Composition of neutral lipids from erythrocytes of common mammals

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ABSTRACT The neutral lipids of the erythrocytes were investigated in several common mammals: cow, dog, goat, horse, pig, rabbit, rat, and sheep. Cholesterol content was determined by gas–liquid, thin-layer, and column chromatography, the last in conjunction with the IR spectrophotometry. The three methods yielded similar results. In every species investigated, cholesterol was the major neutral lipid; cholesteryl esters, triglycerides, and free fatty acids were detected only in trace amounts. It is concluded that these substances may have been contaminants from plasma lipoproteins or leukocytes rather than true constituents of the erythrocyte. In the erythrocytes of all species, cholesterol content was close to 30% of the total lipids extracted from the cells, and the molar ratio of cholesterol to phospholipid was approximately one. The significance of the data is discussed in relation to current concepts of the structure of the cell membrane involving cholesterol–polar lipid complexes.

KEY WORDS erythrocytes · neutral lipids · cholesterol · cholesteryl esters · triglycerides · free fatty acids · cow · dog · goat · horse · pig · rabbit · rat · sheep · cholesterol–phospholipid ratios · membranes

NEUTRAL LIPIDS make up a significant percentage of the total lipids in mammalian erythrocytes (1–4), but reviews by van Deenen and de Gier (5) and others (6, 7) point out that considerable controversy still exists about the relative proportions of the neutral lipids in the erythrocyte. Most current reports (3, 8, 9) agree that cholesterol is the major neutral lipid present and that other compounds such as cholesteryl esters, triglycerides, and free fatty acids are present in much smaller quantities (3, 5, 8–10). Furthermore, some of the more recent investigations indicate that little if any neutral lipid other than cholesterol is present in nonnucleated erythrocytes (4, 10, 11).

The composition of the erythrocyte membrane has achieved new importance because of the current interest in the structure and function of the plasma membrane (12). The neutral lipid composition has therefore been carefully reinvestigated in mammals by modern chromatographic and spectrophotometric techniques. The species investigated included cow, dog, goat, horse, pig, rabbit, rat, and sheep. This paper describes the analytical procedures, presents data on the neutral lipid composition of the erythrocytes in these species, and discusses the findings in relation to current concepts of the membrane structure which involves cholesterol–polar lipid complexes.

MATERIALS

All solvents used in this work were redistilled from glass stills and deoxygenated by bubbling nitrogen through them before use. Analytical standards for cholesterol, cholesteryl oleate, triolein, and oleic acid were obtained from Applied Science Laboratories Inc., State College, Pa. Silica Gel HR was obtained from Brinkmann Instruments Inc. (Westbury, N.Y.), $MgSiO_2$ from Allegheny Industrial Chemical Company (Butler, N.J.), and Silicic Acid (Grade HA for column chromatography) from Bio-Rad Laboratories (Richmond, Calif.). Sephadex was obtained from Pharmacia Fine Chemicals Inc. (New Market, N.J.).

Cholesterol analysis by gas–liquid chromatography (GLC) was performed on the F & M Scientific Model-402 Gas Chromatograph equipped with a hydrogen flame detector. The stationary phase, 3.8% SE-30 (methylpolysiloxy gum) on Diatoport S, was also sup-

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatographic.

plied by F & M. IR spectra were determined with a Perkin-Elmer Corp. Model 521 double-beam recording spectrophotometer. The spectra were recorded from solution in CS_2 in sealed liquid cells with NaCl windows. Densitometry of thin-layer plates was performed on equipment supplied by Photovolt Corp. (New York), which included a plate scanner, a photomultiplier, and a recorder with an electronic integrator.

METHODS

All the animals in this investigation were maintained at this laboratory by standard animal husbandry methods, except for the horse and pig, samples from which were obtained from local slaughter houses. Only mature animals were selected for sampling. Cow, dog, goat, horse, and sheep were analyzed in duplicate, whereas single experiments were performed on pig, rabbit, and rat samples. Samples from rabbit and rat were pools from two and three animals, respectively; all other samples were from single animals.

Blood was drawn by venipuncture into sterile plastic blood bags as reported earlier (11) for the large animals in this series, or directly into plastic centrifuge tubes for the small animals. Heparin was used as an anticoagulant. The blood was cooled immediately to 0°C in ice and kept at that temperature throughout further processing.

The cells were separated from the plasma by centrifugation at 2500 rpm for 20 min in a refrigerated centrifuge. The plasma was removed by aspiration, and the packed cells were washed three or four times with an equal volume of 310 milliosmolar phosphate buffer, pH 7.4. After each recentrifugation the supernatant solution was removed by aspiration, together with the top 2 or 3 mm of packed cells. This procedure removed practically all the white cells from the final erythrocyte preparation; in most of the samples, smears prepared from the final packed cell preparation contained no detectable leukocytes. The cells were always extracted immediately after the final recentrifugation.

Extraction Procedure

A volumetric pipette of appropriate size, usually 20 ml, was used to transfer the packed erythrocytes to the extraction flask. The cells were added slowly to cold methanol, which was being stirred vigorously by a magnetic stirrer, to give a volume ratio of 17:1, methanol/packed cells. After the cells were added, the flask was filled to volume with cold chloroform, and vigorous stirring was continued for 5 min. This technique produced a 2:1, chloroform–methanol extract of the packed cells having a final ratio of 50:1, cells to extraction medium.

The solution was then filtered through a fast, prewashed filter paper (Whatman 41H, or Schleicher & Schuell Sharkskin) into a round-bottomed boiling flask. The solvents were removed from the filtrate by rotary evaporation at low temperature (<15°C) under reduced nitrogen pressure. Before the flask became totally dry, the lipids were transferred to a small volumetric flask (2–5 ml) with chloroform–methanol 19:1. If the total weight of the crude extract was needed, a small aliquot was now removed with a micropipette, transferred to a tared vial, and weighed to constant weight on a Mettler M5SA microbalance. The crude lipid extract was subjected to Sephadex column chromatography for further purification.

Sephadex Column Chromatography

Nonlipid and water-soluble materials present in chloroform–methanol tissue extracts can be conveniently removed by column chromatography on Sephadex (13–15). The procedure followed in this work was essentially that described by Siakotos and Rouser (14), with only slight modification. The Sephadex (G-25, coarse) was washed and columns were prepared as described (14) except that the prewashing of the column was simplified by carrying out the elution scheme once after packing the columns in methanol–water 1:1. Columns 2.5 × 10 cm were fitted with Teflon needle-valve stopcocks (Kontes Glass Co., Vineland, N.J.) which facilitated adjustment of the flow rate.

Three fractions were collected by means of the four different solvent mixtures described by Siakotos and Rouser (14); only Fraction I, which was eluted with 170 ml of chloroform–methanol 19:1 saturated with water and contained the purified lipid extract, was investigated further in this work.

The first fraction was collected at a rate of 1–2 ml/min and the following fractions at 2–4 ml/min. A column was allowed to stand in methanol–water 1:1 for at least 48 hr before reuse, and 500 ml of chloroform–methanol 19:1 saturated with water were passed through the column immediately before the sample was applied.

The solvent was removed from Fraction I as described above for the initial red cell extract. Again, rotary evaporation was stopped before the flask became dry, and the lipids were transferred with chloroform to a glass-stoppered, 5 ml, graduated cylinder. At this stage an aliquot was weighed to determine the total lipid content of the extract. The remainder was stored at low temperature (−10°C) until it was subjected to the analysis described below.

Analyses

Phospholipid phosphorus was determined as previously described (11). The percentage of cholesterol in the

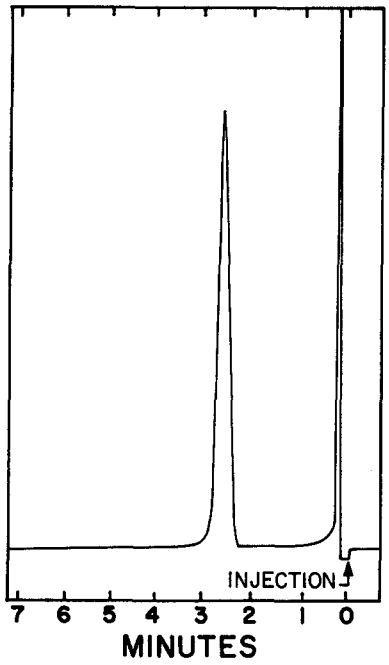

FIG. 1. The GLC pattern of cholesterol eluted from a column after the injection of 8 μg of the total lipid extract obtained from dog erythrocytes. Column packing was 3.8% SE-30 on Diatoport S. Full scale sensitivity was 10^{-6} amp. Other parameters of the instrument and the column are described in the text.

erythrocyte was determined by three independent procedures involving gas–liquid, thin-layer, and column chromatography.

Gas–Liquid Chromatography. A sample of the purified total lipid extract was subjected to GLC directly without any pretreatment or preliminary separation. The stationary phase was 3.8% SE-30 on Diatoport S at 210°C in a column 2 ft × 1/8 inch I.D. The injection port was maintained at 235°C and the flame detectors at 250°C. The helium flow rate was 80 ml/min. Samples containing approximately 0.5–2 μg of cholesterol were analyzed and the peak areas were converted to micrograms by comparison with a standard curve prepared from runs made with purified cholesterol. The reproducibility of the injection procedure was checked by triplicate injection of 2.5 μg of cholesterol standard, which yielded peak heights of 4.85, 4.73, and 4.81 inches with identical base widths. The erythrocyte samples showed similar reproducibility when injected in triplicate.

The presence of the other complex lipids in the sample did not appear to influence the elution of the cholesterol from this column under the conditions described. Fig. 1 is a typical cholesterol elution pattern after injection of the total lipid extract from dog erythrocytes. The standard curve was linear over the range 0.1–5 μg used in this determination. In addition, the injection of half or two or three times the amount of crude lipid extract yielded cholesterol peaks of corresponding size when compared to injections in the standard range.

Thin-Layer Chromatography. Thin-layer chromatographic (TLC) plates were prepared as described by Rouser, Galli, Lieber, Blank, and Privett (16), with Silica Gel HR and 10% $MgSiO_2$ at a layer thickness of 0.25 mm.

We treated them as follows to remove any contaminant from the solvent front. The plates were air-dried and placed in the developing tank; the solvent was allowed to rise up the plate to the top. The plates were left in the tank for 15 min after the solvent reached the top of plate; if this procedure was not followed, a dark band that appeared along the entire solvent front tended to obscure trace components in chromatographed samples.

After the TLC plate was removed from the predevelopment tank, it was air-dried for 5 min, activated for 30 min at 120°C in an oven, and allowed to cool in air for 20 min before the samples or standards were applied by means of Lang-Levy micropipettes of appropriate volume, usually 10 μl. If the TLC plate was to be analyzed by means of a scanning densitometer, samples of 2–5 μg were applied as single spots. When large amounts were used for the investigation of trace components, up to 1.5 mg was applied as a row, about 1 cm long, of overlapping spots.

The developing solvent (17) was petroleum ether–diethyl ether–acetic acid, 85:15:2, which was used also for the preliminary washing. The solvent front was allowed to travel 15 cm from the origin. The development time was 25 min at 20°C and did not vary more than ±1 min for any plate in a series. After the plates had been removed from the developing chamber, they were air-dried for 5 min, sprayed with a charring reagent, 0.6% by weight of potassium dichromate in 55% H_2SO_4 (18), and heated for 20–30 min at 185°C in a forced-draft oven.

The plates were photographed on Polaroid film as previously described (11) and also viewed under UV light in case any additional spots could be thus detected. Fig. 2 shows the TLC analysis of large amounts of erythrocyte lipids from sheep, cow, and rat.

The cholesterol in the developed TLC plates was determined by densitometry. Samples and standards were applied in alternate lanes on the plate and the areas of the sample spots compared with a calibration curve for the areas of cholesterol standards on the same plate.

Silicic Acid Column Chromatography. Small columns containing 0.5 g of silicic acid were prepared as previously described (11). Samples of erythrocyte total lipid extract containing 1–3 mg of cholesterol were applied to the column in chloroform. The neutral lipid fraction (primarily cholesterol) was eluted with 10 ml of

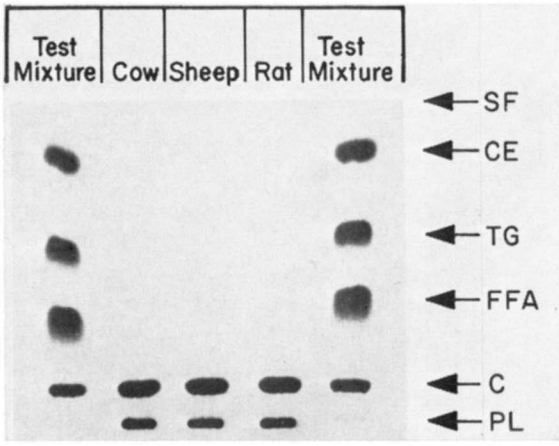

FIG. 2. A TLC separation of the neutral lipids of cow, sheep and rat. To the center lanes about 1.5 mg of each total lipid extract was applied and to the two outside lanes 400 μg of the test mixture containing 100 μg each of cholesterol (C), cholesteryl oleate (CE), triolein (TG), and oleic acid (FFA). Spots were made visible by charring. No spots for neutral lipids other than cholesterol could be detected after charring, but faint spots could be detected under UV light. SF, solvent front; PL, phospholipid (at origin).

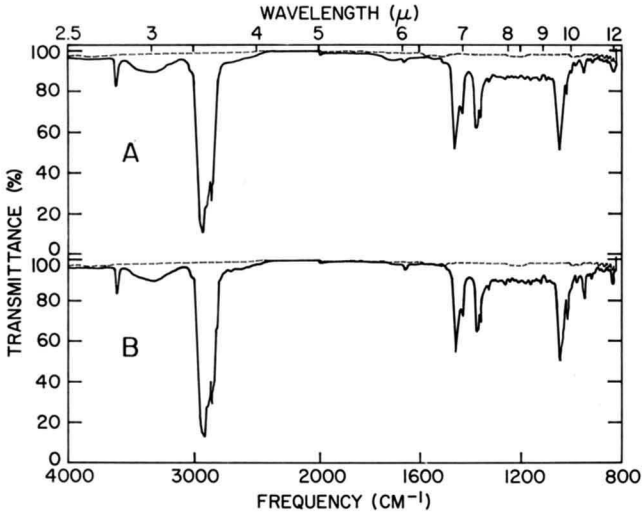

FIG. 3. IR spectra of cholesterol standard obtained from a commercial source and a sample isolated from erythrocytes. Curve *A*, dog erythrocyte neutral lipid fraction obtained by chloroform elution during silicic acid column chromatography of the total lipid extract. Curve *B*, standard sample. Both spectra obtained on samples in CCl_4 solutions at 100 mg/ml in cells (0.1 mm light path) with NaCl windows.

chloroform and collected in tared vials, and the solvent was removed by evaporation under nitrogen. Sample weights were determined either by weighing or by IR spectrophotometry (19). The latter method estimated purity besides yielding quantitative data (Fig. 3).

The results obtained by the three methods were in good agreement for all samples. In erythrocytes of the cow, for example, cholesterol comprised 30.2, 28.4, and 27.9% of the total lipid extract by GLC, IR spectrophotometry, and TLC analysis, respectively. In duplicate determinations on the same sample, the results were 30.9, 29.0, and 28.5%, respectively. In general, the results obtained by one method did not differ by more than $\pm 5\%$ from those obtained by the other two. The duplicate determinations of total lipid extracted from cow, dog, goat, horse, and sheep erythrocytes were within $\pm 2\%$ of each other after Sephadex chromatography (Table 1).

RESULTS

Table 1 presents data on the total erythrocyte lipid of the species investigated in this work. Table 2 gives the cholesterol in mg/ml of packed cells and percentage of the total lipid extracted. Since the neutral lipids of the samples were at least 99% cholesterol in each case, this table also gives accurate values for neutral lipid in percentage of total lipids.

The other neutral lipids in the erythrocyte were not so easily quantified. In the amounts of sample available, silicic acid column chromatography and IR spectrophotometry (Fig. 3) revealed no other neutral lipids. GLC showed only that the cholesterol present in the erythrocyte was eluted as a single peak (Fig. 1). The same results were obtained initially when TLC methods were used, and it appeared that no components other than cholesterol were present.

However, when large loads (>1 mg) were applied to TLC plates and the plates were viewed under UV light after charring, several samples showed faint spots in locations corresponding to those expected for cholesteryl esters, triglycerides, and free fatty acids. Utilization of this technique in a semiquantitative way made it possible to place upper limits on the amounts of these components present in the erythrocyte. For this pro-

TABLE 1 TOTAL ERYTHROCYTE LIPID OF VARIOUS COMMON MAMMALS

Species	Sample No.	No. of Washes	Total Lipid*
			mg/ml of packed cells
Cow	1A	3	4.15
	1B	4	4.27
Dog	2A	3	5.16
	2B	4	5.00
Goat	3A	3	5.69
	3B	4	5.89
Horse	4A	3	4.70
	4B	4	4.82
Pig	5	3	4.19
Rabbit	6	3	4.42
Rat	7	3	4.86
Sheep	8A	4	4.44
	8B	10	4.40

* Excluding gangliosides.

TABLE 2 ERYTHROCYTE CHOLESTEROL IN VARIOUS COMMON MAMMALS

Species	Sample No.	Cholesterol*	
		% of total lipid	*mg/ml of packed cells*
Cow	1A	28 8	1.20
	1B	29.5	1.26
Dog	2A	28.2	1.46
	2B	28.0	1.40
Goat	3A	27.5	1.57
	3B	28.6	1.68
Horse	4A	28.7	1.35
	4B	29.3	1.41
Pig	5	27.5	1.15
Rabbit	6	29.9	1.32
Rat	7	26.1	1.27
Sheep	8A	27.8	1.24
	8B	28.2	1.24

* Values given in table are the average of the results obtained by the three different analytical methods.

cedure, standard test mixtures of the four main neutral lipids prepared from standard samples of cholesterol, cholesteryl oleate, triolein, and oleic acid, each of which was at least 99% pure, were used.

Detection limits were assigned to each component on the basis of the lowest amount which gave a detectable spot. In no species was the amount of any lipid other than free cholesterol more than 0.3% of the total neutral lipids or more than 0.15% of the total lipid in the erythrocyte.

DISCUSSION

Hanahan, Watts, and Pappajohn (8) reported that only 80% of the neutral lipids in bovine erythrocytes was cholesterol; 4% consisted of cholesteryl esters, 10% of triglycerides, and 6% of unidentified hydrocarbons. Vacca, Waring, and Nims (20), Irie, Iwanga, and Yamakawa (21), and de Gier and van Deenen (3) also reported the presence of neutral lipids other than cholesterol in the erythrocytes of various species in amounts appreciably larger than those reported here. Vacca et al. (20) detected mono- and diglycerides in lipid preparations obtained from dog erythrocytes, but none of these compounds were detected in this work, where the detection limit was 0.05%.

In agreement with Hanahan et al. (8) we found the cholesterol fraction in all species, on the basis of the IR spectrum and the GLC characteristics, to be free from any isomers or analogues of cholesterol. Admittedly, these techniques do not isolate or detect such compounds in trace amounts. However, as shown in Fig. 1, no evidence could be found for the "oxycholesterol" reported by Irie et al. (21) in appreciable quantities in dog erythrocytes.

While the high levels of erythrocyte neutral lipids other than cholesterol reported by others may be in error, the presence of minute amounts of such compounds was affirmed in this work. The question then arose whether such compounds truly exist in vivo in the erythrocyte membrane. It is well established now that all of the polar lipids in the erythrocyte are located exclusively in the stroma or membrane, and can be recovered in toto from the lyzed "ghost" (22). The cells extracted here were washed in a manner considered sufficient to free them from circulating plasma proteins and other formed blood components (23). However, the possibility remained that serum lipoproteins, which have large amounts of neutral lipid, could still be bound to the erythrocyte and (or) other formed bodies of the blood in sufficient amounts to contribute the amounts of neutral lipids other than cholesterol detected here.

Calculations showed that if only 0.1% of the original lipoprotein remained bound to the erythrocyte after four washings, it could contribute enough neutral lipid other than cholesterol to account for the levels reported in this work. This hypothesis was checked in a single experiment carried out on sheep erythrocytes. Instead of the usual four washings, sheep cells (Sample 8B) were washed 10 times with isotonic phosphate buffer and then extracted by the procedure described above. No differences could be detected between the phospholipid or cholesterol content of the total lipid extract from these cells and from those washed only three times. Neutral lipids other than cholesterol, on the other hand, could no longer be detected at the previous levels or indeed detected at all with the methods used in this investigation.

Thus, it may be that neutral lipids other than cholesterol are not an integral component of the erythrocyte membrane, and that previous reports to the contrary may have resulted from failure to remove all of the plasma lipoprotein or white cells from the erythrocyte preparation. This conclusion may not apply to the nucleated erythrocytes of birds and amphibians, which may contain other organelles that could contribute neutral lipid to the total lipid extract of the erythrocyte. However, no experiments were performed on erythrocytes from these species.

Another interesting point can be drawn from this investigation, provided that the erythrocyte membrane can be considered typical of the plasma membrane of cells in general: the lipids in the membrane may exist as a phospholipid–cholesterol complex like that first proposed by Finean (24) and later modified by Vandenheuvel (25). Table 3 lists the ratio of cholesterol to phospholipid for the erythrocytes of the species analyzed here. In every case the ratio is relatively close to unity for moles of phosphorus to moles of cholesterol per cell, calculated on the basis of the mean cell volume given by

TABLE 3 CHOLESTEROL: PHOSPHOLIPID MOLE RATIOS OF ERYTHROCYTES OF COMMON MAMMALS

Species	Sample No.	Cholesterol	Phospholipid Phosphorus	Cholesterol: Phospholipid Ratio
		moles/cell × 10^{16}		
Cow	1A	1.79	1.95	0.92
	1B	1.88	1.99	0.94
Dog	2A	3.17	3.30	0.96
	2B	3.04	3.40	0.89
Goat	3A	0.80	0.82	0.98
	3B	0.85	0.80	1.07
Horse	4A	1.67	1.81	0.92
	4B	1.74	1.85	0.94
Pig	5	1.73	2.00	0.87
Rabbit	6	3.10	3.61	0.86
Rat	7	2.04	2.81	0.73
Sheep	8A	1.06	1.37	0.77
	8B	1.06	1.27	0.83

Ponder (26). Vandenheuvel (27) has shown that cholesterol is the only neutral lipid with a steric configuration that can form such complexes. Hence, it is possible that all of the cholesterol and the phospholipids, and perhaps all of the glycolipids, in the membrane are in the form of complexes of cholesterol and polar lipid that are in some way necessary to the physical integrity of the membrane. It has been shown that peroxidation of the lipids in the erythrocyte causes hemolysis (28, 29), which may result from disruption of the cholesterol–polar lipid complex in the membrane.

On the other hand, nothing in these results precludes the possibility that plasma lipoproteins, free fatty acids, or other substances are bound in vivo to the erythrocyte and may well have some physiological significance. The major conclusion to be drawn from this work is that the lipids intimately associated with membrane structure are cholesterol, the phospholipids, and the glycolipids, and that other neutral lipids apparently play no significant role.

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