# ISOLATED HEPATOCYTES FROM HYPERCHOLESTEROLEMIC RATS SECRETE DISCOIDAL LIPOPROTEINS

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Received 25 January 1982

## 1. Introduction

Abnormal high density lipoproteins have been reported in plasma from human subjects with LCAT deficiency [1] and cholestasis [2]. These lipoproteins differ from normal plasma HDL in that they are deficient in core lipids, discoidal in shape and are apo-E rich rather than being spherical and having apo-AI as their major apoprotein. Substantial evidence exists which shows that abnormal lipoproteins can accumulate in the plasma of patients having other disorders in which secondary LCAT deficiency occurs [3]. This enzyme is believed to be responsible for the conversion of nascent discoidal HDL to spherical serum HDL, catalyzing this conversion in vitro [4]. This transformation is accomplished by the accumulation of an apolar core of cholesterol ester derived from the 2 major surface lipid constituents of the discoidal bilayer, cholesterol and phospholipid. In [4] a reduction in the Apo-E content of the resultant spherical HDL particles was observed with an increase in the apo-E content of the incubated human plasma VLDL fraction as a consequence of this LCAT mediated conversion.

De novo synthesis of discoidal, apo-E rich HDL by isolated perfused rat livers was shown [5] when an inhibitor of LCAT (DTNB) was added to the perfusate. These nascent discoidal lipoproteins subsequently proved to be a good substrate for LCAT [5].

*Abbreviations:* LCAT, lecithin–cholesterol acyl-transferase; VLDL, very low density lipoproteins; HDL, high density lipoproteins; DTNB, 5,5′-dithionitrobenzoic acid; EDTA, ethylenediaminetetraacetic acid; FC, free (unesterified) cholesterol; PL, phospholipid

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Discoidal, apo-E rich HDL accumulate in the plasma of guinea pigs in response to dietary cholesterol [6]. In this instance, an excess of free cholesterol may evoke a functional deficiency of LCAT by virtue of the higher cholesterol to phospholipid molar ratios (2:1) in the discoidal HDL of these cholesterol-fed animals [7,8].

Here we show that isolated hepatocytes from hypothyroid–hypercholesterolemic as opposed to normal rats, synthesize de novo both spherical and discoidal apo-E containing lipoproteins in the absence of any added LCAT inhibitor. We conclude that, in rats, an increased dietary and hepatic lipid load can induce the secretion and accumulation of discoidal lipoproteins in the extracellular milieu of hepatocyte preparations.

## 2. Materials and methods

Male, Long-Evans rats (Canadian Breeding Farms, St Constant PQ) (250–300 g body wt) were maintained on a thrombogenic diet (ICN Biochemicals, Montreal PQ) containing 40% butter fat, 2% cholate and 0.3% propylthiouracil for 45 days. These animals are hypothyroid [9], develop fatty livers and severe hypercholesterolemia [10].

### 2.1. *Preparation of isolated hepatocytes*

Isolated hepatocytes from hypercholesterolemic rats were prepared as in [11] with extensive cell washing (5–6 washes) to remove particulate fat. The hepatocytes from 2 livers were incubated at 37°C as a suspension for up to 24 h in 600 ml of Waymouth's MB 752/1 medium supplemented with 17.5% heat-inactivated, delipoproteinated horse serum, 10 000 U

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00145793/82/0000–0000/$02.75  

penicillin/ml and 0.05 mM streptomycin sulphate. The gas phase was 95% $O_2$:5% $CO_2$. Zero time was taken as the time of addition of neutralized solution of 1 mCi $^3$H-labelled amino acid mixture (New England Nuclear, Boston MA) to the incubation medium.

### 2.2. *Separation and isolation of the nascent lipoproteins*

After 24 h incubation a sample of the medium was centrifuged at 200 × *g* for 10 min to pellet the hepatocytes. Particulate fat [12] and membrane fragments [13] were removed from the cell-free medium by ultracentrifugation in a Beckman L5-50 ultracentrifuge at 100 000 × *g* for 30 min. Ethylenediaminetetraacetic acid (EDTA) and sodium azide ($NaN_3$) were added to 0.01% and 0.02% final conc. respectively, before concentration of the medium as in [11]. Concentrated incubation medium (20 ml) was applied to a 1.25 m × 2.5 cm Sephacryl-300 column (Pharmacia Fine Chemicals, Uppsala) and eluted with 0.9% NaCl containing 0.01% EDTA and 0.02% $NaN_3$ at pH 7.3 and 1.5 ml/min flow rate. Column fractions (5.3 ml/tube) were pooled as indicated and concentrated for rat apoprotein quantitation. Lipid analysis and electron microscopy were performed on lipoproteins isolated from the pooled column fractions by ultracentrifugation at 1.21 g/ml for 40 h at 100 000 × *g* [14,15]. All isolated lipoproteins were extensively dialysed against the column eluting buffer prior to analysis.

### 2.3. *Electron microscopy*

Negative stains of freshly isolated lipoproteins from pooled column eluates were prepared on Formvar-coated 200 mesh copper grids with 2% sodium phosphotungstate (pH 7.2). Photographs were taken with a Phillips 200 electron microscope within 48 h of staining.

### 2.4. *Lipid analyses*

Samples of the ultracentrifugally isolated lipoproteins from the pooled column fractions were prepared and analyzed for individual lipids as in [16] on a Hewlett-Packard model 5840A automatic gas chromatograph.

### 2.5. *Electroimmunoassays*

Electroimmunoassay of rat apo-B, -E and -AI were carried out as in [17,18]. The purified monospecific anti-rat apoprotein immunoglobulins showed no reactivity towards the delipoproteinated horse serum used here.

### 2.6. *Electrophoresis*

The electrophoretic mobilities of the nascent lipoproteins were determined by agarose gel electrophoresis of the concentrated, pooled, column eluates as in [19]. Urea–polyacrylamide gel electrophoresis was performed on the ultracentrifuge-isolated lipoproteins after delipidation in ethanol:ether (3:1) at −20°C [20] and solubilization in Tris–glycine buffer (pH 8.9) containing 7 M urea, 1% β-mercaptoethanol and 1% SDS [21]. Radioactivity associated with the apoproteins was determined after slicing and oxidizing the indicated regions of the gels as in [22].

## 3. Results and discussion

Gel filtration of the concentrated medium after a 24 h incubation of hepatocytes from hypercholesterolemic rats results in the elution profile shown in fig.1.

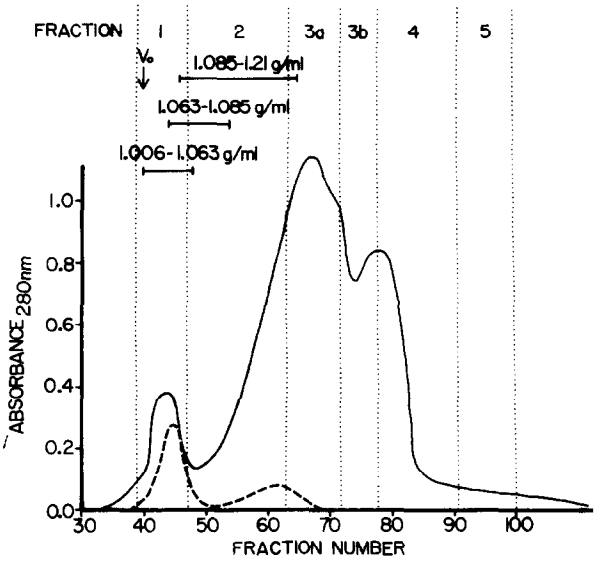
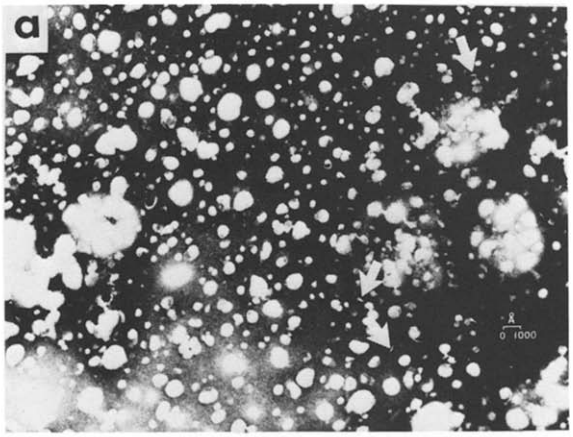
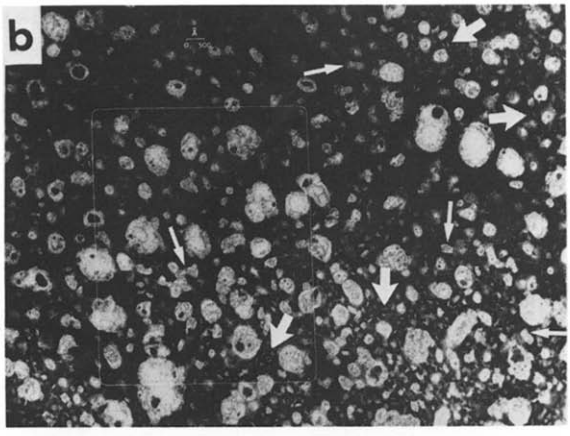



Fig.1. Elution profile of the concentrated incubation medium after 24 h incubation of hepatocytes from hypercholesterolemic rats on a Sephacryl-300 column (——). The profile is essentially that of the horse serum proteins, the secreted rat proteins being obscured due to their relatively small contribution to the overall medium protein content. 5.3 ml/tube was collected: (→) column void volume ($V_o$) and inner volume ($V_i$); (:) pooled eluates labelled fractions 1,2,3a,3b,4 and 5; (– – –) Elution profile of ultracentrifugally isolated rat serum lipoproteins of <1.21 g/ml; (|——|) elution range of 3 separate preparations of rat serum lipoproteins isolated at the density ranges indicated.

Nascent lipoproteins isolated from the pooled column eluates. Fractions 1 and 2 are shown in fig.2a and 2b,c, respectively. Fraction 1 is almost exclusively composed of spherical lipoproteins having a larger mean diameter (table 1), than the corresponding fraction from incubations of normal hepatocytes, (354 ± 5 Å) [9]. The discoidal particles observed in fraction 2 are absent in normal rat hepatocyte incubations and do not arise as a result of propylthiouracil treatment alone [9]. The dimensions of the discoidal lipoproteins (table 1) closely resemble those in [7] for discoidal HDL (252 × 51 Å) in the plasma of hypercholesterolemic guinea pigs. A slightly smaller particle (191.1 × 45.6 Å) was reported [5] in the perfusates of DTNB-treated rat livers and in human patients with cholestasis (190 × 44 Å) [2]. De novo synthesis of discoidal HDL by perfused livers from normal and hypercholesterolemic African green monkeys was shown in [23]. The tendency of the discoidal lipoproteins to stack into rouleau formations, (fig.2b,c) is



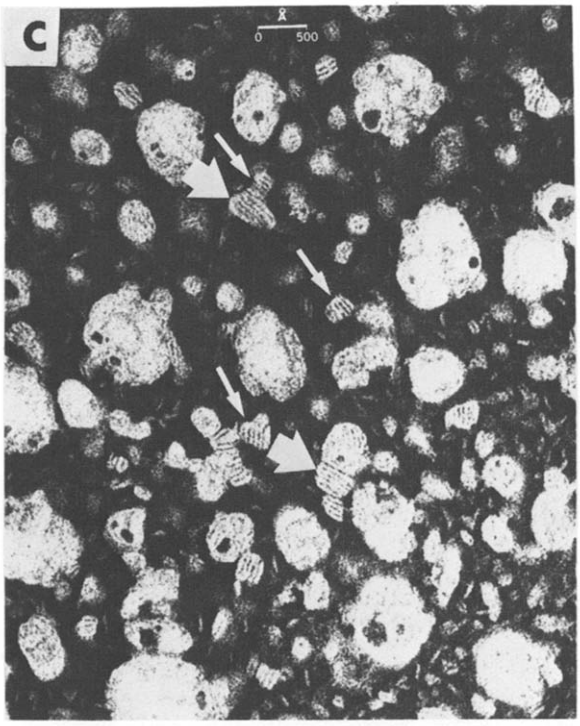




Fig.2. Electron micrographs of nascent lipoproteins from an incubation of hepatocytes from hypercholesterolemic rats: (a) lipoproteins isolated from fraction 1, ×24 000; (b) lipoproteins isolated from fraction 2, ×48 000; (c) higher magnification (×131 200) of the demarcated area in (b). Rouleau formations of discoidal particles in fraction 2 are indicated by small arrows (b,c). Individual discoidal particles viewed edge on can be seen in fraction 2 with very few in fraction 1 (medium arrows in (a,b)). Occasionally rouleaux of double length are observed in fraction 2 (large arrows in (c)) as well as other large aggregates.

characteristic of negatively stained preparations of these abnormal lipoproteins obtained from animal [5,7] or human studies [1,2].

Isolated perfused livers from hypercholesterolemic rats secrete increased quantities of lipids and apo-E [9] accounting, at least in part, for the elevated levels of these components in the sera of these animals. Hepatocytes from hypercholesterolemic rats continue to secrete increased quantities of lipids even after 24 h incubation in a lipid-deficient medium [9]. The latter system, by virtue of the longer incubation times possible, is more useful in studying hepatic HDL biosynthesis as the intracellular pool of nascent HDL is probably small [24]. Here, a significant amount of apo-E was secreted, after 24 h incubation, into frac-

Table 1
Properties of lipoproteins secreted into fractions 1 and 2

| | Fraction 1 | Fraction 2 |
|---|---|---|
| Shape | Spherical | Discoidal |
| Dimensions (A) | | |
| Diameter | 562 ± 12[a] | 227 ± 3[b]<br>202 ± 5[c] |
| Length | – | 54 ± 0.9[b]<br>56 ± 1.2[c] |
| Composition | μg secreted into each fraction | |
| | mg cell protein/24 h | |
| Free cholesterol | 4.7 ± 2.5 (3) | 2.2 ± 1.0 (3) |
| Phospholipid | 5.8 ± 2.4 (3) | 3.0 ± 1.0 (3) |
| Cholesterol ester | 7.6 ± 3.0 (3) | 1.6 ± 0.7 (3) |
| Triglyceride | 6.9 ± 2.0 (3) | 0.7 ± 0.2 (3) |
| Apo-B | 4.1 ± 1.8 (3) | 1.5 ± 0.4 (3) |
| Apo-E | 1.1 ± 0.4 (3) | 1.3 ± 0.6 (3) |
| Apo-AI | n.d. (2) | Trace (2) |

[a] Dimensions of 420 particles were measured
[b] Dimensions measured for 40 discoidal particles stacked in rouleaux
[c] Dimensions measured for 20 individual particles

Each value represents the mean ± SEM for (no. expt) or no. of particles measured; n.d. = not detected

tion 2 which is largely composed of discoidal particles (table 1). Only trace quantities of the major serum HDL apoprotein (apo-AI) could be detected immunologically in this fraction. This observation leads us to conclude that these discoidal particles more closely resemble the abnormal Lp-E particles observed in human LCAT deficiency [1] and cholestasis [25,26], than they resemble Lp-X particles. The β-migration of lipoproteins in fraction 2 on agarose gel electrophoresis (fig.3) has been observed for Lp-E particles in human cholestasis [26].

Urea–polyacrylamide gel electrophoresis of the apolipoproteins isolated from fractions 1 and 2 are shown in fig.4. The lack of C apoproteins observed in these fractions has been reported for incubations of normal hepatocytes [11]. Apo-E separation into ⩾2 bands on urea–polyacrylamide gels was observed in [10]. In fraction 1, approximately equal proportions of radioactivity are associated with apo-B and a thick band migrating as the lower protein band of the purified apo-E (gel region 5, fig.4). In fraction 2, in contrast, the majority of the radioactivity within the gel is associated with the latter band (gel region 5, fig.4). This band is unlikely to be apo-AI as only small, trace quantities of this apoprotein could be detected immunologically in fractions 1 and 2. Inspection of the gel patterns also indicates that this highly labelled band migrates slightly slower than apo-AI. It is of interest to note that the band in gel region 4 corresponding to the slower migrating band of apo-E has little associated radioactivity. This observation parallels [10] where, in isolated perfused livers from hypercholesterolemic rats, significantly more incorporation of radioactive amino acids into the faster migrating apo-E band was found after only 4 h perfusion. This suggests a very high synthetic rate for apo-E in these animals. A 2-fold increase in apo-E mRNA was shown in rats fed an atherogenic diet [27]. Much of the newly synthesized apo-E was not actively secreted into the medium but significantly expanded the intracellular pool of this apoprotein within the hepatocytes of hypercholesterolemic rats [27]. This may explain the low radioactivity associated with apo-B in fraction 2 when compared to apo-E despite the almost equal secretion rates of these 2 apoproteins into this column fraction (table 1).

The molar ratios of unesterified cholesterol to phospholipid of the lipoproteins isolated from fractions 1 and 2 from 24 h incubations of normal and hypercholesterolemic hepatocytes are given in table 2. The lipoproteins secreted by normal hepatocytes are always spherical in shape [9,11] and have FC:PL molar ratios of <1.0. However the corresponding ratios in incubations of hypercholesterolemic hepatocytes are 1.5 and 1.4 for fractions 1 and 2, respectively. A molar ratio of cholesterol/phospholipid of 1:1 or greater completely inhibited LCAT activity in vitro [8]. This observation is consistent with the hypothesis that the disc-to-sphere conversion is retarded which leads to the accumulation of discoidal

Table 2
Free cholesterol:phospholipid molar ratios of nascent lipoproteins from incubations of hepatocytes from normal and hypercholesterolemic rats

| | Normal | Hypercholesterolemic |
|---|---|---|
| Fraction 1 | 0.61 ± 0.09 (4) | 1.53 ± 0.18 (3) |
| Fraction 2 | 0.60 ± 0.08 (4) | 1.36 ± 0.17 (3) |

Each value represents the mean ± SEM of (no. expt)

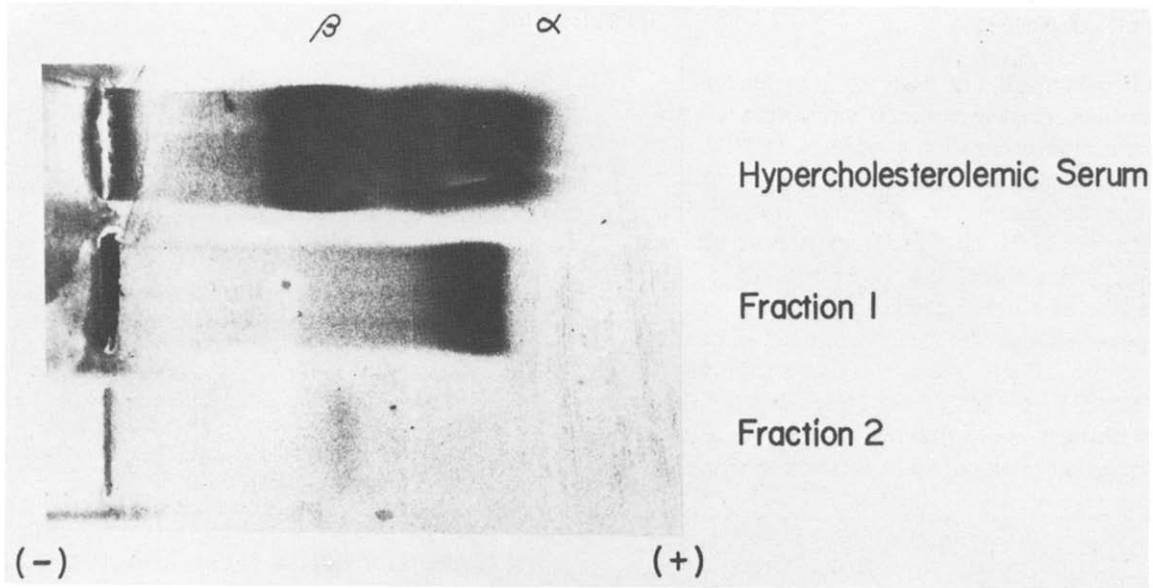



Fig.3. Agarose gel electrophoresis stained with Sudan black of hypercholesterolemic rat serum and fractions 1 and 2 of the medium after 24 h incubation of hepatocytes from hypercholesterolemic rats. The less intensely staining β-migrating band in fraction 2 is typical of lipoproteins containing little or no non-polar lipids [5].

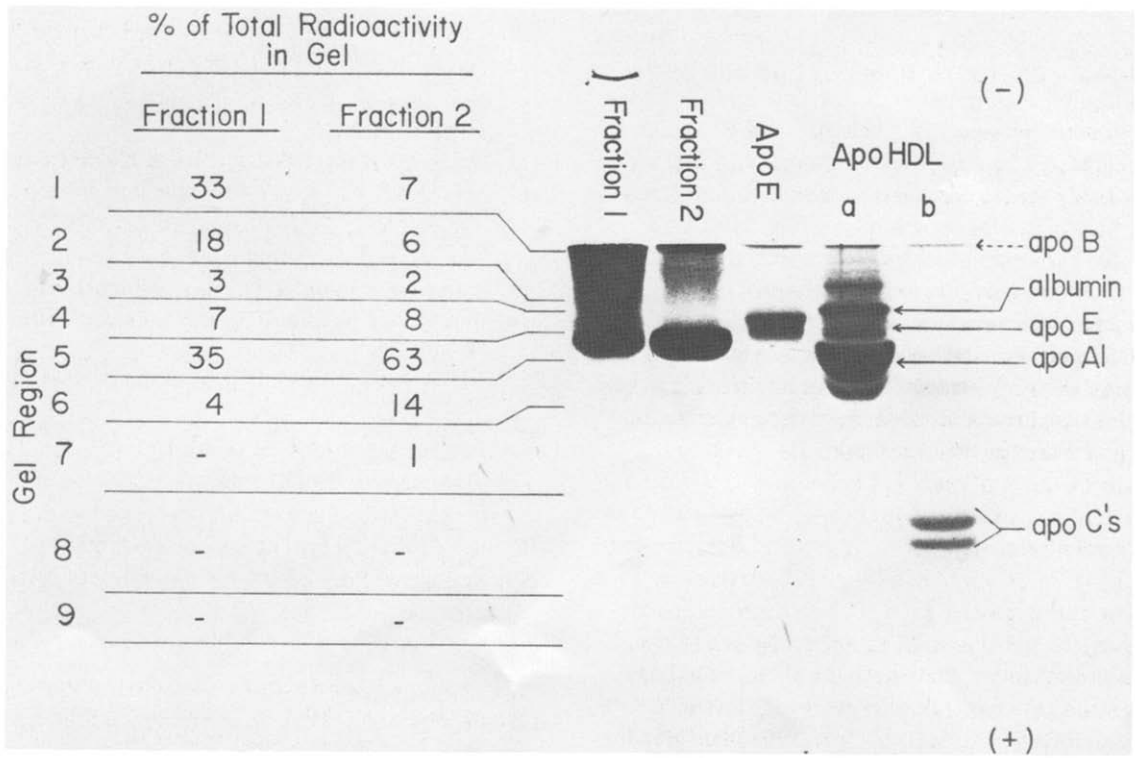



Fig.4. Urea–polyacrylamide gel electropherograms of the apolipoproteins isolated from fractions 1 and 2. The identity of the apoprotein bands was established by comparison with the mobility of purified apoproteins in the 3 right lanes. These latter apoproteins were prepared by isoelectric focussing of rat apo-VLDL, (apo-E) and rat apo-HDL (fractions a,b). The migration of apo-B is indicated by the dotted arrow at the stacking-running gel interface. The % radioactivity associated with the various gel regions between the solid lines is given for the apoproteins isolated from fractions 1 and 2. The total radioactivity due to the incorporation of $^{3}$H-labelled amino acids present in the gel of fraction 1 was 23 200 cpm and 31 700 cpm for fraction 2.

particles in incubation media from hypercholesterolemic hepatocytes. A similar mechanism was proposed for the accumulation of discoidal HDL in the sera of cholesterol-fed guinea pigs [6]. It is significant to note that nascent discoidal HDL with a FC:PL molar ratio of 0.65 isolated from perfused rat livers is, in contrast, a good substrate for LCAT [5]. The lipoproteins secreted into fraction 1 are spherical despite their high free cholesterol-to-phospholipid molar ratio. These lipoproteins have significantly more core lipids than those of fraction 2 (table 1) and thus it would appear that the amount of non-polar lipid present is an important factor in determining particle morphology.

The induction of hypercholesterolemia in rats results in the hepatic synthesis and secretion of cholesterol and apo-E rich spherical and discoidal lipoproteins, the latter resembling particles observed in LCAT-deficient patients [1] or cholestasis [2,26]. The possibility cannot be excluded that hepatocytes from the fatty livers of these animals synthesize or secrete LCAT at a much lower rate than those from normal animals. A reduced secretion of LCAT could in itself result in the accumulation of discoidal lipoproteins in the medium. However, the very high FC:PL molar ratio reported here for these discoidal particles, which is probably due to an excess of free cholesterol, renders these particles poor substrates for any LCAT secreted by the hepatocytes. This observation strengthens the hypothesis that in incubations of hypercholesterolemic hepatocytes an LCAT-deficient, or functionally deficient state occurs which results in the accumulation of discoidal particles in vitro and would suggest that these abnormal lipoproteins can accumulate in the sera of the intact animal.

## Acknowledgements

The authors thank Dr W. C. Breckenridge for performing the gas chromatographic analyses and many helpful discussions. We also thank Dr G. Faulkner for performing the electron microscopy. E. K. holds a Medical Research Council of Canada graduate studentship. This work was supported by grant MA5999 from the MRCC.

## References

[1] Utermann, G., Menzel, H. J. and Langer, K. H. (1974) FEBS Lett. 45, 29–32.
[2] Hamilton, R. L., Havel, R. J. and Williams, M. C. (1974) Fed. Proc. FASEB 33, 351.
[3] Sabesin, S. M., Kuiken, L. B. and Ragland, J. B. (1975) Science 190, 1302–1304.
[4] Norum, K. R., Glomset, J. A., Nichols, A. V., Forte, T., Albers, J. J., King, W. C., Mitchell, C. D., Applegate, K. R., Crony, E., Cabana, V. and Gjone, E. (1975) Scand. J. Clin. Lab. Invest. 35, suppl. 142, 31–55.
[5] Hamilton, R. L., Williams, M. C., Fielding, C. J. and Havel, R. J. (1976) J. Clin. Invest. 58, 667–680.
[6] Guo, L. S. S., Meng, M., Hamilton, R. L. and Ostwald, R. (1977) Biochemistry 16, 5807–5812.
[7] Sardet, C., Hansma, H. and Ostwald, R. (1972) J. Lipid Res. 13, 624–639.
[8] Fielding, C. J., Shore, V. G. and Fielding, P. E. (1972) Biochim. Biophys. Acta 270, 513–518.
[9] Krul, E. S. and Dolphin, P. J. (1982) in preparation.
[10] Noel, S.-P., Wong, L., Dolphin, P. J., Dory, L. and Rubinstein, D. (1979) J. Clin. Invest. 64, 674–683.
[11] Krul, E. S., Dolphin, P. J. and Rubinstein, D. (1981) Can. J. Biochem. 59, 676–686.
[12] Chapman, M. J., Mills, G. J. and Taylaur, C. E. (1973) Biochem. J. 131, 177–185.
[13] Akesson, B. (1980) Lipids 15, 677–681.
[14] Radding, C. M. and Steinberg, D. (1960) J. Clin. Invest. 39, 1560–1569.
[15] Havel, R. J., Eader, H. A. and Bragdon, J. H. (1955) J. Clin. Invest. 34, 1345–1353.
[16] Kuksis, A., Myher, J. J., Geher, K., Hoffman, A. G. D., Breckenridge, W. C., Jones, G. J. L. and Little, J. A. (1978) J. Chromatog. 146, 393–412.
[17] Dolphin, P. J. (1981) J. Lipid Res. 22, 971–989.
[18] Wong, L. and Rubinstein, D. (1978) Can. J. Biochem. 56, 161–166.
[19] Maguire, G. F. and Breckenridge, W. C. (1975) Clin. Biochem. 8, 161–168.
[20] Scanu, A. M. and Edelstein, C. (1971) Anal. Biochem. 44, 576–588.
[21] Rubenstein, B. and Rubinstein, D. (1972) J. Lipid Res. 13, 317–324.
[22] Nestruck, A. C. and Rubinstein, D. (1976) Can. J. Biochem. 54, 617–628.
[23] Johnson, F. L., St. Clair, R. W. and Rudel, L. L. (1981) Arteriosclerosis 1, 381a.
[24] Hamilton, R. L. (1978) in: Disturbances in Lipid and Lipoprotein Metabolism, pp. 155–171, American Physiological Society.
[25] Danielsson, B., Ekman, R. and Petersson, B.-G. (1975) FEBS Lett. 50, 180–184.
[26] Seidel, D. (1978) The Lipoprotein Molecule, pp. 227–235, Plenum, New York.
[27] Lin-Lee, Y.-C., Tanaka, Y., Lin, C.-T. and Chan, L. (1981) Biochemistry 20, 6474–6480.