Low density lipoprotein (LDL) internalization by mutant type C Niemann-Pick (NPC) fibroblasts results in uptake of excess total cholesterol. Uptake of excess lipoprotein cholesterol appears to be mediated by the specific LDL receptor pathway. Associated with excessive LDL-cholesterol uptake is a lesion in early intracellular cholesteryl ester synthesis. In vitro acyl-CoA:cholesterol acyltransferase activity is normal in cell-free extracts of mutant cells. The ability of exogenous sterols to enhance intracellular esterification of [3H]mevalonate-derived [3H]cholesterol was severely limited in mutant cell cultures suggesting that in vivo activation and/or expression of activated acyl-CoA:cholesterol acyltransferase may be compromised by the primary mutation of type C Niemann-Pick disease. After 2 days of LDL uptake, rates of intracellular cholesteryl ester synthesis in mutant cells paralleled the rates of esterification in normal cells suggesting that specific early in vivo expression of the acyltransferase may be affected in this disorder.

Previous studies have indicated that the unique errors of cholesterol processing associated with type C Niemann-Pick disease are closely linked with the primary genetic mutation of human metabolic disorder (1). Cellular cholesterol homeostasis in normal cultured fibroblasts has been elegantly shown to be tightly regulated through the expression and control of major regulatory mechanisms associated with the LDL uptake pathway (2–5). Reciprocal controls in the expression of the LDL receptor (6), 3-hydroxy-3-methylglutaryl coenzyme A reductase (7), and acyl-CoA: cholesterol acyltransferase (ACAT) (8) interact to limit both the total uptake of LDL-derived cholesterol and to maintain the cellular levels of unesterified cholesterol within the narrow range needed for membrane synthesis and cell growth. In heterozygous and homozygous type C Niemann-Pick fibroblasts, excessive uptake of LDL cholesterol and deficiency in the esterification of the internalized cholesterol indicate that major regulatory mechanisms associated with the LDL uptake pathway have been compromised by the primary mutation of this disorder. The present studies characterize the cholesterol processing lesions of mutant type C Niemann-Pick fibroblasts.

MATERIALS AND METHODS

Cells and Tissue Culture—Normal and mutant type C Niemann-Pick fibroblasts originated from the National Institute of General Medical Science, Human Genetic Mutant Cell Repository (Camden, NJ). Homozygous and heterozygous type C Niemann-Pick cells were also obtained from the Dept. of Pediatrics, Colorado Health Science, Denver, CO. Cells were maintained in Eagle’s minimal essential medium with 10% fetal bovine serum, 1% L-glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml) as described. Cell monolayers were harvested by treatment with 0.05% trypsin for 5 min. Specific experimental culture conditions are described in the appropriate sections.

Lipoprotein-deficient Serum, Native LDL, Modified LDL—Lipoprotein-deficient serum (>1.21 g/ml) was prepared from fetal bovine serum by ultracentrifugation from KBr/serum (9) and was purchased from Meloy Laboratories (Springfield, VA). The cholesterol content of this serum was determined to be less than 5% of normal serum. Human LDL was sequentially ultracentrifuged from fresh KBr/plasma (9) and was also obtained from Meloy Laboratories. Lysine-modified acetyl-LDL was prepared by mixing LDL with acetic anhydride at 0 °C as previously outlined (10). The modification of the arginy1 residues of LDL was carried out with 0.1 mM 1,2-cyclohexanediol as described (11). 125I-LDL was prepared by the iodine monochloride method as specifically outlined by Goldstein et al. (12). The reaction was carried out by mixing 7.5 µg of LDL with 3.0 mCi of Na125I. The final specific radioactivity of the 125I-LDL was 100 dpm/ng of protein and >99% of the radioactivity was precipitable with 10% trichloroacetic acid and 3.5% was extractable into chloroform/methanol (2:1).

Other Materials—[3H]Acetate (75–100 Ci/mmol), [9,10-3H]oleate (2–20 Ci/mmol), and [5-3H]mevalonate (2–10 Ci/mmol) were purchased from New England Nuclear. 25-Hydroxycholesterol was obtained from Research Plus, Inc., Bayonne, NJ. Other chemicals were purchased from Sigma.

Lipid Analysis—Total fibroblast homogenates were extracted with chloroform/methanol (2:1), and the level of radioactivity associated with [3H]cholesterol, cholesterol [3H]oleate, or [3H]cholesterol ester was analyzed by thin layer chromatography in a solvent of n-hexane/ether/glacial acetic acid (90:10:1) as previously outlined (13). Radioactivity associated with the respective lipid spots was scraped, and the radioactivity was counted in 10 ml of Aquasol by liquid scintillation spectroscopy.
The nature of the mechanism mediating cellular uptake and accumulation of excess lipoprotein cholesterol was characterized in mutant type C Niemann-Pick (NPC) fibroblasts (Table I). Studies confirming the specificity and receptor nature of the LDL uptake pathway have included criteria such as (a) blocked uptake of LDL-cholesterol through a modification of the lysine (10) or arginine (11) residues of the lipoprotein molecule, (b) saturation kinetics for uptake of LDL (14), and (c) requirement of Ca²⁺ for binding of LDL to its receptor (15). Cellular cholesterol accumulation by mutant NPC cells specifically required the presence of native LDL. No accumulation of cholesterol was observed in mutant as well as normal cell cultures when the lysine or arginine moieties of the LDL molecule were blocked. Uptake of LDL was saturable and cholesterol accumulation was blocked by the chelating agent, EGTA. Judged by the criteria above, excessive intracellular uptake of LDL cholesterol by type C Niemann-Pick fibroblasts appeared to be mediated by the LDL receptor pathway operable in normal cells.

In mutant NPC fibroblasts, the normal cellular homeostatic response that limits intracellular cholesterol synthesis in the presence of exogenous cholesterol (16) appears intact (Table II). [²⁵³]HAcetate incorporation into endogenously synthesized cholesterol was effectively suppressed in mutant and normal cells by the presence of 100 µg/ml LDL in the culture medium. This specific reduction of acetate incorporation into de novo synthesized cholesterol by LDL has been shown to be due to a suppression of hydroxymethylglutaryl-CoA reductase (17). The regulation of hydroxymethylglutaryl-CoA reductase is tightly linked to the cellular uptake and accumulation of exogenous sterol (18) and has been shown to be modulated through synthetic (19), covalent (20), and catabolic (21) mechanisms. The degree of suppression of the enzyme in cultured fibroblasts depends on the cumulative uptake of LDL-cholesterol (12).

The specific interaction of [¹²⁵]I-LDL with cultured fibroblasts has been studied to measure the levels of LD-channel receptor in the plasma membrane and to ascertain the involvement of the receptor in the internalization of the lipoprotein (6, 22). When normal and mutant NPC fibroblasts were first cultured in lipoprotein-deficient medium for 48 h and subsequently incubated for 6 h at 37 °C with fresh medium containing 20 µg/ml [¹²⁵]I-LDL, receptor specific binding, uptake, and catabolism of [¹²⁵]I-LDL were essentially the same in control and mutant cell cultures. Following 24 h of incubation with [¹²⁵]I-labeled LDL, cells were harvested and analyzed for [¹²⁵]I-acetate incorporation into unesterified cholesterol. Measurements were made on triplicate cultures of five separate cell lines.

We have previously shown that deficient esterification of exogenously derived cholesterol is a uniform lesion specifically expressed in mutant NPC fibroblasts (1, 13, 23, 24). The normal cellular response to an influx of exogenous sterols is enhanced intracellular synthesis of cholesteryl esters (17) catalyzed by acyl-CoA cholesterol acyltransferase (ACAT) (8). In mutant type C Niemann-Pick cells, defective cholesterol esterification is not associated with a discernible deficiency of ACAT activity measured in vitro in cell-free extracts (Table IV). In vitro ACAT activity was found to be elevated 30-fold in both mutant and normal cell extracts when cholesterol-depleted cells were incubated with 100 µg/ml LDL or 10% fetal bovine serum.

The underlying molecular basis regulating enhanced intracellular cholesterol esterification and ACAT activation in response to the cellular uptake of exogenous sterols is not well understood. Elevation in a substrate pool of cholesterol available to ACAT, covalent modification of ACAT, and modulation of the membrane environment of ACAT have all been suggested to play a role in regulating intracellular cholesterol esterification (25, 26). In vitro assays of ACAT activity in cell-free extracts do not allow one to readily distinguish many of the potential in vivo regulatory influences affecting ACAT expression intracellularly. Alternatively, in vivo-stimulated esterification of endogenously derived cholesterol, which can be induced secondarily through the cellular uptake of exogenous sterols (8, 27), offers two advantages over in vitro assays of ACAT: (a) the enzyme is monitored in its natural environment and (b) modifications in the levels of endogenous cholesterol esterified by the uptake of exogenous sterols likely

#### Table I

**LDL receptor-mediated nature of lipoprotein cholesterol uptake and accumulation in mutant type C Niemann-Pick fibroblasts**

| Culture conditions | Total cellular cholesterol (nmol/mg protein) | Normal | Mutant |
|--------------------|---------------------------------------------|--------|--------|
| Lipoprotein deficient serum/6 days |                               |        |        |
| +10 µg/ml LDL/24 h  | 28 ± 4                                      | 28 ± 5 |        |
| +50 µg/ml LDL/24 h  | 80 ± 10                                     | 102 ± 10|        |
| +100 µg/ml LDL/24 h | 98 ± 10                                     | 142 ± 15|        |
| +200 µg/ml LDL/24 h | 110 ± 10                                    | 149 ± 10|        |
| +100 µg/ml lysine-blocked LDL/24 h | 110 ± 10              | 154 ± 10|        |
| +100 µg/ml arginine-blocked LDL/24 h | 42 ± 15                 | 30 ± 10 |        |
| +100 µg/ml LDL and 2 mM EGTA/24 h | 30 ± 5                         | 30 ± 6  |        |

#### Table II

**Synthesis of [³H]cholesterol from [³H]acetate in fibroblasts cultured in presence and absence of LDL**

| Culture conditions | [³H]Cholesterol (pmol/³Hacetate/mg protein) |
|--------------------|--------------------------------------------|
| Lipoprotein-deficient culture |                       |
| medium/6 days |                                   |
| +LDL (100 µg/ml), 24 h | 1800 ± 1000                  | 1800 ± 900     |

| Culture conditions | [³H]Cholesterol (pmol/³Hacetate/mg protein) |
|--------------------|--------------------------------------------|
| Normal (5) |                                   |
| Niemann-Pick C (5) |                       |

In vivo regulatory influences affecting ACAT expression intracellularly. Alternatively, in vivo-stimulated esterification of endogenously derived cholesterol, which can be induced secondarily through the cellular uptake of exogenous sterols (8, 27), offers two advantages over in vitro assays of ACAT: (a) the enzyme is monitored in its natural environment and (b) modifications in the levels of endogenous cholesterol esterified by the uptake of exogenous sterols likely

#### Table III

**Synthesis of [³H]cholesterol from [³H]acetate in fibroblasts cultured in presence and absence of LDL**

Stock cells were maintained as described in Eagle's minimal essential medium for 4 days with 10% fetal bovine serum. Cells were harvested and seeded at a level of 2 x 10⁵ cells in 25-cm² flasks in 3.0 ml of McCoy's medium supplemented with 10% lipoprotein-deficient serum for 2 days. The cell medium was replaced with fresh medium and supplemented as indicated with native or modified LDL at various concentrations. Following 24 or 48 h of incubation, cells were harvested, and the mass levels of total cholesterol were determined by an enzymatic, fluorometric method described previously (1).
The procedures developed and outlined by Goldstein and Brown (11) were employed. Stock cells were cultured in 35-mm petri dishes at an initial density of 3 x 10⁶ cells for 48 h in 3 ml of Eagle's minimal essential medium and 10 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% (w/v) glucose, and 10% (w/v) fetal bovine lipoprotein-deficient serum at pH 7.4. Fresh medium ± LDL was added to the cell monolayers for 24 h. Medium was withdrawn and cells were washed and subsequently incubated for 6 h at 37°C in fresh medium containing 20 μg/ml 125I-LDL. Cell cultures were quickly brought to 4°C and medium was removed and saved. After washing, cell monolayers were treated with heparin (10 μg/ml) for 1 h at 4°C. Medium was removed and saved. Cells were quickly washed three times with buffer and cell monolayers were dissolved in 1 ml of 0.5 N NaOH. Cell surface-associated 125I-LDL represented the radioactivity released when cells were incubated with heparin. Intracellular 125I-LDL represented total radioactivity remaining associated with the cell monolayers after heparin treatment. Catabolic excretion of 125I-LDL represents the trichloroacetic acid-soluble 125I radioactivity found in the medium. This was specifically determined by adding 0.250 ml of medium from cells incubated with 125I-LDL/6 h to 0.100 ml of bovine serum albumin (25% w/v) and 0.550 ml of H₂O. Following addition of 0.100 ml of trichloroacetic acid (100% w/v), the solution was vigorously vortexed and allowed to stand at 4°C overnight. Ambient centrifugation at 5000 × g/15 min gave a clear supernatant which was further treated with KI, H₂O₂, and chloroform as described (11). Receptor-specific interactions were vorked from the difference in radioactivity found in the absence and presence of excess nonradioactive LDL (500 μg/ml medium). Two normal and two Niemann-Pick C cell lines were tested in triplicate cell cultures.

Table I

| Receptor-mediated cellular responses | LDL preincubation | Cell cultures | 1 | 2 | 3 | 4 |
|-------------------------------------|-------------------|--------------|---|---|---|---|
| 100 μg/ml/24 h                      |                   |              | 1 | 2 | 3 | 4 |
| Cell surface binding                |                   |              | 1 | 2 | 3 | 4 |
| +                                   | 740 ± 250         |              | 350 ± 200 | 380 ± 80 | 440 ± 170 | 440 ± 170 |
| Cellular uptake                     |                   |              | 90 ± 20 | 40 ± 15 | 170 ± 40 | 160 ± 50 |
| +                                   | 1700 ± 200        |              | 1110 ± 140 | 1200 ± 160 | 1100 ± 60 | 1100 ± 60 |
| Cellular catabolism and excretion   |                   |              | 300 ± 30 | 170 ± 30 | 670 ± 30 | 850 ± 50 |
| +                                   | 4200 ± 200        |              | 3900 ± 1000 | 1800 ± 400 | 1800 ± 800 | 1800 ± 800 |
|                                     | 300 ± 250         |              | 300 ± 100 | 500 ± 200 | 700 ± 100 | 700 ± 100 |

Table IV

In vitro activity of acyl-CoA:cholesterol acyltransferase (ACAT) in cell-free extracts of fibroblasts cultured in presence and absence of LDL

Cells were cultured as described with Eagle's minimal essential medium and 10% (v/v) lipoprotein-deficient fetal bovine serum for 3 days and subsequently seeded in 75-cm² flasks at a density of 5 x 10⁶ cells and cultured an additional 48 h in McCoy's medium with 10% (v/v) lipoprotein-deficient serum. Cell cultures were in turn incubated with fresh medium ± LDL (100 μg/ml) or complete fetal bovine serum (10% v/v) for the indicated time intervals. Cell monolayers were washed, harvested, and stored at −80°C. Frozen cell pellets were suspended in 0.100 ml of 10 mM phosphate buffer and 4 mM dithiothreitol, pH 7.4, to provide a protein concentration of 1-2 mg/ml. This was specifically determined by adding 0.250 ml of medium from cells incubated with 125I-LDL/6 h to 0.100 ml of bovine serum albumin (25% w/v) and 0.550 ml of H₂O. Following addition of 0.100 ml of trichloroacetic acid (100% w/v), the solution was vigorously vortexed and allowed to stand at 4°C overnight. Ambient centrifugation at 5000 × g/15 min gave a clear supernatant which was further treated with KI, H₂O₂, and chloroform as described (11). Receptor-specific interactions were vorked from the difference in radioactivity found in the absence and presence of excess nonradioactive LDL (500 μg/ml medium). Two normal and two Niemann-Pick C cell lines were tested in triplicate cell cultures.

Table V

In vitro activity of acyl-CoA:cholesterol acyltransferase (ACAT) in cell-free extracts of fibroblasts cultured in presence and absence of LDL

Cells were cultured as described with Eagle's minimal essential medium and 10% (v/v) lipoprotein-deficient fetal bovine serum for 3 days and subsequently seeded in 75-cm² flasks at a density of 5 x 10⁶ cells and cultured an additional 48 h in McCoy's medium with 10% (v/v) lipoprotein-deficient serum. Cell cultures were in turn incubated with fresh medium ± LDL (100 μg/ml) or complete fetal bovine serum (10% v/v) for the indicated time intervals. Cell monolayers were washed, harvested, and stored at −80°C. Frozen cell pellets were suspended in 0.100 ml of 10 mM phosphate buffer and 4 mM dithiothreitol, pH 7.4, to provide a protein concentration of 1-2 mg/ml. Total cell extracts (0.025 ml) were incubated with 0.020 ml of stock cofactor-substrate solution (25 mM ATP, 2.5 mM CoA, 80 mM MgCl₂, and 1.5 mM [14C]oleate with specific activity of 370 dpm/pmol in 3.5% bovine serum albumin and 50 mM Tris-HCl, pH 7.4) and with 0.170 ml of stock buffer solution (250 mM sucrose, 50 mM Tris-HCl, pH 7.4, 2 mM diethiothreitol, 50 mM KF, and 0.1 mM phenylmethylsulfonyl fluoride) for 2 h at 37°C. Incubations were subsequently analyzed for [14C]oleate incorporation into cholesteryl [14C]oleate. Assays were all carried out in triplicate.

Culture conditions

| Lipoprotein-deficient medium/5 days | Normal (5) | Niemann-Pick (5) |
|------------------------------------|------------|-----------------|
| Acyl-CoA:cholesterol               |            |                 |
| acyltransferase activity           |      pmol cholesterol | [14C]oleate/mg protein/h |
| Normal (5)                         | 30 ± 10    | 40 ± 20         |
| +LDL (100 μl/ml)/6 h               | 200 ± 100  | 200 ± 60        |
| +LDL (100 μl/ml)/24 h              | 1000 ± 200 | 800 ± 100       |
| +Fetal bovine serum (10%)/24 h     | 800 ± 200  | 900 ± 400       |

represents in part cellular activation of ACAT activity beyond simple elevations in total cholesterol available for esterification (3). It has been previously shown, for example, that exogenous sterols enhance esterification of endogenously derived cholesterol even under conditions where cellular levels of unesterified cholesterol are severely depleted (8). When expression of ACAT activity was monitored in intact normal and mutant fibroblasts through the specific esterification of [3H]mevalonate-derived [3H]cholesterol, a deficiency in the ability of exogenous sterols to stimulate the esterification of the de novo cholesterol was observed with the mutant cultures (Table V). The intracellular pool of cholesterol was labeled in normal and mutant cells by incubating the cultures with 1 μM [3H]mevalonate for 24 h. Under these conditions, cellular mass levels of unesterified cholesterol were not elevated and little of the [3H]mevalonate-derived [3H]cholesterol was esterified. The addition of exogenous sterols (LDL-cholesterol, cholesterol, 25-hydroxycholesterol) to the culture medium of normal fibroblasts caused an extensive net stimulation in the incorporation of [3H]mevalonate-derived [3H]cholesterol radioactivity into newly synthesized [3H]cholesterol esters. Stimulated [3H]cholesterol ester synthesis was also observed when normal fibroblasts were cultured with lipoprotein prior to labeling of the endogenous [3H]cholesterol pool with [3H]mevalonate. In contrast to the high level of stimulation in cholesteryl ester synthesis seen in normal cells, uptake of exogenous sterols by mutant type C Niemann-Pick fibroblasts was associated with a much smaller level of enhanced [3H]cholesterol ester formation which ranged from 10–40% of normal (Table V).

Further indirect means of measuring ACAT activity in vivo involved monitoring the cumulative and daily rate of total intracellular cholesteryl ester synthesis induced in cultured fibroblasts over a 4-day period by the cellular uptake of LDL cholesterol (Fig. 1). During the 1st day of incubation with LDL, cholesteryl [3H]oleate synthesis in mutant cells was 6% of normal. [3H]Oleate incorporation into newly synthesized triglycerides was normal in normal NPC cells during this period (data not shown). By day 4, the total cumulative level of cholesterol esterification in mutant cells had reached 50%
The esterification of \(^{[3]H}\)mevalonate-derived \(^{[3]H}\)cholesterol in fibroblasts cultured in presence and absence of exogenous sterols

Cells were cultured in 10% (v/v) lipoprotein-deficient serum for 4 days and subsequently seeded in 25-cm\(^2\) flasks at 2 \times 10^3 cells for 2 days in McCoy's medium and 10% (v/v) lipoprotein-deficient serum. Culture medium containing 10% complete fetal bovine serum was added to some of the cultures for 2 or 3 days as designated. Experiments were initiated by the addition to all the cell cultures of fresh McCoy's medium containing 1 mm \(^{[3]H}\)mevalonate (18 pmol/pmnl) prepared by mixing 0.100 ml of 300 mm \(^{[3]H}\)mevalonate in ethanol with 30 ml of media. Cells were incubated with \(^{[3]H}\)mevalonate for a total of 48 h. Cell cultures treated with 10% fetal bovine serum retained the complete serum supplement during the entire final 48 h of incubation with \(^{[3]H}\)mevalonate. To the remaining cell cultures, sterol additions were made as noted during the final 24 h of incubation with \(^{[3]H}\)mevalonate. The addition of non-lipoprotein cholesterol and 25-hydroxycholesterol was carried out by mixing 0.012 ml of stock ethanolic sterol solutions with 3.0 ml of McCoy's medium prior to the addition of 3.0 ml of this solution and 1 mm \(^{[3]H}\)mevalonate to the cell monolayers. Cell layers were washed, harvested, and subsequently analyzed for incorporation of \(^{[3]H}\)mevalonate into \(^{[3]H}\)cholesterol and \(^{[3]H}\)cholesterol ester. In the absence of exogenous sterols, incorporation of \(^{[3]H}\)mevalonate into esterified \(^{[3]H}\)cholesterol ranged between 7 and 11 nmol/mg of protein/48 h for both normal and mutant Niemann-Pick C fibroblasts. The level of \(^{[3]H}\)mevalonate incorporation into free \(^{[3]H}\)cholesterol declined approximately 60% upon the addition of the exogenous sterols presumably because of the secondary feedback inhibition of squalene synthase (28). The values in parentheses refer to the number of individual cell lines tested for each experimental manipulation. Individual cell lines were tested in triplicate.

| Cell culture | \(^{[3]H}\)Mevalonate-generated \(^{[3]H}\)cholesterol ester synthesis | Normal | Niemann-Pick C |
|--------------|---------------------------------------------------------------|--------|---------------|
| Lipoprotein-deficient medium/6 days | 50 ± 10 | 30 ± 10 |
| +LDL (100 \(\mu\)g/ml)/24 h (5) | 1000 ± 300 | 100 ± 70 |
| +cholesterol (50 \(\mu\)g/ml)/24 h (2) | 400 ± 40 | 80 ± 10 |
| +25-Hydroxycholesterol (5 \(\mu\)g/ml)/24 h (2) | 1200 ± 150 | 500 ± 100 |
| Fetal bovine serum/2 days (2) | 800 ± 100 | 100 ± 20 |
| Fetal bovine serum/3 days (2) | 1500 ± 300 | 400 ± 150 |

of normal. When the daily rates of cholesteryl \(^{[3]H}\)cholesterol formation were compared in normal and mutant cultures during the 4 days of incubation with LDL, cholesterol esterification in mutant cells was found to be 6% of normal at day 1 and 37% of normal by day 2. During the remaining 48 h of incubation, cholesteryl ester synthesis in mutant cells remained 10-30-fold higher than baseline levels and was equal to that found in normal cells during this period of time.

**DISCUSSION**

We have shown that mutant type C Niemann-Pick (NPC) fibroblasts are deficient in expression of two of the specific regulatory responses associated with cellular cholesterol homeostasis. These anomalous regulatory responses represent excessive uptake and deficient esterification of LDL-derived cholesterol. The metabolic consequence of these lesions is excessive cellular accumulation of LDL-derived cholesterol that is stored predominantly as unesterified cholesterol. A close metabolic proximity of these two lesions to the primary genetic mutation of type C Niemann-Pick disease is suggested by the observation of partial manifestations of these lesions in heterozygous NPC fibroblasts (1).

Several notable features characterize the errors in cholesterol processing in type C Niemann-Pick fibroblasts: (a) although the excessive accumulation of LDL-cholesterol indicates less regulation in the uptake of the lipoprotein, internalization of LDL-cholesterol nevertheless appeared to be mediated by the specific LDL-receptor pathway (Table I); (b) uptake of excess LDL-cholesterol by mutant fibroblasts may be associated with less effective suppression of LDL receptor-mediated \(^{125}\)I-LDL uptake in affected cells as compared to control fibroblasts (Table III); (c) following uptake of LDL-cholesterol by mutant fibroblasts, intracellular cholesteryl ester synthesis is blocked in spite of normal in vitro levels of ACAT activity and a high intracellular accumulation of unesterified cholesterol (Table IV); (d) exogenously derived sterols were largely ineffective in stimulating the esterification of endogenous \(^{[3]H}\)cholesterol in mutant fibroblasts (Table V); (e) the deficiency in cholesterol esterification associated with LDL uptake by NPC fibroblasts diminished as the incubation interval of mutant cells and lipoprotein was extended (Fig. 1).

The apparent involvement of specific LDL receptor-mediated endocytosis in the accumulation of excess lipoprotein-cholesterol by mutant cells suggests that a critical response in the uptake pathway is compromised by the NPC mutation. Excessive receptor-mediated uptake of \(^{125}\)I-LDL by mutant cells previously exposed to LDL may imply that normal down-regulation of the LDL receptor is delayed in affected cells. Alternatively, the prolonged expression of LDL uptake may reflect some other alteration in the properties of the receptor or the uptake pathway itself. Further elucidation of the perturbations in this pathway will require kinetic studies of LDL uptake in mutant NPC fibroblasts.

Although normal levels of ACAT activity were found in cell-free extracts of type C Niemann-Pick cells, intracellular expression of ACAT activity appeared blocked in these mutant cells. Although other possible lesions cannot be ruled out at this time, studies to date suggest that activation or expression of ACAT catalytic activity may, particularly during the early phases of cholesterol uptake, be compromised by the NPC mutation. This possibility is supported by the following findings: (a) exogenous sterols were deficient in stimulating the esterification of \(de novo\) derived cholesterol in mutant cells; (b) impairment in cholesteryl ester synthesis in affected cultures was primarily observed during the first 48 h of incubation with LDL-cholesterol and was followed by normal rates of cholesterol esterification during the latter stages of LDL processing.

The \(in vivo\) failure of ACAT expression in mutant NPC fibroblasts may reflect a direct or indirect lesion in ACAT activation. The mechanism of activation may be comprised itself or the exogenously derived sterols may not reach the intracellular site which triggers activation. Accumulation of LDL-derived cholesterol in mutant NPC fibroblasts has been shown to occur primarily within perinuclear inclusions reminiscent of secondary lysosomes (1, 23). If this abnormal cellular sequestering of cholesterol does indeed prove to represent abnormal lysosomal storage of the sterol, intracellular cholesterol accumulation may reflect a block in the ability of the sterol to be transported out of these organelles for further processing. On the other hand, the possibility of lysosomal deposition of excess exogenous cholesterol may also reflect a secondary cellular response to blocked esterification. If it can be assumed that the cellular uptake of non-lipoprotein cholesterol does not require passage through lysosomes as does LDL-cholesterol (29, 30), then failure of \(^{[3]H}\)cholesterol to be esterified in mutant NPC cells (23) may indicate that the processing error is postlysosomal.
It has been shown that the affinity of the surface receptor for LDL is substantially affected when cell binding is studied at 4 °C rather than at 37 °C (22). Such fluctuations in temperature affect the fluidity of membranes as would also elevated levels of unesterified cholesterol (31). In analogous fashion, overloading of membranes with cholesterol that can- not be processed by ACAT may also possibly begin to compromise regulatory responses of receptor-mediated LDL uptake in NPC fibroblasts. Previous tissue culture studies have shown that a specific inhibition of ACAT leads to a reduction in cholesteryl ester synthesis and a corresponding increase in intracellular levels of unesterified cholesterol (32, 33). This intracellular inhibition in cholesteryl ester formation did not, however, appear to be associated with an abnormal or elevated uptake of LDL-cholesterol (32, 34).

In conclusion, a malfunction of cellular cholesterol homeostasis has been found in cultured type C Niemann-Pick fibroblasts which appears relevant to the primary mutation of this inborn error of metabolism. The significance of lesions in both the uptake and esterification of lipoprotein-derived cholesterol fibroblasts remains to be established. The mutual appearance of both errors may reflect a common or causal relationship with the NPC mutation. It also remains to be established how these tissue culture observations relate to the clinical pathogenesis of this disorder.

Acknowledgments—We acknowledge Rufus Seabron for technical assistance and Carol Kosh for help in preparation of the manuscript.

REFERENCES

1. Kruth, H. S., Comly, M. E., Butler, J. D., Vanier, M. T., Fink, J. K., Wenger, D. A., Patel, S., and Pentchev, P. G. (1986) J. Biol. Chem. 261, 16769-16774
2. Goldstein, J. L., and Brown, M. S. (1976) Curr. Top. Cell. Regul. 11, 147-181
3. Goldstein, J. L., and Brown, M. S. (1977) Annu. Rev. Biochem. 46, 897-930
4. Brown, M. S., and Goldstein, J. L. (1983) Annu. Rev. Biochem. 52, 223-251
5. Brown, M. S., and Goldstein, J. L. (1986) Science 232, 34-47
6. Brown, M. S., and Goldstein, J. L. (1975) Cell 6, 307-316
7. Brown, M. S., Dana, S. E., and Goldstein, J. L. (1974) J. Biol. Chem. 249, 789-796
8. Brown, M. S., Dana, S. E., and Goldstein, J. L. (1975) J. Biol. Chem. 250, 4025-4027
9. Havel, R. J., Eder, H. A., and Bragdon, J. N. (1955) J. Clin. Invest. 34, 1345-1353
10. Basu, S. K., Goldstein, J. L., Anderson, C. G. W., and Brown, M. S. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 3178-3182
11. Mahley, R. W., Innerarity, T. L., Pitas, R. E., Weisgraber, K. H., Brown, J. H., and Gross, E. (1977) J. Biol. Chem. 252, 7279-7287
12. Goldstein, J. L., Basu, S. K., and Brown, M. S. (1983) Methods Enzymol. 98, 241-260
13. Farnham, P. G., Comly, M. E., Kruth, H. S., Patel, S., Festa, M., and Wintroub, H. (1986) J. Biol. Chem. 261, 2772-2777
14. Brown, M. S., Faust, J. R., and Goldstein, J. L. (1975) J. Clin. Invest. 55, 783-793
15. Goldstein, J. L., and Brown, M. S. (1974) J. Biol. Chem. 249, 5135-5162
16. Brown, M. S., Dana, S. E., and Goldstein, J. L. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 2162-2166
17. Goldstein, J. L., Dana, S. E., and Brown, M. S. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 4288-4292
18. Goldstein, J. L., and Brown, M. S. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 2804-2806
19. Luskey, K. L., Faust, J. R., Chin, D. J., Brown, M. S., and Goldstein, J. L. (1983) J. Biol. Chem. 258, 8462-8469
20. Kennelly, P. J., and Rodwel, V. W. (1985) J. Lipid Res. 26, 903-914
Regulatory Lesion in Uptake and Esterification of LDL-cholesterol

21. Chin, D. J., Gil, G., Faust, J. R., Goldstein, J. L., Brown, M. S., and Luskey, K. L. (1986) Mol. Cell. Biol. 5, 634–641
22. Goldstein, J. L., Basu, S. K., Brunschede, G. Y., and Brown, M. S. (1976) Cell 7, 85–95
23. Pentchev, P. G., Comly, M. E., Kruth, H. S., Vanier, M. T., Wenger, D. A., Patel, S., and Brady, R. O. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 8247–8251
24. Vanier, M. T., Rousson, R., Zeitouni, R., Pentchev, P. G., and Louisot, P. (1985) Proceedings of the International Symposium "Enzymes of Lipid Metabolism Part II" Strasbourg, France, (Freusz, L., and Gah, S., eds) pp. 791–802, Plenum Press, New York
25. Spector, A. A., Mathur, S. N., and Kaduce, T. L. (1979) Prog. Lipid Res. 18, 31–53
26. Suckling, K. E., and Strange, S. F. (1985) J. Lipid Res. 26, 647–671
27. Brown, M. S., Dana, S. E., and Goldstein, J. L. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2925–2929
28. Brown, M. S., and Goldstein, J. L. (1980) J. Lipid Res. 21, 505–517
29. Goldstein, J. L., Dana, S. E., Faust, J. R., Beaudet, A. L., and Brown, M. S. (1975) J. Biol. Chem. 250, 8487–8495
30. Goldstein, J. L., Brunschede, G. Y., and Brown, M. S. (1975) J. Biol. Chem. 250, 7854–7862
31. Yeagle, P. L. (1985) Biochim. Biophys. Acta 822, 267–287
32. Goldstein, J. L., Faust, J. R., Dygos, J. H., Chorvat, R. J., and Brown, M. S. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 1877–1881
33. McGookey, D. J., and Anderson, R. G. W. (1983) J. Cell Biol. 97, 1156–1168
34. Tabas, I., Weiland, D. A., and Tall, A. R. (1986) J. Biol. Chem. 261, 3147–3155