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Triacylglycerol Lipase Activities of Cultured Rat L6 Myoblasts

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Abstract

The utilization of exogenous triacylglycerol by fusing and non-fusing rat L6 myoblasts grown in culture was investigated. Although small quantities of triacylglycerol were accumulated by both cell lines during an incubation of 2 h, no evidence could be found for the presence of lipoprotein lipase, either in the cells or released into the medium. Cell homogenate studies confirmed the absence of lipoprotein lipase but revealed the presence of an acid lipase having a pH optimum at 4·6. Acid lipase activity was mainly associated with a 15 000 *g* pellet and was capable of hydrolysing triolein at maximum velocity in the millimolar range. Unlike lipoprotein lipase, acid lipase was strongly inhibited by serum and preliminary investigations suggest that the inhibitory component of serum is located amongst the higher density lipoproteins. It is likely that the acid lipase is of lysosomal origin and is responsible for the hydrolysis of internalized triacylglycerol for subsequent utilization by the cell.

Introduction

The uptake of lipid by cells in tissue culture has been widely investigated to determine the relative contribution of lipoprotein triacylglycerols (TAG) and free fatty acids (FFA) to the cellular TAG content. Certain cells, namely mouse fibroblasts (Bailey *et al.* 1973) and bovine aortic endothelial cells (Howard 1977) have been shown to take up TAG from the medium without prior hydrolysis, especially where the TAG content of the medium is high (Oram *et al.* 1980). In cells such as mesenchymal rat heart (Chajek *et al.* 1978*a*, 1978*b*) and fibroblast-like stromal cells (Rothblat and DeMartinis 1977) uptake of TAG proceeds via lipoprotein lipase (triacylglycero-protein acylhydrolase, E.C.3.1.1.34), whereas in Ehrlich ascites tumour cells (Brenneman and Spector 1974) and human fibroblasts (de la Llera *et al.* 1979) both mechanisms are important.

We have used both fusing and non-fusing lines of cultured myoblasts to investigate these aspects in a system that might give a clue to the events occurring in skeletal muscle. Limited information is available on the uptake of lipid by skeletal muscle but it has been established that lipoprotein lipase does play a role (Borensztajn *et al.* 1975; Tan *et al.* 1977; Lithell and Boberg 1978). Further, the finding that skeletal muscle homogenates are capable of hydrolysing TAG having long-chain fatty acids (Jato-Rodrigues *et al.* 1974) together with the observation that, under certain conditions, TAG uptake does not closely follow lipoprotein lipase activity (Kaciuba-Uscilko *et al.* 1980) gives support to the idea that skeletal muscle can accumulate TAG without prior hydrolysis.

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The present paper describes attempts to demonstrate the presence of lipoprotein lipase in L6 myoblasts, or in their culture medium, and the measurement of the uptake of fatty acid-labelled TAG by these cells. Although lipoprotein lipase activity could not be demonstrated, a small amount of TAG was taken up by the cells. Further, we have identified the presence of TAG acyl-hydrolase (E.C.3.1.1.3) activity in these cells and some of the properties of this enzyme are described.

Materials and Methods

Cell Culture and Subcellular Fractionation

Fusing and non-fusing lines of rat L6 myoblasts were used. Cultures were routinely grown as monolayers in Eagle's Medium Dulbecco Modified Single Strength supplemented with 10% (v/v) fetal calf serum and 0·3% (v/v) kanamycin sulfate (10 mg/ml). For lipid uptake studies, the cells were grown to confluency in 60-mm plastic dishes at 37°C in an atmosphere of 95% air–5% carbon dioxide.

When large numbers of cells were required for the homogenate studies cells were plated at a density of $1·5 \times 10^4$ cells/cm^2 in 1-litre Roux culture bottles and were grown to confluency at 37°C in an atmosphere of 95% air–5% carbon dioxide. The medium was not changed during this period. Cells were detached from the culture bottles by means of trypsin digestion. The cells were washed twice with Krebs–Ringer–bicarbonate, pH 7·4, containing 1% (w/v) bovine serum albumin and resuspended in 10 mM Tris-HCl, pH 7·0. Homogenization of the cells was carried out using an Ultra-turrax driven at maximum speed for two bursts each of 5 s duration. This homogenate was used either without further treatment or was subjected to the following fractionation. The homogenate was centrifuged at 400 *g* for 5 min and the supernatant obtained was removed and then centrifuged at 15 000 *g* for 20 min. The resultant pellets were each resuspended in their original volume of 10 mM Tris-HCl, pH 7·0. In those experiments where the 15 000 *g* pellet was further fractionated the pellet was resuspended in 1 ml of a solution containing 0·25 M sucrose, 2 mM EDTA and 10 mM Tris-HCl, pH 7·0, and then applied to a Percoll self-forming gradient having a starting density of 1·045 g/ml (Belsham *et al.* 1980). Centrifugation at 12 000 *g* for 20 min resulted in three fractions designated 'light', 'intermediate' and 'dense'. These fractions were washed twice to remove the Percoll and finally were resuspended in 0·5 ml of 10 mM Tris-HCl, pH 7·0. Generally, fractions were tested for lipase or acid phosphatase activity within 2 h of preparation. Fractions could be stored in liquid nitrogen for at least 2 months without loss of activity, but rapidly lost activity when kept at 2°C.

Uptake of Labelled TAG

The culture medium was removed from the freshly confluent cells (≃0·5 mg protein) plated in 60-mm dishes and the cells were washed with Eagle's medium. The incubation was started by adding 2 ml of medium composed of 1·82 ml Eagle's Dulbecco Modified Single Strength, 0·17 ml sheep serum, 0·006 ml [^{3}H]triolein (stock anhydrous preparation) and 80 mg bovine serum albumin. The concentration of triolein in the reaction mixture was 0·2 mM. Incubations were performed at 37°C in an atmosphere of 95% air–5% carbon dioxide. At the time indicated, the medium was removed and analysed for [^{3}H]FFA (Nilsson-Ehle and Schotz 1976) and the cells were washed twice in Eagle's medium. The cells were detached from the dish by means of trypsin digestion, washed once in 0·9% (w/v) NaCl solution and extracted with chloroform : methanol, 2 : 1 (v/v), and the radioactivity was determined. Control experiments performed at either 2 or 37°C indicated that an active uptake process was involved.

Assay of Lipase Activities

The determination of lipoprotein lipase activity was carried out essentially as described by Nilsson-Ehle and Schotz (1976). However, in this case the L6 cells were homogenized in 50 mM NH_4OH–NH_4Cl, pH 8·1, containing 0·05 mg/ml heparin instead of 10 mM Tris-HCl, pH 7·0. Protein content of the whole homogenate was approximately 6 mg/ml. The reaction (total volume 0·2 ml) was started by the addition of 0·1 ml of cell homogenate to a solution containing 1·13 μmol [^{3}H]triolein (approximately 1×10^7 dpm per μmol), 6·7 μmol Tris-HCl, pH 8·1, 3 mg bovine serum albumin and sheep serum to a final concentration of 8·5% (v/v). The incubation was done at 37°C for 90 min and was terminated and the [^{3}H]oleic acid released during the reaction was extracted as described by Belfrage and Vaughan (1969) as modified by Nilsson-Ehle and Schotz (1976). The radioactivity of an aliquot of the upper phase was determined by liquid scintillation counting.

The determination of acid lipase was performed on various subcellular fractions using the method described above but with the following modifications. The reaction was started by the addition of 0·025 ml of various subcellular fractions to a solution containing 1·13 μmol [^{3}H]triolein, 5 μmol citrate–phosphate buffer, pH 4·6, and 3 mg bovine serum albumin. In some experiments (Fig. 1) citrate–phosphate buffer, pH 4·1–6·2, or Tris-HCl buffer, pH 8·1, were used.

Assay of Acid Phosphatase

Acid phosphatase activity was measured on various subcellular fractions using *p*-nitrophenolphosphate at pH 4·8 (Linhardt and Walter 1965).

Serum and Serum Fractions

Blood samples were obtained from the jugular vein of sheep or by cardiac puncture of lightly anaesthetized rats. Serum was obtained from these samples by centrifugation (1800 *g* for 20 min). Total lipoproteins from sheep serum and very low density lipoproteins from rat serum were prepared by the method of Alaupovic *et al.* (1972). Samples were stored in liquid nitrogen until required.

Protein Determination

Protein was determined by the method of Lowry *et al.* (1951).

Neutral Lipid Composition of Myoblasts

The neutral lipid composition of the cells was determined by thin-layer chromatography (Krell and Hashim 1963) on chloroform–methanol extracts of the homogenized cells. The percentage distribution of the major lipid components as assessed by densitometry after charring with 3% (w/v) cupric acetate in 8% (w/v) perchloric acid was: phospholipids, 47%; cholesterol, 23%; cholesterol esters, 22%; TAG, 6%; FFA, 2%.

Reagents

Anhydrous emulsions of [^{3}H]triolein with glycerol and phosphatidylcholine were prepared as described by Nilsson-Ehle and Schotz (1976) and stored at 4°C until used. Tri-[9,10-^{3}H]oleylglycerol was purchased from Amersham Australia Pty Ltd, Sydney. Unlabelled triolein was purchased from Calbiochem-Behring Corp., California. Prior to use, bovine serum albumin (fraction V, Sigma Chemical Co., Missouri) was dissolved in the appropriate buffer and heated at 56°C for 20 min to inactivate endogenous lipases. Heparin (158 USP J-A units/mg) and Triton X-100 were purchased from Sigma Chemical Co., Missouri. *p*-Nitrophenylphosphate was obtained from Sigma Chemical Co., Missouri, as Sigma 104 phosphatase substrate. Protamine sulfate (A grade) was purchased from Calbiochem-Behring Corp., California. Culture media, fetal calf serum, antibiotics and trypsin were obtained from the Commonwealth Serum Laboratories, Parkville, Vic. Percoll was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

Results

Fusing and non-fusing lines of L6 myoblasts were used throughout. Thorough analyses of those parameters measured revealed that there were no differences between these two lines. Consequently, for simplicity, the results for these cells have generally been pooled or single representative experiments have been presented.

The uptake of TAG by L6 myoblasts was investigated by incubating cells in culture medium supplemented with ^{3}H-labelled triolein emulsion (Table 1). Cells accumulated small quantities of TAG (about 0·2 nmol TAG per 0·5 mg cell protein) during the 2 h incubation by a process that was temperature- and time-dependent (data not given). The percentage distribution of the ^{3}H-label in the lipid extracts of the cells following incubation is given in the following tabulation (values are means ± s.e. for five experiments): phospholipids, 1·0 ± 0·2; FFA, 5·7 ± 0·3; TAG, 88·1 ± 1·6; other 5·2 ± 1·3. At this relatively short incubation time (2 h) most of the label associated with the cells was TAG. However, the percentage of [^{3}H]FFA of the cells was considerably greater than that of the culture medium at the end of the incubation

(Table 1) where less than 0·5% of the label was present as FFA. This value for the culture medium, was not significantly different from the medium containing no cells.

Table 1. Uptake of TAG by fusing and non-fusing lines of L6 myoblasts in culture
The details of the TAG uptake experiments are given in the Methods section. Values are the means ± s.e. of between three and six individual experiments

Cell type	Time of incubation (h)	TAG uptake (nmol)	Label added present as free fatty acid in medium (%)
Non-fusing	0	0·02 ± 0·01	0·50 ± 0·10
	2	0·24 ± 0·03	0·54 ± 0·10
Fusing	0	0·03 ± 0·01	0·30 ± 0·02
	2	0·18 ± 0·02	0·50 ± 0·02
No cells	0	—	0·23 ± 0·05
	2	—	0·41 ± 0·05

Lipase activity was measured on the cell homogenate using conditions that are known to be optimal for lipoprotein lipase activity (Nilsson-Ehle and Schotz 1976). However, cell homogenates exhibited only low activity at pH 8·1 [0·32 ± 0·09 (s.e.) nmol fatty acid min^{-1} mg^{-1} protein, $n = 5$)] and the lipase was only partially inhibited by NaCl and protamine sulfate (45 and 37% inhibition respectively) at concentrations known to eliminate lipoprotein lipase activity.

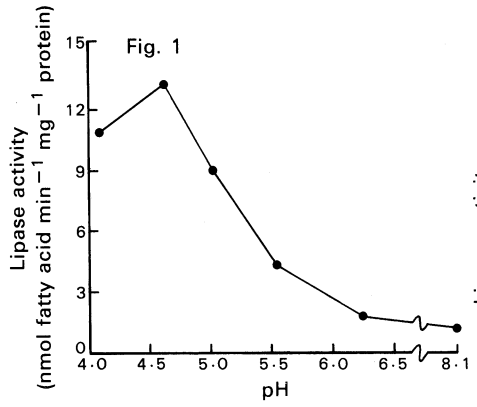
Table 2. Distribution of acid lipase amongst various fractions of L6 myoblasts

Fraction	Protein distribution (%)	Relative specific activity[A] of Acid lipase	Acid phosphatase
Whole homogenate	100 ($n = 12$)	1·00 ($n = 11$)	1·00 ($n = 3$)
400 *g* pellet	25·0 ± 2·3	1·29 ± 0·16	0·65 ± 0·13
15 000 *g* supernatant	51·1 ± 2·4	0·30 ± 0·02	0·54 ± 0·05
15 000 *g* pellet	24·9 ± 1·7	2·32 ± 0·22	3·00 ± 0·20
15 000 *g* pellet[B]			
Percoll light	12·5 ± 3·4	1·07 ± 0·31	0·94 ± 0·18
Percoll intermediate	3·9 ± 0·5	3·70 ± 0·3	1·42 ± 0·20
Percoll dense	1·9 ± 0·7	1·5[C]	0·29 ± 0·08

[A] RSA of acid lipase and acid phosphatase refer to the specific activity of that enzyme in a particular fraction divided by the specific activity in the whole homogenate. The specific activities of acid lipase and acid phosphatase in the whole homogenates were 5·53 ± 1·00 nmol fatty acid min^{-1} mg^{-1} protein and 26·7 ± 4·7 nmol P_i min^{-1} mg^{-1} protein, respectively.
[B] The data in the subfractionation of the 15 000 *g* pellet represents the mean ± s.e. of three experiments.
[C] Single value only.

In order to characterize the lipase present in these cells activity was investigated over a wide range of pH values. Fig. 1 reveals the presence of an acid lipase having high activity near pH 4·6 and therefore all further work was done at this pH.

The distribution of acid lipase activity in various fractions of L6 myoblasts was investigated (Table 2). In the initial stage of the separation a broad centrifugal fraction sedimenting between 400 and 15 000 *g* was found to have the greatest relative specific activity (R.S.A.). A similar distribution pattern was also observed for the lysosomal marker enzyme acid phosphatase. Significant activities were associated with the 400 *g* pellet and as they were somewhat variable it is likely that they resulted either from aggregation of other particulate fractions or from incomplete homogenization of the cells. Further fractionation of the 15 000 *g* pellet on a Percoll self-forming gradient resulted in three fractions, designated 'light', 'intermediate' and 'dense'. A fraction having intermediate density contained only about 4% of original protein but had a R.S.A. of 3·7 for acid lipase. Although this fraction too had the greatest R.S.A. of the subfractions for acid phosphatase, a loss of activity was apparent compared with the 15 000 *g* pellet. In view of the relatively poor recovery of both types of activities in these Percoll fractions and considering the reasonable enrichment found in the 15 000 *g* pellet, this fraction was used in the following experiments.



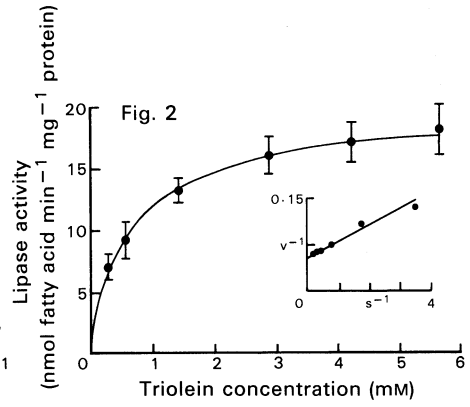


Fig. 1. Effect of pH on lipase activity of L6 myoblast homogenates. L6 myoblasts were homogenized in 10 mM Tris-HCl buffer, pH 7·0. Protein content of the whole homogenate was approximately 6 mg/ml. The reaction (total volume 0·2 ml) was started by the addition of 0·025 ml cell homogenate to a solution containing 1·13 μmol [^{3}H]triolein (approximately 1 × 10^7 dpm/μmol), 3 mg bovine serum albumin and 5 μmol citrate–phosphate buffer, pH 4·1–6·2, or 6·7 μmol Tris-HCl buffer, pH 8·1. The incubations were done at 37°C for 80 min and were terminated and the free fatty acids extracted as described under Methods.

Fig. 2. Effect of triolein concentration on acid lipase activity of a particulate fraction of L6 myoblasts. The reaction (total volume 0·2 ml) was started by addition of 0·025 ml 15 000 *g* particulate fraction to a solution containing 0·06–1·13 μmol [^{3}H]triolein (approximately 1 × 10^7 dpm/μmol), 3 mg bovine serum albumin and 5 μmol citrate–phosphate buffer, pH 4·6. The incubations were performed at 37°C for 90 min and were terminated and the free fatty acids extracted as described under Methods. Error bars show the standard error for three separate cell preparations. The inset shows data from the same experiments but presented as a double-reciprocal plot.

The effect of substrate concentration on acid lipase activity in the particulate fraction (15 000 *g* pellet) was investigated over the concentration range 0·28–5·65 mM triolein (Fig. 2). Lipase preparations from L6 myoblasts exhibited Michaelis–Menten-type kinetics, maximum activity being observed at about 3–4 mM triolein and having an apparent K_m of about 0·7 mM.

The effect of various substances on acid lipase activity in the 15 000 *g* pellet is shown in Table 3. Serum, when present at 8·5% (v/v), caused a marked inhibition of lipase activity, considerably greater than that observed at pH 8·1 with the whole homogenate. At the concentrations used, NaCl and protamine sulfate each inhibited lipase activity but the inhibition was not as great as that reported for lipoprotein lipase at pH 8·1 (Tan *et al.* 1977). The addition of Triton X-100 in low concentrations has been reported to be necessary for obtaining maximum activity when certain particulate fractions are used as a source of lipase (Rindler-Ludwig *et al.* 1977); however, no such requirement was observed here. High concentrations of Triton X-100 (0·4 mg/ml) strongly inhibited lipase activity, presumably by affecting the structure of the triolein substrate emulsion.

Table 3. Effect of various substances on the lipase activity of a particulate fraction of L6 myoblasts at pH 4·6

A particulate fraction (15 000 *g*) suspended in 10 mM Tris-HCl, pH 7·0, of protein content of 1–2 mg/ml, was used. Each incubation (total volume 0·2 ml) was started by the addition of 0·025 ml of particulate fraction to the standard incubation medium as described under Methods. The final concentration of the various substances added is indicated in parentheses. All incubations were performed for 90 min at 37°C and were terminated and the fatty acid extracted as described in Methods

	Activity (%) (mean ± s.e.)	Number of experiments
Control	100[A]	
Plus serum (8·5%)	13 ± 3	7
Plus NaCl (1 M)	32 ± 7	6
Plus protamine sulfate (4·5 mg/ml)	39 ± 4	6
Plus Triton-X-100 (0·013 mg/ml)	93 ± 7	4
Plus Triton-X-100	8 ± 2	4

[A] Equivalent to 15·0 nmol fatty acid min^{-1} mg^{-1} protein.

It has been shown above that 8·5% (v/v) serum strongly inhibits lipase activity. This concentration of serum was selected because it is often the amount used to activate lipoprotein lipase (Nilsson-Ehle and Schotz 1976). Fig. 3*a* shows the effect of serum concentration on acid lipase activity of the 15 000 *g* pellet. Concentrations as low as 1% (v/v) caused some inhibition and at no concentration tested was there activation. In an attempt to ascertain the component of serum responsible for this inhibition and also to determine the kinetics of the inhibition some preliminary investigations were performed. The addition of serum (or lipoprotein subfractions of serum) was investigated over a range of substrate concentrations (Fig. 3*b*). At all substrate concentrations tested, the addition of rat or sheep serum (8·5%, v/v) resulted in almost complete inhibition of lipase activity. When the concentration of sheep serum was chosen so as to obtain about 50% inhibition at 5·65 mM triolein (serum diluted with an equal volume of 0·7%, w/v NaCl) a sigmoidal-shaped curve was obtained, presumably resulting from the high ratio of serum to substrate at the low substrate concentrations. The addition of total lipoproteins isolated from an equivalent amount of sheep serum also

caused strong inhibition and at 5·65 mM triolein was almost identical to its serum equivalent. No inhibition was observed when isolated VLDL was added. The results suggest that the serum inhibitor(s) of acid lipase are the higher density lipoproteins.

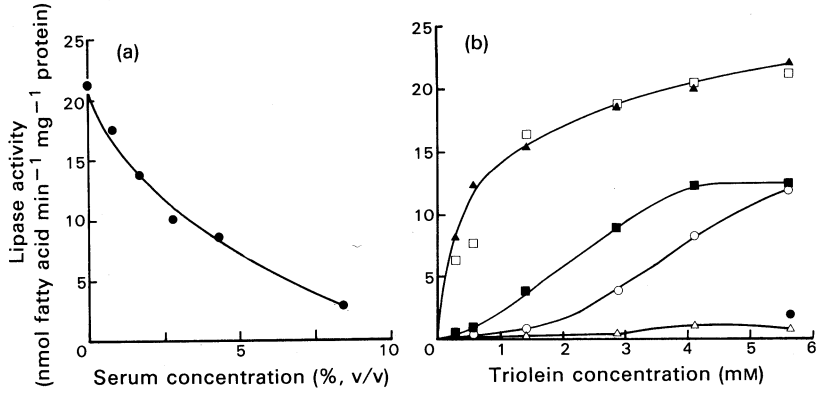


Fig. 3. Effect of serum and serum components on acid lipase activity of a particulate fraction of L6 myoblasts. (*a*) The incubation and procedures were as described in Fig. 2 except that 1·13 μmol [^{3}H]triolein was used and the serum concentration was varied from 0 to 8·5% (v/v). (*b*) The incubation and procedures were as described in Fig. 2 except for the additions shown. ▲ No additions. ● 8·5% (v/v) sheep serum. △ 8·5% (v/v) rat serum. ○ 4·3% (v/v) sheep serum. ■ Total lipoproteins from sheep serum [equivalent of 4·3% (v/v) sheep serum]. □ VLDL from rat serum [equivalent of 4·3% (v/v) rat serum].

Discussion

It is well established that many cells in tissue culture (e.g. mouse lymphoblasts and fibroblasts, Bailey 1977) derive much of their cellular lipid content from serum present in the incubation medium and recently this has also been shown for embryonic chick muscle cells in culture (Sandra and Ionasescu 1980). Using lipid-depleted media, Horwitz *et al.* (1978) showed that L6 myoblasts were capable of growth and proliferation only when an exogenous source of long-chain fatty acids were supplied. The initial studies reported here were designed to show that L6 myoblasts were capable of taking up TAG from the incubation medium. Although the uptake of TAG was low it was significant and comparable to values found for TAG uptake by other cells (Howard 1977; Chajek *et al.* 1978*b*). The pathway for the uptake of TAG by L6 myoblasts was investigated in order to determine whether or not the TAG was taken up intact or following extracellular hydrolysis. Since no hydrolysis of medium TAG could be detected during the 2 h incubation it is suggested that these cells do not have a functionally active lipoprotein lipase for the utilization of medium TAG. The experiments were performed in such a way that had FFA been released to the medium by lipase action it is likely that it would have remained bound to the albumin present in the medium (Chajek *et al.* 1978*b*), and thus be detected. The absence of lipoprotein lipase was confirmed using whole-cell homogenates of L6 cells under incubation conditions known to be optimal for its detection. Therefore, L6 myoblasts would appear to be similar to certain other cell types such as bovine aortic endothelial cells (Howard 1977; Vance *et al.* 1982) and mouse fibroblasts L-929 cells (Bailey *et al.* 1973) where TAG uptake is not the result of secretion of a cellular lipase.

The cells do, however, contain a very active lipase having a pH optimum near 4·6. The low pH optimum and its subcellular distribution paralleling that of acid phosphatase strongly suggest that it is similar to the lysosomal enzymes found in a number of other cells. For example human leucocytes (Ringler-Ludwig *et al.* 1980) and human placenta (Burton and Mueller 1980) have all been shown to contain a lipase having a pH optimum between 3·8 and 5·2 and to be located in a particulate fraction corresponding to a lysosomal fraction (Beaudet *et al.* 1974).

Even though we were able to obtain a subcellular fraction having high relative specific activities for acid lipase and for acid phosphatase, in view of its low protein content this fraction contained only a small amount of the total activities. As acid lipase was present in all Percoll fractions with a relative specific activity equal to or greater than that of the original whole homogenate, it was concluded that, for the purposes of this study, the 15 000 *g* pellet was most suitable. Further, it is known that lysosomes are heterogeneous and exhibit a large range of buoyant densities (Pertoft *et al.* 1978).

The factor(s) in serum responsible for the inhibition of lipase activity is not known, other than it appears to reside in the higher density lipoprotein fractions. Oram *et al.* (1980) have also observed an effect of serum on one of the lipases found in human fibroblasts. The acid lipase measured at pH 4·0 was inhibited by 75% when the incubation medium contained 67% serum. We have shown here that much lower concentrations of serum are effective in inhibiting the acid lipase of L6 myoblasts.

Although cardiac muscle contains a number of lipases, the hydrolysis of endogenous TAG has recently been demonstrated by the presence of an acid lipase having the same subcellular distribution as the lysosomal marker enzyme *N*-acetyl-glucosaminidase (Severson *et al.* 1981). The role of this lipase in cardiac muscle lipid metabolism has not yet been fully assessed but it might be important for the supply of energy requirements under certain conditions.

If the myoblast cells do derive much of their cellular lipid from the medium TAG then the acid lipase present in these cells might be involved in the degradation of TAG to FFA and glyceride esters as the first step in the re-utilization of the lipids. We have noted the low TAG content of these cells (see Methods) compared with the relatively large proportion of cholesterol esters and suggest that this may be due to the presence of the very active acid TAG lipase system in these myoblast cells. Further studies are necessary to determine whether or not the turnover of TAG in these cells is able to maintain the high cholesterol ester content of these cells.

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