Hypolipidemic effect of β,β'-methyl-substituted hexadecanedioic acid (MEDICA 16) in normal and nephrotic rats

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Abstract Treatment of normal or puromycin aminonucleoside-nephrotic rats, kept on a balanced Purina chow diet, with β,β'-tetramethyl-substituted hexadecanedioic acid (MEDICA 16) (Bar-Tana, J., G. Rose-Kahn, and M. Srebnik. 1985. *J. Biol. Chem.* **260:** 8404–8410) resulted in an acute reversible inhibition of liver lipogenesis and cholesterogenesis with a concomitant hypolipidemic effect which was sustained as long as the drug was administered. The hypolipidemic effect in normal and nephrotic rats consisted of 70–80% and 40–60% reduction in plasma VLDL-triacylglycerols and cholesterol, respectively, with a respective increase in the HDL-cholesterol/(VLDL + LDL)-cholesterol ratio. The observed hypolipidemic effect was accompanied by a 10-fold decrease in VLDL-apoC-III content with a concomitant enrichment of the VLDL fraction by VLDL remnants having an increased apoB-100/apoB-48 ratio. The pharmacological reduction of VLDL by MEDICA 16 may offer a treatment mode of choice for selected hyperlipidemic states.—**Bar-Tana, J., G. Rose-Kahn, B. Frenkel, Z. Shafer, and M. Fainaru.** Hypolipidemic effect of β,β'-methyl-substituted hexadecanedioic acid (MEDICA 16) in normal and nephrotic rats. *J. Lipid Res.* 1988. **29:** 431–441.

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The capacity of long chain fatty acids and their respective CoA thioesters to act as inhibitors of the lipogenic pathway (1) has initiated the design of inhibitory, nonmetabolic long chain fatty acyl analogues to be exploited as hypolipidemic effectors. β,β'-Methyl-substituted dicarboxylic acids (MEDICA) of C_{14}–C_{18} chain length ($HOOC-CH_2-C(CH_3)_2-(CH_2)n-C(CH_3)_2-CH_2-COOH$) (2) appear to fulfill this role (2, 3), with MEDICA 16 (n = 10) being the most potent of the series. Thus, the ω-carboxyl function interferes with the esterification of the dicarboxylic acid into neutral lipids and phospholipids while still allowing for an ATP-dependent CoA-thioesterification at either carboxylic end (4), and the β,β'-substitution prevents the β-oxidative catabolism of MEDICA compounds by either peroxisomal or mitochondrial systems. As effectors of lipid synthesis, MEDICA compounds were found to potently inhibit liver ATP-citrate lyase in vivo (2) or in cultured rat hepatocytes (3) with a concomitant dose-dependent decrease in liver acetyl-CoA and malonyl-CoA content. Inhibition of the lyase was followed by 80% inhibition of the incorporation of ^3H_2O or acetate into liver esterified fatty acids and 3-β-hydroxysterols under conditions of fat-free carbohydrate-rich feeding (2). Glucose, palmitate, or acetate oxidation as well as the gluconeogenic flux from lactate or the esterification of glycerol into lipids in the presence of added palmitate remained unaffected in vivo or in culture (2, 3). Inhibition of liver lipogenesis and cholesterogenesis by MEDICA compounds was not accompanied by an anorectic or a cathartic effect with reduction in overall net caloric intake (2).

In light of these features of MEDICA compounds it became of interest to evaluate their potential use as hypotriglyceridemic–hypocholesterolemic effectors in the rat in vivo under conditions of a balanced diet which still allows for the production of lipoproteins from exogenous fatty acids and cholesterol. The hypolipidemic effect reported here in the normal as well as the nephrotic rat appears to implicate MEDICA compounds in the catabolism of plasma lipoproteins in addition to their established role in the synthesis of liver lipids.

Abbreviations: PAN, puromycin aminonucleoside; MEDICA, β,β'-methyl-substituted dicarboxylic acid; MEDICA 16, β,β'-methyl-substituted hexadecanedioic acid; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; TLC, thin-layer chromatography; CM, chylomicrons.

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EXPERIMENTAL PROCEDURES

Animals

Male albino rats of the Hebrew University strain, weighing 150–200 g, were pair-fed nightly meals of ground Purina chow diet (0.1 g/g body weight) consisting of 75–80% of their ad libitum ration and containing 52.5% (w/w) carbohydrates, 18.1% (w/w) proteins, 4.6% (w/w) fat, 5.9% (w/w) cellulose, and 8.5% (w/w) salt–vitamin mixture. Unless otherwise stated, MEDICA 16 was administered by adding the finely powdered drug to the diet for the time periods specified. Dosage was expressed as percent (w/w) of the administered diet. MEDICA 16-treated and nontreated rats consumed the whole food ration under the feeding conditions employed, and the gain in weight following 5 days of treatment amounted to 20.0 ± 3.1 g (n = 12) and 18.2 ± 4.0 (n = 12) for the two respective groups.

Unless otherwise stated, nephrosis was induced by two successive intravenous injections of puromycin aminonucleoside (PAN) (5) at a dose of 7.5 mg/100 g body weight and 5 mg/100 g body weight on the first and third day, respectively.

Plasma triacylglycerol and cholesterol

Total plasma triacylglycerols and cholesterol during follow-up were determined in tail vein blood (0.1–0.2 ml) withdrawn from nonfasted rats at 8–10 AM, using 0.1% NaEDTA (pH 7.0) as an anticoagulant. Cholesterol and triacylglycerols were quantified using the Monotest Cholesterol Enzymatic kit (Boehringer Mannheim, Germany, Cat. No. 237574) and the Biopak Triglyceride Enzymatic kit (Biotrol Paris, France, Cat. No. A 01549), respectively. MEDICA 16 added to the incubation mixture of both enzymatic kits at concentrations of up to 1.25 mM did not interfere with the determination of cholesterol or triacylglycerols.

Lipoprotein analyses

Plasma for lipoprotein analyses was obtained from nonfasted rats at 8–10 AM by vena cava puncture under light ether anaesthesia, using 0.1% NaEDTA as an anticoagulant. All procedures involving lipoprotein isolation and characterization were started immediately after blood drawing and carried out at 4°C.

The distribution of cholesterol and triacylglycerol in plasma lipoproteins was determined by a modification of the LRC protocol (6). The plasma was centrifuged in a Beckman 50.3 Ti rotor for 20 min at 30,000 rpm for chylomicron flotation, followed by centrifuging the recovered chylomicron-deficient plasma for 18 hr at 39,000 rpm for VLDL flotation. The respective chylomicron and VLDL fractions were removed by tube slicing. LDL was precipitated from the 1.006 g/ml infranatant by heparin/$MnCl_2$, and the remaining heparin/$MnCl_2$ supernatant was treated with $NaHCO_3$ (7). Chylomicron cholesterol and triacylglycerol contents were estimated by the difference in the cholesterol and triacylglycerol values between the original plasma and the chylomicron-deficient plasma. VLDL, LDL, and HDL cholesterol and triacylglycerol contents were determined by the respective differences in the cholesterol and triacylglycerol values between the chylomicron-deficient plasma (VLDL + LDL + HDL), the 1.006 g/ml infranatant (LDL + HDL), and the heparin/$MnCl_2$ supernatant (HDL).

The composition of plasma lipoproteins was determined in lipoprotein fractions isolated by sequential density ultracentrifugation as described by Havel, Eder, and Bragdon (8). Chylomicrons, VLDL, LDL, and HDL were isolated by successive flotations for 20 min at 30,000 rpm (chylomicrons), 18 hr at 50,000 rpm at a salt density of 1.006 g/ml (VLDL), 20 hr at 50,000 rpm at a salt density of 1.063 g/ml (LDL), and 44 hr at 50,000 rpm at a salt density of 1.21 g/ml (HDL), respectively. The isolated lipoprotein fractions were refloated at their respective densities and were dialyzed against 400 volumes of 0.15 M NaCl–2 EDTA (pH 7.2). The lipoprotein-protein content of the isolated fractions was determined by the method of Lowry et al. (9) using bovine serum albumin as standard. The lipoprotein-cholesterol and triacylglycerol contents were determined by the respective enzymatic kits. For apoprotein analysis, the respective dialyzed lipoprotein fractions were delipidated with 20 volumes of chloroform–methanol 2:1 followed by 10 volumes of diethylether (10). The protein precipitate was dissolved in 0.1 M sodium phosphate (pH 7.4) containing 1% SDS and 5% 2-mercaptoethanol and heated for 2 min at 100°C. The proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 11% and 4% gels (11). Alternatively, the delipidated lipoproteins were solubilized in 0.015 M Tris–HCl buffer (pH 8.2) containing 6 M urea and subjected to isoelectric focusing (LKB, 2117 Multiphor) between pH 4.0 and 6.0 in 7.5% polyacrylamide gels containing 6 M urea (12). The apoproteins were quantitated by Coomassie blue staining followed by photodensitometry of the stained bands. The differential binding of the stain to apoE, apoC, and apoA-I, and the linearity of dye binding were determined by calibrating the specific dye absorbance of the electrophoresed individual purified apoproteins. The calibration of the dye absorbance of VLDL-apoB was determined by SDS-PAGE of VLDL in 11% gels and the estimation of its apoB content by tetramethylurea precipitation (13). The binding of the stain by VLDL-apoB-100 was assumed to be equal to that of VLDL-apoB-48. The relative binding of the stain amounted to 1.0, 1.25, 3.2, and 10.8 for apoE, apoB, apoC, and apoA-I, respectively, and the binding of the dye was linear within the protein range subjected to SDS-PAGE or isoelectric focusing. The stain binding by apoA-IV was assumed to be the average of that of apoB, E, C, and A-I. Rat VLDL-apoE, VLDL-apoC, and HDL-apoA-I were isolated as previously described (14–16).

VLDL metabolism

Plasma VLDL production was determined in rats injected intravenously under light ether anaesthesia with 0.5 ml of 20% Triton WR-1339 in saline (17). The production of VLDL in the awakened animals was determined by the accumulation of plasma VLDL-triacylglycerol during the period of 40–50 min following the injection of Triton.

The incorporation of ^3H_2O into liver and plasma VLDL lipids was determined by injecting rats intraperitoneally with 10 mCi of ^3H_2O (0.36 Ci/mol) followed 120 min later by sampling the liver and isolating plasma VLDL by sequential density ultracentrifugation as described above. The liver and VLDL lipids were extracted by chloroform-methanol as previously described (2), and the lipid extract was fractionated by TLC as previously described (2, 3). The lipid bands were scraped off the plate and counted in 25% Lumax scintillation fluid in toluene.

Materials

MEDICA 16 was synthesized as previously described (2). Puromycin aminonucleoside and Triton WR-1339 were from Sigma. Ampholyte was from LKB (Sweden). Lumax was from Lumac Application Laboratory (Holland). ^3H_2O was from the Nuclear Research Centre (Negev, Israel). [9,10-^{3}H]Palmitate and [1,2-^{3}H]cholesterol were from Amersham Corp. (U.K.).

RESULTS

Normal rats

Repeated administration of MEDICA 16 to rats fed a Purina balanced diet resulted in a dose-dependent hypotriglyceridemic-hypocholesterolemic effect which reached a 60–80% decrease in plasma triacylglycerols and cholesterol (**Fig. 1**). The hypolipidemic effect was already established during the first day of treatment (Fig. 1), and was sustained as long as the drug was administered; it was reversed upon withdrawing the drug (not shown).

The plasma lipoprotein profile of rats treated by 0.25% (w/w) MEDICA 16 for 5 days is shown in **Table 1**. The 65% decrease in total triacylglycerol concentration was accounted for by a respective decrease in the triacylglycerol of chylomicrons and VLDL. The observed decrease in plasma cholesterol consisted of a 75% decrease in VLDL-cholesterol together with a 35% decrease in HDL-cholesterol, while the minor LDL-cholesterol increased threefold. The hypotriglyceridemic-hypocholesterolemic effect was accompanied by a respective decrease in the protein content of plasma VLDL (Table 1). However, since the decrease in the lipid content of VLDL was more pronounced than that of VLDL-protein, a significant increase was observed in the protein/triacylglycerol ratio and the protein/cholesterol ratio of VLDL in MEDICA 16-treated rats.

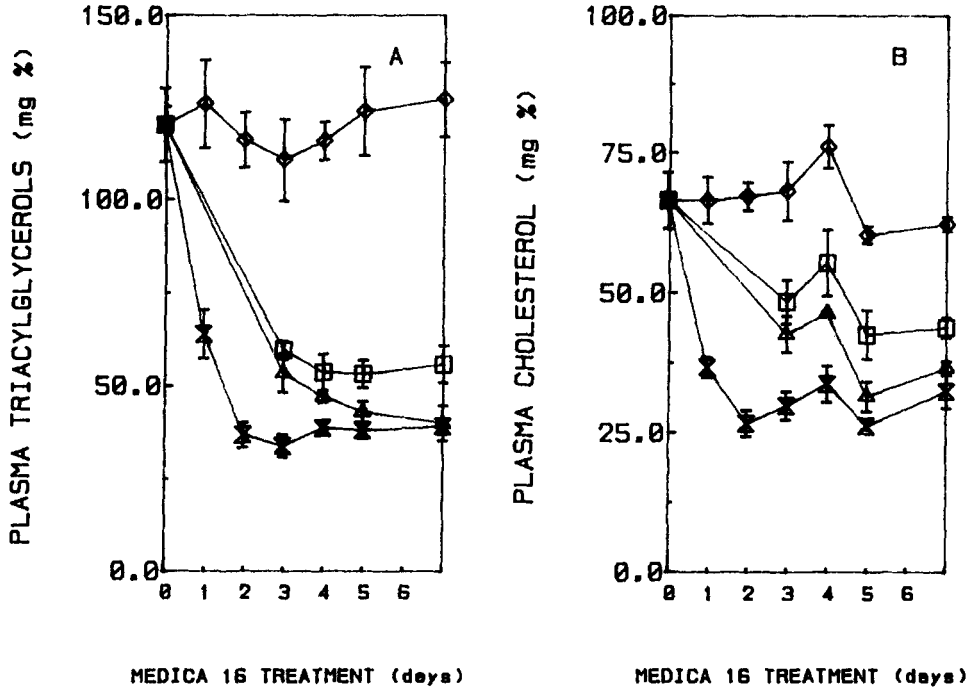

Fig. 1. The hypolipidemic effect of MEDICA 16 in normal rats: time and dose curves. Total plasma triacylglycerol (A) and cholesterol (B) values during follow-up of MEDICA 16-treated and nontreated animals were determined as described in Methods; mean ± SD (n = 5); (◇) 0.0 (nontreated); (□) 0.0625%; (△) 0.125%; and (⧖) 0.25% (w/w) of MEDICA 16 added to the diet.

TABLE 1. Plasma triacylglycerol and cholesterol distribution and the composition of lipoproteins in MEDICA 16-treated normal rats

Composition	Nontreated	MEDICA 16-Treated
Triacylglycerols (mg/dl)		
Total	141.1 ± 30.3	51.3 ± 9.9[a]
Chylomicrons	62.2 ± 19.2	20.1 ± 6.0[a]
VLDL	76.7 ± 10.8	25.0 ± 3.9[a]
LDL	2.1 ± 0.6	2.2 ± 0.3
HDL	3.6 ± 0.3	3.0 ± 0.3
Cholesterol (mg/dl)		
Total	58.0 ± 13.5	32.3 ± 6.6[a]
VLDL	23.2 ± 5.3	6.1 ± 3.1[a]
LDL	1.6 ± 0.2	5.1 ± 2.0[a]
HDL	30.4 ± 4.9	20.6 ± 4.2[a]
Protein (mg/dl)		
VLDL	13.27 ± 3.32	6.60 ± 1.03[a]
HDL	73.14 ± 11.78	54.78 ± 11.17[a]
Composition ratios		
VLDL-protein/VLDL-triacylglycerols	0.173 ± 0.021	0.264 ± 0.096[a]
VLDL-protein/VLDL-cholesterol	0.570 ± 0.070	1.080 ± 0.110[a]
VLDL-cholesterol/VLDL-triacylglycerols	0.169 ± 0.025	0.224 ± 0.030[a]
HDL-protein/HDL-cholesterol	2.406 ± 0.225	2.659 ± 0.154
HDL-triacylglycerols/HDL-cholesterol	0.209 ± 0.086	0.298 ± 0.054

Male rats fed a Purina chow diet were treated with 0.25% (w/w) MEDICA 16 added to the diet for 5 days. The distribution of cholesterol and triacylglycerol in plasma lipoproteins was determined by the modified LRC protocol as described in Methods. The lipoprotein composition was determined in the washed-dialyzed lipoprotein fractions isolated by sequential density ultracentrifugation as described in Methods. The protein content was calculated from the respective composition ratios and the lipid content of the respective particles; mean ± SD (n = 5).

[a]Significantly different from the respective nontreated value; $P < 0.05$.

The apoprotein composition in MEDICA 16-treated rats is shown in **Table 2**. The most significant changes were observed in the compositions of VLDL (**Fig. 2**A, B, C) and HDL (Fig. 2D, E) while that of LDL remained unaffected. Thus, the VLDL-apoB-100/VLDL-apoB-48 ratio amounted to 1.00 and 0.46 in MEDICA 16-treated and nontreated rats, respectively, thus pointing to a relative enrichment in apoB-100 as a result of MEDICA 16 treatment (Fig. 2B). Furthermore, the fractional content of VLDL-apoC decreased by 7.5-fold with a concomitant 9-fold increase in the apoE/apoC ratio of VLDL in MEDICA 16-treated rats. The specificity of the MEDICA 16 effect with respect to VLDL-apoC could be further realized by analyzing the plasma concentration of each of the VLDL-apoproteins as calculated by multiplying the fractional abundance of the respective VLDL-apoproteins (Table 2) by the total VLDL-protein of treated and nontreated rats (Table 1). Thus, the plasma concentrations of (apoB-100 + apoB-48), apoE, and apoC in VLDL amounted to 3.5, 5.8, and 4.0 mg/dl of plasma in nontreated rats as compared to 2.7, 3.6, and 0.3 mg/dl of plasma in MEDICA 16-treated rats. The observed decrease in VLDL-apoC was further analyzed by isoelectric focusing of the VLDL apoproteins (Fig. 2C). The relative abundance in terms of densitometric units amounted to 52, 116, 35, and 110 as compared to 23, 40, 8, and 66 for apoC-II, apoC-III-0, apoC-III-1, 2, and apoC-III-3 of nontreated and MEDICA 16-treated VLDL, respectively. Thus, in the light of the relative abundance of apoC-III, most of the overall decrease induced by MEDICA 16 treatment in VLDL-apoC was accounted for by the respective decrease in apoC-III. However, no preferential enrichment was observed in VLDL-apoC-II.

The apoprotein composition of the HDL fraction in MEDICA 16-treated rats was characterized by a twofold decrease in the fractional abundance of HDL-apoC and a twofold increase in the fractional abundance of HDL-apoE with a concomitant fourfold increase in the apoE/apoC ratio. The plasma concentrations of HDL-apoA-I, HDL-apoE, and HDL-apoC in nontreated rats were 23.2, 11.0, and 25.6 mg/dl of plasma, respectively, as compared to 16.4, 18.2, and 9.5 mg/dl, respectively, in MEDICA 16-treated rats. The relative abundance of the HDL-apoC subfractions in terms of densitometric units were 90, 126, 13, and 68 as compared to 62, 51, 2, and 51 for apoC-II, apoC-III-0, apoC-

TABLE 2. Apolipoprotein composition of MEDICA 16-treated normal rats

Composition	% Composition	
	Nontreated	MEDICA 16-Treated
VLDL		
ApoB-100	8.3	20.5
ApoB-48	18.1	20.5
ApoE	43.4	54.9
ApoC	30.2	4.1
ApoB-100/apoB-48	0.46	1.0
ApoE/apoC	1.44	13.4
LDL		
ApoB-100	63.4	66.7
ApoB-48	9.5	6.8
ApoE	27.2	26.6
HDL		
ApoE	15.1	33.2
ApoC	35.0	17.4
ApoA-I	31.7	29.9
ApoA-IV	18.1	19.5
ApoE/apoC	0.43	1.9

Male rats fed a Purina chow diet were treated with 0.25% (w/w) MEDICA 16 added to the diet for 5 days. The individual lipoprotein fractions of pooled plasma of five to ten treated and nontreated rats were isolated by sequential density ultracentrifugation as described in Methods and the apoprotein composition was determined by SDS-PAGE as described in Methods. The photodensitometric values were corrected for the differential binding of the stain as described in Methods; mean of four experiments. (The % composition presented did not vary by more than 20% of the respective individual composition values.)

III-1, 2, and apoC-III-3 of nontreated and MEDICA 16-treated rats, respectively (Fig. 2E), thus resulting in a 1.4-fold increase in the HDL-apoC-II/HDL-apoC-III ratio.

Nephrotic rats

Nephrosis may offer a hyperlipidemic model system for studying the potential of hypolipidemic agents. Indeed, PAN-induced nephrosis in rats (**Fig. 3**) resulted in 10- to 20-fold progressive increase in plasma triacylglycerol and cholesterol levels as previously reported (18–20), which was sustained at least for 14 days without reverting spontaneously to the normal level. PAN-induced nephrosis was accompanied by the appearance of a major LDL fraction which contributed about 35% to plasma cholesterol, thus making possible the evaluation of MEDICA effect on LDL-cholesterol in the rat. The induction of PAN-nephrosis in MEDICA 16-treated rats resulted in a restrained hyperlipidemic state compared to that observed in nontreated rats (Fig. 3). The effect exerted by MEDICA 16 could not be ascribed to interference of the drug with the induction of PAN-nephrosis, since MEDICA treatment was found to reverse the lipid pattern in an already established PAN-nephrotic state. Thus, treatment of PAN-nephrotic rats by MEDICA 16 for 5 days, starting on the 9th day following the induction of nephrosis by PAN, resulted in an almost complete reversion of plasma triacylglycerol back to the normal level, with a concomitant 50% decrease in plasma cholesterol (**Fig. 4**).

The plasma lipoprotein profile accounting for the hypolipidemic effect in MEDICA 16-treated nephrotic rats is shown in **Table 3,** and may be compared to that observed in normal rats (Tables 1 and 2). The hypotriglyceridemic effect induced by MEDICA 16 could be ascribed to an 80% decrease in the triacylglycerol content of chylomicrons and VLDL, while the overall hypocholesterolemic effect resulted from 65%, 70%, and 50% decreases in chylomicron-, VLDL-, and LDL-cholesterol, respectively, with no change in HDL-cholesterol. The relatively high fractional abundance of HDL-cholesterol in the nephrotic rat (Table 3) (18) taken together with the lack of MEDICA 16 effect on HDL-cholesterol accounts for the limited overall decrease in total plasma cholesterol in MEDICA 16-treated nephrotic rats (Fig. 4), with a concomitant increase in the HDL-cholesterol/(VLDL + LDL)-cholesterol ratio from 0.43 to 1.09.

The abundance of VLDL-apoB, VLDL-apoE, and VLDL-apoC in PAN-nephrotic rats amounted to 46.8, 56.8, and 35.5 mg/dl of plasma, respectively, in nontreated rats as compared to 12.4, 17.1, and 6.5, respectively, in MEDICA 16-treated rats (Table 2 and **Table 4**). Thus, the specific decrease in VLDL-apoC induced by MEDICA 16 treatment appears to be somewhat masked in the nephrotic rat by the overall decrease in the content of VLDL proteins. Also, similar to the increase in the fractional abundance of VLDL-apoB-100 induced by MEDICA 16 in normal rats, the apoB-100/apoB-48 ratio in nephrotic rats amounted to 0.88 and 1.9 in nontreated and MEDICA 16-treated nephrotic rats, respectively. It is worth noting that the increase in the fractional abundance of apoB-100 due to nephrosis was further accentuated by MEDICA 16 treatment, and the combined effect of nephrosis and MEDICA 16 treatment resulted in apoB-100 becoming the major apoB conponent of VLDL in MEDICA 16-treated nephrotic rats. The HDL-apoprotein profile observed in nephrotic rats was characterized by a significant decrease in the fractional abundance of apoE and apoA-IV as previously described (18, 19). Treatment of nephrotic rats with MEDICA 16 resulted in an increase in HDL-apoE at the expense of HDL-apoC, while apoA-I remained essentially unaffected.

VLDL metabolism in MEDICA 16-treated rats

The hypolipidemic effect of MEDICA 16 with respect to VLDL was further pursued by studying the synthesis and secretion of liver VLDL in normal and nephrotic MEDICA 16-treated rats. As shown in **Table 5,** ^3H_2O incorporation into total liver lipids and plasma VLDL lipids was potently inhibited by MEDICA 16 in PAN-nephrotic

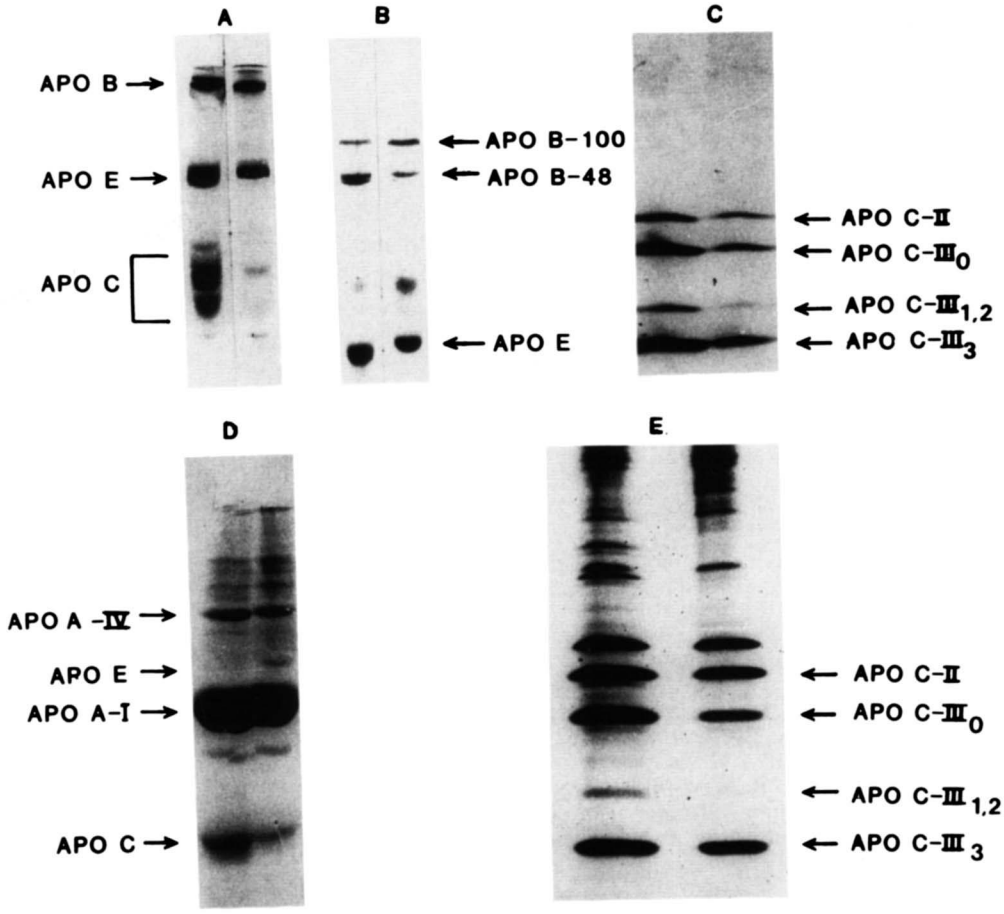

Fig. 2. The apoprotein composition of VLDL and HDL in MEDICA 16-treated rats. Rats were treated with 0.25% (w/w) MEDICA 16 for 5 days. Pooled plasma VLDL and HDL from five to ten nontreated (left lane) or treated (right lane) rats were isolated, delipidated, and analyzed by SDS-PAGE or isoelectric focusing as described in Methods. A, SDS-PAGE of VLDL-apoproteins in 11% gels (100 μg); B, SDS-PAGE of VLDL-apoproteins in 4% gels (100 μg); C, Isoelectric focusing of VLDL-apoproteins (200 μg); D, SDS-PAGE of HDL-apoproteins in 11% gels (100 μg); E, Isoelectric focusing of HDL-apoproteins (150 μg).

rats, thus reflecting the established capacity of MEDICA compounds to inhibit VLDL production (2, 3) under conditions of nephrosis where de novo lipogenesis and cholesterogenesis constitute a major flux in liver lipids synthesis (21). It it worth noting that inhibition of ^3H_2O incorporation into plasma VLDL lipids was actually much more pronounced than the reduction in the mass of the lipid constituents of plasma VLDL (Table 5 vs. Table 3); the difference presumably reflects the specific inhibition of liver lipogenesis by MEDICA 16 while the esterification flux of endogenous fatty acids remains unaffected (2, 3).

The hypolipidemic effect observed in normal rats kept on a carbohydrate-rich, fat-free diet, where de novo lipogenesis and cholesterolgenesis constituted a major route for liver VLDL production, was similarly accounted for by 65% inhibition in liver VLDL production (**Table 6**) with a concomitant inhibition in ^3H_2O incorporation into liver triacylglycerol and cholesterol (2). However, the hypolipidemic effect of MEDICA 16 with respect to plasma VLDL could not be exclusively ascribed to inhibition of plasma VLDL production since it could still be verified under conditions of starvation, where VLDL production was already repressed and could not be further inhibited by MEDICA 16 treatment (Table 6).

DISCUSSION

Treatment of normal rats fed a balanced Purina chow diet with MEDICA 16 resulted in an acute and reversible hypolipidemic effect that was sustained as long as the drug was administered. The hypolipidemic effect amounted to

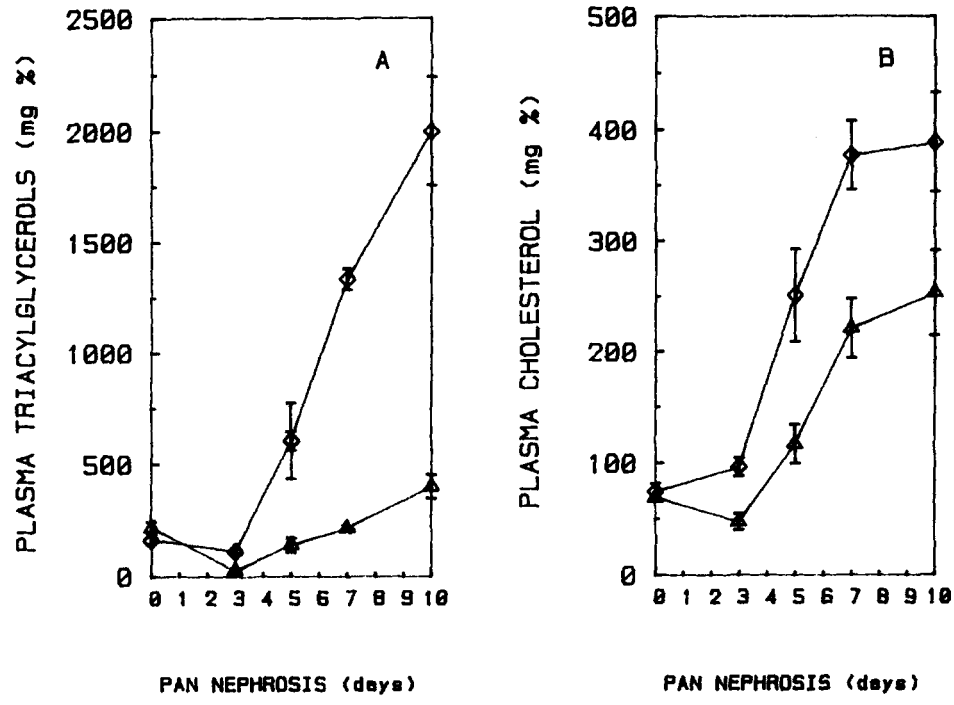

Fig. 3. PAN-nephrosis in MEDICA 16-treated rats. Nontreated rats (◇) or rats treated with 0.25% (w/w) MEDICA 16 added to the diet from the 1st day (△) were injected intravenously once with 10 mg of PAN/100 g body weight on the 1st day. Plasma total triacylglycerol (A) and cholesterol (B) were determined on the specified days following PAN administration as described in Methods; mean ± SD (n = 5).

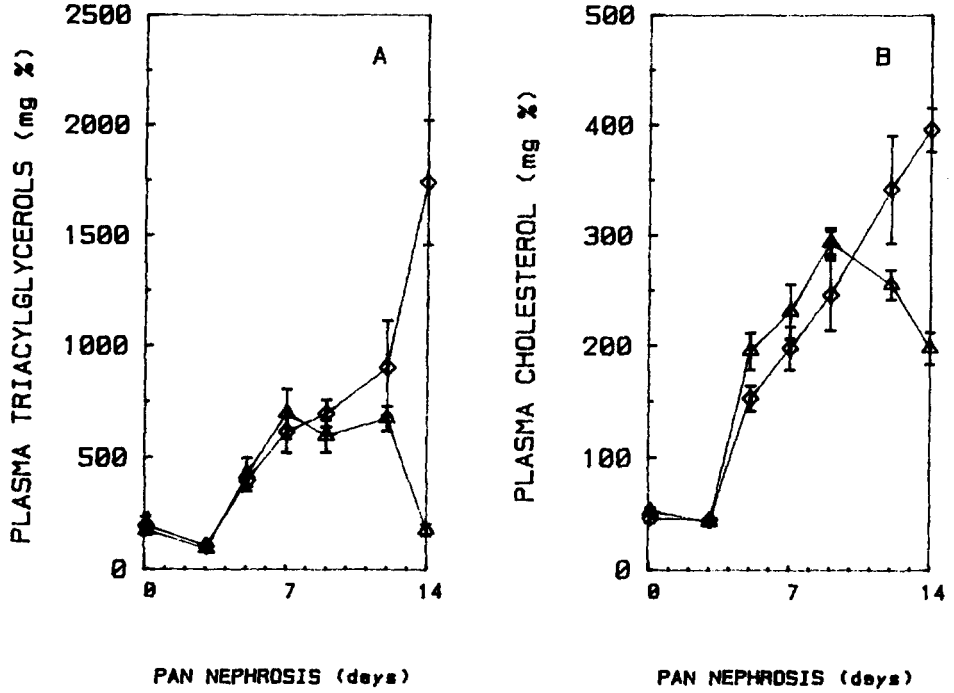

Fig. 4. The hypolipidemic effect of MEDICA 16 in PAN-nephrosis. PAN nephrosis was induced by intravenous injections of PAN on the 1st day and the 3rd days as described in Methods. MEDICA 16-treated rats (△) were dosed with 0.25% (w/w) MEDICA 16 added to the diet from the 9th day on. Nontreated rats (◇) were fed the normal diet. Plasma total triacylglycerol (A) and cholesterol (B) were determined on the specified days as described in Methods; mean ± SD (n = 4).

TABLE 3. Plasma triacylglycerol and cholesterol distribution and the composition of lipoproteins in MEDICA 16-treated PAN-nephrotic rats

Composition	Nontreated	MEDICA 16-Treated
Triacylglycerols (mg/dl)		
Total	1128.7 ± 529.9 (9)	254.1 ± 122.9 (9)[a]
Chylomicrons	500.7 ± 233.4 (9)	106.8 ± 55.3 (9)[a]
VLDL	801.3 ± 393.1 (9)	170.8 ± 70.1 (9)[a]
LDL	24.8 ± 10.2 (9)	9.1 ± 4.6 (9)[a]
HDL	11.0 ± 0.6 (9)	8.0 ± 1.4 (9)
Cholesterol (mg/dl)		
Total	414.7 ± 101.5 (9)	246.5 ± 42.8 (9)[a]
Chylomicrons	48.5 ± 13.6 (9)	15.9 ± 13.0 (9)[a]
VLDL	124.4 ± 55.9 (9)	36.8 ± 19.9 (9)[a]
LDL	156.4 ± 61.8 (9)	78.3 ± 39.6 (9)[a]
HDL	121.8 ± 17.9 (9)	125.1 ± 17.7 (9)
Protein (mg/dl)		
Chylomicrons	56.08 ± 26.14 (9)	42.61 ± 22.07 (9)
VLDL	141.03 ± 69.19 (9)	36.04 ± 14.79 (9)[a]
LDL	97.44 ± 38.50 (9)	37.43 ± 4.36 (9)[a]
HDL	231.30 ± 34.00 (9)	222.43 ± 31.47 (9)
Composition ratios		
CM-protein/CM-triacylglycerols	0.112 ± 0.044 (7)	0.399 ± 0.124 (7)[a]
CM-cholesterol/CM-triacylglycerols	0.065 ± 0.033 (7)	0.064 ± 0.024 (7)
VLDL-protein/VLDL-triacylglycerols	0.176 ± 0.133 (5)	0.211 ± 0.103 (5)
VLDL-cholesterol/VLDL-triacylglycerols	0.093 ± 0.030 (5)	0.085 ± 0.028 (5)
LDL-protein/LDL-cholesterol	0.623 ± 0.076 (9)	0.478 ± 0.110 (9)
LDL-triacylglycerols/LDL-cholesterol	0.242 ± 0.096 (5)	0.180 ± 0.067 (5)
HDL-protein/HDL-cholesterol	1.899 ± 0.191 (9)	1.778 ± 0.134 (9)
HDL-triacylglycerols/HDL-cholesterol	0.117 ± 0.057 (5)	0.074 ± 0.012 (5)

PAN-nephrotic rats fed a Purina chow diet were treated with 0.25% (w/w) MEDICA 16 for 5 days starting on the 9th day as described in Fig. 4. The distribution of cholesterol and triacylglycerol in plasma lipoproteins was determined by the modified LRC protocol as described in Methods. The lipoprotein composition was determined in the washed-dialyzed lipoprotein fractions isolated by sequential density ultracentrifugation as described in Methods. The protein content was calculated from the respective composition ratios and the lipid content of the respective particle; Mean ± SD (number of animals).

[a]Significantly different from the respective nontreated value; $P < 0.05$.

70–80% and 40–60% reduction in plasma triacylglycerol and cholesterol, respectively, and was due essentially to a decrease in the lipid content of chylomicrons and VLDL. The decrease in the triacylglycerol and cholesterol content of the triacylglycerol-rich lipoproteins was accompanied by specific significant changes in the composition of VLDL which consisted of a tenfold decrease in its apoC content and a twofold increase in its apoB-100/apoB-48 ratio. The decrease in VLDL-apoC reflected an overall decrease in plasma apoC rather than a selective transfer of apoC from VLDL to HDL. In the light of the fractional abundance of VLDL-apoC-III, most of the decrease in VLDL-apoC could be accounted for by that of apoC-III. The extent of the hypolipidemic effect of MEDICA 16 in PAN-nephrotic rats reflects the lipid-lowering potential of MEDICA compounds in pathological hyperlipidemic states.

The hypolipidemic effect of MEDICA 16 is qualitatively similar to that observed in starvation with respect to the fractional enrichment in VLDL-apoE and VLDL-apoB-100 (22–24), and the concomitant decrease in liver apoC secretion (25). However, the MEDICA effect could not be ascribed to reduction in the overall net caloric intake as a result of either anorectic or cathartic effects of the drug. Thus, the food comsumption of MEDICA 16-treated rats kept under ad libitum feeding conditions was similar to that of nontreated, age-matched rats, whether normal or nephrotic. Moreover, the hypolipidemic effect was observed here under pair-feeding conditions, where both nontreated and MEDICA 16-treated rats were offered only 75–80% of their ad libitum daily ration and the offered ration was fully consumed by both experimental groups. Also, the daily amount of stool excretion was similar in treated and nontreated rats. Hence, the hypolipidemic effect of MEDICA 16 reflects a metabolic modality rather than being due to a decrease in overall net caloric intake.

The hypolipidemic effect of MEDICA compounds with respect to plasma VLDL was previously observed in rats fed a carbohydrate-rich fat-free diet (2) and was ascribed there

TABLE 4. Apolipoprotein composition of MEDICA 16-treated PAN-nephrotic rats

Composition	% Composition	
	Nontreated	MEDICA 16-Treated
VLDL		
ApoB-100	15.6	22.6
ApoB-48	17.6	11.9
ApoE	41.6	47.5
ApoC	25.2	18.1
ApoB-100/apoB-48	0.88	1.90
ApoE/apoC	1.65	2.62
LDL		
ApoB-100	44.0	56.2
ApoB-48	5.4	9.2
ApoE	50.6	34.6
HDL		
ApoE	4.1	15.7
ApoC	43.5	32.4
ApoA-I	47.8	50.8
ApoA-IV	4.4	1.1
ApoE/apoC	0.09	0.48

PAN-nephrotic rats fed a Purina chow diet were treated with 0.25% (w/w) of MEDICA-16 in the diet for 5 days as described in Fig. 4. The individual lipoprotein fractions of pooled plasma of five to eight treated and nontreated rats were isolated by sequential density ultracentrifugation as described in Methods, and their respective apoprotein composition was determined by SDS-PAGE followed by photodensitometry of the Coomassie blue-stained bands; mean of two experiments. (The % composition presented did not vary by more than 10% of the respective composition values of the two experiments.)

to MEDICA inhibition of the lipogenic and cholesterogenic fluxes by MEDICA compounds acting as reversible, citrate-competitive inhibitors of liver ATP-citrate lyase as well as irreversible inhibitors of the cholesterogenic pathway at a site beyond the HMG-CoA reductase (2, 3). The inhibition of lipogenesis and cholesterogenesis by MEDICA 16 was confirmed here in nephrotic rats (Table 5), and may indeed account for the hypolipidemic effect of MEDICA compounds under conditions of carbohydrate-rich, fat-free diet or nephrosis where endogenous lipogenesis and cholesterogenesis constitute a major flux of liver lipid synthesis. The present results indicate, however, that the hypolipidemic effect of MEDICA 16 may still be expressed under conditions where VLDL synthesis is already repressed by starvation and cannot be further inhibited by MEDICA 16 (Table 6), thus implicating MEDICA compounds in VLDL catabolism apart from their established involvement in VLDL synthesis. Moreover, the increase in VLDL-apoB-100/apoB-48 ratio and the decrease in VLDL-apoC content in MEDICA 16-treated rats further point to the relative enrichment of the VLDL fraction by VLDL remnants (26, 27). Also, the consistent increase in the LDL fraction which accompanies the decrease in VLDL in MEDICA 16-treated normal rats, as well as the restrained decrease in LDL in MEDICA 16-treated nephrotic rats under conditions of a pronounced decrease in their VLDL, seem to corroborate the presumed role of MEDICA 16 in VLDL catabolism. Furthermore, the hypochylomicronemic effect of MEDICA 16 (Tables 1 and 3) points to the hypolipidemic capacity of this drug under conditions where lipogenesis and cholesterogenesis are replaced altogether by exogenous dietary lipids. Since MEDICA 16 does not affect chylomicron synthesis, assembly, and secretion into mesenteric lymph (Frenkel, B., et al., unpublished results), the observed hypochylomicronemic effect could not be accounted for by MEDICA inhibition of chylomicron production. Preliminary results have indeed indicated that the fractional clearance rates of palmitate- or cholesteryl ester-labeled VLDL and chylomicron particles prepared in normal rats and injected into MEDICA 16-treated rats were increased 6- to 10-fold. Hence, the hypolipidemic effect of MEDICA

TABLE 5. The incorporation of ^3H_2O into liver and plasma VLDL in MEDICA 16-treated and nontreated PAN-nephrotic rats

Fraction	^3H_2O Incorporation into Liver Lipids (μmol/g liver per 120 min)		^3H_2O Incorporation into Plasma VLDL (μmol/ml plasma per 120 min)	
	Nontreated	MEDICA 16-Treated	Nontreated	MEDICA 16-Treated
Total lipids	76.9 ± 22.8	21.8 ± 4.1[a]	1.63 ± 0.15	0.12 ± 0.01[a]
Triacylglycerols	30.5 ± 18.0	4.7 ± 2.8[a]	0.44 ± 0.05	0.03 ± 0.00[a]
Phospholipids	21.4 ± 10.4	6.0 ± 2.3[a]	0.11 ± 0.01	0.01 ± 0.01[a]
FFA	15.4 ± 6.2	4.0 ± 2.1[a]	0.84 ± 0.41	0.08 ± 0.06[a]
Cholesterol	2.5 ± 0.0	1.7 ± 0.6[a]	0.03 ± 0.01	n.d.
Cholesteryl ester	1.9 ± 0.2	0.7 ± 0.1[a]	0.01 ± 0.01	n.d.

PAN nephrosis was induced by two intravenous injections of PAN on the first and third days as described in Methods. MEDICA 16 (0.25%, w/w) was added to the diet from the 9th day on. Nontreated rats were fed the vehicle diet. On the 14th day the rats were injected with ^3H_2O and the incorporation of radioactivity into liver lipids and plasma VLDL was determined as described in Methods. The liver weight amounted to 11.0 ± 0.9 g (5.5% of body weight) and 11.8 ± 1.2 g (5.9% of body weight) in nontreated and MEDICA 16-treated rats, respectively; mean ± SD (n = 4); n.d., not detectable.

[a]Significantly different from the respective nontreated value, $P < 0.05$.

TABLE 6. Plasma VLDL production in MEDICA 16-treated rats

Starvation Period	Basal Plasma Triacylglycerols (mg/dl)		Plasma VLDL Production (mg triacylglycerol/min per dl of plasma)	
	Nontreated	MEDICA 16-Treated	Nontreated	MEDICA 16-Treated
	160 ± 40	49 ± 7	9.0 ± 1.1	3.1 ± 0.5
24 hr	80 ± 24	42 ± 15	2.4 ± 0.7	3.0 ± 0.6
48 hr	66 ± 20	44 ± 12	2.7 ± 0.7	2.9 ± 0.6

Male rats weighing 150 g were fed a carbohydrate-rich, fat-free diet ad libitum followed by starvation for the indicated time. MEDICA 16 was added to the powered diet at a concentration of 0.25% (w/w) and was continued during the starvation period (45 mg MEDICA 16/day) in 1% methylcellulose administered by an intragastric tube. Basal plasma triacylglycerols were determined in tail blood samples of ether-anaesthesized rats. Plasma VLDL production was determined by the difference in plasma triacylglycerol 40–50 min following the injection of Triton WR-1339 as described in Methods; mean ± SD (n = 4).

16 with respect to plasma VLDL in rats kept on a balanced Purina diet may be ascribed to both inhibition of liver VLDL production and activation of plasma VLDL catabolism. The activation of catabolism of the triacylglycerol-rich particles could be of major relevance either during the transition from the normolipidemic steady state of the nontreated animal to the hypolipidemic steady state of the MEDICA 16-treated animal, in the course of the decrease of VLDL under physiological conditions where lipogenesis and cholesterogenesis are already repressed (e.g., starvation), or in the course of the hypochylomicronemic effect induced by MEDICA 16 treatment. The possible role of the decreased plasma apoC-III in the activation of plasma VLDL and chylomicron catabolism (28, 29) in MEDICA 16-treated rats is now under investigation.

The overall pattern induced by MEDICA 16 treatment in rats is remarkably similar in many respects to that recently described in human subjects with a genetic deficiency in apoC-III and apoA-I (30, 31). In both cases the hypolipidemic effect with respect to VLDL was extensive and accompanied by apoC-III deficiency with a concomitant significant increase in the fractional clearance rate of normal VLDL particles injected into apoC-III-deficient subjects. The HDL fraction was, however, preserved upon MEDICA 16 treatment, while it was essentially absent under conditions of a genetic deficiency in apoA-I, thus resulting in premature atherosclerosis. The pharmacological reduction of VLDL-apoC-III by MEDICA 16 may thus help in dissecting the contribution made by apoC-III to the overall metabolism of lipoproteins under conditions where apoA-I is conserved.

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REFERENCES

1. Numa, S., and T. Tanabe. 1984. Acetyl-coenzyme A carboxylase and its regulation. *In* Fatty Acid Metabolism and its Regulation. S. Numa, editor. Elsevier Science Publishers, B. V., Amsterdam. 1–27.
2. Bar-Tana, J., G. Rose-Kahn, and M. Srebnik. 1985. Inhibition of lipid synthesis by β,β'-tetramethyl-substituted, C_{14}–C_{22}, α,ω-dicarboxylic acids in the rat in vivo. *J. Biol. Chem.* **260:** 8404–8410.
3. Rose-Kahn, G., and J. Bar-Tana. 1985. Inhibition of lipid synthesis by β,β'-tetramethyl-substituted, C_{14}–C_{22}, α,ω-dicarboxylic acids in cultured rat hepatocytes. *J. Biol. Chem.* **260:** 8411–8415.
4. Vamecq, J., E. De Hoffmann, and F. Van Hoof. 1985. The microsomal dicarboxylyl-CoA synthetase. *Biochem. J.* **230:** 683–693.
5. Fiegelson, E. B., J. W. Drake, and L. Recant. 1957. Experimental aminonucleoside nephrosis in rats. *J. Lab. Clin. Med.* **50:** 437–446.
6. Manual of Laboratory Operations: Lipid Research Clinics Program. 1974. Vol. 1. Lipid and Lipoprotein Analysis. DHEW Pub. No. (NIH) 75–628.
7. Bachorik, P. S., R. E. Walker, and D. G. Virgil. 1984. High-density-lipoprotein cholesterol in heparin-$MnCl_2$ supernates determined with the Dow enzymic method after precipitation of Mn^{2+} with HCO_3^-. *Clin. Chem.* **30:** 839–842.
8. Havel, R. J., H. Eder, and J. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
9. Lowry, O. H., N. H. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
10. Osborne, J. C., Jr. 1986. Delipidation of plasma lipoproteins. *Methods Enzymol.* **128:** 213–222.
11. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
12. Mills, G. L., P. A. Lane, and P. K. Weech. 1984. A Guidebook to Lipoprotein Technique. R. M. Burdon, and P. H. van Knippenberg, editors. Elsevier, New York. 273–276.
13. Kane, J. P., R. L. Hamilton, and R. J. Havel. 1975. Apoprotein composition of very low density lipoproteins of human serum. *J. Clin. Invest.* **56:** 1622–1634.
14. Fainaru, M., R. J. Havel, and K. Imaizumi. 1977. Radioimmunoassay of arginine-rich apolipoprotein of rat serum. *Biochim. Biophys. Acta.* **490:** 144–155.
15. Fainaru, M., R. J. Havel, and T. E. Felker. 1976. Radioim-

munoassay of apolipoprotein A-I of rat serum. *Biochim. Biophys. Acta.* **446:** 56–58.
16. Jackson, R. L., and G. Holdsworth. 1986. Isolation of properties of human apolipoproteins C-I, C-II, and C-III. *Methods Enzymol.* **128:** 288–297.
17. Otway, S., and D. S. Robinson. 1967. The use of a nonionic detergent (Triton WR 1339) to determine rates of triglyceride entry into the circulation of the rat under different physiological conditions. *J. Physiol.* **190:** 321–332.
18. Marsh, J. B., and C. E. Sparks. 1979. Lipoproteins in experimental nephrosis: plasma levels and composition. *Metabolism.* **28:** 1040–1045.
19. Gherardi, E., L. Vecchia, and S. Calandra. 1980. Experimental nephrotic syndrome in the rat induced by puromycin aminonucleoside. Plasma and urinary lipoproteins. *Exp. Mol. Pathol.* **32:** 128–142.
20. Gherardi, E., and S. Lalandra. 1982. Plasma and urinary lipids and lipoproteins during the development of nephrotic syndrome induced in the rat by puromycin aminonucleoside. *Biochim. Biophys. Acta.* **710:** 188–196.
21. Shafrir, E., and T. Brenner. 1979. Lipoprotein lipid and protein synthesis in experimental nephrosis and plasmapheresis. I: Studies in rat in vivo. *Lipids.* **14:** 695–702.
22. Bell-Quint, J., T. Forte, and P. Graham. 1981. Synthesis of two forms of apolipoprotein B by cultured rat hepatocytes. *Biochem. Biophys. Res. Commun.* **99:** 700–706.
23. Marsh, J. B., and C. E. Sparks. 1982. The effect of fasting on the secretion of lipoproteins and two forms of apo B by perfused rat liver. *Proc. Soc. Exp. Biol. Med.* **170:** 178–181.
24. Davis, R. A., J. R. Boogaerts, R. A. Borchardt, M. Malone-McNeal, and J. Archambault-Schexnayder. 1985. Intrahepatic assembly of very low density lipoproteins. Varied synthetic response of individual apolipoproteins to fasting. *J. Biol. Chem.* **260:** 14137–14144.
25. Quarfordt, S. H., J. Hanks, F. Shelburne, and B. Schirmer. 1982. Differing uptake of emulsion triglyceride in fed and fasted rat liver. *J. Clin. Invest.* **69:** 1092–1098.
26. Sparks, C. E., and J. B. Marsh. 1981. Metabolic heterogeneity of apolipoprotein B in the rat. *J. Lipid Res.* **22:** 519–527.
27. Sparks, C. E., D. J. Rader, and J. B. Marsh. 1983. Metabolism of two forms of apolipoprotein B of VLDL by rat liver. *J. Lipid Res.* **24:** 156–166.
28. Brown, W. V., and M. L. Baginsky. 1972. Inhibition of lipoprotein lipase by an apoprotein of human very low density lipoprotein. *Biochem. Biophys. Res. Commun.* **46:** 375–381.
29. Windler, E., and R. J. Havel. 1985. Inhibitory effects of C apolipoproteins from rats and humans on the uptake of triglyceride-rich lipoproteins and their remnants by the perfused rat liver. *J. Lipid Res.* **26:** 556–565.
30. Ginsberg, H. N., N. Le, I. J. Goldberg, J. C. Gibson, A. Rubinstein, P. Wang-Iverson, R. Norum, and W. V. Brown. 1986. Apolipoprotein B metabolism in subjects with deficiency in apolipoproteins C-III and A-I. *J. Clin. Invest.* **78:** 1287–1295.
31. Forte, T. M., A. V. Nichols, and R. M. Krauss. 1984. Familial apolipoprotein A-I and apolipoprotein C-III deficiency. *J. Clin. Invest.* **74:** 1601–1613.