. Pancreatic lipase was also used to learn whether the inhibition could be demonstrated with other known lipases. The results clearly indicate that this was the case. As will be shown later (Fig. 5), pancreatic

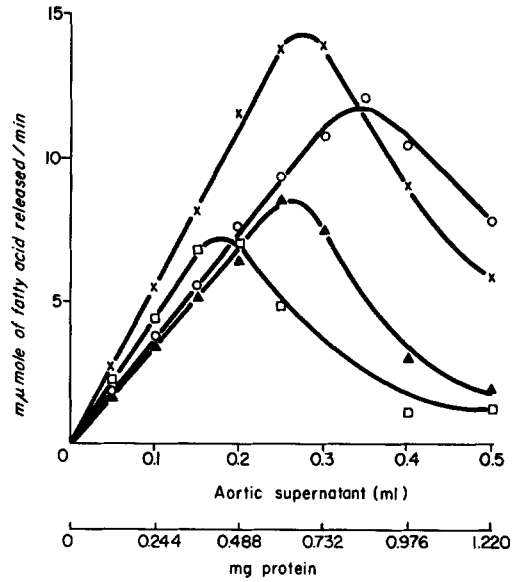

FIG. 4. Effect of concentrations of aortic supernatant on the hydrolysis of glycerol tridecanoate and trioleate. Various amounts of aortic supernatant (2.44 mg of protein per ml) were incubated for 30 min at 37°C under the assay conditions described in the test. Symbols are the same as in Fig. 2.

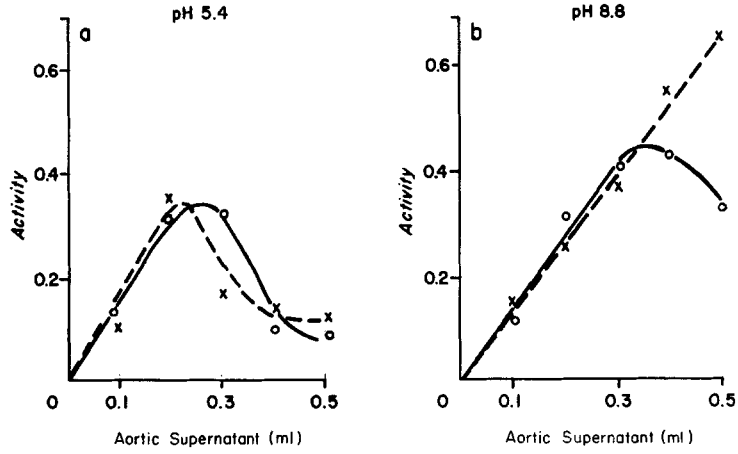

FIG. 5. Effect of dialysis on aortic lipase activities. Aortic supernatant (10 ml) was dialyzed against 0.003 M phosphate buffer (pH 7.0) for 24 hr at 0°C. After adjusting the volume to 10 ml with the same buffer, lipolytic activity was measured. Activity is expressed as absorbance at 440 nm. O—O, before dialysis; ×---×, after dialysis.

TABLE 2 EFFECT OF BOILED AORTIC SUPERNATANT ON THE HYDROLYSIS OF GLYCEROL TRIOLEATE

Addition of Boiled Aortic Supernatant (ml)	Percentage of Control Activity					
	Aortic Lipase				Pancreatic Lipase	
	pH 5.4		pH 8.8		pH 8.8	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Control	100	100	100	100	100	100
0.1	78	86	101	97	105	99
0.2	43	37	101	106	102	98
0.3	15	23	42	41	77	82
0.4	14	15	25	15	53	43
0.5	10	5	11	11	19	22

Aortic supernatant was boiled for 30 min, and the volume was adjusted to that of the original supernatant by addition of 0.01 M phosphate buffer (pH 7.0). Different amounts of boiled supernatant were added to the incubation mixture containing 0.1 ml of the same unboiled supernatant, and the samples were incubated for 30 min at 37°C. When pancreatic lipase was used, 0.1 ml of enzyme solution (500 mg of pancreatic lipase per ml of 0.01 M phosphate buffer [pH 7.0]) was incubated for 5 min at 37°C under the standard assay conditions described in the text. The results are expressed as percentage of the control activities (mμmole of oleic acid released per min) of aortic supernatant at pH 5.4 and 8.8, and of pancreatic lipase, i.e. 3.24, 3.99, and 98.5, respectively.

lipase appeared to be inhibited by the susbtance which inhibits alkaline lipase activity.

Removal of Alkaline Lipase Inhibitor

Aortic supernatant which contained acid and alkaline lipases was dialyzed against 0.003 M phosphate buffer (pH 7.0) at 0°C for 48 hr, and the activities of the supernatants before and after the dialysis were compared (Fig. 5). Both lipases remained active during dialysis; however, the inhibitory effect on alkaline lipase disappeared, although acid lipase appeared to be more inhibited after dialysis. When the same aortic supernatant was dialyzed against deionized water under the identical conditions, alkaline lipase retained its full activity accompanied by the disappearance of its inhibitory substance, whereas acid lipase activity was completely lost. The results, therefore, suggest that different inhibitory substances exerted effects on different lipases. One inhibitor appears to be heat-stable and nondialyzable, and it inhibits acid lipase; the other inhibitor, which is heat-stable and dialyzable, inhibits alkaline lipase. Pancreatic lipase, on the other hand, was not inhibited with dialyzed aortic supernatant (boiled) indicating that it was inhibited by the substance which inhibited alkaline lipase, but not acid lipase. However, since an extremely small amount of BSA (0.01 mg) was found to cause strong inhibition of acid lipase (Fig. 6), the possibility that the inhibition of acid lipase might merely be due to the presence of other proteins in the aortic supernatant has not been ruled out.

Substrate Specificity

Various triglyceride substrates dispersed in 5% gum arabic solution were hydrolyzed by aortic lipases (Table 3). The rate of hydrolysis of saturated triglycerides by both lipases decreased with increasing chain length of the fatty acid, and glycerol tristearate was not hydrolyzed under the conditions employed. Triglycerides esterified with unsaturated fatty acids of the same number of carbon atoms were hydrolyzed at similar rates. Glycerol triarachidonate was not hydrolyzed. Although the method employed is not particularly suitable for estimation of fatty acids with less than 10 carbon atoms (9), we have found that glycerol trihexanoate and glycerol trioctanoate appeared to be hydrolyzed at rates comparable or even higher than those of glycerol tridecanoate. Under the incubation conditions employed cholesteryl oleate was not hydrolyzed by the supernatants.

Inhibitors and Activators

The results of the effect of various compounds on the hydrolysis of glycerol trioleate by aortic lipases are given in Table 4 and Fig. 6. Ca^{++} activated both enzymes 20–25% (see Table 3). However, no compounds tested other than $CaCl_2$ appear to activate acid lipase; most of them, in fact, were found to be inhibitory. The inhibition of both lipases by NH_4^+ and plasma, the moderate inhibition by Na^+, the lack of stimulation by sodium heparin, and the strong inhibition of acid lipase by BSA without significant effect on alkaline lipase (Fig. 6) indicate that the lipases are not to be considered as lipoprotein lipase (12). Sulfhydryl reagents were more inhibitory on acid lipase than on alkaline lipase. Sodium fluoride, which is known to inhibit adipose tissue lipase (13) also inhibited aortic acid lipase. EDTA at a concentration of 1×10^{-3} M tended to inhibit acid lipase slightly but had no effect on alkaline lipase. 0.1 ml of

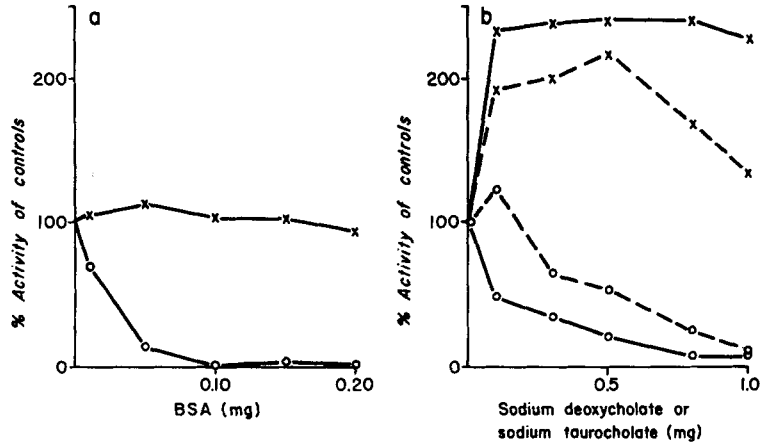

FIG. 6. Effect of BSA, sodium deoxycholate, and aosium taurocholate on aortic lipases. Aqueous solutions of BSA, sodium deoxycholate, and sodium taurocholate were prepared in concentrations of 1 mg/ml, 10 mg/ml, and 10 mg/ml, respectively. Different volumes of these solutions were incubated as described in Table 4. The values represent the averages of three separate experiments. BSA: O—O, pH 5.4; ×—×, pH 8.8. Sodium deoxycholate: O—O, pH 5.4; ×—×, pH 8.8. Sodium taurocholate: O---O, pH 5.4; ×---×, pH 8.8.

human plasma was definitely inhibitory for both lipases and retained the same effect even after dialysis. It is worth mentioning that the amounts of BSA (10 mg), sodium deoxycholate (10 mg), and sodium taurocholate (10 mg), which have been used by many investigators (14), were found to be completely inhibitory for both aortic lipases under the conditions employed. More detailed studies revealed, however, that aortic alkaline lipase was activated more than twofold with 0.5 mg of deoxycholate or taurocholate, whereas BSA (up to 0.2 mg) was without effect. On the other hand, amounts of deoxycholate, taurocholate, and BSA which optimally enhanced alkaline lipase activity, were inhibitory for acid lipase.

Products of Hydrolysis

The products of hydrolysis of glycerol trioleate by aortic lipases and pancreatic lipase were separated by thin-layer chromatography (Fig. 7). After 60 min of incubation at 37°C with aortic supernatant, and after 5 min of incubation with pancreatic lipase, the major products were found to be free oleic acid and glycerol 1,2-dioleate. Although a small amount of glycerol monooleate consistently appeared after the enzymatic cleavage, no accumulation of glycerol 1,3-dioleate could be detected under the conditions employed. An accumulation of glycerol 1,3-dioleate was observed when glycerol trioleate was incubated with lipase of rat liver lysosomes under

TABLE 3 HYDROLYSIS OF DIFFERENT TRIGLYCERIDES BY ACID AND ALKALINE LIPASE

Glycerol Ester	Physical State at 37°C*	Final Concentration	Relative Rates of Hydrolysis			
			pH 5.4		pH 8.8	
			Expt. 1	Expt. 2	Expt. 1	Expt. 2
		m_M				
Tridecanoate	L	7.2	129	143	146	151
Trilaurate	S	7.2	55	47	59	65
Trimyristate	S	7.2	10	24	47	47
Tripalmitate	S	7.2	23	28	24	18
Tristearate	S	3.6	0	1	0	0
Trioleate	L	3.6	100	100	100	100
Trilinoleate	L	3.6	73	69	101	106
"	L	5.4	109	101	—	—
Trilinolenate	L	3.6	126	134	104	107
Triarachidonate	S	3.6	0	2	4	2
Cholesteryl oleate	S	3.6	0	0	0	0

Various triglyceride substrates were prepared in 5% gum arabic solution as described in the text, and an optimal concentration of each substrate was used as indicated. Incubations were with 0.1 ml of aortic supernatant for 60 min at 37°C. The results are expressed as the percentage of the specific activity with glycerol trioleate as substrate. The specific activities at pH 5.4 and 8.8 were 13.5, and 16.2, respectively.

* S, solid; L, liquid.

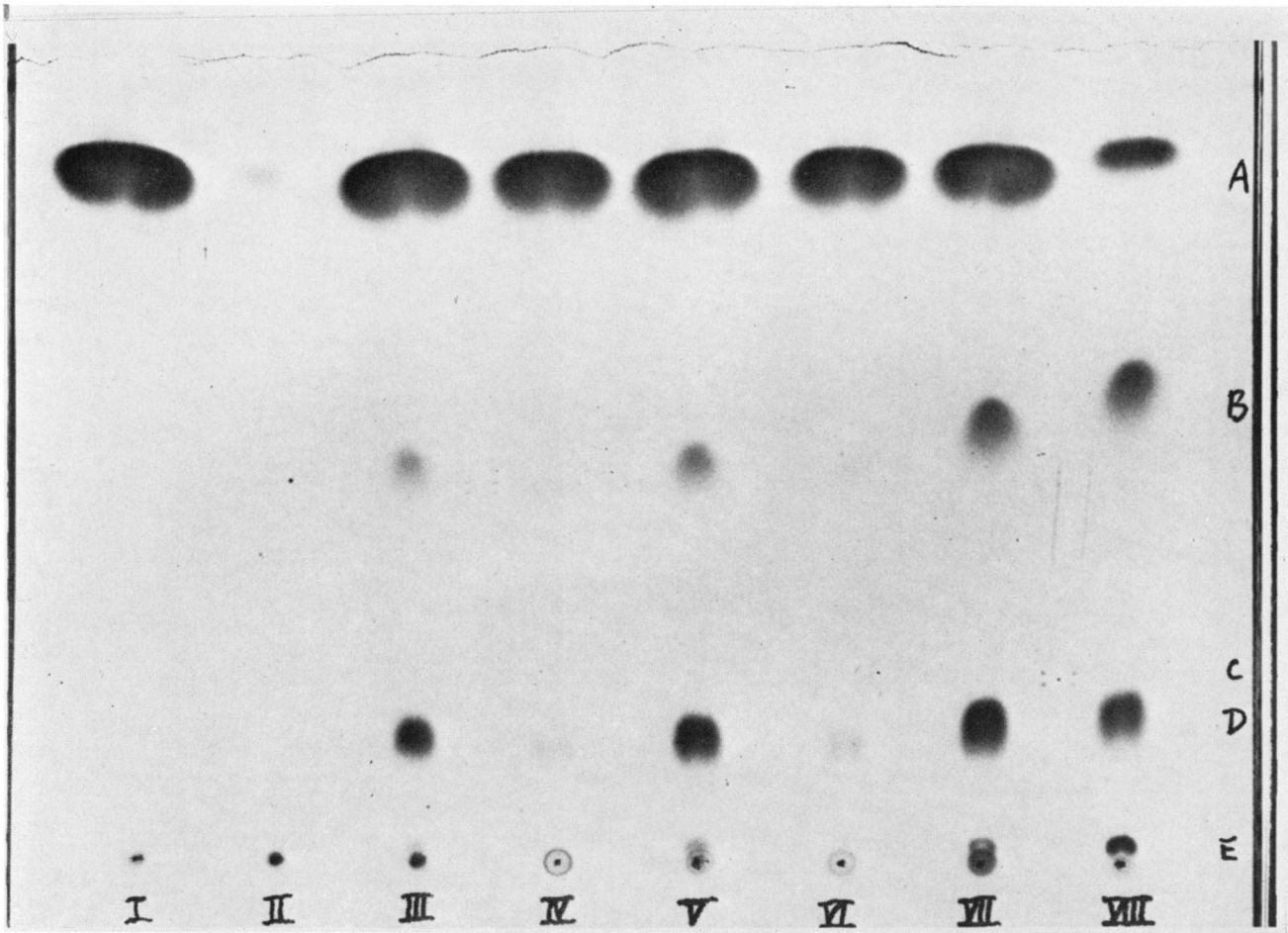

FIG. 7. Thin-layer chromatogram of products of hydrolysis of glycerol trioleate by aortic and pancreatic lipases. Incubations were for 60 min at 37°C with 0.1 ml of aortic supernatant or with pancreatic lipase as described in Table 2. The separation and other procedures were described in the text. Concentration of glycerol trioleate was 3.6 mM. *A*, glycerol trioleate; *B*, oleic acid; *C*, glycerol 1,3-dioleate; *D*, glycerol 1,2-dioleate; *E*, glycerol monooleate; I, substrate alone; II, aortic supernatant alone; III, pH 5.4; IV, pH 5.4 with 0.5 ml of boiled supernatant; V, pH 8.8; VI, 8.8 with 0.5 ml of boiled supernatant; VII, pH 8.8 with 0.5 mg of sodium deoxycholate; VIII, pH 8.8 with pancreatic lipase.

the similar incubation conditions,[1] this did not seem to be the result of isomerization of glycerol 1,2-dioleate to 1,3-dioleate. It was also noted that the addition of 0.5 mg of sodium deoxycholate to the incubation medium for aortic alkaline lipase, increased the accumulation of free oleic acid, glycerol 1,2-dioleate, and glycerol monooleate (15). Thus the results confirmed the earlier finding (Fig. 6) that 0.5 mg of sodium deoxycholate stimulated the hydrolysis approximately 2.4 times, presumably increasing the hydrolysis of both glycerol trioleate and glycerol 1,2-dioleate. As is shown, addition of boiled aortic supernatant almost completely inhibited the breakdown of glycerol trioleate by both lipases. The chromatogram shows both the purity of the substrate and also a minimal contamination of endogenous triglycerides (no more than 20 μg/0.1 ml) in the supernatant prepared from human aortas. It seems obvious from Fig. 3 that such a small amount of endogenous triglycerides could not significantly influence the lipolysis. No differences in mode of action between the acid and alkaline lipases were noted.

DISCUSSION

Lipase with an alkaline pH optimum has been reported by others to be present in aortic tissue homogenates. Patelski et al. (5) prepared an aqueous extract of lipase from acetone-butanol powder of pig aortas and from fresh aortas of rabbit and rat. They reported that the enzyme from the aortas of the pig, rabbit, and rat had a pH optimum of 8.3 with specific activities (mμmole of fatty acid released per mg of protein per min) of approximately 50, 148, and 627, respectively. Maier and Haimovici (6, 7) prepared aortic homogenates of aortas of man, rabbit, and dog in iced water, after first rinsing the tissue with saline. They failed, however, to demonstrate lipase activity in these homogenates. Adams et al.

[1] Personal observation.

TABLE 4 EFFECT OF VARIOUS COMPOUNDS ON THE HYDROLYSIS OF GLYCEROL TRIOLEATE BY AORTIC LIPASES

Addition	Final Concentration	Percentage of Control Activity	
		pH 5.4	pH 8.8
	M		
None (control)	—	100	100
$CaCl_2$	1×10^{-3}	123(113–130)*	126(121–132)
$(NH_4)_2SO_4$	2×10^{-1}	27(21–33)	70(63–75)
NaCl	1×10^{-1}	71(65–76)	90(86–95)
NaF	1×10^{-3}	46(40–49)	98(96–102)
EDTA	1×10^{-3}	80(74–88)	103(98–107)
Iodoacetate	5×10^{-3}	77(71–84)	99(96–104)
"	5×10^{-2}	61(50–70)	74(72–78)
p-Chloromercuribenzoate	1×10^{-5}	72(64–74)	91(83–97)
N-Ethyl maleinide	1×10^{-3}	79(79–80)	92(90–95)
Undialyzed human plasma	0.01 ml	0	105(99–113)
"	0.10 ml	0	0
Dialyzed human plasma	0.10 ml	0	0
Sodium heparin	10 μg	101(94–105)	98(95–104)

The concentrations of the various compounds were such that 0.2 ml of each added to the incubation mixture (total volume of 1.0 ml) gave the final concentrations as indicated. Incubations were for 60 min at 37°C with 0.1 ml of aortic supernatant and with 3.6 mM glycerol trioleate under the assay conditions described in the text. The results are expressed as percentages of the activities of the controls as shown in Table 2. The values represent the averages of three separate experiments.

* The range of values of three experiments is shown in the parentheses.

(4) recently demonstrated esterase and lipase activities in human aortic homogenates prepared either in distilled water or in 0.15 M Krebs–Ringer phosphate buffer (pH 7.4). However, since their results were expressed only in terms of relative values to their controls, no direct comparison of their data to ours can be made. It is interesting that their previous report (3) as well as the recent one (4) stated that "98% of lipase activity was soluble and lost from unfixed sections of human aorta during 'dummy' histochemical incubation."

We confirmed their finding that more than 80% of alkaline lipase activity could be extracted in supernatant (I) from homogenates prepared in a hypotonic buffer medium (Table 1). This solubility of the enzyme, however, raises the question of whether the small but inevitable contamination of blood might have contributed to the lipase activity (16) reported here in human aortas. We tested this possibility with fresh samples of whole blood, plasma, and serum obtained from male and female human subjects or with homogenates of whole blood (fresh or from autopsied subjects within 12–24 hr of death) prepared in the same manner as those of aortas. None of these samples showed any significant lipase activity under the assay conditions employed. Also the observation (Table 1) that only 23% of the lipase activity was released into a sucrose medium after a single homogenization as opposed to 82% in buffer medium, appears to deny this possibility. At the same time, the aortic alkaline lipase inhibitor does not appear to be the same inhibitor with glycoprotein nature found in plasma by Hollett and Meng (17).

Acid lipase, on the other hand, seems not to have been reported in aortic tissue. Our evidence which strongly suggests the existence of this enzyme may be summarized as follows. (*a*) Two pH optima were found in aortic tissue extract. (b) Acid lipase activity was not observed when the aortas were homogenized in deionized water, whereas the alkaline lipase activity was unaffected by the homogenizing medium. (*c*) The buffered preparations which contained acid and alkaline lipase activity, when dialyzed against deionized water at 0°–4°C, lost the acid lipase activity within 48 hr without loss of the alkaline lipase activity. However, both acid and alkaline activities were retained when the preparation was dialyzed against dilute buffer under the same conditions. (*d*) There was a differential release of the two activities into a supernatant fraction (34,800 *g*, 120 min). (*e*) There were different responses of the two lipases to various agents. The effect of deionized water on acid lipase was so unusual that this fact alone might explain the failure of many investigators to demonstrate the existence of this enzyme in aortas. In this connection it might also have been due to this effect of water that Eisenberg, Stein, and Stein (18) found only one pH optimum (pH 7.9–8.6) for phospholipases in human and rat aortic homogenates prepared in ice-cold distilled water. Although the physiological significance of acid lipase is yet to be elucidated, its presence in lysosomes of rat liver and kidney (14), in rabbit polymorphonuclear leukocytes (19, 20), and in peritoneal macrophages (21), has clearly been demonstrated. It is interesting to note that Patrick and Lake (22) recently reported that the livers and spleens of patients with

Wolmans' disease (in which there is an accumulation of triglycerides) lacked acid lipase activity, which is normally found in these organs.

The results obtained for the effect of substrate concentration (Fig. 3) clearly showed differences between two substrates. If, however, the concentration is plotted by weight rather than by molarity, differences in optimal concentrations and substrate inhibition then do not appear to be significant. For instance, glycerol tridecanoate (mol wt 554.9) at a concentration of 12.0 mM and trioleate (mol wt 885.4) at a concentration of 7.2 mM are approximately equivalent to concentrations of 6.66 mg/ml and 6.36 mg/ml, respectively.

Both lipases of aortic wall could be classified as true lipases (23) in that they catalyzed the hydrolysis of different triglycerides at fairly similar rates when the triglycerides were liquid at the incubation temperature (37°C). Therefore, the data presented in Table 3 should be interpreted as a reflection of physical state of substrates rather than chemical specificities (23). Thus, lipase activity could be measured with either medium-chain triglycerides or with long-chain triglyceride as substrate. However, this does not eliminate a possibility that the 40–50% higher hydrolysis rate, obtained with glycerol tridecanoate rather than with those of long-chain triglycerides which are liquid at 37°C, might reflect some participation of nonspecific aortic esterase known to exist in that tissue (4, 6, 7). Although it is not clear why the acid lipase is so strongly inhibited by a low concentration of BSA or bile salts as well as by many other compounds which are known to stimulate lipase activity, it is interesting that acid lipase was found to be more resistant to the substrate inhibition than alkaline lipase in vitro (Fig. 3). This observation leads us to speculate that when lipids start to deposit in the arteries and exceed certain amounts, the mechanism to dispose of them might have to rely upon the activity of acid lipase. Similar substrate inhibition has been reported by Johnson and Moskowitz (24) who found that cholesteryl esterase from pig aorta was strongly inhibited by high concentrations of cholesteryl oleate or linoleate.

It was a surprising observation that the aortic supernatant itself appeared to contain substances which strongly inhibit acid and alkaline lipases. The possibility that the inhibition observed was due to the method employed was ruled out by the following observations: (*a*) known amounts of decanoic or oleic acid (0–1400 mμmole), which were added to the incubation mixture with 0–0.5 ml of aortic supernatant, were completely recovered, and (*b*) the addition of various amounts of aortic supernatant or BSA to the reaction mixture at the end of the incubation period, did not alter the recovery of the released fatty acid. The inhibitory effect was found in homogenates as well as in supernatant fractions prepared from male human aortas, indicating its presence in aortic tissue. As has been shown in Fig. 5, the inhibitor of alkaline lipase, which is easily dialyzable, seems to resemble a lipase inhibitor in human urine reported by Nachlas and Blackburn (25) in that it also inhibits pancreatic lipase in vitro. On the other hand, the inhibitor of acid lipase resembles the lipase inhibitor found in plasma by Hollett and Meng (17) in that it is heat-stable and nondialyzable. The chemical nature of the inhibitory substances and their mode(s) of action are unknown at present. However, the inhibitory substance of acid lipase does not seem to be fatty acid because the addition of stearic acid (up to 500 mμmole) to the incubation mixture did not affect the lipolysis under the assay conditions employed. We have not been able to determine the exact localization of the two lipases and of the inhibitors owing to the difficulties encountered in subcellular fractionation of aorta (26, 27). However, the properties of the two lipases described in this paper suggest that the acid lipase is lysosomal, while the alkaline lipase is microsomal (14, 28).

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References

1. Kritchevsky, D. 1967. *In* Atherosclerotic Vascular Disease. A. N. Brest and J. H. Moyer, editors. Meredith Publishing Co., New York. 1–7.
2. Zemplenyi, T. 1964. *In* Advances in Lipid Research. R. Paoletti and D. Kritchevsky, editors. Academic Press Inc., New York. **2:** 235–293.
3. Adams, C. W. M., Y. H. Abdulla, O. B. Bayliss, F. R. Mahler, and M. A. Root. 1968. *Progr. Biochem. Pharmacol.* **4:** 218.
4. Adams, C. W. M., O. B. Bayliss, Y. H. Abdulla, F. R. Mahler, and M. A. Root. 1969. *J. Atheroscler. Res.* **9:** 87.
5. Patelski, J., Z. Waligora, S. Szulc, D. E. Bowyer, A. N. Howard, and G. A. Gresham. 1968. *Progr. Biochem. Pharmacol.* **4:** 287.
6. Maier, N., and H. Haimovici. 1965. *Proc. Soc. Exp. Biol. Med.* **118:** 258.
7. Maier, N., and H. Haimovici. 1965. *Circ. Res.* **17:** 178.
8. Hayase, K., and A. L. Tappel. 1969. *J. Biol. Chem.* **244:** 2269.
9. Duncombe, W. G. 1963. *Biochem. J.* **88:** 7.
10. Miller, G. L. 1959. *Anal. Chem.* **31:** 964.
11. Marchis-Mouren, G., L. Sarda, and P. Desnuelle. 1959. *Arch. Biochem. Biophys.* **83:** 309.
12. Korn, E. D. 1955. *J. Biol. Chem.* **215:** 1.
13. Mann, J. T., III, and S. B. Tove. 1966. *J. Biol. Chem.* **241:** 3595.
14. Mahadevan, S., and A. L. Tappel. 1968. *J. Biol. Chem.* **243:** 2849.

15. Kates, M. 1960. *In* Lipid & Metabolism. K. Bloch, editor. John Wiley & Sons, Inc., New York. 165–237.
16. Searcy, R. L., S. Hayashi, and J. E. Berk. 1968. *Clin. Biochem.* **1:** 311.
17. Hollett, C., and H. C. Meng. 1957. *Fed. Proc.* **16:** 60.
18. Eisenberg, S., Y. Stein, and O. Stein. 1968. *Biochim. Biophys. Acta.* **164:** 205.
19. Elsbach, P., and M. A. Rizack. 1963. *Amer. J. Physiol.* **205:** 1154.
20. Elsbach, P., and H. J. Kayden. 1965. *Amer. J. Physiol.* **209:** 765.
21. Cohn, Z. A., and E. Wiener. 1963. *J. Exp. Med.* **118:** 991.
22. Patrick, A. D., and B. D. Lake. 1969. *Biochem. J.* **112:** 29p.
23. Desnuelle, P., and P. Savary. 1963. *J. Lipid Res.* **4:** 369.
24. Johnson, L. D., and M. S. Moskowitz. 1968. *Circulation.* **38:** VI-II.
25. Nachlas, M. M., and R. Blackburn. 1958. *J. Biol. Chem.* **230:** 1051.
26. Whereat, A. F. 1966. *J. Lipid Res.* **7:** 671.
27. Stein, Y., O. Stein, and B. Shapiro. 1963. *Biochim. Biophys. Acta.* **70:** 33.
28. Hayase, K., and A. L. Tappel. 1970. *J. Biol. Chem.* **245:** 169.