Effect of Previous Dietary Intake on the Fatty Acid Composition of the Plasma Cholesterol Esters

BERNARD J. FAIRHURST, M.D.* AND CHRISTINE WATERHOUSE, M.D.†

THE EFFECT of diet on cholesterol metabolism has been widely studied in recent years, particularly the relationship between the degree of saturation of dietary fat and serum cholesterol.[1,2] Much of the work in this field has been restricted to measuring total serum cholesterol, and little attention has been paid to the changes that might occur within cholesterol esters. Esters normally constitute 75 per cent of the circulating cholesterol and are unique among the fatty acid esters because of their high polyunsaturated fatty acid content. Kinsell and co-workers[3] have shown that the fatty acid composition of the cholesterol esters could be altered by changes in the type of dietary fat. Ethyl linoleate added to a basic diet increased the percentage of cholesterol esterified with linoleic acid, whereas the addition of ethyl oleate increased the percentage of cholesterol oleate.

Swell[4] has suggested that the composition of serum cholesterol esters may be as important as total serum cholesterol in the genesis of atheroma. He found that cholesterol oleate in atheromatous plaques accounted for 45 per cent of the cholesterol esters compared with 27 per cent in healthy media and 25.8 per cent in serum.

These factors have prompted us to study the plasma cholesterol esters under carefully controlled dietary conditions.

METHODS

Patient Material

Nine patients were studied on a metabolism ward where constant weighed diets were given. Three patients with no metabolic abnormalities were used as control subjects, three patients had metastatic carcinoma, one patient had chronic alcoholism and two patients had active cirrhosis (Table 1). Three basic diets were given: (1) *Low calorie diet:* This varied from 687 to 1,202 calories and was of normal composition. Protein provided 15 to 22 per cent of the calories, fat 32 to 45 per cent of the calories and carbohydrate 38 to 50 per cent of the calories. (2) *High carbohydrate diet:* 200 to 250 gm. of Dexin® (partially hydrolyzed starch) and fruit juice were added to the first diet so that carbohydrate provided 68 to 76 per cent of the calories (except for patient P. C. in whom carbohydrate provided only 61 per cent of the calories). The protein and fat content of the diet remained constant. (3) *High fat diet:* Dairy products of equal caloric value were substituted for the added carbohydrate in the previous diet, to produce a diet in which fat provided 68 to 76 per cent of the calories. The calculated linoleic acid content of the low calorie diet varied from 1.5 to 5.8 gm., i.e., greater than 1 per cent of total caloric intake. When the added fat was given in the third diet, the calculated linoleic acid content of the diets increased 2 to 2.5 gm. a day. Each dietary period lasted from five to seven days. At the end of each period, plasma was taken for analysis after the patient had fasted overnight for fifteen hours.

All patients on the low calorie diet lost weight. As measured by caloric balance,[5] the high carbo-

From the Department of Medicine, The University of Rochester School of Medicine and Dentistry, Rochester, New York.

* Senior Buswell Fellow in Medicine, The University of Rochester School of Medicine and Dentistry. Present address: Sheffield Royal Infirmary, Sheffield, England; † Associate Professor of Medicine, The University of Rochester School of Medicine and Dentistry.

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TABLE I
Patient Material and Dietary Intake

Patient	Diagnosis	Age (yr.)	Weight (kg.)	Low Calorie Diet			High Carbohydrate Diet			High Fat Diet		
				Calories	Carbohydrates (gm.)	Fat (gm.)	Calories	Carbohydrates (gm.)	Fat (gm.)	Calories	Carbohydrates (gm.)	Fat (gm.)
P. R.	Control subject	43	55.33	1,202	138	50	2,188	384	50	2,174	138	158
S. M.	Control subject	85	51.84	687	70	31	1,704	319	32	1,705	79	137
A. W.	Control subject	31	67.69	1,000	94	52	2,017	343	53	1,995	93	163
P. C.	Carcinoma	59	51.89	1,199	85	67	2,199	335	67	2,180	85	176
R. K.	Carcinoma	52	47.41	829	86	37	1,746	335	38	1,738	93	127
D. H.	Carcinoma	38	67.19	700	64	32	1,717	313	33	1,719	72	139
J. S.	Chronic alcoholism	44	58.26	1,077	104	57	2,098	354	58	1,982	108	154
T. H.	Cirrhosis	40	67.57	740	72	32	1,728	318	32	1,735	76	139
E. R.	Cirrhosis	57	87.15	716	67	32	1,733	316	33	1,736	74	140

hydrate and high fat diets proved to be slightly hypocaloric for the majority of patients. Body fat loss was the same, however, in patients on these two diets, even though the tendency to lose weight was greater when the calories were predominantly supplied as saturated fat.

Plasma Lipid Extraction and Free Fatty Acid Determination

Lipids were extracted from 10 ml. of plasma with methanolchloroform (2:1 V/V).[6] Free fatty acids were titrated with alkali following heptane-isopropanol acid extraction.[7]

Separation and Measurement of Cholesterol Esters

The plasma extract, containing the lipid from 6 ml. of plasma, was placed on a washed silicic acid column in petroleum ether. The columns were prepared by the method described by Marinetti and co-workers.[8] The first fraction, eluted from the column with 70 ml. of petroleum ether, contained only the cholesterol esters. This fraction was then used for the separation of the various esters by modification of the method described by Gabby and Waterhouse.[9] An aliquot containing between 80 and 100 μg. of cholesterol was applied to a silicic acid impregnated paper, 19 by 20 cm., along a starting line 3 cm. from the bottom of the paper. The sides of the paper were stapled to form a cylinder and this was placed in a Mason jar containing 50 to 60 ml. of 7.5 per cent benzene in hexane. After ninety minutes at room temperature, when the benzene-hexane mixture had risen to within 3 cm. of the top of the paper, the latter was removed from the jar and allowed to stand in the air until dry. It was stained in a weak solution of Rhodamine G and then washed three times in distilled water to remove excess stain. The papers were again dried and veiwed under ultraviolet light. Four distinct groups of cholesterol esters were visualized. The saturated esters moved in one band which had the greatest RF value. Cholesterol oleate appeared beneath the saturated esters, and was followed by cholesterol linoleate and finally cholesterol arachidonate (Fig. 1). Gabby and Waterhouse[9] have shown that in this system there is very little separation of cholesterol arachidonate and cholesterol linolenate. Since the latter constitutes less than 1 per cent of the cholesterol esters normally,[4] it can contribute little cholesterol to the cholesterol arachidonate area on the paper. The areas containing the separated esters were cut from the paper, folded and placed in test tubes. A similar area free of cholesterol was cut from each chromatogram and treated exactly as the other samples to determine a paper blank. The cholesterol was eluted by the method described by Searcy et al.,[10] using a saturated solution of ferrous sulfate in glacial acetic acid. Two milliliters of this solution was added to each tube which was then stoppered and left to stand overnight. One milliliter was then taken from each tube and to it was added 0.45 ml. concentrated sulfuric acid to develop the color. After ten minutes the optical density of the solution was measured against a re-

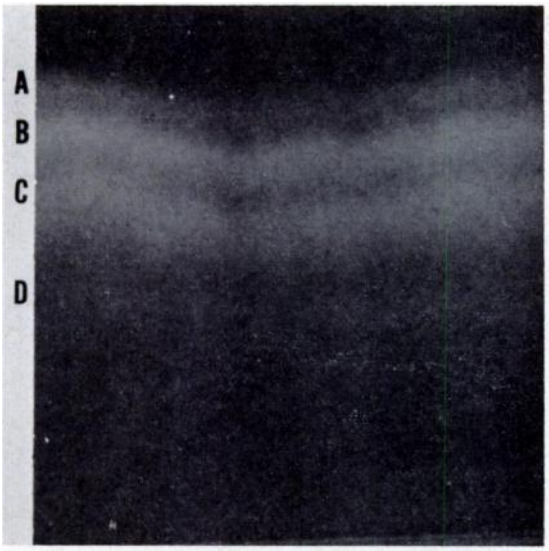

Fig. 1. Photograph of a chromatogram taken under ultraviolet light 2537A. The chromatogram has been stained with Rhodamine G and air dried. A, saturated esters of cholesterol. B, cholesterol oleate. C, cholesterol linoleate. D, cholesterol arachidonate.

agent blank in a Beckman spectrophotometer at a wavelength of 490 mμ using 1.5 ml. cuvettes. A standard containing 20 μg. of cholesterol in 1 ml. of the ferrous sulfate solution was used. The amount of cholesterol in the final solution represented half the cholesterol originally present on the paper prior to elution. The optical density of the paper blank was equivalent to between 3.3 and 3.8 μg. of cholesterol. The range of the blank values, equivalent to 0.5 μg. of cholesterol, limited the accuracy of the method when determining quantities of cholesterol on the paper less than 15 μg.

Recovery and Reproducibility

Known amounts of cholesterol linoleate, cholesterol palmitate and cholesterol oleate in chloroform were chromatographed and the cholesterol eluted as described. The recovery ranged from 78.7 to 97.4 per cent with a mean recovery of 87.7 per cent (Table II).

Five aliquots of the same cholesterol ester fraction obtained from a column were chromatographed and the cholesterol esters measured. The results are shown in Table III. In view of the difficulty mentioned previously in measuring small amounts of cholesterol, the values for the saturated esters and cholesterol arachidonate are variable, with limited reproducibility. Values for cholesterol linoleate and cholesterol oleate showed a narrow range with good reproducibility. The results in the patients studied will be restricted to the measurements of cholesterol linoleate and cholesterol oleate.

RESULTS

Plasma ester cholesterol, cholesterol linoleate and cholesterol oleate levels in the patients studied under the three dietary conditions are shown in Table IV.

(1) *Low calorie diet:* All the patients, except the two with cirrhosis, had the following cholesterol ester pattern. Cholesterol linoleate ranged from 43.7 to 52 per cent—mean 47.2 per cent, and cholesterol oleate from 20.8 to 31.5 per cent—mean 27.8 per cent of the total esters. These values agree with those given by Swell and co-workers,[4] who found that cholesterol linoleate accounted for 43.3 per cent and cholesterol oleate for 25.8 per cent of the cholesterol esters in normal people. Similar values have been reported by Schrade et al.[11] The two patients with cirrhosis had abnormal ester patterns, the cholesterol linoleate being 37.8 and 36.7 per cent, while the cholesterol oleate was 40.8

TABLE II
Cholesterol Ester Recovery from Paper

Ester	Amount Added (μg.)	Amount Recovered (μg.)	Per Cent Recovery*
Cholesterol linoleate	45.4	40.1	88.3
	45.4	40.5	89.2
	45.4	42.8	94.3
	56.8	48.1	84.7
	56.8	51.8	91.2
	56.8	49.8	87.7
Cholesterol palmitate	9.4	7.8	83.0
	9.4	7.4	78.7
	9.4	8.3	88.3
	15.1	13.3	88.0
	15.1	13.6	90.0
	15.1	14.7	97.4
Cholesterol oleate	19.3	16.0	82.9
	19.3	16.5	85.5
	24.1	21.6	89.6
	24.1	21.3	88.4
	28.9	24.9	86.2
	28.9	24.7	88.5

* Mean recovery = 87.7.

TABLE III
Results on Five Aliquots of the Same Cholesterol Ester Fraction Expressed as Micrograms of Cholesterol

Specimen	Aliquot					Mean
	1	2	3	4	5	
Cholesterol arachidonate	10.5	9.7	11.6	13.1	15.5	12.1
Cholesterol linoleate	38.9	37.9	36.4	37.6	39.8	38.1
Cholesterol oleate	25.1	23.3	23.5	23.8	24.6	29.1
Saturated esters	14.6	17.5	14.0	12.6	10.3	13.8

and 35.6 per cent. This abnormality of cholesterol esters in cirrhosis has been noted previously.[12]

(2) *High carbohydrate diet:* All the patients on this diet had an obvious fall in cholesterol linoleate levels. There was a mean fall of 22 mg. per 100 ml. which was significant by the t test ($P < 0.01$). In seven cases there was a small rise in the cholesterol oleate level, with a mean rise of 5.1 mg. per 100 ml. This figure was not significant ($P > 0.05$). The total ester cholesterol level fell in all cases, with a mean fall of 23 mg. per 100 ml. ($P < 0.05$).

(3) *High fat diet:* On this diet the patients had a cholesterol ester pattern similar to that found with a low calorie diet. In seven patients the cholesterol oleate level was higher when they were on a high fat diet than when they were on a low calorie diet. The mean elevation was 6.3 mg. per 100 ml., which was not significant ($P > 0.05$). Total ester cholesterol levels in patients on a high fat diet did not differ significantly from those with the low calorie diet.

Free Fatty Acids

In Table v the plasma levels of free fatty acids are presented for patients on the three diets under study. It can be seen that the

TABLE IV
Effect of Diet on Plasma Cholesterol Esters

Patient	Diagnosis	Low Calorie Diet			High Carbohydrate Diet			High Fat Diet		
		Total Cholesterol Esters	Cholesterol Linoleate	Cholesterol Oleate	Total Cholesterol Esters	Cholesterol Linoleate	Cholesterol Oleate	Total Cholesterol Esters	Cholesterol Linoleate	Cholesterol Oleate
P. R.	Control subject	163	73	47	89	25	38	162	78	49
S. M.	Control subject	133	69	35	106	27	52	145	61	46
A. W.	Control subject	159	76	33	153	60	37	154	75	34
P. C.	Carcinoma	124	55	39	99	26	47	115	50	39
R. K.	Carcinoma	99	47	30	91	35	35	102	44	35
D. H.	Carcinoma	137	68	34	131	56	38	195	80	57
J. S.	Chronic alcoholism	141	61	44	126	45	52	169	70	62
T. H.	Cirrhosis	150	57	61	136	46	71	152	56	58
E. R.	Cirrhosis	156	57	56	134	43	55	150	54	56

NOTE: All values are expressed as milligrams of cholesterol per 100 milliliters of plasma. Total ester cholesterol was determined gravimetrically from the column, each value representing the mean of five plasma samples taken from the patient during a tracer study over a period of two hours. The cholesterol content of ester fraction was derived from the equation: milligrams cholesterol ester $\times$ 0.6 = milligrams cholesterol.

TABLE V
Effect of Diet on Fasting Free Fatty Acids

Patient	Diagnosis	Low Calorie Diet (mEq./L.)	High Carbohydrate Diet (mEq./L.)	High Fat Diet (mEq./L.)
P. R.	Control subject	0.6	0.3	0.4
S. M.	Control subject	0.7	0.7	0.9
M. W.	Control subject	0.7	0.4	0.6
P. C.	Carcinoma	0.6	0.4	0.6
R. K.	Carcinoma	0.7	0.6	0.8
D. H.	Carcinoma	1.5	0.4	1.1
J. S.	Chronic alcoholism	0.9	0.4	0.6
T. H.	Cirrhosis	0.9	0.6	0.8
E. R.	Cirrhosis	1.5	1.0	1.5
Mean		0.9	0.55	0.8

fasting free fatty acid levels are lower in patients on the carbohydrate diet (mean 0.55 mEq. per L.) compared with those on the low calorie diet (mean 0.9 mEq. per L.) and the high fat diet (mean 0.8 mEq. per L.).

The free cholesterol level was determined, but there was no significant difference on the three diets.

COMMENTS

The significant finding in this study is the reduction in serum cholesterol linoleate levels following a high carbohydrate intake. The fall in this cholesterol ester accounts for the parallel decrease in total ester cholesterol. The first explanation to consider is impaired production of cholesterol linoleate due to either an absolute or a relative lack of linoleic acid available for esterification. Hirsch et al.[13] noted a fall in the plasma cholesterol linoleate level when a fat free-high carbohydrate diet was given. They suggested that with very low or absent exogenous fat, the body stores provided the fatty acids necessary for cholesterol esterification. Since linoleic acid represents less than 12 per cent of the fatty acids present in the adipose tissue, the amount of cholesterol esterified with linoleic acid falls from the normal figure of about 50 per cent to one approaching the linoleic acid content of adipose tissue. The concept that the cholesterol ester fatty acid pattern approaches that of adipose tissue when exogenous fat is limited, will not explain the findings in this study however, since the dietary fat was identical in both the low caloric and high carbohydrate diets. Furthermore, the low levels of fasting free fatty acid observed in the high carbohydrate feeding implied diminished mobilization of fat stores during this diet.

A relative lack of linoleic acid in the fatty acid pool available for cholesterol esterification must also be considered. Lipogenesis resulting from a high carbohydrate intake would produce predominantly oleic acid and saturated fatty acids but no linoleic acid. This might account for the low level of cholesterol linoleate in the plasma, but it would not explain the reduction of the total cholesterol ester level which was seen in all instances. Since it has been shown that fasting plasma triglyceride levels fail to rise normally after a high carbohydrate intake in patients with either carcinoma or cirrhosis of the liver,[14] it seems even less likely that the ester changes seen here can be solely due to increased lipogenesis resulting from a high carbohydrate intake.

Excessive loss of cholesterol linoleate from the plasma must also be considered as a possible explanation. Similar cholesterol ester changes have been produced by neomycin in man.[15] This decrease in plasma cholesterol linoleate while the other esters remained constant was associated with a significant increase in fecal bile acids. It thus appeared that the increase in cholesterol catabolism to bile acids occurred at the expense of cholesterol linoleate. It is of interest that in the human adrenal gland, stimulation with ACTH results in depletion of ester cholesterol with the linoleate falling proportionately more than the other esters.[16] It has been shown in experimental animals that starch can induce hypocholesterolemia when compared with isocaloric diets of free sugars[17] and that this hypocholesterolemia is accompanied by an increased excretion of bile acids.[18] Since the high carbohydrate content of the diet given to our patients was achieved by the addition of 200 to 300 gm. of Dexin, which is a partially hydrolyzed starch, it seems possible that increased bile acid excretion may have occurred.

Either impaired esterification or increased degradation of cholesterol could explain the fall in ester cholesterol observed in this study after feeding a high carbohydrate diet. However, this would only explain the change in ester pattern if the cholesterol esters are *not* metabolized as a homogeneous group. In other words, the present study lends support to the thesis that the metabolism of cholesterol in the esterified form may well differ according to the fatty acid to which it is attached.[19,20]

SUMMARY

A method to determine the individual cholesterol esters in the plasma is described. The cholesterol esters were determined in a group of patients under three dietary conditions, *viz.*, low calorie, high carbohydrate and high fat diets. The significant finding was a fall in cholesterol linoleate levels after ingestion of the high carbohydrate diet. Possible explanations for the change are discussed. It is concluded that the plasma ester cholesterol is not a homogeneous compartment and that the individual cholesterol esters are handled differently by the body.

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