

A novel method for measurement of triglyceride lipase activity: suitable for microgram and nanogram quantities of tissue

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Summary The measurement of triglyceride lipase activity in microgram and nanogram quantities of tissue is reported. The method involves quantitation of glycerol released from a triglyceride substrate, which is shown to provide a value of approximately one-third of that obtained by quantitation of free fatty acid release. Influences on glycerol release, including pH optimum, NaCl inhibition, and activation by serum and heparin are characterized. Two separate assays are described for the measurement of glycerol that yield identical results with nanogram quantities of tissue. The advantage of one assay is its simplicity, while the advantage of the other is that it can be adjusted to measure very small tissue samples (nanogram) with the use of microanalytical procedures (i.e., enzymatic amplification of the NAD⁺ product of glycerol analysis) (13, 14). Sensitivity of the method is demonstrated by the analysis of triglyceride lipase activity in nanogram samples of single soleus muscle fibers. Measurement of picomole quantities of glycerol produced by lipase activity in single muscle fibers represents at least a 1,000-fold increase in sensitivity compared to currently available methods. — Young, D. A., D. S. King, M. Chen, B. Norris, and P. M. Nemeth. A novel method for measurement of triglyceride lipase activity: suitable for microgram and nanogram quantities of tissue. *J. Lipid Res.* 1988. 29: 527-532.

Supplementary key words lipoprotein lipase • triglyceride lipase • triglycerides • glycerol • skeletal muscle • microchemistry

Triglyceride lipase activity in rat heart exists as several distinct enzyme pools: capillary-bound lipoprotein lipase, which can be released by heparin perfusion, and tissue residual or heparin non-releasable neutral lipase (1-3).

Much of the residual lipase activity exhibits serum dependence like lipoprotein lipase, and it has been proposed that this may only be a precursor of capillary-bound lipoprotein lipase. However, there is also a serum-independent triglyceride lipase activity which would seem to be a distinct enzyme (4, 5). This is a subject that requires further investigation because, although a good deal is known about hydrolysis of extracellular triglyceride by lipoprotein lipase, little is known about regulation of intracellular triglyceride metabolism.

We are interested in the use of triglycerides as fuel during endurance exercise. Studies using rat muscle have shown that the oxidative type of muscle contains higher levels of lipase activity than the glycolytic type (6, 7). This is in accordance with the finding that endurance-trained human muscle utilizes greater amounts of intracellular triglyceride during exercise than untrained (normal) muscle (8). In order to study the cellular regulation of lipolysis in skeletal muscle and possibly other tissues, it is necessary to be able to measure triglyceride lipase activity in small discrete tissue samples. Currently available assays that depend on the extraction and measurement of free fatty acids generated during triglyceride hydrolysis (9, 10) do not provide sufficient sensitivity. Much greater sensitivity could be achieved by avoiding the extraction procedure and exploiting microfluorometric methods. We have developed a lipase assay that measures the glycerol product of triglyceride hydrolysis, coupled with a purine nucleotide-linked enzymatic amplification procedure (11, 12), for use with microgram and nanogram quantities of tissue.

METHODS

Whole muscle assays

The developmental work for this assay was performed using rat tissue, since lipase activity has been extensively

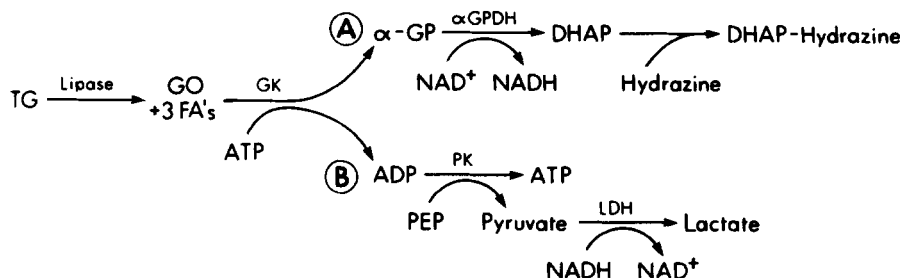


Fig. 1. Pathways used for the measurement of triglyceride lipase. In pathway A glycerol was measured as described in Methods, and in pathway B according to Garland and Randle (14). Abbreviations are: ADP, adenosine diphosphate; ATP, adenosine triphosphate; DHAP, dihydroxyacetone phosphate; FA, fatty acid; GO, glycerol; GK, glycerol kinase; α -GP, α -glycerophosphate; α -GPDH, α -glycerophosphate dehydrogenase; LDH, lactate dehydrogenase; NAD⁺, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide (reduced); PEP, phosphoenolpyruvate; PK, pyruvate kinase; and TG, triglyceride.

TABLE 1. Comparison of the sensitivity and reproducibility of glycerol measurement at the picomole level

Predicted	Observed	Predicted	Observed
	<i>nmol</i>		<i>pmol</i>
5.14	5.11 ± 0.12	3.52	3.50 ± 0.11
10.28	10.23 ± 0.08	7.04	7.08 ± 0.07
15.79	15.69 ± 0.10	15.15	15.21 ± 0.13

Known amounts of a spectrophotometrically calibrated glycerol standard were used to calculate the predicted values, and an average of the final readings observed in the fluorometer was used to calculate the observed values. Each observed value is the average of at least five determinations ± SEM. Glycerol was assayed according to the method of Garland and Randle (Fig. 1B, ref. 14) as described in Methods for whole muscle (nmol) and single fiber assays (pmol).

investigated in these animals (6, 7, 9). Soleus and heart muscle was obtained from male Sprague-Dawley rats between 8 and 10 AM. The rats had free access to Purina chow prior to being killed. The muscle was trimmed of visible connective tissue and homogenized at 0°C in 25 mM phosphate buffer (KH₂PO₄-K₂HPO₄) at pH 7.4. The homogenates were prepared just prior to assay at a concentration of 50 mg/ml.

The lipase reagent was prepared on the day of the assay and contained 50 mM phosphate buffer (pH 7.4), 7.5 mM emulsified triolein, 15% fasted dialyzed rat serum, 5 mg/ml bovine serum albumin (BSA), 0.1 mg/ml sodium dodecyl sulfate, and 1.5 U/ml heparin. The triolein emulsion was prepared by sonication with a Branson model

W-185 sonicator essentially according to the method of Nilsson-Ehle and Schotz (10). The emulsion contained 100 mM phosphate buffer (pH 7.4), 15 mM triolein, 20% fasted rat serum, 0.5 mg/ml BSA, and 0.2 mg/ml Triton X-100. The emulsion was sonicated on ice for 4 min at setting 4 with a microtip, and stored at -20°C.

The lipase reaction was carried out with 100 μl of 50 mg/ml homogenate and 200 μl of lipase reagent at 37°C for 30 min. The reaction was stopped by the addition of 30 μl of 3 N perchloric acid. Zero-time blanks were run for each sample by adding muscle homogenate to lipase reagent already containing perchloric acid. The deproteinized samples were centrifuged at 1,000 g for 10 min, and the supernatant was enzymatically assayed for glycerol content by a modification of the spectrophotometric method of Wieland (13), in which the sensitivity was enhanced for use in the more sensitive fluorometer (Fig. 1A). One hundred μl of supernatant was added to 1 ml of glycerol reagent consisting of 1 M hydrazine hydrate (pH 9.8), 0.2 M glycine (pH 9.8), 1 mM EDTA, 2.5 mM MgCl₂, 0.2 mM NAD⁺, 0.5 mM ATP, 0.25 U/ml glycerol kinase (*Candida mycoderma*), and 2 U/ml glycerophosphate dehydrogenase (rabbit muscle). The samples were read in the fluorometer before and after addition of glycerol kinase.

Due to its simplicity, and because it is easier to measure an increase in fluorescence than a decrease, this method of determination was used routinely for nanomole quantities of glycerol. However, it was not used for single fiber

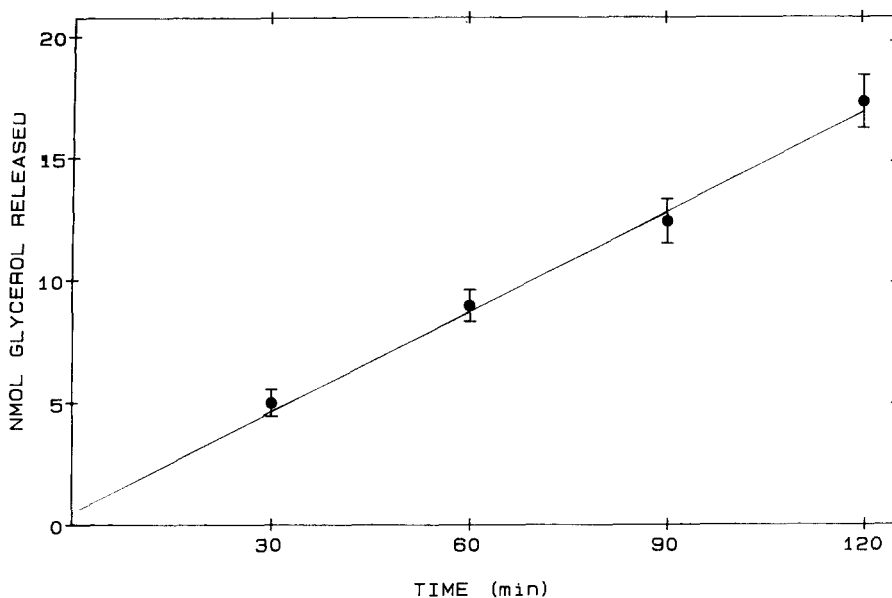


Fig. 2. Rate of glycerol release from 50 mg/ml soleus muscle homogenate incubated in the presence of 5 mM emulsified triolein, 10% fasted rat serum, and 1 U/ml of heparin (final concentrations) at 37°C as described in Methods. Each point represents the average of four muscle samples.

analyses because the femtomole quantities of NADH require amplification by enzymatic cycling (11, 12), and hydrazine is known to interfere with this process (11). Therefore, the glycerol liberated by single fiber triglyceride lipase activity was measured by an alternative method described below (Fig. 1B), modified from Garland and Randle (14). When tested with 10 and 20 nanomole glycerol standards, both methods yielded equivalent results (i.e., 9.81 ± 0.11 vs. 10.09 ± 0.08 , and 20.12 ± 0.15 vs. 20.16 ± 0.12 nmol, respectively). Further, the method of glycerol measurement described by Garland and Randle (14) had the additional benefit of being adaptable to measurement of glycerol levels in the picomole range (Table 1) as described above.

Single fiber analysis

Individual skeletal muscle fibers were isolated from freeze-dried rat soleus muscle using microdissection techniques (11). Portions of approximately 0.25–1.5 μg were weighed on a quartz fiber torsion balance (11) on the day of the assay. The assay reagents were pipetted, using calibrated micropipettes, into droplets of mineral oil to avoid evaporation of the small volumes. The oil droplets were maintained in tiny wells drilled into Teflon racks (11). The fiber samples were rapidly and sequentially pushed through the oil onto a 1.5- μl drop of preincubation solution containing 50 mM phosphate buffer (pH 7.4). After 30 min, two 0.5- μl aliquots were removed from each

sample and placed in separate Teflon racks. One rack of samples received 0.5 μl of lipase reagent containing 2 M NaCl (tissue blank). The NaCl inhibits triglyceride lipase activity, and thereby provides a reliable assessment of the tissue blank in each fiber. The other rack received 0.5 μl of reagent containing 2 U/ml heparin (lipase sample). Following a 30-min incubation, the reactions were stopped with 0.2 μl of 1 N perchloric acid. After 10 min, 5 μl of the glycerol reagent was added to each sample. It consisted of 100 mM Tris-HCl (pH 8.1), 0.025% BSA, 4 mM MgCl_2 , 100 μM phosphoenolpyruvate, 100 μM ATP, 100 μM NADH, 10 $\mu\text{g/ml}$ glycerokinase, 5 $\mu\text{g/ml}$ pyruvate kinase, and 20 $\mu\text{g/ml}$ lactate dehydrogenase. The reaction reached completion by 30 min, at which time 2 μl of 0.3 N HCl was added to destroy the excess NADH. The NAD^+ formed during the glycerol reaction was enzymatically amplified using the cycling techniques of Lowry (12). Details of the cycling technique have recently been described specifically for this assay system (15). The difference between the samples and the tissue blanks was determined fluorometrically. Glycerol standards (approximately 10–30 μM at the first step) were carried through the entire assay sequence.

Materials

All chemicals were obtained from Sigma Chemical Co. Enzymes were obtained from either Sigma or Boehringer-Mannheim.

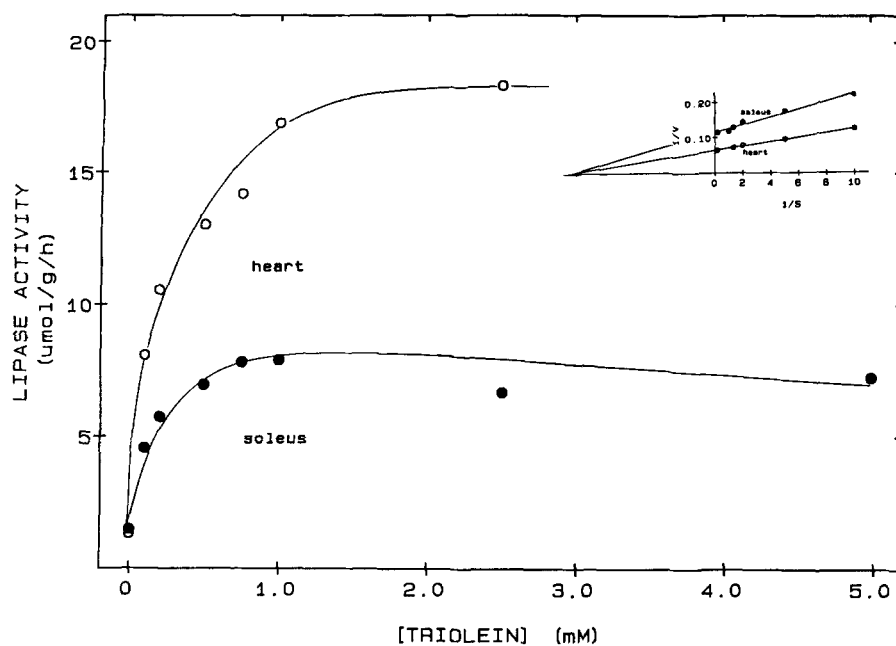


Fig. 3. Dependence of heart and soleus triglyceride lipase activity on triolein concentration. Concentrations given are the final concentrations during the assays. Inset shows double-reciprocal plot of the same data. Assays were performed as described in Fig. 2 and in Methods. Each point represents the average of two to five muscle samples.

RESULTS AND DISCUSSION

Whole muscle and heart

Initial experiments demonstrated that triglyceride lipase activity could be accurately measured by quantitating the amount of glycerol released during the incubation period. The rate of glycerol release was linear for up to 120 min (Fig. 2). No glycerol was released from the assay mixture in the absence of homogenate. The activities were consistent with those previously reported in rat muscle using the free fatty acid release assay, when the latter values are divided by 3 (6, 9). This suggests that the tissue homogenates are capable of releasing all three fatty acids contained in the triglyceride molecule. Also, the amount of glycerol released was not increased by the addition of up to 10 U/ml of esterase (hog liver) to the reaction mixture, indicating that triglyceride, and not di- or monoglyceride cleavage was the rate-limiting step for glycerol formation. Triglyceride lipase activity was dependent on addition of the emulsified substrate, with maximal activity being exhibited in the presence of 1 mM triolein. The V_{max} of the heart and soleus enzymes differed by almost twofold (9.2 vs. 15.9 $\mu\text{mol/g}\cdot\text{hr}$) while the apparent K_m of both enzymes for triolein substrate was approximately 100 μM (Fig. 3).

The specificity of the lipase reaction was tested by examining the serum and heparin dependence of glycerol liberation in rat soleus and heart muscle homogenates. It was found that the activity was greatly dependent on the presence of both of these substances (Table 2). This suggests that glycerol is indeed being released by the action of lipoprotein lipase and tissue triglyceride lipase, both of which are known to be activated by serum and heparin. Furthermore, it was found that when rat hindlimb mus-

TABLE 2. Dependence of triglyceride lipase activity on presence of serum and heparin in the assay reagent

	Soleus	Heart
Serum and heparin	7.72 \pm 0.44 (16)	16.51 \pm 1.34 (12)
No serum	0.92 \pm 0.15 (5)	3.73 \pm 0.60 (4)
No heparin	2.10 \pm 0.45 (4)	3.41 \pm 0.63 (4)
Perfused soleus	5.08 \pm 0.64 ^a (4)	

Homogenates were prepared from rat soleus or heart at a concentration of 50 mg/ml. Enzyme activity is expressed as $\mu\text{mol/g}$ (wet wt.) \cdot hr \pm SEM. Numbers in parentheses indicate number of muscles assayed.

^aRat hindlimb was perfused for 30 min with 5 U/ml of heparin to remove capillary-bound lipoprotein lipase. Homogenates were prepared from perfused muscle as described in Methods. Enzyme activity was measured in the presence of serum and heparin.

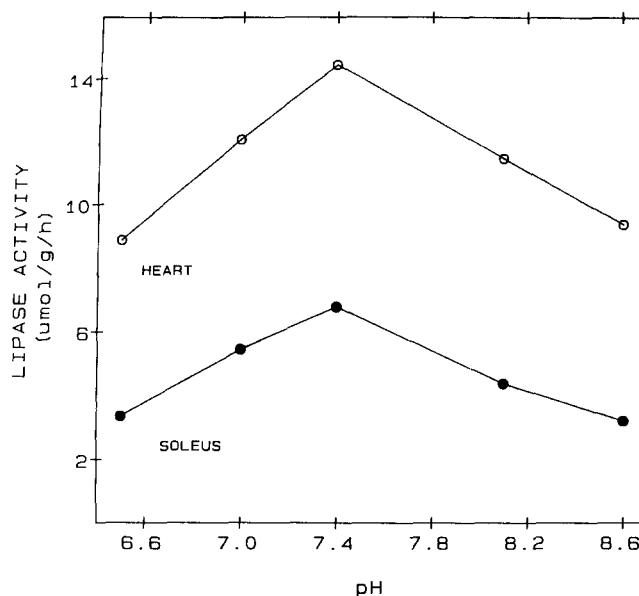


Fig. 4. The pH optimum of triglyceride lipase in heart and soleus homogenates. In each case the same buffers were used in the tissue homogenization, triolein emulsion, and lipase reagent. The buffers used were phosphate (pH 6.6, 7.0, and 7.4) and Tris-HCl (pH 8.1 and 8.6). Each point represents the average of four muscle samples.

cles were perfused for 30 min with 5 U/ml of heparin, approximately 33% of total activity was lost from the soleus muscle (Table 2). The lost activity presumably represents capillary-bound lipoprotein lipase, and the percentage of activity lost during heparin perfusion agrees with previous findings in rat hindlimb muscle (6).

The pH optimum for lipase activity in whole muscle homogenates was found to be approximately 7.4 (measured with either phosphate or Tris-HCl buffer) (Fig. 4). This is consistent with reports that the pH optimum of lipoprotein lipase is approximately 8, whereas tissue residual lipase has a neutral pH optimum (1, 4, 5). Therefore a mixture of the two enzymes, such as we have in whole homogenates, might be expected to exhibit peak activity somewhere between these two values. Indeed, the pH optimum of lipoprotein lipase in the heparin perfusate from rat hindlimb was found to peak at pH 8-8.5 (data not shown). Finally, we tested the inhibition of triglyceride lipase by high concentrations of NaCl and found nearly complete inhibition at concentrations above 0.4 M NaCl (Fig. 5). We exploited this well-known property of lipase in order to determine the tissue blank for single fiber analyses.

Single soleus muscle fibers

Individual soleus muscle fibers were dissected free of all visible connective tissue and capillaries. The average triglyceride lipase activity was 36.1 \pm 2.9 $\mu\text{mol/g}$ (dry wt.) \cdot hr (n = 23). This is consistent with the homogenate

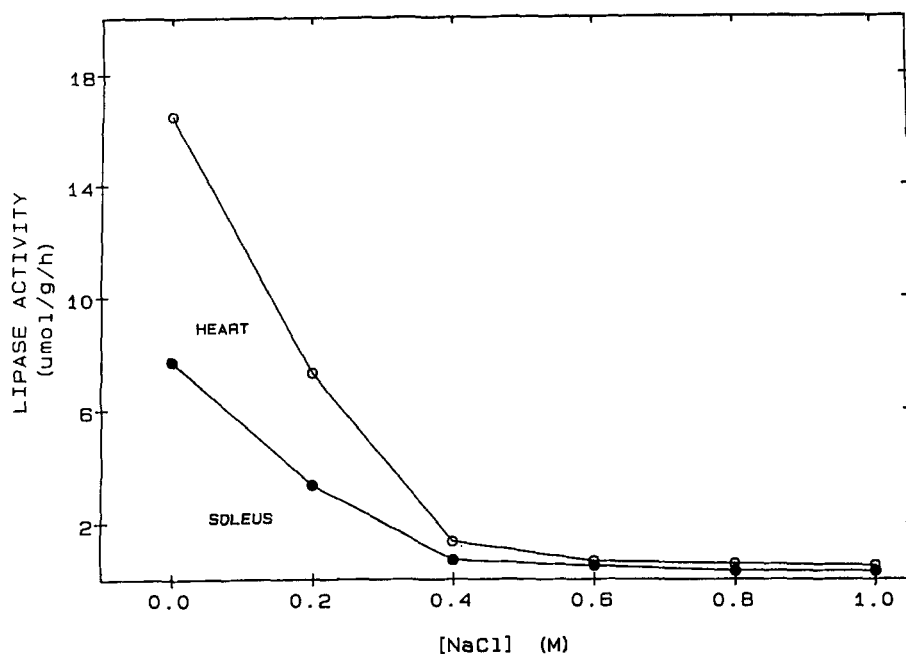


Fig. 5. Inhibition of triglyceride lipase in heart and soleus homogenates by NaCl. Concentrations given are the final concentrations during the assay. Each point represents the average of three muscle samples.

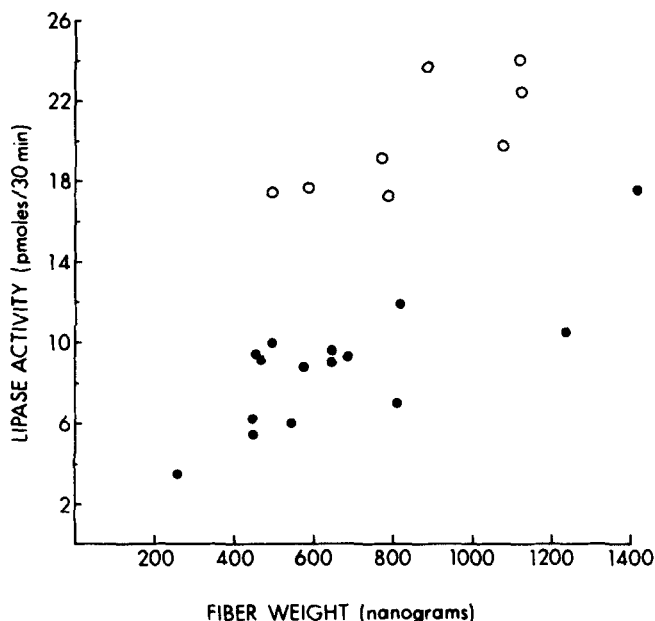


Fig. 6. The relationship between triglyceride lipase activity and weight of individual soleus muscle fibers. Lipase activity was determined by measurement of glycerol release as described in Methods for assay reaction in Fig. 1B. Each point represents the net amount of glycerol released (total release - tissue blank) by one fiber. Soleus fibers fell into two groups, a low activity group (closed circles) and a high activity group (open circles). Both groups exhibited significant correlation between lipase activity and fiber weight ($r = 0.79$, $P < 0.001$ and $r = 0.75$, $P < 0.05$, respectively).

values, taking into account that single fiber weight is expressed as dry weight, and fresh muscle weight is approximately 75% water. Interestingly, the fibers seemed to fall into two groups with respect to lipase activity, a low activity group ($28.4 \pm 1.9 \mu\text{mol/g} \cdot \text{hr}$, $n = 15$) and a high activity group ($50.3 \pm 4.1 \mu\text{mol/g} \cdot \text{hr}$, $n = 8$). Both groups exhibited significant correlations between lipase activity and fiber weight ($r = 0.79$, $P < 0.001$ and $r = 0.75$, $P < 0.05$, respectively), indicating that the enzyme is uniformly distributed in freeze-dried single fibers (Fig. 6). If soleus muscle, which is considered relatively homogeneous, has detectable differences in single fiber lipase activity, it should prove interesting to measure lipase activity in single fibers obtained from muscle of mixed fiber types. ■

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