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Properties and Metabolism of 2-Alkylalkanoates. Cholesteryl 2-Methylalkanoates*

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ABSTRACT: Substitution of a methyl group at the α-methylene carbon of methyl esters of laurate, myristate, and palmitate results in characteristic infrared spectra that readily distinguishes them from the straight-chain homologs, and facilitates their unequivocal identification when separated from complex lipid mixtures by gas–liquid partition chromatography (glpc). Spectral changes observed with the methyl esters are also evident in the cholesterol esters of 2-methylalkanoates and also serve in their identification. No significant absorption of either cholesterol-4-C^{14}, as determined by radioassay, or of 2-methylalkanoate, as determined by glpc, could be demonstrated when given to the rat as cholesteryl-4-^{14}C 2-methyl alkanoate, either incorporated in normal rat chow, fed by peroral intubation, or perfused *in situ* through isolated intestinal loops. These studies thus support the thesis that neither cholesterol nor long-chain fatty acids is readily transported across the intestinal mucosa as the cholesterol ester, as such, and would minimize the role of pinocytosis as a mechanism for the absorption of fatty acid esters, at least in the case of cholesterol esters.

Substitution of alkyl or aryl groups at the methylene carbon adjacent to the carbonyl group of fatty acids is known to alter the metabolic pathways of fatty acids and their ester derivatives. Metabolic anomalies associated with α- or 2-alkyl fatty acids apparently arise because of steric hindrance to β-oxidation, with resultant compensatory ω-oxidation of such acids, and in the case of esters of 2-alkyl substituted acids, to inhibition of hydrolysis by esterases (*cf.* Carter, 1941; Weitzel, 1951; Tryding, 1957a). Glycerides of 2-methylstearic, *e.g.*, have been shown to be resistant to hydrolysis by pancreatic lipase (Tryding, 1957b), and after feeding 2-methylstearic acid-1-^{14}C, 1–2% of the radioactivity was recovered in the urine of rats as 2-methyladipic and 2-methylsuccinic acid (Tryding and Westoo, 1957). The long-chain 2-methylalkanoic acids are readily separated in complex lipid mixtures by gas–liquid partition chromatography (glpc)[1] (Napier, 1963), and with verification of structure by infrared spectra, metabolic studies of these nonnaturally occurring acids are facilitated. Furthermore, when these novel acids are esterified with ^{14}C-labeled alcohols such as sterols, methanol, and glycerides, the fate of both moieties of the effectively double-labeled compound may be unequivocally followed, particularly as related to the transport of lipids and the stereospecificity of hydrolases and esterases.

The present communication summarizes the properties and characteristic infrared spectra of both the methyl and cholesterol esters of 2-methyllauric, 2-methylmyristic, and 2-methylpalmitic acids, as well as studies of the absorption of cholesteryl-4-^{14}C 2-methylalkanoates. Evidence is presented which supports the thesis that neither long-chain fatty acids nor cholesterol are transported as cholesterol esters, as such, across the intestinal mucosa.

Experimental Section

Syntheses. The 2-methylalkanoic acids were synthesized by the diethyl methylmalonate procedure described by Cason *et al.* (1953).[2] Samples collected from

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[1] Abbreviations used: glpc, gas–liquid partition chromatography; tlc, thin layer chromatography; BSP, bromsulfonphthalein.

[2] The novel alkyl migration in dialkyl ketones, to produce 2-alkyl substituted acids in the presence of SeO_2 (Sonoda and Tsutsumi, 1959), was initially employed at the suggestion of Dr. Hugh S. Wiggins. Although the reaction was indeed found applicable to the synthesis of long-chain 2-methyl fatty acids, the yields of the 2-methyl fatty acids were found to be in approximately a 1:1 ratio with the straight-chain isomer as determined by glpc (*e.g.*, 2-tridecanone → 2-methyllauric + tridecanoic).

TABLE I: Elemental Analyses.[a]

	% C		% H		% O		Mp (°C)	Mp Amide (°C)
	Calcd	Found	Calcd	Found	Calcd	Found		
dl-2-Methyllauric	72.83	72.22	12.23	12.88	14.93	14.73	23.0–24.0[b]	90.7
dl-2-Methylmyristic	74.32	74.60	12.47	12.58	13.20	12.93	35.5–36.0[c]	96.2
dl-2-Methylpalmitic	75.50	75.46	12.61	12.53	11.83	11.59	45.5–46.3[d]	100.5
Cholesteryl 2-methyllaurate	82.41	82.11	12.10	11.93	5.49	6.06	55.2	...
Cholesteryl 2-methylmyristate	82.56	83.01	12.21	11.86	5.24	5.27	58.0	...
Cholesteryl 2-methylpalmitate	82.69	82.93	12.30	12.19	5.01	5.03	63.8	...

[a] See footnote 4 of text. [b] Stallberg-Stenhagen (1946), 23.3–24.1°. [c] Greer and Adams (1930), 34–36°; Weitzel and Wojahn (1951), 39.5–40.0°. [d] Greer and Adams (1930), 45.5–47.5°.

repeated fractional distillations were converted to their methyl esters with anhydrous methanolic HCl (Stoffel *et al.*, 1959), and assayed for purity by glpc and infrared spectrophotometry (Grubb Parsons 2A double-beam spectrophotometer, KBr prism).

Cholesterol-4-^{14}C was added to the acyl chloride,[3] the solution heated at 70° for 1 hr under an atmosphere of nitrogen, and the ester which precipitated on cooling was washed with water and ethanol, and then recrystallized from acetone until a constant melting point was obtained. The purity of the esters was checked by hydrolysis with 2% KOH (in absolute ethanol) at 70° for 16 hr, and the fatty acids then extracted with hexane after acidification with HCl. The extracts were dried under vacuum and titrated with 0.02 N tetramethylammonium hydroxide in absolute ethanol, using thymol blue as an indicator. The cholesterol (Zlatkis *et al.*, 1953) to fatty acid mole ratio was thus calculated to be 1:1. Chromatography of the esters on silicic acid columns (Hirsch and Ahrens, 1958) or thin layer silica gel G plates (Malins and Mangold, 1960) resulted in 95–98% recovery of radioactivity as the cholesterol ester. The specific activity of the cholesterol esters was approximately 18,000 dpm/μmole. Melting points were recorded uncorrected with a capillary apparatus (Table I).[4]

Radioactivity of lipid fractions was determined with a Packard 318 X dual-channel liquid scintillation spectrometer, using a solution of 0.4% 2,5-diphenyloxazole and 0.1% 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene in toluene as scintillator. Lipids resolved by thin layer chromatography (tlc) (petroleum ether–diethyl ether–acetic acid, 90:10:1, v/v) were washed from the plates, after drying, directly into counting vials with scintillation fluid. No measurable quenching was observed because of the insoluble silica gel in the bottom of the vials. Autoradiographs of tlc plates were obtained by exposure to Kodak Royal Blue Medical X-ray film.

[3] Cholesterol-4-^{14}C was obtained from New England Nuclear Corp., Boston, Mass.

[4] Elemental analyses were performed by Spang Microanalytical Laboratories, Ann Arbor, Mich.

Intestinal Perfusions. Fasting, male Sprague-Dawley rats, weighing between 150 and 200 g, were anaesthetized with subcutaneous injections of sodium pentobarbital (60 mg/kg). Similar to the technique of Fullerton and Parsons (1956), a cannula was ligated in the intestine 3-cm distal to a ligature at the ligament of Treitz. Approximately 20–30-cm caudal to the ingoing cannula, the intestine was again ligated, and an outgoing cannula was secured proximal to the lower ligature. The abdominal incision was closed, and the ingoing cannula connected to a 50-ml syringe driven at a calibrated rate of 0.764 ml/min with a Harvard Model 600 infusion apparatus. Solutions were infused for a period of 5–10 min to permit washing and equilibration of the intestine, and multiple 10-min samples were then collected from the outgoing cannula.

The solutions were prepared by dissolving 100 mg of the cholesterol ester in 400 mg of oleic acid and 400 mg of a glyceride mixture containing approximately equal parts of triolein, diolein, and monoolein. The mixture was then diluted to 100 ml with an aqueous solution of 1% sodium taurocholate and 0.01% bromsulfonphthalein (BSP), adjusted to pH 7.3 with 0.1 N NaOH, and centrifuged for 5 min at 1000*g* to clarify the emulsion. The opalescent, homogeneous subnatant solution was then removed by aspiration and used for the perfusion studies. With this method of preparation, the final concentration of cholesterol esters was approximately 20 mg/100 ml. BSP was determined by adding 5 ml of 0.005 N NaOH to 0.2 ml of the perfusate, and recording the absorbance at 575 mμ (Gaebler, 1945).

Balance Studies. Rats were fasted for a period of 24 hr and given water *ad libitum*. The cholesterol ester (100 mg) was dissolved in 0.5 ml of olive oil in a counting vial, and fed quickly by peroral intubation. The tubing was washed with an additional 0.5 mg of olive oil to ensure transfer of the perfusate into the stomach, withdrawn, and rinsed with chloroform–methanol (2:1) into the vial to record any residual radioactivity. The animals were then placed in all-glass balance cages (Roth *et al.*, 1948), and fed *ad libitum* a synthetic normal diet (Wooley and Sebrell, 1945) which eliminated radioactive quenching and markedly eased the extrac-

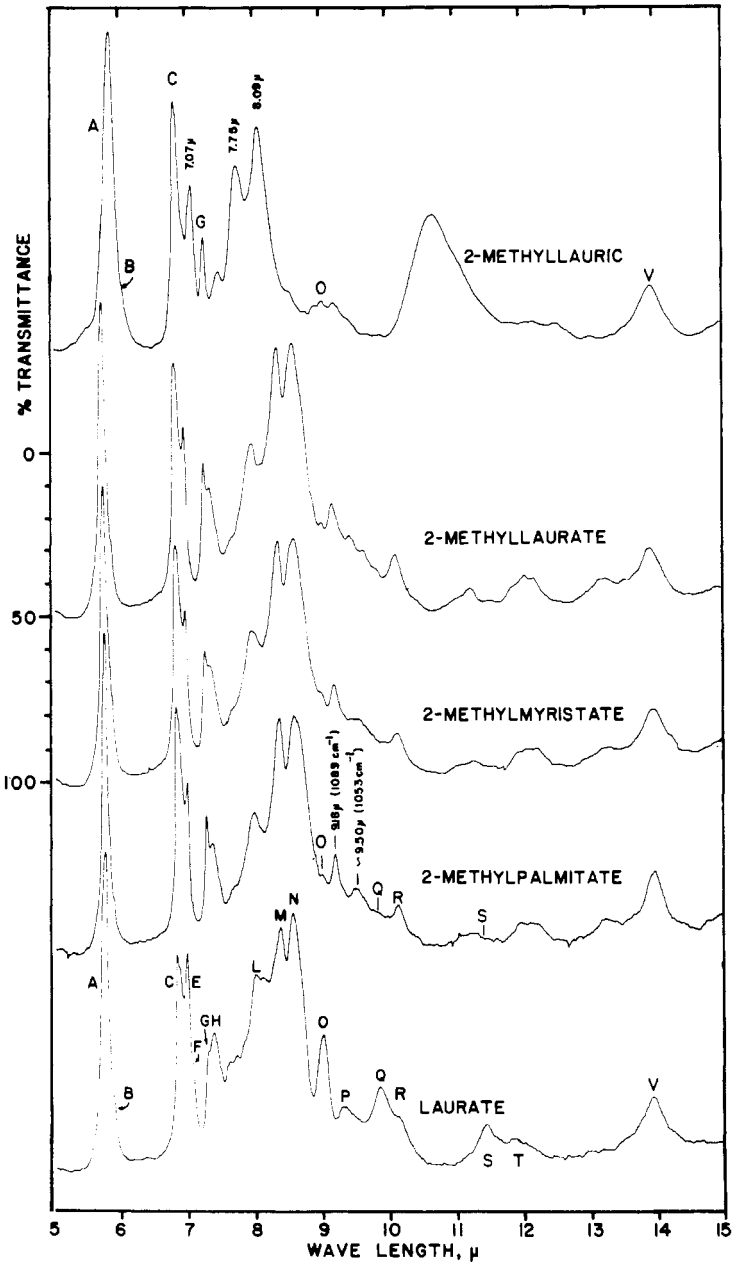

FIGURE 1: Infrared spectra of the methyl esters of the 2-methyl homologs of laurate, myristate, and palmitate as compared to an unsubstituted fatty acid (laurate) and nonesterified 2-methylalkanoic (2-methyllauric). KBr cell length, 0.03–0.05 mm.

tion and separation of fecal lipids as compared to commercial rat pellets. Feces and urine were collected at 24-hr intervals for 5 days. Expired CO_2, collected in sodium hydroxide traps, was monitored at 12 hr intervals by liquid scintillation counting of the Hyamine carbonate (Passmann *et al.*, 1956). The rats were then sacrificed with ether and the tissues excised.

Lipids were extracted from chow, tissue, or feces by repeated homogenization in a Waring blendor with chloroform–methanol (2:1) according to the method of Folch *et al.* (1957), and concentrated *in vacuo* preparatory to analyses by chromatography and liquid scintillation counting.

Results

Infrared Spectra. As reported by Freeman (1952), and illustrated by 2-methyllauric acid (Figure 1), alkyl

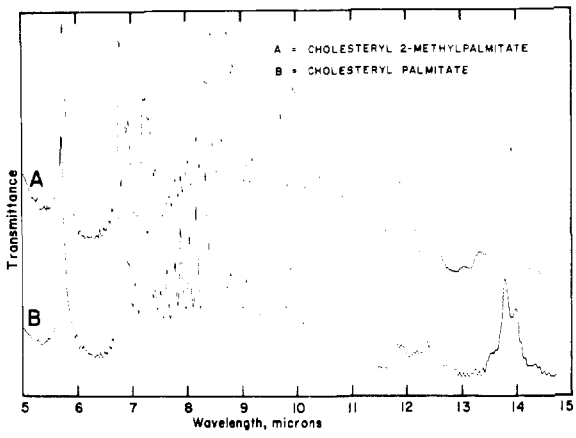

FIGURE 2: Infrared spectra of the cholesterol esters of 2-methylpalmitate and palmitate (4 mg of ester/400 mg of KBr).

substitution at the 2-position is readily identified by a reversal in intensity of the 7.75- and 8.09-μ bands, as compared to straight-chain acids. Reversal of intensity of the 6.83- (band C) and 7.07-μ bands is also observed, which is associated with alkyl substitution at the α-methylene carbon (Guertin *et al.*, 1956). It has been noted, however, that since the 6.83-μ band is a function of the methylene chain (C–H scissor), and the 7.07-μ band attributed to C–H scissor of the methylene adjacent to the carbonyl grouping (Francis, 1950), that increased chain length will contribute to stronger absorption at 6.8 μ (greater than 14 carbon atoms) and hence nullify this reversal in intensity.

Comparison of film spectra of the methyl esters of 2-methyllaurate, 2-methylmyristate, and 2-methylpalmitate, however, does not reveal an obvious reversal of intensity attributed to chain length. The symmetrical C–H bending of the methyl groups (7.27 μ, band G) is markedly enhanced in both the free acid as well as in the esters of the 2-methyl substituted acids, and readily distinguishes the 2-methylalkanoate esters from the straight chain (laurate) homologs. Here, one observes a marked reversal of intensity of the 7.27- and 7.36-μ bands (band H) in the 2-methyl fatty acid esters as compared to methyllaurate. This reversal is not altered with chain length. Similarly, comparison of the 6.83- and 6.98-μ bands (bands C and E) in the 2-methylalkanoates, as compared to laurate, also demonstrates a marked reversal of intensity, which is still quite apparent with increasing chain length. Other qualitative characteristics of the 2-methylalkanoates which aid in their identification are the marked diminution of bands F (7.05 μ), O (9.00 μ), P (9.31 μ), Q (9.84 μ), and S (11.40 μ), and the appearance of a sharp band at 9.18 μ (1089 cm^{-1}), and a diffuse band at about 9.50 μ (1053 cm^{-1}).

The diminution of the scissoring mode attributed to the α-methylene grouping at 7.05 μ is also seen in the cholesterol esters of 2-methyl fatty acids (Figure 2), and is analogous to that reported by Jones (1962) for laurate deuterated in the α-methylene position. At 7.27 μ, a sharpening of the doublet is also observed in cholesteryl 2-methylpalmitate, which is attributed to the one additional CH_3 grouping, as compared to cholesteryl palmitate. This doublet, in lieu of a single peak at 7.27 μ, would be analogous to that reported in the case of neo and iso fatty acids (Sobotka and Stynler, 1950), and has been previously noted in cholesterol and ergosterol derivatives (Rosenkrantz *et al.*, 1952), and assigned to the CH_3-isopropyl grouping in the C_{21} side chain.

The solid spectra of cholesteryl 2-methylpalmitate also exhibit a series of progression bands between 7.5 and 8.0 μ which, in the case of cholesteryl palmitate, have been previously assigned to wagging of the methylenes (in the fatty acid side chain) in the more stable *trans* conformation (Labarrère *et al.*, 1958). Although the eight progression bands appearing in cholesteryl palmitate are also observed in the 2-methylpalmitate ester, their relative intensities are considerably altered, and three additional bands appear as weak shoulders. These changes in the band progressions are probably related to altered crystal structure. In cholesteryl 2-methylpalmitate, relatively strong bands are present at 8.79, 8.90 and 9.98 μ, and a pair of bands are noted at 13.63 and 13.92 μ which are reversed in intensity, in place of the doublet of cholesteryl palmitate at 13.7 to 13.98 μ. Further studies of related ester derivatives, however, are necessary to establish the structural significance of these spectral changes.

Balance Studies. Rats trained (Turner *et al.*, 1960) to eat, during a 10-min period, a prepared 2-g commercial chow pellet containing 100 mg of cholesteryl-4-^{14}C 2-methylalkanoate showed no significant radioactivity in the respiratory CO_2, urine, or tissues for periods up to 6 hr after feeding. Conversely, at least 1 and 5% of the fed radioactivity, respectively, was found after 6 hr in the serum and liver of animals fed cholesteryl-4-^{14}C palmitate.

As shown in Table II, between 80 and 95% of the radioactivity was recovered from the feces and gastrointestinal tract over a 5-day period when cholesteryl 2-methylmyristate was fed by intubation. As a comparison, no more than 70% of the radioactivity fed as cholesteryl-4-^{14}C palmitate was recovered in the feces and intestinal tract.

The highest total level of fed radioactivity found in lipid extracts of tissues from 22 rats given cholesteryl-4-^{14}C 2-methylalkanoates by intubation was found to be 3.6% after a 5-day period (rat 1, Table II). Most of the radioactivity was found in the liver (1.7%), blood (1.0%), lung (0.5%), and intestinal wall (0.7%), and only negligible levels were detected in the heart, kidneys, testes, adrenals, pancreas, spleen, and brain. Silicic acid chromatography of lipid extracts from the liver, and subsequent tlc resolution of the lipids, showed that at least 88% of the recovered radioactivity was in the free cholesterol fraction. In the remaining animals, the maximum level of radioactivity was found to be in the liver (0.3%), blood (0.2%), lung (0.2%), and intestinal wall (0.2%).

To explore the possible origin of this radioactivity in the tissues, the pooled sera from 12 animals, collected

TABLE II: Per Cent Recovery of Fed Cholesterol-4-^{14}C Esters in Rat Feces.[a]

	Cholesterol Ester								
	2-Methylmyristate							Palmitate	
Rat no.	1	2	3	4	5	6	7	8	9
Net amount fed (mg)	62.2	92.8	93.0	73.1	75.8	90.6	93.8	70.0	98.9
Feces									
24 hr	32.1	89.2	86.4	58.0	35.9	59.1	50.7	52.7	54.0
48 hr	23.6	3.5	4.9	20.9	45.3	16.4	18.2	9.8	9.1
72 hr	20.9	0.8	1.6	4.9	3.1	3.7	10.2	2.0	3.2
96 hr	0.3	0.8	1.4	0.7	0.5	1.5	0.7	0.9	2.4
120 hr	0.0	0.4	0.4	0.5	0.4	3.8	0.4	0.8	0.9
Intestinal contents	7.5	0.3	0.4	0.4	0.4	0.0	0.0	0.0	0.0
% Total recovered	84.4	95.0	95.1	85.4	85.6	84.5	80.2	66.1	69.6

[a] Approximately 100 mg of cholesterol-^{14}C ester (18,000–20,000 dpm/μmole) was dissolved in 0.5–1.0 ml of olive oil and fed by peroral intubation to fasting rats. The residual ester was rinsed from the tube and then assayed, to calculate the net amount fed to each animal.

6 hr after feeding cholesteryl-4-^{14}C 2-methylpalmitate, was analyzed. It was found that of the total fed radioactivity recovered in the serum (less than 0.3%), the radioactivity in the cholesterol ester and free cholesterol fractions was in the same ratio as the concentration of cholesterol esters to free cholesterol; *i.e.*, approximately 3:1. The specific activity (cpm/mg cholesterol), however, was found to be the same in both fractions, which would suggest compartmentalization and pooling of free cholesterol-4-^{14}C. Analyses of the fatty acids of sera and other tissues by glpc did not reveal the presence of the fed 2-methylalkanoate in any of the animals studied, even though as little as 1 μg, or 0.2% relative concentration, of the fatty acids may be detected. The sensitivity of glpc to detect absorption of 2-methylpalmitic acid, when fed as the free acid, is illustrated in Figure 3.

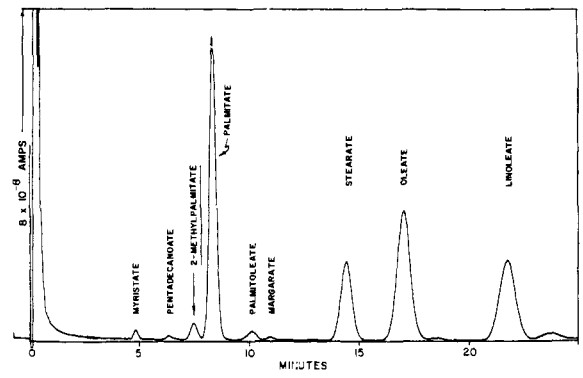

FIGURE 3: Glpc of rat serum fatty acids 3 hr after feeding 25 mg of 2-methylpalmitic acid dissolved in 0.5 ml of olive oil. Column: 8 ft × 0.25 in., 17% EGS on Chromosorb W, 185°, argon ionization detector.

Intestinal Perfusions. As compared to the volume of perfusate and the concentration of the aqueous soluble BSP, total radioactivity was recovered from solutions of cholesteryl-4-^{14}C 2-methylalkanoates perfused *in vivo* for periods of 40–60 min (Table III).

The decreased solubility of cholesteryl palmitate in the perfusion mixture, as compared to the cholesteryl 2-methylalkanoates, did not permit perfusion of comparably higher concentrations of the straight-chain ester. Under these conditions, nevertheless, a loss in radioactivity was evident when solutions containing cholesteryl-4-^{14}C palmitate were perfused. Furthermore, at the termination of the perfusion period, extraction and silicic acid chromatography of the serum lipids demonstrated that the specific activity of both the cholesterol ester and free cholesterol fractions were comparable, which would be compatible with absorption and pooling of cholesterol-4-^{14}C when presented to the mucosa as the palmitate ester. On the other hand, no radioactivity was detected in the serum or tissues of animals perfused with solutions containing cholesteryl-4-^{14}C 2-methylalkanoates, even at the higher (up to 30 mg/100 ml) concentrations of esters perfused.

Recovery of Cholesteryl 2-Methylalkanoates. Tlc separation of lipids extracted from the perfusates showed radioactivity only in the cholesterol ester fraction, as assayed by autoradiography of the plates or liquid scintillation counting of scrapings from the plates. Quantitative glpc of the cholesterol ester fatty acids eluted from the tlc plates showed total recovery of the 2-methylalkanoate, when calculated as counts per minute in cholesterol esters per mole of 2-methylalkanoate in cholesterol esters; that is, the specific activity did not change as a result of perfusion. Infrared assay of samples of the 2-methylalkanoates, eluted as methyl esters by glpc, verified unequivocally the recovery of unaltered 2-methylalkanoate in the perfusates.

TABLE III: Recovery of Perfused Cholesterol Esters in Rat.[a]

Expt No.	Cholesterol-4-^{14}C Ester	Specific Activity cpm/mg of Ester	Intestinal Length (cm)	Perfusion Medium: Infusion Rate (ml/10 min)	Perfusion Medium: cpm/ml	Perfusion Medium: Ester (mg/100 ml)	% Recovered: Volume	% Recovered: BSP	% Recovered: cpm
1	2-Methylmyristate	16,280	24.0	7.98	4,678	28.8	96.8	96.1	97.8
2	2-Methylmyristate	16,280	17.0	7.40	4,211	25.9	91.0	93.2	86.2
3	2-Methylpalmitate	18,250	27.9	7.40	3,655	20.0	93.1	97.7	93.4
4	2-Methylpalmitate	18,250	33.6	7.98	2,787	15.3	98.6	...	93.6
5	Palmitate	20,300	37.0	7.45	606	3.0	94.6	95.8	64.1
6	Palmitate	20,300	32.5	8.20	582	2.9	99.0	101.8	69.0

[a] At least four perfusates were collected, after 10-min periods, in each experiment. The individual samples were then measured for total volume collected, radioactivity, and BSP. The sum of the data from the individual periods was used to calculate recovery, since no significant difference between infusion periods within an experiment was observed.

Analyses of intestinal contents pooled from 12 rats sacrificed 6 hr after feeding cholesteryl 2-methylpalmitate by intubation did not show any alteration of the compound, and cholesteryl 2-methylalkanoates were also recovered unaltered in fecal samples collected up to 48 hr after feeding. On the other hand, alteration was evident in those animals that excreted significant radioactivity after a period of 72 hr, and thus appeared to be a function of the variable transient time through the gastrointestinal tract (Table II).

This alteration was illustrated, *e.g.*, in rat 1, Table II, whereby 20.9% of the fed radioactivity was not excreted until after 72 hr, and 7.5% of the fed radioactivity remained in the intestinal tract after 5 days. Silicic acid chromatography of the pooled lipids extracted from the 72-hr fecal sample and intestinal contents showed the separation of four major radioactive fractions which were tentatively identified by tlc as follows: (a) cholesteryl-4-^{14}C 2-methylmyristate, 36%; (b) sterol-4-^{14}C 2-methylmyristate, 45%; (c) neutral sterols (principally coprostanol), 12%; and (d) residual lipids eluted from the silicic acid column with methanol, 6%. Hydrolysis of fraction b resulted in the release of 2-methylmyristate, as assayed by glpc, but digitonin-precipitable material was not present, even though a positive test for cholesterol was obtained with the ferric chloride–glacial acetic acid reagent. In contrast to these results, of the 11% total radioactivity excreted after 72 hr in rat 7, less than 6% was not identified as cholesteryl-4-^{14}C 2-methylmyristate.

The variation in the excretion time of the fed radioactivity between different animals was not understood; but it was evident that even in those animals that excreted radioactivity 72 hr after feeding that neither cholesterol-4-^{14}C or 2-methylmyristate were present at a significant level in the tissues.

Discussion

The characteristic spectra of methyl substitution at the α-methylene carbon should serve to augment spectral structural assignments associated with the straight-chain fatty acid esters (*cf.* Jones, 1962). Unfortunately, most detailed infrared studies of branched chain fatty acids have been of the free acids, which limits comparative analyses of spectra of the methyl esters. In any event, the use of these spectra to compliment glpc in the unequivocal identification of these acids when following metabolic events in complex lipid mixtures is apparent. This was illustrated, *e.g.*, by the appearance of 2-methylpalmitate in rat serum lipids 3 hr after feeding the free acid, and confirms previous studies with ^{14}C labeled acids, such as 2,2-dimethylstearic (Bergström *et al.*, 1954) and 2-methylstearic (Tryding and Westoo, 1957), that 2-alkyl fatty acids are readily absorbed.

Mueller (1915) has previously shown that the ratio of cholesterol to cholesterol ester in chyle was not altered when either cholesterol or cholesterol esters were fed to dogs, and that fed cholesterol ester (Mueller, 1916) resulted in increased free cholesterol within the intestinal lumen. Thus, the absorption of cholesterol appeared to require hydrolysis of cholesterol esters and esterification of free cholesterol. That hydrolysis of fed cholesterol esters is required for the absorption of cholesterol, as suggested by Swell *et al.* (1955), was further supported by the report that neither *in vitro* hydrolysis of cholesteryl trimethylacetate by pancreatic cholesterol esterase, nor absorption of cholesterol when fed as cholesteryl trimethylacetate to lymph fistula rats, could be demonstrated (Vahouny and Treadwell, 1958). Our studies with cholesterol esters of long-chain, 2-methyl fatty acids would not only exclude the transport of either long-chain fatty acids or cholesterol

mediated as cholesterol esters, but would also be compatible with the thesis that cholesterol must be in the free form for optimal absorption.

Subsequent to the completion of our studies, Hyun *et al.* (1964) reported that cholesteryl-4-^{14}C 2-ethylcaproate was hydrolyzable by pancreatic cholesterol esterase preparations. Furthermore, feeding cholesteryl-4-^{14}C 2-ethylcaproate to lymph fistula rats resulted in recovery of 6.8% of the cholesterol-4-^{14}C in lymph, 2.4% in the small intestine, and 51.4% in the feces and intestinal contents after a period of 24 hr, with a total recovery of 60.6% after 24 hr. As determined by the resistance of cholesteryl 2-ethylcaproate to saponification, these authors concluded that 2-ethylcaproate was not in the lymph or intestine as the ester. The low recovery of radioactivity found in the feces and intestinal contents after feeding the short-chain ester, 2-ethylcaproate, would not be in agreement with our studies using the long-chain 2-methylalkanoates, and indeed represents recovery that was comparable to our data for the natural-occurring cholesteryl palmitate. It is possible that these differences may be resolved on the basis of chain length of the fatty acid, the alkyl group at the 2-position (*i.e.*, methyl *vs.* ethyl), or the experimental conditions. More recently, however, Vahouny *et al.* (1964) reported that 98.1% of fed cholesteryl-4-^{14}C 2,2-methylethylcaproate was recovered from the feces and intestinal contents of rats studied for a period of 12 hr. The latter report would thus be comparable with our studies using the long-chain cholesteryl 2-methylalkanoates.

Unequivocal evidence for the transport of fatty acids across the intestinal mucosa as phospho-, tri-, or diglyceride at significant physiological levels has yet to be demonstrated. It is of interest to note, however, that fatty acids have been shown to be rapidly transported as the free acid, 1- or 2-monoglyceride (Skipski *et al.*, 1959), but not as the cholesterol ester. The latter observation would present evidence that pinocytosis is not a major mechanism for the absorption of lipids, at least in the case of cholesterol esters, and further strengthen the argument that the absorption of fatty acid esters appears to be stereospecific at an enzymic level.

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