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Research Article

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Effect of Dietary Fat on the Fecal Excretion of Cholesterol and Its Degradation Products in Man

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ABSTRACT Fecal bile acid and neutral sterol excretion rates were determined in five healthy young men when serum cholesterol changes were induced by isocaloric substitution of an unsaturated (safflower oil) for a saturated fat (butter). The isotope balance method was used after the intravenous injection of cholesterol-4-^{14}C. A feces extraction method is presented which permits essentially complete separation of fecal neutral sterols and bile acids.

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INTRODUCTION

Since the original observations by Kinsell and coworkers (1) and by Groen and his associates (2) that the feeding of saturated fats in the diet elevates whereas the feeding of unsaturated fats lowers the serum cholesterol concentration, there have been numerous reports confirming these effects in man. There has not yet been a definitive explanation of the mechanism of the hypocholesteremic effect of polyunsaturated fat in the diet. Theoretically this action could result from any or a combination of the following alterations in cholesterol metabolism: (*a*) a decrease in endogenous cholesterol synthesis; (*b*) a reduction in absorption of cholesterol and (or) bile acids; (*c*) an increase in fecal excretion of cholesterol and (or) bile acids; and (*d*) a redistribution of blood cholesterol between plasma and other body tissues, either directly or by way of an alteration in lipoprotein metabolism.

Dietary unsaturated fats have been reported to result in increased cholesterol synthesis in rats using in vivo (3–6) and in vitro (6–8) incorporation of labeled acetate into cholesterol, and also using an indirect method (9) measuring carcass, blood, and various tissue cholesterol contents. On the other hand, decreased synthesis rates have been reported to result from unsaturated fat ingestion in man (10) and gerbils (11) using in vivo incorporation of labeled mevalonic acid into blood cholesterol, and also in the rabbit (12) using an indirect method measuring the serum cholesterol concentrations and specific activity values. In still other stud-

ies the type of fat ingested made no apparent difference in cholesterol synthesis as determined by the incorporation of labeled precursor (acetate or mevalonate) into cholesterol in man (13), in the rat in both in vivo (14, 15) and in vitro studies (14, 16), and in indirect studies in man using either the blood and liver cholesterol concentrations (17) or the fecal steroid excretion rates (18). Therefore, from the published studies it appears that the effect of dietary fat saturation on cholesterol synthesis is not clearly understood.

There have been several reports of increased absorption of dietary cholesterol in the rat (19–24) and in man (23) when unsaturated fat is present in the intestine. Wood, Lee, and Kinsell (25) reported that polyunsaturated fat in the diet of formula-fed human subjects reduced their cholesterol absorption. In an unpublished study we have found no significant difference in the amount of a single test dose of cholesterol-4-^{14}C absorbed in humans when safflower oil was substituted for butter in the diet. It appears that the effect of dietary unsaturated fat on cholesterol absorption is not well delineated.

Early reports of augmented fecal excretion of bile acids in man (26–31), of nonacidic or neutral sterols in man (31, 32), the rat (9, 33, 34), and the rabbit (35), or of both bile acids and neutral sterols in man (36–38) and the rat (19, 39–41) agreed that an increased fecal excretion of cholesterol accompanied the hypocholesteremic effect of substituting polyunsaturated for saturated fatty acids in the diet. Hellman, Rosenfeld, Insull, and Ahrens (42) suggested that this increase fecal loss accounted for the fate of the cholesterol leaving the plasma under these conditions. Because of the difficulties in obtaining quantitative recoveries of the various intestinal bacterial transformation products of fecal bile acids and sterols (43–47) and in distinguishing exogenous plant sterols from endogenous sterols (48) with the analytical methods used in these studies, some doubt has been cast upon the conclusions (46–49). An isotope balance method, introduced by Hellman and coworkers in 1957 (42), has been used more recently by other investigators (50, 51), who have confirmed the earlier reports of increased fecal bile acid and neutral sterol excretion in humans ingesting polyunsaturated fats.

Several groups of investigators have found no significant differences in the fecal excretion of bile acids or neutral sterols in the rat (52–54) or in the rabbit (55). Others have found no significant differences in the turnover rate of biliary cholic acid (56, 57), in the excretion of neutral steroids (58), or in the fecal excretion of bile acids and neutral sterols (59, 60) in men given saturated or unsaturated fats in the diet. Spritz, Ahrens, and Grundy (60) suggested that, by exclusion, the hypocholesteremic effect of polyunsaturated fat is due to a redistribution of cholesterol from plasma into other body tissues. However, attempts to demonstrate a significant increase in the cholesterol content of various organs and tissues during the ingestion of unsaturated fats in monkeys (61), rats (62, 63), and rabbits (55, 64) have failed to confirm this hypothesis. In man, Frantz and Carey (17) found a decrease in hepatic cholesterol concentration during isocaloric substitution of polyunsaturated for saturated dietary fat. Thus, the mechanism of the hypocholesteremic action of dietary polyunsaturated fats remains undetermined.

The present paper presents the effects of isocaloric substitution of a polyunsaturated fat (safflower oil) for a saturated fat (butter) on the fecal excretion of cholesterol-4-^{14}C and its degradation products using the isotope balance method in five healthy young men. A reciprocal relationship is demonstrated between the changes in serum cholesterol concentration and the rate of fecal bile acid and neutral sterol excretion, and supports the hypothesis that the hypocholesteremic effect of dietary polyunsaturated fatty acids is associated with an increase in cholesterol excretion (as bile acids and sterols) in man.

METHODS

Experimental design. Five healthy young male college students were fed controlled diets during three consecutive experimental periods of approximately 16 days each. The subjects were paid volunteers between the ages of 20 and 29 yr, had negative histories of any significant metabolic or hepatic disease, had normal weights for their height and age, and were normocholesterolemic. The basic diet (before adjustment for the body weight of each subject) provided 3200 cal/day with approximately 40% being supplied as fat. The fat content consisted of a basal portion (three glasses of milk and two servings of meat daily), and an experimental portion (either butter or safflower oil) added to such items in the diet as pancakes, cereals, potatoes, and breads. The foods used in the diets were evaluated by the use of standard food tables. Since

the five subjects weighed 72.3, 66.8, 102.3, 68.2, and 65.7 kg, respectively, the total dietary intake was adjusted for each individual to maintain a constant (± 1.0 kg) body weight during the course of the experiment. The subjects were divided into two groups (A and B), each group receving the butter containing diet (B) during one period and the safflower oil containing diet (S) during another period; the sequence was reversed for the two groups during the first two experimental periods. During the third period they continued eating the same type of diet they were fed in the second period but were given a daily period of exercise on a treadmill (2 hr on a 10% grade at 3.5 mph). To provide the additional energy required for this work the diets were increased to provide approximately 4200 cal/day, keeping the relative proportions of the various dietary constituents the same as before. The subjects were fed all meals at a special metabolic kitchen in the Laboratory of Physiological Hygiene and were instructed to consume no other foods during the course of the experiment. Daily weights and records of the food not consumed were kept for individual subjects to provide a check on their cooperation.

With every meal each subject was given a tablet containing a known amount of chromic oxide to serve as an inert marker in the collected feces (65, 66), which permitted feces collected on a calendar basis to be corrected to a true 24 hr period (and expressed as equivalent days). During the entire experiment we recovered 99.9% of the administered chromic oxide, resulting in 243.7 equivalent days' recovery in 244 calendar days. Once during each experimental period each subject was given a gelatin capsule containing small colored glass beads; from the time of their appearance in the eliminated feces (which ranged from 18 to 48 hr) a measure of the time required for a complete transit of the gastrointestinal tract was made for each individual.

All feces passed during the experiment were collected by the subjects in individual plastic bags, which were appropriately marked and stored in a freezer in the laboratory. 4-day pooled samples for each subject were prepared by homogenization in an electric blender and suitable aliquots were kept frozen (in sealed containers) until the time of processing and radioactivity measurement. Most stools were collected while the subjects were in the vicinity of the metabolic kitchen (e.g., at meal times), but the subjects were also given the necessary equipment for stool collection while away from that area. Each subject kept a record of his bowel movements during the experiment to assist in verifying the completeness of the collection.

On the second day of the experiment each subject was given a single intravenous injection of 24 μc of cholesterol-4-^{14}C as a suspension in saline. We prepared this by dissolving the appropriate amount of labeled crystalline cholesterol of high specific activity (35 mc/mmole) in 2 ml of absolute ethanol, sterilizing by heating to 85°C in a sealed glass vial for 2 hr and suspending the ethanol solution of cholesterol in 30 ml of sterile physiological saline solution by vigorous shaking immediately before injection into the subject. Venous blood samples were obtained before breakfast (fasting) at intervals throughout the experiment.

Methods of analysis. The actual composition of the diets was determined by chemical analysis of homogenized representative diets as described by Anderson, Grande, Matsumoto, and Keys (67). The content of water, ash, nitrogen, total lipid, carbohydrate, and total unsaponifiable matter was obtained. Fatty acid analysis was made by gas-liquid chromatography and the amounts of saturated, monounsaturated, and polyunsaturated glycerides were calculated.

Serum total cholesterol concentration was determined by the method of Abell, Levy, Brodie, and Kendall (68). The ^{14}C radioactivity was measured in petroleum ether extracts of serum obtained by that method using a liquid scintillation spectrometer [1] with 0.3% 2,5-diphenyloxazole in toluene as the counting medium. The degree of pulse quenching was then determined by recounting the samples after the addition of a ^{14}C internal standard; appropriate corrections for quenching and efficiency of counting were made to obtain sample disintegrations per minute (dpm). The specific activity of serum cholesterol was determined by dividing the sample radioactivity (dpm) by the amount of cholesterol in the sample (milligrams) obtained by spectrophotometric measurement at 620 mμ wavelength [2] using the Liebermann-Burchard color reaction.

Aliquots (approximately 2 g) of the 4 day pooled feces samples were extracted by reflux boiling with absolute ethanol (60 ml) for 2 hr, and were separated into ethanol extract and nonextractable residue by filtration. The extract, reduced to 20-ml volume, was subjected to alkaline hydrolysis by reflux boiling for 1 hr after adding 1.2 ml of 33% aqueous potassium hydroxide solution. Separation of the hydrolyzed extract into the two principal groups of compounds providing loss of cholesterol from the body (bile acids and nonsaponifiable sterols) was carried out by multiple (4–6) extraction by petroleum ether, followed by extraction (twice) of the petroleum ether fraction by 0.1 N aqueous sodium hydroxide solution. As a check on the completeness of this separation, feces obtained from a man previously given cholic acid-24-^{14}C was extracted by this method and 99.9% of the radioactivity in the feces was recovered in the alkaline ethanol fraction. In addition, labeled cholesterol was added to a sample of normal feces and extracted, and all the radioactivity was recovered in the petroleum ether fraction. To check on the completeness of the initial ethanol extraction, the nonextractable residue was reextracted several times and the yield was less than 1% additional radioactivity.

The materials available for radioactivity measurements were the following: ethanol extract, nonextractable residue, petroleum ether fraction (nonsaponifiable or neutral

[1] Tri-Carb Liquid Scintillation Counting System, model 314 EX, Packard Instrument Company, Inc., La Grange, Ill.

[2] Coleman Junior Spectrophotometer, model 6-A, Coleman Instruments, Inc., Maywood, Ill.

sterols), and aqueous alkaline ethanol fraction (bile acid containing fraction). We made measurements of the ^{14}C radioactivity in duplicate aliquots of these four fractions by converting the carbon to CO_2 by the wet carbon combustion method of Van Slyke and Folch (69, 70), collecting this in an ionization chamber using a special glassware system,[3] and determining the radioactivity using a vibrating-reed electrometer.[4]

The amount of chromic oxide in the stool samples was determined colorimetrically by a combination of the method of Schürch, Lloyd, and Crampton (71) and that of Allen (72). Dividing the amount of inert indicator in a given sample by the amount ingested per day gave the number of days represented by that sample (as equivalent days).

Method of calculating net sterol and bile acid excretion. The isotope balance method introduced by Hellman and associates in 1957 (42) is based upon two assumptions: first, that the 4-carbon atom of the cholesterol molecule is not metabolized to any significant extent in the body or in the intestinal lumen (73–78) and, therefore, the metabolic products of cholesterol metabolism (bile acids and sterols) will contain the ^{14}C label of the administered cholesterol-4-^{14}C; and second, that the fecal end products of cholesterol metabolism have specific activities that closely parallel that of the serum cholesterol (42, 50, 59, 77–82).

Dividing the radioactivity in a given sample by the average serum cholesterol specific activity during the time period represented by the feces sample provides a measure of the amount of cholesterol excreted in that sample (as bile acids or sterols).

$$\text{Amount excreted (mg)} = \frac{\text{radioactivity in sample (dpm)}}{\text{average serum cholesterol specific activity (dpm/mg)}}.$$

Since the feces may remain in the large bowel for hours to days after passage through the small intestine (where bile acid and sterol excretion and reabsorption occur) it is necessary to make a correction for this delay; this was done by using the gastrointestinal transit time as determined by the glass beads. The serum cholesterol specific activity values were plotted on a semi-logarithmic graph; the mid-point of the time for a given sample was found, the colonic lag time (in days) was subtracted from this, and the specific activity value corresponding to that day was used as the average for that particular sample in the equation above. The amount excreted in a given sample, divided by the number of equivalent days determined for that sample by the chromic oxide measurements, yielded the average daily fecal excretion rate of bile acids or sterols in that sample.

[3] Model GW-1, Carbon-14 Glassware System, Nuclear-Chicago Corporation, Chicago, Ill.

[4] Model 6000 Dynacon, Dynamic Condenser Electrometer, Nuclear-Chicago Corporation.

RESULTS

Reliability of feces extraction method. The completeness of ^{14}C extraction from feces was shown by serial reextraction of the nonextractable residue fraction; of the total radioactivity present, 95% was extracted initially, but only 1% and less than 0.3% were obtained by the second and third reextractions. The method yielded an average 96% of the total radioactivity in the ethanol extract, the values for the residue ranging from 0 to 9% with an average of 4%. That the method effected virtually complete separation of bile acids and nonsaponifiable sterols was suggested by finding over 99% of the radioactivity from added cholesterol-4-^{14}C in the sterol fraction and over 99% of the radioactivity in feces of a patient given cholic acid-24-^{14}C in the bile acid fraction. Satisfactory reproducibility of extraction was demonstrated by quadruplicate processing of a representative sample and obtaining total radioactivity values (dpm/g of stool sample) of 29,350, 30,400, 28,668, and 29,933, respectively. Duplicate radioactivity measurements of the various fractions fell within a range of $\pm 3\%$.

Analysis of diets used. The composition of the two basic diets used in this experiment is shown in Table I. It can be seen that the major difference in the two diets is the type of fatty acids present, with the butter-containing diet (B) having a greater amount of saturated and a lesser amount

TABLE I

Energy and Nutrients in the Diets Served Daily

Diet*	Total calories	Protein		Carbohydrate		Fat		Glycerides‡				(2S-P)	Cholesterol	Plant sterols
								S	O	P	Other			
		g	*%cal*	*g*	*%cal*	*g*	*%cal*	*%cal*					*mg*	*mg*
B	3280	92.5	11.3	401.3	48.9	145	39.8	24.0	13.3	2.1	0.4	46	347	420
S	3280	92.5	11.3	401.3	48.9	145	39.8	10.0	8.7	19.9	1.2	0	197	720

* The diets contained either butter fat (B) or safflower oil (S).

‡ Glycerides expressed as % of total calories for saturated (S), oleic (O), polyunsaturated (P), and other fatty acids.

of polyunsaturated fatty acids than the safflower oil-containing diet (S). The quantity (2S-P), with S and P expressed as the per cent of total calories as saturated and polyunsaturated fatty acid glycerides, respectively, was 46 for the B diet and 0 for the S diet. There was more cholesterol in the butter diet than in the safflower oil diet, 347 and 197 mg/day, respectively. The differences in the two diets resulted from the type of experimental fat used (either 103 g of butter or 100 g of safflower oil). The plant sterol content of the two diets was 420 mg/day for the B diet and 720 mg/day for the S diet, giving a difference of 300 mg between these two diets. During the third experimental period (exercise) the proportions of the various dietary components remained the same as in the preceding period although the total amounts were increased to provide the additional energy required.

Serum cholesterol concentration. The values of the serum total cholesterol concentration for each subject are illustrated graphically in Fig. 1. In all three subjects of group A there was a significant reduction in cholesterol level when the butter-containing diet (B) in period 1 was replaced by the safflower oil-containing diet (S) in period 2. This reduction was prompt and the maximum effect was observed within 10 days of the diet change. There was an average 28.7% reduction from the average 174 mg/100 ml during period 1 to the average 124 mg/100 ml during period 2. During the third period (exercise) there were slight (average 10%) but probably insignificant increases (from 124 to 137 mg/100 ml). In the two subjects of group B there was a prompt and significant reduction in serum cholesterol concentration when they changed from their preexperimental (ad lib.) diets to the S diet of period 1; there was an average 27.6% reduction from 196 before to 142 mg/100 ml during the latter part of period 1. When the diet was changed to the butter-containing one in period 2 there was a nearly identical increase from the average 142 to 203 mg/100 ml in the latter part of period 2; again this change was prompt and reached its maximum within 10 days after the diet change. During the third period (exercise) there were no significant changes in serum cholesterol levels in these two subjects.

Thus it can be seen that the magnitude of changes in serum cholesterol concentration resulting from the substitution of one diet for the other was the same regardless of the sequence of the diets. It is also evident that the preexperimental diets of the subjects had effects similar to the butter-containing diet (B) since there were no

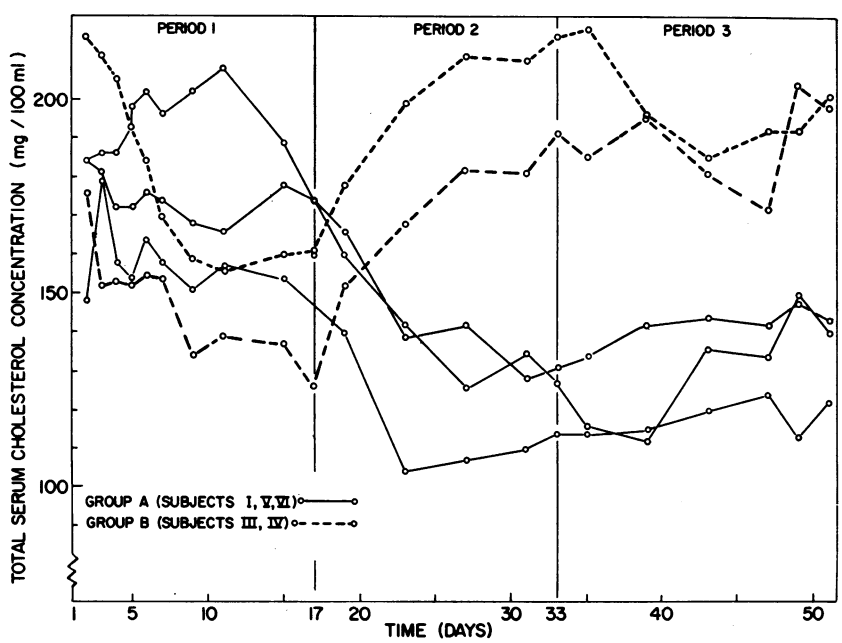

FIGURE 1 Total serum cholesterol concentration (mg/100 ml).

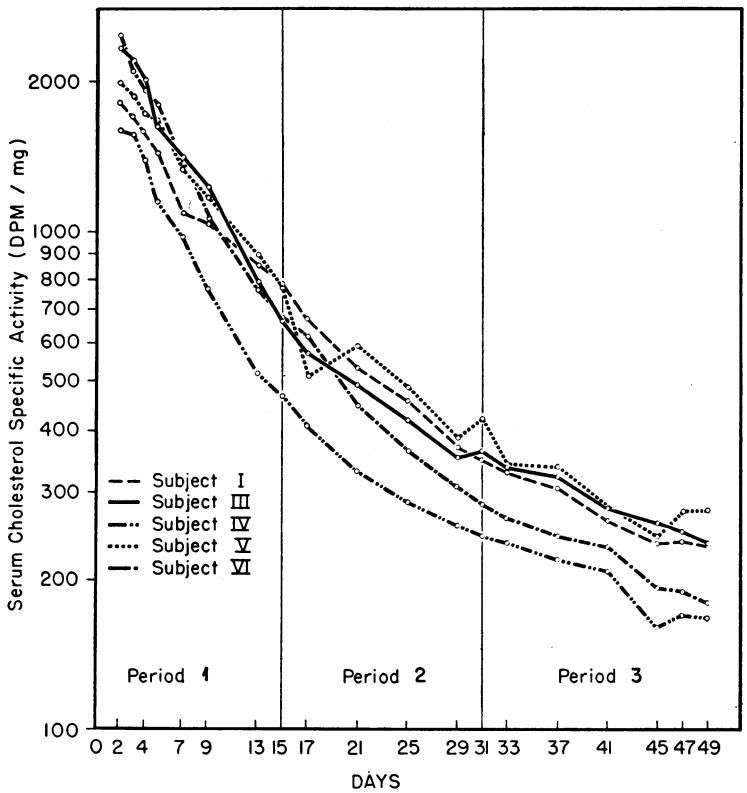

FIGURE 2 Semilog. Plot of serum cholesterol specific activity values.

significant changes during period 1 in group A and since there were equal effects obesrved in group B on changing from ad lib. diet to safflower oil diet (period 1) and on changing from S to B diet (period 2).

There was an over-all average 28% difference in the serum cholesterol level with these two diets, or an average difference of 53.2 mg/100 ml. Using the equation of Keys, Anderson, and Grande (83) to predict the effect of dietary fat on serum cholesterol concentration (Δcholesterol = 1.35 [2ΔS − ΔP]), we obtain a value of 62 mg/100 ml. Since this equation applies to middle-aged males, and since the effects of diet on cholesterol concentration are less marked in younger individuals with lower blood cholesterol levels, the predicted change in serum cholesterol concentration agrees satisfactorily with the observed change (53 mg/100 ml) in these five subjects. The difference in the cholesterol content of the two diets (347 and 197 mg/day, respectively) would be expected to make little significant difference in the blood cholesterol levels (5 mg/100 ml at the most) (84).

Serum cholesterol specific activity. The values for the serum total cholesterol specific activity (dpm/mg) for each of the five subjects are illustrated on a semi-logarithmic graph in Fig. 2. There does not appear to be any significant difference in the curves for the subjects of group A (I, V, and VI) as compared with those of group B (III and IV) and there is no observable break in the curves when dietary fat is changed (at the transition from period 1 to 2). Since complete equilibration between serum and tissue cholesterol requires at least 30 days (85) and since the diet change was made before this time (on day 17) it is possible that the processes of equilibration overshadowed the change in turnover rate and therefore the slope of the curve was not noticeably altered by the different diet ingested in period 2.

Fecal bile acid and neutral sterol excretion. The values for the average daily fecal steroid excretion (total, neutral sterols, and bile acids) in each 4-day pooled feces sample are listed for the individual subjects in Table II. It can be seen that there was a considerable degree of variability

TABLE II

*Fecal Steroid Excretion Rate (mg/day) in 4-Day Pooled Feces Samples in Individual Subjects**

Subject	Period 1			Period 2			Period 3		
	T	NS	BA	T	NS	BA	T	NS	BA
Group A		B-diet			S-diet			S-diet + exercise	
I	1124	678	444	1204	629	555	1782	878	521
	897	585	352	877	535	309	1566	971	588
	919	543	379	1051	711	489	1296	848	499
				1156	712	523	1192	722	502
	(980)	(602)	(392)	(1072)	(647)	(469)	(1459)	(855)	(528)
V	889	342	551	1390	482	831	1290	487	832
	565	183	382	782	267	415	951	351	628
	1033	372	667	1221	442	700	1108	420	691
				1019	360	660	1124	427	706
	(829)	(299)	(533)	(1103)	(388)	(652)	(1118)	(421)	(714)
VI	1138	573	488	1222	601	635	1225	651	596
	1049	489	531	1369	608	809	1415	776	607
	853	375	462	1429	765	669	1114	639	471
				1156	593	580	1244	745	507
	(1013)	(479)	(494)	(1294)	(642)	(673)	(1250)	(703)	(545)
Group B		S-diet			B-diet			B-diet + exercise	
III	903	552	351	856	417	446	945	510	432
	983	509	468	721	326	404	988	502	463
	1339	658	700	912	455	485	852	407	421
				925	491	485	1061	454	577
	(1075)	(573)	(506)	(854)	(422)	(455)	(962)	(468)	(473)
IV	1144	738	370	1320	707	566	951	465	461
	1361	770	586	1086	533	514	1066	560	421
	1041	507	494	1089	614	432	804	498	294
				1042	576	448	995	554	398
	(1182)	(672)	(483)	(1134)	(608)	(490)	(954)	(519)	(394)

T, total (alcohol extract); NS, neutral sterol fraction; BA, bile acid fraction.

* Average values for each subject given in parentheses.

within a given subject during a constant diet as well as between different subjects ingesting the same diet. In addition, there appears to be a distinct difference between the values obtained during the butter-containing diet and those obtained during the safflower oil-containing diet in the individual subjects. The average daily fecal excretion of bile acids and unsaponifiable sterols (milligram/day) during the three consecutive experimental periods is illustrated for each of the five subjects in Fig. 3. The lower (black) part of each bar represents the sterol fraction, the upper (open) part represents the bile acid fraction, and the total is given by the vertical height of the bar. The sequence of the diets is shown above the two groups (A and B). It can be seen that each subject had a greater total fecal steroid excretion rate during the safflower oil-containing diet (S) as compared to the butter-containing diet (B), regardless of the sequence of these two diets. This increase was fairly equally distributed between the neutral sterol and the bile acid fractions. The per cent of the total found in the bile acid fraction is listed in Table III, and ranged from 40 to 60% in the several subjects (over-all average was 49%); however, there was no significant variation in the proportion of bile acids during the three experimental periods in any given subject. Since there was a considerable range in the body weights of the five subjects (from 66 to 102 kg) the fecal excretion rates were calculated for a standard weight of 70 kg for each subject and are shown in Fig. 4. This demonstrates even more clearly the apparent increase in neutral sterol and bile

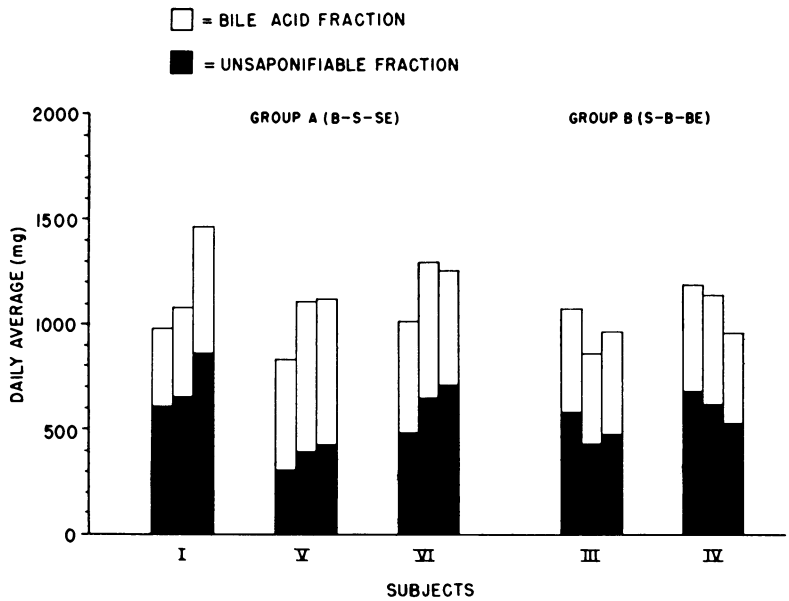

FIGURE 3 Average daily fecal steroid excretion.

acid excretion during the safflower oil dietary period. When statistical evaluation was made of the total fecal steroid excretion rates obtained from the 4-day pooled feces samples in a given subject, a significant difference ($P < 0.01$) was obtained in one of the five subjects (VI) when comparing the two dietary periods (butter vs. safflower oil). However, because of the small sample size ($n = 3$ in one period and 4 in the other) and the considerable degree of variability within a given subject under constant dietary conditions, this method of statistical analysis of the data was not felt to be adequate for determining the significance of the observed differences. Therefore, other statistical tests were employed using all five subjects as a group, as described below.

TABLE III
Per Cent of Total Daily Fecal Steroid Excretion in the Bile Acid Fraction

Subject	Period 1	Period 2	Period 3	Over-all average
Group A				
I	39	42	38	40
V	64	63	63	63
VI	51	51	44	49
Group B				
III	47	50	50	49
IV	42	45	43	43

The feces were collected from the beginning of the experiment, but since it requires at least 3 days for the specific activity of serum ester cholesterol to reach that of serum free cholesterol (after which they have nearly similar values and decline at nearly identical rates) (86–90), only those stools collected after the 5th day were used in measuring fecal excretion rates. As a result there were three 4-day pooled samples in period 1, four in period 2, and four in period 3 for each individual subject. Since three subjects were on the butter diet (B) and two were on the safflower oil diet (S) during period 1, the number of 4-day pooled samples available for statistical analysis was 17 for the B diet and 18 for the S diet. For the third period (exercise) there were 12 for the S diet and 8 for the B diet.

The over-all average fecal steroid excretion rates during the B and S diets are listed in Table IV. There was an 18.7% (181 mg/day) greater total excretion rate during the safflower oil diet than during the butter diet, 1147 ± 44.8 and 966 ± 42 mg/day, respectively. This difference was statistically significant ($P < 0.01$). For the neutral sterol and bile acid fractions there were significant increases (94 and 91 mg/day, respectively) during the safflower oil diet ($P < 0.05$ for both fractions). The averages of the fecal steroid excretion rates, corrected to a standard 70 kg weight for each sub-

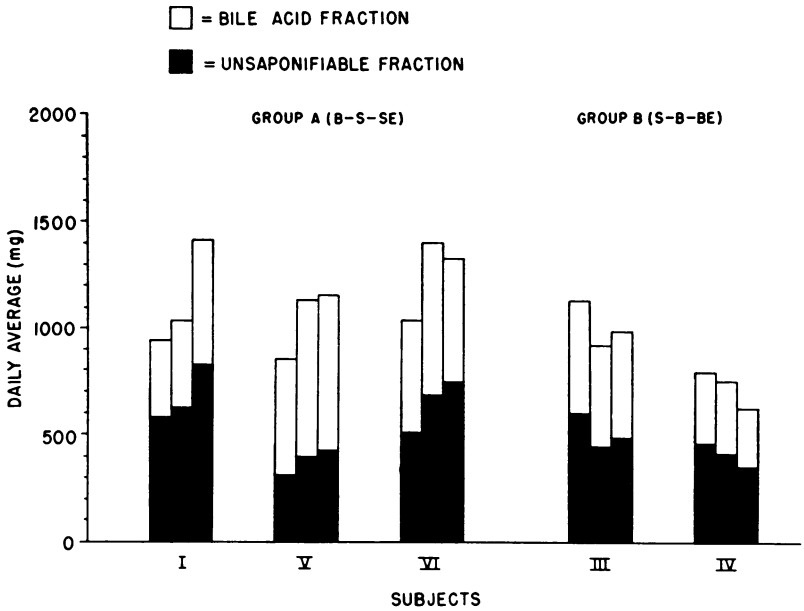

FIGURE 4 Average daily fecal steroid excretion, standardized to 70 kg body weight.

ject, are listed in the lower half of Table III. Again there was a greater excretion rate during the safflower oil diet, 23% higher, and the differences are statistically significant for both the neutral sterol and the bile acid fractions ($P < 0.02$ and < 0.04, respectively) as well as for the total ($P < 0.01$). These statistical evaluations were made using an uncorrelated means test,[5] which compared the average of all of the 4 day pooled samples during one diet with all of those during the other diet, and included both the intra-individual as well as the inter-individual variability. By using another statistical test, a correlated means test,[6] which minimizes the effect of intra-individual variability and uses mainly the inter-individual variability, we found significant differences between the fecal steroid excretion rates during the S and B diets ($P = 0.018$, 0.012, and 0.04 for the total, neutral sterol, and bile acid fractions, respectively). Thus, it is evident that in these five subjects considered as a group there was a significantly greater

[5] $$t = \frac{\bar{X}_a - \bar{X}_b}{\sqrt{\frac{(SD_a)^2}{N_a} + \frac{(SD_b)^2}{N_b}}}$$

[6] $$t = \frac{\bar{\Delta}}{SE_\Delta} \quad \text{where} \quad SE_\Delta = \sqrt{\frac{N\Sigma\,\Delta^2 - (\Sigma\,\Delta)^2}{N^2(N-1)}}$$

TABLE IV

*Average Daily Fecal Steroid Excretion Rate (mg/day) during Butter and Safflower Oil Diets**

Fecal steroid fraction	Butter diet (B)		Safflower oil diet (S)		Difference (S-B)		Significance of difference
	Mean	± SE	*Mean*	± SE	*Mean*	%	*P*
Total	966	42	1147	45	+181	19	<0.01
Neutral sterol	486	33	580	33	+ 94	19	<0.05
Bile acid	473	19	564	36	+ 91	19	<0.05
			Standardized to 70 kg body weight				
Total	901	39	1111	59	+210	23	<0.01
Neutral sterol	449	28	556	32	+107	24	<0.02
Bile acid	447	25	552	44	+105	24	<0.04

* The average values obtained by considering the data from all five subjects as a single group.

TABLE V

*Average Daily Fecal Steroid Excretion Rate (mg/day) during Periods 2 and 3**

Fecal steroid fraction	Period 2		Period 3, exercise		Difference (3 − 2)		Significance
	Mean	± SE	*Mean*	± SE	*Mean*	%	*P*
Group A, safflower oil diet							
Total	1156	57	1276	64	+120	10	>0.15
Neutral sterol	559	43	660	58	+101	18	>0.15
Bile acid	598	44	596	31	− 2	0	>0.90
Group B, butter diet							
Total	994	64	958	36	− 36	4	>0.60
Neutral sterol	515	42	494	18	− 21	4	>0.60
Bile acid	473	26	433	28	− 40	8	>0.30

* The average values obtained by considering the data from the subjects in each group separately.

fecal excretion of both neutral sterols and bile acids during the ingestion of the safflower oil-containing diet.

The average fecal steroid excretion rates during the second and third (exercise) periods are listed in Table V for group A (S diet) and group B (B diet) separately. There was a tendency for a greater excretion rate during period 3 in the subjects ingesting the safflower oil-containing diet, but the average differences were not statistically significant. However, in one subject (I) there was a definite increase during the exercise period (from 1072 to 1459 mg/day). In the two subjects on the B diet there was a tendency for a decreased excretion rate during the third period, but these were not statistically significant. Using the correlated means test we found that the over-all differences between individual subjects during these two experimental periods were not significant ($P > 0.60$, > 0.30, and > 0.80 for the total, neutral sterol, and bile acid fractions, respectively). Thus it appears that, with the exception of one subject, the addition of exercise and an increase in the total caloric intake and amount of fat ingested made no significant difference in the fecal excretion of cholesterol and its degradation products.

Relationship between the changes in blood cholesterol content and fecal steroid excretion. Since red blood cell cholesterol concentration does not vary significantly over wide ranges in plasma cholesterol concentration (91) we calculated the plasma cholesterol content in each subject during the three experimental periods by multiplying the plasma volume (PV in ml $= 927 + 31.5 \times$ kg body weight) (92) by the serum total cholesterol concentration (milligram/milliliter). The plasma cholesterol content calculated in the above manner is listed for each subject in Table VI A during each of the three experimental periods. The total fecal steroid excretion for each subject during each of the three experimental periods is listed in Table VI B. The changes in plasma cholesterol content and the net changes in total fecal steroid excretion for each subject are listed in Table VI C. In all three subjects of group A there were decreases in

TABLE 6 A

Plasma Cholesterol Content during Three Experimental Periods

Subject	Period 1	Period 2	Maximum change*	Period 3
	g	g	g	g
I	6.17	4.13	4.05, 10 days	4.21
V	4.87	3.35	3.20, 7 days	3.66
VI	5.23	4.05	3.94, 14 days	4.26
III	4.06	5.60	5.52, 10 days	5.74
IV	6.60	8.80	8.67, 10 days	8.19

* To high or low point in period 2.

TABLE 6 B

Total Fecal Steroid Excretion during Three Experimental Periods

Subject	Period 1	Period 2	Period 3
	g	g	g
I	15.68	17.15	23.34
V	13.26	17.65	17.89
VI	16.21	20.70	20.00
III	17.20	13.66	15.39
IV	18.91	18.14	15.26

TABLE 6 C

Changes in Plasma Cholesterol Content and Total Fecal Steroid Excretion

Subject	Change in plasma cholesterol content				Change in total fecal steroid excretion			
	Period 1–2	To maximum change in period 2*	Period 2–3	Period 1–3	Period 1–2	To maximum change in period 2‡	Period 2–3	Period 1–3
	g				g			
Group A								
I	−2.04	−2.12 (10 days)	+0.07	−1.96	+1.47	+0.92 (10 days)	+6.19	+9.13
V	−1.52	−1.67 (7 days)	+0.31	−1.21	+4.39	+1.92 (7 days)	+0.24	+9.02
VI	−1.18	−1.29 (14 days)	+0.22	−0.97	+4.49	+3.93 (14 days)	−0.70	+8.28
Mean	−1.58	−1.69	+0.20	−1.38	+3.45	+2.26	+1.91	+8.81
Group B								
III	+1.54	+1.45 (10 days)	+0.14	+1.68	−3.54	−2.21 (10 days)	+1.73	−5.35
IV	+2.20	+2.08 (10 days)	−0.61	+1.59	−0.77	−0.48 (10 days)	−2.88	−4.42
Mean	+1.87	+1.76	−0.24	+1.64	−2.15	−1.35	−0.58	−4.88

* To low point in group A and to high point in group B during period 2.

‡ Calculated by multiplying the difference in average excretion rate in period 1 and 2 times the number of days to the maximum serum cholesterol change in period 2.

the plasma cholesterol pool and reciprocal increases in fecal steroid excretion during the safflower oil diet (period 2), averaging − 1.58 and + 3.45 g, respectively. The maximum effect on the plasma cholesterol occurred between 7 and 14 days after the diet change; the increased fecal steroid excretion during this period of time was adequate to account for the plasma cholesterol loss in two of the three subjects. The two subjects in group B had increased plasma cholesterol pools (average = + 1.87 g) during period 2 (butter diet) which were more than accounted for by the decreased fecal steroid excretion (average = − 2.15 g). During period 3 (exercise) there were minimal changes in plasma cholesterol content but the trend in fecal excretion during period 2 continued. Throughout the entire experiment the safflower oil diet in group A resulted in a net loss (average 1.38 g) in the plasma cholesterol content which was more than accounted for by a net increase (average 8.81 g) in fecal steroid loss. The butter diet in Group B resulted in a net gain (average 1.64 g) in plasma cholesterol content which was more than balanced by a net decrease (average 4.88 g) in fecal steroid excretion. Thus, the changes in plasma cholesterol level resulting from the sequential feeding of these two diets (S and B) are more than adequately accounted for by the reciprocal changes in fecal neutral sterol and bile acid excretion.

DISCUSSION

From the numerous reports in the world literature it is well established that the substitution of polyunsaturated for saturated fat in the diet results in a reduction in the blood cholesterol concentration in man. The fatty acid composition of the dietary fat appears to be the predominant factor in this effect (93), although the content of plant sterols (94) and cholesterol (95) may play additional roles. Because of the conflicting reports in the literature it is difficult to make definitive conclusions as to the effect of dietary fat on endogenous cholesterol synthesis or on exogenous cholesterol absorption.

The earlier reports of augmented fecal excretion of bile acids, neutral sterols, or both during the ingestion of unsaturated vs. saturated fats seemed to provide an explanation for this hypocholesteremic effect. More recently, however, doubt has arisen over these conclusions because of the recognition of the methodological difficulties in quantitative recovery and isolation of the various fecal end products of cholesterol metabolism after their alteration by intestinal bacterial action. The isotope balance method, which does not necessitate the isolation and purification of these compounds, should provide a solution to this problem. However, there have been reports from several groups of investigators using this technique which come

to opposite conclusions as to the effect of dietary fat upon fecal sterol and bile acid excretion (42, 50, 51, 59, 60).

One of the principles underlying the isotope balance method employed in this study is that the 4-carbon of labeled cholesterol is not significantly altered by body metabolism or passage through the intestinal tract. There have been several reported studies in man and experimental animals confirming this concept (73–78). Recently Ahrens and associates (96, 97) have cast doubt upon the validity of fecal neutral steroid measurements by this method as a result of finding significant losses of both plant sterols and cholesterol in some, but not all, of their patients being fed liquid formula diets. They attributed these losses to degradation of sterols to nonsteroidal compounds by bacterial action in the small intestine, although they did not isolate or identify these postulated compounds. They found no evidence of degradation of the steroid nucleus of bile acids and there was no suggestion that the type of dietary fat affected the sterol losses. Whether their results represent a general phenomenon or are limited to some liquid formula fed hypercholesteremic patients will have to await further studies using regular diets and metabolically normal subjects. In our present study we cannot entirely rule out some undetected losses of neutral sterols; if there had been such losses we have no reason to believe that they would be different in a given individual when one diet was substituted for the other, although this possibility cannot be ruled out.

Several investigators have demonstrated that plasma ester and free cholesterol specific radioactivities are very nearly the same after 3–5 days and decline at virtually identical rates (42, 86–89). In another study (unpublished) we have found that the serum free and total cholesterol specific activities are practically identical after 5 days. Therefore, because of the convenience of the extraction method of Abell and coworkers (68) we have used the serum total cholesterol specific activity in this study.

It has been repeatedly demonstrated that the specific activities (SA) of the fecal end products of cholesterol metabolism approximate the serum cholesterol specific activity, demonstrating their origin from plasma cholesterol. Hellman and coworkers (42) showed that plasma and fecal cholesterol specific activities approximate each other, which suggested that fecal cholesterol is largely derived from a pool in isotopic equilibrium with plasma cholesterol. Frantz and associates (79) found that fecal digitonin-precipitable sterols had specific activities equal to blood cholesterol SA two days earlier. Rosenfeld and Hellman (81) reported that fecal deoxycholic acid and plasma ester cholesterol specific activities were approximately equal after the 9th day of their experiment. Avigan and Steinberg (59) found that the fecal sterol and deoxycholic acid specific activities closely paralleled the serum cholesterol SA. Lindstedt (82) showed that biliary cholesterol SA closely followed the serum free cholesterol SA. Wood, Shioda, and Kinsell (50) reported that the specific activities of biliary free cholesterol, cholic acid, and chenodeoxycholic acid were similar to that of the plasma free cholesterol even when there was a sharp change in the plasma cholesterol specific activity curve resulting from substitution of polyunsaturated for saturated fat in the diet.

In the present study we have shown that the feces extraction method used provides a reliable and quantitative recovery of over 96% of the total fecal radioactivity with virtually complete separation of the two major classes of compounds providing excretory loss of cholesterol in the feces (bile acids and neutral sterols). We have corrected for the colonic delay of the feces, thereby using the serum cholesterol specific activity value that more closely corresponds to the time the fecal sample actually passed through the small intestine. Since fecal elimination is a discontinuous or irregular function with respect to time, we have attempted to minimize the error in a calendar day feces collection by use of the inert indicator, which permitted a more accurate estimation of the number of days' passage through the intestinal tract represented by a given pooled feces collection.

The changes in serum cholesterol level observed in this study were of similar magnitude regardless of the sequence of feeding the two diets, averaging 53.2 mg/100 ml (or 28%) lower during the safflower oil diet as compared with the butter diet. The subjects fed saturated fat in the first experimental period had no significant change in cholesterol level during this period, and those fed unsaturated fat in the first period had serum cholesterol reductions which were essentially the same as the

elevations observed when they were fed saturated fat in the second period. This suggests that the preexperimental diets of these subjects had fatty acid compositions similar to the saturated fat diet (B) used in the experiment. The over-all average difference in cholesterol levels between the two experimental diets, 53 mg/100 ml, compares favorably with the difference predicted from the fatty acid composition of the diets, 62 mg/100 ml. Our subjects would be expected to show somewhat less effect than that predicted by the equation of Keys and associates (83), since they were younger and had lower blood cholesterol levels than the middle-aged men used in the derivation of the equation. When exercise was added to the regimen (in the third period) and the subjects were kept in caloric balance by providing additional food intake, there were no significant changes in blood cholesterol levels, which confirmed the previous studies reported by Taylor, Anderson, and Keys (98) and by Taylor (99). In addition to the difference in fatty acid composition of the two experimental diets, there was also a greater amount of plant sterols in the safflower oil-containing diet, amounting to 300 mg digitonin-precipitable material per day. If this were entirely β-sitosterol we would expect to see no significant effect on the serum cholesterol concentration in view of the quantity of dietary β-sitosterol required to affect blood cholesterol levels (100). With the total plant sterol content of these two diets (420 and 720 mg/day) we would expect to see no more than minimal effects (1 to 5 mg/100 ml changes) on the serum cholesterol levels of these subjects as a result of the plant sterols (101).

There were no observable changes in the slopes of the serum cholesterol specific activity curves when the dietary fats were changed. However, the diet change was made before the time required for complete equilibration between the plasma cholesterol pool and various tissue cholesterol pools, so that any change in the slope of the curve may have been masked by the processes of this equilibration. Wood and associates (50) have shown a distinct steepening of the negative slope of the plasma free cholesterol specific activity curve when dietary fat was changed from palmitate-oleate to trilinolein. In another report Grundy and Ahrens (49) observed a slight temporary upward deflection in the specific activity time curve when corn oil replaced butter oil in the diet. Therefore, it is difficult to resolve this problem on the basis of the evidence provided by the reported serum cholesterol decay curves alone.

In all five of our subjects there appeared to be a greater fecal excretion of neutral sterols and bile acids during the ingestion of the safflower oil containing diet as compared to the butter containing one, regardless of the sequence of these two diets. In any given subject there were either three or four pooled feces samples per dietary period, and the variability in fecal steroid excretion rate was such that a statistical test of the difference between the means of the two dietary periods in a given subject (5 df) was not considered a satisfactory method of evaluating the results (although in one subject $P < 0.01$ and in another subject $P = 0.05$). We considered it more satisfactory to evaluate the data from all five subjects treated as a group, comparing the data from the subjects when ingesting one diet with the data from the subjects when ingesting the other diet. Using first an uncorrelated means test, which included both the inter-individual and the intra-individual variability, we found the average total fecal steroid excretion rate to be 19% higher (181 mg/day) during the safflower oil ingestion, and this difference was highly significant ($P < 0.01$). There were also significant differences ($P < 0.05$) in bile acid (91 mg/day) and neutral sterol excretion (94 mg/day) during this diet. Using next a correlated means test, employing mainly the inter-individual variability, we again found the differences in fecal excretion rate during these two diets to be significant ($P = 0.018$, 0.012, and 0.04 for the total, neutral sterol, and bile acid fractions, respectively). Since part of the variability between subjects may have been due to their different body weights (from 66 to 102 kg) the excretion rates were corrected to a standard 70 kg weight for each subject; again, significant increases were seen during safflower oil ingestion ($P < 0.01$, < 0.02, < 0.04 for the total, neutral sterol, and bile acid fractions, respectively). In all subjects the changes in fecal steroid excretion rate were in the opposite direction from the changes in serum cholesterol concentration. With one exception (subject I), the addition of exercise to the regimen did not result in any significant changes in fecal neutral sterol or bile acid excretion. In this exception there was

a further increase in excretion rate from period 2 (safflower oil) to period 3 (exercise). It is not readily understood why this patient responded in this manner, although the direction of the response (increased excretion) was the same as that observed in all subjects when ingesting the safflower oil.

Our finding of from 40 to 60% of the total cholesterol excretory products in the bile acid fraction is in keeping with the results of other investigators using similar experimental designs (42, 50, 51, 59). There were no significant differences in the proportion of bile acids during the three experimental periods in a given individual subject, but there were significant differences between the five subjects. The magnitude of the bile acid excretion (473–564 mg/day) was also similar to that reported by the above investigators. However, in the four patients reported by Spritz and associates (60) the bile acid fraction accounted for only 20–40% of the total, and the total fecal steroid excretion (599–778 mg/day) was definitely lower than in our study (966 mg/day) and that in the four studies referred to above (979–1520 mg/day).

When we examined the relationship between the changes in (*a*) serum cholesterol content, and (*b*) fecal steroid excretion, we found a reciprocal relationship between these two. During the ingestion of safflower oil the net decrease in serum cholesterol content was more than adequately balanced by the net increase in fecal steroid losses. The increased serum cholesterol content during the ingestion of butter in the two subjects fed safflower oil during the preceding experimental period was more than accounted for by the net decrease in fecal steroid excretion. The changes in blood cholesterol level resulting from a change in dietary fat were prompt and reached their maximum within about 10 days; in three of the five subjects the changes in fecal steroid excretion during the first 10 or 11 days after the diet change were adequate to account for the blood cholesterol changes. After the maximum effect on the blood cholesterol level had been reached the altered fecal steroid excretion rate continued for as long as the particular diet was ingested, showing that the dietary fat induced a persistent rather than a transient change in fecal neutral sterol and bile acid excretion. Continuation of the augmented fecal loss during the safflower oil diet suggests that after a certain amount of cholesterol had been removed from the plasma pool (probably lost from the body) there was either a compensatory increase in endogenous cholesterol synthesis or a shift of cholesterol from tissue pools into the plasma and thence out into the feces. Continuation of the diminished fecal steroid excretion rate during the butter diet suggests that after retention of a certain amount of cholesterol in the plasma pool there was a compensatory decrease in endogenous cholesterol synthesis, or a shift of cholesterol from plasma into other tissues.

The findings in our present study are in close agreement with those reported by Wood, Shioda, and Kinsell (50), Sodhi and coworkers (51), and Hellman and associates (42) using the isotope balance method and, with many of the earlier reports, using nonisotopic methods. However, Spritz and coworkers (60), in a study of three hypercholesteremic patients and one normal subject fed liquid diets containing 40% of calories as fat, were unable to find any significant changes in the excretion of total fecal steroids when plasma cholesterol levels were altered by exchange of dietary fats. Likewise, Avigan and Steinberg (59), in a study of five hypercholesteremic and one normocholesteremic subjects fed liquid formula diets providing 60% of calories as fat, found no consistent correlation between the effects of dietary fats on serum cholesterol level and fecal bile acid and neutral sterol excretion. However, there was such a large variation in excretion rate from sample to sample that it would have been difficult to detect small changes resulting from the dietary fat. It is noteworthy that in three of their six subjects there was a greater fecal steroid excretion rate during the unsaturated fat than during the saturated one, whereas in the other three subjects it was lower. In one subject there was a definitely significant increase during the period on unsaturated fat (from 3.4 to 5.3 g/day, a truly amazing fecal steroid excretion rate). It is difficult to explain why the results of these two studies differ from those of our present study and those of the three reports cited above (42, 50, 51). Perhaps the older ages of their subjects, their use of hypercholesteremic patients, or their use of liquid formula diets may be responsible for the differences. In addition to biological differences between hypercholesteremics and normal subjects, varia-

bility between subjects of any homogeneous group, and alterations in intestinal bacterial flora resulting from changes in the diet, there may well be other ill defined variables involved in the different results reported in the above mentioned two groups of studies. However, in each of 11 patients investigated by Wood, Shioda, and Kinsell (50) and in the five subjects in our present report there was an increase in fecal neutral sterol and bile acid excretion during the ingestion of polyunsaturated fats.

The present study demonstrates that in these five normal young men the isocaloric substitution of safflower oil for butter in the diet resulted in a prompt reduction of serum total cholesterol concentration and a prompt augmentation in the fecal excretion of the end products of cholesterol metabolism, which was of sufficient magnitude to account for the lowered cholesterol levels. It must be emphasized that a direct cause and effect relationship cannot be established from this study and that much further investigation will be required in order to establish with certainty the mechanism of the hypocholesteremic action of dietary unsaturated fats. However, the results of this study do lend support to the hypothesis that the hypocholesteremic action of dietary unsaturated fats is associated with an augmentation in fecal steroid excretion. The possibility that such an association represents a cause and effect relationship remains an open question.

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