[Chem. Pharm. Bull. 25(10)2669—2675(1977)]

UDC 615.272.4.015.42.076.9 : 577.153.07

Enhancement of Oleate-rich Cholesteryl Ester after Removal of Cholesterol Diet from Cholesterol-fed Rabbits[1]

TATSUYA TAKANO and MASAO SATO[2]

Department of Physiological Chemistry, Hiroshima University School of Medicine[3]

(Received February 21, 1977)

Oleate-rich cholesteryl ester (CE) in serum and arterial wall increased after cholesterol administration. Concentration of oleic acid in CE increased further, after removal of cholesterol diet. This paper suggested that CE hydrolytic enzymes as well as synthetic enzymes might involve in the accumulation of oleate-rich CE in serum and arterial wall.

Keywords——cholesteryl ester; oleic acid; cholesterol; serum; aorta; atherosclerosis; fatty acid

Introduction

It is well known that, in atheromatous aorta, cholesterol and its esters accumulate and the ratio of oleate to linoleate of cholesteryl ester (CE) tends to be higher than that of normal counterparts.[4] Recently, CE, accumulated in arterial wall, has been observed as a liquid crystal in arterial smooth muscle cells and was called lipid inclusion bodies.[5] There are also circumstantial evidence that cholesterol and CE, accumulated in arterial wall, are derived from serum lipoproteins.[6] We have pointed out[7] that CE, retaining in arterial wall, might be a residual form of lipoproteins in lysosomes. If saturated fatty acid (FA) of CE increased in serum lipoprotein, it is easy to believe that such kind of CE might be hard to be eliminated by CE hydrolytic enzymes, according to the FA-specificity of the enzymes. Consequently, deposit of CE may be observed in arterial wall as saturated FA-rich CE (lipid inclusion bodies). In view of the facts, it seems of importance to elucidate the control mechanism of compositional change of FAs of CE, during and after cholesterol feeding. Present experiments suggested that CE hydrolytic enzymes might play an important role on a compositional change of FAs as well as synthetic enzymes.

Materials and Methods

Source of Rabbit Serum——Unless otherwise specified, all animals were female albino rabbits purchased from Miyamoto Rabbitry. The initial weight of the animals were about 2.4 kg. When not on a special diet, the animals were maintained on regular rabbit chow (RC-4 Oriental Kobo Kogyo). For the cholesterol feeding experiments, the rabbit chow was impregnated with 1% cholesterol, according to Wang, *et al.*[8] The diet was fed *ad libitum* to the animals for a period of 150 days. The animals were maintained, thereafter, on the basal diet for additional 30 days. Daily consumption of rabbit chow of each animal was 100 g, which

1) This study was supported by Grant-in-Aid from Ministry of Education of Japan and from Kotobuki Seiyaku.

2) Traveling fellow from Kotobuki Seiyaku.

3) Location: *1–2–3 Kasumi, Hiroshima-shi, Hiroshima.*

4) *a*) D.S. Goodman, *Physiological Rev.*, **45**, 747 (1965); *b*) O.W. Portman, *Adv. Lipid Res.*, **8**, 41 (1970).

5) *a*) W. Insull Jr., Y. Hata. J.D. Meakin, L. Marchant, C.W. Andrews, and B. Bazek, *Atheroscl.*, **19**, 555 (1974); *b*) Y. Hata, J. Hower, and W. Insull Jr., *Am. J. Path.*, **75**, 423 (1974).

6) *a*) E.B. Smith and R.S. Slater, "Ciba Found. Sym. Atherogenesis; Initiating Factors," 39 (1973); *b*) E.B. Smith and R.H. Smith, *Atheroscl. Rev.*, **1**, 119 (1976).

7) T.J. Peters, T. Takano, and C. de Duve, "Ciba Found. Sym. Atherogenesis; Initiating Factors," 214 (1973).

8) C.I. Wang, L.E. Shaefer, and D. Holessberg, *J. Mt. Sinai Hosp. NY.*, **21**, 19 (1954).

is equivalent of 1 g cholesterol per day. In each group, three rabbits were examined. The serum samples were collected from ear vein on 0, 90, 100, 110, 130, 150, 165 and 180 days of experimental date. Normal samples were collected from rabbits before cholesterol feeding. At 180 days, these animals were sacrificed and the aorta from the heart to the iliac bifurcation was removed. The adventitia was carefully dissected from the segments, which were opened longitudinally and any adherent blood clot was removed. Pieces of tissue were washed in ice cold Hanks' solution, blotted on absorbent paper and weighed. Strips of aorta were cut into slices approximately 0.5 mm thick by electric slicer (Hotta Rika S.S.). Slices were homogenized by polytron homogenizer (Kinematica) and were used for the extraction of lipids.

Analytical Techniques——Lipids were extracted from serum and aortic homogenate by the method of Folch, *et al.*[9] To extract lipid fraction, 3.3 ml of chloroform: methanol (2: 1) was added on 150 μl of suitably diluted sample. After vigorous mixing, 0.5 ml of distilled water were added. Samples were shaken vigorously for 30 sec, centrifuged briefly to separate phases. The lower chloroform layer, 2 ml aliquot, was transfered into a 4 ml conical centrifuge tube, and chloroform was removed under gas stream of nitrogen. The extracted lipids were dissolved in 25 μl of benzene: hexane (1: 1), and were deposited onto the silicic acid thin-layer chromatography (TLC) plate (20 cm × 20 cm, Eastman Kodak 6060, plastic plate with fluorescence indicator). Standard mixture, cholesterol, cholesteryl pentadecanoate and trimargarin (Applied Sci. Labs. Inc.) were run alongside of these samples. Chromatogram were developed in benzene: cyclohexane: acetic acid (60: 60: 0.1). Cholesterol and CE portions of the chromatogram were cut out and soaked in 2 ml of chloroform: methanol (2: 1) for 2 hr with occasional shaking. Chloroform: methanol fraction, 1.5 ml aliquot, was taken to dryness over the 70° water bath. These cholesterol and CE fractions were saponified by 0.5 ml of ethanol with 50 μl of 33% KOH at 60° for 15 min. After vigorous mixing with 1.5 ml of hexane, and subsequently 0.5 ml of distilled water for 30 sec. Samples were centrifuged briefly. Upper organic layer, 1 ml aliquot, was transfered into another tube and hexane was removed under gas stream of nitrogen. For the colorimetric assay of cholesterol, these samples were dissolved in 2 ml of *o*-phthalaldehyde reagent (50 mg *o*-phthalaldehyde in 100 ml of acetic acid), 1 ml of concentrated sulfuric acid was added and mixed.[10] After 10 min, 550 nm was read by spectrophotometer. For a standard and a recovery test, 0—50 μg of cholesterol (Applied Sci. Labs. Inc.) and 0—100 μg of cholesteryl oleate (Applied Sci. Labs. Inc.) were run alongside of these samples.

For the analysis of FA composition of CE, extraction of lipids and separation of CE from other lipids were carried out in the same way as was described previously.[11] Extracts from the portion of CE from TLC plate dissolved in 6 ml of 5% sulfuric acid in methanol and incubated at 70° for 90 min in water bath. Methylated FAs were extracted by 1.5 ml of pet-ether (30—60°). One ml of pet-ether fraction was taken to dryness and apply on GLC for FA analysis. Chromatography was carried out on 1 ml glass column filled with 10% diethyleneglycol succinate polyester on acid wash chromosorb W (DMCS) 60/80 mesh (Nihon Chromato. Works Ltd.). Temperature program was 125—190°, 2°/min. The instruments used was Hitachi gas chromatograph (type 163) with Hewlett-Packard integrator (type 3380A). As an internal standard, 20 μg of cholesteryl pentadecanoate was added in the samples, before extraction of lipids. To demonstrate a FA distribution profile of CE were arranged according to the FA specificity of CE hydrolytic enzymes from right to left in the following figures.

Results

Cholesterol and CE in Serum after Administration of Cholesterol Diet

Free cholesterol and CE in serum increased progressively throughout the period on the experimental diet to a plateau value during 110 and 150 days. The plateau value for free

Table I. Free and Esterified Cholesterol in Serum

Days	Free cholesterol (mg/ml)	Cholesteryl esters (mg/ml)
0	0.18±0.07	0.81±0.31
90	2.63±0.09	13.61±2.25
100	3.35±0.55	18.70±4.72
110	4.12±1.06	19.29±3 39
130	3.86±0.34	24.03±3.24
150	5.93±1.98	27.38±8.57

9) J. Folch, M. Lees, and G.H.S. Stanley, *J. Biol. Chem.*, **226**, 497 (1957).
10) L.L. Rudel and M.D. Morris, *J. Lipid Res.*, **14**, 64 (1973).
11) *a*) T. Takano, W.J. Black, T.J. Peters, and C. de Duve, *J. Biol. Chem.*, **249**, 6732 (1974); *b*) D. Deykin and D.S. Goodman, *J. Biol. Chem.*, **237**, 3649 (1962).

Table II. FA Composition of CE in Serum after Administration of Cholesterol Diet

Days	FA composition of CE in serum (mg/ml)						
	C 16:0	C 16:1	C 18:0	C 18:1	C 18:2	C 18:3	C 20:4
0	0.07±0.01	0.00±0.00	0.01±0.01	0.07±0.00	0.13±0.01	0.01±0.00	0.00±0.00
90	0.92±0.04	0.32±0.02	0.19±0.01	1.80±0.19	1.35±0.08	0.84±0.00	0.03±0.00
100	1.34±0.21	0.39±0.06	0.30±0.06	2.51±0.45	1.99±0.33	1.16±0.19	0.04±0.01
110	1.33±0.25	0.45±0.11	0.26±0.05	2.69±0.61	2.03±0.36	1.13±0.19	0.05±0.00
130	1.63±0.30	0.46±0.12	0.28±0.03	3.13±0.55	2.65±0.37	1.68±0.30	0.05±0.01
150	1.92±0.45	0.59±0.04	0.34±0.08	3.66±0.71	2.95±0.49	1.55±0.20	0.06±0.02

and CE was about 4—6 mg/ml and 19—27 mg/ml respectively. Since, concentration of the free and CE from normal serum was 0.18 mg/ml and 0.81 mg/ml, they increase 21—33 times and 24—34 times of normal value respectively (Table I). These results suggested that CE must be synthesized after cholesterol feeding. In order to examine what composition in FAs of CE was predominantly synthesized during these synthetic period, quantitative analysis on FA composition of CE was carried out. After cholesterol feeding, most of FAs of CE in serum increased progressively. Among these FAs, however, oleic acid increased predominantly during these period (Table II). In plateau period from 110 to 150 days, concentration of oleic acid was almost 38—52 times of normal value, though concentration of linoleic acid was only 15—22 times. These results suggested that CE synthetic enzymes may play an important role in synthesis of serum CE, which will be discussed afterward. Suppose, enhancement of oleate-rich CE had been simply depending upon CE synthetic enzyme, FA composition should have been constant, once CE increased in serum. Table II showed, however, that concentration of oleic acid increased gradually in parallel with increase of CE accumulated in serum. These data suggested that CE hydrolytic enzymes, which can preferentially hydrolyze polyundaturated FA of CE, such as linoleate, may also involved in accumulation of oleate-rich CE in serum, as well as CE synthetic enzymes. To demonstrate a participation of the CE degradation enzymes, FA composition of CE was arranged according to the specificity of CE hydrolytic enzymes (Fig. 1). FAs, arranged on the right hand, can be preferentially hydrolyzed by CE hydrolytic enzymes. In normal serum, concentration of linoleic acid was remarkably high. After administration of cholesterol diet, however, distribution of FAs tended to shift from right to left, suggesting that FA, which is remarkably resistant against CE hydrolytic enzymes, predominantly remained in serum. Consequently

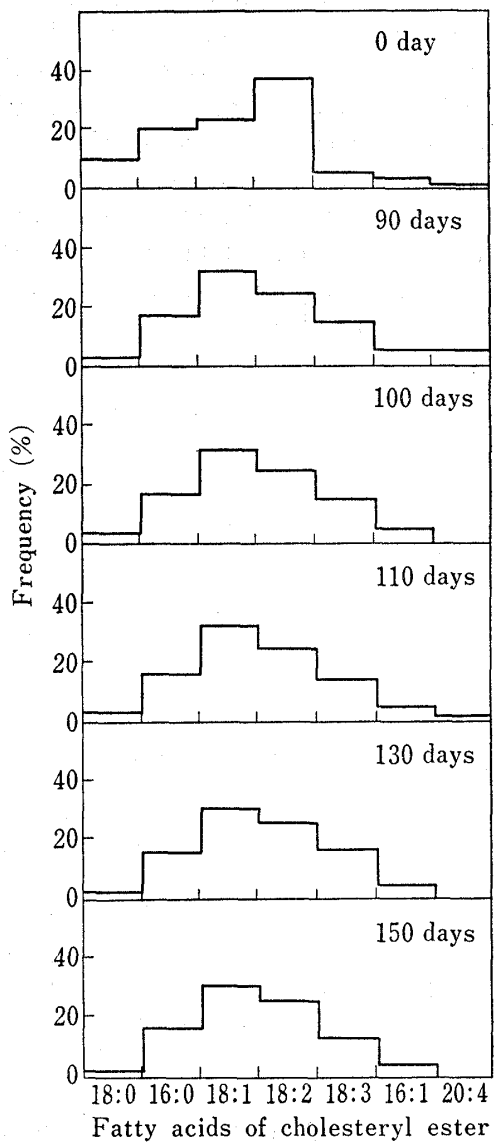

Fig. 1. FA Composition of CE in Serum was arranged according to the FA Specificity of CE Hydrolytic Enzymes from Right to Left

Serum samples were collected at 0—150 days after cholesterol administration.

oleate-rich CE was increased gradually in parallel with increase of CE concentration, even in plateau period of 110 to 150 days.

Cholesterol and CE in Serum after Removal of Cholesterol Diet from Cholesterol-fed Rabbits

To demonstrate the participation of CE degradation enzymes, further, compositional change of FAs of CE was examined after removal of cholesterol diet from cholesterol-fed animals. In this condition, both cholesterol and CE was reduced in serum rapidly. As far as CE concerned, 40% for 15 days and 67% for 30 days, it was eliminated from serum (Table III). Compositional change in FAs of CE was examined during these short periods, when CE was eliminated from serum. Most of FAs was reduced in parallel with reduction of CE in serum. Among these FAs, however, elimination of oleate was slower than linoleate and other polyunsaturated FAs (Table IV). For example, 33% of oleate for 15 days and 58% for 30 days was reduced, on the other hand, 44% of linoleate for 15 days and 80% for 30 days was reduced after removal of cholesterol diet. These results suggested that polyunsaturated FAs of CE, such as linoleate, was preferentially eliminated from serum in this particular condition. To demonstrate the participation of CE hydrolytic enzymes, distribution of FAs in CE was arranged according to the specificity of CE hydrolytic enzymes as was described previously (Fig. 2). Distribution in FAs of CE tends to shift from right to left further, which means that CE, which is remarkably resistant against CE hydrolytic enzymes, retained in serum after preferential degradation of polyunsaturated FAs by CE hydrolytic enzymes. Consequently, high amount of oleate-rich CE might be observed in serum after removal of cholesterol diet.

Table III. Free and Esterified Cholesterol in Serum after Removal of Cholesterol Diet

Days	Free cholesterol (mg/ml)	Cholesteryl esters (mg/ml)
0	5.93±1.98	27.38±8.57
15	3.03±0.83	16.31±8.54
30	1.16±1.04	8.95±4.48

Table IV. FA Composition of CE in Serum after Removal of Cholesterol Diet from Cholesterol-fed Rabbits

Days	FA composition of CE in serum (mg/ml)						
	C 16:0	C 16:1	C 18:0	C 18:1	C 18:2	C 18:3	C 20:4
0	1.92±0.45	0.59±0.04	0.34±0.08	3.66±0.71	2.95±0.49	1.55±0.20	0.06±0.02
15	1.09±0.30	0.37±0.09	0.16±0.01	2.48±0.73	1.65±0.45	0.79±0.22	0.00±0.00
30	0.66±0.23	0.21±0.06	0.11±0.03	1.57±0.52	0.61±0.24	0.41±0.09	0.01±0.00

Cholesterol and CE in Atheromatous Arterial Wall

Assuming that CE, accumulated in atheromatous aorta, may be derived from residual materials of lipoprotein, penetrated from blood stream. It may be important to compare FA composition of CE from atheromatous aorta with that from serum. In normal arterial wall, Fig. 3 shows that concentration of linoleate is the highest in any other FA moiety. Once, atheroma was induced by experimental diet, composition of FA changed from normal counterparts and shift from right to left (Fig. 3). In another words, CE, which is resistant against CE hydrolytic enzymes, deposited in arterial wall as residual products. Suggested that, preferential degradation of polyunsaturated FAs by CE hydrolytic enzymes, must be taken

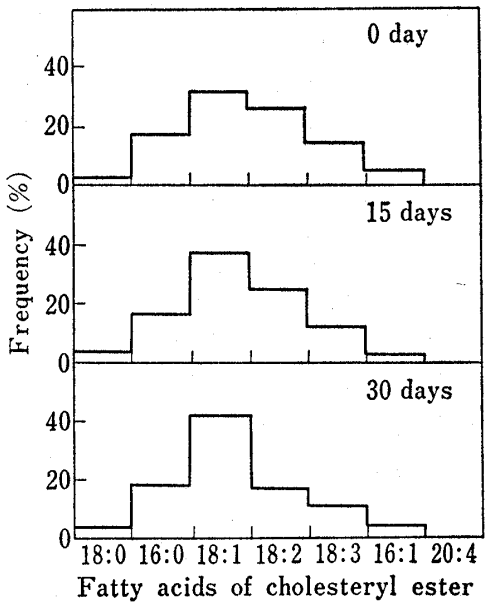

Fig. 2. FA Composition of CE in Serum after Removal of Cholesterol Diet from Cholesterol-fed Animals

Order of FA is same with that illustrated in Fig. 1. Serum samples were collected at 0, 15 and 30 days after removal of the experimental diet.

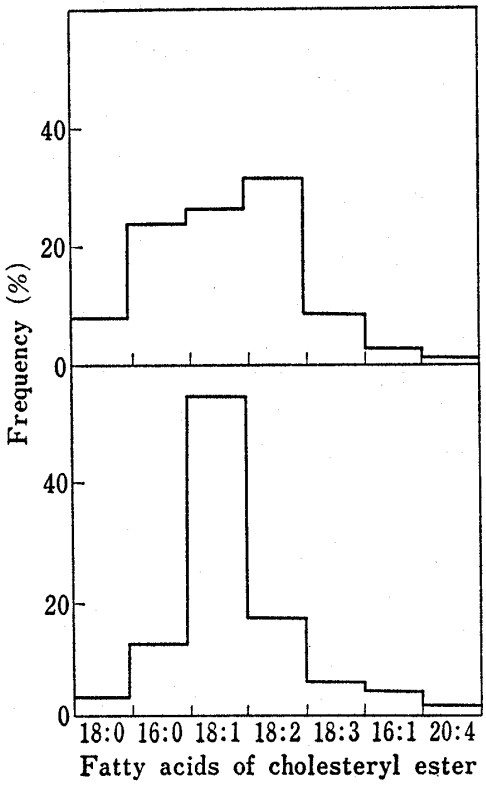

Fig. 3. FA Composition of CE from Normal and Atheromatous Arterial Wall

Order of FA is same with that illustrated in Fig. 1. Upper figure; normal. Lower figure; atheromatous aorta.

plase in arterial wall, as was observed in serum CE. Possible mechanism will be discussed in following section.

Discussion

These data have shown that, measured in terms of FA composition of CE in serum and arterial wall, CE hydrolytic enzymes as well as synthetic enzymes may be involved in accumulation of oleate-rich CE after cholesterol feeding. Several worker[12] has pointed out that high proportion of saturated FA of CE can be observed in atheromatous aorta from human specimens and experimental animals. There are circumstantial evidence that CE, accumulated in atheromatous aorta, derived from lipoprotein of blood stream.[6] Smith, *et al.*,[13] however, pointed out that FA composition of CE from atheromatous aorta are different from that from lipoproteins; concentration of oleate from atheromatous aorta is higher than that from lipoprotein. The difference might be explained by preferential degradation of polyunsaturated FAs in CE of lipoprotein in arterial cells. FA composition in atheromatous aorta, was rich in these FAs that are resistant against CE hydrolytic enzymes (Fig. 3). In addition, the distribution revealed more similarity to that from serum after removal of cholesterol feeding (Table IV, Fig. 2) than that from serum on 150 days experimental diet (Table II, Fig. 1). After removal of cholesterol diet from cholesterol-fed animals, 40% of CE for 15 days and 67% for 30 days was reduced from blood stream (Table III). In this condition, degradation

12) *a*) A.V. Chobanian and F. Manzur, *J. Lipid Res.*, **13**, 201 (1972); *b*) D.B. Zilversmit, C.C. Sweeley, and H.A.I. Newman, *Circulation Res.*, **9**, 235 (1961); *c*) D.S. Goodman, *Physiological Rev.*, **45**, 747 (1965).
13) E.B. Smith, D.H. Evans, and M.D. Dawnham, *J. Atheroscl. Res.*, **7**, 171 (1967).

or elimination of CE from serum must be taken place during these short period. These results might be able to be interpreted that, in serum after removal of cholesterol feeding, CE hydrolytic enzymes might involve in preferential degradation of polyunsaturated FAs of CE. Consequently, oleate-rich CE was accumulated in this fraction.

It is important to know a participation of CE synthetic enzymes on accumulation of oleate-rich CE as well as hydrolytic enzymes. Enhancement of oleate-rich CE might be also explained by the enzymes, because FA specificity of the enzymes are high in order, oleic> palmitic>stearic>linoleic acid in liver,[14] and oleic>linoleic>arachidonic>palmitic>stearic acid in aortic intima.[15] Serum CE increased progressively throughout the period on the experimental diet to a plateau value, which is 24—34 times of normal one (Table I). These data suggested that CE must be synthesized and accumulated in serum after cholesterol administration. FA composition of CE indicated that oleate-rich CE was accumulated in serum from cholesterol-fed animals (Table II), in agreements with the observation of Zilversmit, *et al.*[16] It might be explained, therefore, that oleate-rich CE also increased after synthesis by CE synthetic enzymes. Enhancement of oleate-rich CE in serum, however, can not be able to explained simply by CE synthetic enzymes even in these condition, because concentration of oleic acid in CE increased progressively throughout the period on experimental diet. FA composition of CE should have been constant throughout the period on experimental diet, if synthetic enzyme alone had involved in enhancement of oleate-rich CE. As shown in Table II, however, proportion of oleic acid of CE was not constant during these plateau period, suggested that not only CE synthetic enzymes, but also hydrolytic enzymes might involve in accumulation of oleate-rich CE in serum. Fig. 1 might support this concept. In another word, oleate-rich CE might be retained in serum after preferential hydrolysis of polyunsaturated FA of CE, at the same time, oleate-rich CE was synthesized by CE synthetic enzymes, according to their FA specificity. After rapid synthesis of CE, equilibrium of synthesis and degradation of CE in serum must be maintained by these enzymes during plateau period 110—150 days after cholesterol administration. Consequently, oleate-rich CE must be accumulated gradually in serum. It is still not clear where synthesis and hydrolysis of CE in serum was taken place. It must be believed that liver or adipose tissue mainly synthesized it and excreted it out into blood stream. Participation of serum lecithin cholesterol acyltransferase on the synthesis of oleate-rich CE must be eliminated, because FA specificity of the enzyme was supposed to be higher on polyunsaturated FA.[17] On the other hand, degradation of CE in serum is less clear, since no hydrolytic enzyme on CE was detected in serum. One can not neglect that lipoprotein lipase[18] might involve in hydrolysis of CE in serum. These results, however, suggested that CE hydrolytic enzymes as well as synthetic enzymes involved in increase of oleate-rich CE. High concentration of oleate-rich CE in serum might stimulate atherosclerosis in arterial wall, because arterial CE hydrolytic enzymes might be

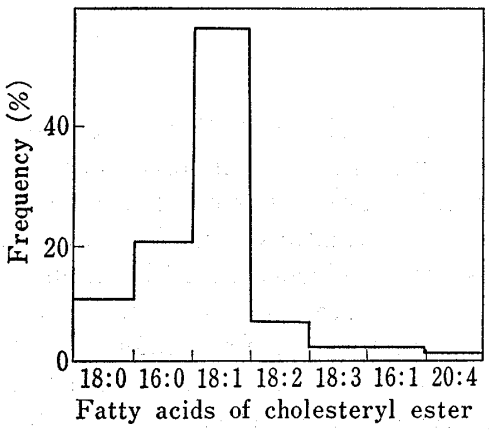

Fig. 4. FA Composition of CE from Lipid Inclusion Bodies prepared from Atheromatous Aorta

Order of FA is same with that illustrated in Fig. 1.

14) D.S. Goodman, D. Deykin, and T. Shiratori, *J. Biol. Chem.*, **239**, 1335 (1964).
15) H.V. Kothari and D. Kritchevsky, *Lipids*, **10**, 322 (1974).
16) D.B. Zilversmit, C.C. Weeley, and H.A.I. Newman, *Circulation Res.*, **9**, 235, 747 (1965).
17) J.A. Glomset, *Biochim. Biophys. Acta*, **65**, 128 (1962).
18) E.D. Korn, *Methods of Biochem. Analysis*, **7**, 145 (1959).

hard to hydrolyze them to eliminate from arterial cell, once oleate-rich CE lipoprotein penetrated into arterial wall. Oleate-rich CE in lipoprotein, therefore, might stimulate accumulation of CE in arterial wall (Fig. 3).

Consequently, CE might come out as a liquid crystal "lipid inclusion body" which is containing high concentration of oleic acid in CE, as is shown in Fig. 4.

To elucidate the mechanism of accumulation of oleate-rich CE in atheromatous arterial wall and serum, however, further experiments must be required.

Acknowledgement We are particularly indebted to Prof. Sadahiko Ishibashi for helpful advise and discussion.