CHANGES IN RABBIT LIPOPROTEIN PROPERTIES BY DIETARY CHOLESTEROL, AND SATURATED AND POLYUNSATURATED FATS

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SUMMARY

Plasma lipids and chemical, electrophoretic and electron microscopic properties of VLDL, LDL and HDL are examined in rabbits fed a control diet (group I) or diets containing 1% cholesterol (group II), 1% cholesterol + 5% coconut oil (group III) or 1% cholesterol + 5% corn oil (group IV).

The diets II, III and IV resulted in hypercholesterolemia, hypertriglyceridemia and hyperphospholipidemia. The lipid–protein composition of VLDL, LDL and HDL is changed by these diets. There is a marked increase in the total cholesterol content of all lipoprotein fractions of the high fat dietary groups II, III and IV.

The electrophoretic mobilities of the VLDL and LDL II and III are reduced while the respective mobilities in the corn oil group IV are nearly “normal”. In contrast to the control LDL fraction I which is not precipitated by heparin, the LDL fractions of the dietary groups II, III and IV are readily precipitated. The apoprotein pattern of the lipoproteins in polyacrylamide gel differs distinctly between the dietary groups, most bands appearing in group IV. An abnormal stacking of lipoprotein particles in electron micrographs of VLDL, LDL and HDL of groups II and III can be observed. In contrast, these lipoprotein fractions of rabbits of the corn oil group IV have morphological properties that are similar to those of the lipoproteins of the control group. It is suggested that these findings are related to the marked reduction of atherosclerosis in rabbits fed a diet with polyunsaturated fat as compared with rabbits on cholesterol and cholesterol–coconut oil diets.

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INTRODUCTION

Diets reducing serum cholesterol by their high contents of polyunsaturated fat have in some studies been shown to reduce also the mortality from coronary heart disease.\(^1\)\(^-\)\(^4\)

The serum cholesterol lowering effect of polyunsaturated fats, originally observed by Kinsell et al.\(^5\), seems to be due to a redistribution of cholesterol between serum and tissues\(^6\) and/or an accelerated entero-hepatic circulation of bile salts with a consequent increase in their excretion\(^7\). Wigand\(^8\) was the first to show that the divergent effects of saturated and polyunsaturated fats described in humans can also be demonstrated in rabbits.

Using different “cholesterol vehicles” in experimental atherosclerosis of rabbits, Kritchevsky et al.\(^9\)\(^-\)\(^11\) found that the severity of atheromata varies inversely with the iodine number of the fat added to the diet. These findings were essentially confirmed by Vles et al.\(^13\), Kloeze et al.\(^14\), Bortz et al.\(^15\) and Stange et al.\(^12\). In these experiments there was a poor correlation between the cholesterolemic action and the atherogenicity of these fats. To clarify this discrepancy it was speculated that different dietary fatty acids induce structural changes in their triglyceride molecules and that these changes in turn alter the lipoprotein conformation and thereby reduce its atherogenicity.\(^9\)\(^,\)\(^16\)

Changes in rabbit and guinea pig lipoprotein structure by dietary cholesterol have been reported by Gofman et al.\(^17\), Camejo et al.\(^18\)\(^,\)\(^19\) and Shore et al.\(^20\) as well as Sardet et al.\(^21\), but so far little is known about the influence of saturated or unsaturated fats.\(^22\) With regard to the crucial role played by lipoproteins in atherogenesis,\(^5\)\(^0\) we investigated the very low density lipoprotein (VLDL), the low density lipoprotein (LDL) and the high density lipoprotein (HDL) fractions of rabbits fed a control, 1% cholesterol, 1% cholesterol + 5% coconut oil, and a 1% cholesterol + 5% corn oil diet. Results presented in this paper indicate that these dietary fats influence lipoprotein and apoprotein structure. These structural changes of lipoproteins possibly affect the development of atherosclerosis in rabbits.

METHODS

Animals and feeding schedule for experimental diets

Thirty male New Zealand white rabbits weighing 3–4 kg were kept in individual, wire bottomed cages throughout the experimental period of 18 weeks. They were divided at random into 4 groups receiving their respective diets and water ad libitum. The feeding schedule during the experimental period of 18 weeks is shown in Fig. 1.
Changes in Rabbit Lipoprotein Properties

Fig. 1. The feeding schedule for experimental diets in four groups (I-IV) of rabbits for the dietary period of 18 weeks.

According to this schedule, group I rabbits (n = 6) received a control diet consisting of commercial rabbit chow (Georg Plange Co., Soest, W.-Germany) for 18 weeks. Group II animals (n = 5) were fed the control diet containing 1% cholesterol for the same period of time. Group III (n = 4) had the control diet + 1% cholesterol during the first 9 weeks, then had additional 5% coconut oil (iodine number = 10) as saturated fat for the second 9 weeks. Group IV animals (n = 6) followed the same dietary regimen as group III, except that coconut oil was replaced by 5% of the unsaturated fat corn oil with the iodine number of 126 (Fig. 1).

The animals were bled every 3 weeks from the ear veins into tubes containing EDTA to a final concentration of 0.01%. Plasma was isolated by centrifugation at 3,000 rpm for 10 min. In these samples triglycerides, phospholipids, cholesterol, the SGOT and the SGPT were determined. At the end of the dietary period the animals were weighed and then bled from the carotid artery. The individual plasma samples were pooled before the lipoprotein fractions of groups I-IV respectively were isolated. All blood samples were drawn from the rabbits in a not fasted state.

Preparation of lipoproteins

Lipoproteins were isolated by standard flotation technique. After ultracentrifugation the tubes were cut by a slicing device. The supernatant and infranatant fractions were adjusted to the desired density by the addition of crystalline KBr. Autooxidation of lipoproteins was prevented by addition of 0.01% EDTA. Chylomicrons were removed by centrifugation at 15,000 rpm for 15 min at 4°C in a Sorvall centrifuge RC 2 B, using the SS 34 rotor.

Plasma was fractionated into 3 density classes: the fraction of $d < 1.006$ g/ml (very low density lipoproteins, VLDL), the fraction of $d = 1.006-1.063$ g/ml (low density lipoproteins, LDL) and the fraction of $d = 1.063-1.210$ g/ml (high density lipoproteins, HDL). Ultracentrifugation was performed in a Beckmann L-2 65 B model with a rotor Type 60 Tl at 60,000 rpm for 22 hr (VLDL and LDL) or 24 hr.
(HDL) at the respective densities. All fractions were recentrifuged and dialysed against 0.15 \( M \) NaCl, containing 0.01\% EDTA and sodium azide (NaN\(_3\)).

**Electrophoresis**

Lipid electrophoresis was carried out according to Kahlke\(^{24}\) in 0.8\% agarose with a 0.025 \( M \) veronal buffer containing 0.1\% albumin. Lipoprotein bands were fixed in 2\% acetic acid and stained in 60\% alcohol with sudan black for 2 hr.

Immunelectrophoresis was performed according to Noble\(^{25}\) with 1\% agarose and a barbital buffer of pH 8.5, with an ionic strength of 0.05.

Lipoprotein precipitation by polyanionic compounds originally described by Burstein et al.\(^{26}\) was accomplished as was recently described by Wieland and Seidel\(^{27}\) after agarose gel electrophoresis. After the electrophoretic run the agarose slides were placed in "solution 1", a bath of 0.1 mole/1,000 ml MgCl\(_2\) containing 1.5 g/1,000 ml sodium heparin\(^{26,27}\). In control animals this solution was shown to precipitate VLDL selectively. This is comparable with the results in humans\(^{26,27}\). After 30 min, the same slides were placed in "solution 2", containing 0.2 mole/1,000 ml CaCl\(_2\) and 6 g/1,000 ml sodium dextran sulphate (Pharmacia, Upsala, Sweden), which as in the case of human lipoproteins\(^{26,27}\) precipitates the VLDL, LDL and HDL fractions of rabbits. The precipitated bands were made visible under oblique light.

Polyacrylamide gel electrophoresis was essentially performed according to the method of Kane\(^{28}\). 0.2 ml tetramethylurea was used to delipidate 0.2 ml lipoprotein solution. Thioglycol, mercaptoacetic acid and EDTA were added to the mixture as reducing agents to obtain a better resolution of the individual bands. After a 20 min incubation period the lipid phase and an insoluble apoprotein, probably corresponding to the human apoprotein B, were removed by high speed centrifugation at 20,000 r.p.m. for 1 hr, to avoid interference of insoluble material with electrophoresis. Persulphate was used as a catalyst in the 15\% separating gel, whereas the stacking gel photopolymerized with riboflavin. The gels were stained in 20\% acetic acid and methanol with 0.05\% Coomassie Brilliant Blue (Serva Co., Heidelberg, W. Germany) and destained by diffusion in a bath containing 10\% acetic acid and methanol for up to 2 days. To allow a grossly quantitative comparison of the peptide patterns the same amount of protein of about 100–150 \( \mu \)g of the different fractions was applied to the gel. Densitometric scanning (Bender and Hobein, Zurich, Switzerland) was always accomplished under comparable conditions, but no attempts for exact quantitative estimation were made.

**Chemical analysis**

All lipids were determined by standard tests (Boehringer Co., Mannheim, W. Germany). The triglyceride estimation is based on the method of Eggstein and Kreutz\(^{29}\). Total cholesterol was determined according to Watson, Leffler\(^{30}\). Phosphorus was estimated as described by Zilversmit and Davis\(^{31}\). The factor 25 was used to convert phosphorus into phospholipid. Protein was assayed by the method
TABLE 1
SERUM CHOLESTEROL (X ± S) OF GROUP I-IV RABBITS DURING THE DIETARY PERIOD OF 18 WEEKS

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum cholesterol (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>I</td>
<td>83±32</td>
</tr>
<tr>
<td>II</td>
<td>128±29</td>
</tr>
<tr>
<td>III</td>
<td>75±16</td>
</tr>
<tr>
<td>IV</td>
<td>85±34</td>
</tr>
</tbody>
</table>

Electron microscopy

The freshly isolated lipoprotein fractions were diluted to appropriate concentrations in order to avoid artifactual clustering of the particles. Observations in the electron microscope were done after negative staining at 4°C and at room temperature with a 1% solution of phosphotungstic acid, adjusted to pH 7.1 with KOH. Usually a drop of the suspension was placed on a formvar and carbon coated grid. After 10–20 sec the grid was drained onto a filter paper and immediately covered with a drop of stain for about 40 sec, then excess stain was removed with filter paper. Alternatively the staining solution was added to the specimen suspension in a test tube, and then a grid was dipped into the mixture and excess liquid was removed. Grids were dried in air and immediately examined in a Siemens Elmiskop 101 electron microscope, equipped with a cooling device, at an accelerating voltage of 80 kV. Pictures were taken at magnifications ranging from 20,000 to 50,000.

RESULTS

Plasma lipids

At the beginning of the experimental period plasma cholesterol levels varied...
between 75 and 128 mg/100 ml in group I, II, III and IV rabbits (Table 1). Plasma cholesterol of the control group I remained constantly low for the whole dietary period, declining slightly from 83 to 51 mg/100 ml (Table 1). The cholesterol group II showed an increase in plasma cholesterol to 2035 mg/100 ml after 9 weeks (Table 1), while in the second part of the experiment there was a decrease to 1444 mg/100 ml. In the coconut oil group III and the corn oil group IV plasma cholesterol showed an increase after 3 and 6 weeks on the cholesterol diet and a decrease after 9 weeks to 1759 mg/100 ml in group III and to 1260 mg/100 ml in group IV. The addition of coconut oil in group III prompted an increase in serum cholesterol to 2629 mg/100 ml, while in the corn oil group IV plasma cholesterol showed a less pronounced rise to 2002 mg/100 ml after 18 weeks of the experiment (Table 1).

Plasma triglyceride concentrations varied between 149 and 203 mg/100 ml in group I, II, III and IV rabbits at the beginning of the experimental period (Table 2). In the control group I plasma triglycerides showed only minor changes throughout the experiment of 18 weeks. In groups II, III and IV triglycerides were increasing during the first 6 weeks of the experiment and decreasing to control values after 9 weeks (Table 2). During the second half of the dietary period of 18 weeks, however, triglyceride concentrations in the hypercholesterolemic groups II, III and IV rose again, the most marked increase being initiated by coconut oil (group III in Table 2). No differ-

### TABLE 3

SERUM PHOSPHOLIPIDS ($\overline{x} \pm S$) OF GROUP I-IV RABBITS DURING THE DIETARY PERIOD OF 18 WEEKS

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum phospholipids (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>I</td>
<td>145±25</td>
</tr>
<tr>
<td>II</td>
<td>162±20</td>
</tr>
<tr>
<td>III</td>
<td>134±30</td>
</tr>
<tr>
<td>IV</td>
<td>137±57</td>
</tr>
</tbody>
</table>

### TABLE 4

CHEMICAL COMPOSITION (%) OF THE VERY LOW DENSITY LIPOPROTEINS (VLDL) OF GROUP I-IV RABBITS

<table>
<thead>
<tr>
<th>Group</th>
<th>Chemical composition (%) of VLDL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>protein</td>
</tr>
<tr>
<td>I</td>
<td>11.5</td>
</tr>
<tr>
<td>II</td>
<td>6.4</td>
</tr>
<tr>
<td>III</td>
<td>7.5</td>
</tr>
<tr>
<td>IV</td>
<td>7.2</td>
</tr>
</tbody>
</table>
TABLE 5
CHEMICAL COMPOSITION (%) OF THE LOW DENSITY LIPOPROTEINS (LDL) OF GROUP I-IV RABBITS

<table>
<thead>
<tr>
<th>Group</th>
<th>Chemical composition (%) of LDL</th>
<th>protein</th>
<th>triglycerides</th>
<th>cholesterol</th>
<th>phospholipids</th>
<th>protein/lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td>28.4</td>
<td>11.0</td>
<td>43.4</td>
<td>17.2</td>
<td>0.40</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td>16.1</td>
<td>4.1</td>
<td>54.4</td>
<td>25.4</td>
<td>0.19</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td>15.0</td>
<td>9.9</td>
<td>52.9</td>
<td>22.2</td>
<td>0.18</td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td>18.8</td>
<td>10.9</td>
<td>48.6</td>
<td>21.7</td>
<td>0.23</td>
</tr>
</tbody>
</table>

ence could be found when additional corn oil was given, as compared with the cholesterol group II (Table 2). At the end of the experimental period plasma triglyceride concentrations were 339, 511 and 313 mg/100 ml in groups II, III and IV respectively.

Plasma phospholipids showed control values of 134 to 211 mg/100 ml at the beginning and in group I during 18 weeks of the dietary period (Table 3). The cholesterol diet increased plasma phospholipids to 792, 615 and 541 mg/100 ml in groups II, III and IV after 9 weeks. As compared to group II rabbits coconut oil (group III) and corn oil (group IV) effected a further increase of the phospholipids to 941 and 788 mg/100 ml respectively (Table 3).

SGOT and SGPT did not increase during the dietary period in any of the rabbit groups I-IV. The mean values were between 6 and 40 mU./ml.

Chemical composition of rabbit lipoproteins
After differential ultracentrifugation of serum VLDL, LDL and HDL all fractions were tested for purity by immunoelectrophoresis and gave a single precipitin band against polyvalent anti-rabbit serum (Behringwerke, Marburg, W. Germany).

The control VLDL (Table 4) contains 11.5 % protein, 55.4 % triglycerides, 11.5 % cholesterol and 21.6 % phospholipids. The protein/total lipid ratio is 0.13. In comparison with the control group this ratio is decreased to 0.07 in the cholesterol group II

TABLE 6
CHEMICAL COMPOSITION (%) OF THE HIGH DENSITY LIPOPROTEINS (HDL) OF GROUP I-IV RABBITS

<table>
<thead>
<tr>
<th>Group</th>
<th>Chemical composition (%) of HDL</th>
<th>protein</th>
<th>triglycerides</th>
<th>cholesterol</th>
<th>phospholipids</th>
<th>protein/lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td>45.0</td>
<td>17.9</td>
<td>10.7</td>
<td>26.4</td>
<td>0.82</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td>57.0</td>
<td>3.1</td>
<td>19.4</td>
<td>20.5</td>
<td>1.32</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td>42.3</td>
<td>23.3</td>
<td>20.2</td>
<td>14.2</td>
<td>0.73</td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td>53.4</td>
<td>10.2</td>
<td>28.0</td>
<td>8.4</td>
<td>1.15</td>
</tr>
</tbody>
</table>
Fig. 2. Agarose gel electrophoresis of the VLDL of group I–IV rabbits (arrow = origin).

and to 0.08 in both the cholesterol–coconut oil group III and the cholesterol–corn oil group IV (Table 4). This increase of the lipid moiety of the VLDL is most striking in the cholesterol fraction with its approximately 6-fold increase in group II, III and IV VLDL (Table 4). The cholesterol content of these groups varies between 62 and 65 %, but is only 11.5 % in the control group I. In contrast, the protein, triglyceride and phospholipid portion of the VLDL molecule is decreased in the high cholesterol groups. The protein content of group II, III and IV VLDL only accounts for 6.4–7.5 % as compared with 11.5 % in the control VLDL (Table 4). The respective values for the VLDL triglyceride contents are 5.7 % in group II, 18.8 and 18.6 % in groups III and IV respectively, as compared with 55.4 % in group I. The phospholipid is slightly increased to 25.4 % in group II, but is decreased to 8.7 and 11.4 % in groups III and IV respectively.

The chemical composition of the LDL fraction (Table 5) in the control group was found to be 28.4 % protein, 11.0 % triglycerides, 43.4 % cholesterol and 17.2 % phospholipids, making up a protein/total lipid ratio of 0.40 (Table 5). Similar to the VLDL fraction, this ratio is decreased to 0.19, 0.18 and 0.23 in groups II, III and IV respectively. The changes induced by the diets II, III and IV are characterized by a lower protein and triglyceride content in the LDL fraction contrasting to the increase in cholesterol and phospholipid content (Table 5).

The HDL fraction (Table 6) of the control animals contains 45 % protein, 17.9 % triglycerides, 10.7 % cholesterol and 26.4 % phospholipids, resulting in a protein/total lipid ratio of 0.82 (Table 6). This ratio is raised to 1.32 and 1.15 in groups II and IV, but is as low as 0.73 in group III (Table 6). As was already observed in the VLDL and LDL
fractions there is a marked increase in cholesterol content of 19.4%, 20.2% and 28.0% in groups II, III and IV respectively. The phospholipid portion is only 20.5, 14.2 and 8.4% in the respective groups II, III and IV. The findings in the percentual contribution of protein and triglycerides in the HDL molecules are less consistent (Table 6).

**Electrophoresis**

The mobilities of the isolated lipoprotein fractions were tested in 0.8% agarose gel. The fractions to be compared were run in the same gel at the same time to obtain identical conditions.
Fig. 5. Polyanionic precipitation after 1% agarose gel electrophoresis of VLDL I, II, LDL I, II, III, IV with solution 1 (MgCl₂ 0.1 mole/1000 ml + sodium heparin 1.5 g/1000 ml) and LDL I with solution 2 (CaCl₂ 0.2 mole/1000 ml + sodium dextran sulfate 6 g/1000 ml).

The VLDL of group I migrated into the typical pre-ß position (Fig. 2), while VLDL II and III were found in the ß-position of control LDL (Fig. 3). VLDL IV displayed an accelerated mobility when compared with VLDL II and III, but was slightly slower than VLDL I (Fig. 2). A similar pattern was observed in the LDL (Fig. 3), where the mobility of LDL I and IV is faster than that of LDL II and III. The HDL I
fraction (Fig. 4) migrates to the characteristic α-position, HDL II, III and IV were found to be slower (Fig. 4).

Precipitation of lipoproteins

The technique of selectively precipitating lipoproteins by polyanionic com-
Fig. 7. Electron micrograph of negatively stained VLDL of control rabbit. All micrographs are of preparations stained with 1% potassium phosphotungstate, pH 7.1, at the same magnification (×100,000). Bars in Figs. 7–16 indicate 0.1 μm.

Pounds$^{26,27}$ was shown to be also applicable to rabbit lipoproteins. In control lipoprotein fractions of group I, the heparin solution 1 precipitated exclusively VLDL, but not LDL (Fig. 5), solution 2 precipitated all groups of lipoproteins. In contrast to normal LDL of group I rabbits (Fig. 5), the LDL fractions of groups II, III and IV could be precipitated by solution 1 (Fig. 5), indicating an increased tendency of these lipoproteins to form complexes with heparin at an ionic strength which is normally inadequate to permit a visible precipitation of LDL in the gel$^{127}$.

Polyacrylamide gel electrophoresis

To facilitate the explanation of the pattern of bands in polyacrylamide gel, a tentative nomenclature using consecutive numbers corresponding to the increasing mobility of the bands is proposed (Fig. 6). VLDL IV displays the most bands and is therefore used as reference (Fig. 6). The VLDL fraction of the corn oil group IV displays 12 bands 1–12. Characteristically two broad bands 4 and 7 can be detected in the VLDL and LDL fractions of the dietary groups II, III and IV. Band 4 is also found in the VLDL and LDL fractions of the control group I (Fig. 6). VLDL of the control group I is characterized by four bands 1, 2 and 4 and a very faint band near the origin of the separating gel. In the cholesterol and cholesterol–coconut oil fed groups II and III four new bands 6, 7, 8, 9 appear (Fig. 6), while band 1 is lacking. In VLDL IV another four bands 5, 10, 11 and 12 are found (Fig. 6). In the LDL fraction a similar
CHANGES IN RABBIT LIPOPROTEIN PROPERTIES

Fig. 8. VLDL preparation of cholesterol fed rabbit (group II).

Fig. 9. The same preparation as in Fig. 8. Arrows indicate fusion of particles.
apoprotein pattern is observed (Fig. 6). It should be noted that, as in human LDL, an apoprotein B-like fraction of LDL does not enter the gel. LDL of the control group I is separated into three bands 4, 7 and 8 (Fig. 6). In LDL II four additional bands 6, 7a, 9 and 10 appear, in the LDL III the bands 1, 2 and 3 can also be demonstrated. In LDL IV the additional bands 10, 11 and 12 are characteristic (Fig. 6).

**Electron microscopy**

*Very low density lipoproteins*

When observed in the electron microscope (Fig. 7) negatively stained preparations of VLDL of control rabbits (group I) appear to consist of more or less round particles rather heterogeneous in size. The diameter of most particles ranges from 30 up to 70 nm; however, large particles with a diameter up to 100 nm are also present to an extent varying from one micrograph to another. No definite statement on the proportion of particles with different diameter can be made as the diameters of individual particles vary a great deal between the above mentioned limits. On contact with each other the lipoprotein particles of this fraction may undergo some distortion in shape; however, no fusion of individual particles can be observed. VLDL particles of rabbits fed with cholesterol (Group II) and those of rabbits fed with cholesterol and coconut oil (group III) display striking morphological differences from those of
control animals. VLDL preparations of group II (Figs. 8 and 9) display particles very heterogeneous in size and shape. Square or rectangular or disc-like particles with a great tendency to form elongated or branched stacks, and more or less round particles frequently surrounded by flattened discs, can be observed. The disc-like particles may fuse with each other in the stacks or more frequently with the large round particles (Fig. 9, arrows), forming rosette-like structures. Because of the morphological changes and fusion no adequate measurement of the size of the various particles of this group can be made, however, they appear on average larger than those of control VLDL. Group III VLDL resembles closely group II VLDL as can be seen in Fig. 10. In contrast to these findings in groups II and III, VLDL of rabbits fed with cholesterol and corn oil (Fig. 11) display no significant morphological alteration. This preparation shows mostly round individual particles like those of the control group I (Fig. 7), the average size being slightly larger.

Low density lipoproteins

Negatively stained LDL of groups II and III also differ significantly from those of groups I and IV. While LDL particles of the control group I (Fig. 12) are more or less spherical with a mean diameter of about 26 nm, those of groups II (Fig. 13) and III (Fig. 14) appear ellipsoidal or square with mean minimum and maximum diameters of 20 and 36 nm respectively, and display a great tendency to form elongated or branched stacks. Large particles with a diameter from 65 up to 100 nm surrounded by
Fig. 12. LDL preparation of control rabbit.

Fig. 13. LDL of cholesterol fed rabbit (group II). Arrow indicates fusion of particles.
Fig. 14. LDL preparation of rabbit fed with cholesterol and coconut oil (group III). Arrow indicates fusion of particles.

Fig. 15. LDL of rabbit fed with cholesterol and corn oil (group IV).
the small square particles are also present (Figs. 13 and 14). Fusion of small particles with the large ones can also be observed. Contrary to groups II and III, the LDL of the cholesterol-corn oil group IV (Fig. 15) show round individual particles with a mean diameter of 28 nm, being on the average slightly larger than control LDL of group I (Fig. 12).

**High density lipoproteins**

In the electron microscope the HDL fraction of control rabbits consists of particles ranging from 9 up to 15 nm in diameter which appear to be composed of several subunits (Fig. 16). HDL preparations of group II rabbits appear very different from those of the control group I. Large round particles of subunit structure are scattered among smaller ones, comparable to the control group I (Fig. 16). The latter tend to form rouleaux of disc-shaped particles with a periodicity of 6 nm and mean diameters of 5 and 15 nm respectively. Similar aspects are detectable also on group III HDL. In contrast to this, the HDL of group IV appears very similar to that of the control group (Fig. 16).

**DISCUSSION**

Polyunsaturated fat like corn oil inhibits the development of cholesterol induced
atheromatosis in the rabbit\textsuperscript{9–12,15,39}, rat\textsuperscript{35} and rhesus monkey\textsuperscript{36,37}. Up to now, no explanation can be offered for this effect, since polyunsaturated fat has no lowering effect on serum cholesterol in experimental animals fed cholesterol\textsuperscript{15}.

The present results confirm those of the literature\textsuperscript{15,38,40,41,56} that diets with cholesterol and mixtures of cholesterol and either saturated or unsaturated fat lead to hypercholesterolemia, hypertriglyceridemia and hyperphospholipidemia in rabbits. Hypertriglyceridemia after cholesterol feeding is apparently caused by an inhibition of lipoprotein lipase by VLDL rich in cholesterol\textsuperscript{42,56}. Additional saturated or unsaturated fat gives rise to serum cholesterol levels which considerably exceed those of rabbits on a cholesterol diet. This is no additive effect, since Steiner \textit{et al.}\textsuperscript{41} have shown that unsaturated fat alone does not increase serum cholesterol, while saturated fat effects only minor elevations of serum cholesterol in rabbits. It is proposed by Merrill\textsuperscript{40} that the hypercholesterolemic effect of saturated and unsaturated fat in rabbits fed cholesterol is caused by an increased absorption and retention of cholesterol. As the serum cholesterol in rabbits fed a diet with cholesterol and corn oil is more elevated than with cholesterol alone, serum cholesterol levels cannot be the only determining factor of the severity of atheromatosis in rabbits. Another factor might also be the alterations of serum lipoproteins after cholesterol, cholesterol-coconut oil and cholesterol–corn oil feeding.

It has been shown by several groups\textsuperscript{43–46} that the plasma lipoproteins are in a very dynamic and closely interrelated state. This could be demonstrated for chylomicrons and their cholesterol rich remnants\textsuperscript{49} as well as for VLDL, the intermediate lipoprotein and LDL\textsuperscript{43–48}. On the other hand, VLDL and LDL can change their physico-chemical properties as a result of experimental diets\textsuperscript{17–20,49}.

Gofman\textsuperscript{17} was the first to describe a new lipoprotein with a flotation constant of $S_f \geq 10–30$ in rabbits fed 3 g cholesterol per week as compared with the $S_f \leq 5–8$ component found in control rabbits. Gofman thought that these lipoprotein changes have a "direct bearing" on the development of atheromatosis in the rabbit. Similar results were obtained by Schumaker\textsuperscript{49}. More recently several groups\textsuperscript{16–21} demonstrated that a cholesterol diet most strikingly changes the VLDL fraction. Camejo \textit{et al.}\textsuperscript{18,19} show that a marked increase in cholesteryl ester content in the $d < 1.019$ fraction is the most prominent change. They have also demonstrated changes in the apoprotein content of the $d < 1.019$ fraction by polyacrylamide gel electrophoresis, in that the apoproteins found in the $d = 1.019–1.063$ fraction appear in the $d < 1.019$ fraction after cholesterol feeding. The same authors\textsuperscript{19} could also separate the fraction of $d < 1.019$ by gel filtration into a VLDL-1 and VLDL-2 fraction in cholesterol fed rabbits. Both VLDL 1 and 2 have the same apoprotein composition. Shore \textit{et al.}\textsuperscript{20} have also described cholesterol induced changes in the apoprotein composition of VLDL in rabbits. They demonstrated an arginine rich apoprotein which corresponds to a human apolipoprotein being especially found in human Type III hyperlipoproteinemia\textsuperscript{20}.

Cholesterol (group II rabbits) feeding results in lipoprotein alterations which are well established\textsuperscript{18–20}. The present results confirm the finding that the proportion of cholesterol in the VLDL fraction is considerably increased, while the protein and tri-
glyceride portion is decreased. This change is also found in rabbit VLDL fractions after cholesterol–coconut oil and cholesterol–corn oil feeding. Both diets with cholesterol and cholesterol–coconut oil induce a change of VLDL electrophoretic mobility to the \( \beta \)-position. The number of apoproteins increases in the VLDL fraction after both diets. A similar augmentation of apoproteins has been reported for the guinea pig\(^2\). Electron microscopy reveals on the average larger particles of variable size in the VLDL of rabbits fed diets II and III. These observations were already made by Camejo\(^{18,19} \) and Shore\(^20\). Camejo\(^{18,19} \) also observed an aggregation tendency in the VLDL fraction after cholesterol feeding. Probably as a result of the longer dietary period we have found even polygonal deformations and a stacking in the VLDL fractions of rabbits fed cholesterol (group II rabbits) and cholesterol plus coconut oil (group III rabbits). These alterations were also found in the LDL and HDL fractions of the corresponding dietary groups. Moreover, in the HDL fraction we observed a second population of larger particles.

As was mentioned above, rabbits fed cholesterol and corn oil show significantly less atheromatosis than would result from the feeding of cholesterol alone. In the present study, we observed the cholesterol content of the aorta and the aortic plaque covered area to be only half in group IV as compared with groups II and III\(^12\). This is possibly caused by the effect of corn oil on the physico-chemical properties of VLDL and LDL. It is demonstrated that corn oil induces new apoprotein bands in polyacrylamide electrophoresis being exclusively present in group IV rabbits. The faster moving polypeptides in the corn oil group IV resemble apo-C III lipoproteins\(^5\) which are known to be inhibitors of the lipoprotein lipase\(^6\). Simultaneously, VLDL and LDL fractions show a nearly "normal" electrophoretic mobility in agarose gel. Just as striking is the nearly "normal" electron microscopic appearance of the VLDL and LDL particles in the corn oil group IV.

The meaning of the morphological alterations of lipoprotein particles described above is not clear. They cannot be simply attributed to an effect on lipoprotein particles by negative staining technique because of their reproducibility and absence in control preparations. They probably reflect some changes in intrinsic physico-chemical properties of the particles, such as the type of fatty acids present in the particle shell\(^16,53\). Similar alterations have been observed in the LDL and HDL fractions of patients with LCAT deficiency\(^5\) and in LDL fractions of patients with obstructive jaundice\(^52,55\). The stacking phenomenon was also observed in cholesterol fed guinea pigs after a long-term feeding period of 10–12 weeks\(^2\). In patients with Type II hyperlipoproteinemia the same stacking phenomenon was described\(^9\). Here it was proposed that this plays a role in the development of severe atherosclerosis in these patients. This is supported by present results in rabbits.

The lipoprotein changes presented seem to have considerable implications as to the process of atherosclerosis in rabbits. As recently postulated by Zilversmit\(^5\) atherosclerosis may be due to the action of lipoprotein lipase in the arterial endothelium converting triglyceride rich VLDL or intermediate particles into cholesterol rich LDL. It is proposed that heparin forms a link between the endothelial cell surface and
the lipoprotein lipase complex, binding the triglyceride rich lipoproteins for the degradation process and releasing the newly formed LDL into the blood stream. The higher affinity of heparin to VLDL than to LDL therefore is a prerequisite for a proper function of this mechanism. Experiments with heparin precipitation presented in this paper show the same relation in heparin affinity as described in humans\textsuperscript{26,27} for the VLDL fractions I–IV and the LDL fraction I. All VLDL fractions are precipitated by the heparin solution I, while the control LDL fraction I is not precipitated. In contrast, high cholesterol LDL of the rabbit groups II, III and IV are precipitated by heparin salt concentrations that do not precipitate the control LDL I. This new property of high cholesterol LDL is accompanied by the appearance of additional bands in polyacrylamide gel. The strong tendency of high cholesterol LDL to form complexes with heparin may interfere with the release of newly formed LDL from the endothelial site, thereby increasing the local LDL concentration, which in turn would lead to an increased influx of LDL particles along the transendothelial concentration gradient. Moreover, LDL particles would compete with VLDL particles for the binding sites of the arterial and capillary wall and would remain for a longer period of time linked there. Thereby the high cholesterol LDL particle could interfere with the VLDL catabolism to "intermediate lipoproteins" and LDL. Actually this hypothesis is supported by the 30-fold increase of the VLDL concentration compared with a 2 to 4-fold increase in LDL after cholesterol feeding\textsuperscript{20}. At the same time there is also a marked shift in the VLDL $S_f$ values from $S_f$ 28–70 to $S_f$ 33–350\textsuperscript{18}. The findings of Fielding\textsuperscript{12} and Huang and Kako\textsuperscript{56} that hypercholesterolemic VLDL is a poor substrate for lipoprotein lipase would also be in agreement with this hypothesis. Moreover the three fast moving bands of VLDL IV resembling the C III peptides in humans may further inhibit the metabolic conversion of VLDL to LDL\textsuperscript{59} reducing thereby the local concentration at the arterial wall in the cholesterol–corn oil group IV rabbits.

Beside LDL particles, VLDL particles can also intrude into the arterial wall as recent investigations suggest\textsuperscript{58}. This intrusion of high cholesterol VLDL would additionally increase cholesterol deposits in the arterial wall.

If the strong tendency of LDL and VLDL particles of groups II and III to form stacks is also found \textit{in vivo}, a network of "agglutinating" VLDL and LDL particles at the arterial endothelium would result. In contrast, VLDL and LDL IV particles of the cholesterol–corn oil group IV do not display the stacking tendency. Therefore, they may be less "dangerous" to the arterial wall.

It may be concluded that the results presented in this paper fit well into one of the current concepts about atherosclerosis\textsuperscript{59} and suggest new mechanisms in the cholesterol-induced atheromatosis of the rabbit and its partial prevention by dietary polyunsaturated fat.

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