

## Lysosomal triacylglycerol lipase activity in L6 myoblasts and its changes on differentiation

Victor S. SAURO, Henry J. KLAMUT, Chao-Hsiung LIN and Kenneth P. STRICKLAND\*  
*Department of Biochemistry, Health Sciences Centre, University of Western Ontario, London, Ontario, Canada N6A 5C1*

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L6 myoblasts, before fusion, accumulate large stores of neutral lipid when cultured in medium supplemented with fatty acid. Upon fusion to terminally differentiated myotubes, a noticeable decrease in these neutral-lipid stores was observed. Triacylglycerol lipase activity was examined in L6 myoblasts at various stages of cell differentiation to assess a possible role for this enzyme in the above phenomenon. In this first study to demonstrate lipolytic activity in cultured muscle cells, the activity was found to be totally dependent on the presence of a detergent, either Cutscum or Triton X-100, during homogenization. The inhibition by many thiol-specific reagents [*N*-ethylmaleimide, *p*-chloromercuribenzoate, iodoacetate, 5,5'-dithiobis-(2-nitrobenzoic acid)] suggest that a thiol group is at or near the active site. The observed acidic pH optimum (5.5–6.0), the acute inhibition by chlorpromazine (a lysosomal lipase inhibitor) and the distribution of lipolytic activity upon cell fractionation (which co-sediments with acid phosphatase, a lysosomal marker enzyme) suggest that the lipase may be of lysosomal origin. Under the optimal conditions described, the triacylglycerol lipase activity of L6 myoblasts was determined to be  $2.9 \pm 0.4$  nmol of oleic acid released/min per mg of DNA. This activity increased 3-fold, to  $9.0 \pm 1.6$  nmol/min per mg, in the myotube phase. This increase in lipolytic activity may be responsible for the observed decrease in neutral-lipid stores of differentiating myoblasts.

It is generally agreed that muscle cells utilize fatty acid as a source of energy while in the resting state (Blaise-Smith & Finch, 1979; Hochachka *et al.*, 1977). Muscle cells are also capable of synthesizing triacylglycerols, and store these neutral lipids in endogenous cytoplasmic droplets (Oduote *et al.*, 1981; Kabara & Tweedle, 1981). In order to mobilize these energy stores, an intracellular lipase is required to hydrolyse the triacylglycerols and liberate esterified fatty acids, which can then be further oxidized in the mitochondria (Slavin *et al.*, 1975; Stearns *et al.*, 1979). There have been many studies demonstrating the existence of lipolytic activities isolated from various muscle tissues, but all studies to date have been performed on whole tissue (Wallach, 1968). Little information is available on this activity in the pre-differentiation muscle cell (myoblast). B. D. Sanwal, C. Wright & S. A. Hill (personal communication) have shown that L6 myoblasts, a permanent

cell line derived from rat skeletal muscle by Yaffe (1968), accumulate neutral-lipid droplets when the cells are grown in medium supplemented with fatty acid. Furthermore, they observed a significant decrease in the number of lipid droplets when the myoblasts fuse to form multinucleated myotubes. In order to understand this phenomenon, a study on the lipolytic activity of L6 myoblasts in the pre-fusion and post-fusion phases was undertaken, since it is possible that an enhanced lipolytic activity present in the myotube phase might be responsible for the observed decrease in neutral lipid.

### Materials and methods

#### Materials

$\alpha$ -Minimal Essential Medium and horse serum were obtained from Flow Laboratories (Rockville, MD, U.S.A.). Tri[9,10(n)-<sup>3</sup>H]oleoylglycerol (sp. radioactivity 1 Ci/mmol) was purchased from Amersham-Searle (Arlington Heights, IL, U.S.A.). Phosphatidylcholine and trioleoylglycerol

\* To whom reprint requests should be addressed.

were obtained from Serdary Research Laboratories (London, Ontario, Canada). Formula 947 liquid-scintillation-counting 'cocktail' was obtained from New England Nuclear (Lachine, Quebec, Canada). All other chemicals and solvents were of reagent grade.

#### *Cells and cell culture*

L6 myoblasts were routinely cultured in  $\alpha$ -Minimal Essential Medium supplemented with 50  $\mu$ g of gentamycin/ml of medium, 10% (v/v) horse serum and 16 mM-glucose at 37°C and an atmosphere of 5% (v/v) CO<sub>2</sub> in water-saturated air. Cells were plated at an initial density of  $3.9 \times 10^5$  cells/100 mm-diameter culture dish in 10 ml of supplemented medium. The medium was removed on day 3, the cells were washed with citrate/saline (134 mM-KCl/23 mM-sodium citrate) buffer (pH 7.8) and 10 ml of fresh medium was added. Subculturing was also done 3 days after the initial plating. This involved the detachment of the cells with 0.1% (v/v) trypsin.

#### *Cell fusion*

Myoblast fusion was estimated by determination of the percentage of nuclei within myotubes as described by Morris & Cole (1972). Cells were fixed with methanol and stained with Giemsa stain. Only cells containing more than two nuclei were considered myotubes.

#### *Cell harvest*

Cells were harvested in ice-cold Dulbecco's phosphate-buffered saline A, pH 7.8 (Flow Laboratories) by gentle detachment from the culture dish with a rubber policeman after rinsing the cells twice with the same medium. The cell suspension was centrifuged at 1000g for 5 min and the cell pellet was stored at -20°C.

#### *Triacylglycerol lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) preparation*

L6 cell pellets were stored for up to 8 weeks at -20°C without significant loss of enzyme activity. Cell pellets were thawed and resuspended in 0.1 M-sodium phosphate buffer, pH 6.0, containing 0.75% (w/v) Cutscum detergent (di-iso-octylphenoxyethanol, supplied by Fisher Scientific Co., Toronto, Ontario, Canada), and homogenized in a Potter-Elvehjem homogenizer with 20 strokes of a tight-fitting pestle. The homogenate was centrifuged at 20000g for 15 min and the supernatant was used as the enzyme source after suitable dilution.

#### *Triacylglycerol lipase assay*

A stable emulsion of fatty acid-labelled triacylglycerol was prepared by the procedure of Nilsson-

Ehle & Schotz (1976), by using tri[9,10(n)-<sup>3</sup>H]-oleoylglycerol. This emulsion was used as the substrate for the triacylglycerol lipase assay. The assay mixture consisted of 0.1 M-sodium phosphate buffer, pH 6.0; 0.48  $\mu$ mol of trioleoylglycerol, containing 0.1  $\mu$ Ci of tri[9,10(n)-<sup>3</sup>H]oleoylglycerol; 0.12 mg of phosphatidylcholine; 1.5% (w/v) bovine serum albumin (fatty-acid free); 12.5% (v/v) glycerol; and 0.05 ml of enzyme preparation, containing up to 1 mg of protein, in a total volume of 0.25 ml. The reaction was stopped after incubation at 37°C for 60 min in a shaking water bath with 3.25 ml of chloroform/methanol/heptane (141:125:100, by vol). Non-esterified fatty acids were separated and quantified by liquid/liquid partition by the method of Belfrage & Vaughan (1969). A portion of the upper methanol/water phase was counted for radioactivity (Beckman scintillation counter, model LS 7500) to determine the oleic acid released. Activity was expressed as nmol of oleic acid released/min per mg of protein or per mg of DNA.

#### *Subcellular fractionation*

Cell pellets stored at -20°C were thawed and suspended in 5 vol. of 0.25 M-sucrose/1 mM-EDTA and homogenized with 20 strokes of a tight-fitting pestle in a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 15 min at 800g, the supernatant removed and the pellet resuspended in an additional 5 vol. of 0.25 M-sucrose/1 mM-EDTA. The homogenization step was repeated and the homogenate was centrifuged again at 800g for 15 min. The resulting pellet was stored at -20°C and the pooled supernatants were centrifuged at 10000g for 15 min. The supernatant was removed and the pellet resuspended in 0.25 M-sucrose/1 mM-EDTA and centrifuged again at 10000g for 15 min. The resultant pellet was stored at -20°C and the pooled supernatants were centrifuged at 90000g for 100 min. The supernatant was removed and stored at -20°C. The pellet after resuspension in 0.25 M-sucrose/1 mM-EDTA and centrifugation at 90000g for 100 min was also stored at -20°C, and the supernatant was discarded.

#### *Acid phosphatase (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.2) assay*

Acid phosphatase was assayed with  $\beta$ -glycerophosphate (Sigma Chemical Co., St. Louis, MO, U.S.A.) as substrate by the procedure described by Leighton *et al.* (1968).

#### *Protein and DNA determinations*

Protein was determined by the method described by Bradford (1976) with bovine  $\gamma$ -globulin (Bio-Rad Laboratories, Richmond, CA, U.S.A.) as

standard. DNA was determined by the colorimetric procedure described by Burton (1956).

## Results

### Characterization of cell culture system

L6 myoblasts proliferate until confluence is reached 3 days after the initial plating. They then begin fusing to form myotubes. Over 90% of the cells are multinucleated after 6 days (Fig. 1). Fig. 1 also shows that protein contents significantly increase during fusion, in contrast with DNA contents, which are not significantly changed from day 3 to day 6. When incubated in medium supplemented with fatty acid, myoblasts accumulate large numbers of lipid droplets (Fig. 2), which were noticeably decreased upon cell fusion into myotubes.

### Characterization of triacylglycerol lipase

The detection of any significant activity was totally dependent on the presence of a non-ionic detergent during homogenization (Fig. 3). The optimal concentration of the detergent Cutscum was found to be 0.75% (w/v). Triton X-100 produced similar results, but yielded a lower specific activity. One peak of lipolytic activity was observed in the acidic range, with the optimal pH being 5.5–6.0 (Fig. 4). All further lipase assays were carried out at pH 6.0 in sodium phosphate buffer (0.1M). Under these conditions, the assay was linear with respect to time for 60 min and with protein to 1 mg per incubation.

The effects of various compounds that are known to inhibit or activate various lipases were studied. The results in Table 1 show the inhibitory effects of the various thiol-specific reagents, *p*-chloromercuribenzoate, *N*-ethylmaleimide, 5,5'-dithiobis-(2-nitrobenzoic acid) and iodoacetate, which suggests that there is a thiol group at or near the active site. This is further supported by the observation that dithiothreitol (1.0mM) partially reverses the inhibition by *N*-ethylmaleimide (1.0mM). Table 1 also shows that the chelating agents EDTA and EGTA, along with high concentrations of NaCl (1.0M) and CaCl<sub>2</sub> (10mM), have little effect on the triacylglycerol activity. The lysosomal lipase inhibitor chlorpromazine (Ruth *et al.*, 1980; Jensen *et al.*, 1980; Traynor & Kunze, 1976) strongly inhibited the triacylglycerol lipase activity at 5.0mM. Horse serum at high concentrations (1%, v/v) did not inhibit the activity *in vitro*. Therefore it is highly unlikely that the cellular uptake of a serum protein triacylglycerol lipase inhibitor is responsible for the accumulation of neutral lipid in myoblasts, as was shown in cultured fibroblasts by Gorin *et al.* (1982).

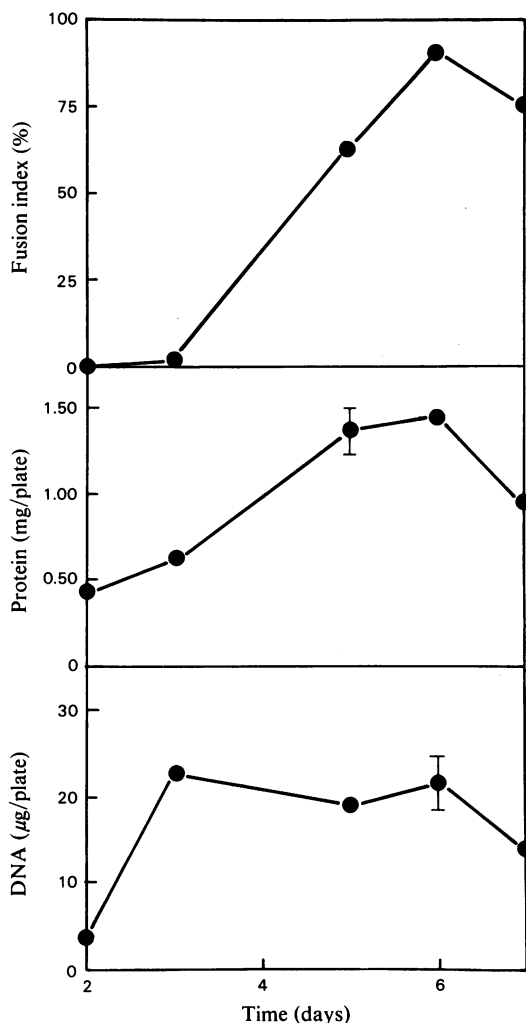


Fig. 1. *Growth parameters of cultured L6 myoblasts* L6 myoblasts were routinely cultured as stated in the Materials and methods section. After 2, 3, 5, 6 and 7 days of culture, cells were washed twice with ice-cold Dulbecco's phosphate-buffered saline A, harvested and pooled from eight 100mm-diameter plates for determination of protein and DNA. At the same times, two culture dishes were removed for determination of the percentage of fusion (i.e. fusion index). Results are means  $\pm$  S.E.M. for six separate experiments; where no error bars are shown, the errors are less than the symbol height.

### Localization of triacylglycerol lipase activity in sub-cellular fractions

The distribution of lipolytic activity and acid phosphatase activity in the various fractions, 800g pellet, 10000g pellet, 90000g pellet and 90000g supernatant, were similar in myoblast and myotube preparations (Fig. 5). The lipolytic activity

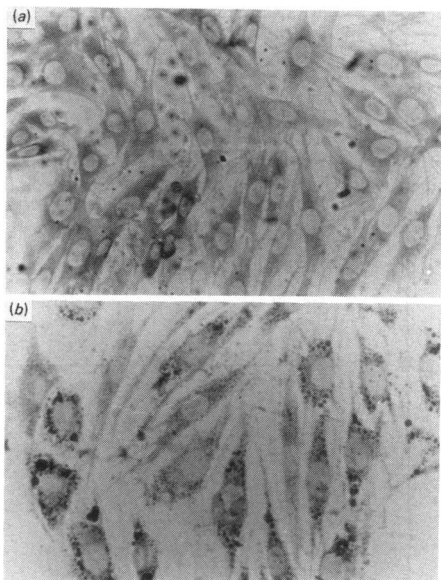


Fig. 2. Lipid accumulation in differentiating L6 myoblasts. L6 myoblasts were grown in culture medium ( $\alpha$ -Minimal Essential Medium, 10% horse serum, 50  $\mu$ g of gentamycin/ml of medium and 16 mM-glucose) only (a) or supplemented with 100  $\mu$ g of oleic acid (dissolved in 0.015% Tween 20 to emulsify the oleic acid)/ml of culture medium (b). After 3 days, the cells had reached confluence and fresh culture medium (without supplemented oleic acid) was added. On day 4, the culture medium was removed, the cells were washed twice with ice-cold Dulbecco's phosphate-buffered saline A, pH 7.8, fixed with 10% (v/v) phosphate-buffered formalin, pH 7.5, and stained for neutral lipid with Sudan Black (Humason, 1972). Magnification  $\times 600$ .

was predominantly found (over 80% of the total activity) in the 90000g pellet. This distribution of lipase activity was similar to that of the lysosomal marker enzyme acid phosphatase (Leighton *et al.*, 1968).

#### Changes of triacylglycerol lipase activity with age of cultured cells

In order to study the time-dependent changes in triacylglycerol lipase activity in this system, the activity is better expressed relative to DNA, the amount of which, as stated above, remains constant during the fusion process. Fig. 6 shows that the triacylglycerol lipase activity in myotubes is 3 times that of myoblasts and that the increase in activity occurs simultaneously with cell fusion.

#### Discussion

This study has confirmed the original observation made by B. D. Sanwal, C. Wright & S. A. Hill

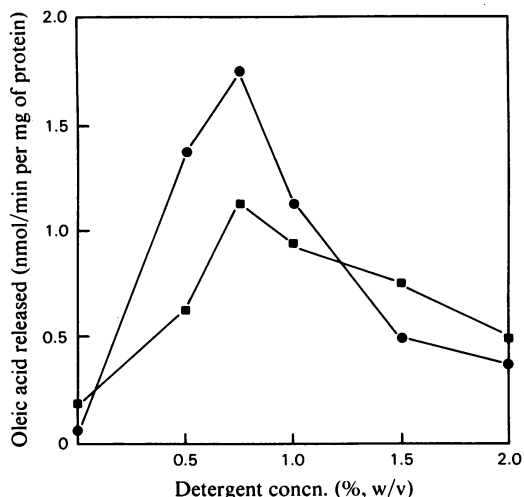


Fig. 3. Effect of detergents on triacylglycerol lipase activity in L6 myoblasts

The effects of two non-ionic detergents, Cutscum (●) and Triton X-100 (■), on the triacylglycerol lipase activity of L6-cell extracts were studied. Cells were homogenized in the presence of the detergent with 20 strokes of a tight-fitting pestle in a Potter-Elvehjem homogenizer. Controls to ensure that the detergents were not activating the triacylglycerol lipase were performed by using cells homogenized with no detergent present but assayed in the presence of the detergent. Each value is the mean of duplicate determinations done in one experiment. Two or more independent experiments for each detergent produced similar results.

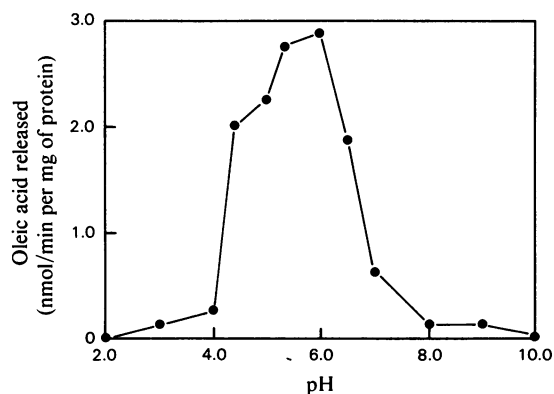


Fig. 4. Effect of pH on triacylglycerol lipase activity in L6 myoblasts

Triacylglycerol lipase was prepared from L6 myoblasts and assayed by using the procedures described in the Materials and methods section, except that the assay was done at a wide range of pH values in the following buffers (final concn. 0.1 M): glycine/HCl (pH 2.0–3.0), citric acid/sodium citrate (pH 4.0–5.5),  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$  (pH 6.0–6.5), triethanolamine hydrochloride/NaOH (pH 7.0–8.0),  $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$  (pH 9.0–10.0).

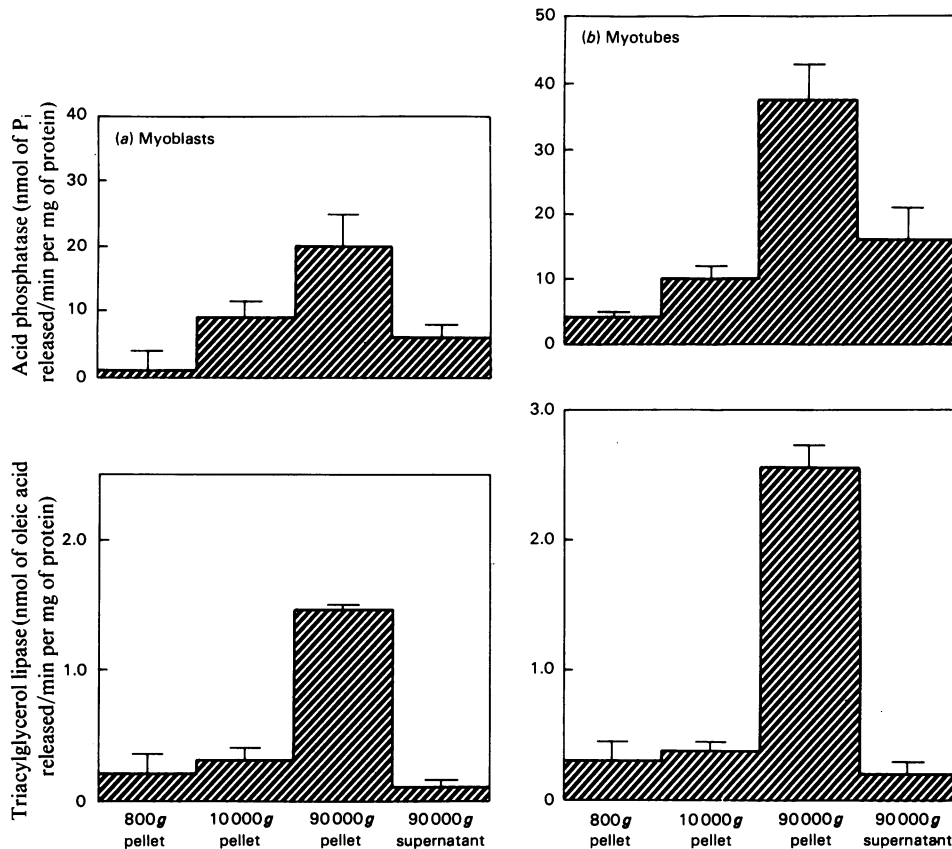


Fig. 5. Distribution of triacylglycerol lipase activity and acid phosphatase activity in subcellular fractions of L6 myoblasts and myotubes

L6 cells were fractionated by differential centrifugation by the procedure described in the Materials and methods section. The 800g pellet, 10000g pellet, 90000g pellet and 90000g supernatant were assayed for acid phosphatase and triacylglycerol lipase in myoblast (a) and myotube (b) cell preparations. Results are means  $\pm$  S.E.M. for four separate experiments.

(personal communication) of accumulated neutral lipids in L6 myoblasts after incubation of cell cultures with oleic acid; also confirmed is the finding of a decrease in the content of neutral lipids as the L6 myoblasts fuse to form myotubes. The formation of Sudan Black-stainable cytoplasmic droplets appeared to be dependent on the concentration of exogenous fatty acid, since an intermediate number of stainable droplets could be seen at lower concentrations of fatty acid in the culture medium (results not shown). Preliminary experiments using L6 myoblasts incubated in the presence of  $^{14}\text{C}$ -labelled oleic acid show that most of the radioactivity (67%) recovered in the lipid extract was found in the triacylglycerol fraction, with only a small percentage (15%) in the phospholipid fraction. When the same cultures were harvested after fusing to form myotubes, most of the radioactivity (50%) of the lipid extract could be found in the phospholipid fraction, with a concurrent decrease

occurring in the triacylglycerol fraction (results not shown). These findings are essentially the same as those of Blaise-Smith & Finch (1979), using primary cultures of embryonic-chick muscle. The results suggest that one of the functions of the neutral-lipid droplets is to supply fatty acids for the synthesis of phospholipid during myoblast differentiation. In agreement with Blaise-Smith & Finch (1979), evidence was also obtained which suggests that some of the triacylglycerol stores (40%) could have been used as an energy source. These findings have resulted in the investigation reported here of an intracellular triacylglycerol lipase that may be responsible for the hydrolysis of the neutral-lipid stores and thus explain the significant decrease in the lipid droplets upon fusion of the myoblasts into myotubes.

Previous studies on rat skeletal muscle (the source from which L6 cells were derived) were unable to detect any lipolytic activity towards long-

Table 1. *Effects of various compounds on triacylglycerol lipase activity in L6 cells*

Triacylglycerol lipase was assayed by the procedure stated in the Materials and methods section. All assays were performed in duplicate, with differences between replicates of less than 5%. The effects of all compounds were identical on lipase activity isolated from myoblasts and myotubes.

Compound	Final concn. (mM)*	Triacylglycerol lipase activity (% of control)
<i>p</i> -Chloromercuribenzoate	0.5	5
Iodoacetate	0.5	6
5,5'-Dithiobis-(2-nitrobenzoic acid)	0.5	8
<i>N</i> -Ethylmaleimide	1.0	24
<i>N</i> -Ethylmaleimide + dithiothreitol	1.0	77
NaF	0.5	10
Hg <sup>2+</sup>	1.0	2
Chlorpromazine	5.0	8
EDTA	1.0	95
EGTA	1.0	100
CaCl <sub>2</sub>	10	105
NaCl	1.0M	97
Horse serum	1.0% (w/v)	96

\* Unless otherwise stated.

chain (acyl chains in excess of 12 carbon atoms) triacylglycerols (Wallach, 1968). Jato-Rodriguez *et al.* (1974) first reported the existence of a long-chain triacylglycerol lipase present in mouse skeletal muscle, which required pretreatment with a non-ionic detergent to detect activity. To our knowledge, the present study is the first to report a long-chain triacylglycerol lipase activity in L6 myoblasts. The enzyme has similar properties to that described by Jato-Rodriguez *et al.* (1974) for mouse skeletal muscle. There is a requirement for cell pretreatment with a non-ionic detergent to detect activity, and an intact thiol group appears to be essential for optimal activity.

The acidic pH optimum and the inhibition by chlorpromazine are findings that are consistent with the view that the triacylglycerol lipase activity is of lysosomal origin. Further support for this is provided by the co-sedimentation of the lipolytic activity with acid phosphatase activity. A lysosomal triacylglycerol lipase has been previously reported in both rat liver (Hayase & Tappel, 1970) and rat kidney (Mahadevan & Tappel, 1968). Our studies on L6 myoblasts indicate that the dominant, if not only, lipolytic activity towards long-chain triacylglycerol (in this study, trioleoylglycerol) is the acid triacylglycerol lipase described.

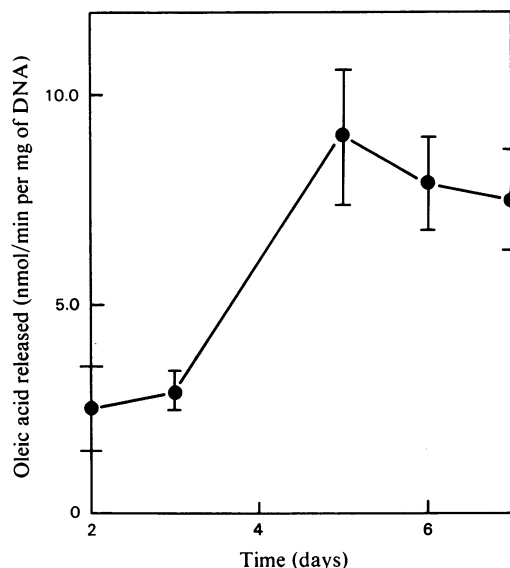


Fig. 6. *Time-dependent changes in triacylglycerol lipase activity in differentiating L6 myoblasts*

L6 cells were cultured as described in the Materials and methods section. Cells were harvested and pooled from eight 100 mm-diameter dishes after 2, 3, 5, 6 and 7 days. Triacylglycerol lipase activity was assayed as described in the Materials and methods section. Results are means  $\pm$  S.E.M. for four separate experiments. A highly significant increase ( $P < 0.01$ ) in activity is observed between days 3 and 5, based on comparison by Student's *t* test.

The results also show the well-documented (Sanwal, 1979) increase in protein content which is associated with myoblast differentiation. However, because the cells at this stage of development are no longer proliferating, the DNA contents remain essentially constant. Therefore, when the triacylglycerol lipase activity is expressed relative to DNA, a significant 3-fold increase in activity is observed in myotube extracts compared with myoblast extracts. Myoblasts possess a low basal rate of triacylglycerol lipase activity, which suggests that the lipase is present at this early stage of development. The nature of this increased activity, whether due to the inactivation of an inhibitor, to the production of an activator or to an increase in synthesis of the enzyme during fusion, is unknown at this time. We suggest that the enhanced lipolytic activity observed in myotubes is sufficient to be responsible for the observed decrease in neutral lipids in fusing myoblasts. To substantiate this claim further, the endogenous synthesis of triacylglycerols during myoblast differentiation should be studied to verify that some alteration in the synthesis rate of neutral lipid does not occur simultaneously with the enhanced lipolytic activity.

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## References

- Belfrage, P. & Vaughan, M. (1969) *J. Lipid Res.* **10**, 341–344
- Blaise-Smith, P. & Finch, R. A. (1979) *Biochim. Biophys. Acta* **572**, 139–145
- Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254
- Burton, K. (1956) *Biochem. J.* **62**, 315–323
- Gorin, E., Gonen, H. & Dickbuch, S. (1982) *Biochem. J.* **204**, 221–227
- Hayase, K. & Tappel, L. (1970) *J. Biol. Chem.* **245**, 169–175
- Hochachka, P. W., Neely, J. R. & Driedzic, W. R. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 2009–2014
- Humason, G. L. (1972) in *Animal Tissue Techniques* (Kennedy, D. & Park, R. B., eds.), pp. 307–308, W. H. Freeman and Co., San Francisco
- Jato-Rodriguez, J. J., Hudson, A. J. & Strickland, K. P. (1974) *Biochim. Biophys. Acta* **348**, 1–13
- Jensen, G. L., Baly, D. L., Brannon, P. M. & Bensadoun, A. (1980) *J. Biol. Chem.* **255**, 11141–11148
- Kabara, J. J. & Tweedle, C. D. (1981) *Neurochem. Res.* **6**, 619–631
- Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J. W., Fowler, S. & de Duve, C. (1968) *J. Cell Biol.* **37**, 482–513
- Mahadevan, S. & Tappel, L. (1968) *J. Biol. Chem.* **243**, 2849–2854
- Morris, G. E. & Cole, R. J. (1972) *Exp. Cell Res.* **75**, 191–199
- Nilsson-Ehle, P. & Schotz, M. C. (1976) *J. Lipid Res.* **17**, 536–541
- Odusote, K., Karpati, G. & Carpenter, S. (1981) *Muscle Nerve* **4**, 3–9
- Ruth, R. C., Owens, K. & Weglicki, W. (1980) *J. Pharmacol. Exp. Ther.* **212**, 361–367
- Sanwal, B. D. (1979) *Trends Biochem. Sci.* **4**, 155–157
- Slavin, G., Wills, E. J., Richmond, J. E., Chanarin, I., Andrews, T. & Stewart, G. (1975) *J. Clin. Pathol.* **28**, 701–710
- Stearns, S. B., Tepperman, H. M. & Tepperman, J. (1979) *J. Lipid Res.* **20**, 654–662
- Traynor, J. R. & Kunze, H. (1976) *Biochim. Biophys. Acta* **424**, 246–252
- Wallach, D. P. (1968) *J. Lipid Res.* **9**, 200–206
- Yaffe, D. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **61**, 477–483