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A serum protein inhibitor of acid lipase and its possible role in lipid accumulation in cultured fibroblasts

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Histochemical examination of L929 fibroblasts indicates massive accumulation of intracellular lipids in cells grown in medium supplemented with 10% calf serum. The present study suggests that the accumulation of triacylglycerols in these cells may be due to the inhibition of acid lipase activity by a serum component present in the culture medium. This is based on the following observations. (*a*) Acid lipase appears to be the major intracellular enzyme responsible for triacylglycerol catabolism in L929 cells. (*b*) The acid lipase is strongly inhibited by either human of calf serum. Several lines of evidence show that the inhibitor is a serum protein: it is heat-labile, non-dialysable and is destroyed by trypsin. It is present mainly in Cohn's fraction IV and has mol.wt. approx. 50000. (*c*) Lipid accumulation in intact cells is reduced when cells are grown on a limited supply of serum (2%) and is elevated by the addition of Cohn's fraction IV, freed of lipoproteins, to the growth medium.

Triacylglycerols in cultured fibroblasts are known to be derived from several sources: synthesis from glucose *de novo*; incorporation of non-esterified fatty acids from the medium; uptake of intact triacylglycerol molecules (Geyer, 1967; Mackenzie *et al.*, 1967; Moskowitz, 1967; Klintworth & Hijmans, 1970; Baily *et al.*, 1973).

In cultures of L929 fibroblasts, incorporated triacylglycerols were shown to be further metabolized and converted into other cell glycerolipids (Baily *et al.*, 1973). Studies with double-labelled triacylglycerols indicated that the conversion of triacylglycerols into phospholipids occurs with the conservation of both glycerol and non-esterified fatty acid moieties (Baily *et al.*, 1973), presumably via the diacylglycerol pathway (Hokin & Hokin, 1959). Hydrolysis of the incorporated triacylglycerols to non-esterified fatty acids was found to be a relatively minor process (Baily *et al.*, 1973). The enzymes involved in intracellular lipid hydrolysis in L929 cells have not been previously investigated

The present study was undertaken to characterize the lipolytic activities catalysing hydrolysis of intracellular triacylglycerols in these cells and to examine their possible role in the regulation of cellular triacylglycerol metabolism. In the course of the present investigation, an inhibitory action of a serum protein on acid lipase was noted. The characterization of this inhibitor, and experiments suggesting its possible role in lipid accumulation in the intact cells, are presented.

Materials and methods

Cell culture

The L929 cells used in the present study were obtained from the American Type Culture Collection. L929 is a cloned strain developed from the parenteral L cells, which were originally derived from mouse subcutaneous tissue. Cultures were grown in a CO_2/air (1:19) environment in Dulbecco's modified-Eagle's medium (Grand Island Biological Co., Grand Island, NY, U.S.A.) containing 10% calf-serum (Bio-Lab, Jerusalem, Israel).

Preparation of acetone-dried powder

Cells grown to early confluence were washed three times with phosphate-buffered saline, pH 7.2, gently detached from the flasks with a rubber scraper and suspended in a small volume of phosphate-buffered saline. The cells were extracted and converted into an acetone-dried powder as described by Hall *et al.* (1973) and stored at −15°C. Immediately before use, the powder was resuspended in 0.05 M-Tris/HCl buffer, pH 7.2 or 8.0, to a final protein concentration of about 10 mg/ml and used as the source of lipolytic activities.

Determination of lipolytic activities

Fatty acid-labelled trioleoylglycerol emulsion, prepared by the procedure of Nilsson-Ehle & Schotz (1976), was used as the assay substrate. Lipolytic activities were measured in an assay mixture

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consisting of 0.1 M-citrate buffer, pH 4.4, 2.26 μmol of [9,10(n)-^{3}H]trioleoylglycerol (Amersham International, Amersham, Bucks., U.K.), 0.12 mg of phosphatidylcholine (Sigma Chemical Co., St. Louis, MO, U.S.A.), 1.5% bovine serum albumin (Pentax, Santa Monica, CA, U.S.A.; fatty acid-poor); 12.5% (v/v) glycerol (Frutarum, Haifa, Israel) and 0.05 ml of enzyme source in a total volume of 0.25 ml. After incubation for 30 min at 30°C the reaction was stopped by adding 3.25 ml of methanol/chloroform/heptane (141 : 125 : 100, by vol.) (Nilsson-Ehle & Schotz, 1976). The fatty acids liberated were separated and determined by the method of Belfrage & Vaughan (1969). The release of fatty acids under these conditions is linear with regard to incubation time and amount of enzyme. Inclusion of excess amounts of lecithin up to 0.4 mg/assay did not affect the lipase assay system.

Preparation of delipidized serum

Delipidized human serum proteins were prepared by the method of Rothblat *et al.* (1976). The proteins were dissolved in phosphate buffer (pH 7.4) to give a protein concentration similar to that of the original serum.

Serum freed of lipoprotein

Lipoproteins were removed from serum by ultracentrifuge flotation in the presence of KBr (d < 1.25; Havel *et al.*, 1955).

Gel-filtration chromatography

Cohn's fraction IV (3 ml) from which lipoproteins were previously removed was applied to a column (1.5 cm × 87 cm) of Sepharose-6B (Pharmacia Fine Chemicals). The column was equilibrated with 0.1 M-Tris/HCl buffer (pH 8.0) containing 0.1 M-NaCl. Elution was carried out at a flow rate of 15 ml/h and fractions (3 ml) were collected; 40 μl of each fraction was assayed for inhibition activity.

Assay of trypsin inhibitor

Trypsin (0.05 μg; Worthington) was incubated with 10 μg of [^{3}H]globin as described by Ciechanover *et al.* (1978) and in the presence or absence of the inhibitor as specified, in a total volume of 100 μl at 30°C for 30 min. The reaction was stopped by the addition of 10 mg of albumin followed by the addition of 0.9 ml of 10% trichloroacetic acid. Radioactivity in the soluble non-precipitable fraction was measured. α_1-Antitrypsin, partially purified from human plasma, was obtained from Sigma Chemical Co.

Triacylglycerol determination

The triacylglycerols were split by saponification and the glycerol moiety was enzymically determined. Triacylglycerols were specifically extracted from cellular lipids as described by Dole & Meinertz (1960). The upper phase was evaporated to dryness in a stream of N_2 and redissolved in a small volume of light petroleum (b.p. 60–80°C). The samples were incubated with 0.5 ml of alcoholic 0.5 M-KOH at 65°C for 45 min. After cooling, 1.0 ml of 0.15 M-$MgSO_4$ was added, the samples were centrifuged and the clear solution served for glycerol determination. The assay system for glycerol determination contained, in 1.0 ml, 0.1 M-triethanolamine/0.004 M-$MgSO_4$ buffer, pH 7.6, 0.15 μM-NADH, 1.65 μM-ATP, 0.55 μM-phosphoenolpyruvate, 20 μg of lactate dehydrogenase and 10 μg of pyruvate kinase. The reaction was started by the addition of 20 μg of glycerol kinase and the change in the absorbance at 340 nm was measured in a Gilford spectrophotometer (model 2400-S).

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

Results

L929 fibroblasts grown in a medium supplemented with 10% calf serum show a remarkable accumulation of intracellular lipids.

Fig. 1 illustrates a typical histochemical appearance of logarithmically dividing L929 fibroblasts, stained with Oil Red O. Identical micrographs were observed in about 20 independent cultures grown under the same conditions. As can be seen, lipids accumulated within the cells in a typical morphological pattern. The possibility that lipid accumulation in these cells is a result of the uptake of lipoproteins from the medium is unlikely, since similar results were observed when L929 cells were grown on medium containing 10% calf serum from which lipoproteins were previously removed by ultracentrifuge flotation.

An alternative possibility that fat accumulation in L929 fibroblasts is due to enhanced uptake of fatty acids is also unlikely. Similar rates of incorporation of fatty acids were observed in fat-accumulating L929 cells and in 3T3 cells, which do not accumulate lipids (results not shown).

The following experiments were designed to determine whether the fat accumulation in L929 cells is due to any defect in the lipolytic activity of these cells.

Acetone-dried powders prepared from cultured L929 fibroblasts were assayed for lipolytic activity. Fig. 2 depicts a typical result obtained in one of four independent preparations. It is clear that the cell extract contained two distinguishable lipolytic activities. These are an acid lipase with pH optimum of 4.4 and an alkaline lipase activity with pH optimum at about 8.5. No neutral pH activity could be detected in these cells.

The acid lipase activity was found to be markedly

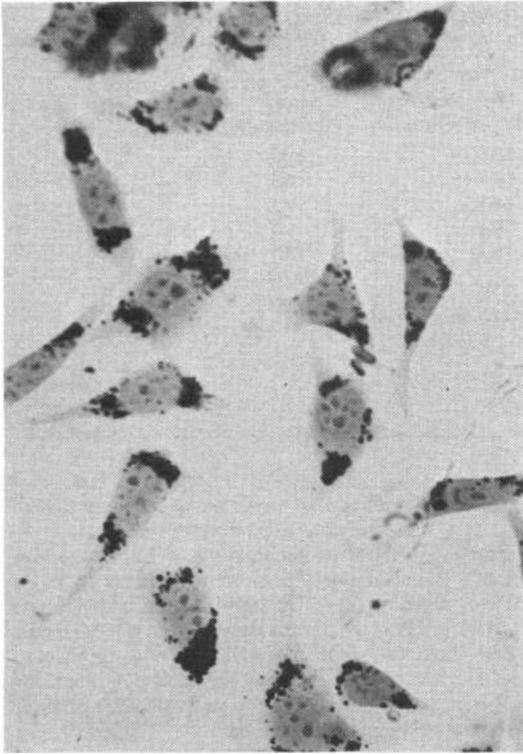

Fig. 1. *Accumulation of lipids in L929 fibroblasts*
L929 cell cultures grown on medium containing 10% serum were washed three times with phosphate-buffered saline (pH 7.2). The cultures were stained with Oil Red O and counterstained with haematoxylin. The brilliant-red spots indicative of triacylglycerol appear as black particles in the photograph. Magnification × 350.

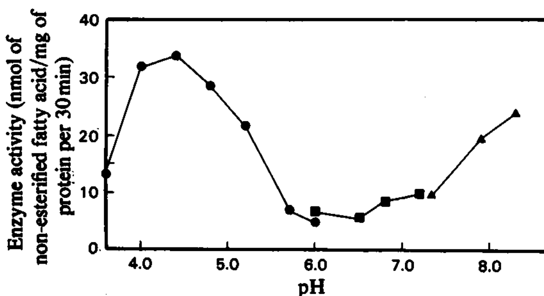

Fig. 2. *Influence of pH on lipolytic activity of L929 fibroblasts*
Acetone-dried powder extract of L929 cells was used as the enzyme source. Lipolytic activity was determined as described in the Materials and methods section except for the use of the different buffers. The assay buffers (0.1 M) were citrate buffer (●), phosphate buffer (■) and Tris/HCl buffer (▲). Each point represents the mean of duplicate determinations.

inhibited by serum. Fig. 3 shows that the addition of as little as 5 μl of either calf or fasted-human serum to the assay mixture caused an about 50% reduction of lipolytic activity. Similar results were repeatedly obtained in five separate experiments.

To define the serum component responsible for the inhibition of acid lipase activity, fasted-human serum was treated by different procedures as described below, before its addition to the lipase assay. As shown in Table 1, serum dialysed overnight against phosphate-buffered saline (pH 7.2) retained all the inhibitory activity. Boiling the serum for 5 min abolished most of the inhibition. These results indicated that the acid lipase inhibitor present in the serum is either of a protein nature or is a component attached to the serum proteins.

The possible involvement of a serum lipid component attached to protein in the acid lipase inhibition *in vitro* was ruled out by the finding that the removal of serum lipoproteins by ultracentrifuge flotation failed to affect inhibitory activity (Table 1). This procedure leaves non-esterified fatty acids bound to serum albumin. We therefore examined the effect of solvent-delipidized serum on the lipase activity. Human serum was treated with acetone/ethanol (1:1, v/v) and serum proteins were then dissolved in buffer to the same protein concentration as in the original serum. As shown in Table 1, this fraction, too, retained all the inhibitory activity present in the serum. These results clearly excluded any lipid component present in the serum from being responsible for the inhibitory effect of serum on acid lipase.

The possibility that the serum inhibitor is a protein component was further examined by the addition of trypsin-treated serum to the acid lipase assay.

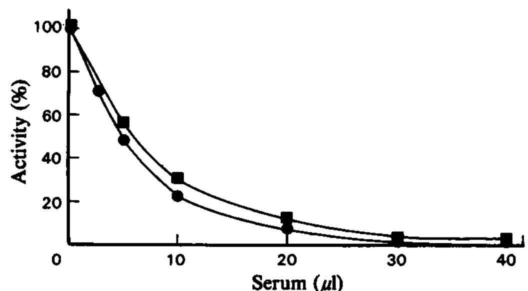

Fig. 3. *Effect of serum on acid lipase activity*
The acid lipase activity in acetone-dried powder extract from L929 fibroblasts was assayed in the absence and presence of fasted-human serum (■) or calf serum (●). The activity in the absence of serum was 27.4 nmol of fatty acid/mg of protein per 30 min.

Table 1. *Effects of various treatments of serum on its inhibition of acid lipase*
All treatments were performed on human serum and 5 μl samples were added to the assay mixture of acid lipase.

Addition	Activity (nmol of fatty acid/mg of protein per 30min)	Activity (%)
None	28.9	100
Serum (untreated)	14.4	50
Dialysed serum*	11.85	41
Boiled serum supernatant†	26.9	93
Serum freed of lipoprotein‡	14.6	50
Delipidated serum proteins‡	13.3	46

* The serum was dialysed overnight against phosphate-buffered saline (pH 7.2).
† Serum was boiled for 5 min and centrifuged at 15000 rev./min for 20 min.
‡ Sera were treated as described in the text.

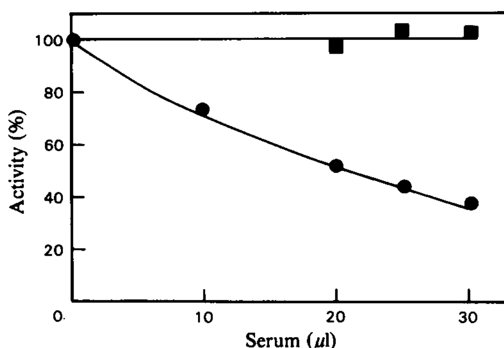

Fig. 4. *Effect of trypsin treatment of human serum on the inhibition of acid lipase*
Serum (250 μl) diluted 1:8 was incubated with 0.11 mg of trypsin (Worthington) for 4 h at 30°C before its addition to the acid lipase assay mixture. No trypsin activity was observed under the acid lipase assay conditions, i.e., at pH 4.4. ●, Untreated serum; ■, trypsin-treated serum. The lipase activity in the absence of serum was 53.4 nmol of fatty acid/assay.

Human serum was incubated with trypsin for 4 h at 37°C before its addition to the lipase assay. Fig. 4 illustrates a typical result obtained in four independent experiments. It is clear from the data that the activity of the inhibitor was completely destroyed by protein digestion. Trypsin added to acid lipase at pH 4.4 did not affect the lipolytic activity. This clearly indicates that the inhibitor is a serum protein component.

Table 2 illustrates the distribution of the acid lipase inhibitor in the Cohn fractions (Cohn *et al.*, 1946) of human serum.

Table 2. *Distribution of acid lipase inhibitor in Cohn fractions of human serum*
Fraction I–III is a combination of Cohn fractions I–III. Equal amounts of protein from the fractions were added to the acid lipase assay and lipolytic activity was measured as described in the Materials and methods section. Activity in the absence of added fraction was 31.7 nmol of fatty acid released/30 min. A representative result of one of four separate experiments is shown.

Amount of protein added (μg)	Inhibition of acid lipase activity (%)		
	Fraction I–III	Fraction IV	Fraction V
110	1	52	0
220	22	76	0
330	39	93	0

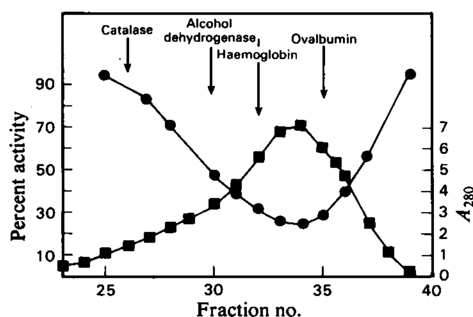

Fig. 5. *Gel-filtration chromatography of acid lipase inhibitor*
Cohn fraction IV of human serum freed from lipoproteins was applied to a Sepharose-6B column as described in the Materials and methods section. ●, Acid lipase activity; ■, absorption at 280 nm. The column was calibrated with the following markers: ovalbumin (mol.wt. 45000); haemoglobin (64500); alcohol dehydrogenase (148000); catalase (240000).

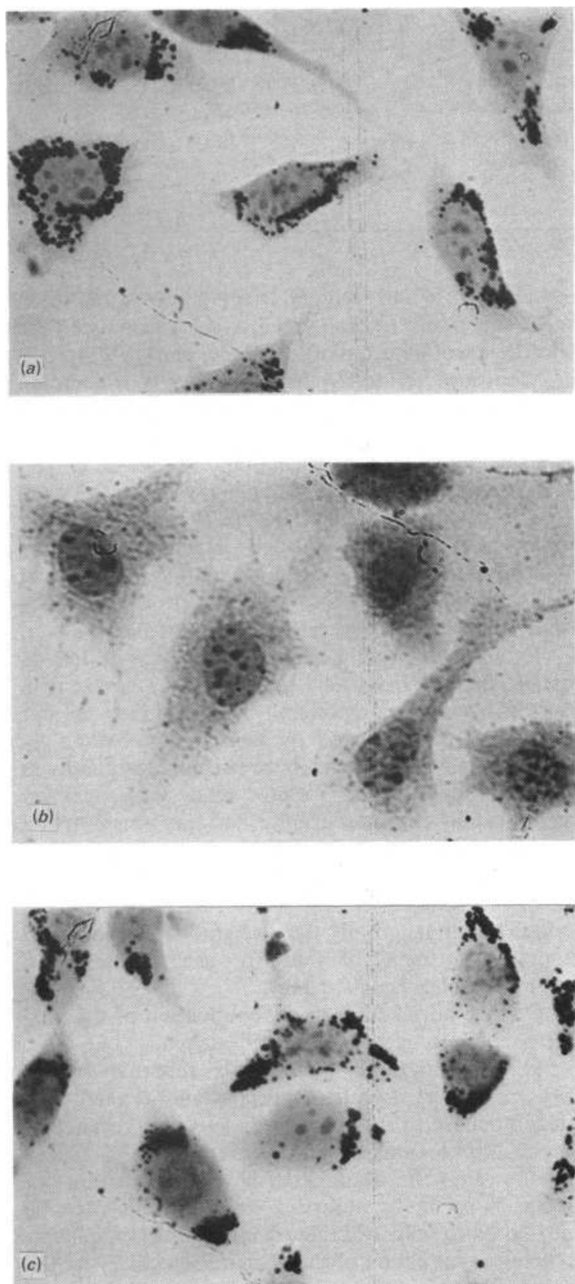

Fig. 6. *Histochemical appearance of L929 cells grown under different conditions*

Cells were grown on medium containing (*a*) 10% calf serum; (*b*) 2% calf serum; (*c*) 2% calf serum and 0.9mg of fraction IV/ml. Lipoproteins were removed from serum and fraction IV by ultracentrifuge flotation before their addition to the growth medium. The cultures were stained with Oil Red O and counterstained with haematoxylin. Magnification ×460.

The highest specific activity of the inhibitor was located in Cohn fraction IV. The combined fractions I–III seem to contain much less inhibitor protein, and fraction V had no inhibitor activity (Table 2).

Fraction IV was freed from lipoproteins by ultracentrifuge flotation and dialysed overnight against 0.9% NaCl. This fraction, which retained most of the inhibitor activity, was then applied to a column of Sepharose-6B equilibrated with 0.1 M-Tris/HCl buffer, pH 8.0, containing 0.1 M-NaCl. The eluate was monitored by absorption at 280nm. As shown in Fig. 5, the inhibitor was eluted in a single peak with an estimated mol.wt. of 50000.

Since serum α_1-antitrypsin inhibitor, which has about the same molecular weight, is also precipitated in Cohn fraction IV, we examined the possibility that these two inhibitors are similar.

The ratio of lipase inhibitor/antitrypsin activity in Cohn fraction IV was compared with that of a partially purified preparation of α_1-antitrypsin (Sigma Chemical Co.). The significant difference in the activity ratio between the two preparations shown in Table 3 favours the notion that the two inhibitors are distinct protein entities.

The question arises whether the presence of the acid lipase inhibitor in the growth medium may affect lipid breakdown within the intact cells and thus cause fat accumulation. Histochemical observations illustrated in Fig. 6 show that reduction in the amount of the inhibitor in the growth medium by decreasing the serum concentration from 10% to 2% only, almost abolishes the fat accumulation in the intact cells (Figs. 6*a* and 6*b*). These results are in accord with chemical determinations of the triacylglycerol content in these cells (Table 4). Furthermore, when lipoprotein-free fraction IV was added to the growth medium containing 2% serum, the cells started again to accumulate a remarkable amount of fat (Fig. 6*c* and Table 4).

These experiments *in vivo* suggest a possible relationship between intracellular fat accumulation and the presence of the acid lipase inhibitor in the growth medium.

An attempt was made to examine whether enzyme derived from cells grown in 10% serum was already inhibited. Acid lipase activities extracted from three separate cultures of cells grown in medium containing either 2% or 10% serum were 33 ± 6.7 nmol of fatty acid/mg of protein and 29 ± 5 nmol of fatty acid/mg of protein (means ± S.D.) respectively. Thus no difference could be discerned in activities of an acetone-dried powder preparation of acid lipase from cells exposed to high or low serum concentration.

Final conclusions on the possibility of complex-formation between enzyme and inhibitor must await full purification of the inhibitor, however.

Table 3. *Relative activities of acid lipase inhibitor and antitrypsin in fraction IV and α_1-antitrypsin preparations*
One unit is defined as the amount required for 50% inhibition of trypsin or acid lipase activity.

	Lipase inhibitor activity (μg of protein/unit)	Antitrypsin activity (μg of protein/unit)	Antilipase/antitrypsin ratio
Fraction IV	95	9.89	9.6
α_1-Antitrypsin	90	0.10	900

Table 4. *Content of triacylglycerols in L929 cells grown under different conditions*
Cells were grown in medium containing 10% serum for 2 days. The growth medium was then changed as indicated in the Table. After an additional 4 days of growth the cells were harvested and homogenized in phosphate-buffered saline. Triacylglycerols were extracted by the method described by Dole & Meinertz (1960) and quantitatively determined as described in the Materials and methods section.

Growth condition	Triacylglycerols (nmol/mg of protein)
10% serum	13.52 ± 0.073 (5)
2% serum	6.56 ± 0.152 (5)
2% serum + fraction IV (0.9 mg/ml)	11.94 ± 0.083 (5)

Discussion

The results demonstrate that L929 fibroblasts grown on medium supplemented with 10% calf serum accumulate massive amounts of intracellular lipids.

A high content of lipids was also found in 3T3-L_1 fibroblasts, a sub-line capable of being differentiated into adipocyte-like cells (Green & Kehinde, 1974). However, the morphological lipid distribution in the two cell types is completely different.

The histochemical pattern of lipid accumulation in L929 cells resembles that found in human skin fibroblasts from patients with a genetic deficiency of acid lipase (Kyriakides *et al.*, 1972; Cortner *et al.*, 1976).

Two lipolytic activities, which differ in their pH optima, are present in L929 cell extract: an acid lipase and an alkaline lipase. No neutral lipase activity could be observed in these cells. The alkaline activity is slightly enhanced by the presence of serum and is inhibited by protamine sulphate and 1 M-NaCl (results not shown), and so, probably, is lipoprotein lipase activity. Since lipoprotein lipase is not considered to be active within the cell, it seems likely that the hydrolysis of intracellular triacylglycerols in these cells is catalysed mainly by an acid lipase, an enzyme known to be largely located in the lysosomes (Mahadevan & Tappel, 1968; Hayase & Tappel, 1970). In cultured human fibroblasts, as in L929 fibroblasts, acid lipase plays a major role in the catabolism of intracellular triacylglycerols. In these cells too, neutral lipase activity, if it exists, is on the borderline of detectability (Beaudet *et al.*, 1974).

Contrary to L929 fibroblasts and to human fibroblasts, Littlefield L mouse fibroblasts and 3T3 cells, which do not accumulate lipid, contain considerable neutral lipase activity (Lengle & Geyer, 1973; Adebonojo *et al.*, 1977).

The results presented here clearly show that acid lipase activity from L929 cells is strongly inhibited by the serum. Similar inhibition by serum of acid lipase activity from other tissues such as rat kidney and liver has been observed (results not shown).

Evidence is provided that the inhibitory effect of serum on L929-cell acid lipase activity is due to a serum protein component. The inhibitor is not dialysable, is destroyed by heat treatment and by trypsin digestion. The inhibitor precipitates mainly in Cohn fraction IV. It is eluted as a single peak on gel-filtration chromatography and has an estimated mol.wt. of approx. 50000.

Cohn fraction IV contains several proteins, most of them classified as α_1 glycoproteins, which have a roughly similar molecular weight. The biological function of most of these proteins is unknown (Schulze & Heremans, 1966).

Further purification and identification of the acid lipase inhibitor is now being undertaken.

Experiments with intact cells reported in the present paper indicate a correlation between lipid accumulation in the intact cells and the presence of an acid lipase inhibitor in the growth medium. These results suggest that excessive accumulation of intracellular lipids observed in L929 cells may be attributed to failure of the cells to hydrolyse lipids as a result of inhibition of the acid lipase activity by the serum factor.

In the intact cells, inhibition of acid lipase activity by the serum protein requires its introduction to the intracellular environment, presumably to the lysosomal fraction where acid lipase is located (Mahadevan & Tappel, 1968). Very little information is currently available on protein uptake by fibroblasts. Uptake of intact low-density lipoproteins by fibroblasts has been reported (Goldstein & Brown, 1977). To clarify the physiological role of the protein inhibitor, it remains to be established whether the inhibitor protein can be taken up by

these cells. A further problem is that if the inhibitor is endocytosed, is it resistant enough to the action of lysosomal proteinases to exert its effect within the lysosome.

As mentioned above, the inhibitor protein present in the serum affects acid lipase activity from various tissues. Fat accumulation in different cell types might depend, therefore, on the ability of the inhibitor to be taken up by the cell, and on the presence of additional lipases within the cells that are not affected by the inhibitor.

The physiological significance of the presence of an active acid lipase inhibitor in normal serum remains to be determined.

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