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# Notes on Methodology

## Cholesterol measurement in normal and lipemic sera: elimination of an extraneous chromogen

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» Unusually high and variable cholesterol values were obtained in an attempt to apply the method of Zlatkis *et al.* (1) to normal and lipemic sera (Table 1). Further investigation demonstrated that the variability could be reduced by extraction and saponification of the lipids prior to color development; the values so obtained were in agreement with those found using the method of Sperry and Webb (2). The resulting procedure, incorporating these findings, permits the rapid determination of cholesterol in both tissues and sera at macro and micro levels.

*Procedure.* The lipids are coprecipitated with the proteins from a sample containing from 0.2 to 10 mg of cholesterol by the addition of trichloroacetic acid to a final concentration of from 5% to 10%. After centrifuging and discarding the supernatant liquid, the total lipids are extracted from the residue with 2 ml of 0.1 N potassium acetate in absolute alcohol. The extraction is repeated twice with 2 ml of absolute alcohol each time, and the three extracts are combined. Saponification is carried out by adding 0.2 ml of 33% (w/w) KOH to the combined alcohol extracts and incubating at 40° for 1 hour. The nonsaponifiable lipids are subsequently extracted into 10 ml of high boiling petroleum ether, using the technique described by Abell *et al.* (3), and a suitable aliquot of the petroleum ether extract is evaporated to dryness. A standard and a blank are carried through the saponification, petroleum ether extraction, and drying steps. The dried residues are dissolved in glacial acetic acid, and color development and measurement are carried out, using the ferric chloride reagent and the technique described by Zlatkis *et al.* (1).

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TABLE 1. EFFECT OF EXTRACTION AND SAPONIFICATION ON CHOLESTEROL VALUES FOR HUMAN SERA

| Sample No. | Without Extraction* | With Extraction and Saponification | | |
|---|---|---|---|---|
| | | Spectrophotometric† | Fluorometric† | Sperry and Webb‡ |
| | *mg/100 ml* | *mg/100 ml* | *mg/100 ml* | *mg/100 ml* |
| 1 | 249 | 152 | 146 | 146 |
| 2 | 268 | 162 | 158 | 159 |
| 3 | 321 | 198 | 190 | 200 |
| 4 | 259 | 152 | 148 | 156 |
| 5 | 273 | 166 | 163 | 164 |
| 6 | 285 | 178 | 171 | 189 |
| 7 | 270 | 172 | 166 | 175 |

* Direct method of Zlatkis *et al.* (1).
† Sample preparation and measurement as described in the text.
‡ See Ref. 2.

By reducing the volume tenfold, the same sample preparation technique may be carried out at a micro level, and the cholesterol measured with a Farrand Model A fluorometer (4). Because of the increased sensitivity of the fluorometric measurement, the original sample need only contain from 2 to 100 μg of cholesterol. The reagent of Albers and Lowry (4) and their method of addition have been modified as follows: Acetic anhydride, 1,1,2-trichloroethane, and sulfuric acid are mixed in the proper proportion immediately before use. One milliliter of the reagent is added to the dried residues with immediate and thorough mixing. This modification results in an approximately twofold increase in the galvanometer deflection for the same amount of cholesterol, and requires only one manipulation instead of two (Fig. 1). Lang-Levy constriction pipettes (5) are used for all additions and transfers in the micromethod and a "buzzer" (6) is used for mixing.

The procedures employed in the sample preparation

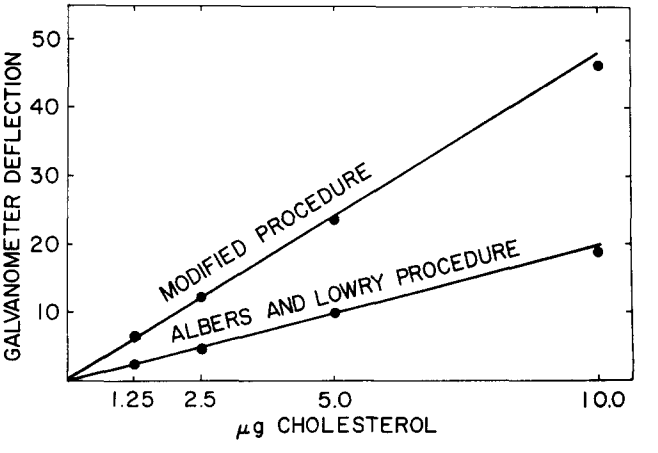


FIG. 1. Increased sensitivity using proposed reagent and method of addition.

have been demonstrated to be quantitative by various authors and by the data included herein. Tourtellotte *et al.* (7), Robins *et al.* (8), and Zilversmit and Davis (9) have demonstrated that lipids are quantitatively coprecipitated with proteins by trichloroacetic acid at a final concentration of from 5% to 10%. The use of this procedure makes possible the precipitation of lipids from various types of samples. Robins *et al.* (8) and Albers and Lowry (4) have shown that lipids are quantitatively extracted from the protein precipitate by alcoholic potassium acetate and absolute alcohol, as described. Abell *et al.* (3) have shown that cholesterol is quantitatively extracted into petroleum ether from an equal volume of 50% alcohol.

Table 2 shows the apparent cholesterol level of pooled rat sera when measured directly, and the level in various fractions prepared according to the method described in the text. The petroleum ether extract yields a value which is in good agreement with the value obtained by the method of Sperry and Webb (2). In addition, $C^{14}$-cholesterol added to the original samples is quantitatively recovered in the petroleum ether extract, indicating that the decrease in the cholesterol level was not due to loss of the sterol but rather to the removal of interfering substances prior to measurement. This is confirmed by the spectral data (Fig. 2). The spectrum of the petroleum ether fraction closely parallels that of cholesterol and produces a lower absorbency at 560 mμ than the directly measured serum or the unsaponified alcohol extract. Equivalent amounts of both the trichloroacetic acid precipitate and the saponifiable material contribute to the absorbency of 560 mμ, and hence produce the high value for cholesterol when serum is measured directly.

TABLE 2. CHOLESTEROL VALUES OF VARIOUS FRACTIONS OF RAT SERUM AND RECOVERY OF ADDED $C^{14}$-CHOLESTEROL

| Sample | A | B | A | B |
|---|---|---|---|---|
| Fraction | Cholesterol | | | |
| | *mg/100 ml* | | *cpm* × $10^{-3}$* | |
| Serum† | 137 | 67 | 906 | 907 |
| Unsaponified ethanol extract‡ | 128 | 65 | 924 | 924 |
| Petroleum ether extract‡ | 102 | 57 | 917 | 912 |
| Digitonin precipitate§ | 98 | 51 | | |

* $C^{14}$-cholesterol was added to the serum sample and an aliquot was removed for direct determination of cholesterol and counting. The remainder of the sample was fractionated as described in the text and aliquots of the fractions were removed for cholesterol measurement and counting. Counting was done in a liquid scintillation counter using the solvent described by Werbin *et al.* (10).

† Analysis by direct method (1).

‡ Sample preparation as described in text. Color development and measurement as described in Ref. 1.

§ Analysis by the method of Sperry and Webb (2).

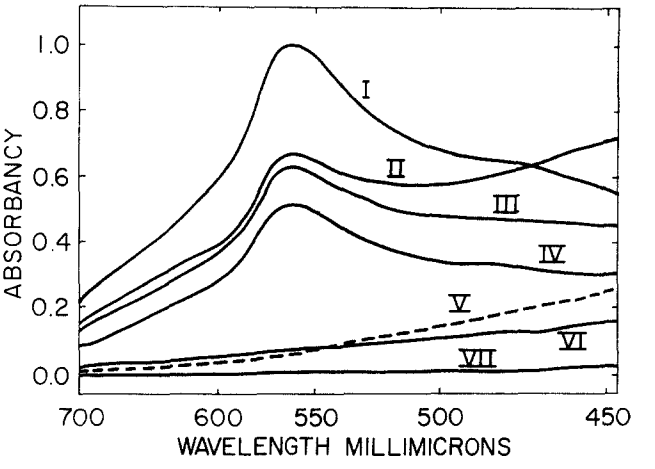


FIG. 2. Spectrum of cholesterol, serum, and fractions obtained during sample preparation. Color development and measurement as described in Ref. 1. *I*, cholesterol; *II*, serum; *III*, unsaponified ethanol extract; *IV*, petroleum ether extract; *V*, redissolved trichloroacetic acid precipitate after extraction of lipids; *VI*, saponified ethanol extract after removal of nonsaponifiable material; and *VII*, reagent blank.

The exact nature of the interfering material has not been established, but the need for its removal prior to measurement is evident from the preceding experiments. The method of Sperry and Webb (2) removes extraneous material by digitonin precipitation of the sterol, which requires approximately 13 hours for completion. The present procedure allows analysis to be completed in a single day, and may be applied to tissues as well as sera on either a macro or micro level.

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