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The lipoprotein lipase of cow's milk

EDWARD D. KORN

Laboratory of Cellular Physiology and Metabolism,
National Heart Institute,
Bethesda 14, Maryland

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SUMMARY

Cow's milk has been found to contain a lipoprotein lipase similar to those previously demonstrated in post-heparin plasma, adipose tissue, and heart. Upon separation of the milk, most of the enzyme was found in the skim milk, although some was present in the cream from which it could be isolated with a relatively high specific activity. The triglycerides of cream were not hydrolyzed by the lipoprotein lipase, and it is supposed that the enzyme does not function in milk but rather in the lactating mammary gland.

Several years ago, Quigley *et al.* (1) briefly reported evidence indicating the presence of a lipoprotein lipase in particles (the milk "microsomes" of Morton (2)) isolated from the buttermilk from churned washed cream. As part of our continuing interest in lipoprotein lipase, it was decided to reinvestigate this finding. We have confirmed that particles from cream do contain lipoprotein lipase but find that most of the enzyme is present not in the cream but in the skim-milk fraction. The properties of milk lipoprotein lipase are very similar to those of lipoprotein lipase prepared from adipose tissue, heart, and post-heparin plasma.

METHODS

Unpasteurized skim milk and cream (40% fat) were obtained from a commercial dairy. Chylomicrons were isolated from chyle collected from the cannulated thoracic duct of a rat fed olive oil (3). The coconut oil emulsion used was Ediol.[1] Activated coconut oil refers to the lipoprotein-triglyceride complex formed when Ediol and serum are incubated together (4). The activated coconut oil was isolated centrifugally and washed repeatedly by centrifuging through 0.15 M NaCl. Raw or autoclaved cream were occasionally used as substrate.

[1] Obtained from Schenley Laboratories, Inc., Lawrenceburg, Indiana. Composition: coconut oil, 50%; sucrose, 12.5%; glyceryl monostearate, 1.5%; polyoxyethylene sorbital monostearate, 2.0%.

In the usual assay, the formation of formaldehydogenic products was followed as previously described (5). Unless otherwise specified, the incubation tube contained bovine serum albumin, 10%, pH 8.5; ammonium sulfate, 0.05 M; enzyme; and substrate in a total volume of 1 ml. Tubes were incubated at 37°, and aliquots of 0.05 ml were removed at 0, 30, and 60 minutes. After oxidation with periodate, formaldehyde was determined colorimetrically with chromotropic acid. The results are reported as increase in absorbancy at 570 mμ. An absorbancy of 0.025 corresponds to 0.01 μmole of formaldehyde (equivalent to 0.01 μmole of α-monoglyceride or 0.005 μmole of glycerol).

In those experiments in which the formation of free fatty acids was also followed, the incubation volume was 10 ml. Aliquots of 2 ml were removed after 0, 30, and 60 minutes of incubation, extracted according to Dole (6) (except for the use of twice as much 1 N H_2SO_4), and titrated according to Gordon (7).

Protein was determined by the method of Lowry, *et al.* (8).

RESULTS

Distribution of Enzyme Between Skim Milk and Cream. One gallon of whole milk was separated into skim milk and cream (about 50% fat). The cream was cooled to 2° and churned by mechanical stirring, and the buttermilk was separated by filtering through cheesecloth. The buttermilk was centrifuged at 25,000 x *g* for 1 hour to give a reddish-brown gelatinous

precipitate (milk "microsomes") and a clear supernatant fraction. Aliquots of the whole milk, skim milk, buttermilk, and a suspension of the particulate fraction obtained from the buttermilk were assayed for lipoprotein lipase activity. Two-thirds of the enzyme was found in the skim milk. Of that present in the cream, most was accounted for in the buttermilk and less than 10% was found in the particulate fraction. It is this particulate fraction that was examined by Quigley *et al.* (1).

Solubilization of the Lipoprotein Lipase of Skim Milk. When skim milk was centrifuged for 1 hour at 114,000 x *g*, two-thirds of the total protein and two-thirds of the lipoprotein lipase were found in the gelatinous precipitate. When the milk was first dialyzed against water, 0.1 M NaCl, or 0.02 M trisodium citrate, all of the lipoprotein lipase and all but a small percentage of the protein remained in solution after centrifugation for 2 hours at 114,000 x *g*. Such preparations could be stored at 2° for approximately 2 weeks with little or no loss of activity. In the experiments described below, skim milk dialyzed against 0.1 M NaCl for 18 hours was used.

Preparation of Cream Particles and Extraction of Lipoprotein Lipase. One gallon of raw cream was diluted to approximately 32 liters with distilled water at 30°. The diluted cream was put through a cream separator adjusted so that the cream was recovered in its original volume. This washing procedure was repeated twice more. The cream was then cooled to 2° and churned, and the particles were isolated as described before. This material was stable for at least several weeks when stored at −15°. The lipoprotein lipase could not be extracted from the particles by water, 0.1 M NaCl, or 0.02 M trisodium citrate. It was neither soluble in nor rendered more soluble by 5%, 10%, or 20% butanol. Approximately 25% of the enzyme could be extracted from acetone-dried particles by 0.025 M ammonia. Serum extracted one-third of the enzyme from both fresh and frozen particles. Approximately 60% of the lipoprotein lipase was extracted from either fresh or frozen particles with 0.025 M ammonia, the method of choice. The lipoprotein lipase in the ammonia extracts remained in solution when centrifuged at 114,000 x *g* for 1 hour. Such preparations were very unstable at 2°, losing at least 60% of their activity overnight.

Substrate Specificity. Skim milk was found to catalyze the hydrolysis of the triglycerides of chylomicrons, activated coconut oil, or a mixture of coconut oil and serum (Fig. 1). The rates of hydrolysis as a function of substrate concentration were very similar for all three substrates. No formaldehydogenic

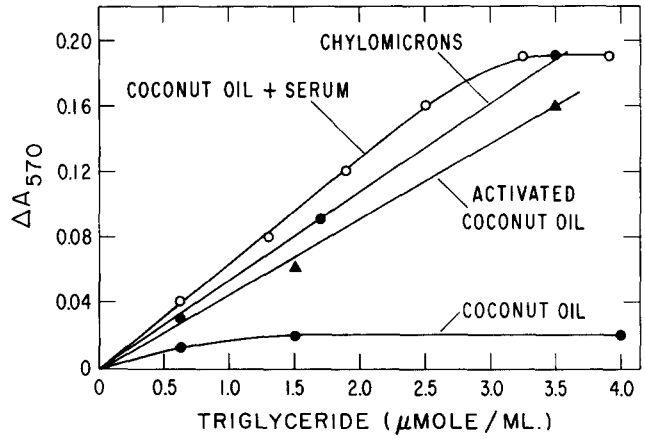

FIG. 1. Substrate specificity of lipoprotein lipase of skim milk. All vessels contained 0.05 ml of skim milk and were incubated for 1 hour.

TABLE 1. SUBSTRATE SPECIFICITY OF LIPOPROTEIN LIPASE OF SKIM MILK

Substrate	ΔA_{570}/hr
Coconut oil	0.02
Coconut oil + serum, 0.005 ml	0.13
Coconut oil + serum, 0.01 ml	0.20
Cream	0.00
Cream + serum, 0.01 ml	0.08

All tubes contained 0.05 ml of skim milk and 5 μmoles of triglyceride. Serum alone does not catalyze the hydrolysis of either coconut oil or cream.

products were formed when coconut oil or cream was incubated with skim milk in the absence of serum. Addition of serum to the incubation mixture, however, activated the hydrolysis of both substrates (Table 1), although cream was still a poor substrate compared to chylomicrons. Raw and autoclaved cream behaved similarly as substrates. The rate of hydrolysis was directly proportional to the concentration of enzyme (skim milk) and to the time of incubation (Fig. 2). All of these results were confirmed by the determination of free fatty acids. The lipoprotein lipase of skim milk had a specific activity (μmoles HCHO/min/mg protein) of about 0.025.

The lipoprotein lipase of the ammonia extract of the particles prepared from washed cream had the same substrate specificity as the enzyme in skim milk (Table 2). Several preparations had a specific activity of from 0.7 to 1.7 μmoles of HCHO/min/mg protein. This is approximately 10 to 20 times the specific activity of the lipoprotein lipase in ammoniacal extracts of acetone powders of chicken adipose tissue, the source with the next highest specific activity.

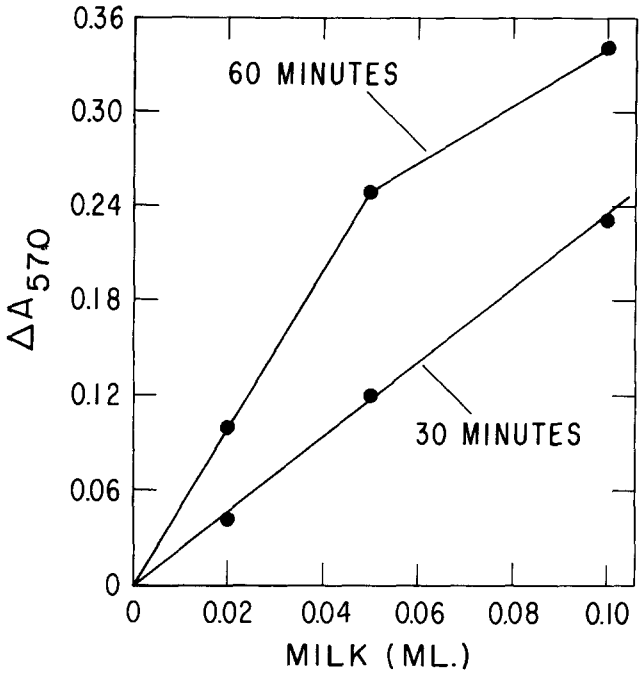

FIG. 2. Activity as a function of milk concentration. Substrate was coconut oil plus serum, 5.0 μmoles of triglyceride/ml.

Activation by Serum. The ability of serum to activate coconut oil for hydrolysis by lipoprotein lipase is due to its lipoproteins (4). All of the activity resides in the supernatant fraction obtained when serum is centrifuged for 16 hours at 114,000 x *g* at a salt density of 1.21. Previously, it had been found that all of the activity resided in the α-lipoproteins (4), but we now find that β-lipoproteins are also active. Both the lipoproteins that float when serum is centrifuged at a salt density of 1.063 and serum that contains no α-lipoproteins (both chemically and immunologically) are active. β-Lipoproteins also activate coconut oil for hydrolysis by lipoprotein lipase of adipose tissue (9). No explanation is apparent for the discrepancy between these and the earlier results.

Requirement for a Fatty Acid Acceptor. Another of the distinguishing characteristics of the lipoprotein lipases of adipose tissue, heart, and post-heparin plasma is the necessity for a fatty acid acceptor. That this is also true for the enzyme in skim milk is shown by the effect of increasing albumin concentration on the rate of hydrolysis (Fig. 3). Calcium chloride at a concentration of 0.02 M was about one-third as effective as albumin but was inhibitory at higher concentrations. The lipoprotein lipase of cream particles also shows an absolute dependence on the presence of fatty acid acceptors.

Inhibitors. As with lipoprotein lipase from other sources, the enzyme in milk is inactivated at relatively low ionic strengths (Table 3). It appears that the sensitivity to ionic strength may vary somewhat with different substrates and that the preparation from cream may be more sensitive than that from skim milk, but these matters have not been further examined. Polycations inhibit the lipoprotein lipase of milk (Table 4) as they do lipoprotein lipases from other sources.

Formation of Free Fatty Acids. In a series of experiments, chylomicrons and coconut oil plus serum were incubated with skim milk, and the production of free fatty acids (FFA) and formaldehydogenic compounds followed simultaneously. The results are summarized in Table 5. If the only products of hydrolysis were free fatty acids and glycerol, the FFA/HCHO ratio would be 1.5. Higher ratios must be due to the presence of partial glycerides that are less (α-monoglycerides, FFA/HCHO=2.0) or not at all (β-monoglycerides, diglycerides) formaldehydogenic.

When chylomicrons were the substrate, the ratio FFA/HCHO averaged 1.8. In previous studies, a value of 1.6 ± 0.3 was found by using lipoprotein lipase from hen adipose tissue, and a much higher value, 4.2 ± 1.4 was found for pancreatic lipase (3). A value as low as 1.8 can be obtained only if an ap-

TABLE 2. SUBSTRATE SPECIFICITY OF THE LIPOPROTEIN LIPASE OF CREAM PARTICLES

Substrate	ΔA_{570}/hr
Coconut oil	0.04
Coconut oil + serum	0.22
Activated coconut oil	0.21

All tubes contained 5 μmoles of substrate and 0.02 ml of the ammonia extract of washed cream particles.

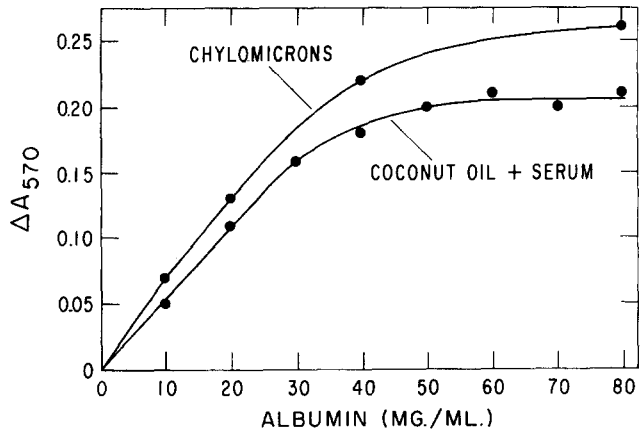

FIG. 3. Requirement for a fatty acid acceptor. All vessels contained 0.05 ml of dialyzed skim milk and 5 μmoles of substrate.

TABLE 3. INHIBITION OF LIPOPROTEIN LIPASE BY NaCl

Enzyme	Substrate	NaCl Concentration	ΔA_{570}/hr
		M	
Skim milk	Chylomicrons	0	0.24
		0.25	0.12
		0.5	0.06
		1.0	0.00
	Activated coconut oil	0	0.23
		0.5	0.13
		1.0	0.01
Cream particle extract	Activated coconut oil	0	0.21
		0.2	0.11
		0.4	0.02

All tubes contained 5 μmoles of triglyceride. 0.05 ml of skim milk or 0.02 ml of the ammoniacal extract of washed cream particles were used as the source of enzyme.

preciable percentage of the free fatty acids are derived from the β-position.

When coconut oil plus serum was the substrate, however, the observed FFA/HCHO ratio was much higher. In control experiments using coconut oil alone as substrate, the production of free fatty acids was never more than 10% of that found with coconut oil plus serum. It is apparent, then, that the percentage of free fatty acids derived from the β-position was less in these experiments than in those in which chylomicrons were the substrate. This is due not to a decrease in the rate of hydrolysis of β-esters (the rates of formation of formaldehydogenic products are the same for the two substrates (Fig. 1)) but to an increase in the rate of hydrolysis of α-esters.

DISCUSSION

The experimental data reported in this paper would seem to establish unequivocally the presence in milk of a lipoprotein lipase with properties similar to, if not identical with, the lipoprotein lipases of post-heparin plasma, adipose tissue, and heart. There are many studies in the literature, the most recent of which are those of Jensen *et al.* (12) and Forster *et al.* (13), on the esterases of milk. Forster and his collaborators have distinguished three such enzymes: A-esterase, which hydrolyzes only aromatic esters; B-esterase, which hydrolyzes aliphatic and aromatic esters but not choline esters; and C-esterase, which is a specific choline esterase. Of these, the only one that must be considered here is the B-esterase.

TABLE 4. INHIBITION OF LIPOPROTEIN LIPASE BY POLYLYSINE

Polylysine·HBr	ΔA_{570}/hr
mg	
0	0.26
0.2	0.26
0.3	0.13
0.5	0.10
0.75	0.04

All tubes contained 0.05 ml of skim milk, 0.02 ml of serum and coconut oil (5 μmoles of triglyceride).

The substrate usually employed in studies of B-esterase is tributyrin (not a substrate for lipoprotein lipase (14)). With tributyrin as substrate, the rate of hydrolysis is approximately 20 μEq/hr/ml of milk (13). Triglycerides of long-chain fatty acids are also hydrolyzed (12) but at a diminished rate that has never been reported. In the experiments reported in this paper, the rate of hydrolysis of activated coconut oil emulsions was about 100 μEq/hr/ml of milk. The rate of hydrolysis of coconut oil without activation was less than 10 μEq/hr/ml of milk. From these calculations and the data on substrate specificity and inhibitors, it would seem necessary to conclude that, in addition to esterases A, B, and C, milk contains a lipoprotein lipase.

In a previous publication (3), it was reported that, in contrast to pancreatic lipase, lipoprotein lipase from

TABLE 5. STOICHIOMETRY OF THE HYDROLYSIS CATALYZED BY LIPOPROTEIN LIPASE

Experiment*	Substrate	Incubation time	Ester bonds hydrolyzed†		FFA/HCHO†
		min	*μEq/ml*	%	*μEq/mole*
1	Chylomicrons	30	0.92	6	1.6
		60	1.93	13	1.8
2	Chylomicrons	30	1.96	13	2.1
		60	3.10	20	1.8
3	Chylomicrons	30	0.99	22	2.2
		60	1.86	42	1.7
4	Chylomicrons	30	1.58	35	1.7
		60	3.05	67	1.6
5	Coconut oil	30	2.62	17	3.75
	+ serum	60	4.10	27	3.02
6	Coconut oil	30	2.56	17	3.7
	+ serum	60	4.83	32	3.7
7	Coconut oil	30	2.54	17	3.8
	+ serum	60	5.01	33	3.0
8	Coconut oil	30	2.69	18	4.6
	+ serum	60	4.13	27	3.9

* Substrate concentration: 5 μmoles of triglyceride/ml in tubes 1, 2, 5, 6, 7, 8. 1.5 μmoles of triglyceride/ml in tubes 3 and 4. Enzyme concentration: 0.05 ml of milk/ml in tubes 1, 3, 5, 6, 7, 8. 0.10 ml of milk/ml in tubes 2 and 4.

† The values obtained with coconut oil may be 6% to 9% low because 70% of the fatty acids are lauric acid (10) of which only 90% would have been extracted and titrated (11).

hen adipose tissue catalyzes the hydrolysis of all three ester bonds of the triglycerides of chylomicrons at essentially identical rates. The differences in the FFA/HCHO ratios found in the present experiments with chylomicrons and activated coconut oil as substrates indicate that the relative positional specificity of a lipase may be dependent on the physical and/or chemical nature of the substrate as well as on the enzyme.

Lipoprotein lipase, although present in high concentration in milk, would appear not to function there. The lipase is unable to hydrolyze the triglycerides of cream and would be destroyed in the digestive tract in any case. A more likely supposition is that the lipase functions in the mammary gland and that its appearance in milk is a reflection of the rupturing of cells that occurs during the secretion of milk. Indirect evidence has led to the hypothesis that the lipoprotein lipases of adipose tissue and heart are involved in the uptake into the tissue of chylomicron triglycerides (12). Most of the long-chain fatty acids of cream are not synthesized in the mammary gland (13). The lipoprotein lipase of the mammary gland might be involved in the removal of glycerides from the plasma. On the other hand, the enzyme might be supposed to have another metabolic role, transfer or otherwise, within the cell. Studies have been initiated on the mammary gland of lactating guinea pigs with which it is hoped to answer some of these questions.

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