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Title: THE EFFECT OF A SALMON DIET ON THE ABSORPTION OF DIETARY CHOLESTEROL, PLASMA LIPID LEVELS, AND THE FECAL EXCRETION OF NEUTRAL STEROIDS AND BILE ACIDS

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The influence of a diet containing salmon flesh and its oil on plasma lipids, cholesterol absorption, as well as fecal neutral and acidic steroid excretion was studied. Two female and four male normolipidemic subjects were fed diets containing approximately 40% of their caloric intake from salmon oil, polyunsaturated vegetable oil or saturated fat. Each diet was fed for four weeks. Venous blood was drawn twice weekly for the determination of plasma cholesterol and triglycerides. C^{14} cholesterol was administered during the last week of each dietary period to determine the absorption of dietary cholesterol. Fecal neutral and acidic steroids were determined in feces collected following the administration of the isotope. Compared to the saturated fat diet, plasma cholesterol

was significantly lower following the salmon and linoleic acid diets. The salmon diet produced significantly lower plasma triglycerides than the polyunsaturated vegetable oil and saturated fat diets. The absorption of cholesterol and the fecal excretion of neutral and acidic steroids were not significantly altered by diet.

The Effect of a Salmon Diet on the Absorption of Dietary Cholesterol, Plasma Lipid Levels, and the Fecal Excretion of Neutral Steroids and Bile Acids

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THE EFFECT OF A SALMON DIET ON THE ABSORPTION OF DIETARY CHOLESTEROL, PLASMA LIPID LEVELS, AND THE FECAL EXCRETION OF NEUTRAL STEROIDS AND BILE ACIDS

INTRODUCTION

It has been shown that elevated levels of plasma cholesterol correlate with an increased incidence of coronary heart disease (CHD) (1-5). Therefore, it seems that lowering plasma cholesterol in humans may be an effective means of preventing this prominent cause of death in the United States.

Although many aspects of the diet-heart hypothesis are not proven, it may be desirable to follow a diet low in saturated fat and cholesterol for the prevention of CHD. To lower plasma cholesterol levels and to decrease CHD, Americans are encouraged to reduce dietary fat consumption from 40% to 30% of total caloric intake, and to increase dietary polyunsaturated fatty acids and decrease dietary cholesterol (6). There is no evidence that a low fat diet is detrimental (7).

Eskimos, even though their diet is high in cholesterol and fat (7-9), have a lower incidence of death from cardiovascular diseases than mainland Americans. The marine oils in the typical Eskimo diet have a fatty acid composition that is different from that found in animal or vegetable fat (10-13). Fish is also commonly eaten by the Japanese, who have one of the lowest death rates in the world from CHD (14). Marine oils have substantial amounts of long-

chained polyunsaturated fatty acids, as do some vegetable oils. Unique to marine oils, however, are fatty acids with the ω3 configuration[1] (e.g. 20:5ω3, eicosapentaenoic acid, and 22:6ω3, docosahexaenoic acid) whereas the polyunsaturated fatty acids of other fats are usually of the ω6 and ω9 configuration.

Oils high in polyunsaturated fatty acids have been found to lower plasma cholesterol (15, 16). Salmon oil may produce even lower plasma cholesterol levels than polyunsaturated vegetable oils (17). Like other fish oils, salmon oil is polyunsaturated, and its fatty acid constituents are mainly of the ω3 type rather than the ω6 and ω9 types found in vegetable oils and animal fats.

The mechanism by which oils high in polyunsaturated fatty acids lower cholesterol is unknown. The purpose of the research reported in this thesis is to determine if plasma cholesterol drops with the feeding of a diet high in salmon and its oil, and to determine if the mechanism of this decrease is caused by a diminished absorption of dietary cholesterol. I consider this to be a practical research topic because salmon along with other fish that also contain ω3 fatty acids can be easily incorporated into people's everyday diets.

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[1]The ω nomenclature is used to refer to the position of the first double bond from the methyl end of the fatty acid, ω3 meaning that the first double bond occurs at the third carbon from the methyl end of the fatty acid.

REVIEW OF LITERATURE

Effect of Polyunsaturated Vegetable Oils on Blood Lipids

As early as 1952, Kinsell et al. (18) showed that serum cholesterol dropped an average of 100 mg% in normal subjects receiving formula diets containing large amounts of vegetable oil. A formula diet containing an equivalent amount of animal fat gave serum cholesterol levels comparable to those from a mixed diet. Ahrens et al. (19), who fed fats of plant and animal origin to normo- and hypercholesteremic patients in successive five-week periods, observed lower serum lipid concentrations with a corn oil formula than with a formula containing an equivalent amount of saturated fat. Beveridge et al. (20) administered formula diets containing animal or vegetable fat to healthy male subjects for five months. An increase from 28% to 58% of the total calories in the form of vegetable oil lowered plasma lipid levels, whereas a similar increase in animal fat significantly raised them. The higher polyunsaturated nature of marine oils may possibly be the reason why Eskimos, living in their own cultures, have serum cholesterol levels as much as 50% lower than mainland Americans of comparable age (21).

The fatty acid composition of various foods is shown in Table 1. Presented in Table 2 are the structures of

Table 1. Fatty acid composition of various foods[1] (% of total fatty acids)[2].

Fatty Acid	Egg	Beef	Soy	Corn	Safflower	Sunflower	Cottonseed	Peanut
Saturated	36	44	15	13	12	12	25	18
Monounsaturated	48	51	23	25	08	20	21	47
Polyunsaturated								
Dienes	13	03	51	57	79	63	50	29
Trienes or Greater	02	02	07	tr	0	0	0	0
C20:5ω3	tr	01	0	0	0	0	0	0
C22:6ω3	tr	01	0	0	0	0	0	0

Fatty Acid	Olive	Coconut	Cocoa Butter	Salmon	Cod	Menhaden	Mackerel
Saturated	11	86	60	27	27	27	26
Monounsaturated	76	07	33	46	24	24	22
Polyunsaturated							
Dienes	07	tr	03	01	01	02	1.4
Trienes or Greater	0	0	0	20	51	23	24
C20:5ω3	0	0	0	08	18	13	6.8
C22:6ω3	0	0	0	06	30	09	11.6

[1]Data from Nutritive Value of American Foods #456. Agricultural Research Service U.S.D.A.; and Stansby, M. E., Nutritional properties of fish oils. World Rev. Nutr. Diet 11:46, 1969; and Malmros, H. and G. Wigand, The effect on serum cholesterol of diets containing different fats. Lancet II:1, 5, 1957.

[2]Fatty acids listed are those that are relevant to this thesis and do not necessarily add up to 100%.

Table 2. Names and structures of some fatty acids in foods.

Docosahexaenoic Acid (22:6ω3)

C-C-C=C-C-C=C-C-C=C-C-C=C-C-C=C-C-C=C-C-C-COOH

Eicosapentaneonic Acid (20:5ω3) (Trimndonic)

C-C-C=C-C-C=C-C-C=C-C-C=C-C-C=C-C-C-C-COOH

Linolenic Acid (18:3ω3)

C-C-C=C-C-C=C-C-C=C-C-C-C-C-C-C-C-COOH

Linoleic Acid (18:2ω6)

C-C-C-C-C-C=C-C-C=C-C-C-C-C-C-C-C-COOH

Oleic Acid (18:1ω9)

C-C-C-C-C-C-C-C-C=C-C-C-C-C-C-C-C-COOH

Stearic Acid (18:0)

C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-COOH

Palmitoleic Acid (16:1ω7)

C-C-C-C-C-C-C=C-C-C-C-C-C-C-C-COOH

Palmitic Acid (16:0)

C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-COOH

Myristic Acid (14:0)

C-C-C-C-C-C-C-C-C-C-C-C-C-C-COOH

some fatty acids in foods.

Effect of Marine Oil on Blood Lipids

In a review Stansby (17) reported that fish oils, in general, decreased plasma cholesterol levels in rats and mice. In addition, Kingsbury et al. (22) found that in three healthy male subjects a diet containing 25 g of cod liver oil (which consists of 5 g of 20:5ω3 and 22:6ω3) produced a 31-95 mg% drop in plasma cholesterol, whereas a diet containing an equal amount of corn oil produced a 25 mg% drop.

Peifer et al. (23) concluded that the effect of fish oil on lowering plasma cholesterol in rats was not predictable by its iodine number. Menhaden oil fractions containing 19-33% polyunsaturated fatty acids, compared to corn oil with the same percentage of unsaturated fatty acids, produced lower plasma cholesterol levels after four weeks (134 ± 21 mg% vs. 297 ± 19 mg%). Peifer et al. (24) compared the hypocholesteremic effect of linoleic acid and linolenic acids with tuna oil in hypercholesteremic rats. Diets containing 3.3% fish oil lowered serum cholesterol from a mean of 318 ± 6 mg% to a mean of 140 ± 35 mg% after five weeks. The diet containing 3.3% linolenate lowered serum cholesterol to 175 ± 21 mg% and the one containing 3.3% linoleate produced a drop to 238 ± 33 mg%.

Plasma triglyceride levels fell when 200 g/day portions of mackerel were incorporated into an ovo-lacto

vegetarian diet (25). Ruiter et al. (26) also found that a diet containing mackerel oil at 20% of calories reduced plasma triglyceride levels by 50% in piglets. Ahrens et al. (27) observed that menhaden oil reduced serum cholesterol in two hyperlipidemic subjects an average of 275 mg%, and serum triglycerides an average of 359 mg%.

Postulated Mechanisms by Which Vegetable Oils Lower Blood Lipids

Several mechanisms by which polyunsaturated vegetable oils lower plasma cholesterol have been postulated, but not proven. For example, Goldsmith, Hamilton and Miller (28), who fed isocaloric diets containing 90 g of butter or corn oil to six hyperlipidemic subjects, found a 20-25% increase in fecal bile acid excretion with the corn oil diet, but not with the butter diet. Lewis (29) studied bile acid excretion during the administration of sunflower seed, cottonseed and hydrogenated coconut oils to three patients with complete bile fistulas. Sunflower seed oil given orally increased the fecal bile acid excretion from approximately 7 g/24 hr to approximately 23 g/24 hr. Cottonseed oil administered intravenously raised the fecal bile acid excretion by approximately 8 g/24 hr. Both oils decreased the serum cholesterol levels by an average of 33 mg%. Hydrogenated coconut oil did not affect bile acid excretion, but caused a rise in serum cholesterol. Haust and Beveridge (30) found that bile acid excretion was

increased by 50% in young adult male subjects fed a formula with 60% of the total calories as corn oil when compared to a similar formula but with butterfat. Moore et al. (31) observed a significant increase in the fecal excretion of neutral steroids and bile acids in young male subjects fed a mixed food diet with 40% of the calories supplied as safflower oil as compared to a similar diet containing the same amount of butterfat. The total bile acid and neutral steroid fecal excretion increased from 966 ± 42 mg/day on the butterfat diet to 1147 ± 45 mg/day on the safflower oil diet. In contrast, Ali, Kuksis and Beveridge (32), who fed formula diets containing either corn oil or butterfat at 35% and 60% of the total calories to three male subjects, found that at either level of fat, bile acid excretion was greater on the butterfat diet.

Connor et al. (15) postulated that polyunsaturated fats may cause a loss of cholesterol from the tissues. In six men fed cholesterol-free formula diets with 40% of the total calories as corn oil or cocoa butter, an increase in fecal steroid excretion was noted on the corn oil diet which was twice as much as could be accounted for by the drop in plasma cholesterol. No changes were observed with the cocoa butter diet. Similar results were obtained by Frantz and Carey (33). Twelve healthy men with normal serum cholesterol levels who were eating an institutional diet were divided into two groups of six men each. One group received one ounce of hydrogenated

coconut oil prior to each meal while the other group received the same amount of corn oil. Serum cholesterol levels fell an average of 9% and liver cholesterol, as measured by punch biopsy, fell an average of 25% in the men fed the corn oil. No consistent effect was noted in the men fed the coconut oil.

Wood, Shioda and Kinsell (34) found that increased excretion of total steroids occurred on a polyunsaturated fat diet and suggested that their data supported the idea that increased excretion of steroids is associated with decreased absorption and reabsorption of steroids. However, Nestel et al. (16) concluded that cholesterol balance (cholesterol absorbed = H^3 given - H^3 in feces) was not different in healthy men who had been given a diet with a high polyunsaturated to saturated fat ratio (P/S) when compared to healthy men given a diet with a lower P/S. These results suggest that there is no difference in the absorption of cholesterol on either diet. Tanaka and Portman (35) indicated that regardless of the level of dietary cholesterol, cholesterol absorption in thirteen squirrel monkeys was approximately 75% when they were fed a diet high in polyunsaturated oil and approximately 50% when they were fed a diet high in butterfat. The polyunsaturated diet produced lower plasma cholesterol levels in the monkeys (221 ± 13 mg%) than the diet high in butterfat (444 ± 74 mg%). Sklan et al. (36) demonstrated that the absorption of exogenous, but not endogenous

cholesterol, increased in chicks fed diets containing 15% olive oil. In human subjects receiving diets high in polyunsaturated oil, Grundy and Ahrens (37) observed that only one out of eleven subjects showed a decreased absorption of neutral and acidic steroids. Beveridge, Haust and Connell (38) postulated that the β-sitosterol and other steroids in vegetable fats may decrease the absorption of cholesterol.

Fish oil, which is composed largely of polyunsaturated fat, may act in a similar manner as vegetable oil in lowering plasma cholesterol. However, there is some evidence, as stated earlier, that fish oil produces a greater hypocholesteremic effect than vegetable oil. The mechanism by which marine oils lower serum cholesterol is not known. Perhaps it acts to lower plasma cholesterol in a number of ways, as does vegetable oil.

Diet-Heart Hypothesis Controversy

Much controversy exists on the effect of dietary cholesterol on CHD. Reduction of dietary cholesterol and saturated fats leads to a decreased level of plasma cholesterol (39, 40), whereas dietary cholesterol ranging in amounts from 200-1000 mg/day will produce a 15-30% elevation of plasma cholesterol in humans (18, 41-43). However, prevention of CHD by lowering plasma cholesterol has not been proven. In two different studies (44, 45) niacin and clofibrate reduced plasma cholesterol levels by 15-30%

in humans, but even with this decrease no effect on the course of CHD was noted after five years of observation. In contrast, a positive correlation was demonstrated between decreased incidence of new events of myocardial infarction and lowered plasma cholesterol in hypercholesteremic humans (1). In addition, regression of this disease by decreased dietary cholesterol in laboratory animals, including primates, has been shown (2-5). In parts of the world where the incidence of CHD is rare, diets low in fat and cholesterol are typically consumed (7).

A decrease in plasma cholesterol alone may not be the only factor involved in lowering the incidence of CHD. During World War II, food rationing in Northern Europe led to a reduced proportion of total calories from dietary fats, including cholesterol. Total caloric intakes were also reduced and a higher percentage of calories came from carbohydrates. During this time, the incidence of CHD and other diseases fell. The incidence of these diseases rose again after the war (46). These observations stimulated interest in the role of the diet in the development of CHD. However, they do not show cholesterol as having an effect as an independent variable. Factors that challenge the belief that lowered plasma cholesterol alone leads to a decreased incidence of CHD include individual and genetic differences in susceptibility to CHD (47).

Digestion and Absorption of Fats

Dietary lipids are mainly in the form of triglycerides. Food also contains cholesterol and its esters, phospholipids, and the fat soluble vitamins. After food is ingested, it travels down the esophagus into the stomach where a gastric lipase may act to release short and medium chain fatty acids from the triglyceride molecule (48). Hydrochloric acid and pepsin break up fat emulsions leaving large globules of fat. Fat is then released to the duodenum which elicits the release of enterogastrone, a hormone which decreases gastric secretion and motility, thereby slowing the emptying of the stomach contents into the duodenum. The slow entrance of the fat into the duodenum prevents exceeding the capacity of the pancreatic lipolytic enzymes. Pancreatic enzymes that handle fat include glycerol ester hydrolase (lipase) and cholesterol esterase. Bile acids aid in the emulsification of lipids in the lumen, thus increasing their surface area so that the action of the lipases are more efficient. Bile acids also shift the pH of the lumen contents to 6, which is optimum for pancreatic lipase activity. The resulting free fatty acids of chain length greater than ten, and 2-monoglycerides, along with conjugated bile salts, phospholipids, free cholesterol, and the fat soluble vitamins, form mixed micelles which are organized structures of hydrophilic and hydrophobic moieties that are necessary to solubilize lipids. These micelles are

subsequently absorbed. The fatty acids of chain length less than ten carbons are absorbed directly into the portal blood. Supposedly, the primary role of the mixed micelle in lipid absorption is to overcome the resistance of the unstirred water layer while lipid absorption occurs as simple diffusion through the mucosa in equilibrium with the micelle (49). Other researchers have suggested that the uptake occurs either by pinocytosis of the micelle (50, 51) or that the individual lipids partition into the cell membrane during direct interaction of the micelle with the cell membrane (52-54). Chylomicrons are formed in the intestinal cell and contain resynthesized triglycerides, phospholipids, free and reesterified cholesterol, cholesterol esters, and proteins. These chylomicrons enter the lymph through lacteals and travel through the thoracic duct to the left inominate junction and into the general circulation. Eventually they are cleared of triglycerides by lipoprotein lipase (LPL) at peripheral blood vessels. The chylomicron remnants are rapidly cleared by the liver (54).

Absorption of Cholesterol

Absorbed cholesterol comes from three main sources: sloughed mucosal cells, biliary and dietary cholesterol. Approximately 20-50 percent of dietary cholesterol, which is composed of the free and esterified forms, is absorbed (55). Hydrolysis of the esters by pancreatic

cholesterol esterase and its cofactor taurocholate (48), is necessary before intestinal absorption can occur. Free cholesterol is incorporated into the mixed micelles and absorbed into the mucosa. After the lipid enters the mucosa, cholesterol is reesterified with fatty acids from pools of exogenous and endogenous sources and associated into chylomicrons. The chylomicrons are absorbed into the lymph as discussed previously.

It seems plausible that fewer cholesterol molecules esterified with ω3 fatty acids could be incorporated into chylomicrons than polyunsaturated vegetable fatty acid esters because of the bulkier shape of the ω3 fatty acids, as suggested by Spritz and Mishkel (56). Fewer cholesterol esters would lead to a net decrease of cholesterol moving into the lymph. This is possible since there is a selective incorporation of 20:5ω3, one of the main fatty acids in salmon fat, into the plasma cholesterol esters (21, 25). However, there is no evidence that this selectivity occurs in lymph cholesterol too. Another possibility is that this type of "steric hindrance" could occur at the level of the micelle, the fatty acids themselves being bulky thus possibly crowding out cholesterol from the micelle. Cholesterol absorption has been reviewed in detail by Treadwell and Vahouny (55).

Enterohepatic Circulation of Bile Acids

The conversion of cholesterol to bile acids occurs exclusively in the liver and is a major pathway for the degradation of cholesterol in mammals (57). Bile acids leave the liver and are conjugated with glycine or taurine by amide linkage to the carboxyl group of the side chain (58). These compounds are known as conjugated bile acids. At this point the bile acids have begun enterohepatic circulation which involves several main events. These are, in addition to the secretion of newly synthesized primary bile salts into the biliary canaliculi: transport to the gall bladder for storage and concentration; emptying into the duodenum thus mixing with chyme and pancreatic juice; involvement in lipid digestion and absorption; transport down the small intestine, and active transport of most of the bile acids in the ileum. In the colon deconjugation and dehydroxylation occur due to bacterial enzymes. Passive absorption in the colon of some of the secondary bile acids occurs, which are then transported via the portal vein to the hepatocyte where they may be reconjugated and finally resecreted again into the bile (58).

Fat soluble vitamins and hormones undergo a slight degree of enterohepatic circulation, but it is quantitatively and physiologically insignificant. The enterohepatic circulation of cholesterol is dependent on the bile acid cycle (58).

MATERIALS AND METHODS

This investigation was conducted at the University of Oregon Health Sciences Center in the Clinical Research Center (CRC). The study was given approval by the human subjects committee at that institution.

Two male and four female volunteers, ages 21-55 years, participated in the study. Except for one subject, the subjects' plasma cholesterol values, prior to this research, were less than 220 mg%. The subjects' plasma triglyceride values were less than 140 mg%. These and other data on the subjects are included in Table 3.

This experiment lasted for a total of twelve weeks with three four-week dietary periods and a three-week "wash-out" period between the polyunsaturated fat phases. A different fat was fed in each period. The linoleic acid diet contained safflower oil and stripped corn oil;[1] the saturated diet included peanut oil and cocoa butter; and the salmon diet used salmon flesh and oil, which contain polyunsaturated fats from the ω3 family. The diets also differed in sources of cholesterol and protein. The cholesterol was from salmon flesh and oil in the salmon diet and from egg yolk for the other two diets. The percentage of total energy from protein, fat, and carbohydrate in each of the three diets were equivalent. Non-formula food items on each diet were identical in bread,

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[1]Stripped corn oil contains fewer plant sterols and less Vitamin E.

Table 3. Subject data

Subject	Age (yrs)	Sex	Weight[1] (kg)	Height (cm)	Plasma Cholesterol[1] (mg%)	Plasma Triglycerides[1] (mg%)
1	51	F	73.2	163	251	103
2	27	M	81.7	182	125	36
3	21	F	53.2	165	162	62
4	24	M	76.9	187	136	41
5	54	F	66.3	157	211	112
6	53	F	52.4	158	218	44

[1]Prior to study.

fruit, and vegetable exchanges (59), but differed in amounts, depending on the subjects' caloric needs. Information and composition of the formulas and diets are shown in Tables 4, 5 and 6. Daily throughout the study the subjects were given a multiple vitamin and mineral supplement,[2] and 50 mg of ferrous sulfate[3] to compensate for the blood loss during twice weekly samplings. Vitamin E,[4] 50 IU, was given daily during all dietary periods to cover the increased need of this vitamin during the highly polyunsaturated fat diets, the salmon and linoleic acid feedings. The recommended daily allowances (RDA '73) for all nutrients were met. The caloric need of each subject was determined using the Mayo Clinic Boothby and Berkson Food Nomograms (Post. Grad. Med. 9:106-115, 1951). Subjects were weighed each morning and ate their three meals each day at the CRC throughout the study. The amount of formula consumed by each subject was determined by caloric need and was kept constant throughout each formula

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[2]Multiple vitamin and mineral supplement: Vitacaps M Filmtabs, given daily in all dietary periods. Ingredients: Vitamin C, 50 mg; Niacinamide, 20 mg; Ca Pantothenate, 5 mg; Vitamin A (acetate and palmitate, 1.5 mg; B_6, 0.5 mg; Ergocalciferol, 10 mcg; Cyanocobalamin, 2 meg; Iron, 10 mg; Zinc, 1.5 mg; Iodine, 0.15 mg; Magnesium, 5 mg; Copper, 1 mg; Manganese, 1 mg. Abbott Laboratories, N. Chicago, Ill.

[3]Iron supplement: Fer-in-Sol, given daily in all diet periods, 50 mg. Ingredients: Ferrous sulfate. Mead Johnson, Evansville, Indiana.

[4]Vitamin E supplement: Aquasol, given daily, all diets 50 IU. Ingredients: dl tocopherol. USV Laboratories, Tuckahoe, N.Y.

Table 4. Total diet information.

	Salmon Diet	Linoleic Acid Diet	Saturated Diet
Fat Source	Salmon oil and flesh	Stripped corn oil Safflower oil	Peanut oil Cocoa butter
Salmon, cooked (g)	340		
Salmon oil (g)	82		
Iodine value	157[1]	122[1]	64[1]
P/S	1.4	3.6	0.3
Cholesterol (mg/day)	500	500	500
Percent total kilocalories provided by formula		48	48
Percent total kilocalories provided by salmon flesh and oil	45		
Kilocalories	As needed to maintain weight.		
Other foods	Fruit, vegetable and bread exchanges.[2]		

[1] Determined by Dr. William Harris on actual sample.

[2] As needed to maintain weight.

Table 5. Sample composition of formulas fed during the linoleic acid and saturated diet periods[1] (g).

Linoleic Acid Diet	Total	CHO	PRO	FAT
Stripped corn oil[2]	83	-	-	83
Safflower oil[3]	12	-	-	12
Egg yolk mix[4]	49	5	6.8	12
Calcium caseinate	53	0.1	50	.80
Corn starch	17	15	0.1	-
Sucrose	33	33	-	-

Saturated Diet	Total	CHO	PRO	FAT
Peanut oil[3]	40	-	-	40
Cocoa butter[5]	55	-	-	55
Egg yolk mix[4]	49	5	7	12
Calcium caseinate	53	0.1	50	.80
Corn starch	17	15	0.1	-
Sucrose	33	33	-	-

[1]Sample composition for subject on a 2,600 Kcal/day requirement.

[2]Distillation Products Industries, Rochester, N.Y.

[3]Portland Wholesale, Portland, Oregon

[4]Fleischmans Lab, New York, N.Y.

[5]Boldmann Chocolate Co., Union City, Calif.

Table 6. Composition of total diet[1] (% of caloric intake)

	Salmon Diet	Linoleic Acid Diet	Saturated Diet
Protein	15	15	15
Carbohydrate	45	45	45
Starches	27	27	27
Sucrose	14	14	14
Other Simple Sugars	4	4	4
Fat	40	40	40
Saturated Fatty Acids	8	6	16
Monounsaturated Fatty Acids	20	14	19
Polyunsaturated Fatty Acids	11	20	5
(ω6) (Linoleic acid)	1	19	4
(ω3) C20:5	4	0	0
(ω3) C22:6	4	0	0

[1]Data calculated from Nutritive Value of American Foods, Agricultural Handbook #456. Agricultural Research Service, USDA.

diet period. If the subjects gained or lost weight, their caloric intake was adjusted accordingly by the addition or removal of bread, fruit and vegetable exchanges.

The order of feeding the diets was randomized. Because of a carryover effect of polyunsaturated fat incorporated into the cell membranes of the body, a "wash out" period followed the linoleic acid and salmon diets. This was a three-week period during which the subject returned to his or her usual diet.

During each dietary period venous blood was drawn twice weekly from fasting subjects into heparinized Vacutainer tubes. Plasma cholesterol and triglyceride levels were monitored during each dietary period.

During the last week of each dietary period cholesterol absorption was determined by administering to the subjects a test meal at breakfast. This meal contained cholesterol C^{14} and β-sitosterol H^{3} along with 40 radio opaque pellets, and supplied 800 kilocalories (Table 7). Stools were collected after each test meal until at least 32 pellets were recovered, marking the end of the absorption test meal stools. The same stools were used for determining fecal neutral steroids and bile acids. After weighing, the stools containing pellets were mixed with water for 8 minutes in paint cans on a paint can shaker. Total mixed stool weights were obtained. Aliquots of the mixed stools were transferred into bottles and stored at -15°C until analyzed.

Table 7. Cholesterol absorption test meal.

Isotope: 3.0 μCi cholesterol-4-C^{14} and 3.0 μCi β-sitosterol H^3 in 150 mg β-sitosterol and 0.5 mg crystalline cholesterol. Source of isotope: Amersham Corp.

Food Mixed with Isotope

Salmon Diet	Linoleic Acid Diet	Saturated Diet
50 g salmon meat	18 g egg yolk	18 g egg yolk
5 g salmon oil	5 g peanut oil	5 g peanut oil

Food Added to Make Meals Equivalent in Kcal, Protein, Carbohydrate, Fat, and Cholesterol Content

Salmon Diet	Linoleic Acid Diet	Saturated Diet
2 bread exchanges	Formula[1]	Formula[2]
100 g apple juice		
150 g salmon meat		
13 g salmon oil		

Nutrient Composition of Test Meal

800 Kcal, 45 g of protein, 42 g of carbohydrate, 50 g of fat, and 150 mg of cholesterol

[1]The formula consisted of stripped corn oil (36 g), peanut oil (4 g), calcium caseinate (44 g), dextrimaltose (20 g), corn starch (10 g) and sucrose (11 g).

[2]The saturated diet formula differed from the linoleic acid diet formula by containing cocoa butter (27 g) and peanut oil (13 g) instead of the stripped corn oil and 4 g of peanut oil.

Determination of Plasma Cholesterol and Triglycerides

For these two determinations 0.5 ml of plasma was treated with 10 ml of isopropanol and approximately 2 g of a zeolite mixture (800 g of zeolite, 200 g of Lloyd reagent,[5] 20% cupric sulfate, and 40% calcium hydroxide). After the samples were mixed and allowed to stand for 30 minutes, they were centrifuged at 2500 rpm for 5 minutes. The supernatent was used for determining cholesterol and triglycerides according to a method described in a lipid research clinic procedure manual (60). Plasma cholesterol and triglyceride values were determined using the samples collected at the end of each week.

Assay for Cholesterol Absorption

Five-tenths gram of the stool mixture was saponified with 20 ml of 10 N NaOH in 90% ethanol in a hot oil bath (100°C) for one hour. Samples were cooled and 10 ml of water were added. The samples were extracted three times with 50 ml of hexane. The hexane fraction was removed by aspiration and used for the determination of the cholesterol and β-sitosterol isotopes. These hexane extracts were evaporated to dryness, quantitatively transferred with hexane to scintillation vials, and evaporated again to dryness. After the addition of 10 ml of toluene

[5]Lloyd reagent is a hydrated aluminum silicate.

scintillation fluid, the vials were counted in a Hewlett Packard Tricarb Scintillation Spectrometer (Avondale, Penn.). Cholesterol absorption was determined by subtracting counts from the stool from those given in the test meal.

Neutral Steroid and Bile Acid Determination

The fecal excretion of neutral steroids and bile acids was determined by the method of Miettinen and Grundy and their associates (61, 62). Five-tenths gram of mixed stools were saponified and extracted as for the cholesterol absorption test, except that before saponification high specific activity radioactive cholesterol (cholesterol-4-C^{14}) and bile acid (deoxycholic acid-24-C^{14}) (5000 cpm in 0.5 ml of each) were added to each sample as internal standards. A scintillation vial containing 0.5 ml of cholesterol-4-C^{14} and another containing 0.5 ml of deoxycholic acid-24-C^{14} was prepared for the purpose of calculating recovery values. Hexane extracts were saved for the determination of neutral steroids (cholesterol and its microbially altered products, coprostanol and coprostanone; and including plant sterols, campesterol, stigmasterol, and β-sitosterol). The aqueous phase was used for the determination of bile acids, deoxycholic acid and lithocholic acid.

Assay for Fecal Neutral Steroids

The hexane extracts were evaporated to dryness on a flash evaporator and quantitatively transferred to 15 ml conical tubes with three 2-ml hexane washings. The combined washings were evaporated under nitrogen and applied to 0.5 mm-thick florisil plates[6] which had been activated at 100°C for one hour. Plates were developed with a heptane ether (45:55) solvent and visualized under UV light after having been sprayed with a saturated solution of Rhodamine G. Visible bands were scraped individually into test tubes, extracted five times with 5-ml portions of ether, and centrifuged. The supernatant was poured into 50 ml glass-stoppered test tubes and evaporated to dryness under nitrogen. Two milliliters of cholestane in ethyl acetate (260 ul/ml) were added as the standard for gas-liquid chromatography (GLC). One milliliter of this solution was placed in a counting vial and evaporated to dryness under oxygen. Ten milliliters of scintillation fluid were added and the vials were counted in the scintillation spectrometer. The remaining 1 ml of cholestane solution was evaporated to dryness under nitrogen and 0.4 ml of dimethyl foramide-hexamethyl disilazane trimethyl chlorisilane (40:40:1) was added. Samples were reacted one-half hour at room temperature. One to three microliters of sample was injected into the gas-liquid chromatograph.

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[6]Brinkman Instruments, Inc., Des Plaines, Ill.

The neutral steroids were run on a SE 30 methyl silicon column which was 4 mm in diameter, 120 cm tall and at 230°C. The injector and detector temperatures were at 260°C. Helium was used as a carrier gas at a flow rate of 80 mm/min. The retention times, in minutes, for the neutral steroids were: cholesterol, 2.30; campesterol, 3.01; stigmasterol, 3.28; β-sitosterol, 3.80; coprostanol, 1.84; coprocampesterol, 2.40; coprostigmasterol, 2.65; and coprostigmastanol, 3.04.

Assay for Dietary Plant Sterols

The procedure was the same as for fecal neutral steroid determination except that a 5 g mixed food aliquot was used. The fecal excretion of plant sterols was compared with the intake.

Assay for Bile Acids

After extracting the aqueous phase with hexane, 5 ml of 2 N NaOH were added to each sample which were then saponified in a household pressure cooker at 120 kilopascals for three hours. After saponification the samples were cooled and 10 ml of water were added. This mixture was adjusted to pH 2 with concentrated HCl. The samples were then extracted with 50 ml of chloroform-methanol (2:1) once, and then with 30 ml of chloroform three more times. The extracts were evaporated to dryness using the flash evaporator. The residue was dissolved

in 25 ml of chloroform-methanol (2:1) and a 10 ml aliquot was transferred to a glass stoppered test tube and evaporated to dryness under nitrogen. The samples were methylated with 4 ml of 5% HCl in anhydrous methanol by refluxing for two hours at 100°C. The samples were evaporated to reduce volume and spotted on 0.5 mm-thick silica gel H plates[7] that had been pre-run in 10% acetic acid in methanol and activated at 120°C for one hour. The plates were developed with benzene, visualized with iodine vapor, and the fatty acid methyl esters were discarded. The plates were then developed in a mixture of 60 ml of isooctane, 20 ml of isopropanol, and 0.5 ml of acetic acid and visualized again with iodine vapor. The methyl esters of bile acids were scraped into test tubes and extracted with 5 ml of methanol six times. Extracts were collected in 50 ml conical tubes and 1 ml of 5α-cholestane (0.25 mg in 1 ml solution) was added as the internal standard for GLC. The samples were evaporated to dryness and the residues were dissolved in 10 ml of ethyl acetate. One 5-ml aliquot was used for counting and the other for GLC. The ethyl acetate solution was evaporated to dryness under nitrogen and 0.4 ml of TMS mixture (dry pyridine-hexamethyl disilazone-trimethyl chlorisilane, 9:3:1) was added. The samples were reacted for 30 minutes at room temperature and 1-3 ul were injected into the gas-liquid chromatograph.

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[7]Brinkman Instruments, Inc., Des Plaines, Ill.

The bile acids were run on the same column as discussed under "Assay for Fecal Neutral Steroids" and under the same conditions except that the column temperature for the bile acids was 240°C. The retention times, in minutes, for the bile acids were: lithocholic acid, 2.12; and deoxycholic acid, 2.54.

RESULTS AND DISCUSSION

Results

The percent absorption of dietary cholesterol by each subject during the last week of each of the three dietary periods is shown in Table 8. The subjects' fecal excretion of bile acids and neutral steroids is presented in Tables 9 and 10, respectively. A two-way analysis of variance (ANOVA) revealed that cholesterol absorption, and the fecal excretion of bile acids and neutral steroids were not significantly altered by diet.

Weekly changes in the plasma cholesterol and triglyceride values, respectively, for the salmon diet are given in Figures 1 and 2; for the linoleic acid diet, in Figures 3 and 4; and for the saturated diet, in Figures 5 and 6. The subjects' plasma cholesterol and triglyceride levels on the last day of each dietary period are given in Table 11. Statistical analysis of the plasma lipid data, using a student's paired t-test, showed that at the end of the four-week dietary periods, the salmon and linoleic acid diets gave a significantly lower mean plasma cholesterol level ($p < 0.025$ and $p < 0.05$, respectively) than the saturated diet. However, no significant difference in mean plasma cholesterol was found between the salmon and linoleic acid diets. The highest plasma cholesterol levels in each of the subjects occurred at the end of the

Table 8. Percent of cholesterol (cholesterol-4-C^{14}) absorbed during the last week of the three diet periods.[1,2]

Subject	Salmon Diet	Linoleic Acid Diet	Saturated Diet
1	22	23	28
2	31	42	58
3	48	43	44
4	47	54	47
5	48	24	23
6	20	19	27
Mean ± S.D.	36 ± 13	34 ± 14	38 ± 14

[1]Normal range of cholesterol absorption is 20-50% (55, 63)

[2]Mean values in all three dietary periods were not significantly different.

Table 9. Fecal bile acid[1] excretion during the last week of the three diet periods (mg/day).[2]

Subject	Salmon Diet	Linoleic Acid Diet	Saturated Diet
1	1571	1680	1245
2	62	365	1579
3	426	743	464
4	604	724	413
5	703	706	350
6	1030	576	486
Mean ± S.D.	733 ± 520	799 ± 454	756 ± 521

[1]Fecal bile acids include lithocholic and deoxycholic acids.

[2]Mean values in all three dietary periods were not significantly different.

Table 10. Fecal neutral steroid[1] excretion during the last week of the three diet periods (mg/day)[2]

Subject	Salmon Diet	Linoleic Acid Diet	Saturated Diet
1	2841	1680	2087
2	1975	1562	1274
3	1498	1563	1712
4	980	1996	1243
5	2608	2388	2072
6	2846	2761	2019
Mean ± S.D.	2125 ± 774	1992 ± 493	1735 ± 393

[1]Neutral steroids quantified in this table include cholesterol, coprostanol and coprostanone.

[2]Mean values in all three dietary periods were not significantly different.

Figure 1. Changes in plasma cholesterol over the four-week salmon dietary period.

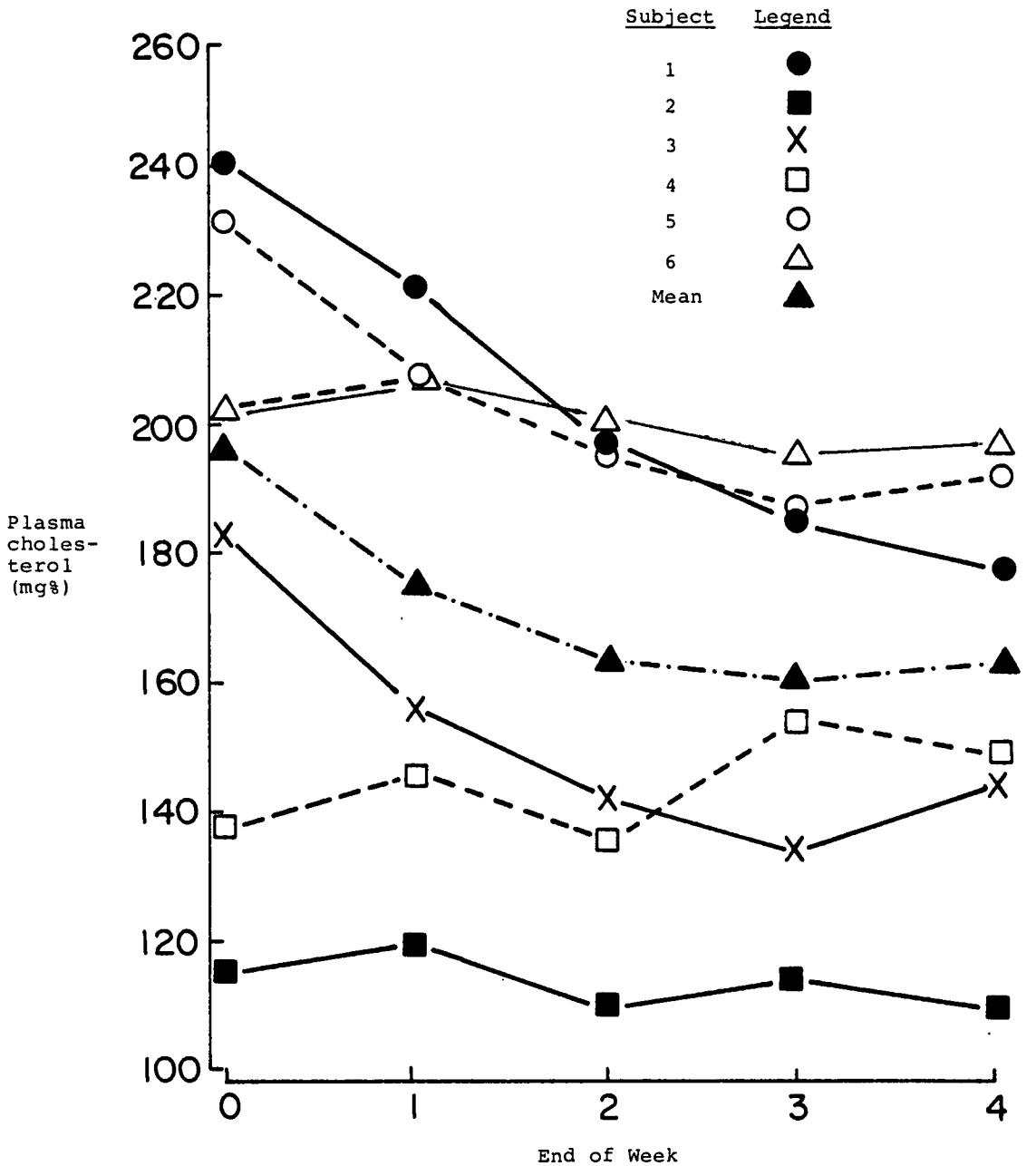


Figure 2. Changes in plasma triglicerides over the four-week salmon dietary period.

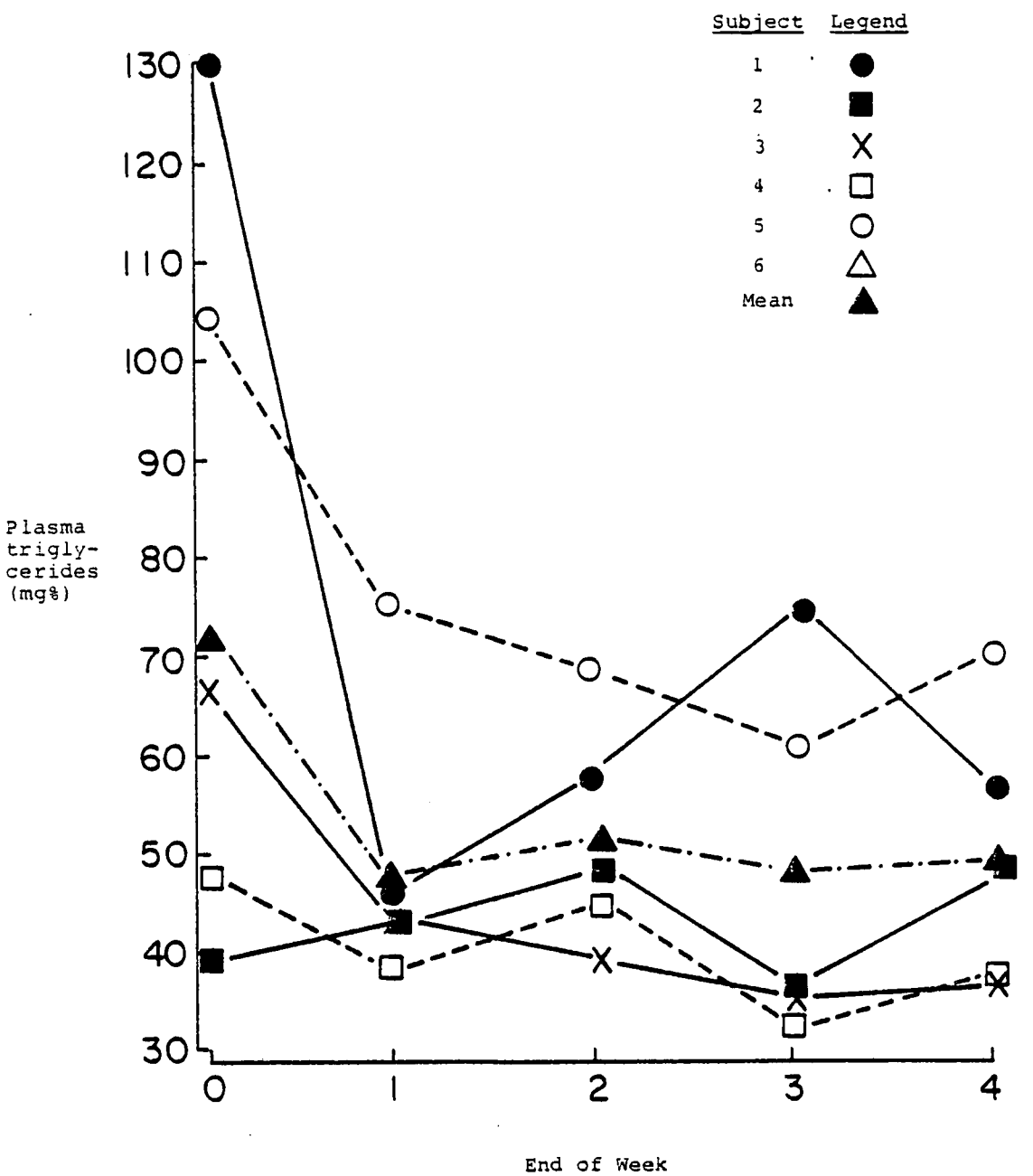


Figure 3. Changes in plasma cholesterol over the four-week linoleic acid dietary period.

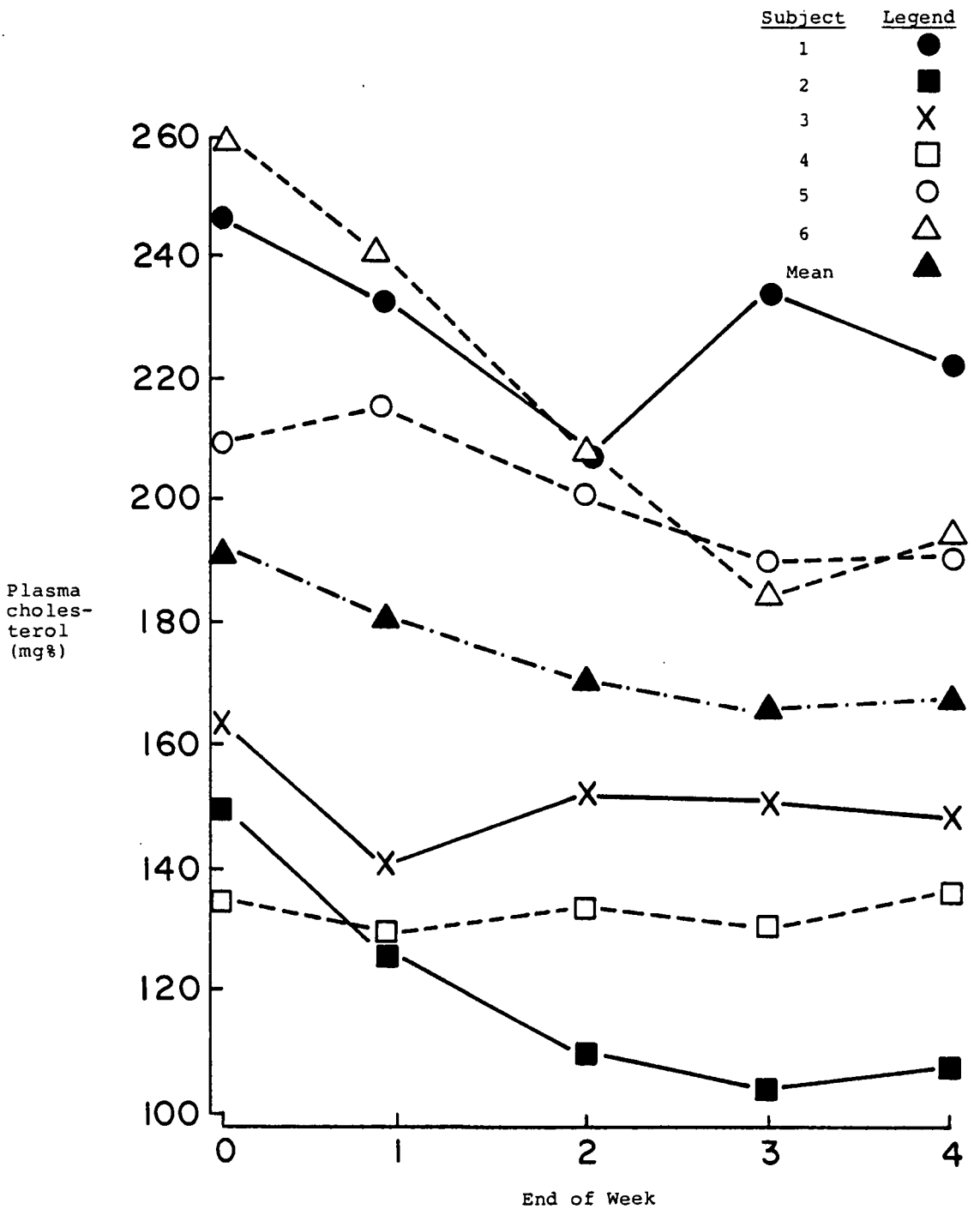


Figure 4. Changes in plasma triglycerides over the four-week linoleic acid dietary period.

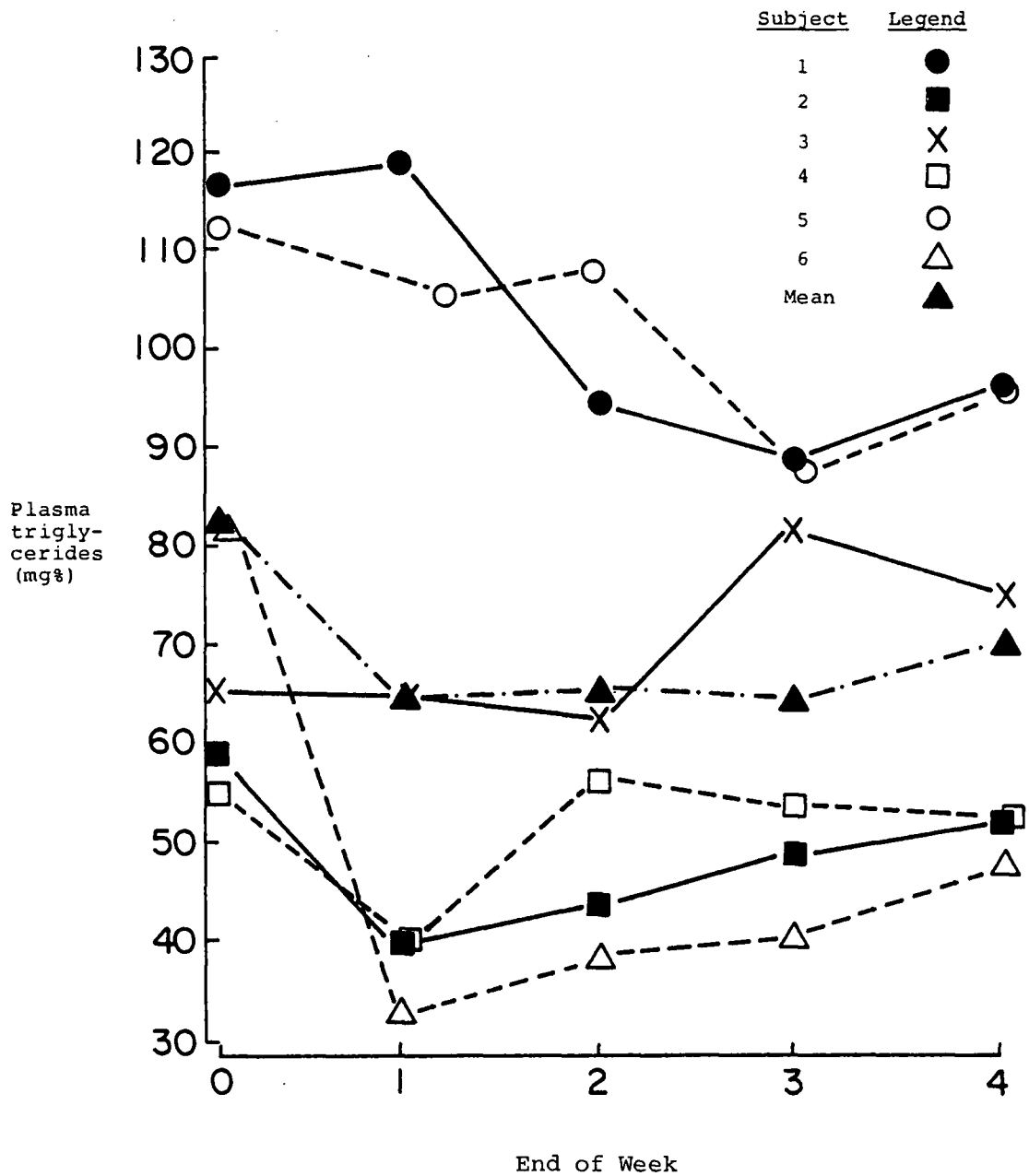


Figure 5. Changes in plasma cholesterol over the four-week saturated dietary period.

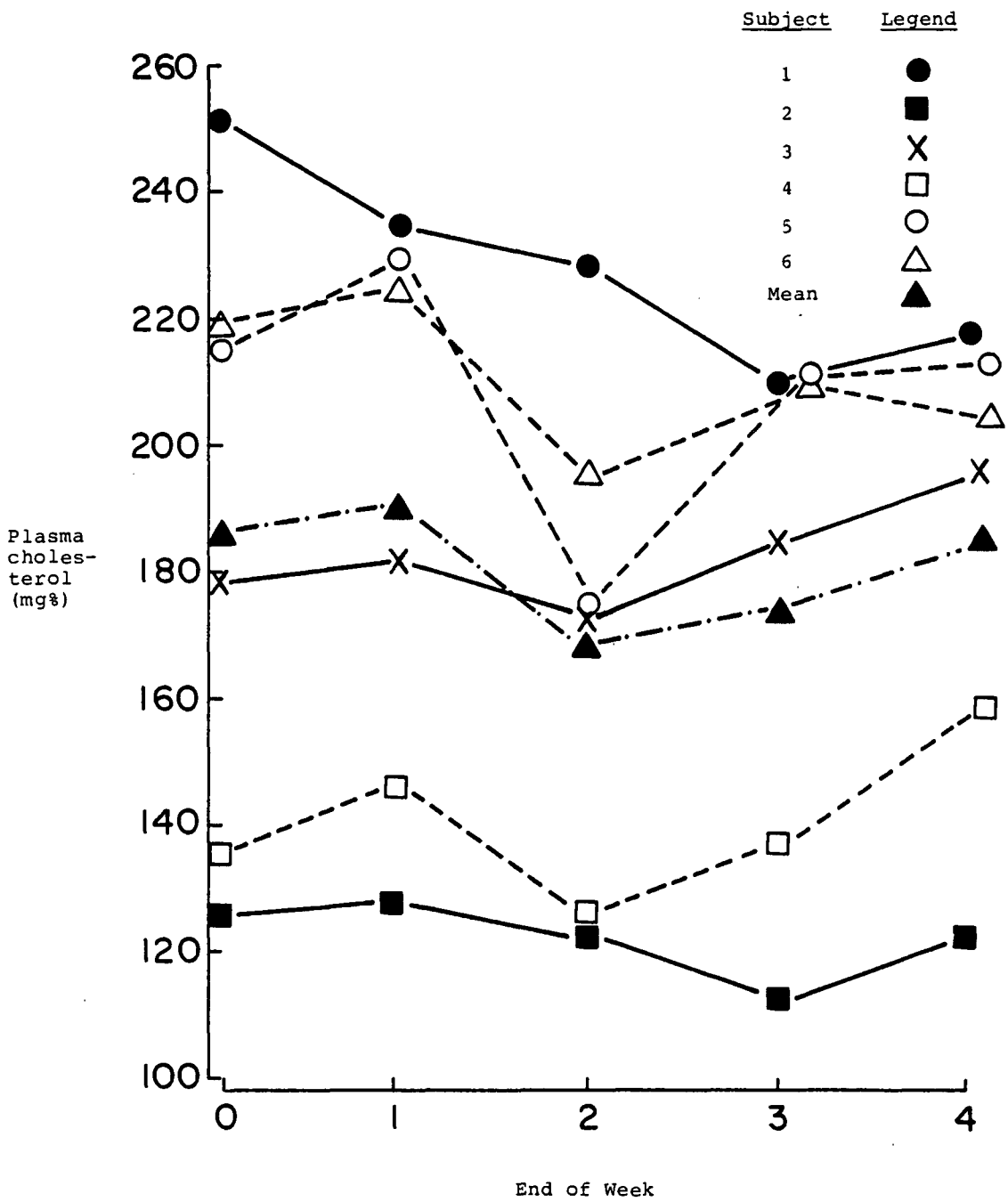
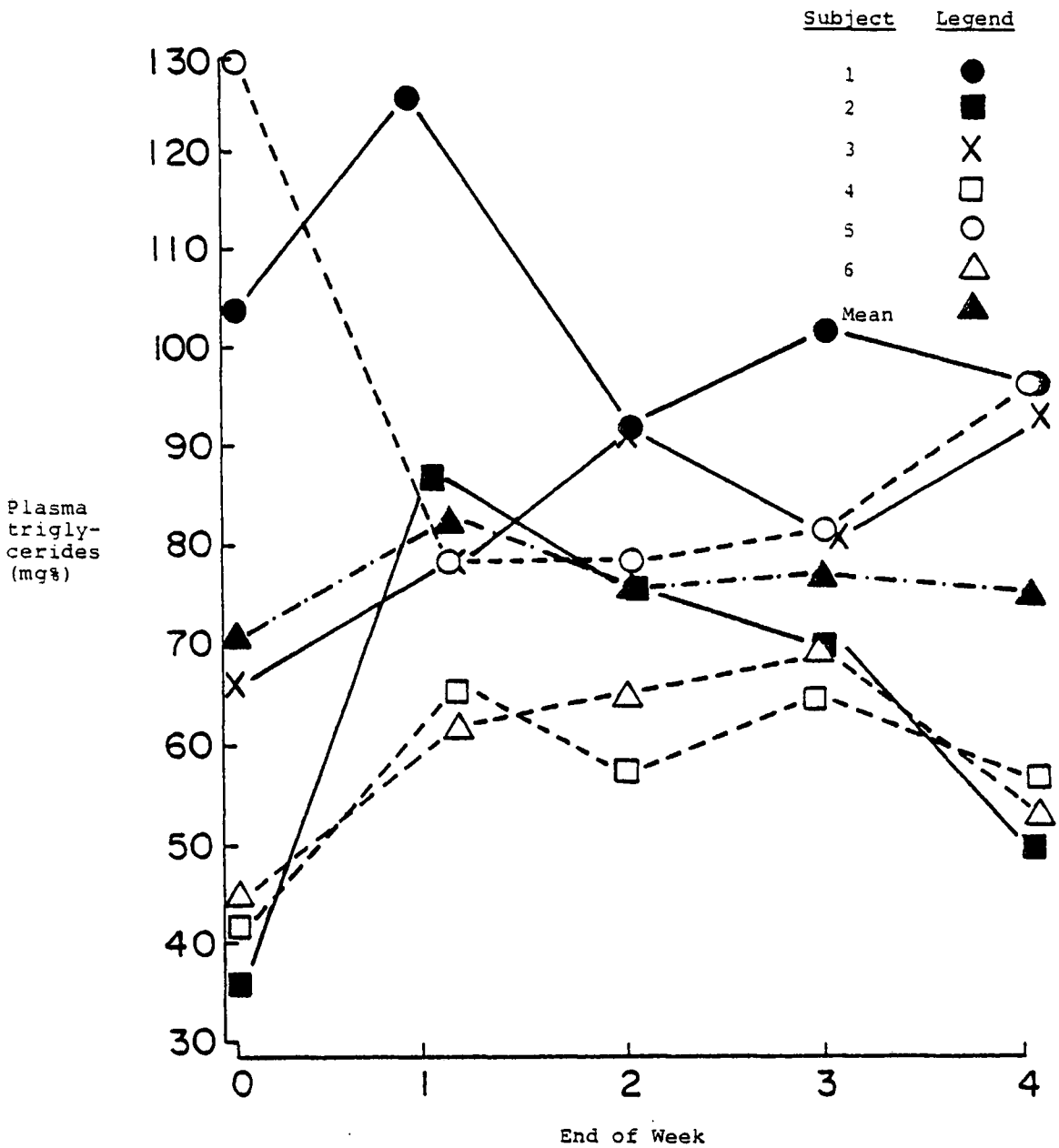


Figure 6. Changes in plasma triglycerides over the four-week saturated dietary period.



saturated dietary period except for Subject #1. Mean plasma triglycerides were significantly lower at the end of the salmon dietary period than at the end of the linoleic acid ($p < 0.05$) and saturated dietary periods ($p < 0.01$) (Table 11). No significant difference in plasma triglycerides was found between the linoleic acid and saturated diets. The lowest plasma triglyceride levels in each of the subjects occurred at the end of the salmon dietary period.

Discussion

Even though plasma cholesterol fell to significantly lower levels at the end of the linoleic acid and salmon dietary periods than during the last week of the saturated dietary period (Table 11), the absorption of cholesterol was not significantly changed by these dietary treatments (Table 8). The cholesterol absorption values for each subject fell within or near the average range for cholesterol absorption (55, 63). These findings are consistent with those reported by Nestel *et al*. (16) who found no difference in cholesterol absorption on either a highly polyunsaturated fat diet or a saturated one. However, Grundy and Ahrens (37) found, during metabolic studies on cholesterol, that one subject out of their eleven showed decreased absorption of cholesterol on a highly polyunsaturated fat diet.

In addition, the fecal excretion of bile acids (Table 9) and neutral steroids (Table 10) was not

Table 11. Plasma cholesterol and triglyceride levels in subjects at the final week of each dietary period (mg%).[1]

Subject	Cholesterol			Triglycerides		
	Salmon Diet	Linoleic Acid Diet	Saturated Diet	Salmon Diet	Linoleic Acid Diet	Saturated Diet
1	179	221	219	57	119	96
2	108	110	122	35	51	49
3	146	151	194	36	76	93
4	147	135	158	37	51	57
5	191	190	211	70	96	96
6	198	194	204	43	47	52
Mean ± S.D.	162 ± 34[2]	167 ± 42[3]	185 ± 37[2,3]	46 ± 14[3,4]	73 ± 29[3]	75 ± 23[4]

[1]Venous blood was drawn twice weekly to determine cholesterol and triglyceride values. Values in this table represent the final drawing of the week.

[2]Statistically significant difference at $p < 0.025$.

[3]Statistically significant difference at $p < 0.05$.

[4]Statistically significant difference at $p < 0.01$.

significantly increased on the salmon or linoleic acid diets either, which has been suggested as a possible mechanism for lowering plasma cholesterol. Consistent with these findings were those from a study by Ali et al. (32) who fed formula diets containing either corn oil or butterfat at 35% and 60% of the total caloric intake to three male subjects. Ali et al. found that, at either level of fat, fecal bile excretion was not increased by the diet containing corn oil.

Apparently, the unique fatty acids in the salmon diet did not cause a significantly greater depression of plasma cholesterol than the ω6 fatty acids of the linoleic acid diet (Table 11). However, plasma cholesterol levels at the end of the salmon dietary period were lower ($p < 0.025$) than those found at the end of the linoleic acid dietary period ($p < 0.05$) when these two are compared to the values found at the end of the saturated dietary period (Table 11). Perhaps the lowering of plasma cholesterol by these two diets was due to a redistribution of plasma lipids, for example, from the plasma to peripheral or hepatic tissue. However, Frantz and Carey (33) showed that a diet supplemented with corn oil lowered not only plasma cholesterol, but liver cholesterol as well. Perhaps polyunsaturated oils enhance the utilization of dietary cholesterol thereby shunting it into metabolic pathways causing a drop in the plasma content. The polyunsaturated nature of the salmon and vegetable fatty acids

alone, rather than the ω3 configuration of salmon oil fatty acids, may be responsible for the lowering of plasma cholesterol. Spritz and Mishkel (56) postulated that dietary polyunsaturated fats may alter the spatial configuration of the lipids into which they are incorporated because they occupy more space than saturated fatty acids. They would, therefore, cause fewer lipids to be accommodated by the low density lipoprotein (LDL) fraction, thus lowering plasma cholesterol levels. However, in a review by Stansby (17), a substantial amount of evidence is given showing that fish oils in general lower plasma cholesterol to a greater degree than vegetable oils. Perhaps fish oils, with their greater proportion of tetraenoic fatty acids than vegetable oils, or maybe some as yet unidentified compounds in fish oil are responsible for the lower plasma cholesterol levels.

Plasma triglycerides were significantly lower at the end of the salmon dietary period ($p < 0.01$) than at the end of the linoleic acid and the saturated fat dietary periods ($p < 0.05$) (Table 11). These findings are consistent with those of Ruiter et al. (26) and Ahrens et al. (27) who found plasma triglyceride levels to fall as much as 50% on a fish oil diet. Triglycerides in salmon oil and flesh are mainly esterified with ω3 fatty acids. The human body can incorporate fatty acids absorbed from the diet into its own depot fats (64) and also into circulating lipids. There is a selective incorporation of 20:5ω3

fatty acid (Table 2) into plasma cholesterol esters (21, 25). Possibly this selectivity is favorable enough that plasma triglycerides disassociate in favor of esterification with the cholesterol molecule. Possibly the lower plasma triglyceride levels on the salmon diet are due to an increased clearance or decreased production of triglycerides. LPL is specific for cleaving triglycerides from intact chylomicrons and very low density lipoprotein (VLDL). Less LPL activity was found in the adipose tissue of rats fed saturated fats than in those fed polyunsaturated fats (65). Therefore, on a polyunsaturated fat diet more LPL could be present possibly causing increased clearance of triglycerides. Also, triglycerides made with polyunsaturated fatty acids may be a better substrate for LPL.

Even though the mean level of plasma cholesterol at the end of each dietary period was significantly lower on the last week of the salmon dietary period than on the last week of the saturated dietary period (Table 11), individual differences at the end of these dietary periods were not all that great. Individual variability must be considered when analyzing the data. Subjects 1, 2, and 3, who showed greater differences in plasma cholesterol levels at the end of these dietary periods (Table 11) have shifted the mean difference to a point of significance. Individual variability can possibly be a genetic difference in the metabolic handling of dietary lipids or perhaps due to differences in physical activity of the subjects

which has been suggested to be a factor affecting plasma cholesterol levels (66, 67, 68). Plasma triglycerides were consistently and more obviously lower for each individual at the end of the salmon dietary period than at the end of either of the other dietary periods (Table 11). Perhaps the lowering of plasma triglycerides by the salmon diet is less subject to individual variability. Individual differences in bile acid (Table 9) and neutral steroid (Table 10) excretion also varied dramatically in some subjects during different dietary periods. Again, individual variability in the metabolism of dietary fats may play a role in determining these differences.

SUMMARY AND CONCLUSIONS

The influence of a diet containing salmon flesh and its oil on plasma lipids, cholesterol absorption, as well as fecal neutral and acidic steroid excretion was examined using steroid balance studies.

The results of this study were consistent with research in the past that showed fish oil to lower plasma cholesterol and triglycerides. The proposed mechanism by which this drop in plasma lipids occurs is either by decreased absorption of cholesterol or increased fecal excretion of bile acids and neutral steroids. The results of this research showed that these mechanisms were not the cause of the decreased plasma cholesterol and triglycerides. More research is needed to uncover the means by which fish oils and polyunsaturated vegetable oils lower plasma lipids.

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