Pattern of Cholesterol Ester Fatty Acids in Serum in Normal Subjects and in Patients with Essential Hypercholesterolemia

By Per Björntorp, M.D., and Bertil Hood, M.D.

Vegetable oils lower the serum cholesterol level in man, and this effect is probably due to their content of polyunsaturated fatty acids, as has been demonstrated by Kinsell and co-workers, Ahrens and co-workers, Malmros and Wigand, and others.

Separation of total fatty acids in serum has been performed in normal subjects and patients with demonstrated atherosclerosis by Hammond and Lundberg, Schrade and co-workers, and James and co-workers. The 2 first-mentioned studies showed a lower percentage of dienes and tetranea in atherosclerotics as compared with normals, while James and co-workers were unable to find any differences in the serum levels of the unsaturated fatty acids between a group of normals and a group of patients who had suffered a myocardial infarction. In the work of James and co-workers, however, there was no difference in the level of serum cholesterol between the 2 groups.

The high degree of unsaturation in cholesterol ester fatty acids (CEFA) in serum has been demonstrated by Kelsey and Longenecker and Lough and Garton in bovine serum, and in man by Kinsell and co-workers and recently by Lewis. Lewis has also shown, that patients with coronary artery disease have a lower percentage content of diene and tetranea in their CEFA-fraction than normal whites. On the other hand, such patients had a lower percentage of unsaturated CEFA than the Bantu. These cases were investigated under ambulatory conditions and under metabolic ward conditions a similar study was done during the maintenance of various diets. Similar work has also been done by Kinsell and co-workers. Both studies showed a rise in degree of unsaturation in CEFA when unsaturated fat was given and a decrease on a diet with a low content of unsaturated fatty acids.

The main questions in this study have been the following: Is there any difference in the pattern of fatty acids in patients with essential hypercholesterolemia as compared with normals? Is the pattern of fatty acids changed by the substitution of corn oil or corn oil plus pyridoxine for other fats in the diet?

Methods

Twelve patients with essential hypercholesterolemia were investigated. Seven were members of well-studied families with essential hypercholesterolemia and xanthomatosis. All patients had cholesterol values exceeding 325 mg. per cent in repeated determinations. Six presented xanthomas. All showed a clear serum in the fasting state and had a normal uptake of iodine and no signs of biliary obstruction or kidney disease of the nephrotic type.

For comparison, we have used 10 normal subjects who were matched as regards age and sex with the 10 patients studied on ad libitum diet. Subjects consuming moderate amounts of vegetable oils were excluded from this comparison. As normals we have accepted persons who had no familial history of myocardial infarction or gall-bladder disease. Furthermore, the electrocardiogram was normal and repeated serum cholesterol values were less than 300 mg. per cent.

There was no evidence of rapid shifts in weight at the time of sampling. The subjects studied during changes in diet or with addition of pyridoxine were weighed during the entire time of study. In one of them (E.C. fig. 1) there was a definite gain in weight during the heavy dosage of corn oil (7 Kg./18 months).
A detailed dietary history was made. The patients selected for changes in the diet have been only those, who, after several years of experience, have proved themselves reliable and co-operative.

Three subjects were analyzed before and after the substitution of corn oil (100 to 125 Gm. daily) in the diet. One of them (V.F., fig. 2) is included in the 10 patients on ad libitum diet, and in this patient the serum analysis was made 6 weeks after the substitution of corn oil. The other 2 patients were studied 6 months after the substitution of corn oil in the diet and again 1 month after the addition of pyridoxine (100 mg. daily) to the corn oil. One of them (E.C., fig. 1) was studied for 2 periods after the addition of pyridoxine.

A sample of venous blood (25 ml.) was drawn in the fasting state in the morning and was centrifuged. The serum was immediately extracted and then analyzed or stored in —20 C. Serum was extracted according to Bloor in ethanol: ether (3:1, v/v) and re-extracted in chloroform: ethanol (99:1, v/v). Cholesterol, phosphorus, and evaporation residue were determined in the chloroform:

A part of the extract containing approximately 120 mg. of total fat was evaporated to dryness, dissolved in petroleum-ether and the lipids were separated on silicic acid into the following fractions: cholesterol esters, glycerides + unesterified fatty acids + free cholesterol and phosphatides. The solvent from the column was collected in 25 ml. portions of which 10 ml. was evaporated and the residue weighed. This residue was analyzed for free and esterified cholesterol by paper-chromatography and for glycerol before the elution of the glycerides + unesterified fatty acids + free cholesterol.

For further control of the separation, glycerol determinations and paper chromatography of free and esterified cholesterol were performed in aliquots from the cholesterol ester eluate. Only those separations have been accepted where the glyceride fatty acid contamination of the CEFA was less.

*In an earlier part of this investigation the lipid chromatography was performed according to Borgström. This method essentially showed the same results as the method of Hirsch and Ahrens in parallel analyses, but sometimes there were contaminations of glycerides in the cholesterol ester eluate.
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12 ml serum

- Extraction (ethanol; ether, 3:1)
- Re-extraction (chloroform; ethanol, 9:1)

- Determination of weight, phosphorus and cholesterol.
- Chromatography of lipids on SiO₂,
  (elution with 1% di- (elution with di- (elution with ethyl ether in petroleum ether) methanol)
  leum ether + 4% di- (elution with ethyl ether in petroleum ether)

- Cholesterol esters. Glycérides + free Phosphatides
- Determination of cholesterol + unesterified fatty acids.

- Saponification.
- Unsoapifiable matter separated off.
- Acidification.
- Fatty acids extracted with petroleum ether.
- Evaporation.
- Fatty acids dissolved in ethanol.

- Titration of fatty Alkali isomerization acids

Figure 3

Scheme of the procedure.

than 5 per cent of the CEFA. The recovery of cholesterol from the column was determined by comparing the cholesterol figures before and after the chromatographic separation.

The cholesterol esters were saponified, the unsaponifiable matter removed, the soaps hydrolyzed, and the fatty acids extracted and determined by titration. Polyunsaturated fatty acids were determined by the alkali isomerization technic described by Holman and Hayes.¹³

All determinations were made in duplicate. Evaporations were performed in an atmosphere of oxygen-free nitrogen.

Cholesterol was determined according to Theorell,¹⁴ whose method has recently been modified and compared with the method of Sperry and Webb¹⁵ by Cranér and Isaksson.¹⁶ Paper-chromatography of free and esterified cholesterol was performed according to Michaelis.¹⁷ Glycérol was determined according to Lambert and Neish,¹⁸ as modified by Carlsson and Wadström.¹⁹ Scheme of the procedure is seen in figure 3.

Results

Table 1 summarizes the absolute content of dienes in the CEFA in mg. per cent in patients with hypercholesterolemia and in normal subjects. The serum levels in the patients are higher than in the controls (fig. 4). The data in table 1 also show that the group of hypercholesterolemic patients had a some what lower percentage content of dienes in CEFA than the normals on ad libitum diet. There is no consistent difference in the other polyene fractions. The difference in the percentage content of dienes is statistically significant at the 5 per cent level.

In patients in whom the hypercholesterolemia was less marked, full normalization in the serum cholesterol level was achieved after the substitution of a large dose of corn oil for other fats in the diet. This procedure also resulted in a high level of unsaturation in the CEFA fraction (fig. 2).

We were not able, even by using a very large dosage of corn oil during a long experimental period, to normalize the total cholesterol values in one patient (fig. 1) even though an extremely high percentage content of polyunsaturated fatty acids was achieved in the CEFA.*

Addition of pyridoxine to the corn oil regimen produced no certain effect either on the

*The lipid separations of these sera were performed with the method according to Borgström.¹²

Figure 4

Relation between total cholesterol values and absolute amount of CEFA dienes. (Filled circle at 110 mg. per cent; see footnote, at bottom of page.)
Table 1

Analytical Data (mean Values and Standard Deviations) for the Normals and the Hypercholesterolemic Subjects on Ad Libitum Diet

<table>
<thead>
<tr>
<th></th>
<th>Normals</th>
<th>Essential hypercholesterolemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg.%)</td>
<td>237 ±34</td>
<td>399 ±73</td>
</tr>
<tr>
<td>Dienes (% of CEFA)</td>
<td>42.2± 3.2</td>
<td>37.8± 4.2</td>
</tr>
<tr>
<td>Trienes (&quot; )</td>
<td>2.6± 0.9</td>
<td>2.7± 1.0</td>
</tr>
<tr>
<td>Tetraenes (&quot; )</td>
<td>6.1± 0.9</td>
<td>5.9± 1.3</td>
</tr>
<tr>
<td>Pentaenes (&quot; )</td>
<td>2.2± 0.5</td>
<td>2.1± 0.7</td>
</tr>
<tr>
<td>Hexaenes (&quot; )</td>
<td>1.7± 0.7</td>
<td>1.8± 0.7</td>
</tr>
<tr>
<td>Total PUFA (&quot; )</td>
<td>54.9± 3.1</td>
<td>50.3± 3.9</td>
</tr>
<tr>
<td>CEFA Dienes (mg.%)</td>
<td>49 ± 9</td>
<td>71 ±18</td>
</tr>
</tbody>
</table>

level of the total serum cholesterol or on any of the fatty acid fractions (fig. 1).

**Discussion**

This investigation shows that the levels of dienes in the CEFA were higher in patients with essential hypercholesterolemia than in 10 matched normal subjects. The patients had usually a lower percentage content of dienes in the fraction most rich in polyunsaturated acids, i.e., cholesterol esters. A similar trend is also evident from the work of Lewis. Pyridoxine plays a role in the conversion of dienes to tetraenes and trienes to hexaenes. In this study there was no increase in tetraenes and hexaenes when corn oil and pyridoxine were administered and there was no further decrease in the cholesterol level caused by the pyridoxine.

The high absolute level of dienes in CEFA and the inability to achieve normal serum cholesterol values in 1 severe case of essential hypercholesterolemia and xanthomatosis, in spite of intake of a large dosage of unsaturated fat, do not favor the hypothesis of an absolute deficit of unsaturated fatty acids as playing a prominent part in the pathogenesis of this condition. The observations of a very marked increase in the absolute amount of polyunsaturated fatty acids in the cholesterol esters and a still remarkably elevated serum cholesterol after a large intake of unsaturated fat are against such factors as a block in the esterification of cholesterol with polyunsaturated fatty acids. Our data might be interpreted along the following lines: There is an increased level of cholesterol caused by unknown factors. This phenomenon is accompanied by a higher absolute amount of polyunsaturated CEFA than normal. The percentage content is generally lowered and this phenomenon is probably secondary to the high cholesterol level. In this connection it might be of interest with data in situations like hypothyrosis, nephrosis and other conditions where very rapid changes in the serum cholesterol level might be produced.

**Summary**

Sera from 10 patients with essential hypercholesterolemia on ad libitum diet have been analyzed for their content of polyunsaturated fatty acids in the cholesterol esters and the results compared with a matched group of normal subjects. Three cases were analyzed after corn oil substitution for other fats in the diet, and 2 of them were analyzed after addition of pyridoxine to the corn oil regimen. One patient was followed 18 months, and during that time he received a large dosage of corn oil.

Patients with essential hypercholesterolemia had an absolute increase in the amount, but generally a lower percentage content of dienes in their cholesterol esters than normals. After the change to corn oil, the content of polyunsaturated fatty acids increased to levels sometimes considerably above those in the normal subjects, although the total serum cholesterol in one of these cases was by no means normalized. The addition of 100 mg. of pyridoxine to the corn oil regimen did not produce any further decrease in the serum cholesterol level and did not produce any changes in the fatty acid fractions.

The absolute increase in the amount of polyunsaturated fatty acids in the cholesterol esters in essential hypercholesterolemia, particularly after an extremely high intake of unsaturated fat, speaks against factors causing an inhibition in esterification with polyenes as being of central importance.
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Summary in Interlingua

Seros de 10 patientes con hypercholesterolemia esseva analysate pro lor contento de poly-nonsaturated acidos grasse in le esteres de cholesterol. Le resultatos esseva compareate con le resultatos de simile observation durante 18 menses. In le curso de iste marstrand etiam esseva analysate post le addition de pyridoxina al regime de oleo de mais. Un patiente esseva tenite sub observation durante 18 menses. In le curso de iste tempore illo recepeva un forte dosage de oleo de mais.

Patients con hypercholesterolemia essentia montava un augmento absolute de dienns in lor esteres de cholesterol, sed generalmente le procentagc de tal dienns esseva plus baxe que in subjectos normal. Post le addition de oleo de mais, lo contento de poly-nonsaturated acidos grasse montava a nivellos que esseva a vices considerablemente plus alte que illos observate in le subjectos normal. Nonobstante, in un de iste casos le total contento serai de cholesterol esseva nettamente non normalisata. Le addition de 100 mg de pyridoxina al regime a oleo de mais non resultava in un reduction additional del nivello serai de cholesterol. Illo non resultava in ulle alteration in le fractiones de acidos grasse.

Le augmento absolute in le quantitate de poly-nonsaturated acidos grasse del esteres de cholesterol in cases de hypercholesterolemia essential, particularmente post le ingestion de alitissime quantitates de nonsaturated grassias, representa un argumento contra factorcs que causan un inhibition de esterification, in lo que polyenas esseran de importanta central.

References

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