

THE EXTRACTION OF LIPIDS FROM THE RED BLOOD CELLS*

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(Received for publication, May 4, 1936)

In a previous communication (1) it has been shown that lipids may be completely extracted from blood plasma or serum without heat if diluted with 20 volumes or more of alcohol-ether. The maximum cold extraction of whole blood was found to require at least 30 volumes of solvent. No values for the red blood cells were reported at that time, because it was found that heat produced apparently higher values after maximum extraction had been obtained in the cold. A similar situation prevailed in extracts of whole blood. Since heating caused the extraction of colored products from the decomposition of hemoglobin, products which were not present in cold extracts and which contaminated all of the isolated lipids, it was unknown whether the apparently higher lipid values in the heated extracts were due to further extraction of lipids or only to the presence of the contaminating colored material. There having since been found a satisfactory method of separating this colored matter from the lipids, the present report is concerned with the optimum conditions for extracting lipids from the red blood cells, in general according to the same procedures previously used with plasma and serum.

There have been two common methods for estimating the lipid composition of the red blood cells, the direct and the indirect. In the first the red cells are isolated and an extract made of the weighed or measured cells either with or without saline washing. In the second, the lipid content of whole blood and of plasma is separately estimated and values for the red cells are calculated

* This work was aided financially by the Alice F. Richardson Fund of the Kingston General Hospital.

from these in conjunction with the hematocrit reading. Both methods have been extensively used and both have been employed by the author. Values by the indirect method are calculated from at least three experimental figures, each of which is subject to experimental errors which accumulate in the final values for the red blood cells. Hence the direct method is to be preferred, but heretofore it has had the objection that extracts of the red blood cells are invariably deeply colored when prepared in the usual way, this colored material, as already mentioned, having many of the physical properties of lipids.

The red blood cells used in the present investigation were from samples of human blood obtained by mixing together routine specimens received in the laboratories of the Kingston General Hospital. This not only provided a convenient source of material but also eliminated the possibility of individual peculiarities from single samples of blood, and, since values in all experiments tended to be of the same magnitude (*i.e.* about the mean of human red cells), the composite plotting of results was facilitated. The specimens were of oxalated blood and each was allowed to settle and if found unhemolyzed was then used.

The mixed sample was then centrifuged at full speed for $\frac{1}{2}$ to 1 hour or until the red cell layer had become uniformly translucent. This was taken to indicate that the cells had become squarely packed with the elimination of all interstices in which plasma might be present. Repeated washing of such cells with isotonic saline was found to affect their lipid content inappreciably. If differences were encountered between washed and unwashed cells, all the lipids were either increased or decreased to the same relative extent. The lipid composition of plasma is different from that of the red blood cells and inclusion of plasma in a red cell extract would usually increase its neutral fat and cholesterol ester and lower its phospholipid and free cholesterol. When all the lipids of a washed red cell extract were found decreased (or increased) in the same proportion, this must have meant that the saline used in washing was not quite isotonic for the particular sample of cells washed. Hence it was concluded that the saline washing of thoroughly centrifuged red cells is not only unnecessary but may actually introduce an error if the saline is not exactly isotonic with each sample of red blood cells.

A sufficient amount of blood was centrifuged so that, after the plasma was removed, the red cells could be drawn off with a pipette from well below the surface (on which lie the white blood cells of a considerably higher lipid content). Aliquots thus obtained were extracted in various ways but all of the extracts were analyzed by the author's modification of Bloor's oxidative microtechnique as employed in the study of plasma and serum extracts (1). An interesting point in analytical technique arose during the course of this study. A small amount of acetone which had been standing for some time over calcium chloride at the bottom of a large bottle was redistilled and used for the precipitation of phospholipids. The resulting precipitate was of a dirty gray appearance, in contrast to the usual white color, and the calculated phospholipid values were suspected of being low. The acetone was redistilled but this made no difference. The magnesium chloride reagent, which was about 2 years old, was replaced by fresh reagent but this again did not improve the condition. When part of the acetone was allowed to dry again over fresh anhydrous calcium chloride and the results with this compared with the old acetone and with an entirely new dried specimen, it was found that the old acetone had been incompletely dehydrated and that this was responsible for the aberrant low values.

Since the most important factor in the preparation of plasma extracts was found to be the *degree of dilution* in alcohol-ether, this was the first factor studied with the red blood cells. Aliquots of 2 cc. were hemolyzed with an equal volume of distilled water in 125 cc. Erlenmeyer flasks and to this were quickly added amounts of alcohol-ether varying from 5 cc. (a dilution of 2.5 times the original volume of cells) to 120 cc. (a dilution of 60 times). It was found that a finer precipitate of proteins was obtained by quickly adding the solvent to the hemolyzed cells than by adding the hemolyzed cells to the solvent. With dilutions of 10 times or less the proteins tended to clump and had to be broken up with a glass rod, but with greater dilutions a finely divided precipitate was obtained. Unless shaken for a few minutes the protein precipitate even in the greater dilutions tended to settle out and form a solid mass at the bottom of the flask. The extracts were filtered at once without being heated, the precipitate washed once with a small portion of the solvent (so as not to increase solution in the

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smaller volumes), and the filtrate and washings made up to volume.

The results of analyses of these extracts for phospholipid, total fatty acid, total cholesterol, and free cholesterol have been shown in Fig. 1 which is a composite presentation of several experiments.

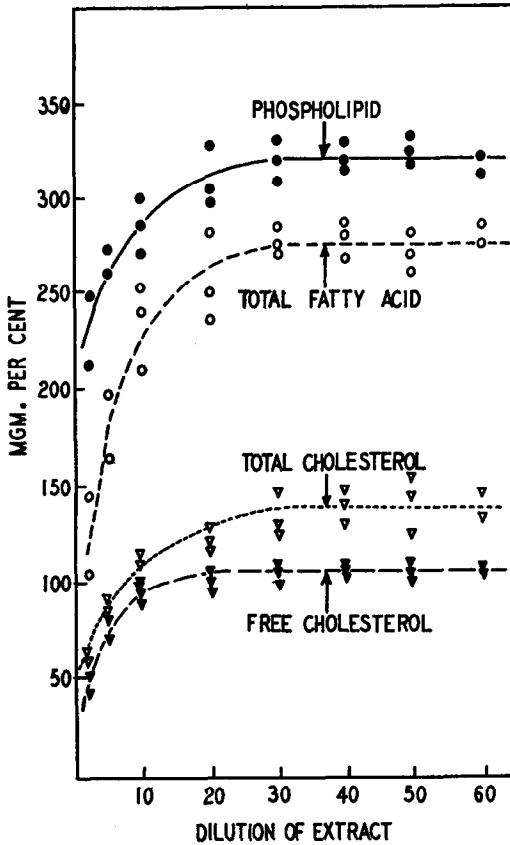


FIG. 1. The relation of lipid composition to the degree of dilution of red blood cells in alcohol-ether.

It may be seen that dilution of the red cells in less than 25 to 30 volumes of alcohol-ether produced incomplete extraction, but that dilutions greater than this did not appreciably affect the recovery. From the data given it may be deduced that neutral fats and cholesterol esters are more difficult to extract with the lesser dilutions than phospholipid and free cholesterol.

The *effect of boiling* the extracts was studied next. 2 cc. of mixed red blood cells were hemolyzed with an equal volume of water and about 60 cc of alcohol-ether quickly added. Some flasks were left as unheated controls and the remainder were placed on the steam bath and allowed to boil gently for varying periods up to 1 hour. Solvent lost by evaporation was replaced from time to time by fresh alcohol-ether. In preliminary experiments difficulty was encountered with superheating, resulting in boiling over. This was finally overcome by continuously stirring the extracts with a glass rod, especially by dislodging particles of protein which tended to stick to the bottom of the flask. After cooling, the extracts were filtered, washed several times with solvent, the residue pressed out, and the combined filtrates made up to volume. The unheated extracts were colorless but a progressively deeper brownish red developed in the heated extracts. 50 analyses on several mixed samples of human red cells demonstrated that boiling produced apparently higher values for all lipids in the red blood cells, just as was previously found in whole blood (1). The colored matter contaminated all of the isolated lipids and was especially marked in the petroleum ether solution of the total fatty acids, resulting in exceptionally high values for neutral fat.

The problem was how to separate this colored matter from the lipids. By evaporating to dryness portions of the colored alcohol-ether extracts, the colored material was found to be soluble in alcohol, ether, acetone, petroleum ether, methyl alcohol, chloroform, and dilute sodium hydroxide solution; it was insoluble in water and dilute hydrochloric or sulfuric acid. When the colored material was dissolved in 0.1 N NaOH and this extracted with petroleum ether, the color remained in the aqueous alkaline medium and did not pass visibly into the petroleum ether. This was considered as a possible method of separating the colored material from the lipids, but unfortunately the extraction of lipids from the colored alkaline solution was incomplete, especially with regard to phospholipid and free cholesterol. Lipids could be fairly well extracted from an alkaline solution of colorless unheated extracts, so that in some manner the presence of the colored matter hindered the extraction.

While this method was being further investigated, a means was accidentally found whereby the extracts could be freed of colored

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substances. A petroleum ether extract of the saponified and acidified total fatty acids and total cholesterol happened to be left standing overnight and next day the colored material was seen to have completely precipitated out. The petroleum ether solution was filtered, the precipitate repeatedly washed with fresh solvent, and the filtrates were quite clear. Total fatty acid and total cholesterol determined in this extract were found to give values identical with those in unheated control extracts and not higher values, as had always been found in the colored extracts. This experiment was repeated a number of times, some 60 analyses in

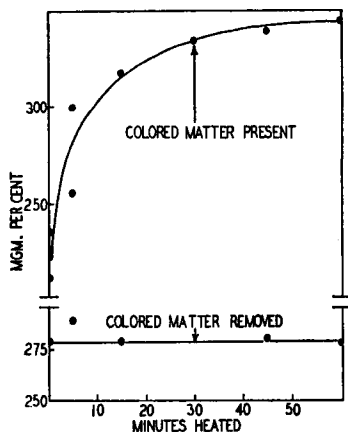


FIG. 2. The total fatty acid content of sufficiently diluted extracts of the red blood cells boiled for varying periods, *before* and *after* removal of the colored material.

all being performed. It was invariably found that when the colored material was thus removed by standing overnight (or longer if necessary) in petroleum ether, the immediately filtered, unheated but sufficiently diluted extracts of the red blood cells contained just as much lipid as the heated ones. A typical experiment illustrating the values for total fatty acid in a colored and a color-free heated extract is shown in Fig. 2. This experiment proved that a sufficient dilution in cold alcohol-ether will extract all lipids from the red blood cells capable of being extracted with this solvent and that heat does not further the extraction of lipids but dissolves colored decomposition products of hemoglobin.

Several *methods of preparation* of the red blood cells for extraction in cold alcohol-ether were next studied. When these cells were added directly to the solvent, the drops from the pipette fell to the bottom in a mass, somewhat resembling the formation of glass beads. Adding alcohol-ether quickly to the untreated cells also produced a clumped precipitate and both of these extracts of untreated red cells gave low lipid values whether or not the massed precipitate was broken up with a glass rod. Hence extraction was carried out on red cells previously (*a*) hemolyzed with half a volume of distilled water, (*b*) hemolyzed with 1 volume of distilled water, (*c*) hemolyzed with 1.5 volumes of distilled water, (*d*) hemolyzed with 2 volumes of distilled water, and (*e*) ground with sufficient sand to produce a semidry mass. These several methods of preparation were found to result in almost equally good extraction. No single experiment demonstrated the relative virtue of any one, but from the mean value of a number of experiments (100 analyses) hemolyzing with an equal volume of distilled water was found to give slightly higher lipid values. Hence this latter procedure was adopted in the routine method.

In preparing an alcohol-ether extract of blood lipids, there are two methods in general use for *making up to volume*. One is the distribution method used by Bloor (see previous paper (1)) in which blood is added to the solvent in a volumetric flask and the extract made to volume with the protein precipitate still in the flask. It is believed that the lipids are distributed through the protein precipitate in the same concentration in which they are distributed through the alcohol-ether. In the second method (filtration method), the proteins are filtered off and washed several times with fresh solvent, the combined filtrates being made up to volume. Those who favor the distribution method claim that it is difficult to wash all of the lipid material out of the protein precipitate. An attempt was made to compare these two procedures: Extracts of the red blood cells were prepared as above and made up to volume, one group by the filtration and one by the distribution method. The volume of filtered extract by the distribution method was carefully noted and the total amount of lipid in this determined. To this was added the amount of lipid in the protein precipitate which was calculated to contain the same relative lipid composition as the filtrate. The sum of these two was then

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compared with values obtained by the filtration method, the results being compared in terms of mg. of lipid per 100 cc. of red cells. Curiously enough, the lipid composition of the red cells determined by these two methods was practically the same. To quote figures from one experiment, the values by filtration and by distribution were respectively, total fatty acids 294 and 289 mg. per 100 cc., total cholesterol 144 and 133, free cholesterol 128 and 127, phospholipid 304 and 327. It would appear that both methods are quite satisfactory; in the one case the lipids are equally distributed through the protein precipitate, and in the other case washing

TABLE I
Comparison of Lipid Values in Red Blood Cells from Defibrinated and Oxalated Blood

The results are expressed in mg. per 100 cc. of red blood cells.

Case No.	Blood	Red blood cells	Total fatty acids	Total cholesterol	Free cholesterol	Phospholipid
		<i>vol. per cent</i>				
1	Defibrinated	44.3	268	178	128	351
	Oxalated	40.0	280	190	140	367
2	Defibrinated	49.1	251	144	120	318
	Oxalated	38.2	313	150	130	395
3	Defibrinated	46.0	247	122	116	244
	Oxalated	39.1	306	145	134	377
4	Defibrinated	45.8	267	120	99	270
	Oxalated	43.3	259	132	105	286
5	Defibrinated	47.7	267	122	114	294
	Oxalated	42.8	248	127	115	316

the precipitate thoroughly removes effectively all of the residual lipid material.

Finally, a study was made of the *effect of anticoagulants* on the lipid content of the red blood cells. The lipid composition was determined as above in oxalated and defibrinated samples of the same blood and five such comparisons are given in Table I. In eighteen out of the twenty analyses of directly (not calculated) estimated lipids, the values were higher in the red cells from oxalated blood than from defibrinated blood. In all cases the volume of the red cells was greater in defibrinated than in oxalated blood. Since many of the erythrocytes are invariably caught in

the clot formed during defibrination, this difference in volume was undoubtedly greater than that actually shown. Total fatty acid was the only lipid found higher (in two out of five cases) in the defibrinated cells. Hence it may be concluded that the lipid content of oxalated red blood cells is greater than that of defibrinated cells. This is probably due to absorption of water from the red cells (2), but there were a number of factors here which are being subjected to further study.

SUMMARY

The following method was found to give complete extraction of lipids in the red blood cells in so far as they are soluble in alcohol-ether. Blood is centrifuged until the red cells are translucent when an aliquot is removed from well below the surface. This is hemolyzed with an equal volume of distilled water and 25 to 30 volumes (per 1 volume of red cells) of alcohol-ether quickly added with shaking. Heating is not only unnecessary but undesirable. The extracts are filtered, the residue thoroughly washed, and the filtrate made up to volume. Distribution and filtration methods give practically the same results, but the values are higher in red cells from oxalated than from defibrinated blood.

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J. Biol. Chem. 1936, 115:37-45.

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