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Fatty Acid Ethyl Ester Formation During Ethanol Metabolism *in vivo.* (28277)

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During recent experiments on the enzymatic hydrolysis of cholesterol esters, we observed that rat liver homogenate fractions capable of hydrolyzing cholesterol esters were also active in effecting hydrolysis of ethyl palmitate(1). Since Margolis and Vaughan have reported the esterification of ethanol and other short-chain alcohols with palmitic acid by adipose tissue microsomes(2), this suggested that formation and breakdown of fatty acid ethyl esters might occur during mammalian metabolism of exogenous ethanol. The present experiments were conducted to test this hypothesis.

Four male Sprague-Dawley rats weighing 190-210 g were each given an intravenous injection of 1 ml isotonic saline containing 60 μc of 1-C^{14}-ethanol. The total amount of ethanol injected into rats No. 1 and 2 was 0.15 ml and into rats No. 3 and 4 was 0.05 ml. In addition, rats 1 and 2 received 1 ml 40% ethanol by stomach tube 30 minutes prior to injection. This was done in order to provide a large load of ethanol for metabolism by rats 1 and 2. Since the relative rates of the pathways of ethanol metabolism are not known, the experiment was designed to examine C^{14}-ethanol metabolism at 2 time intervals, after both a small and large load of ethanol. Rats 1 and 3 were killed by cervical fracture 20 minutes following the injection and rats 2 and 4 after 2 hours. Each rat was then passed through a meat grinder and homogenized in a gallon-size blender with 3 liters of ethanol:acetone, 1:1 (v/v). Significant amounts of blood were not lost during the processing. The suspensions were allowed to stand for 2 days with intermittent shaking, to extract all tissue lipids. Portions of each extract were then evaporated just to dryness with a stream of nitrogen. The residue was dissolved in $CHCl_3$:MeOH, 2:1 (v/v) followed by addition of 1/5th vol. water, collection of the lower $CHCl_3$ phase and evaporation of the $CHCl_3$. This solvent partition removed all non-lipid materials from the residue obtained after ethanol-acetone extraction. The lipid residues were then transferred to small vials with light petroleum ether, the petroleum ether evaporated, and the residues finally dissolved in measured small volumes of benzene.

Control experiments have shown that this series of procedures, including 3 complete solvent evaporations and one $CHCl_3$-MeOH solvent partition, completely removes all unreacted labeled ethanol which might be present in the original extract.

Portions of the resultant total body lipid extract from each rat were then directly assayed for radioactivity in a Packard liquid scintillation counter. Other identical portions were hydrolyzed by heating at 60° for 1 hr under nitrogen with 4% KOH in 90% ethanol. After addition of an equal volume of water and acidification with concentrated HCl, each solution was extracted repeatedly with light petroleum ether, until no further radio-

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TABLE I. Effect of Hydrolysis on Total Body Lipid Radioactivity After C^{14}-Ethanol.

Sample from rat No.:	Total lipid radioactivity (cpm × 10^{-2}) Before hydrolysis	After hydrolysis	Decrease of lipid radioactivity on hydrolysis cpm × 10^{-2}	% of total
1	3,305	1,934	1,371	41.5
2	13,702	8,264	5,438	39.7
3	12,684	7,612	5,072	40.0
4	37,655	30,808	6,847	18.2

activity was obtained. The combined petroleum ether extracts were evaporated and the residues assayed for radioactivity. This provided a measure of the total lipid radioactivity present after hydrolysis and acidification.

The results shown in Table I indicate that hydrolysis was accompanied by a considerable decrease in radioactivity in the total lipid fractions. The loss of lipid C^{14} during hydrolysis was close to 40% of the total lipid C^{14} of samples from rats No. 1, 2 and 3, and about half as much with sample No. 4. Since this "loss" represented lipid-soluble radioactivity which became water-soluble (rather than petroleum ether extractable) after hydrolysis, it seemed probable that this loss consisted of labeled ethanol, present as fatty acid ethyl ester before hydrolysis. It was, of course, also possible that the "loss" of radioactivity after hydrolysis partly consisted of some other water-soluble metabolic product of ethanol.

An attempt was then made to isolate this presumed fatty acid ethyl ester fraction from samples 1 and 2, to demonstrate directly its existence; a modification of the method of silicic acid column chromatography of Horning *et al.*(3) was used. Since most of the total body lipids consist of triglycerides, 2 chromatographies were performed. In the first, a single elution with 30% benzene in hexane collected all lipid less polar than triglyceride, and removed most of the triglyceride. The samples were then applied to columns containing 5 g silicic acid, and elutions conducted serially with 10 ml portions of 25% benzene in hexane. Both samples showed a broad peak of radioactivity eluted between 50 and 90 ml. This location for the eluted radioactivity was different from that observed with known major animal tissue lipids, but corresponded to the location expected for fatty acid ethyl esters. In contrast, cholesterol esters appear largely in the first 50 ml of eluate from similar columns and triglycerides are just beginning to appear by 90 ml. Most other lipids (partial glycerides, free fatty acids, free cholesterol, phospholipids) are only eluted from silicic acid columns with more polar solvents. The radioactivity recovered in these peaks was 60 to 70% of the radioactivity listed in the final column of Table I.

Portions of the radioactive material eluted from the columns were hydrolyzed, and the solutions acidified and extracted with petroleum ether as described above. Only 18% of the radioactivity present before hydrolysis in sample No. 1, and 27% of the C^{14} in sample 2, was recovered in the petroleum ether extracts after hydrolysis and acidification. In similar control experiments using ethyl palmitate-1-C^{14}, fatty acid labeled tripalmitin, or cholesterol esters labeled in the cholesterol and/or fatty acid moieties, the recovery of lipid soluble radioactivity after hydrolysis and acidification was always complete. This therefore demonstrated that most of the radioactivity recovered in the peaks from the silicic acid columns was not present in the fatty acid or other lipid components, consistent with its identification in esters of labeled ethanol with fatty acids.

In a final study, portions of the radioactive material obtained from the silicic acid columns were analyzed by gas-liquid chromatography.‡ The effluent from the columns was collected serially at 3 minute intervals on anthracene packed cartridges(4). Definite peaks of mass were observed in locations expected for fatty acid ethyl esters. The recovery of radioactivity in the anthracene cartridges was 99% of the applied radioactivity for sample 1 and 77% for sample 2. Most of

‡ The gas-liquid chromatographic column contained 10% ethylene glycol-adipate polyester on Celite, and was operated at 198°. The detector was an ionization chamber, and the apparatus was kindly made available by Dr. Arthur Karmen.

the radioactivity corresponded in location to esters of palmitic and oleic acids, with smaller peaks corresponding to linoleic acid. This demonstrated that most of the radioactivity recovered from the silicic acid columns had the volatility expected for fatty acid ethyl esters. Since this radioactive material also behaved during silicic acid chromatography in a manner consistent with this identification, and since most of the C^{14} was no longer petroleum ether extractable after hydrolysis and acidification, it seems highly probable that most of the label in this fraction resided in the ethanol moiety, in ester linkage with fatty acids.

At this point the question might be raised whether these labeled ethyl esters were formed *in vivo* during the metabolism of the administered C^{14}-ethanol, or whether they were artifacts formed *in vitro*. Studies of the amount of fatty acid ethyl esters formed during extraction and processing of specimens with ethanol-acetone have shown that 0.1 to 2% of the total fatty acids are converted to ethyl esters, depending on the conditions employed. The present study was so conducted as to minimize this value. Even assuming a maximum value of 2%, however, together with a maximum value of 20 g total fatty acid per animal(5), this would correspond to a maximum of 0.4 g, or about 1.4 mmole of fatty acid esterified with ethanol *in vitro*. The equivalent amount of ethanol (1.4 mmole) is about 65 mg (or 0.08 ml). If we also assume that almost all the administered C^{14} was still present in the extraction mixture as ethanol, this would correspond to a specific radioactivity of 60 μc in 1500 ml, or 0.040 μc per ml. The maximum *in vitro* incorporation of C^{14}-ethanol into fatty acid esters was thus 0.040 μc/ml $\times$ 0.080 ml, or less than 0.006% of the total administered C^{14}-ethanol. Since the observed recovery of C^{14} in this fraction was about 1% of the administered dose, it is apparent that the ethyl esters must have been formed *in vivo*, and present in the animals at time of extraction. Further information about the turnover rate of the ethyl ester fraction is being sought, to try to define the quanitative importance of this pathway of ethanol metabolism. Other studies will deal with the tissues which participate in this pathway, and the effects of chronic alcoholism and other conditions on its operation. In addition, the present findings, together with the observations of Dhopeshwarkar and Mead(6) that methyl esters of fatty acids, once absorbed, persist in various tissues, raise the possibility that simple esters of this type play some significant role in normal lipid metabolism.

Summary. Long-chain fatty acid ethyl esters containing labeled ethanol have been tentatively identified in total body lipid extracts of rats given C^{14}-ethanol intravenously. Identification has included the isolation of a peak of radioactive material with the expected mobility on silicic acid column chromatography, the loss of most of its lipid-soluble radioactivity after hydrolysis, and demonstration of appropriate volatility of the radioactive material on gas-liquid chromatography. About 40% of the total lipid radioactivity was contained in this fraction.

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