Cholesterol Vehicle in Experimental Atherosclerosis. III. Effects of Absence or Presence of Fatty Vehicle[1]

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Three separate experiments show that cholesterol administered to rabbits in the absence of added fat is more atherogenic than cholesterol fed together with corn oil. When cholesterol is dissolved in the corn oil (by heating) prior to mixing with the diet, it is more atherogenic than when it is suspended in the oil. In every case the lowest serum and liver cholesterol levels were observed in the group not receiving corn oil.

Gas chromatography of the fatty acids of the serum cholesterol esters of pooled sera suggest that there are smaller amounts of unsaturated C_{18} fatty acids in the cholesterol-no fat group. Deficiency in these unsaturated acids may be the cause of the high atherogenicity of the cholesterol-no fat diet. There is also a lower proportion of unsaturated fatty acids in the triglycerides of this group.

Heating of corn oil 10 min. at 160–200°C.) causes an increase of titratable fatty acid in the oil (0.005 m-equiv./g. to 0.088 m-equiv./g. or 0.14% FFA to 2.5% FFA). The relatively large amounts of free unsaturated fatty acids in the heated oil may be the cause of the greater atherogenicity of the solution, as compared to the suspension, of cholesterol in corn oil.

FEEDING RABBITS CHOLESTEROL without added fat will result in the development of atheromatous lesions (1–4). In early experiments we compared the atheromata in rabbits fed cholesterol without added fat, with relatively saturated fat, or with relatively unsaturated fat (5) and found that cholesterol fed without other dietary lipid caused the most severe atheromata. Bortz, Larsen, and Civin (6) have recently confirmed these results. To extend these observations we then compared the atherogenicity of cholesterol suspended in corn oil with that of cholesterol dissolved in corn oil and of cholesterol without fat (7). Again the cholesterol administered in the absence of fat proved to be the most atherogenic.

The work described in this report was undertaken to expand these early observations. Three separate feeding experiments were carried out, the serum and liver lipids were assayed, and the atherosclerotic lesions were evaluated. In all cases the initial observation, namely, the increased atherogenicity of cholesterol administered without added fat, was confirmed.

Experimental

In each experiment four groups of Dutch-Belted rabbits weighing 1.5 to 2.0 kg. were used. The animals were maintained on the experimental diets for eight weeks, bled by heart puncture, and killed. The diets consisted of a) Purina rabbit Chow, b) the Chow augmented with 2% cholesterol, c) Chow with 2% cholesterol dissolved in 6% corn oil, and d) Chow with 2% cholesterol suspended in 6% corn oil.

The 2% cholesterol diet was prepared by dissolving the sterol in chloroform and pouring the hot solution on the Chow while the Chow was being tumbled in a cement mixer. Mixing was continued until the Chow was thoroughly coated. This diet was then kept in the air until free of solvent odor. The cholesterol-corn oil diets were prepared in the same way. The corn oil used was the regular commercial product purchased from A. E. Staley Manufacturing Company, iodine number 127.

Aortas were inspected visually for atherosclerotic lesions and graded on a 0–4 scale. The thoracic aorta and the aortic arch were graded separately.

Serum cholesterol was determined by the method of Trinder (8). The livers were weighed and homogenized; and an aliquot was taken for lipid analysis. The liver aliquot was homogenized in alcohol-ether 3:1, and the extract was dried over anhydrous Na_2SO_4. The dried solution was diluted in a volumetric flask, total liver lipids were determined gravimetrically, and liver cholesterol was determined colorimetrically by using the Trinder method.

For gas chromatography, aliquots of pooled sera from each group were extracted with methanol-chloroform and the extracted lipid was subjected to silicic acid chromatography (9). The cholesterol esters were separated, the fatty acids were converted to the methyl esters, and the resulting fatty acid methyl esters were subjected to gas chromatography. Gas chromatography was carried out, using a Barber-Colman gas chromatograph. For detection an argon cell ionization cell was used; radium foil acted as the ionization source. The lipid phase consisted of diethylene glycol succinate polyester, adsorbed on siliconized chromosorb W (Johns-Manville). The chromatograph was developed by using argon gas at a pressure of 30 p.s.i. and a flow rate of 100 cc./min.

Results and Discussion

Autopsy data on the rabbits in the three experiments are shown in Table I. The results consistently show that the rabbits fed cholesterol in the absence of added corn oil had the most severe atheromata and the lowest serum and liver cholesterol levels. The results also show consistently higher atherogenicity for the solutions, as compared with the suspensions, of cholesterol in corn oil. The cholesterol-corn oil suspension was prepared by mixing the sterol and the oil, then adding this suspension to the Chow. The cholesterol-corn oil solution was prepared by heating the suspension until all the sterol had dissolved, then adding this to the Chow. As soon as the hot solution came in contact with the cold Chow, the cholesterol crystallized out and mixing was maintained until all the food had become coated. Two possible changes in the "solution" diet could account for the differences in atheromata observed: a change in the cholesterol or in the vehicle. The most likely change in the cholesterol would be oxidation to 7-hydroxycholesterol or some similar compound, but recent work has shown rigorously purified cholesterol

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TABLE I

Autopsy Data on Rabbits Fed 2% Cholesterol With and Without 6% Corn Oil: Experiments 1, 2, and 3

Exp. No.	Diet	No. of rabbits	Weight gain	Liver weight	Liver lipid	Liver cholesterol	Serum cholesterol	Atheromata	
								Arch	Thoracic
			g.	*g.*	*% wet wt.*	*% wet wt.*	*mg.%* [a]	*score*	*score*
1	Cholesterol–no oil	5 [b]	334	73.4	3.0	0.6	2195 (1040–3500)	3.4	2.9
2	Cholesterol–no oil	8	242	96.9		1.8	1378 (750–2550)	3.4	3.0
3	Cholesterol–no oil	8	152	77.5			1555 (1090–2550)	3.4	3.2
1	Cholesterol–oil solution	7 [b]	656	110.0	10.4	1.8	2248 (725–3075)	2.7	2.1
2	Cholesterol–oil solution	6 [b]	− 80	93.5		6.9	1970 (1525–2590)	3.4	2.4
3	Cholesterol–oil solution	8	140	95.6			2493 (1765–3970)	3.1	2.6
1	Cholesterol–oil suspension	8 [b]	424	99.1	11.4	2.5	2318 (1420–3260)	2.1	1.4
2	Cholesterol–oil suspension	7 [b]	34	97.6		7.3	1752 (790–2650)	2.6	1.6
3	Cholesterol–oil suspension	10 [b]	116	87.8			2170 (1100–3040)	2.9	2.2
1	Normal	5	518	51.0	1.3	0.1	55 (25–88)	0.1	0.1
2	Normal	8	126	63.9		0.1	128 [c] (45–500)	0.1	0.1
3	Normal	6	498	72.3			67 (48–85)	0.10	0.0

[a] Ranges in parentheses.
[b] Number of rabbits surviving until end of experiment. Experiment 1: originally nine rabbits in this group; Experiment 2: originally eight rabbits in this group; Experiment 3: originally 12 rabbits in this group.
[c] Average relatively high because of one lipemic rabbit (without atheromata) with cholesterol level of 500 mg. %.

to be more atherogenic than relatively impure commercial material (10). The other possible cause for the observed differences would be a change in the composition of the cholesterol vehicle. The explanation, discussed later in this report, is that the heating of corn oil (to effect solution of cholesterol) may be responsible for the increase in atherogenicity.

The Chow which was the basis of the atherogenic diet contains about 2.2% of ether-alcohol extractable material. This material has an iodine number of 115. Gas chromatography of the fatty acids of this Chow lipid shows the principal composition to be 22.8% palmitic acid, 2.5% stearic acid, 24.0% oleic acid, 48.8% linoleic acid, and 1.8% trace acids. These values compare well with earlier findings of Evans *et al.* (11), who found Purina Chow to contain 2% lipid of iodine value 120.7; 70% of the component fatty acids were unsaturated. The fatty acid composition of the corn oil used was 15.3% palmitic, 5.2% stearic, 30.5% oleic, 47.6% linoleic, and 1.4% traces including arachidic.

In an effort to learn more about the fatty acids of the cholesterol esters and triglycerides present in the sera of these rabbits, pooled sera from each group in each of the first two experiments were subjected to silicic acid chromatography and the fatty acids from the cholesterol esters and triglycerides thus obtained were analyzed by gas chromatography. The data are presented in Table II. Calculations were based on the assumption that the recovered acids represented all of the fatty acids.

In the first experiment considerably less unsaturated acid was found in the serum cholesterol ester fraction of the group fed no corn oil than in any of the other three groups. In view of reports that rabbits fed cholesterol plus unsaturated fat exhibit less atheromata than rabbits fed cholesterol plus saturated fat (5, 6, 12) our results may perhaps be explained as functions of the amount of available unsaturated fatty acid. As would be expected, the triglycerides contained a smaller proportion of unsaturated fatty acids than did the cholesterol esters.

In the second experiment an appreciable amount of palmitoleic acid was found in the pooled sera of the group on the corn oil-free diet. This acid also was present in the pooled triglyceride fatty acids of this group. Inasmuch as these data represent pooled sera, a significant amount of an anomalous fatty acid in any one serum may affect the results as shown. The percentage of C-18 unsaturated acids in this sample was lower than that of the rabbits on cholesterol dissolved in corn oil or of the controls. As in the previous experiment, a larger proportion of saturated fatty acids was generally found in the triglycerides. A new series of experiments in which fatty acids will be studied in individual sera is under way. It may also be noteworthy that the most consistent results were obtained with the two samples of pooled normal-rabbit serum.

The differences between the fatty acid distribution in the serum cholesterol esters of the two groups fed cholesterol and 6% corn oil were slight. The effect of heating on the composition of the corn oil was investigated as a possible cause of the increased atherogenicity of the heated corn oil-cholesterol diet. Nishida, Takenaka, and Kummerow (13) had found that heated corn oil was more atherogenic for cholesterol-fed chickens than was fresh oil. They attributed their results to "toxicity" of the heated oil and possibly to reduced availability of the essential fatty acids which facilitate cholesterol absorption (14). For solution of cholesterol in corn oil the oil is maintained at elevated temperatures for 10–15 min. Since it has been reported that free unsaturated fatty acids aggravate cholesterol atherosclerosis in rabbits (15), we titrated the free fatty acids in corn oil before and after it had been heated for 10 min. at 160–200°C. We found that the fresh oil had 0.005 ±0.0003 m-equiv. free acid per gram (0.14% free fatty acid), and the heated oil 0.088 ±0.02 m-equiv. free acid per gram (2.5% free fatty acid). The results represent the average of three different titrations of each sample. The greater content of free fatty acid may explain the increased atherogenicity of the heated corn oil.

Examination of the liver cholesterol data shows that the liver cholesterol levels were the lowest in

TABLE II
Gas Chromatography of Cholesterol Ester and Triglyceride Fatty Acids from Sera of Rabbits on Various Atherogenic Regimens: Experiments 1 and 2

Exp. No.	Acid		Diet							
	No. of carbon atoms	No. of double bonds	Cholesterol–no fat		Cholesterol–solution		Cholesterol–suspension		Normal	
			CE [a]	TG [b]	CE	TG	CE	TG	CE	TG
			%	%	%	%	%	%	%	%
1	14	0	.....	.....	9.4	11.3	.....	13.5	.....	.....
2	14	0	2.4	2.4	.....	.....	7.9	.....	.....	.....
1	16	0	37.0	46.4	15.7	21.9	18.2	33.0	20.8	35.4
2	16	0	20.1	35.9	22.4	29.3	25.2	27.1	23.8	30.2
2	16	1	9.0	3.7	.....	.....	.....	.....	.....	.....
1	18	0	4.9	11.7	1.6	3.6	2.9	5.5	3.8	4.7
1	18	1	43.5	31.5	51.7	28.5	53.0	32.3	34.0	37.9
1	18	2	14.5	10.5	31.5	34.7	25.8	15.7	41.5	21.9
2	18	0	1.4	4.0	3.5	4.3	1.7	3.8	1.7	4.8
2	18	1	42.3	26.6	50.6	44.3	48.6	34.3	38.4	35.4
2	18	2	24.8	27.4	23.5	22.1	16.6	34.8	36.2	29.5
1	% Saturated acids		41.9	58.1	26.7	33.2	21.1	52.0	24.6	40.1
2	% Saturated acids		23.9	42.3	25.9	33.6	34.8	30.9	25.5	35.0
1	% Unsaturated acids		58.0	42.0	83.2	63.2	78.8	48.0	75.5	59.8
2	% Unsaturated acids		76.1	57.7	74.1	66.4	65.2	69.1	74.6	64.9

CE[a] = Cholesterol ester.
CE[b] = Triglyceride.

groups with the highest atheromata. Nishida *et al.* (13) found a similar trend in chickens fed cholesterol with heated or fresh corn oil. Furthermore the most atherogenic diet produced the lowest serum cholesterol levels although it must be pointed out that even these so-called low levels were considerably above normal serum cholesterol levels. It is probable then that the higher atherogenicity observed for the fat-free diet may be caused by insufficient amounts of the unsaturated fatty acids with which cholesterol is preferentially esterified (16,17), thus retarding normal circulation and metabolism of this sterol. The atherogenicity of the heated fat may be increased by changes affecting the transport of cholesterol and the composition of the *beta*-lipoprotein. Nishida *et al.* (13) found that the serum of the chickens fed cholesterol plus heated corn oil was practically free of the S_f 20–400 classes of lipoproteins. Thus all the lipoprotein was present as the cholesterol-rich S_f 0–20 fraction (18). Although we have not done lipoprotein analyses of the sera obtained in this experiment, previous analyses showed that rabbits fed cholesterol in the absence of fat had lower serum lipoprotein levels than those fed cholesterol in corn oil but that a larger proportion of the lipoprotein was present as the S_f 0–20 class (5). The effect of free fatty acids on serum lipid composition in both normal and cholesterol-fed animals merits further scrutiny.

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The Complex Nature of Castor Sensitivity

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The castor seed antigen CB-1A, prepared by the basic lead acetate method of Spies *et al.*, has been subjected to column chromatography on diethylaminoethyl cellulose. Antigenic differences between fractions were found by the Schultz-Dale technique, which indicated the possible existence of six antigenic or allergenic components.

Cross-reactions between castor pollen, castor blossoms, and castor seed meal were indicated by the Schultz-Dale method.

It would appear that allergy to castor pomace may actually be sensitivity to any one or more of the antigenic components of the pomace, including both pollen and female blossoms.

ALLERGY to castor seed protein has been recognized since 1914 when Alilaire (1) described the allergy and attributed it to sensitivity to the toxic albumin ricin. Ratner and Gruehl (2) showed that ricin was not responsible for the allergenicity of castor proteins, and the classical studies of Spies, Coulson,

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