again centrifuged, decanted, inverted, and allowed to drain for at least 5 minutes. To the washed digitonide precipitate add 5 ml of the orcinol reagent and 2 ml of the acid ferric chloride reagent. Cap the top of the tube with parafilm and mix thoroughly. It is best to set up at least 2 standards and run them through the entire procedure. We use a stock standard containing 2.50 mg of cholesterol per ml, and a working standard containing 0.10 mg of cholesterol. Both of these standards are made up in acetone-alcohol and are stable indefinitely. Two blanks are prepared, one containing orcinol and ferric chloride, and the second, in addition, containing digitonin (1 ml of a 1% solution). The latter is included to check for completeness of removal of excess digitonin by the isopropyl wash. The two blanks should yield identical readings, within the limits of accuracy of the colorimeter. All the tubes are heated in a boiling water bath for 20 minutes and cooled. It is necessary for the water bath to be evenly heated throughout. Differing temperatures in different sections of the water bath will give poor results. The colorimetric readings are made at 540 mμ wave length. Some change in color will occur if the tubes are left in direct sunlight; otherwise, no demonstrable change occurs if the readings are made within 2 hours after cooling. The number of duplicate determinations of free and total cholesterol carried out in this laboratory by this method exceeds ten thousand. Duplicates vary by less than ± 3%.

Summary. 1. A method for quantitative chromatographic separation of cholesterol esters from other blood lipids is presented. 2. A colorimetric micromethod for determination of the iodine number of plasma lipid is described. 3. A more stable reagent and a more stable color reaction than the Liebermann-Burchard reaction for the determination of plasma cholesterol is presented. 4. Employment of the above methods, together with determination of total lipids by the method of Bragdon, permits determinations of net unsaturation of the fatty acids esterified with cholesterol in the plasma.

Grateful acknowledgement is made to Merck, Sharp and Dohme for generous supplies of Digitonin.

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Plasma Cholesterol Ester Fatty Acid Composition in Relation to Diet.* (24198)

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In our previous paper it was noted that in a young normal adult male subject during the intake of 80 g of ethyl linoleate daily, the level of plasma cholesterol fell in a remarkable fashion and the iodine value of the cholesterol ester fatty acids at times exceeded 250(1). The present report includes data dealing with fatty acid composition of the plasma cholesterol esters in 4 male and one female subjects during the course of controlled studies.

Methods. Cholesterol esters were fractionated by column chromatography as described by Michaels, *et al.*(2) and unsaturated fatty acids determined by the Michaels modi-

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TABLE I. Patient EMAR.

		Plasma total lipids	Total cholesterol	Cholesterol ester fatty acids				
				Saturated	Mono-enoic	Di-enoic	Tri-enoic	Tetra-enoic
Diet:	10/28	553	195	18.4	38.5	35.2	3.2	3.6
Coconut oil, 60 g	/31	562	175	30.8	22.5	38.8	2.4	4.0
Protein, 104 g	11/ 4	522	150	28.6	17.7	43.7	3.6	5.0
Carbohydrate, 300 g	/ 7	500	157	9.3	47.6	34.8	2.7	4.3
	/11	603	180	16.6	60.4	20.2	2.7	3.6
	/14	572	163	0	67.3	23.8	3.3	4.1
Diet:	11/18	537	165	0	72.5	21.1	3.1	2.3
Ethyl oleate, 60 g	/21	475	128	0	68.5	20.9	4.1	5.0
Protein, 104 g	/25	447	116	0	74.5	19.1	3.1	1.7
Carbohydrate, 300 g	/27	472	120	0	0	0	0	0
	/29	441	118	0	72.7	19.4	4.3	2.5
	12/ 2	481	135	0	84.6	9.2	2.3	2.3
	/ 4	503	133	5.9	68.5	13.9	3.6	3.7
	/ 6	503	128	0	67.1	16.5	4.4	4.4
Diet:	12/ 9	522	134	0	72.9	19.8	4.6	1.8
Ethyl linoleate, 60 g	/11	453	123	0	51.3	40.8	3.2	3.2
Protein, 104 g	/13	462	136	0	47.6	45.1	2.0	3.5
Carbohydrate, 300 g	/16	453	126	0	36.6	55.4	3.1	3.4
	/18	391	103	0	6.5	82.5	2.9	5.9
	/20	412	110	0	30.5	58.4	5.2	4.2
	/23	412	118	11.2	6.7	63.3	3.8	4.3
	/25	428	111	0	0	0	0	0
	/27	387	105	15.9	0	69.4	5.2	11.6
	/30	384	105	11.6	0	68.6	8.2	7.8

fication of the method of Riemenschneider(3). Total lipids were determined by the method of Bragdon(4). Plasma cholesterol was determined by a modification of the method of Schoenheimer and Sperry(2).

Observations. The first patient, EMAR, a 61-year-old male with osteoporosis, but with no obvious manifestations of vascular disease, was placed on a chemically constant formula diet, which, for periods of 3 weeks each, contained coconut oil, ethyl oleate, and ethyl linoleate, respectively. The ethyl oleate was 99.8% pure; the ethyl linoleate was more than 95.0% pure. The remainder of the diet was constituted as shown in Table I. All of these diets were thoroughly fortified with essential vitamins and minerals and with crude liver. Protein was derived from non-fat milk solids. Prior to institution of the study, the patient had been on a diet containing relatively large amounts of saturated fat.

During the period on coconut oil, total cholesterol averaged 170 mg % and total lipids 552 mg %. During the period on ethyl oleate, total cholesterol averaged 130 mg% and total lipids averaged 457 mg %. During the period on ethyl linoleate, total cholesterol averaged 117 mg % and total lipids 430 mg %. The final values on linoleate were 105 and 384 respectively.

During the period on ethyl oleate, the mono-enoic acid in the cholesterol ester approached 80% and the di-enoic acid approximated 20%. The ratio was approximately reversed during ingestion of ethyl linoleate.

Within the limits of experimental error, the contents of penta-enoic and hexa-enoic acids did not vary during this study, and were in each instance less than 5% of the total. Except for the final portion of the study, the tri- and tetra-enoic acids were 5% or less.

Stool sterol excretion at all times was less than one gram daily, and did not appear to vary predictably in relation to the kind of fat which was fed.

Patient HMEN, aged 55, a diabetic with evidence of considerable vascular disease, received during a period of 32 days, ethyl linoleate in amounts varying from 50 to 100 g daily, and, for 28 days, 100 g daily of ethyl oleate. The composition of the diet is shown in Fig. 1. As in the previous patient, the mono-enoic acid content of the esters was high during oleate ingestion, and the di-enoic

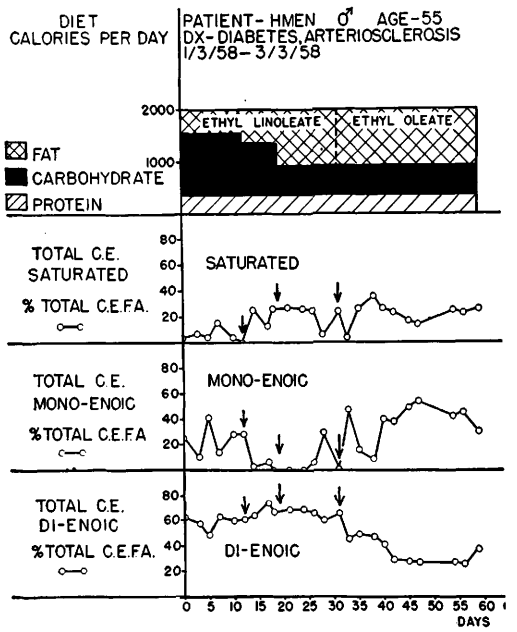

FIG. 1. Cholesterol ester fatty acids in patient HMEN.

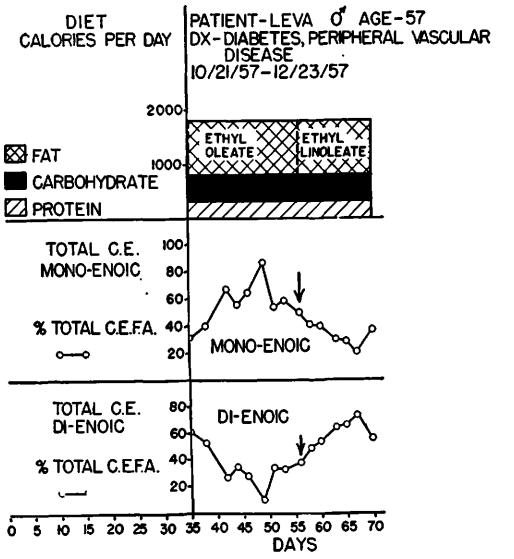

FIG. 2. Effects of oleate and linoleate on cholesterol ester fatty acid composition in patient LEVA.

content was low. The opposite of this was observed during the linoleate intake.

Content of tri-enoic, tetra-enoic, penta-enoic and hexa-enoic acids were low throughout, and, within the limits of error of the method, bore no obvious relationship to the type of fat that was fed.

Patient LEVA, a 57-year-old male mild diabetic, with extensive vascular disease, as in the case of the 2 preceding patients had a relatively high mono-enoic acid and low di-enoic acid content of the cholesterol esters during oleate ingestion and the opposite pattern during linoleate ingestion (Fig. 2). Tri-, tetra-, penta-, and hexa-enoic acid content was low, and, within the error of the method, bore no relationship to the fat which was fed.

The fourth patient, EROS, a middle-aged female, received a totally fat-free, iso-caloric diet. She, like the other patients, was in nitrogen equilibrium or positive balance throughout the study.

In Table II, it is apparent that the mono- and di-enoic acid content of her cholesterol esters followed the same pattern as that noted during oleate administration in the previous patients. Tetra-enoic acids averaged more than 5% of the total cholesterol ester fatty acids.

The fifth patient, AMOR, aged 47, differed from the other diabetics in that his diabetes was severe and totally insulin-dependent. During a 3-week portion of the study he received all of his fats in the form of coconut oil. During the remaining 4 weeks, he received fat mixtures containing the amounts of saturated fats, oleic acid and linoleic acid, respectively, shown in Table III. The progressive hypercholesteremia and hyperlipidemia noted during the coconut oil ingestion is apparent, and the return of lipid values toward normal during the intake of fat containing appreciable amounts of linoleic acid is equally apparent. The stool sterol excretion did not vary significantly during intake of the different fats, nor did the total stool fats. The patient was maintained in weight and nitrogen equilibrium throughout the study.

In the case of the mono-enoic and di-enoic fatty acids, the response pattern is essentially identical with the 3 patients described above. During the period of coconut oil ingestion, tetra-enoic acid content of the cholesterol esters averaged in excess of 5% of total fatty acids.

TABLE II. Patient EROS.

		Plasma total lipids	Total cholesterol	Cholesterol ester fatty acids				
				Saturated	Mono-enoic	Di-enoic	Tri-enoic	Tetra-enoic
Diet:	1/ 3	878	256	22	0	65	3.9	5.6
Protein, 97 g	/ 6	1016	210	41	0	44	5.5	5.6
Carbohydrate, 300 g	/ 9	1372	228	10.5	46	30	3.8	5.0
	/13	1453	260	4.5	60	25	3.3	5.4
	/16	1484	263	40	26	24	3.3	5.9
	/20	1316	248	5	63	21	3.8	5.4
	/23	1275	235	20	46	24	4.8	5.2
	/27	1103	235	18	52	22	2.4	4.5
	/30	1062	198	25	40	22	4.4	4.9
	2/ 3	1031	196	33	54	19.5	4.4	5.5

In this patient, as in most but not all patients studied to date, the saturated fatty acid content of the cholesterol esters was highest during intake of saturated fat.

Discussion. Only 2 consistent observations emerge from these studies: (1) Plasma cholesterol esters mirror to a significant degree the kind of fat which is fed under the experimental conditions used, and in the type of patient studied. (2) Fall in plasma cholesterol and total lipids observed in all individuals during intake of relatively large amounts of lineoleic acid was associated with a relatively high level of di-enoic acid in the cholesterol ester.

In one patient who received a high carbohydrate, fat-free diet, the cholesterol ester composition was much the same as in the patients who received oleate. A much higher plasma total lipid level was observed in this patient than in patients receiving oleate.

In none of these patients, even during high linoleate intake, were cholesterol ester fatty acid iodine values in excess of 180 noted, (as opposed to values of 250 in a young normal male, previously reported). Subsequent studies in normal young adults may help to establish the nature of the polyunsaturated fatty acids responsible for this high iodine value.

Summary. Administration of large amounts of purified linoleic acid preparations

TABLE III. Patient AMOR.

		Plasma total lipids	Total cholesterol	Cholesterol ester fatty acids				
				Saturated	Mono-enoic	Di-enoic	Tri-enoic	Tetra-enoic
Dietary fat:	9/30	844	205	4.6	71.5	21.8	.5	1.3
Coconut oil, 100%	10/ 2	934	229	43.2	16.7	34.1	.8	4.1
	/ 3	906	230	40.7	15.7	33.0	3.0	5.1
	/ 7	1119	269	51.7	.0	34.9	4.5	6.5
	/ 9	1412	305	31.4	31.3	25.0	4.8	5.1
	/10	1178	325					
	/14	1394	395	17.1	49.2	26.5	2.0	4.5
	/17	1250	365	9.6	48.5	29.6	.6	6.1
Dietary fat:	10/21	1400	395	7.3	57.5	26.5	3.1	4.8
Linoleic, 22.6%	/24	1562	431	.98	50.2	36.9	1.8	3.8
Oleic, 27.4%	/28	1394	407	0	57.1	44.8	1.9	3.4
Saturated, 45%								
Dietary fat:*	10/30	1500	350	5.2	.0	81.4	4.7	6.2
Linoleic, 45%	/31	1428	375	15.8	10.7	64.7	2.7	4.8
Oleic, 20%	11/ 4	1212	312	.0	21.1	74.8	1.2	4.0
Saturated, 29%	/ 7	1353	343	3.8	2.1	82.6	4.9	4.4
	/11	1084	285	5.9	17.2	70.0	1.7	3.3
	/14	1037	269	5.5	10.2	77.5	1.9	3.3
	/18	1103	250	.0	21.8	69.8	1.7	3.0

* "Swirl" (Procter and Gamble).

to diabetic and non-diabetic patients results in a marked increase in the di-enoic acid content of the cholesterol esters, in association with a lowering in the mono-enoic acid content. When ethyl esters of oleic acid are substituted for the ethyl linoleate the opposite pattern is observed. A high carbohydrate, fat-free diet produces much the same pattern of cholesterol ester composition as that seen during oleate ingestion.

Grateful acknowledgement is made to Procter and Gamble Research Labs. for supplies of purified oleate and linoleate.

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Ketosis in the Rat Fetus.* (24199)

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Although placental transfer of many substances has been demonstrated(1-3), such studies of ketone bodies have not been made. Since ketosis may readily develop in the pregnant animal when deprived of insulin(4,5) or when food intake is limited(5-7), it seemed of interest to determine if the ketone body level in fetal blood was related to that in maternal blood.

Methods. Sprague-Dawley rats pregnant 17 to 20 days and fed Purina Chow were used. Five of these rats were "totally" pancreatectomized(8) after 17-hour fast and tested 23 hours later. They were not fed postoperatively. Controls consisted of pregnant normal rats fasted 17 and 40 hours. Maternal blood samples for glucose determinations were taken from the tail. Immediately thereafter, mother rats were anesthetized with ether. Fluid was drawn from amniotic cavity with a hypodermic needle and syringe. Fetal blood samples were obtained by removing the fetus from the uterus, cutting open the chest and heart, and collecting the blood on a spot plate with heparin. Generally a single fetus was used for each determination. After obtaining fetal blood samples, blood for determination of maternal levels of ketone bodies and fat was drawn from the aorta with a syringe and needle. Glucose was measured by the method of Haslewood and Strookman(9), ketone bodies by the method of Bessman(10) and total lipids by the method of Bragdon(11).

Results. There was a marked elevation of glucose, ketone bodies, and fat in blood of the pancreatectomized rats (Fig. 1). Blood ketone bodies of the fasted normal pregnant rats were also increased, whereas their glucose levels were less than those in the fed rats. There was no significant difference in blood ketone body levels between pancreatectomized and 40-hour fasted normal pregnant rats. Preliminary observations indicate that the marked ketosis observed in the fasting pregnant rat does not occur before 14th day of gestation. Blood fats of some of the fasted normal pregnant rats were also increased.

In agreement with observations of others (1,2), maternal and fetal blood glucose levels were essentially the same. In the diabetics maternal glucose levels (260-420 mg/100 ml) averaged 60 mg/100 ml higher than fetal levels. These data reflect the known rapid transfer of glucose across the placenta(2). Glucose levels in the amniotic fluid of both normals and diabetics were 20 to 30 mg/100 ml lower than those in the fetal blood.

Maternal blood ketone body levels ranged from 0.6 to 114 mg/100 ml (Fig. 1). Over

* The excellent technical assistance of Barbara Guarco and Theresa R. Clary is gratefully acknowledged.