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Abnormal Neutral Lipase Activity in Acid-Lipase-Deficient Cultured Human Fibroblasts

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Acid and neutral lipase activities of homogenized or sonicated cultured fibroblasts were examined using [2-^{3}H]glycerol triolein, glycerol tri[1-^{14}C]oleate or cholesterol [1-^{14}C]oleate as substrates. In normal fibroblasts, optimal conditions for acid lipase activity were pH 4.5–5.0, 0.15–0.2 mM triacylglycerol, and 0.25% Triton X-100. Fatty acid release was linear to 2 h and between 0.2–2.0 mg fibroblast protein/ml. For the neutral lipase, activity was optimal at pH 6.0–7.0, >1.5 mM triacylglycerol or cholesterol oleate (suspended in 8 mg albumin/ml), and 160 μg phosphatidylserine/ml. The reaction was linear to 60–120 min, and up to 1.0 mg protein/ml. In contrast to the situation at neutral pH, very little [^{3}H]glycerol was released under acid conditions, suggesting little monoglyceride lipase activity at acid pH. Acid lipase activity in fibroblasts from Wolman's disease (WD) or cholesterol ester storage disease (CESD) patients was <1% of activity in normal fibroblasts. Neutral lipase activity (as % of control values) was <15%, <15%, and undetectable when measured as fatty acid release or diglyceride or monoglyceride appearance, respectively, and was less than 25% of controls when cholesterol oleate was the substrate. Neutral lipase activity in mixtures of control and WD fibroblast homogenates was similar to that predicted from individual activities. The triacylglycerol content of the control and mutant cells was within normal limits, and cholesterol content was only slightly elevated, indicating that endogenous dilution of substrate was not the reason for the low activity in mutant cells. Hydrolysis of labeled endogenous triacylglycerol was reduced by >75–80% in the WD cells, indicating that low levels of activity measured with the exogenous substrate were not due solely to lack of accessibility to the neutral lipase. These results suggest a close relationship between the acid and neutral activities of the normal cell in that mutations which affect one apparently affect the activity of the other.

Abbreviations

CESD, cholesterol ester storage disease
FCS, fetal calf serum
MEM, minimal essential medium
WD, Wolman's disease

Several investigators have described lipase activity with an acid pH optimum (3.5–4.5) in homogenates or subcellular fractions of cultured mammalian fibroblasts (1, 3, 12–14, 19). Tri- and diacylglycerols, cholesterol ester, and *p*-nitrophenyl or 4-methylumbelliferyl-linked fatty acids are substrates. The acid lipase activity is probably localized in lysosomes (16) and is deficient in two human inherited diseases of neutral lipid metabolism, WD, and CESD (2, 20). Although the clinical phenotype is quite different, both diseases are characterized by the intralysosomal accumulation of triacylglycerol and cholesterol esters (8).

There is less certainty about the presence and relative amounts of other fibroblast lipases active with triacylglycerol and/or cholesterol esters. Several studies of acid lipase activity, in which the effects of pH on activity included the neutral range, have found very little or no detectable neutral activity (1, 3, 9). On the other hand, at least three studies have clearly demonstrated fibroblast lipase activities with pH optima in the neutral range. Lengle and Geyer (14) reported three pH optima (pH 4.5, 6.5, and 8.5) for lipase activities in mouse fibroblasts; 80% of the activity was at pH 6.5 and was localized in the cytosol. Oram *et al.* (19) also reported three pH optima (pH 4, 6, and 8) for lipase activities in human fibroblasts; the activity at pH 4 was at least twice the other activities and accounted for more than 50% of total cell lipase activities. The neutral activities were not affected by the lipid composition of the medium. Hoeg *et al.* (12) observed neutral (pH 7) lipase activity in cultured fibroblasts from normal subjects and from subjects with CESD and WD.

Using as a starting point the reaction conditions for neutral lipase activities of Lengle and Geyer (14) and Oram *et al.* (19), and of Beaudet *et al.* (1) for acid lipase, we determined optimal conditions for measurement of acid (pH 4.5) and neutral (pH 6.5) lipase activities in cultured fibroblasts from normal subjects and subjects with WD or CESD. Acid lipase activities were less than 1% of controls in both WD and CESD lines; neutral triacylglycerol and cholesterol ester lipase activity in the same lines were less than 25% of the activities in control lines under all conditions tested.

MATERIALS AND METHODS

Materials. Glycerol tri[1-^{14}C]oleate (99.7 mCi/mmole), and cholesterol [1-^{14}C]oleate, (58 mCi/mmole) were obtained from NEN Canada, Lachine, PQ; [2-^{3}H]glycerol triolein (500 mCi/mmole) was from Amersham, Oakville, ON. Triton X-100, fatty acid-poor bovine serum albumin, cholesterol oleate, and *p*-nitrophenyl laurate were from Sigma Chemical Co., St. Louis, MO. Phosphatidylserine and glycerol triolein were from Serdary Research Laboratories, London, ON. Fibroblast cultures from donors with WD (GM 2109, GM 1606) and CESD (GM 0863) were from the Human Genetic Mutant Cell Repository, Camden, NJ. Fibroblast cultures from normal subjects were from healthy volunteers.

Fibroblast culture. The fibroblasts were grown to confluency in MEM (Gibco Canada Ltd., Burlington, ON) containing 10% fetal calf serum (Flow Laboratories, Mississauga, ON). No antibiotics were added. Cells were harvested by trypsinization (0.25% trypsin) or by scraping with a rubber policeman, and after washing twice with phosphate buffered saline, were stored frozen (−20°C) in 0.85% saline. Before measurement of protein and enzyme activities, the cells were disrupted by three 10-sec periods of sonication (Sonic Dismembrator, Artek Systems Corporation, Farmingdale, NY).

Enzyme assays. Except where indicated otherwise, enzyme activities were assayed under the following conditions: for acid lipase with natural substrate, 0.1 ml reaction mixture contained 0.1 M sodium acetate buffer, pH 4.5, 0.25 mg Triton X-100, 150 μM

triolein (4.5 mCi/mmole glycerol tri[1-^{14}C]olein, or 20 mCi/mmole [2-^{3}H]glycerol triolein) and 20–200 μg fibroblast sonicate. Incubation was for 2 h at 37°C. Acid lipase activity with *p*-nitrophenyl laurate was measured as described by Beaudet *et al.* (1). For neutral lipase activity, the 0.1 ml reaction mixture contained 0.1 M HEPES buffer, pH 6.5, 1.5 mM triolein (0.45 mCi/mmole of glycerol tri[1-^{14}C]oleate or 2 mCi/mmole [2-^{3}H]glycerol triolein) or 1.5 mM cholesterol oleate (0.45 mCi/mmole cholesterol [1-^{14}C] oleate), 16 μg phosphatidylserine, 0.8 mg fatty acid-poor bovine serum albumin and 20–100 μg of fibroblast sonicate. Incubation was for 2 h at 37°C.

Hydrolysis of triacylglycerol or cholesterol ester substrate was measured by one of two different methods, determined by the position of the label in the substrate. The two methods gave similar hydrolysis rates. For the [2-^{3}H]glycerol triolein substrate where glycerol, monoacylglycerol, and diacylglycerol formation were measured, the reaction was stopped by addition of 1.5 ml chloroform-methanol (2:1, v/v) and 0.2 ml 1 N HCl. [^{3}H]glycerol radioactivity was determined in the upper phase. [^{3}H]di- and mono-olein in the lower phase were separated from [^{3}H]triolein by thin layer chromatography on precoated silica gel plates, 250 μg thick (Mandel Scientific Co., Ville St. Pierre, PQ). The plates were activated for 30 min at 100°C before spotting and were developed in petroleum ether-diethyl ether-glacial acetic acid (70:30:1, v/v/v). The labeled compounds were located by reference to standards, the silica gel scraped from the plate, and radioactivity determined after suspension in 10 ml Ready-Solv HP (Beckman Instrument Co., Toronto, ON) and counting in a Searle Delta 300 Liquid Scintillation Spectrometer.

When fatty acid release was the only product measured, the reaction was stopped by addition of 1.5 ml isopropanol-3 N H_2SO_4 (40:1, v/v) and fatty acid separated as described by Scholtz *et al.* (21).

Hexosaminidase and 5′-nucleotidase were measured as previously described (23). Lactate dehydrogenase was measured using the Sigma Chemical Company 226-UV kit (Sigma Chemical Co., St. Louis, MO).

Cholesterol (7), triacylglycerol (17), and protein (15) were measured by micromodifications of published techniques.

RESULTS

No consistent differences were observed in either acid or neutral lipase activities when fibroblasts were harvested by trypsinization or scraping and both activities were stable to frozen storage at −20°C for at least 3 wk.

In normal fibroblasts, the optimal conditions for measurement of acid lipase activity with labeled triolein were pH 4.5–5.0 (Fig. 1A), a triacylglycerol concentration of 0.15–0.2 mM (fig. 1B) and 2.5 mg Triton X-100/ml assay mixture. Although the relative proportions of di- and monoacylglycerol formed under these conditions were not always the same, the trends suggested that the limited hydrolysis of di- to monoacylglycerol had a similar pH optimum and Triton X-100 requirement. Fatty acid release was linear with time to 2 h (Fig. 1C) and between 0.2–2.0 mg fibroblast protein/ml assay mixture (Fig. 1D).

For neutral lipase activity, optimal activity was between pH 6.0–7.0 (Fig. 2A). Under the conditions used, the enzyme of normal cells could not be saturated with triacylglycerol (Fig. 2B) or cholesterol-oleate (Fig. 2C), though the rate tended to fall off above 1.5 mM substrate. The enzyme in WD cells appeared to be saturated around 4 mM triacylglycerol, but not with cholesterol oleate at any concentration. A substrate concentration of 2.0 mM was used routinely. The reaction was stimulated 2-fold by phosphatidyl serine (Fig. 2D); it was linear with time to 1 h and fell off only slightly in the next 2 h (Fig. 2E). In spite of the fact that the substrate concentration was not saturating, reaction rate was linear with protein concentration to 1 mg/ml (Fig. 1F). Albumin, used to suspend the substrate had little effect on product formation in the range 2–16 mg/ml (results not shown). Apart from the differences in activity with varying substrate concentration, the optimal

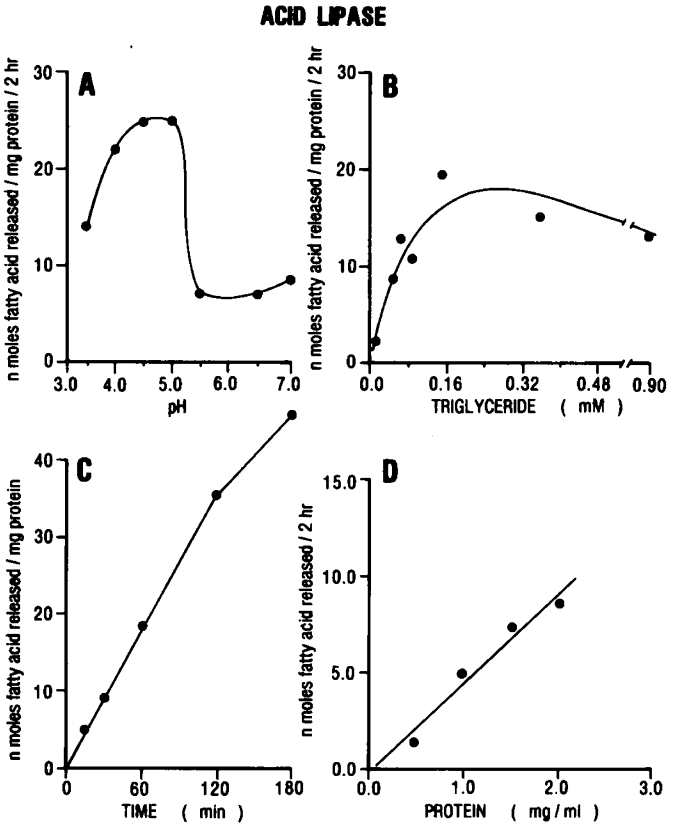

Fig. 1. Formation of [1-^{14}C]oleic acid from glycerol tri[1-^{14}C]olein by acid lipase. Variations in activity with (*A*) pH; (*B*) substrate concentration; (*C*) incubation time; and (*D*) protein concentration.

conditions for measurement of neutral enzyme activity were similar for the WD and CESD cells.

The amounts of [2-^{3}H]glycerol diolein and monoolein formed paralleled fatty acid formation, exhibiting similar optima with varying pH. Release of [2-^{3}H]glycerol was observed only under neutral conditions (Table 1) suggesting that the monoglyceride lipase activity of these cells is active only in the neutral pH range.

Under the conditions tested, the neutral activity was not sensitive to L-isoproterenol. Incubation of normal fibroblasts for 1 h in MEM containing 10% fetal calf serum and 0.15 mg L-isoproterenol/ml had little effect on either acid (control, 70 ± 3; L-isoproternol-treated, 65 ± 1 nmole [1-^{14}C]oleic acid released·mg protein^{-1}·2h^{-1}) or neutral (control, 392 ± 29; L-isoproterenol-treated, 394 ± 56 nmole [1-^{14}C]oleic acid released·mg protein^{-1}·2h^{-1}) lipase activities measured subsequently *in vitro*.

As expected, both the WD and CESD cell lines had barely detectable acid lipase activity, with either artificial (*p*-nitrophenyl laurate) or natural (glycerol tri[1-^{14}C]olein)substrates (Table 2). The unexpected finding was that lipase activity under neutral conditions, whether measured as [1-^{14}C]oleic acid or [2-^{3}H]glycerol released, or as di- or monoacylglycerol formed was less than 15% of control values (Table 3). This difference persisted over the pH range 5.0–7.0, and with varying concentrations of substrate (up to 7 mM, Fig. 2), phosphatidyl serine (0–320 μg/0.1 ml) and fibroblast protein (0.25–1.0 mg/ml). The difference was readily apparent, though less marked (approximately 25% of normal), when cholesterol [1-^{14}C]oleate was used as substrate. The difference was present through the 6–8 subcultures examined and in preconfluent and postconfluent (7–14 day) cells, and in fresh and frozen fibroblast preparations. The activities of three other enzymes were measured to see whether there was a generalized depression of all enzyme activities in the mutant cells. The activities of 5′-nucleotidase and lactate dehydrogenase were similar to controls; hexosaminidase activity was 1.5–2-fold higher.

This difference in neutral lipase activities between control and WD or CESD fibroblasts was not due to dilution of the exoge-

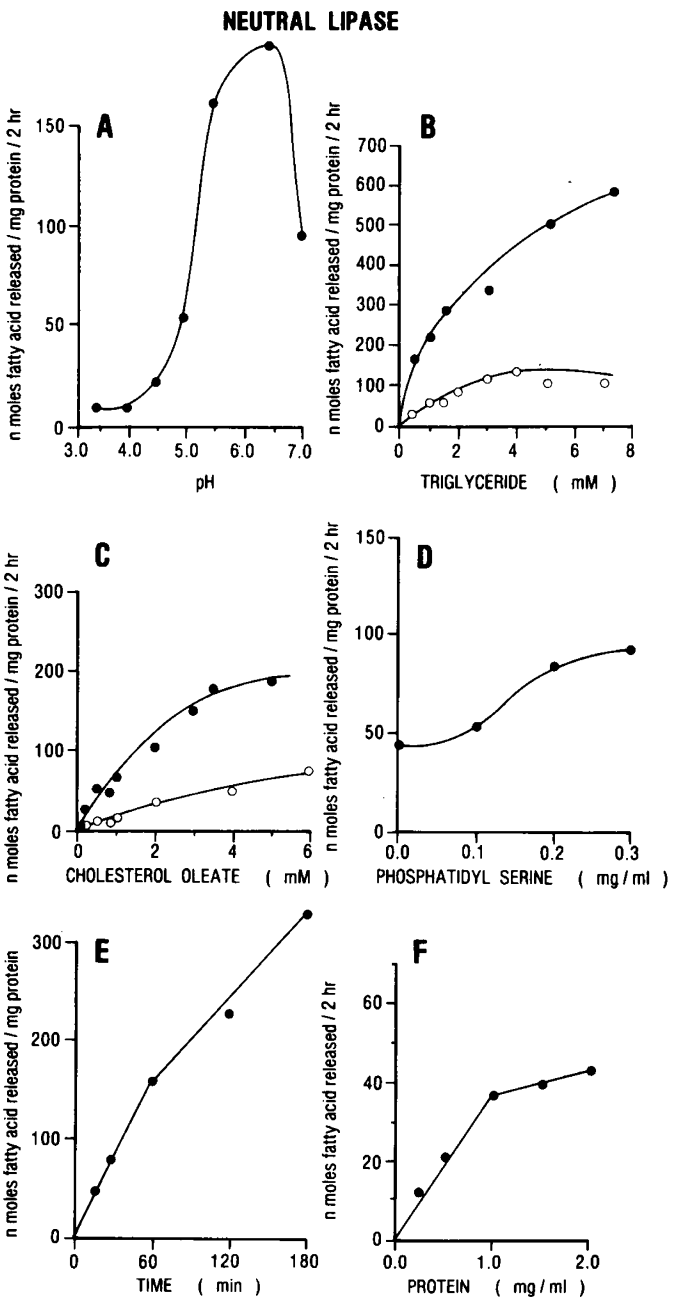

Fig. 2. Formation of oleic acid from glycerol tri[1-^{14}C]olein or [2-^{3}H] glycerol triolein by neutral lipase. Variations in activity with (*A*) pH; (*B*) triglyceride concentration; (*C*) cholesterol oleate concentration; (*D*) phosphatidyl serine; (*E*) incubation time; and (*F*) protein. (●——●) normal fibroblasts and (○——○) WD fibroblasts.

Table 1. *A comparison of acid and neutral lipase activities of normal fibroblasts measured with glycerol tri[1-^{14}C]olein or [2-^{3}H]glyceroltriolein*

	Acid conditions[1]		Neutral conditions	
Product formed	pH 4.5	pH 6.5	pH 4.5	pH 6.5
[2-^{3}H]glycerol released from [^{3}H]triacylglycerol	0.3[2]	0.2	4.2	65
[1-^{14}C]oleic acid from [^{14}C] triacylglycerol	69	2	22	220

[1] Acid and neutral conditions refer to conditions of incubation for the acid and neutral enzymes, respectively, see "Materials and Methods."

[2] nmole product released · mg protein^{-2} · 2 h^{-1}.

Table 2. *Acid-lipase activity of normal and acid-lipase deficient human fibroblasts*

	Substrate	
Cell line	*p*-Nitrophenyl laurate	Glycerol tri[1-^{14}C]olein
Control[1]	3.4[3] (2.4–4.5)	74[4] (30–112)
WD[2]	0	0
CESD	0	0.9

[1] Control cells, mean and range (n = 3).

[2] Wolman's disease (WD) and cholesterol ester storage disease (CESD) cells, mean of duplicate determinations at two separate subcultures.

[3] μmole *p*-nitrophenyl formed · h^{-1} · mg protein^{-1}.

[4] nmole [1-^{14}C]oleate formed · 2 h^{-1} · mg protein^{-1}.

Table 3. *Neutral lipase activity of control and acid-lipase deficient human fibroblasts*

	Triacylglycerol substrate[1]			Cholesterol oleate substrate[1]
Cells	Fatty acid	Diacylglycerol	Monoacylglycerol	Fatty acid
Control (n = 11)	212 (85–393)	61 (33–136)	26 (7–85)	88 (51–119)
WD[2]	31 (7–90)	7 (2–13)	2 (0–7)	23 (15–29)
CESD[3]	14 (1–28)	12 (5–19)	3 (0–3)	

[1] Mean and range for control cells. Values for mutant cells are the mean of duplicate determination at two different subcultures. All values are expressed as nmole lipid product formed · 2 h^{-1} · mg protein^{-1}.

[2] WD, Wolman's disease.

[3] CESD, cholesterol ester storage disease.

Table 4. *Triglyceride and cholesterol content of normal and acid-lipase deficient cultured human fibroblasts*

Cell line	Triacylglycerol	Cholesterol
Control 1	37[1]	52
Control 2	19	32
WD[2]	29	68
CESD[3]	23	74

[1] Each value is the mean of replicate determinations on lipid extracts of the cultured cells, expressed as μg lipid/mg fibroblast protein.

[2] WD, Wolman's disease.

[3] CESD, cholesterol ester storage disease.

nously added substrate by endogenous stored neutral lipid. The triacylglycerol content of control and acid-lipase deficient cells was similar (Table 4), and contributed <3% to the total substrate pool available for hydrolysis in the assay. The cholesterol content of the deficient cells was 1-2-fold higher than controls but contributed <12% to the total available substrate pool.

Low neutral lipase activity of WD cells was also evident when the hydrolysis of endogenous substrate was examined (Table 5). These experiments were complicated by the labeling of phospholipid also. Nevertheless, triglyceride disappearance, [2-^{3}H]glycerol release, and di- and monoacylglycerol formation of the WD cells were only 2–25% that of the control cells.

The possibility of an inhibitor in the lipase-deficient cells that was not present in the controls, or of an activator in the controls that was not present in the lipase-deficient cells, was examined by mixing experiments (Table 6). Activity in the mixture was similar to that predicted from the individual activities.

Table 5. *Hydrolysis of endogenous neutral glycerolipids by normal and acid-lipase deficient cultured human fibroblasts*[1]

Method of measuring hydrolysis	Activity in WD cells as % of control cells
Triacylglycerol disappearance	20%
[2-^{3}H]glycerol release	25%
[2-^{3}H]diacylglycerol formed	4%
[2-^{3}H]monoacylglycerol formed	2%

[1] 10×10^6 confluent fibroblasts were incubated with minimal essential medium (MEM) plus 10% FCS containing 0.2 μmole oleic acid/ml; 5 μg insulin/ml; and 1 μCi (1 μmole) [^{3}H]glycerol/ml. After 48 h, the medium was removed, and MEM plus 10% FCS containing 30 μmole unlabeled glycerol/ml was added for 0.5 h. The medium was removed and the cells harvested by scraping. Control cells contained 52,000 dpm/mg protein; 30% of the label was in neutral lipid and 70% in phospholipid. Wolman disease's (WD) cells contained 31,000 dpm/mg protein; 44% of the label was in neutral lipid and 56% in phospholipid. The sonicated cells were incubated for 2 h at pH 6.5 under neutral lipase conditions, as described in "Materials and Methods."

Table 6. *Neutral lipase activity in control and lipase-deficient cells before and after mixing*

Cells	Protein (mg/ml)	Fatty acid	Diglyceride	Monoglyceride
Control	1.0	228[1]	35	16
	0.5	180	33	30
WD[2]	1.0	10	2	1
Mixture[3]-Control	0.5	161	31	9
WD	0.5			

[1] nmole product formed $\cdot 2\ h^{-1} \cdot$ mg protein^{-1}.

[2] WD, Wolman's disease.

[3] Equal amounts of control and WD fibroblast sonicates were mixed together before incubation.

DISCUSSION

In this study, we confirmed the presence, in cultured fibroblasts, of a neutral lipase with optimum activity between pH 6 and 7. The failure of several groups to detect this activity during studies of the acid lipase, in which the effects of pH were studied up into the neutral range, is likely due to inappropriate reaction conditions, in particular low substrate concentrations and the use of detergents. We have also documented the apparent lack of a monoglyceride-hydrolysing capability in cultured fibroblasts under acid lipase conditions. Purified hepatic acid lipase has no monoacylglycerol lipase activity (24) and so several tissues may not have an acid monoacylglycerol-hydrolysing capability. This observation could be useful in separating the contributions of acid and neutral activities to overall tissue lipase activities in that only the neutral lipase is capable of releasing [^{3}H]glycerol from [^{3}H] glycerol-labeled triacylglycerol.

Since the original reports of hepatic acid hydrolase deficiency in WD (20) and of acid lipase deficiency in CESD (2, 22), several laboratories have reported very low acid hydrolase activities in cultured fibroblasts from WD or CESD subjects using either natural or artificial substrates (1, 3, 6, 10). Neutral lipase activity has rarely been examined. In their original report on the WD defect, Patrick and Lake (20) reported that hepatic neutral lipase was normal. The activity was measured using 15 mM cholesterol oleate or cholesterol acetate as substrate, in the presence of 16% Triton; such conditions would markedly inhibit the neutral lipase activity of fibroblasts. Sloan and Frederickson (22) reported that hepatic neutral lipase activity was normal in CESD subjects. The activity that they measured at pH 5.5 was 65%, and at pH 7.4 was 1% of the activity measured at pH 4.0, a pattern that is distinctly different from that seen in cultured fibroblasts. Hoeg *et al.* (12) studied neutral lipase activity in liver and cultured fibroblasts from subjects with Wolman's disease and controls. Fibroblast activity was only 10% of the activity observed in our studies, probably as a result of quite different methods of substrate preparation. The authors reported values for the mutant cells that were similar to controls. The presence of normal hepatic neutral lipase activity in patients with WD could be explained by the presence of different lipases in different tissues or different reaction conditions. The difference in results with fibroblasts between Hoeg *et al.* and ourselves probably relates to different reaction conditions also. If higher rates are a reflection of more physiologic conditions, then our reaction conditions would seem to be more appropriate. In any case, there is a marked difference in neutral lipase activity between WD and control cells, suggesting a significant, probably genetic, relationship between acid and neutral lipase activities.

The exact relationship between the fibroblast neutral and acid activities is not known, and possibilities include a common subunit or segment. Studies of purified lipase from liver (24), suggest a dimeric or multimeric aggregate structure. The placental enzyme (4) was isolated in two forms of differing molecular weight but with similar kinetic properties and substrate specificities. The authors suggested one form might be a multimer of the other, but only one protein band was seen on SDS-polyacrylamide gel electrophoresis.

Another possibility is that both activities are catalysed by the same enzyme protein, or by a protein with the same primary structure but differing in post-translational modification (11, 18). In the latter circumstance, one activity would be the precursor of the other. Both activities could be physiologically relevant, and reflect normal function in two or more subcellular locations that differ widely in environment. Alternatively, only the acid activity may be physiologically relevant, and the neutral activity in an *in vitro* phenomenon. This seems less likely in view of the low activity in WD cells with the endogenous substrate. Post-translational modification of several lysosomal hydrolases has been demonstrated and in some cases, the precursors of the final product have enzyme activity also (11, 18).

If a portion, or indeed all of the enzyme protein is common to both the acid and neutral activities, then this portion, at least, must be present in WD and CESD cells to support the modest levels of neutral activity observed. That this is so is supported by the observation that both mutant cell lines used in our study contain immunologically cross-reacting material to antibodies to acid lipase (5).

Whatever the mechanism may be, the results of this study support a genetic link between acid and neutral lipase activities in cultured fibroblasts. Whether such a link is peculiar to these lipase activities only, or is common to other lysosomal hydrolases that have neutral counterparts elsewhere in the cell remains to be determined.

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