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The effect of dietary fatty acids on coprostanol excretion by the rat*

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SUMMARY

By means of gas-liquid chromatographic analysis, coprostanol excretion has been studied in rats fed diets containing either no fat or varying amounts of linoleic acid, palmitic acid, or oleic acid. Coprostanol excretion was accelerated by linoleic acid, and depressed by oleic and palmitic acids. The acceleration of coprostanol formation by linoleic acid was demonstrated to occur in the large intestine. In these experiments cholesterol and coprostanol were the only neutral excretion products of cholesterol-4-C^{14}.

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The mechanisms by which variations in the type and quantity of dietary fat alter cholesterol metabolism in both man (1, 2, 3) and experimental animals (4, 5) are unknown. Previous attempts to evaluate the influences of various unsaturated fats on cholesterol metabolism, both in our laboratory (6, 7) and by others (8, 9) have been complicated by two factors—the cumbersome methods required for the separation of various cholesterol excretory products and the fact that the natural sources of unsaturated fats contain a variety of plant sterols (10) that interfere with these determinations. The recent development of relatively simple gas-liquid chromatographic techniques for the separation of sterols (11) and the current availability of highly purified unsaturated fatty acids in quantities sufficient for dietary studies (12) make it possible to re-examine the relationship between dietary fat and certain aspects of cholesterol metabolism.

In order to define some of the mechanisms by which dietary factors regulate the excretion of neutral sterols, the effects of several fatty acids on cholesterol and coprostanol excretion have been studied in the rat. This communication presents evidence that linoleic acid accelerates coprostanol formation and that palmitic and oleic acids inhibit it.

EXPERIMENTAL

Treatment of Animals. Male rats of the Long-Evans strain, weighing 100 to 200 g, were fasted for 24 hours, then placed in individual cages, and fed diets which either were fat-free or contained fatty acids at levels which varied from 4% to 20%.[1] Four days after beginning the experimental diets the rats were anesthetized with ethyl ether, and a solution of cholesterol-4-C^{14} in Tween 20, containing 1.12×10^6 cpm, was injected into the tail vein (13). Feces were subsequently collected from each rat for periods of 2 to 4 days. At the end of the experimental period the rats were killed, and the intestines were removed in one piece and frozen at $-20°$ until analyzed.

METHODS

The samples of feces were dried in an evacuated desiccator for 24 hours, weighed, and pulverized with a mortar and pestle. The powder was transferred with 100 ml of chloroform:methanol 3:1 (v/v) into an Erlenmeyer flask. The lipids were extracted by heating the slurry to boiling. The lipid extract was filtered and dried by warming under a stream of nitrogen. Ten milliliters of 20% potassium hydroxide in ethanol was then added; the samples were gassed with nitrogen, capped, and allowed to sit at room temperature for 90

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† Established Investigator of the American Heart Association.

[1] Each 1,000 g of the fat-free diet contained 250 g of casein, 680 g of glucose, 70 g of Hawk-Oser Salt Mix, 1 bottle Folbesyn® 10 mg folic acid, 0.5 g inositol, 0.1 ml Oleum Percomorphum®. In the diets containing 4% or 5% fatty acids, the acids were added directly to this mixture. In order to keep the protein content similar, each 1,000 g of the 20% fatty acid diets contained 250 g casein, 480 g glucose, 200 g fatty acid, and salts and vitamins as before.

minutes. At the end of the saponification period, 10 ml of water was added, and the solution was extracted twice with 100 ml of petroleum ether by shaking on an International bottle shaker #2 for 5 minutes. The petroleum ether extracts were evaporated to about 50 ml and washed first with 5 ml of 1 N sodium hydroxide in 50% ethanol and then with 5 ml of 50% ethanol. The washed petroleum ether fraction was dried under nitrogen and dissolved in 2 ml of chloroform. One portion was analyzed by gas-liquid chromatography as described below.

The intestines were divided into appropriate segments. The individual segments and their contents were added directly to 20% potassium hydroxide in ethanol and saponified for 90 minutes, following which the neutral sterols of the intestine were analyzed by the same procedure used for the fecal sterols.

The sterols were chromatographed by the method of VandenHeuvel *et al.* (11). Five- to 10 μl-portions of the chloroform solutions were injected into a Research Specialities Company gas-liquid chromatography assembly equipped with an ionization detector. Six-foot $\times$ 4-mm columns packed with 3.5% SE 30 silicone on Chromosorb W, 60 to 80 mesh, were used in these experiments, and the operating temperatures were as follows: column, 230°; detector, 250°; outlet, 300°; and vaporizer, 310°. The argon inlet pressure was 30 psi, and the monitored outflow was 100 ml/minute. The areas recorded under the various peaks were estimated by triangulation.

In some experiments the sterols were collected at the outlet in glass tubes filled with cotton. The materials were eluted from the cotton with petroleum ether, dried, and treated in one of two ways. Some samples were chromatographed on paper using the reversed phase system described by Martin (14); the spots were developed by spraying with either 8% phosphomolybdic acid or phosphotungstic acid in ethanol. Digitonides were precipitated and assayed as described by Sperry and Webb (15). Other samples were added to 10 ml of 0.4% diphenyloxazole in toluene, and assayed for C^{14} in a liquid scintillation counter.

MATERIALS

Cholesterol-4-C^{14} was purchased from the Chicago Nuclear Corporation, Chicago, Illinois; linoleic acid from the Hormel Foundation, Austin, Minnesota; and oleic acid from the J. T. Baker Chemical Company, Phillipsburg, New Jersey; SE 30 silicone was supplied by the General Electric Company, Silicone Products Department, Waterford, New York.

RESULTS

Separation and Quantification of Fecal Sterols. The utilization of the silicone-Celite column described by VandenHeuvel *et al.* (11) was found to provide a satisfactory separation of coprostanol from cholesterol (Fig. 1). Although variations in the silicone content

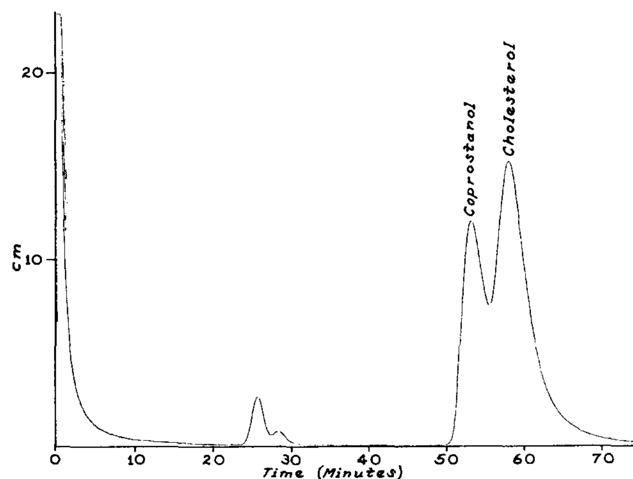

FIG. 1. Separation by gas-liquid chromatography of cholesterol and coprostanol; 10 μg of cholesterol and 10 μg of coprostanol in 10 μl of chloroform were injected into a gas-liquid chromatographic assembly. Conditions: 3.5% SE 30 silicone gum on Chromosorb W (60 to 80 mesh); 6-foot $\times$ 4-mm column; 230°; argon flow, 100 ml/minute; sensitivity 10. Two small contaminants of the coprostanol can be seen at 24 and 28 minutes.

of the column packing material from 0.5% to 5%, and in the column operating temperature from 210° to 250°, markedly altered the appearance time of cholesterol, the relative retention time of coprostanol to cholesterol (0.9) remained constant. The operating temperatures and silicone concentrations used in these experiments were chosen because they provided convenient and clear-cut separation of cholesterol and coprostanol from the many other substances present in extracts of biological materials.

Despite the fact that the peaks for coprostanol (appearance time 48 to 50 minutes) and cholesterol (appearance time 54 to 56 minutes) overlap in the system used here, a linear relationship was demonstrated between the amounts of these materials injected and the area recorded under the curve for quantities ranging from 5 μg to 50 μg (Fig. 2). Of the sterols tested in this system (desmosterol, cholestanol, cholestanone, 7-dehydrocholesterol, cholestenone, epicholesterol, epicoprostanol, methostenol, stigmasterol, and sitosterol), the only peaks which superimposed were those of cholestanol and cholesterol. No other significant contamination of either cholesterol or coprostanol could be demonstrated. The quantity of cholestanol in

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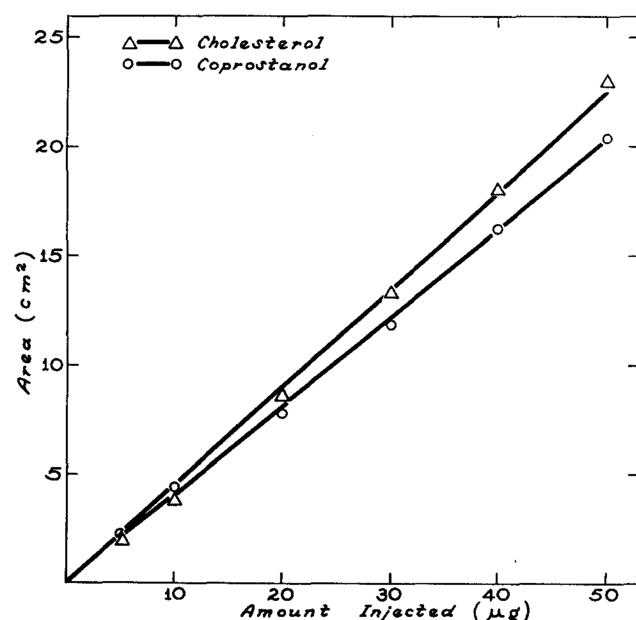

FIG. 2. Relation of varying quantities of cholesterol and coprostanol to area recorded. Varying amounts of cholesterol and coprostanol in 5 μl of chloroform were injected into a gas-liquid chromatography assembly. Conditions as in Figure 1.

feces was in no case sufficient to be detected by the paper chromatographic system of Martin (14), and the degree of contamination of the cholesterol in these experiments was therefore negligible. The fecal sterols found in largest quantities were cholesterol and coprostanol.

Effect of Linoleic and Palmitic Acids. Marked differences in neutral sterol excretion were noted when rats were fed either linoleic or palmitic acid. The addition of linoleic acid to the diet resulted in the excretion of large amounts of coprostanol, whereas the feeding of palmitic acid caused cholesterol to be the predominant sterol in the feces. These effects are demonstrated in the representative chromatograms shown in Figure 3. In Table 1 (experiment 1), the effects on neutral sterol excretion of supplementing the fat-free diet with either palmitic acid oı linoleic acid are summarized. There was little difference among the three groups of rats, either in weight or in the average daily caloric intake. When compared to that of the fat-free group, the average weight of the dried feces was slightly increased in the linoleic acid group, and markedly increased (threefold) in the animals fed palmitic acid.

The most striking differences among the three groups of animals were in the partition of the neutral sterols. The animals fed the fat-free diets excreted an average of 2.2 mg coprostanol and 1.0 mg cholesterol per day. The linoleic acid-fed animals excreted an average of 6.2 mg coprostanol and 4.2 mg cholesterol daily, while the rats fed palmitic acid excreted an average of 1.2 mg coprostanol and 4.0 mg cholesterol per day. The suppression of coprostanol excretion by palmitic acid is particularly noteworthy since it occurred under circumstances in which total neutral sterol excretion was unchanged.

Effect of Linoleic and Oleic Acids. Since palmitic acid has an exceedingly low coefficient of digestibility, coprostanol formation was studied in rats fed smaller amounts of linoleic and oleic acids, fatty acids which have similar coefficients of digestibility (16). Representative chromatograms of fecal sterols from these rats are shown in Figure 4. Once again, the rats fed either no fat or linoleic acid excreted large quantities of coprostanol, whereas the rat fed oleic acid excreted cholesterol predominantly. The results of feeding isocaloric quantities of diets containing these acids to 14 rats are summarized in Table 1 (experiment 2). There were no significant differences in body weight, average daily caloric intake, or weight of the dried feces. Further-

TABLE 1. EFFECT OF FATTY ACIDS ON COPROSTANOL EXCRETION BY THE RAT

Exp.	Diet	Number of Rats	Average Weight	Average Daily Food Intake		Average Daily Weight of Dried Feces	Sterol* Excretion/24 Hours		
							Coprostanol	Cholesterol	Total
			g	*g*	*cal*	*g*	*mg*	*mg*	*mg*
1†	Fat free	5	153	6.8	23.2	0.3	2.2 ± 1.1	1.0 ± 0.2	3.2 ± 1.7
	20% linoleic acid	5	132	4.6	21.5	0.4	6.2 ± 1.6	4.2 ± 2.4	10.4 ± 3.1
	20% palmitic acid	5	149	5.2	24.4	1.0	1.2 ± 0.8	4.0 ± 1.4	5.2 ± 1.8
2‡	Fat free	5	153	10	38	0.6	2.4 ± 1.8	4.9 ± 2.7	7.3 ± 1.7
	5% oleic acid	4	150	10	40	0.5	1.6 ± 0.7	8.9 ± 3.3	10.5 ± 2.7
	5% linoleic acid	5	160	10	40	0.7	7.8 ± 1.4	2.8 ± 1.2	10.6 ± 2.3

* Sterols separated and chromatographed as described in the text.
† Feces collected during the last 4 days of an 8-day experimental period.
‡ Feces collected during the last 2 days of a 6-day experimental period.

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more, there were no consistent differences in total sterol excretion between the three groups. However, again there were marked differences in the average daily coprostanol excretion (fat-free, 2.4 mg; linoleic acid, 7.8 mg; oleic acid, 1.6 mg) and in the average daily cholesterol excretion (fat-free, 4.9 mg; linoleic acid, 2.8 mg; oleic acid, 8.9 mg).

Effect of Mixtures of Linoleic and Oleic Acids. Coprostanol excretion was then studied in rats fed varying amounts of linoleic and oleic acids. The results of feeding these diets to 23 animals are summarized in Table 2. No consistent differences in body weight, average daily caloric intake, weight of the dried feces, or total sterol excretion were observed among the five groups. Again there were marked differences in the average daily coprostanol excretion between the rats fed 4% linoleic acid (5.1 mg) and those fed 4% oleic acid (0.5 mg). It was found that the addition of 0.1% linoleic acid to the 4% oleic acid diet did not increase coprostanol excretion. Furthermore, the addition of 2% linoleic acid to an equal amount of oleic acid also did not elevate fecal coprostanol. Thus, in the presence of oleic acid, linoleic acid does not seem capable of accelerating coprostanol excretion.

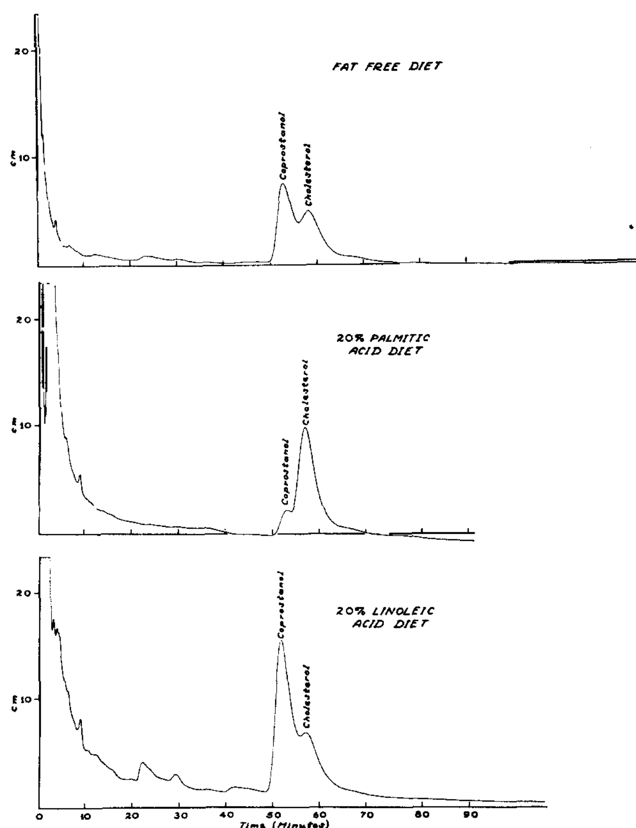

FIG. 3. Separation by gas-liquid chromatography of fecal sterols from rats fed diets which contained either no fat, 20% palmitic acid, or 20% linoleic acid. Five-microliter aliquots of the chloroform extracts were chromatographed under the same conditions described in Figure 1. The peaks labeled cholesterol were identified by comparison of the appearance time in this system (56 minutes) with that of authentic cholesterol (56 minutes). In addition, the material was collected, and an aliquot was rechromatographed in *n*-propanol-kerosene (14) (R_f of unknown, 0.48; R_f of cholesterol, 0.48). The peaks labeled coprostanol were identified by comparison of the appearance time in this system (48 minutes) with that of authentic coprostanol (48 minutes). The migrations of the collected material were also compared in the *n*-propanol-kerosene system (R_f of unknown, 0.34; R_f of coprostanol, 0.35); both the coprostanol and cholesterol gave a blue reaction when sprayed with the phosphomolybdic reagent; cholesterol gave a red color when developed in phosphotungstic acid, and coprostanol did not react at all. The material labeled coprostanol was digitonin precipitable and did not react with Liebermann-Burchard reagent (15).

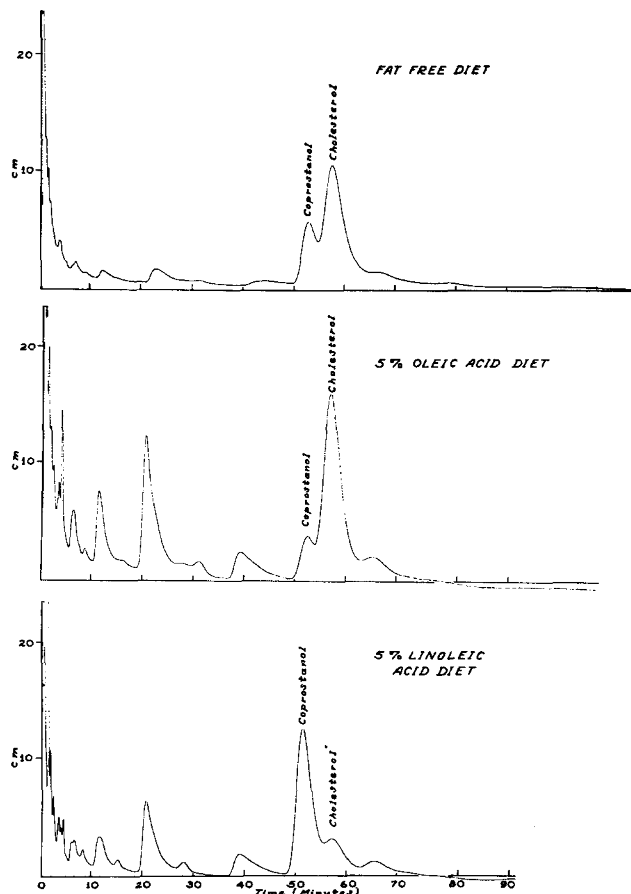

FIG. 4. Separation by gas-liquid chromatography of fecal sterols from rats fed diets which contained either no fat, 5% oleic acid, or 5% linoleic acid. Five-microliter aliquots of the chloroform extracts were chromatographed as described in Figure 1. The identification of the peaks is described in Figure 3.

Influence of Linoleic and Oleic Acids on the Neutral Excretory Products of Cholesterol-4-C^{14}. In order to determine which of the fecal sterols in these experiments were derived from the exchangeable cholesterol pool of the rat, C^{14}-labeled cholesterol was given intravenously to the rats described in the previous section.

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TABLE 2. EFFECT OF VARYING AMOUNTS OF LINOLEIC ACID AND OLEIC ACID ON COPROSTANOL EXCRETION BY THE RAT

	Number of Rats	Average Weight	Average Daily Food Intake		Average Daily Weight of Dried Feces	Sterol Excretion/24 Hours		
						Coprostanol	Cholesterol	Total
		g	*g*	*cal*	*g*	*mg*	*mg*	*mg*
Fat free	5	130	14	53	0.5	3.5 ± 1.5	2.8 ± 1.1	6.3 ± 1.3
4% linoleic acid	4	123	12	47	0.5	5.1 ± 0.5	2.0 ± 0.2	7.1 ± 0.6
2% oleic acid-2% linoleic acid	5	142	14	55	0.6	0.9 ± 0.4	5.4 ± 2.9	6.3 ± 2.7
4% oleic acid-0.1% linoleic acid	5	148	14	55	0.5	0.5 ± 0.4	4.9 ± 0.9	5.4 ± 1.0
4% oleic acid	4	143	14	55	0.6	0.5 ± 0.5	6.5 ± 1.6	7.0 ± 1.2

Four days after starting the experimental diets, 0.4 μmole cholesterol containing 1.12×10^6 cpm cholesterol-4-C^{14} was injected into the tail vein. Feces were collected from the tenth through the fourteenth day. Sterols were separated, chromatographed, and assayed for C^{14} as described in the text.

As shown in Figure 5, when the various chromatographic peaks from representative rats from each of these groups were collected and assayed for C^{14}, the only areas containing significant radioactivity corresponded to cholesterol and coprostanol. Thus, in these experiments cholesterol and coprostanol were the only significant neutral excretion products of cholesterol.

Coprostanol Formation in the Intestine. In order to determine the site of coprostanol formation in these experiments, the intestines from three of the rats reported in Table 1 (experiment 2) were removed, and the coprostanol was measured in various portions of the gastrointestinal tract (Table 3). No coprostanol was found in the entire intestine of the rats fed the fat-free or 5% oleic acid diets. However, a small quantity of coprostanol was found in the ileum of the linoleic acid-fed animals, but in the contents and wall of the colon of this animal, coprostanol accounted for nearly half of the total neutral sterol. Furthermore, the coprostanol present in the large intestine (7.0 mg) corresponded closely to the excretion of coprostanol for the previous 2 days (10.4 mg/day). It was concluded, therefore, that the marked enhancement by linoleic acid of the bacterial reduction of cholesterol to coprostanol occurs in the lower intestinal tract.

DISCUSSION

It has been established both by studies *in vivo* and *in vitro* that the *cis* hydrogenation of cholesterol to form coprostanol in the intact animal is due to microbial action in the large intestine. No coprostanol is excreted by germ-free animals (17), and coprostanol formation can also be abolished by the administration of a number of bacteriostatic agents (18, 19, 20). In addition, the conversion of cholesterol to coprostanol by bacterial cultures isolated from feces has also been demonstrated conclusively (21 to 24), and the formation of coprostanol has been shown by the isotopic studies of Rosenfeld *et al.* (22) to be a direct microbial reduction, not involving a 3-one intermediate.

The factors determining the extent to which cholesterol is converted to coprostanol by the intestinal bacteria are poorly understood. Several studies have appeared reporting the influence of various dietary constituents on the synthesis of coprostanol. Milk diets (25), dietary lactose (26), sodium taurocholate (27), and Tween 80 (27) all markedly inhibit coprostanol formation. On the other hand, the early observations of Dorée and Gardner (28) suggested that the presence of brain in the diet accelerated coprostanol excretion. These observations were extended by Rosenheim and

TABLE 3. EFFECT OF LINOLEIC ACID AND OLEIC ACID ON COPROSTANOL FORMATION IN THE RAT INTESTINE

Intestinal Segment	Fat-Free Diet		5% Oleic Acid Diet		5% Linoleic Acid Diet	
	Coprostanol	Cholesterol	Coprostanol	Cholesterol	Coprostanol	Cholesterol
	mg	*mg*	*mg*	*mg*	*mg*	*mg*
Stomach	0	3.75	0	4.46	0	7.16
Duodenum	0	3.11	0	2.97	0	3.62
Jejunum	0	8.24	0	12.57	0	6.54
Ileum	0	8.10	0	10.14	0.1	11.22
Colon	0	11.89	0	16.76	7.0	8.27

Intestines from the rats reported in Table 2 were removed in one piece at the time of death and immediately frozen at −20°. The intestines were subsequently sectioned, and the intestinal segments plus contents were saponified as a unit; the sterols were extracted and assayed as described in the text.

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Webster (29, 30), who concluded that the glycolipid phrenosin, obtained from brain and other organs, was an essential factor both for the formation of coprostanol and of coprositosterol. Wells (27), however, has reported that coprostanol excretion is unimpaired in rats fed purified synthetic diets for long periods of time.

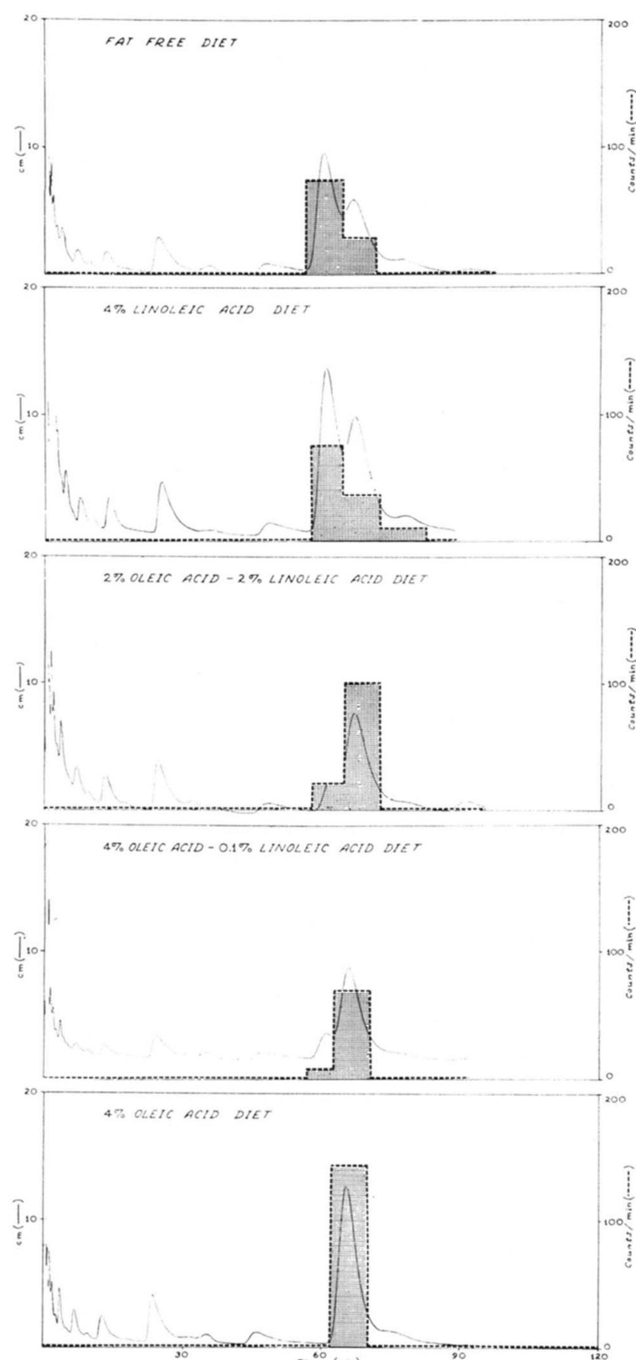

FIG. 5. Separation by gas-liquid chromatography of fecal sterols from rats fed diets which contained varying amounts of oleic and linoleic acids. The chromatographic conditions were as in Figure 1, except that the argon flow was 60 ml/minute; identification of the peaks is described in Figure 3. Each peak was collected in a sample-collecting tube and assayed for C^{14} as described in the text. The only peaks containing significant radioactivity were coprostanol (appearance time, 54 minutes) and cholesterol (appearance time, 62 minutes).

While the data reported here confirm the fact that the animals fed purified or even fat-free diets can synthesize coprostanol, far more striking is the demonstration that the presence of linoleic acid markedly accelerates the formation and excretion of coprostanol. In fact, this effect is of such a magnitude that in the animals fed linoleic acid, coprostanol was the major neutral sterol present in the feces. This enhancement of coprostanol formation by linoleic acid was even more marked when compared with the coprostanol excretion of rats fed diets containing either oleic or palmitic acids, both of which suppressed coprostanol formation. It is possible that the results previously reported by Wells and by Rosenheim and Webster might, in fact, be due to the fat content of the diets employed. The diets in the studies of Wells and Cooper (26, 27) were all supplemented with either methyl linoleate, cod liver oil, or corn oil, and Rosenheim and Webster (29) observed that the brain extracts did not accelerate coprostanol formation in the absence of dietary fat.

The present studies do not furnish evidence as to the mechanism by which linoleic, palmitic, and oleic acids influence coprostanol formation. It would seem unlikely that the effect of linoleic acid is due solely to its action as an essential fatty acid, for although the feeding of saturated fat accelerates the development of essential fatty acid deficiency, oleic acid (in the form of triolein) neither accelerates nor retards the development of this condition (30). Attempts to clarify the mechanisms of these effects are now in progress.

Further studies will likewise be necessary before it is possible to draw any conclusions regarding a possible relationship between the acceleration of coprostanol formation by linoleic acid and the previously demonstrated cholesterol-lowering effect of linoleic acid both in man (12) and the hypercholesterolemic rat (5).

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