Isolation and characterization of four lipolytic preparations from rat skeletal muscle

DONALD P. WALLACH

Research Laboratories, The Upjohn Company, Kalamazoo, Michigan 49001

ABSTRACT Four different lipolytic preparations have been isolated from rat skeletal muscle. Two of these, provisionally designated monopalmitin lipase (MPL) and monomyristin lipase (MML), are associated with insoluble cellular particulate fractions. The other two enzymes, provisionally designated tricaproin lipase (TCL) and monolaurin lipase (MLL), are found in the high-speed supernatant fraction. Taken as a group, these enzymes are capable of hydrolyzing short-chain triglycerides (acyl moieties of C_3 to C_8) and monoglycerides of lauric, myristic, palmitic, stearic, and oleic acids. All of these enzymes have a serine residue at or near the catalytic site as they are strongly inhibited by diisopropyl fluorophosphate. The two particulate preparations contain a sulfhydryl group and are sensitive to *p*-chloromercuribenzoate and *N*-ethylmaleimide, while the soluble preparations are not.

The MLL, MML, and MPL preparations all have alkaline pH optima, while the TCL preparation has an acidic optimum. Buffer type is important: some buffer compounds completely inhibited one or more preparations.

Of the soluble enzymes, MLL withstood heating to 60°C, while TCL is completely inactivated at this temperature. Of the particulate preparations, only MML was stable to lyophilization. It is concluded that there are at least four lipolytic enzymes in rat skeletal muscle. The possible significance of the presence of these enzymes in muscle is discussed.

KEY WORDS rat skeletal muscle · monoglycerides · triglycerides · lipolytic enzymes · substrate specificity · inhibitors · isolation techniques · enzyme stability · optimal conditions

In recent years, an extensive literature which demonstrates that skeletal muscle derives a very important, if not major part of energy requirements from lipid substrates has accumulated. As a survey of all the pertinent literature is beyond the scope of this report, the reader is referred to several extensive reviews, notably those of Fritz (1), Masoro (2), and Drummond and Black (3) (this last-named review is especially interesting for its survey of species other than mammals). In addition, work (4) on rat latissimus dorsi and diaphragm, and more recently on rat diaphragm (5, 6), in man (7, 8), and in dogs (9, 10) provides conclusive evidence that skeletal muscle does utilize considerable amounts of lipid as fuel.

In the light of this extensive work on lipid utilization by skeletal muscle, it is surprising that little attention has been paid to the isolation and characterization of the lipolytic enzymes of skeletal muscle. George and Talesara (11, 12) and George and Vallyathan (13) have demonstrated the presence of lipolytic enzymes in the breast muscle of pigeons (*Columba livia*) and in starlings (*Sturnus roseus*), but they made the assumption (12) that one lipase was present in both the high-speed supernatant fraction and the particulates. They reported that neither tributyrin nor triolein was oxidized by either muscle mitochondria or whole homogenate.

There appear to be no similar reports dealing with the isolation and characterization of lipases from mammalian skeletal muscle. As hydrolysis of glyceride esters is an essential first step to utilization, exploration of the lipolytic mechanism of muscle is important in elucidating patterns of lipid metabolism by this tissue.

MATERIALS AND SOURCES

Sprague-Dawley rats (Upjohn strain) weighing 300–450 g were used throughout this study and were fed on Purina Laboratory Chow from Ralston Purina Co., St.

Abbreviations and terminology: MLL, monolaurin lipase; MML, monomyristin lipase; MPL, monopalmitin lipase; TCL, tricaproin lipase; DFP, diisopropyl fluorophosphate; PCMB, *p*-chloromercuribenzoate; BCME, butyl carbamic acid methyl ester; Tris, tris (hydroxymethyl) amino methane; IPA, 1-imino (1-piperidino) acetonitrile.

Louis, Mo. Monobutyrin, tributyrin, and hexadecyltrimethyl ammonium bromide were purchased from Eastman Organic Chemicals, Rochester, N.Y.; monolaurin and monomyristin from C. P. Hall Co., Chicago, Ill.; and monopalmitin, monostearin, monoolein, monolinolein, and distearin from The Hormel Institute, Austin, Minn. Tricaprylin, tripropionin, tristearin, and *N*-ethylmaleimide were obtained from Calbiochem, Los Angeles, Calif., and tricaproin and diisopropyl fluorophosphate from K & K Laboratories, Inc., Plainview, N.Y. Trilaurin, physostigmine, and neostigmine were obtained from City Chemical Corp., New York, tripalmitin from Fisher Scientific Company, New York, and triolein from Nutritional Biochemicals Corporation, Cleveland, Ohio. Sigma Chemical Co., St. Louis, Mo. supplied *p*-chloromercuribenzoate; BCME (butyl carbamic acid methyl ester) and IPA [1-imino (1-piperidino) acetonitrile] were synthesized in these laboratories. The "Nonisol" series of polyethylene glycol esters of higher fatty acids was kindly donated by Mr. H. J. McElhone of Geigy Chemical Corporation, Ardsley, N.Y., and Tween 20 was obtained from Atlas Chemical Industries Inc., Wilmington, Del. Triton X-200 (sodium salt of alkyl aryl polyester sulfonate) was obtained from Rohm & Haas Co., Philadelphia, Penn., and Maracell E and Marasperse N from the Marathon Division of American Can Company, Neenah, Wis. Victamine C was obtained from Stauffer Chemicals Company, New York, and cetyl betaine from E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.

METHODS

Substrates were prepared by weighing enough compound into a 50 ml beaker to make 20 ml of 0.05 M suspension. 10 ml of an aqueous solution of 0.5% Triton X-200 was added to this, and the mixture was sonicated with a Branson sonifier, tuned to maximal output for 30 sec. This treatment resulted in a homogeneous suspension which was quantitatively transferred to a graduated cylinder and made up to 20 ml in water. Triglycerides with C_{12} and higher acyl groups and monoglycerides with C_{16} or higher acyl groups had to be liquefied by warming before sonication. Made as described, the suspensions are stable for at least 24 hr. All the Nonisol surfactants, with the exception of Nonisol 300, are water-soluble. The latter requires warming and sonication to disperse it adequately.

Assays were conducted as follows: to 18 × 150 mm test tubes were added 300 μmoles of appropriate buffer (as indicated in Table 3, below), followed by addition of 10 μmoles of substrate, the respective enzyme preparations, and water to a final volume of 1 ml. Each mixture was incubated for 30 min at 38°C. At the end of this time, the tubes were removed from the bath and chilled in ice; 7.5 ml of isopropanol–heptane–1 N H_2SO_4 4:1:0.1 was added and the solution was extracted and titrated by the method of Ko and Royer (14). Appropriate corrections were made for enzyme and substrate blanks.

Soluble protein was estimated by a modified biuret method (15) with crystalline bovine serum albumin as the standard. Nitrogen in the particulate preparations was estimated as described by Natelson (15).

Homogenates of skeletal muscle were prepared from the anterior and posterior muscles of the hind legs of rats killed by decapitation and immediately skinned. The muscles were removed as rapidly as possible and chilled ice; when the adipose tissue had solidified it was carefully trimmed from the muscle and discarded. Trimmed muscles were then weighed and homogenized in a Waring Blendor in 4 volumes (v/w) of cold water. The resulting homogenate was then centrifuged at 2500 *g* at 0–3°C for 5 min. The slightly turbid red supernate was decanted and recentrifuged at 35,000 *g* at 0–3°C for 30 min. The residue from the low-speed centrifugation was resuspended in at least 5 volumes of cold water, filtered through one layer of a gauze bandage pad to remove fibrous material, and then centrifuged as before for 5 min at 2500 *g*. The supernatant fluid was decanted and discarded, and the residue was washed once more as described, filtration through gauze being omitted. The residue was then made up in cold water to the initial volume of the homogenate and is designated as "low-speed particulates." This preparation contains the insoluble enzyme activity designated monopalmitin lipase (MPL).[1]

The supernatant fluid from the high-speed centrifugation (35,000 *g*) was carefully decanted from the residue, and the residue was suspended in at least 10 volumes of cold water and centrifuged as before. The supernatant fluid was discarded, and the residue washed once more as described. It was then made up in cold water to 0.2 volume of the initial homogenate and is designated "high-speed particulates." This fraction contains the insoluble enzyme activity designated as monomyristin lipase (MML).[2] Particulates prepared as described had maximal enzymatic activity. No advantage was apparent when they were prepared in either isotonic KCl (0.154 M) or sucrose (0.25 M).

The supernatant fluid from the first high-speed centrifugation was found to contain at least two other soluble lipolytic activities; one, heat-stable, designated as monolaurin lipase (MLL), and the other, heat-labile, designated as tricaproin lipase (TCL). Because these

[1] The specific activity (μmoles of free fatty acid released per mg of Kjeldahl nitrogen per hr) of a typical preparation was 1.4.

[2] The specific activity (defined in footnote 1) of a typical preparation was 2.4.

enzymes may be more conveniently prepared directly from the initial homogenate, the preparative methods for each are outlined as follows:

Monolaurin Lipase (MLL)

(Note. References to volume refer to the volume of the enzyme solution at the beginning of the step described.)

Step 1: Heating. A muscle homogenate, prepared as described, was heated in a flask in a water bath at 63°C with rapid swirling until the temperature of the homogenate had reached 60°C. The mixture was then rapidly cooled in an ice slush to 3°C and centrifuged at 4000 *g* at 0–3°C for 10 min. The resulting clear brown supernate was filtered through glass wool to remove small amounts of fat floating on the surface. The enzyme at this stage is designated EH_1 in Table 1.

Step 2: First Ammonium Sulfate Fractionation. Over a period of 20 min, 0.45 g/ml of crystalline ammonium sulfate was added to EH_1 while the mixture was mechanically stirred. The resulting precipitate was collected by centrifugation and dissolved in 0.2 volume of cold water. The enzyme at this stage is designated ES_1.

Step 3: Alkaline Ammonium Sulfate Fractionation. To ES_1 was added 0.45 volume of saturated ammonium sulfate solution at pH 7.9 (the pH of the solution was measured at a 1:10 dilution at room temperature after addition of concentrated ammonium hydroxide solution). The resulting precipitate was removed by centrifugation and discarded. 0.4 volume of ammonium sulfate solution was added to the supernatant fluid and the precipitate was dissolved in 0.5 volume of water and called ESS_2 in Table 1.

Further attempts at purification have not been reproducible since at this stage of purification the enzyme is quite unstable.

Tricaproin Lipase (TCL)

Step 1: Extraction. The muscle homogenate prepared as described was centrifuged for 10 min at 4,000 *g* and the supernatant fluid was decanted from the residue and designated "crude extract" in Table 2.

Step 2: First Ammonium Sulfate Fractionation. To the crude extract, stirred mechanically in a beaker in ice, 0.4 g/ml of solid ammonium sulfate was added slowly over 15–20 min. The resulting protein precipitate was recovered by centrifugation and made up in cold water to 0.2 volume of the original crude extract at the beginning of this step. This fraction is designated ES_1 in Table 2.

Step 3: First Acetone Fractionation. To ES_1, cold acetone (measured at 0°C) was added dropwise with mechanical stirring to a concentration of 25%. The resulting precipitate was removed by centrifugation and discarded. To the supernate, acetone was added as before to a concentration of 43% (the percentage of acetone was calculated without correction for volume changes or removal of precipitates). The precipitate was recovered by centrifugation and extracted with 0.5 volume of cold water in a Potter-Elvehjem homogenizer. The resulting suspension was centrifuged and the supernatant fluid was set aside. The insoluble residue was re-extracted as before and the supernatant fraction from this extraction was combined with the first. The resulting solution contains the fraction designated EA_2 in Table 2.

At this stage the enzyme, like the monolaurin lipase previously discussed, is unstable, and further attempts to fractionate it by a variety of procedures have not been successful.

TABLE 1 SUMMARY OF A TYPICAL PURIFICATION OF MONOLAURIN LIPASE

Step	Fraction	Units/ ml	Protein	Specific Activity*	Total Units	Recovery
			mg/ml			%
1	EH_1	3.9	5.1	0.76	2045	100
2	ES_1	19.9	11.1	1.80	2097	102.5
3	ESS_2	27.0	6.7	4.05	1423	69.6

* Specific activity indicates μmoles of free fatty acid released per mg of protein per hr.

TABLE 2 SUMMARY OF A TYPICAL PURIFICATION OF TRICAPROIN LIPASE

Step	Fraction	Units/ ml	Protein	Specific Activity*	Total Units	Yield
			mg/ml			%
1	Crude extract	6.1	10.7	0.57	2085	100
2	ES_1	21.6	19.5	1.10	1481	71.1
3	EA_2	18.2	3.3	5.6	1248	59.8

* Specific activity indicates μmoles of free fatty acid released per mg of protein per hr.

RESULTS

pH Optima

The pH optima of these four enzyme preparations for the hydrolysis of their respective substrates is shown in Table 3. Previous experience in this laboratory has indicated that in addition to pH, an important consideration for maximal activity with lipases is the type of buffer employed. In Table 3 the results obtained with the various enzyme preparations in different buffers at their respective pH optima are shown. For the monoglyceride lipases (MLL, MML, and MPL) the best buffer pair for lipolytic activity is ammonium chloride–ammonium hydroxide. With at least two enzymes (MLL and MML), triethanolamine–HCl and Tris–HCl buffers at the con-

TABLE 3 COMPARISON OF OTHER BUFFERS WITH OPTIMAL BUFFER AT OPTIMAL pH

	Enzyme Preparation							
	MLL		MML		MPL		TCL	
Optimal pH	8.45		8.13		8.52		6.39–6.6	
Substrate	Monolaurin		Monomyristin		Monopalmitin		Tricaproin	
Optimal buffer	NH_4Cl–NH_4OH		NH_4Cl–NH_4OH		NH_4Cl–NH_4OH		Imidazole–HCl	
Other Buffers	pH of Incubation	% of Optimal Buffer	pH of Incubation	% of Optimal Buffer	pH of Incubation	% of Optimal Buffer	pH of Incubation	% of Optimal Buffer
N,*N*-(2-hydroxyethyl) glycine–NaOH	8.50	0	8.15	69	8.55	43	6.36	58
Triethanolamine–HCl	8.52	0	8.18	0	8.49	24	6.5	60
Tris–HCl	8.57	0	8.28	0	8.52	10	6.5	45
Imidazole–HCl	8.38	45	8.28	49.1	8.56	35	—	—
Potassium phosphate	—	—	8.15	64	—	—	6.5	43
2-(*N*-Morpholino)ethane sulfonic acid–NaOH	—	—	—	—	—	—	6.34	54

centrations employed completely inhibit hydrolysis. *N*,*N*-bis(2-hydroxyethyl) glycine buffer also completely inhibits the monolaurin lipase, but not the monomyristin lipase.

With the tricaproin lipase, which has an acidic pH optimum, the optimal buffer was imidazole–HCl. None of the buffers investigated completely inhibited this enzyme.

Inhibiting Effects of Albumin and Calcium

End-product inhibition has been observed with a number of lipolytic enzymes, and additions of calcium ions or albumin have been advocated for the removal of free fatty acids from the reaction mixture. The effects of albumin were tested as follows. The substrates and buffers were preincubated at 38°C for 30 min in the presence of sufficient albumin to make a final concentration in the reaction mixture of 2%. Control samples were incubated without added albumin. After the indicated time had elapsed, the tubes were removed from the bath and chilled, and the individual lipolytic preparations were added. The tubes were again incubated for 30 min at 38°C and then chilled, and the fatty acids were extracted and titrated as described.

In all cases the reactions were inhibited by the presence of albumin; TCL and MML were inhibited 54%, MLL was inhibited 41%, and MPL 21%.

When calcium ions were added to the reaction mixture at 1×10^{-2}M and 2×10^{-3}M, the higher concentration was slightly inhibitory to all four preparations. At the lower concentration, calcium ions had no effect.

Tentative Substrate Specificity

Before substrate specificity was investigated, the linearity of the reaction rates with the four preparations were studied. With the preparations MLL, TCL, and MML the reaction was linear with time and zero order for at least 30 min under the conditions described. With MPL, the curve was sigmoid, with little hydrolysis occurring in the first 10 min. After this, rapid hydrolysis occurred, and this portion of the curve was linear for the next 40 min, after which the rate diminished. The initial lag in hydrolysis with this enzyme could be completely abolished by incubation at 45°C. At this temperature, the curve was linear for 20 min. It thus appeared that 30 min at 38°C would be satisfactory for the determination of substrate specificity for all four preparations, particularly as the results are expressed (for insoluble substrates) in terms of the most rapidly hydrolyzed mono- or triglyceride.

The tentative in vitro substrate specificities of the muscle lipases are summarized in Table 4. The results are given in percentages of the rates of hydrolysis of monolaurin for MLL, monomyristin for MML, monopalmitin for MPL, and tricaproin for TCL.

Of the monoglycerides, monolaurin is the most readily hydrolyzed: it is attacked by all enzyme preparations while monolinolein is hydrolyzed by none. These preparations show a moderate to marked specificity for their preferred substrate, as opposed to other monoglycerides. For example, MLL rapidly hydrolyzed only monolaurin out of the monoglycerides tested. The high-speed particulate preparation, MML, hydrolyzed, in addition to monomyristin, only monolaurin and monoolein. The least specific preparation was the MPL of the low-speed particles, which was the only one to attack monopalmitin and monostearin. The soluble preparation TCL hydrolyzed, in addition to monolaurin, only monobutyrin.

It should be borne in mind that these substrates were used as supplied by the manufacturer and that the position of the fatty acid moiety on the glycerol molecule was not specified. Mattson and Volpenhein (16), Entressangles, Sari, and Desnuelle (17), and Vaughan, Berger, and Steinberg (18) have, however, shown that at pH values above 6.0, a 2-monoglyceride would rapidly isomerize to the 1-isomer. As the substrate specificities of the

TABLE 4 SUBSTRATE SPECIFICITIES OF FOUR LIPOLYTIC ENZYMES OF RAT SKELETAL MUSCLE

Substrate	Enzyme and Fraction			
	MML (ESS_2)	MML (High-Speed Particulates)	MPL (Low-Speed Particulates)	TCL (EA_2)
Monoglycerides				
Monobutyrin	9	0	1	17
Monolaurin	100*	88	54	59
Monomyristin	9	100*	92	0
Monopalmitin	0	0	100*	0
Monostearin	0	0	69	0
Monoolein	0	59	85	0
Monolinolein	0	0	0	0
Diglycerides				
Distearin	0	0	0	0
Triglycerides				
Tripropionin	6	0	0	20
Tributyrin	14	13	0	85
Tricaproin	31	53	37	100*
Tricaprylin	18	83	79	0
Trilaurin	0	0	0	0
Tripalmitin	0	0	0	0
Tristearin	0	0	0	0
Triolein	0	0	0	0
Synthetic Esters				
Nonisol 100	284	34	42	356
Nonisol 110	33	65	42	181
Nonisol 200	13	0	0	33
Nonisol 210	16	0	9	0
Nonisol 250	20	0	0	34
Nonisol 300	6	0	0	0
Tween 20	300	52	73	437
Polyethylene glycol 400 monolaurate	135	78	46	395
Polyethylene glycol 400 dilaurate	131	0	0	0

* Results in terms of percentages of the rates of hydrolysis of monolaurin for MLL, monomyristin for MML, monopalmitin for MPL, and tricaproin for TCL.

four enzymes in this report were tested at pH values above 6.0, it may be assumed that the results reported indicate enzyme action on 1-monoglycerides.

The results with triglycerides are remarkable in that no triglyceride with acyl chains longer than C_8 was attacked by any of these enzymes.

Nonisols 100 and 110 and Tween 20, in addition to the last two esters listed in Table 4, are lauroyl esters of polyethylene glycol. As might be expected from the results with monoglycerides, these surfactant compounds were, in general, rapidly hydrolyzed by all the preparations. Nonisol 300, on the other hand, is a stearoyl and Nonisols 200, 210, and 250 are oleoyl esters. Generally, these were much poorer substrates than the laurates. The soluble MLL preparation hydrolyzed all these esters to some extent and was the only one to do so. None of the other preparations hydrolyzed either Nonisol 300 or polyethylene glycol 400 dilaurate. Esterolytic activity associated with the high-speed particulates failed to hydrolyze five of these esters; the monopalmitin lipase, four; and the tricaproin lipase, three.

Thus it appears that while these preparations are far from purified enzymic entities, they do display markedly different specificities.

The results obtained in this specificity study must be interpreted with caution. It is evident that the conditions under which these enzymes were tested in vitro are very different from those obtaining in vivo, and until more is known about the physiological role of these enzymes, the indicated specificities must be regarded as tentative.

Effects of Various Surfactants

The effects of various surfactants were studied with MLL and its substrate, monolaurin. Emulsions were made with the various surfactants as described; the final concentration of surfactant in each case was 0.25%. The surfactants used were the anionic Marasperse N, Maracell E, and Triton X-200, the cationic hexadecyltrimethyl ammonium bromide and Victamine C, and the amphoteric cetyl betaine. Good hydrolysis was evident with the monolaurin substrate made up in all these surfactants, but most extensive hydrolysis was seen with Triton X-200. With other surfactants, the hydrolysis was about 80% of that obtained with Triton X-200.

Influence of Inhibitors

In further studies to characterize these hydrolases, their behavior with various potential inhibitors was investigated (Table 5). All of these preparations are strongly inhibited by diisopropyl fluorophosphate (DFP), which indicates the presence of a serine residue at or near the catalytic sites. The data also suggest that both insoluble particulate preparations (MML and MPL) contain a sulfhydryl group that is important in their catalytic function since both preparations were completely inhibited by PCMB and by *N*-ethylmaleimide at a concentration of 1×10^{-3}M. At this concentration, MLL is insignificantly affected by these reagents, whereas TCL is inhibited 45% by PCMB and only 18% by *N*-ethylmaleimide. This might indicate the presence of a sulfhydryl group at some distance from the catalytic site. Thus these four lipolytic preparations may be differentiated into two groups on the basis not only of their solubility in water, but of their sensitivity to PCMB and *N*-ethylmaleimide as well.

The two soluble, partially purified preparations of TCL and MLL may be further differentiated from each other by the greater sensitivity of the MLL to butyl carbamic acid methyl ester (BCME), and its lesser sensitivity to 1-imino (1-piperidino) acetonitrile (IPA).

The insoluble enzymes MPL and MML may be readily distinguished from each other by the greater

TABLE 5 INHIBITION CHARACTERISTICS OF MONOLAURIN LIPASE (MLL), MONOMYRISTIN LIPASE (MML), MONOPALMITIN LIPASE (MPL), AND TRICAPROIN LIPASE (TCL)

Inhibitor	MLL		MML		MPL		TCL	
	Concn.	% Inhib.	Concn.	% Inhib.	Concn.	% Inhib.	Concn.	% Inhib.
DFP	1×10^{-4} M	100	1×10^{-4} M	100	1×10^{-4} M	100	1×10^{-4} M	100
"	1×10^{-5} M	77	1×10^{-5} M	100	1×10^{-5} M	50	1×10^{-5} M	87
p-Chloromercuribenzoate	1×10^{-3} M	5	1×10^{-3} M	100	1×10^{-3} M	100	1×10^{-3} M	45
"	1×10^{-4} M	—	1×10^{-4} M	74	1×10^{-4} M	42	1×10^{-4} M	—
"	1×10^{-5} M	—	1×10^{-5} M	40	1×10^{-5} M	16	1×10^{-5} M	—
N-Ethylmaleimide	1×10^{-3} M	3	1×10^{-3} M	100	1×10^{-3} M	100	1×10^{-3} M	18
"	1×10^{-4} M	—	1×10^{-4} M	93	1×10^{-4} M	84	1×10^{-4} M	—
"	1×10^{-5} M	—	1×10^{-5} M	31	1×10^{-5} M	18	1×10^{-5} M	—
BCME	1×10^{-3} M	92	1×10^{-3} M	9	1×10^{-3} M	20	1×10^{-3} M	50
IPA	1×10^{-3} M	90	1×10^{-3} M	69	1×10^{-3} M	0	1×10^{-3} M	100
"	1×10^{-4} M	78	1×10^{-4} M	32			1×10^{-4} M	700
"	1×10^{-5} M	51	1×10^{-5} M	0			1×10^{-5} M	51
Physostigmine	1×10^{-3} M	7	1×10^{-3} M	9	1×10^{-3} M	78	1×10^{-3} M	12
Neostigmine	1×10^{-3} M	0	1×10^{-3} M	0	1×10^{-3} M	0	1×10^{-3} M	0

sensitivity of MPL for physostigmine and its insensitivity to IPA, which inhibits MML.

Enzyme Stability

The enzymes in this report have been studied from the standpoint of stability to freezing and thawing and to heat and desiccation techniques. The two soluble preparations TCL and MLL are unstable to repeated freezing and thawing, but may be kept in the frozen state for more than two months without appreciable loss of activity. The particulate preparations MML and MPL, on the other hand, are more stable, and may not only be kept in the frozen state for 6 months or longer without appreciable loss of activity, but may also be frozen and thawed repeatedly without activity loss.

We have examined the heat stability of these preparations by heating aliquots of the enzymes in tubes in a water bath 2°C higher than the temperature being investigated. With a thermometer in the enzyme preparation, the temperature was brought to the desired level, and then the preparation was immediately removed from the bath and chilled in an ice bath. Precipitates which formed in MLL and TCL were removed by centrifugation. The particulate preparations of MML and MPL were tested without centrifugation. The most stable enzyme, MLL, could withstand 60°C, as is indicated in the purification procedure. TCL, in contrast, was completely inactivated at this temperature. Of the particulate preparations, MPL was appreciably less stable than the monomyristin lipase, 68% of the activity disappearing at 55°C compared to only 8% of the MML.

When the four preparations were lyophilized, only the MML remained active, at approximately initial levels. All three other preparations were completely inactivated by this procedure. Acetone powders of both the MPL and MML particulates were prepared; only the MML retained activity afterwards.

DISCUSSION

The four lipolytic preparations described in this report are obviously not purified, discrete enzymes. It is quite possible, therefore, that while each preparation probably contains a predominant lipase, it may also contain more than one such enzyme. The characteristics of each preparation, therefore, with regard to pH, optimal buffer, inhibitors, substrates, and stability, could be regarded as the sum of the behavior of constituent enzymes. With this limitation conceded, however, it is still consistent with the data to state that rat skeletal muscle has at least four lipases with the collective ability to hydrolyze monoglycerides with fatty acid moieties from 12:0 to 18:0 and 18:1 (fatty acids designated by chain length: number of double bonds), and also to hydrolyze triglycerides containing fatty acids from 3:0 to 8:0 [tricaprin (tri- 10:0) was not tested]. No triglyceride containing fatty acids of 12:0 or longer was found to be hydrolyzed either by the four described lipolytic preparations or by crude total homogenates. While inability to show the presence of such a lipase is not definitive proof that it does not exist, this observation is consistent with the conclusions of Masoro and coworkers in monkeys (19) and in fasted rats (20) that the intracellular triglycerides of skeletal muscle do not serve as an energy reservoir for use either during exercise or under extreme fasting conditions. Only when fasting was prolonged to the point of death were the intracellular stores of triglyceride drawn upon and almost totally depleted.

A contrasting view was presented by Havel and coworkers (7, 8). Using exercising athletes in the postabsorptive state, these workers deduced from respiratory quotients and measurements of turnover rates of infused labeled palmitate that fatty acids comprised almost all the fuel of metabolism but that less than half of the fatty acids utilized appeared to be derived from those circulat-

ing in the blood. They suggested that the source of the remaining fatty acids used could be (*a*) from triglyceride stores within the muscles themselves or (*b*) from the abundant adipose tissue depot adjacent to muscle. Lipolysis within such adipose tissue could follow the stimulus of energy demand, and the resulting free fatty acids could be released from these depots to enter muscle by simple diffusion. A necessary corollary to (*a*) would be the presence in skeletal muscle of a triglyceride lipase.

The data in this report suggest another possibility—that monoglycerides may be an important form in which fatty acids enter the muscle cell, since the enzymic machinery to deal with them is present. The amount of information on levels of monoglycerides in blood or tissue is limited. Carlson and Wadström (21), in an interesting study in eight human subjects, found that 12–14 hr after the last meal the total serum glycerides averaged 0.65 mmole/liter of serum. Of this, the concentration of tri-, di-, and monoglycerides was 0.55, 0.05, and 0.02 mmole/liter of serum, respectively. Thus partially hydrolyzed triglycerides constituted approximately 10 moles % of the total glycerides, and of this 10%, approximately 28% was monoglyceride. When an alimentary lipemia was induced by administration of 100 g of fat, followed by intravenous administration of heparin 3 hr later, a pronounced increase in serum monoglyceride was observed within the first 5 min after the heparin dose. The diglyceride appeared to remain relatively constant. Mead and Fillerup (22) indicate that after rats have been fed a fat meal, as much as 20% of the fatty acid esters in their blood plasma consist of mono- and diglycerides. While these figures can be taken only as estimates of the potential size of this pool, and monoglyceride serum or plasma concentrations are not indicative of turnover rates, it does suggest that a not insignificant percentage of blood lipids can be partially hydrolyzed triglycerides.

The author wishes to acknowledge the expert technical assistance of Mr. N. J. Crittenden throughout the course of this study.

Manuscript received 10 July 1967; accepted 6 November 1967.

References

1. Fritz, I. B. 1961. *Physiol. Rev.* **41:** 52.
2. Masoro, E. J. 1966. *Physiol. Rev.* **46:** 67.
3. Drummond, G. I., and E. C. Black. 1960. *Am. Rev. Physiol.* **22:** 169.
4. Fritz, I. B., D. G. Davis, R. H. Holtrop, and H. Dundee. 1958. *Am. J. Physiol.* **194:** 379.
5. Neptune, E. M., Jr., H. C. Sudduth, and D. R. Foreman. 1959. *J. Biol. Chem.* **234:** 1659.
6. Neptune, E. M., Jr., H. C. Sudduth, D. R. Foreman, and F. J. Fash. 1960. *J. Lipid Res.* **1:** 229.
7. Havel, R. J., A. Naimark, and C. F. Borchgrevink. 1963. *J. Clin. Invest.* **42:** 1054.
8. Havel, R. J., L. A. Carlson, L. Ekelund, and A. Holmgren. 1964. *J. Appl. Physiol.* **19:** 613.
9. Issekutz, B., H. I. Miller, P. Paul, and K. Rodahl. 1964. *Am. J. Physiol.* **207:** 583.
10. Spitzer, J. J. 1964. *In* Fat as a Tissue. Lankenau Hospital Proceedings of a Conference. K. Rodahl and B. Issekutz, editors. McGraw-Hill Book Company, Blackiston Division, New York. 215–227.
11. George, J. C., and C. L. Talesara. 1961. *J. Cellular Comp. Physiol.* **58:** 253.
12. George, J. C., and C. L. Talesara. 1962. *J. Cellular Comp. Physiol.* **60:** 33.
13. George, J. C., and N. V. Vallyathan. 1964. *Can. J. Physiol. Pharmacol.* **42:** 447.
14. Ko, H., and M. Royer. 1967. *Anal. Biochem.* **20:** 205.
15. Natelson, S. 1957. Microtechniques of Clinical Chemistry for the Routine Laboratory. Charles C Thomas, Springfield, Ill. 272 and 311.
16. Mattson, F. H., and R. A. Volpenhein. 1962. *J. Lipid Res.* **3:** 281.
17. Entressangles, B., H. Sari, and P. Desnuelle. 1966. *Biochim. Biophys. Acta.* **125:** 597.
18. Vaughan, M., J. E. Berger, and D. Steinberg. 1964. *J. Biol. Chem.* **239:** 401.
19. Masoro, E. J., L. B. Rowell, R. M. McDonald, and B. Steiert. 1966. *J. Biol. Chem.* **241:** 2626.
20. Masoro, E. J. 1967. *J. Biol. Chem.* **242:** 1111.
21. Carlson, L. A., and L. B. Wadström. 1956. *Koninkl. Vlaam. Acad. Wetenschap., Letter. Schone Kunsten Belg., Kl. Wetenschap., Intern. Colloq. Biochem. Probl. Lipiden, 3. Brussels.* 17.
22. Mead, J. F., and D. L. Fillerup. 1957. *J. Biol. Chem.* **227:** 1009.