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THE LIMITED DEPLETION OF CHOLESTEROL FROM ERYTHROCYTE MEMBRANES ON TREATMENT WITH INCUBATED PLASMA

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SUMMARY

About 35% of the cholesterol of human erythrocyte membranes can be removed by the "preincubated plasma" technique (Murphy, J. R. (1962) J. Lab. Clin. Med. 60, 86–109), in which erythrocytes are extracted with plasma that has been preincubated to esterify a portion of its lipoprotein cholesterol. The limitation on the cholesterol depletion is shown not to be a result of insufficient plasma capacity to take up additional cholesterol or of changes in the plasma during the extraction.

The maximum cholesterol depletion from "ghosts" was the same as that from whole cells. "Inside-out" membrane vesicles (Steck, T. L. (1974) in Methods in Membrane Biology (Korn, E. ,ed.), Vol. 2, pp. 245–281) were utilized to determine if the limitation to cholesterol depletion is a result of the remaining cholesterol being located at the membrane inner surface and therefore not accessible to the plasma. No further cholesterol depletion occurred when "inside-out" vesicles, prepared from erythrocytes which were depleted of cholesterol by the usual method, were extracted. Also, "inside-out" vesicles prepared from untreated erythrocytes gave the same cholesterol depletion as is usually attained.

The maximal cholesterol depletion was unaffected by a number of modifications of the extracting preincubated plasma: addition of lysolecithin or albumin, dialysis against isotonic buffer, and variation in pH of the preincubated plasma from 6.0 to 9.0.

It is concluded that the limitation on the cholesterol depletion is a result of a firm binding of the remaining cholesterol.

INTRODUCTION

Murphy [1] and Cooper and Jandl [2] describe a method for reducing the cholesterol content of human erythrocytes under conditions closely resembling those encountered by the erythrocytes in vivo, and therefore seemingly with a minimum of extraneous changes in membrane structure. This method is based on the rapid exchange ($t_{\frac{1}{2}} \simeq 1$ h) of unesterified cholesterol between the cell membrane and the

plasma lipoproteins, first reported by Hagerman and Gould [3]. Murphy, and Cooper and Jandl showed that a net transfer of cholesterol from the membranes to the lipoproteins occurs when the unesterified cholesterol content of the latter is reduced. The reduction of the lipoprotein unesterified cholesterol level was accomplished by preincubating the erythrocyte-free plasma at 37 °C, whereupon lipoprotein cholesterol was esterified through the reaction:

$$\text{cholesterol} + \text{lecithin} \rightarrow \text{cholesterol ester} + \text{lysolecithin} \quad (1)$$

This reaction is catalized by the enzyme lecithin-cholesterol acyltransferase, which is normally present in plasma.

Only up to about 35 % of the cholesterol was removed from the erythrocytes in the experiments of Murphy, and Cooper and Jandl. However, their papers were not primarily concerned with the maximum cholesterol extraction and their experiments were not designed to establish whether 35 % is indeed the maximum extractable by this technique. This question is of interest for two reasons. First, an extraction limitation prevents studies, analogous to those reported on partially cholesterol-depleted erythrocytes [4–10], on erythrocytes from which all cholesterol has been removed. Second, and equally important, it suggests that there are two classes of cholesterol in the erythrocyte membrane, one freely removable and the other firmly bound. Although the data are inconsistent two cholesterol classes are indicated by some of the studies on the exchange of isotopically labelled cholesterol between erythrocytes and lipoproteins [11–14]. Also, Deuticke and Zöllner [6] have postulated two classes of cholesterol to explain certain of its effects on membrane permeability.

EXPERIMENTAL

The general experimental procedure consisted of first "preincubating" erythrocyte-free plasma at 37 °C. An aliquot, typically 0.5 to 1 ml, of washed erythrocytes from the same donor was then added to the plasma, and the suspension shaken at 37 °C for the appropriate time period. The erythrocytes were separated from the plasma by centrifugation, and washed 3 times with isotonic saline. Lipids were then extracted from the erythrocytes and analyzed for cholesterol and for phospholipid.

Experiments were carried out with fresh blood collected in acid-citrate-dextrose anticoagulant from donors specified to have no cholesterol abnormalities. Sterile procedures were used throughout. Garamycin antibiotic (Schering, 0.25 ml per 100 ml plasma) was added to the plasma prior to the preincubation period and again prior to the extraction of the erythrocytes. No bacterial growth was noted with these precautions. Similar cholesterol depletion results were obtained in control experiments using a combination of Mycidradin Sulfate (Upjohn) and Vanococin HCl (Lilly) as antibiotics, and, despite extensive bacterial growth, in the absence of antibiotic.

Preincubation of plasma consisted of keeping erythrocyte-free plasma at 37 °C for 60–72 h, the period found by the earlier workers for maximum cholesterol esterification. Plasma pH ranged from 7.2 to 7.4 in the various samples used, and was found to be unchanged by the preincubation. A portion of the whole blood was stored at 5 °C while the plasma was being preincubated. Erythrocytes were separated

by centrifuging the whole blood at $500 \times g$ for 20 min, and then washed 3 times with sterile, isotonic, pH 7.4 buffered solution (removing the buffy layer). The wash solution, which was also used at times for cell storage, was 150 mmol NaCl, 5 mmol KCl, 11 mmol Na_2PO_4 and NaH_2PO_4, 11 mmol glucose, and 2 mmol adenosine.

Five ml of 5 % glucose was added per 100 ml plasma prior to the extractions of the cell cholesterol by the preincubated plasma. The extractions were done in sealed, half-full, Erlenmeyer flasks, shaken at about 2 vibrations per s.

Some experiments utilized particularly large ratios of plasma to erythrocytes, and therefore necessitated two experimental steps not taken by the previous workers. First, the preincubated plasma was centrifuged ($4000 \times g$ for 20 min) before the addition of erythrocytes. This step presumably completed the removal of platelets. Its omission resulted in the erythrocytes forming a sticky mass when centrifuged after the incubation (particularly with incubation times of 24 h and more). The second modification was required by the finding that a lipoprotein precipitate* formed in the plasma during the incubation. To avoid contamination by this precipitate the erythrocytes were osmotically hemolyzed prior to extraction of lipids and the hemolyzate was centrifuged at $500 \times g$ for 20 min to sediment the lipoprotein. The supernatant solution was then centrifuged ($30\,000 \times g$ for 20 min) to sediment the ghosts, which were then washed 3 times with 0.01 M NaCl. Phospholipid analysis indicated 93 % to 100 % recovery of the membranes. Thin-layer chromatography analysis [16] of the membrane lipid extracts showed no trace of cholesteryl esters, the major constituent of the lipoprotein precipitate. Plasma lipids were extracted with $CHCl_3/CH_3OH$ [18]. Cell lipids were extracted using the $CHCl_3$/isopropanol procedure of Rose and Oklander [17]. Cholesterol was analyzed by the *o*-phthalaldehyde method of Zlatkis and Zak [19] and phospholipid determined from phosphate analysis by the method described by Rouser et al. [20], using dipalmitoyl lecithin as standard. Hemoglobin was determined by the cyanide-ferricyanide method [21]. Determinations were carried out at least in triplicate, with average deviations of about ± 2 %.

In the experiments on possible lysolecithin effects, egg lysolecithin and human serum albumin (fatty acid free, Fraction V) were used as obtained from Sigma Chemical Co., and dialysis of the preincubated plasma was across sausage casing, at 5 °C and against two ten-fold excesses of the previously described isotonic buffer for 4 h and 16 h respectively.

For examination of plasma pH effects, pH was adjusted by the addition of small amounts of 0.15 N HCl or NaOH after the plasma preincubation. The plasma was then centrifuged at $500 \times g$ for 20 min.

In experiments with cell ghosts, the ghosts were freshly prepared, using the method of Dodge et al. [22]. "Inside-out" membrane vesicles were prepared as described by Steck [23]. "Sealed" vesicles were separated from "unsealed" vesicles by

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* This lipoprotein precipitation is presumably similar to that found by Zapol et al. [15] on oxygenating dog plasma. In the present case, the amount of precipitation varied widely from experiment to experiment, typically being about 1 mg each cholesteryl ester and protein, 0.3 mg each of triglyceride and phospholipid, and 0.1 mg of unesterified cholesterol per 100 ml of plasma per 24 h. Since the cholesterol content of human red cells is 1.3 mg/ml spun cells, and since cholesteryl esters analyze as cholesterol in most analytical procedures, the lipoprotein precipitate would result in errors of more than 50 % in cholesterol/phospholipid ratios in experiments run at 1 % hematocrit, and errors of 5 % at 10 % hematocrit.

centrifuging in an 8 % solution of Dextran T-500 (Pharmacia) for 16 h at 22 000 rev./min using the Spinco SW 27.1 rotor. (The Dextran T-110 recommended by Steck is no longer available.) The band separating at the top of the solution was recovered, and the remainder of the vesicles discarded. The procedure resulted in an overall 25 % yield (based on lipid phosphorus) of vesicles, 85 % of which were "inside-out" and "sealed" according to the sialic acid accessibility (neuraminidase, Triton X-100) criterion [23]. Purification from "rightside-out" vesicles by aqueous phase partition, as recommended by Steck, was not carried out since, as will be seen, definitive conclusions could be drawn from the results on the 85 % pure preparation.

RESULTS AND DISCUSSION

A. Is the plasma the limiting factor in the extraction of cholesterol from the erythrocyte

It will be helpful to first consider some data from the previous workers [1, 2]. Their experiments with prolonged plasma preincubation, in which maximal esterification of lipoprotein cholesterol occurred, were carried out at 10 % hematocrit (i.e. with about 10 ml spun erythrocytes per 100 ml plasma). In the preincubation about 25 mg (of an original 45 mg) of lipoprotein cholesterol was esterified per 100 ml of plasma. The cholesterol content of the 10 ml of erythrocytes was decreased by about 4 mg (from an original value of about 13 mg) on subsequent shaking with this plasma. Thus only about 1/3 of the erythrocyte cholesterol was transferred to the lipoproteins, although the amount of plasma lipoprotein esterified during the preincubation was about twice the total erythrocyte cholesterol content. However, these data do not show that the limiting factor in the depletion lies in the erythrocytes and not in the plasma, since it is possible (a) that all of the plasma lipoprotein cholesterol esterified during the preincubation is not replaceable by cholesterol from the erythrocytes, and (b) that the extraction reached an upper limit because of either a deterioration of the lipoproteins during the extraction, or of the formation of substances that inhibited further cholesterol extraction.

In the first experiment these possibilities were examined by using the self-same unit of preincubated plasma to successively extract the cholesterol from three different samples of erythrocytes. In this experiment the cholesterol and cholesteryl ester contents of the plasma were also followed, and the cholesterol and phospholipid of the cells were determined with reference to a non-membranous component, the cell hemoglobin. Referring to Table I, and first considering the data on the cholesterol content of the plasma (column 6 and the footnote) it is seen that the preincubation results are similar to those of the previous workers in that about half of the plasma cholesterol became esterified during the incubations. Also similar to the experience of the previous workers, referring to column 3, the phospholipid to hemoglobin ratios indicate no loss of membrane phospholipid during the extractions.

Table I shows that about 40 % of the membrane cholesterol was removed by extracting for 6 h, and that no additional cholesterol was removed in the next 18 h. Since Table I also shows that the same plasma was then capable of extracting an equal quantity of cholesterol from two additional cell samples, the cessation of cholesterol removal from the cells was clearly not due to exhaustion of the plasma capacity to take up more cholesterol, or to changes in the plasma.

In a second, converse, experiment a given sample of erythrocytes was extracted

TABLE I

CELL AND PLASMA COMPOSITIONS AFTER EXTRACTING SUCCESSIVE FRESH CELL SAMPLES WITH THE SAME INCUBATED PLASMA* (5 % HEMATOCRIT**)

Extraction time (h)	Cells			Plasma	
	Cholesterol (mg/gm Hb)	Phospholipid*** (mg/gm Hb)	Cholesterol / Phospholipid	Cholesterol (mg/100 ml)	Cholesterol ester (mg/100 ml)
0	3.8	9.7	0.39	14	130
		After shaking first cell sample			
6	2.1	9.2	0.23	–	–
24	2.2	9.4	0.23	13	135
		After shaking second cell sample			
6	2.3	9.4	0.24	–	–
24	2.4	10.3	0.23	14	120
		After shaking third cell sample			
6	2.3	9.7	0.24	–	–
24	2.0	9.0	0.22	9	140

* Unincubated plasma contained 30 mg/100 ml cholesterol and 121 mg/100 ml cholesteryl ester.
** i.e. 1.5 gm Hb/100 ml plasma.
*** Computed as dipalmitoyl lecithin from phosphorus analyses.

with successive portions of fresh, incubated plasma for 24-h periods. As shown in Table II, no additional cholesterol depletion occurred after the first extraction. Again it is clear that the cessation of the cholesterol depletion in the first extraction was not due to the plasma's inability to extract more cholesterol.

As a third check on the plasma being the limiting factor in the cholesterol extraction, the rates of cholesterol depletion were determined with different ratios of plasma to cells. As shown by Fig. 1, the rates (and amounts) of cholesterol depletion are independent of the plasma to cell ratio, for ratios between 10 : 1 and 100 : 1. Fig. 1 also shows that the half-time for cholesterol depletion by incubated plasma is about 1 h, similar to that found for exchange of labelled cholesterol between lipoproteins and erythrocytes [3].

The final experiments of this group involved extractions for much longer periods than considered above. Extractions were carried out at 1 % and 5 % hematocrit for periods of 24, 48 and 72 h. As shown by Table III, no additional depletion

TABLE II

CHOLESTEROL DEPLETIONS ON EXTRACTING CELLS WITH SUCCESSIVE VOLUMES OF FRESH PREINCUBATED PLASMA (10 % HEMATOCRIT)

	Cholesterol/phospholipid (g/g)
Original cells	0.43
After extraction with: first volume	0.28
: second volume	0.30
: third volume	0.28

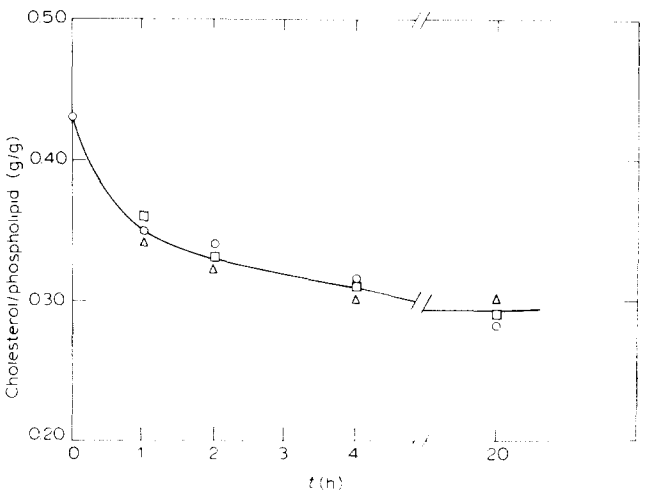

Fig. 1. Cholesterol depletion with various ratios of "preincubated plasma" to erythrocytes, ○ 10 % hematocrit; □ 5 % hematocrit; △ 1 % hematocrit.

occurs on prolonged extraction. Although considerable hemolysis occurred during the 48 and 72 h extractions, the cholesterol to phospholipid ratios of the recovered ghosts were similar to those of the erythrocytes that survived without hemolyzing. (The total phospholipid recovery in these cases accounted for about 70 % of the original phospholipid.)

B. Extraction from cell "ghosts" and from "inside-out" membrane vesicles

Table IV shows a comparison of cholesterol extraction from intact erythrocytes with that from "ghosts" prepared from the same blood sample by osmotic hemolysis. These extractions were done at 1 % hematocrit (i.e., in the case of the ghosts, using 100 ml plasma with the quantity of ghosts prepared from 1 ml of spun erythrocytes). These data show that cholesterol extractability from "ghosts" is no greater, and possibly somewhat less, than that from intact erythrocytes.

The next experiments explored the possibility that the limitation on cholesterol depletion is a result of the location of the remaining cholesterol at the inner membrane surface, where it is not accessible to the plasma lipoproteins. For this purpose use was made of erythrocyte membrane vesicles with "inside-out" orientations, i.e. vesicles

TABLE III

CHOLESTEROL DEPLETION ON EXTRACTION FOR LONG PERIODS

Extraction time (h)	Cholesterol/phospholipid (g/g)	
	1 % Hematocrit	5 % Hematocrit
0	0.36	0.36
24	0.22	0.24
48	0.25	0.22
72	0.23	0.23

TABLE IV

COMPARISON OF CHOLESTEROL EXTRACTION FROM INTACT ERYTHROCYTES AND FROM GHOSTS PREPARED BY OSMOTIC HEMOLYSIS

	Cholesterol/phospholipid (g/g)	
	Intact erythrocytes	Ghosts
Before extraction	0.42	0.42
After 24 h extraction	0.28	0.31
After 48 h extraction	0.29	0.33

in which the normally cytoplasmic surface is in contact with the plasma, and the normally external surface is not. In one experiment erythrocytes were shaken as usual in preincubated plasma to deplete the cholesterol. Inside-out vesicles were then made from these cholesterol depleted erythrocytes and shaken in preincubated plasma. In a second experiment, inside-out vesicles prepared from fresh erythrocytes were shaken in preincubated plasma. The results of these experiments are shown in Table V. It is clear that: (a) no further depletion of cholesterol takes place from membranes that have been turned "inside-out" after an initial maximal cholesterol depletion, and (b) the cholesterol depletion from inside-out vesicles is the same as that for whole cells. Thus the limitation to the cholesterol removal is not a simple result of the residual cholesterol being located at the inner surface of the membrane. This result may perhaps have been anticipated on the basis of the measured rates of "flip-flop" of cholesterol from the inner to outer surfaces of phospholipid bilayer vesicles [24]. The half-time for this process is about 1 h, a period far shorter than the maximum period of 72 h for which cells were shaken in preincubated plasma in this study.

C. Modifications of the extracting incubated plasma

Since the previous results indicated an inherent, about 35 %, limitation on the

TABLE V

CHOLESTEROL EXTRACTION FROM INSIDE-OUT VESICLES

Extraction at 5 % hematocrit for 20 h.

	μg/mg phospholipid		Cholesterol/ phospholipid (g/g)
	Neuraminidase-accessible sialic acid	Total sialic acid	
Cells before incubation	30	29	0.40
Cholesterol-depleted cells	28	30	0.27
Inside-out vesicles from cholesterol-depleted cells	5.2	29	0.27
Above vesicles after incubation	5.4	30	0.28
Inside-out vesicles from unincubated cells	4.8	32	0.41
Above vesicles after incubation	4.6	32	0.27

TABLE VI

CHOLESTEROL EXTRACTION IN SERUM WITH pH ADJUSTED AFTER PREINCUBATION

Shaken at 5 % hematocrit. Cholesterol/phospholipid ratio originally = 0.40.

Serum pH	Incubation time (h)	Cholesterol/phospholipid (g/g)
7.2 (unadjusted)	0	0.40
	4	0.32
	24	0.28
6.0	4	0.30
	24	0.23*
6.8	4	0.30
	24	0.23
7.8	4	0.29
	24	0.24
9.0	4	0.31
	24	0.21*

* Cells were completely hemolyzed after 24 h incubation at these pH extremes.

cholesterol that can be extracted with preincubated plasma, the sensitivity of this limitation to some modifications in the extracting plasma was examined. Murphy [1] has already reported that the amount of cholesterol extracted is not greatly affected by the temperature.

The results of extractions with plasma of various pH values, as obtained by addition of HCl or NaOH after the preincubation, are shown in Table VI. Extensive hemolysis occurred in all the extractions with the pH adjusted samples. However, the cholesterol/phospholipid ratios of the recovered ghosts were similar to those of the cells that survived without hemolysis. (Recovered ghosts and intact erythrocytes here accounted for from about 50 % of the original phospholipids in the extractions at the pH extremes, to about 80 % in the intermediary pH extractions.) Table VI shows that the cholesterol extracted in 4 h is independent of the pH of the extracting plasma, but that on extraction for 24 h more cholesterol was removed with the pH-adjusted plasmas than with the unadjusted plasma. In view of the extensive hemolysis in the cases of the adjusted plasmas, of the fact that greater depletions occurred at pH values both higher and lower than those of the unadjusted plasma, and the fact that depletions for 4-h shaking periods were unaffected by pH, we believe that the pH effects observed for 24-h shaking periods are not relevant to normal erythrocyte membranes but are a result of major membrane deterioration. In any event, the additional depletions attained with the pH adjusted plasmas were only moderate, and not sufficient to either significantly extend the range of studies on properties of cholesterol depleted cells or to modify the indications of two classes of cholesterol.

The final experiments examined whether the cholesterol extraction from the erythrocytes is sensitive to treatments of the preincubated plasma that would alter its lysolecithin content. Eqn. 1 indicates that lysolecithin is produced during the preincubation period in amounts equivalent to the cholesterol esterified. The 15 mg of cholesterol found (Table I) to become esterified per 100 ml of plasma corresponds to

the production of 20 mg of lysolecithin per 100 ml of plasma, a quantity which considerably increases the lysolecithin concentration above its normal level of 10 mg/100 ml [25]. Since lysolecithin distributes between the plasma and erythrocytes [25] and is known to interact with cholesterol [26], it was considered possible that the incorporation of lysolecithin into the erythrocytes was, in some way, responsible for the limitation to the cholesterol depletion. An effect of plasma lysolecithin on the cholesterol depletion was examined by: (a) adding an additional 50 mg of lysolecithin per 100 ml of preincubated plasma; (b) attempting to remove lysolecithin by dialyzing the preincubated plasma against isotonic buffer; (c) attempting to bind the lysolecithin by adding an amount of defatted human serum albumin (6 g per 100 ml of plasma) which, on the basis of the minimum of a 1 : 1 molar binding of lysolecithin to serum albumin [27], is more than sufficient to bind all the lysolecithin produced.

The results of these experiments are shown in Table VII. None of the treatments of the plasma had an effect on the cholesterol depletion. Analysis of the dialyzed plasma showed that no lysolecithin was removed by the dialysis; this may indicate that all of the lysolecithin produced during the incubation is bound to the serum albumin normally present. Both the addition of lysolecithin and of albumin resulted in considerable, about 50 %, hemolysis. However, as before, the cholesterol/phospholipid ratio of ghosts isolated from the plasma was the same as that of cells that survived the incubations.

In summary, our results show that (a) only about 35 % of the erythrocyte cholesterol can be extracted by preincubated plasma, (b) the limitation is not a result of the remaining cholesterol being located at the inner surface of the membrane, and (c) that the limitation is insensitive to a number of modifications of the plasma. Although no detailed study has been reported, it appears that there is also an upper limit to the fraction of the erythrocyte cholesterol that can be extracted by phospholipid liposomes [7, 28]. However, the maximum extractable by the latter is about 60 %, considerably greater than with incubated plasma. A possible reason for the difference is that the liposomes may cause extraneous changes in the membrane, either

TABLE VII

CHOLESTEROL DEPLETION IN SERUM TREATED TO CHANGE LYSOLECITHIN CONTENT

Extracted at 1 % hematocrit.

Serum treatment (after preincubation)	Extraction time (h)	Cholesterol/phospholipid (g/g)
None	0	0.42
None	24	0.28
	48	0.29
Lysolecithin addition	24	0.31
	48	0.29
Dialysis	24	0.28
	48	0.30
Albumin addition	24	0.29
	48	0.28

extracting substances other than cholesterol, or by themselves becoming incorporated into the membrane.

The limited extractability of cholesterol suggests the presence of two classes of cholesterol in the membrane, one removable and one tightly bound. Another possibility is that some structural change, that prevents further cholesterol depletion, occurs in the membrane on removing the first portions of the cholesterol. Studies of the exchange between erythrocyte cholesterol and isotopically labelled plasma lipoprotein cholesterol, in which there is no net change in the cholesterol content of the erythrocytes, should in principle permit a decision between the two possibilities. Unfortunately, the literature is divided between studies that indicate that the exchange continues until isotopic equilibrium is reached and those that indicate that only a portion of the erythrocyte cholesterol is exchangeable with plasma cholesterol [3, 11–14] and that therefore cholesterol exists in different states in the membrane.

There is little in the literature to indicate that 35 % of the erythrocyte cholesterol differs from the remaining 65 %. Badin and Denne report that only about 11 % of the cholesterol binds to streptolysin O [29]. It is perhaps significant that the permeability changes occurring in erythrocyte membranes on the exhaustive cholesterol depletion by phospholipid liposomes only occur after the removal of 30 % of the cholesterol [7].

Shattil and Cooper [30] have shown that the cholesterol/phospholipid ratio of the plasma membrane of reticulocytes, the precursors of erythrocytes, is the same as that of erythrocytes. Therefore, it does not appear that the 65 % non-removable cholesterol is present in a basic structural membrane and the remaining 35 % present only as a result of a subsequent transfer from the plasma lipoproteins.

REFERENCES

1 Murphy, J. R. (1962) J. Lab. Clin. Med. 60, 86–109
2 Cooper, R. A. and Jandl, J. H. (1969) J. Clin. Invest. 48, 906–914
3 Hagerman, J. S. and Gould, R. G. (1951) Proc. Soc. Exp. Biol. Med. 78, 329–332
4 Bruckdorfer, K. R., Demel, R. A., de Gier, J. and Van Deenen, L. L. M. (1969) Biochim. Biophys. Acta 183, 334–345
5 Sha'afi, R. J., Gary-Bobo, C. and Solomon, A. K. (1969) Biochim. Biophys. Acta 173, 141–142
6 Deuticke, B. and Zöllner, Chr. (1972) Biochim. Biophys. Acta 266, 726–731
7 Grünze, M. and Deuticke, B. (1974) Biochim. Biophys. Acta 356, 125–130
8 Vanderkooi, J., Fischkoff, S., Chance, B. and Cooper, R. A. (1974) Biochemistry 13, 1589–1595
9 Cooper, R. A., Arner, E. C., Wiley, J. S. and Shattil, S. J. (1975) J. Clin. Invest. 55, 115–126
10 Kroes, J. and Ostwald, R. (1971) Biochim. Biophys. Acta 249, 647–650
11 Basford, J. M., Glover, J. and Green, C. (1964) Biochim. Biophys. Acta 84, 764–766
12 Bruckdorfer, K. R. and Green, C. (1967) Biochem. J. 104, 270–277
13 Bjornson, L. K., Gniewkowski, C. and Kayden, H. J. (1975) J. Lipid Res. 16, 39–53
14 Bell, F. P. and Schwartz, C. J. (1971) Biochim. Biophys. Acta 231, 553–557
15 Zapol, W. M., Levy, R. I., Kolobow, T., Spragg, R. and Bowman, R. L. (1969) Curr. Top. Surg. Res. 449–474
16 Kwiterovich, P. O., Sloan, H. R. and Fredrickson, D. S. (1970) J. Lipid Res. 11, 322–330
17 Rose, H. G. and Oklander, M. (1965) J. Lipid Res. 6, 128–131
18 Nelson, G. J. (1972) Blood Lipids and Lipoproteins, p. 11, Wiley-Interscience, New York
19 Zlatkis, A. and Zak, B. (1969) Anal. Biochem. 29, 143–148
20 Rouser, G., Fleischer, S. and Yamamoto, A. (1969) Lipids 5, 494–496
21 Van Kampen, E. J. and Zijlstra, W. G. (1961) Clin. Chem. Acta 6, 538–545
22 Dodge, J. T., Mitchell, C. and Hanahan, D. J. (1963) Arch. Biochem. Biophys. 100, 119–130

23 Steck, T. L. (1974) in Methods in Membrane Biology (Korn, E., ed.), Vol. 2, pp. 245–281
24 Smith, R. J. M. and Green, C. (1974) FEBS Lett. 42, 108–111
25 Sato, T. (1973) Chem. Pharmacol. Bull. 21, 176–183
26 Rand, R. P., Pangborn, W. A., Purdon, A. D. and Tinker, D. O. (1975) Can. J. Biochem. 53, 189–195
27 Switzer, S. and Eder, H. A. (1965) J. Lipid Res. 6, 506–511
28 Bruckdorfer, K. R., Edwards, P. A. and Green, C. (1968) Eur. J. Biochem. 4, 506–511
29 Badin, J. and Denne, M. (1973) Protides Biol. Fluids 21, 45–48
30 Shattil, S. J. and Cooper, R. A. (1972) J. Lab. Clin. Med. 79, 215–227