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Simultaneous quantitation of ^{14}C-labelled cholesterol and individual long-chain fatty acids by paper chromatography

As the result of lipid biosynthesis, ^{14}C-labelled fatty acids and cholesterol appear together. Even though cholesterol is generally found in much smaller amounts, its separation from the corresponding fatty acids is often of vital analytical importance owing to its relatively high radioactivity content.

In this laboratory we assay fatty acids by paper chromatography according to methods developed by KAUFMANN and coworkers[1,2]. Comments on the quantitative aspects of these methods have been published previously[3,4] and an example of their use in the assay of ^{14}C-labelled rat liver fatty acids is given in ref. 5. Chromatography is based on a reversed-phase system with undecane as the stationary phase and acetic

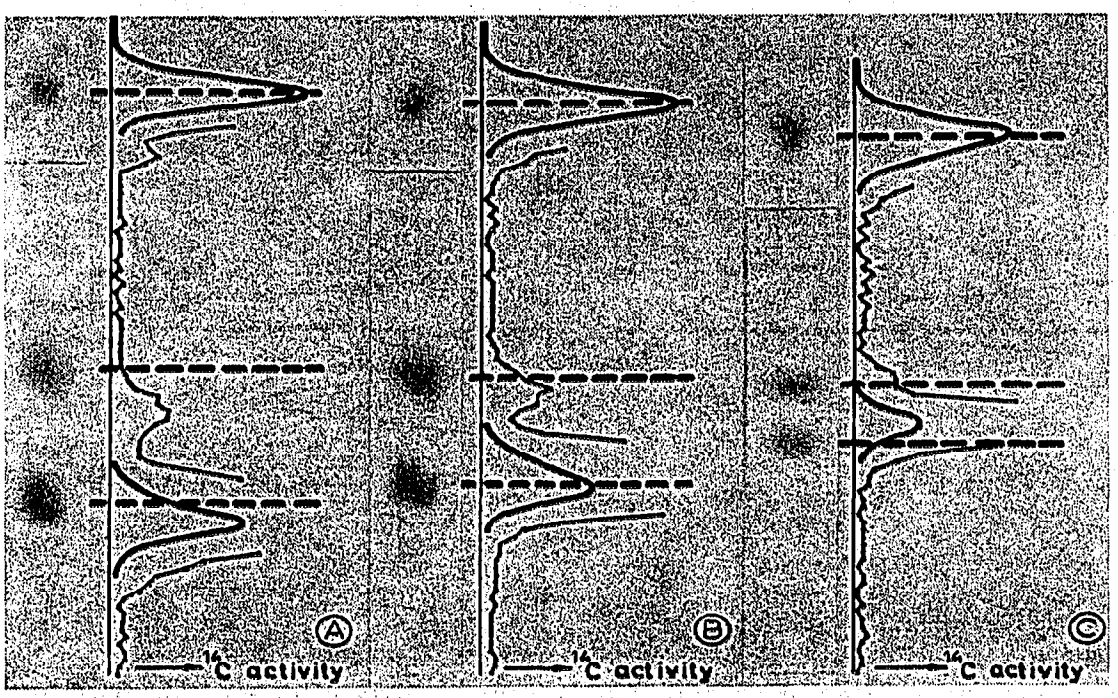

Fig. 1. Paper chromatograms of a sample from Fraction III (free cholesterol, free fatty acids) after silicic acid column chromatography according to LIS *et al.*[8] Lipids were synthesized from ^{14}C-acetate by rat liver slices. Equal amounts of unlabelled palmitic acid and myristic acid were added for the sake of identification. The papers were developed at 25° (from the bottom upwards on the figure; the palmitic spots are thus seen below the myristic spots) with three different acetic acid concentrations in the mobile phase, namely: (A) 80%, (B) 90% and (C) 95%. Spots of a standard ^{14}C-labelled stearic acid, that had been run alongside each of the chromatograms, were cut out and fixed to the paper strips before counting in order to ensure exact correlation between radioactivity and colour. The radioactivity measurements are shown on two different scales, one ten times the other.

acid as the mobile phase. The fatty acids are visualized by transformation into their copper soaps and release of the added cupric ions by treatment with dithio-oxamide to form a green copper complex suitable for quantitative evaluation by densitometry. Critical pairs are evaluated by rechromatography after direct hydrogenation of the

fatty acid mixture on the paper. Radioactivity is measured by automatic scanning intermittently with a special anticoincidence-corrected low-level counter.

KAUFMANN AND KOHLMEYER[6] have shown that cholesterol can move in an undecane–acetic acid system. Notwithstanding PORTER AND TIETZ[7] do not distinguish between saponifiable and unsaponifiable matter before chromatography in a kerosene–acetic acid system, and we must admit that we too have previously[5] failed to realize the degree of interference between cholesterol and palmitic acid radioactivity in our system. It now turns out that in certain circumstances cholesterol and palmitic acid will have exactly the same R_F values.

Results

Results from paper chromatography of the free cholesterol and the free fatty

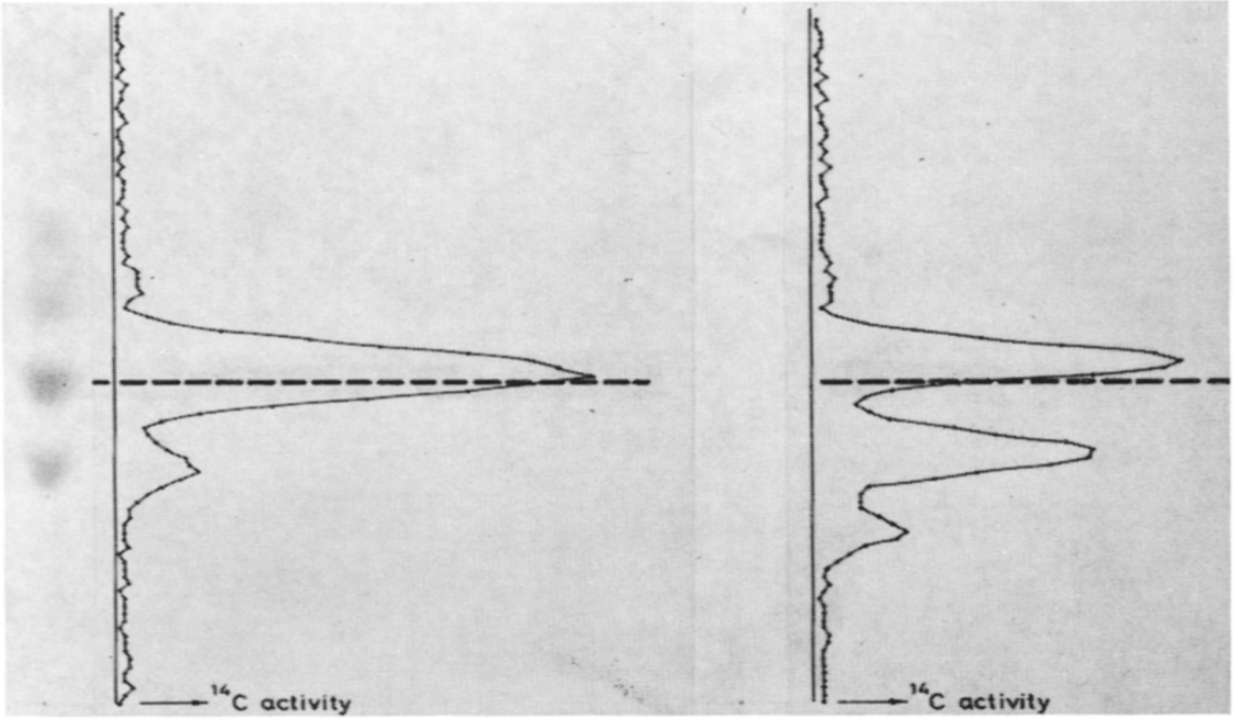

Fig. 2. Paper chromatograms of a sample of total lipids from rat liver after saponification and acidification. Lipids were incorporated from ^{14}C-acetate *in vivo*. The chromatograms were developed on the same sheet with the same conditions as in Fig. 1 and with 95% acetic acid as the mobile phase. On the left-hand side the spots represent from bottom to top: mainly stearic acid, mainly palmitic or oleic acid and myristic acid surrounded by various unsaturated acids with chain lengths of 18 or more. On the right-hand side the fatty acids have been transformed into their methyl esters before chromatography, by refluxing for 2 h at 90° in absolute methanol containing 5% HCl, and thus no colour is seen.

acid fraction of a sample of biologically labelled lipids are shown in Fig. 1. With 80% acetic acid as mobile phase the main radioactivity lies behind the palmitic acid spot; with 90% acetic acid it coincides with the palmitic spot; with 95% acetic acid it lies in front of the palmitic spot. A comparison with the chromatographic behavior of cholesterol-4-^{14}C has among other evidence identified this main radioactive fraction as cholesterol. Four per cent of the radioactivity lies near the myristic acid spot and

has not been identified. It seems to be a degradation product of cholesterol since it also changes its position relative to the fatty acids as the polarity of the mobile phase changes.

Fig. 2 shows how the problem of assaying the individual fatty acids in a biologically labelled sample also containing cholesterol can be solved directly, without separation of saponifiable from unsaponifiable matter before chromatography. Two

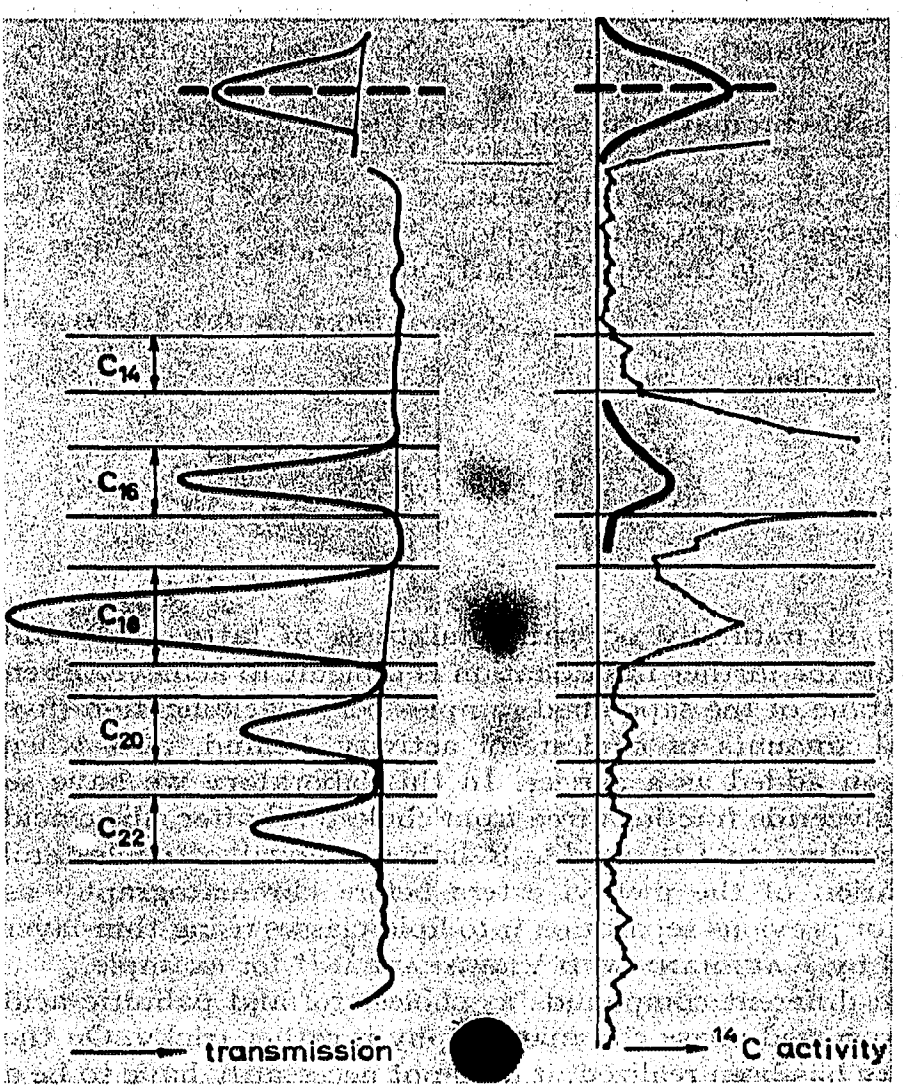

Fig. 3. The same sample as in Fig. 2 chromatographed after direct hydrogenation at the starting point according to KAUFMANN[1]. Densitometry measurements are shown together with radioactivity measurements, the latter on two different scales, one ten times the other. As in Fig. 1, the measurements also include a separate spot of standard stearic acid with a known specific activity. The mobile phase is 95% acetic acid.

chromatograms have been run parallel to each other on the same sheet, one to the left in the usual way, the other after transformation of the fatty acids into their methyl esters. In the former the main amount of radioactivity is seen to have a skew distribution around the palmitic–oleic acids spot; in the latter the colour has disappeared, but on tracing the radioactive areas back to the left hand chromatogram one sees that

the original single main radioactive spot has split into two nearly equal spots, one of which has remained in position, while the other has moved about one step backwards together with the original stearic acid activity. Ninety-five per cent acetic acid was used as the mobile phase and a comparison with (C) in Fig. 1 shows that in the right hand side uncoloured chromatogram in Fig. 2 the leading (seen as the top) radioactive spot is cholesterol, followed by methyl palmitate or oleate and methyl stearate.

In Fig. 3, the sample containing both cholesterol and fatty acids has been hydrogenated on the paper before chromatography. This enables a chain-length analysis of the fatty acids other than the C_{16} and C_{14-16}, which are contaminated by cholesterol.

Based on the three types of chromatograms shown in Figs. 2 and 3 one gets the following quantitative results:

Specific activity of fatty acids: 43 μC/g fatty acid
Specific activity of cholesterol: 33 μC/g fatty acid
Percentage of incorporated ^{14}C in the separate fatty acids:

Myristic	2
C_{14-16}	7
Palmitic and palmit–oleic	57
C_{16-18}	4
Stearic	16
Oleic	9
Unsaturated $> C_{18}$	5

Discussion

A complete separation of naturally occurring mixtures of fatty acids and cholesterol in amounts suitable for further fractionation is difficult to achieve. Even repeated extraction with pentane of the saponified samples before acidification (five times) will leave substantial amounts of cholesterol activity behind, also when unlabelled cholesterol has been added as a carrier. In this laboratory we have so far been unable to obtain triglyceride fractions free from cholesterol after silicic acid column chromatography according to LIS *et al.*[8]. Reliable removal of cholesterol requires either microsublimation of the methyl esters before chromatography as described by STOFFEL *et al.*[9], or previous separation into lipid classes using thin-layer chromatography as described by KAUFMANN AND VISWANATHAN[10] for example.

The fact that two such different compounds as cholesterol and palmitic acid behave so very similarly on reversed-phase chromatography is rather tricky. On the other hand, once this difficulty has been realized, it does not necessarily have to be a disadvantage. The interference can be overcome by simple methylation, and one ends up with supplementary information on cholesterol metabolism.

It should be stressed in favor of direct paper chromatography, that the quantitative data presented above are based on only three chromatograms that have been measured with relatively inexpensive equipment. The results are by no means exhaustive, but not all investigations require full analytical details, and simplified methods are often to be preferred in the assay of systems with large biological variations that call for many repeated experiments. Finally, changing the polarity of the mobile phase as shown in Fig. 1 can be used to test the effectivity of any previous removal of unsaponifiable matter.

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The Danish Atomic Energy Commission
Research Establishment, Risö (Denmark)

HEINZ J. M. HANSEN

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