The production of methyl esters of fatty acids as artifacts during the extraction or storage of tissue lipids in the presence of methanol

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» Recent experiments in this laboratory have involved the extraction of total lipids from samples of thoracic duct lymph obtained from sheep. The lymph, as collected, was citrated (3 ml saturated Na citrate solution per 100 ml lymph), and lipids were extracted from it by two different methods. In the first method, the lymph was poured into excess ethanol and the precipitate (protein containing most of the lipids) was removed by filtration and extracted under reflux with chloroform–methanol 2:1 (v/v). The aqueous ethanolic filtrate was taken to dryness *in vacuo* at about 60°, and to the residue (mainly salts together with a little lipid) was added the chloroform–methanol extract. The mixture was then refluxed, filtered, and taken to dryness. In the second method, the lymph was lyophilized and then refluxed several times with chloroform–methanol 2:1 (v/v). The filtrates were pooled and taken to dryness *in vacuo* at about 60°, after which the residue was refluxed with chloroform and filtered, and the solvent was removed.

The lipid thus extracted was fractionated on a column of silicic acid as described by Garton *et al.* (1). Elution with light petroleum (b.p. 40–60°) containing 0.5% (v/v) diethyl ether yielded a fraction, a portion of which, on alkaline saponification, gave an amount of fatty acids greatly in excess of that required to account for all the cholesterol present initially in the fraction in esterified form. Triglycerides would not be eluted under these conditions but methyl esters of fatty acids, if present, would be eluted.

A sample of the fraction, unhydrolyzed, was subjected to thin-layer chromatography on Kieselgel G

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(E. Merck, A. G., Darmstadt, West Germany), using 3% (v/v) diethyl ether in light petroleum as developing solvent and 2′, 7′ dichlorofluorescein as spray reagent (2). The fraction was thus separated into two classes of lipids corresponding in R_f values to cholesterol esters and to methyl esters of fatty acids. The latter were subjected to gas-liquid chromatography, as described by Garton *et al.* (1), using an Argon Chromatograph (W. G. Pye and Co. Ltd., Cambridge, Great Britain). The mixture contained a variety of components (e.g., palmitate, stearate, oleate), the retention times of which on polymerized ethylene glycol succinate or Apiezon L (Shell Chemicals Ltd., London) corresponded to those of authentic methyl esters of fatty acids. Regardless of which method was used to extract the lymph lipids, as much as 40% of the total fatty acids were found as methyl esters. In lipids extracted from sheep plasma or liver (by the methods used for lymph), methyl esters of fatty acids were also present, though in very much smaller amounts than in the lymph lipids.

When lyophilized lymph was extracted with acetone or chloroform or with mixtures of acetone and chloroform or diethyl ether, no methyl esters were detected in the extracts nor were they present when total lipids were extracted from the lyophilized material according to the procedure used by Rhodes and Lea (3) for liver tissue. In this method, based on that of Folch *et al.* (4), the tissue is homogenized in the cold with a mixture containing chloroform, methanol, and water; after centrifuging, the aqueous methanolic layer is decanted and the chloroform layer is taken from beneath the plug of protein separating the two layers. Removal of the chloroform yields the lipid. The protein and the aqueous methanolic layer from such an extraction of lyophilized lymph were separately dried *in vacuo* at room temperature and then powdered in an agate mortar. The lipid (containing about 80% of triglyceride) obtained from the chloroform layer was then allowed to stand at room temperature in solution in chloroform–methanol with the addition of powdered protein, powdered aqueous methanolic extract, or both powders. Methyl ester formation took place spontaneously only in the presence of the aqueous methanolic extract. Solutions in chloroform–methanol of tripalmitin or olive oil gave similar results. This methanolysis could be greatly accelerated by refluxing the mixture of triglyceride and powdered aqueous methanolic extract. A portion of the powdered extract, dissolved in distilled water, had a pH value of 9.3 due to the presence of carbonate. A further portion of the powdered extract was ashed in a platinum crucible; the ash retained catalytic potency.

Salts likely to be present in lymph (5, 6) were then examined for their possible catalytic effect in promoting alcoholysis when refluxed with tripalmitin and methanol or ethanol; all the cations were ineffective, and bicarbonate ($NaHCO_3$) was the only anion that promoted methyl (and ethyl) ester production, due to its transformation to carbonate. This transformation is effected by heating or lyophilizing a solution of bicarbonate or (more slowly) during the storage of solutions containing bicarbonate. In the presence of methanol, as little as 1 mg Na_2CO_3/100 mg tripalmitin catalyzed the conversion of 80–90% of the palmitic acid residues to methyl palmitate when the mixture was refluxed for 45–60 min. In addition, it was found that anhydrous conditions are not a prerequisite for carbonate-catalyzed methanolysis to occur. On prolonged standing at room temperature of triglycerides in the presence of methanol containing 10% (v/v) water and traces of Na_2CO_3, no methyl ester formation occurred. When such a mixture was refluxed, however, methyl esters were formed, though much more slowly than in the absence of water. It is noteworthy that the effect of small amounts of carbonate in promoting alcoholysis was reported some 40 years ago by Fischer (7).

In further similar experiments, we found that trace amounts of Na_2CO_3 promoted very rapid formation of methyl esters when free fatty acids were refluxed with methanol and that methanolysis of cholesterol esters and egg lecithin also took place under similar conditions, though apparently not as readily as with triglycerides. The larger amounts of methyl esters formed from thoracic lymph lipids in the presence of carbonate can be associated with the high content of triglycerides in lymph compared with that in plasma, though the bicarbonate content of the two tissue fluids is of the same order, namely, 26–30 mEq/liter.[1]

From our observations, it is evident that measures should be taken to eliminate bicarbonate when lipids are being extracted from tissues in the presence of methanol or ethanol. In addition, it is clear that no lipid should be stored in solvent containing methanol or ethanol if there is any chance that traces of carbonate are present or potentially present. Though we have shown that methyl esters can readily arise as artifacts during the extraction of lipids, it should be noted that trace amounts of methyl esters of fatty acids have been reported by Dhopeshwarkar and Mead (8) as normal constituents of the tissue lipids of guinea pigs.

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