Vol. 100, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 15, 1981 Pages 391-399

INTERCONVERSION OF HIGH DENSITY LIPOPROTEINS DURING INCUBATION OF HUMAN PLASMA

Alex V. Nichols, Elaine L. Gong and Patricia J. Blanche

Donner Laboratory, Lawrence Berkeley Laboratory
University of California, Berkeley, CA 94720

Received March 30,1981

SUMMARY: Incubation (6h,37°) of untreated human plasma resulted in marked reductions in concentrations of two major subpopulations (mean sizes: ~84 Å and ~79 Å) of the HDL_3 subclass. These reductions were accompanied by the appearance of larger species with mean particle size (95.2 Å) in the range of the HDL_{2a} subpopulation. In the presence of mercaptoethanol (14 mM), incubation resulted in further reduction of the two HDL_3 subpopulations but now with formation of both larger (95.4 Å) and smaller (77.2 and 75.2 Å) species. Inhibitors of lecithin: cholesterol acyltransferase showed varying effectiveness in modifying the extent and character of the interconversion. With paraoxon (2 mM) the interconversion was much reduced and was associated with the formation predominantly of smaller particles (74.6 Å); with 5,5'-dithiobis-(2-nitrobenzoic acid) (1.4 mM) the interconversion resembled that produced with mercaptoethanol but to a considerably lesser degree. Incubation of HDL, ultracentrifugally isolated from nonincubated plasma containing paraoxon, resulted in negligible interconversion, suggesting that other plasma components participate in the process.

INTRODUCTION

Early studies (1) using analytic ultracentrifugation indicated an apparent interconversion of HDL_3 to HDL_2 species during incubation (24h,37°) of human plasma both in the absence and in the presence of p-hydroxymercuribenzoate, an inhibitor of lecithin:cholesterol acyltransferase (LCAT)(2). By gradient gel electrophoresis (GGE) we have recently demonstrated that HDL_3 subclass to be comprised of up to three major subpopulations, and the HDL_2 subclass to be comprised of up to two major subpopulations (3,4). In the present work, we have applied this technique to the characterization of incubation-induced interconversion of plasma HDL to obtain information on the extent of participation of the different HDL_3 subpopulations and on the specific "HDL_2" and possibly other products formed. We have also evaluated the effects of mercaptoethanol (ME),

Abbreviations: HDL, high density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; GGE, gradient gel electrophoresis; ME, 2-mercaptoethanol; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

0006-291X/81/090391-09$01.00/0

which stimulates LCAT activity with liposomal substrates (5,6), and of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (2) and paraoxon (7), both of which inhibit LCAT activity, on the interconversion.

MATERIALS AND METHODS

Penicillin-streptomycin was purchased from Gibco Laboratories; 5,5'-dithiobis-(2-nitrobenzoic acid) from Sigma Chemical Co.; paraoxon (diethyl p-nitrophenyl phosphate) from Aldrich Chemicals, Inc., and 2-mercaptoethanol from Calbiochem. GGE was performed on the Gel Electrophoresis Apparatus GE 2/4 using 4-30% polyacrylamide gradient gels calibrated with the High Molecular Weight (HMW) Calibration Kit (Pharmacia Fine Chemicals). Cholesterol Reagent Set #124087 was obtained from Boehringer-Mannheim.

Processing and Incubation of Plasma. Blood was obtained from fasting normal healthy male volunteers. Penicillin-streptomycin (50 units/ml-50 µg/ml) was added to the plasma immediately following separation in a refrigerated centrifuge.

Aliquots of untreated plasma and plasma containing either 1.4 mM DTNB, 2 mM paraoxon or 14 mM ME were pipetted into 8 ml glass screw-cap vials, flushed with nitrogen and sealed. Nonincubated samples were stored at 4°C; incubation was performed in a shaking water bath at 37°C.

Isolation of d $\leq$ 1.20 Fraction and Total HDL Fraction. The d $\leq$ 1.20 fraction from all plasma samples was obtained by a single ultracentrifugation (Beckman 40.3 rotor, 114,000 x g, 15°C, 24 h) of 2 ml of the sample plus 4 ml of a NaBr-NaCl solution to give a final density of 1.20 g/ml in the top 1 ml after ultracentrifugation. The total HDL fraction (d 1.07-1.20 g/ml) was isolated from plasma containing 2 mM paraoxon by sequential ultracentrifugation (114,000 x g, 15°C, 24 h), using NaBr-NaCl solutions to obtain required densities.

Analysis of HDL. GGE of the d $\leq$ 1.20 fractions was performed as previously described (4). Samples and reference proteins were electrophoresed at 125 volts (constant voltage) at 10°C for 24 h. Densitometric scans of the gradient gels following electrophoresis and staining were used to identify HDL subpopulations and to estimate the level of HDL material within specific particle size intervals These intervals were previously shown to be characteristic for the different subpopulations of HDL (4) and were designated as follows: 125-98 Å, $(HDL_{2b})_{gge}$; 98-88 Å, $(HDL_{2a})_{gge}$; 88-82 Å, $(HDL_{3a})_{gge}$; 82-78 Å, $(HDL_{3b})_{gge}$; and, 78-72 Å, $(HDL_{3c})_{gge}$. The interval (125-72 Å) which generally contained the total HDL pattern was designated $(HDL)_{gge}$.

Other Analyses. In addition to GGE, some d $\leq$ 1.20 samples were analyzed by analytic ultracentrifugation (8,9). Esterification of free cholesterol was determined using the enzymatic colorimetric endpoint method with the Cholesterol Reagent Set.

RESULTS

GGE patterns representative of HDL distributions in plasma of subjects used in the incubation studies are shown in Fig. 1. The HDL_3 subclass was the predominant HDL component and exhibited two major peaks which were located within the $(HDL_{3a})_{gge}$ and $(HDL_{3b})_{gge}$ intervals. Mean particle sizes of HDL corresponding to these two peaks were approximately 84 Å and 79 Å, respectively.

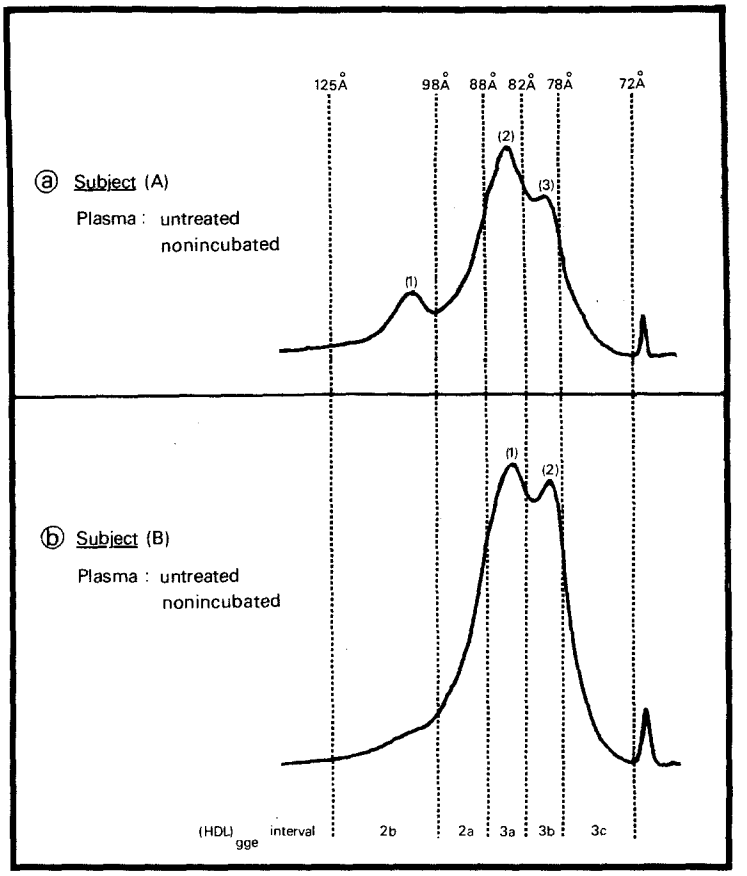

Fig. 1. GGE PATTERNS OF HDL IN PLASMA d ≤ 1.20 FRACTIONS. d ≤ 1.20 fractions were isolated from nonincubated untreated plasma of subjects (A) and (B). Particle sizes (Å) at peaks (identified by number) and percent distribution of area within $(HDL)_{gge}$ intervals are as follows:

	$(HDL_{2b})_{gge}$	$(HDL_{2a})_{gge}$	$(HDL_{3a})_{gge}$	$(HDL_{3b})_{gge}$	$(HDL_{3c})_{gge}$
(a) Subject (A)					
Size (at peak)	102.9±0.7(1)	-	84.0±0.2(2)	78.8±0.4(3)	-
% Area	16.2±0.1	16.2±0.6	33.4±0.2	23.3±0.0	10.6±0.8
(b) Subject (B)					
Size (at peak)	-	-	83.4±0.4(1)	78.7±0.1(2)	-
% Area	10.1±1.0	21.5±1.6	33.6±0.1	26.2±1.3	8.6±1.1

The small peak appearing at the extreme right is residual albumin occurring in the d ≤ 1.20 fraction.

No major differences in GGE patterns were observed in HDL isolated from nonincubated plasma in the presence or absence of LCAT inhibitors (Table I). Incubation of untreated plasma resulted (Fig. 2a) in a marked decrease of material within the $(HDL_{3a})_{gge}$ and $(HDL_{3b})_{gge}$ intervals (from 56.7% of total area at 0 h to 30.4% at 6 h) and the appearance of a new peak (mean size: 95.2 Å)

Table I

GGE ANALYSIS OF HDL ISOLATED FROM NONINCUBATED PLASMA IN PRESENCE OF LCAT INHIBITORS

		$(HDL)_{gge}$ INTERVALS				
		$(HDL_{2b})_{gge}$	$(HDL_{2a})_{gge}$	$(HDL_{3a})_{gge}$	$(HDL_{3b})_{gge}$	$(HDL_{3c})_{gge}$
Plasma: nonincubated, untreated	Size (at peak)	102.9±0.7	-	84.0±0.2	78.8±0.4	-
	% Area	16.2±0.1	16.2±0.6	33.4±0.2	23.3±0.0	10.6±0.8
Plasma + DTNB	Size (at peak)	104.9±1.1	-	85.6±0.5	80.0±0.2	-
	% Area	15.2±0.2	17.9±3.0	33.1±0.0	23.9±0.1	10.0±2.5
Plasma + paraoxon	Size (at peak)	104.5±1.6	-	86.6±1.1	80.9±0.4	-
	% Area	15.5±1.4	18.3±3.2	34.6±1.1	22.4±2.8	9.2±1.0

in the $(HDL_{2a})_{gge}$ interval. Modest additional reduction in $(HDL_{3a})_{gge}$ and $(HDL_{3b})_{gge}$ material (down to 23% of total area) was observed after 24 h incubation. Incubation of plasma with ME (14 mM) for 6 h resulted (Fig. 2b) in a more extensive reduction of material in the $(HDL_{3a})_{gge}$ and $(HDL_{3b})_{gge}$ intervals (from 56.7% of total area to 20.0%) than was observed in untreated plasma after 6 h incubation. While the mean size (95.4 Å) and relative amount of the larger product species appearing within the $(HDL_{2a})_{gge}$ were comparable to those in untreated plasma, there also was an increase of species of smaller particle size (mean size: 77.2 Å, peak 2; 75.2 Å, peak 3) within the $(HDL_{3c})_{gge}$ interval. The additional interconversion stimulated by ME was associated with an increased esterification of free cholesterol (13.2% untreated vs 20.6% with ME). When ME was added to untreated plasma which had previously been incubated for 6 h, and this mixture was incubated an additional 6 h, the results (Fig. 2c) were similar to those obtained when plasma was incubated with ME for 6 h. These observations strongly suggested that the effect of ME was primarily the interconversion of the HDL_3 subpopulations to smaller species. Analytic ultracentrifugal data (Fig. 3) on HDL from incubated plasma containing ME were consistent with interconversion of HDL_3 to both larger and smaller HDL species.

Incubation of plasma with paraoxon, an inhibitor of serine-histidine hydrolase activity and of both LCAT phospholipase and transferase activity (10),

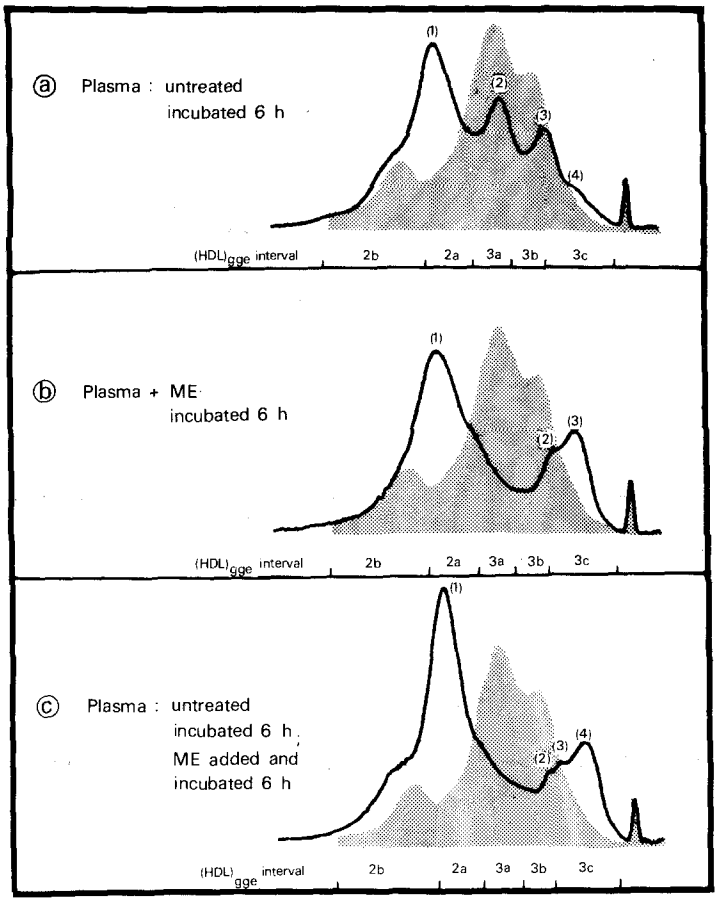

Fig. 2. EFFECT OF INCUBATION ON PLASMA HDL DISTRIBUTION [SUBJECTS (A) AND (B)]. Scans for subject (A) shown above: (a) untreated plasma, incubated 6 h; (b) plasma + 14 mM ME, incubated 6 h; (c) untreated plasma was incubated 6 h, then 14 mM ME was added and mixture was incubated an additional 6 h. Stippled area represents HDL from nonincubated untreated plasma. Particle sizes (Å) at peaks (identified by number) and percent distribution of area within $(HDL)_{gge}$ intervals are as follows:

		$(HDL_{2b})_{gge}$	$(HDL_{2a})_{gge}$	$(HDL_{3a})_{gge}$	$(HDL_{3b})_{gge}$	$(HDL_{3c})_{gge}$
Subject (A)						
ⓐ Plasma: untreated, incubated (6h,37°C)	Size	-	95.2±0.3(1)	83.0±0.1(2)	-	77.4±0.0(3), 74.6±0.2(4)
	% Area	27.4±1.6	30.3±0.8	18.4±0.6	12.0±0.6	11.8±1.1
ⓑ Plasma + ME (6h,37°C)	Size	-	95.4±0.4(1)	-	-	77.2±0.1(2), 75.2±0.0(3)
	% Area	27.6±0.1	32.4±0.1	11.8±0.0	8.2±0.3	20.0±0.1
ⓒ Plasma: untreated, incubated (6h,37°C); ME added, incubated (6h,37°C)	Size	-	95.4±0.1(1)	-	78.5±0.0(2)	77.2±0.1(3), 74.7±0.1(4)
	% Area	30.1±0.3	33.5±0.7	11.2±0.4	7.6±0.4	17.7±0.6

GGE scans for plasma HDL of subject (B) are not shown; particle sizes (Å) at peaks and percent area distribution are as follows:

Subject (B)						
Plasma: untreated, incubated (6h,37°C)	Size	-	94.6	83.0	78.0	-
	% Area	28.5	30.0	17.8	16.5	7.3
Plasma + ME (6h,37°C)	Size	-	93.6	-	-	77.0, 75.4
	% Area	26.1	32.3	9.5	14.0	18.1

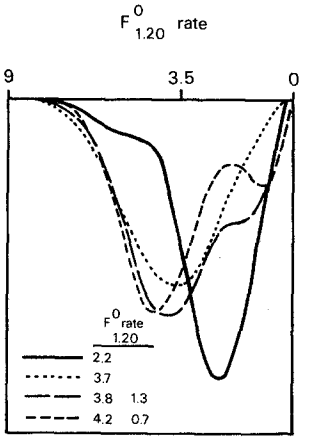

Fig. 3. ANALYTIC ULTRACENTRIFUGAL PATTERNS OF HDL IN PLASMA d < 1.20 FRACTIONS. Patterns shown are: nonincubated untreated plasma (———); plasma incubated 24 h, 37°C (•••••); plasma + ME incubated 6 h, 37°C (— —); plasma + ME incubated 24 h, 37°C (- - - -). $F^0_{1.20}$ is the HDL flotation rate, expressed in svedbergs (10^{-13}cm/sec/dyne/g)(8).

resulted (Fig. 4a) in only a modest reduction (from 56.7% of total at 0 h to 47.3% at 6 h) of $(HDL_{3a})_{gge}$ and $(HDL_{3b})_{gge}$ material. This reduction was associated primarily with the appearance of a distinct peak (mean size: 74.6 Å) within the $(HDL_{3c})_{gge}$ interval. When the sulfhydryl-blocking reagent, DTNB, was used to inhibit LCAT activity, marked reduction in $(HDL_{3a})_{gge}$ and $(HDL_{3b})_{gge}$ material was seen (Fig. 4b) coupled with increases in both larger and smaller species. The increase in larger species was confined primarily to the $(HDL_{2a})_{gge}$ interval where a bimodal peak (mean sizes: 92.0 Å and 89.2 Å) was observed. Smaller species with two peaks (mean sizes: 76.8 Å and 74.5 Å) also appeared in the $(HDL_{3c})_{gge}$ interval. The particle size associated with the latter peak compared closely to that of the small species formed during incubation of plasma with paraoxon. The particle sizes associated with the larger species (92.0 Å and 89.2 Å) were appreciably smaller than those observed for the larger species produced in untreated plasma (95.2 Å) or in ME-containing plasma (95.4 Å). These results indicated that considerable interconversion in HDL distribution can occur in the absence of LCAT activity and that the nature of the interconversion depended strongly on the inhibitor used.

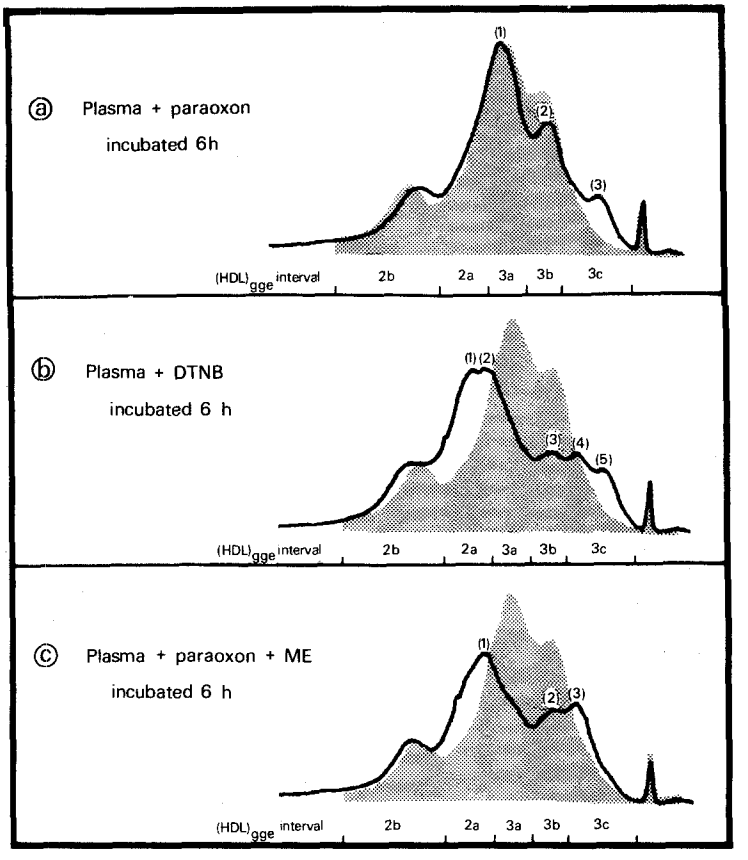

Fig. 4. EFFECT OF INCUBATION ON PLASMA HDL DISTRIBUTION [SUBJECT (A)]. Scans for subject (A) shown above: (a) plasma + 2 mM paraoxon incubated 6 h; (b) plasma + 1.4 mM DTNB incubated 6 h; (c) plasma containing 2 mM paraoxon + 14 mM ME incubated 6 h. Stippled area represents HDL from nonincubated untreated plasma. Particle sizes (Å) at peaks (identified by number) and percent distribution of area within $(HDL)_{gge}$ intervals are as follows:

		$(HDL_{2b})_{gge}$	$(HDL_{2a})_{gge}$	$(HDL_{3a})_{gge}$	$(HDL_{3b})_{gge}$	$(HDL_{3c})_{gge}$
ⓐ Plasma + paraoxon (6h,37°C)	Size	104.6±1.3	-	87.5±0.4(1)	80.2±0.1(2)	74.6±0.1(3)
	% Area	17.0±0.6	20.5±0.7	30.2±1.1	17.1±0.7	15.3±0.4
ⓑ Plasma + DTNB (6h,37°C)	Size	105.6±0.3	92.0±0.1(1), 89.2±0.1(2)	-	79.9±0.0(3)	76.8±0.1(4), 74.5±0.1(5)
	% Area	21.0±0.1	29.8±0.5	20.2±0.3	11.8±0.4	17.0±0.7
ⓒ Plasma + paraoxon + ME (6h,37°C)	Size	105.5±0.6	89.8±0.8(1)	-	79.4±0.4(2)	77.0±0.2(3)
	% Area	20.8±0.8	28.8±0.2	19.0±1.3	15.2±0.7	16.1±0.0

Further complexity of this system was noted when we attempted to evaluate the effect of incubation with ME when plasma LCAT activity was first inhibited by paraoxon. In this case, the GGE pattern appeared (Fig. 4c) similar in character to that observed with ME alone, but all of the shifts were highly atten-

uated. The larger species had a mean particle size of 90.4 Å (vs. 95.4 Å with ME) and the smaller species had a mean particle size of 77.1 Å (vs. 75.2 Å with ME). There was no esterification of free cholesterol during this incubation. Thus, ME stimulated some interconversion even in the presence of paraoxon which was previously shown to be effective in attenuating interconversion. Lastly, separate experiments showed negligible interconversion when HDL, which was ultracentrifugally isolated from nonincubated plasma containing paraoxon, was subsequently incubated (6 h) in the absence of $d \geq 1.20$ plasma proteins and other lipoproteins.

DISCUSSION

Interconversion of HDL_3 subpopulations during incubation of untreated plasma gives rise to "HDL_2" species which by GGE appear as a peak predominantly within the particle size interval of native HDL_{2a} subpopulations. The relevance of this observation to possible origins of HDL_{2a} subpopulations *in vivo* is yet to be established. Comparison of the apolipoprotein and lipid compositions of these product species with those of native HDL_{2a} is in progress. In the presence of ME, an additional pathway for interconversion of HDL_3 subpopulations is stimulated; this pathway results in the formation of smaller particles in the size range of the $(HDL_{3c})_{gge}$ interval. Varying types and degrees of interconversion of HDL_3 subpopulations occur in the presence of two functionally different inhibitors of LCAT. Inhibition of LCAT and possibly other serine-histidine hydrolase activities in plasma by paraoxon was found effective in attenuating interconversion, particularly to larger particles.

The implications of the present observations to work on the relationship of HDL structure and composition to LCAT activity and lipid-transfer protein function are considerable. In many such investigations (11,12), DTNB and ME are used during *in vitro* labelling of plasma cholesterol (by exchange) and cholesteryl esters (by LCAT activity). Clearly, incubation of plasma in the presence of these reagents results in marked alterations in properties of HDL and hence can complicate meaningful interpretation of experimental results.

ACKNOWLEDGEMENTS

This work was supported by the National Institutes of Health Research Grant HL-18574 from the National Heart, Lung and Blood Institute, National Institutes of Health.

REFERENCES

1. Nichols, A.V., Coggiola, E.L., Jensen, L.C. and Yokoyama, E.H. (1968) Biochim. Biophys. Acta 168, 87-94.
2. Glomset, J.A. (1962) Biochim. Biophys. Acta 65, 128-135.
3. Nichols, A.V., Blanche, P.J., Krauss, R.M. and Gong, E.L. (1979) in Report of the HDL Methodology Workshop, San Francisco (K. Lippel, ed.) NIH Publication 79-1661, pp. 303-309.
4. Blanche, P.J., Gong, E.L., Forte, T.M. and Nichols, A.V. (1981) Biochim. Biophys. Acta, submitted.
5. Ho, W.K.K. and Nichols, A.V. (1971) Biochim. Biophys. Acta 231, 185-193.
6. Verdery, R.B. (1981) Biochem. Biophys. Res. Comm. 98, 494-500.
7. Rose, H.G. and Juliano, J. (1979) J. Lipid Res. 20, 399-407.
8. Lindgren, F.T., Jensen, L.C. and Hatch, F.T. (1972) in Blood Lipids and Lipoproteins: Quantitation, Composition, and Metabolism (G.J. Nelson, ed.) Wiley-Interscience, New York, pp. 181-274.
9. Jensen, L.C., Rich, T.H. and Lindgren, F.T. (1970) Lipids 5, 491-493.
10. Aron, L., Jones, S. and Fielding, C.J. (1978) J. Biol. Chem. 253, 7220-7226.
11. Chajek, T. and Fielding, C.J. (1978) Proc. Natl. Acad. Sci. USA 75, 3445-3449.
12. Marcel, Y.L., Vezina, C., Teng, B. and Sniderman, A. (1980) Atherosclerosis 35, 127-133.