

### **Improved procedure for the extraction of lipids from human erythrocytes**

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**SUMMARY** A procedure for the extraction of human erythrocyte lipids using chloroform-isopropanol 7:11 (v/v) is described. It is simple and reproducible, affords almost quantitative extraction of cholesterol and phospholipid, uses a single extraction tube, and yields an extract only slightly contaminated by heme.

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DESPITE THE GENERAL applicability of the lipid extraction procedure of Folch, Lees, and Sloane Stanley (1) employing chloroform-methanol 2:1 (v/v), we have found that its use for extraction of human erythrocyte lipids is unsatisfactory. Values obtained for erythrocyte cholesterol are low and, in addition, extracts are contaminated by a brown pigment that is difficult to remove. Similar difficulties were encountered by Ways and Hanahan (2), who reported poor extraction of phospholipid and contamination of extracts with water-soluble substances. Reed, Swisher, Marinetti, and Eden (3) have reported variability in total lipid extraction with the Folch method and have proposed an alternative method utilizing methanol-chloroform 1:1. Values for human erythrocyte cholesterol obtained with their method were in accord with those of Brun (4) and of Munn (5) using different extraction procedures, while lower and more variable values have been reported by other investigators (6-8). Phospholipids were also well extracted. The method does not require high temperatures (4); it is well supported by studies establishing completeness of extraction and appears to be the most reliable of the methods currently in use.

Nevertheless, the method of Reed et al. (3) has several disadvantages. The procedure requires four initial extractions and three further extractions of the residue. In addition, the initial extract is contaminated by heme pigment and denatured proteins, which are apparently removed by dissolving the dried lipids in redistilled anhydrous chloroform. These pigments cause quenching in liquid scintillation counters and may act to stimulate *in vitro* oxidation of lipids (9). Furthermore, they give rise to high values for cholesterol when this is measured by the  $\text{FeCl}_3\text{-H}_2\text{SO}_4$  method of Zlatkis, Zak, and Boyle (10, 11). In the present study, we have found that heme pigment is extracted by all solvents containing methanol. A simpler procedure employing isopropanol instead of methanol, which extracts little pigment and gives essentially quantitative extraction of cholesterol and phospholipids, is described below.

**Procedure.** Reagent grade chloroform (redistilled) and isopropanol are used. Whole blood treated with trisodium ethylenediaminetetraacetate (5 mg/ml) is centrifuged at  $2280 \times g$  for 30 min. The packed red cells are washed 3 times with 5 volumes of 0.89% NaCl. The buffy coat is removed and 1.0 ml of packed erythrocytes is pipetted by means of a siliconized serologic pipette into a 20 ml tube with a Teflon-lined screw cap. One milliliter of distilled water is added, and the contents of the tube are mixed

with a Vortex Junior Mixer and allowed to stand for 15 min. Isopropanol (11 ml) is added slowly with mixing. The cells gradually turn dark and clump. After 1 hr with occasional mixing, 7.0 ml of chloroform is added and mixed. At the end of another hour, the tube is centrifuged at  $500 \times g$  for 30 min. The extract can be sampled directly for determination of cholesterol by the Zlatkis method provided the solvents are removed by evaporation before the addition of acetic acid. Contaminating flecks of clumped red cells do not affect the results; they can be removed if desired by filtration through glass wool. The total fluid volume in the tube is 19.6 ml. For determination of lipid phosphorus a washing procedure to remove inorganic phosphorus was found to be essential. We transferred the extract into chloroform-methanol 2:1 (v/v) and washed with 0.2 vol of 0.05 N KCl (1), but other procedures would doubtless be applicable. The entire extraction is carried out at room temperature.

Values for erythrocyte cholesterol using the isopropanol extraction in seven hospitalized patients with non-systemic diseases gave a mean of  $1.37 \pm 0.08$  mg/ml packed cells, in good agreement with Reed et al. (3), Brun (4), and Munn (5). Overnight (15 hr) extraction, mechanical blending, and saponification with 20% ethanolic KOH at  $116^\circ$  for 16 hr failed to yield more cholesterol from the extraction residue. Replicate determinations on one preparation of cells gave 1.38, 1.39, 1.40, and 1.35 mg/ml. Recovery of cholesterol- $4\text{-C}^{14}$  from erythrocytes equilibrated with labeled plasma indicated that  $\text{C}^{14}$  lost from the plasma was quantitatively recoverable in the cells by this procedure. Extraction of fatty acids from erythrocytes was similarly found to be satisfactory. Saponification of the extracted residue followed by titration of the liberated fatty acids (12) indicated that a maximum of 2.9% of phospholipids might not have been extracted.

In Table 1 are compared lipid values from one preparation of washed human erythrocytes extracted by seven different solvent mixtures. Mixture 1 (Folch) gave a low value for cholesterol, apparently because of the high proportion of chloroform, since when this ratio is reduced to 7:11 (mixture 2) higher values are obtained. Poor extraction with mixtures 5 and 6 was probably due to the partial phase separation which was observed. Satisfactory cholesterol values were obtained with chloroform-isopropanol, chloroform-ethanol, or ether-ethanol. All procedures gave similar values for erythrocyte phospholipid except for the chloroform-isobutanol and possibly the ether-ethanol mixture.

A more detailed comparison between the Folch and isopropanol extractions of erythrocyte total cholesterol was made using cells from five hospitalized subjects with a variety of illnesses. Washed erythrocytes were prepared from blood which had been defibrinated by shaking with

glass beads. For the Folch extraction, packed erythrocytes (1.0 ml) were lysed in a separatory funnel with 1.0 ml of distilled water. Methanol (15 ml) was added slowly with mixing, followed after 15 min by 30 ml of chloroform. After 1–2 hr at room temperature, 7.0 ml of 0.05 N KCl was added and the phases were allowed to separate overnight at 4°. The lower chloroform phase was then dried in a flash evaporator at 30°, the lipid was dissolved in chloroform, and aliquots were taken for cholesterol determination. Four paired comparisons with the chloroform–isopropanol procedure were made, each time using fresh erythrocytes. The Folch extraction gave lower values, varying from –4.8 to –18.0%, with a mean difference of –11.2%. Five paired comparisons were made using samples of the same cells after 7–10 days storage of whole blood (nonsterile) at 4° in Acid–Citrate–Dextrose Solution (Baxter Laboratories, Morton Grove, Ill.) at a ratio of 1.0 ml of ACD Solution to each 3.2 ml of blood. Again the Folch extraction gave lower values in every case, ranging from –8.6 to –24.0%, mean –19.3% ( $P=0.01$ , 11 values). Thus the Folch extraction as applied by us to human erythrocytes gives consistently low values for cell cholesterol.

The Folch extraction also suffers from the disadvantage of producing lipid extracts which are highly pigmented. Reed et al. (3) have commented on this problem and suggested that the (major) pigment is heme. Spectra of lipid extracts from the same preparation of cells extracted by the Folch and isopropanol procedures are compared in Fig. 1. A peak was observed in both extracts at 400  $m\mu$ , which corresponded to that of hemoglobin in an organic solvent mixture. Absorbancy at 400  $m\mu$  for the Folch extract was nearly three times as great as for the isopropanol extract.

TABLE 1 RELATIVE EFFECTIVENESS OF DIFFERENT SOLVENT MIXTURES FOR EXTRACTION OF HUMAN ERYTHROCYTE LIPIDS

Solvent Mixture*	Total Cholesterol	Phospholipid†
(1) Chloroform–methanol 2:1	1.00‡	3.05
(2) Chloroform–methanol 7:11	1.35‡	3.10
(3) Chloroform–isopropanol 7:11	1.32	3.15
(4) Chloroform–ethanol 7:11	1.35‡	3.27
(5) Chloroform–isobutanol 7:11	0.93	1.82
(6) Chloroform–butanol 7:11	1.20	3.00
(7) Ether–ethanol 2:1	1.32	2.95

\* All extractions were preceded by lysis of the packed erythrocytes with an equal volume of distilled water. Solvent volumes were 18 ml in each instance except for mixture 1, where 45 ml was used. Solvent ratios are volume ratios.

† Micrograms of lipid phosphorus  $\times 0.025$  to give milligrams of phospholipid.

‡ Values corrected for pigment contamination by subtraction of absorbancy obtained from a duplicate aliquot when  $FeCl_3$  is omitted from the color reagent.

That the lesser degree of pigment contamination of extracts prepared by the isopropanol procedure was not an isolated occurrence was shown by paired comparisons made using cells from six patients. Five comparisons were made with fresh cells and six with erythrocytes which had been stored for 7–10 days in ACD Solution as described above. With fresh cells, absorbancy at 400  $m\mu$  was greater in every case in extracts prepared by the Folch procedure, ranging from +23 to +663%, mean +249%. With stored cells, the Folch procedure also gave higher results in five out of six comparisons. Comparisons ranged from –11 to +175%, with a mean of +104%.

To assess the extent to which the isopropanol extraction procedure reduced the contribution of heme to the pigment present in Folch extracts, the quantity of pigment in an isopropanol extract of erythrocyte lipids was compared with that of an extract prepared from hemo-

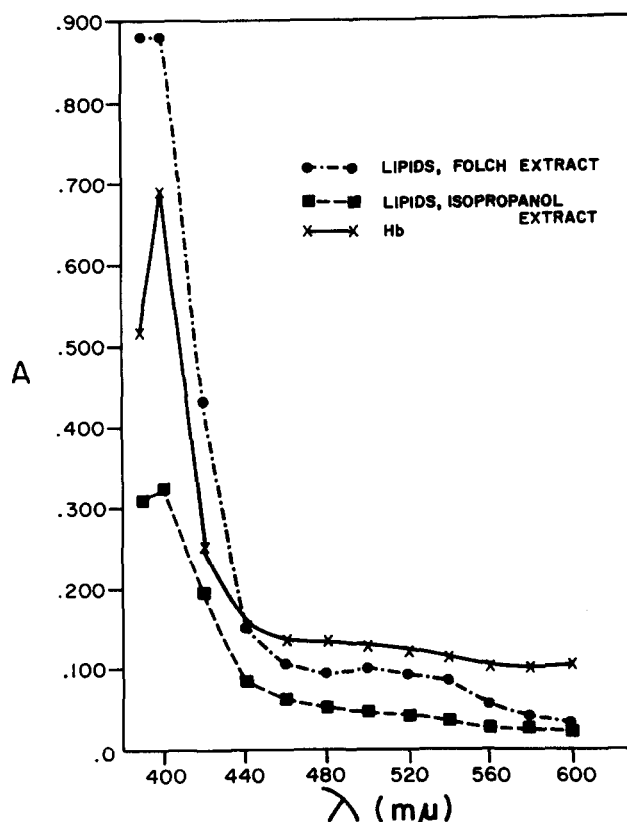


FIG. 1. Spectra of pigmented lipid extracts from human erythrocytes prepared by the Folch and isopropanol procedures. Lipids were extracted from erythrocytes from a normal subject, and dissolved in chloroform at 3.0 mg/ml. Absorbancies were determined against a chloroform blank in a Beckman DU spectrophotometer using a quartz cell with a 1.0 cm light path. Hemoglobin was prepared by osmotic lysis of erythrocytes and dissolved in chloroform–methanol 2:1 v/v by shaking the solution against a large volume of the organic solvent mixture. The quantity of hemoglobin dissolved was not determined.

globin-free erythrocyte ghosts. Erythrocyte ghosts were prepared from a portion of the packed cells of a normal subject using the method of Dodge, Mitchell, and Hanahan (13). Lipids were extracted by the isopropanol and Folch procedures, weighed, and dissolved in chloroform at 1.5 mg/ml. Absorbancies for ghost extracts, made using either procedure, were 0.039. A value of 0.373 was obtained for the Folch extract of fresh erythrocyte lipids, while 0.102 was obtained for the isopropanol extract of whole cells. A second experiment confirmed this result. These findings, together with the spectral evidence (Fig. 1), indicate that the contaminating pigment is, in large part, heme. The isopropanol mixture extracted only 19% of the pigment that could be removed by osmotic lysis.

In the series of extraction mixtures listed in Table 1 the chloroform-methanol (7:11) extract was highly pigmented before washing ( $A > 2.0$ , 1.5 mg lipid per ml), while the chloroform-isopropanol extract was only slightly pigmented ( $A = 0.106$ ). The chloroform-ethanol (7:11) extract was between these extremes ( $A = 0.252$ ). This suggests that the extent of pigment contamination increases with the polarity of the alcohol component and that the lower polarity of isopropanol explains its superiority in this respect.

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