

matoid arthritis, two congestive heart failure, and 10 nonspecific pleural effusion.

Blood and pleural fluid were collected on the same day, defibrinated, and stored for a maximum of nine months at  $-20^{\circ}\text{C}$  until assayed. The B-subunit of the CK-BB and CK-MB isoenzymes was determined with a radioimmunoassay (CK-B RIA kit; Nuclear Medical Systems, Inc., Newport Beach, CA 92663). Its concentration in plasma was considered abnormal when it exceeded  $12.5\ \mu\text{g/L}$ . Samples with above-normal values for CK-B were also analyzed by electrophoresis and only BB and MM fractions were found.

Thirteen of the patients with malignant disease (Figure 1) and 10 of the patients with benign disorders had measurable concentrations ( $\geq 2\ \mu\text{g/L}$ ) of CK-BB in pleural fluid. Abnormal concentrations in plasma were found in four patients: one with adenocarcinoma of the lung (CK-BB  $15.6\ \mu\text{g/L}$ ), one with tuberculosis ( $30.0\ \mu\text{g/L}$ ), one with bacterial pneumonia ( $16.0\ \mu\text{g/L}$ ), and one with nonspecific pleural effusion ( $14.2\ \mu\text{g/L}$ ). Values for CK-BB in pleural fluid exceeding  $12.5\ \mu\text{g/L}$  were found in four patients: two with adenocarcinoma of

the lung ( $19.0$  and  $59.2\ \mu\text{g/L}$ ), one with anaplastic carcinoma of the lung ( $14.0\ \mu\text{g/L}$ ), and one with tuberculosis ( $17.0\ \mu\text{g/L}$ ). The three patients with malignant pleural effusion and a high CK-BB concentration in pleural fluid also had a high pleural fluid/plasma CK-BB ratio (3.7 or higher). In contrast, all patients with benign disorders had a pleural fluid/plasma CK-BB ratio of 1.6 or lower. Tumor cells were found in two of the three malignant effusions with high CK-BB concentrations.

The probable source of the circulating CK-BB in cancer patients is the malignant glandular epithelium (6). The high pleural fluid/plasma CK-BB ratio observed in three of our patients with malignant pleural effusion suggests that CK-BB was produced locally by the tumor.

Our results show that the combination of a high CK-BB concentration in pleural fluid and a high pleural fluid/plasma CK-BB ratio strongly indicates that the effusion is caused by a malignant disease. This finding, however, lacks sensitivity; it was diagnostic in only 20% of patients with malignant pleural effusion.

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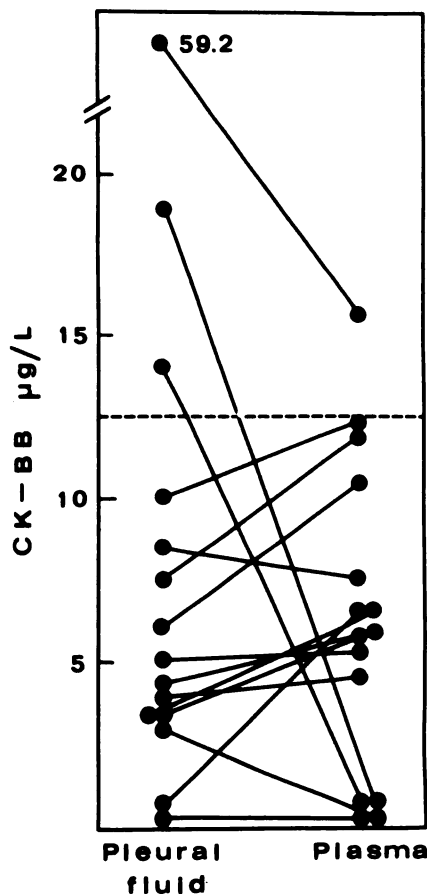


Fig. 1. Concentration of CK-BB in pleural fluid and plasma from 15 patients with pleural effusion caused by malignant disease

The dotted line indicates the upper normal concentration ( $12.5\ \mu\text{g/L}$ ) of CK-BB

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#### Errors in Determination of Fatty Acid Composition of Cholesteryl Esters after Extraction from Serum with *n*-Octane

To the Editor:

The fatty acid composition of serum cholesteryl esters reflects the composition of dietary fat (1, 2) and such data are a useful tool for estimating the dietary fatty acid composition in groups of free-living subjects.

Rockerbie et al. (3) recently described a rapid assay for determining the cholesteryl ester fatty acid composition by using extraction with *n*-octane instead of the time-consuming method involving thin-layer chromatography to separate cholesteryl esters from other lipids in serum.

We have now re-investigated this method and found that fatty acids from serum triglycerides (triacylglycerols) are included with the cholesteryl ester fatty acids in the Rockerbie method. This method is a modification of the procedure described by Peter and Reynolds (4), in which *n*-octane is used to extract the cholesteryl esters from an alkaline aqueous isopropanol mixture. Peter and Reynolds did report that the cholesteryl esters extracted from serum by their method were contaminated with an "insignificant" amount of triglyceride.

To investigate the problem, we carried out two experiments. In Experiment 1, the fatty acids in a pooled sample of fresh serum from normolipemic donors were analyzed by the method of Rockerbie et al. (3) except that the fatty acid methyl esters were separated by gas-liquid chromatography as described earlier (5). If *n*-octane contained substances that interfered with the subsequent chromatographic analysis, it was purified before use. In addition, the lipids of the pooled sample of serum were extracted by the method of Folch et al. (6) and then separated by chromatography on thin layers (0.5 mm) of Silica Gel H (E. Merck, Darmstadt, F.R.G.), with petroleum ether (b.p.  $40-60^{\circ}\text{C}$ )/diethyl ether/glacial acetic acid (160/30/5 by vol) as solvent. Rhodamine 6G was incorporated in the gel layer, to make visible the components of the mixture under ultraviolet light. The area of silica gel corresponding to cholesteryl esters was scraped off the plate and, after extraction with chloroform, the cholesteryl esters were hydrolyzed and the fatty acids methylated and analyzed as described above. The results of the fatty acid analysis are presented in Table 1. It is apparent from this table that, with the Rockerbie method, the proportion of the  $\text{C}_{16:0}$ ,  $\text{C}_{18:0}$ , and  $\text{C}_{18:1}$  fatty acids is higher and that of  $\text{C}_{18:2}$  is lower. This suggested to us that the fraction containing the cholesteryl esters was contaminated with triglyceride.

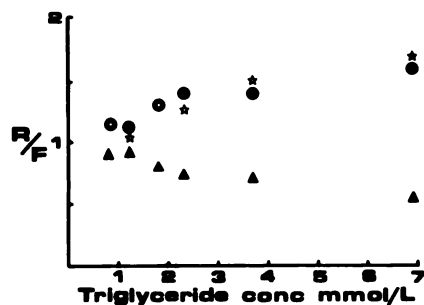
To test this hypothesis, we carried out

**Table 1. Fatty Acid Composition of the Fraction Containing the Cholesteryl Esters from Serum, When Isolated by Folch Extraction Followed by Thin-Layer Chromatography or by the Method of Rockerbie et al. (Experiment 1)**

Fatty acid	Mean proportion, % by weight (and SD) <sup>a</sup>	
	Folch/TLC method	Rockerbie method
C <sub>14:0</sub>	0.7 (0.18)	0.7 (0.19)
C <sub>16:0</sub>	11.3 (0.53)	13.4 (0.62) <sup>b</sup>
C <sub>16:1</sub>	3.9 (0.24)	3.7 (0.24)
C <sub>18:0</sub>	1.2 (0.12)	2.5 (0.15) <sup>b</sup>
C <sub>18:1</sub>	19.6 (0.55)	21.4 (0.39) <sup>b</sup>
C <sub>18:2</sub>	58.0 (0.92)	53.1 (0.89) <sup>b</sup>
C <sub>20:4</sub>	5.6 (0.50)	5.3 (0.35)

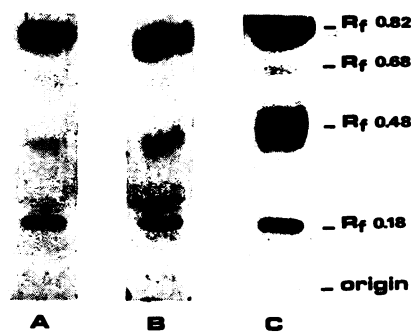
<sup>a</sup> Calculated as a proportion of the sum of the fatty acids listed. <sup>b</sup> Comparisons of Student's *t*-test with the Folch/TLC method: *p* < 0.001.

Experiment 2 to establish the effect of the triglyceride concentration on the composition of the fatty acids of the cholesteryl esters, using the Rockerbie technique. Six different samples of fresh serum with different triglyceride concentrations were analyzed by the methods described above and from the results (Figure 1) it can be clearly seen that the errors associated with the fatty acid analysis of the cholesteryl ester fraction increase with increasing concentrations of triglyceride in the serum. The ratios for C<sub>16:0</sub> were almost identical to those observed for C<sub>18:1</sub>. The fraction containing the cholesteryl esters isolated as in the Rockerbie method from three of the serum samples with different triglyceride concentrations were separated by chromatography on thin layers of Silica Gel H as described above, except that Rhodamine 6G was not incorporated in the gel layer. The different lipid bands on the plate were then made



**Fig. 1. Effect of triglyceride concentration on the estimation of the fatty acid composition of the cholesteryl esters of serum method (Experiment 2)**

Results expressed as ratio of value obtained by the Rockerbie method (R) divided by the value obtained by Folch extraction followed by thin-layer chromatography (F): ☆, C<sub>16:0</sub>; ●, C<sub>18:1</sub>; and ▲, C<sub>18:2</sub>



**Fig. 2. Photograph of a thin-layer chromatographic plate of the cholesteryl fraction of sera extracted as in the Rockerbie method**

A, B, and C correspond to the serum samples with triglyceride concentrations of 0.8, 1.2, and 6.9 mmol/L, respectively, (Figure 1). The bands are as follows: R<sub>f</sub> = 0.18, cholesterol; R<sub>f</sub> = 0.48, triglyceride; R<sub>f</sub> = 0.68, fatty acid isopropyl esters; and R<sub>f</sub> = 0.82, cholesteryl esters

visible by spraying with aqueous sulfuric acid (100 mL/L) and heating for 15 min at 200 °C (7) (Figure 2).

Contamination of the fraction containing the cholesteryl esters is also clearly seen in Figure 2. The cholesteryl esters (R<sub>f</sub> = 0.82) are not only contaminated with triglyceride (R<sub>f</sub> = 0.48) but also with material with an R<sub>f</sub> of 0.68. The extent of the contamination increases with the triglyceride concentration. Free cholesterol (R<sub>f</sub> = 0.18) is also present, but this would not interfere with the fatty acid analysis of the cholesteryl esters.

To characterize the material with an R<sub>f</sub> of 0.68, we repeated the extraction and chromatography procedure with the pooled serum sample used in Experiment 1. The area of the plate corresponding to the material with an R<sub>f</sub> of 0.68 was scraped from the plate, eluted with chloroform, and analyzed by mass spectrometry. The results obtained are consistent with the material being a mixture of isopropyl esters of fatty acids.

Thus it would appear that esterification or transesterification with isopropanol occurs during the initial extraction procedure or in the subsequent removal of solvent, which would contain traces of isopropanol dissolved in the octane. The fatty acid composition of the isopropyl esters (C<sub>14:0</sub>, 1.0%; C<sub>16:0</sub>, 28.6%; C<sub>16:1</sub>, 2.2%; C<sub>18:0</sub>, 9.9%; C<sub>18:1</sub>, 41.5%; C<sub>18:2</sub>, 14.8%; and C<sub>20:4</sub>, 2.0%) would suggest that the fatty acids were more likely to be derived from the triglyceride or free fatty acid fraction of the serum lipids rather than the cholesteryl esters. Triglycerides and free fatty acids contain more C<sub>16:0</sub> and less C<sub>18:2</sub> than do cholesteryl esters (2). It is also possible that a particular class (or classes) of phospholipids or sphingolipids contributes to the isopropyl ester fraction.

Our results suggest that contamination of the cholesteryl ester fraction ex-

tracted by the method of Rockerbie et al. can be attributed to contamination with both triglyceride and isopropyl esters possibly also largely derived from triglyceride. The error associated with the method is more pronounced when sera with high concentrations of triglyceride are analyzed. Value reported by this method—e.g., by ourselves (5)—should be modified. The proportions of palmitic acid (C<sub>16:0</sub>) found in the cholesteryl esters should be decreased and that of linoleic acid (C<sub>18:2</sub>) should be increased, and the extent of the correction would depend on the concentration of triglyceride. However, the method can still be usefully used to compare groups of people with similar concentrations of triglyceride but different cholesteryl ester fatty acid patterns in their serum.

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