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Characterization of Mechanisms for Transfer of Cholesterol between Human Erythrocytes and Plasma†

Yvonne Lange* and Josephine S. D'Alessandro

ABSTRACT: The removal from human erythrocytes of cholesterol (mass) and of [^{3}H]cholesterol which had been introduced into the erythrocyte by exchange was studied. Removal was accomplished by incubating erythrocytes in plasma, the free cholesterol content of which had been lowered by the action of lecithin:cholesterol acyltransferase. It was shown that the exchange of cholesterol between erythrocytes and plasma and the net movement of cholesterol out of the membrane into plasma are characterized by the same rate constant and are driven by cholesterol to phospholipid ratios in cells and plasma. The apparent limitation on cholesterol depletion of erythrocytes observed in experiments of this type is explicable as the result of equilibrium between cholesterol in the membrane and in the plasma, an equilibrium reached when there is still cholesterol left in the cells. It is concluded from this study that all the exchangeable cholesterol in human erythrocytes is available for removal from the membrane.

Recently, great progress has been made in the understanding of the molecular organization of the erythrocyte membrane. It has been established that phospholipids (Bretscher, 1972; Verkleij et al., 1973; Gordesky and Marinetti, 1973; Whiteley and Berg, 1974), proteins (Steck, 1972; Juliano, 1973; Whiteley and Berg, 1974; Amar et al., 1974), and carbohydrates (Nicholson and Singer, 1971; Juliano, 1973) are asymmetrically distributed between the two halves of the membrane bilayer. However, very little is known about the localization of cholesterol in the membrane and its interaction with other membrane constituents. One approach to this question has been through studies of cholesterol exchange between erythrocytes and plasma lipoproteins. Unfortunately, the results of such studies are divided between those that indicate that only a portion of erythrocyte cholesterol is exchangeable with plasma cholesterol (Bell and Schwartz, 1971; d'Hollander and Chevallier, 1972) and those that indicate that all of the cell cholesterol is available for exchange (Basford et al., 1964; Quarfordt and Hilderman, 1970). The presence of two classes of cholesterol in erythrocytes also has been suggested on the basis of permeability studies of cells having altered cholesterol content (Grunze and Deuticke, 1974) and from the results of a study of the correlation between erythrocyte cholesterol content and plasma cholesterol content in abnormal erythrocytes (Cooper et al., 1972).

In a recent paper (Gottlieb, 1976) the extent to which erythrocyte cholesterol could be depleted by incubation with plasma of lowered cholesterol content was studied. In these experiments, a maximum of 35% of membrane cholesterol could be removed. This finding was interpreted as indicating that the remaining 65% could not be removed because it was in a distinct, more firmly bound pool.

In this paper, we show that, in fact, all of the exchangeable cholesterol in the human erythrocyte membrane is available for depletion. This conclusion is based on a study of the removal from erythrocytes of cholesterol (mass) and [^{3}H]cholesterol which had been introduced into the membrane by exchange. We demonstrate that depletion and exchange are the same process, describable by a single rate constant. The limitation on cholesterol depletion observed when erythrocytes are incubated with plasma of lowered cholesterol content is because equilibrium between cholesterol in the membrane and in the plasma lipoproteins has been reached.

† From the Biophysics Division, Boston University School of Medicine, Boston, Massachusetts 02118. *Received February 17, 1977.* This work was supported by grants from the National Institutes of Health (HL 20303 and HL 18623).

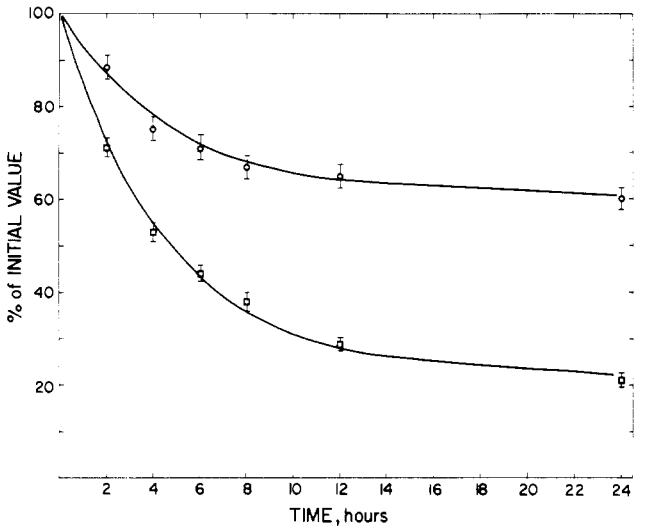

FIGURE 1: Cholesterol depletion from [^{3}H]cholesterol-labeled erythrocytes. Data from one experiment, representative of a total of five experiments. In each experiment seven to eight time points were taken and measurements of the composition of the extracted cell lipids made in duplicate: (○) cell cholesterol/phospholipid; (□) cell [^{3}H]cholesterol/phospholipid. Composition expressed in terms of initial value is plotted against time. The curves are obtained from eq 1 with τ = 4.8 h and B = 61% (upper curve) and 21% (lower curve).

Materials and Methods

Preparation of Erythrocytes and Plasma. Blood from healthy human donors was collected in heparin (4000 USP units/L of blood). Plasma and the "buffy coat" were separated from the cells after 15 min of centrifugation at 1500g. Prior to incubation, the red cells were washed three times in 10 volumes of buffer (buffer A) containing: 150 mM NaCl, 5 mM KCl, 5.5 mM Na_2HPO_4, 0.8 mM NaH_2PO_4, 0.5 mM $CaCl_2$, 5 mM glucose, pH 7.4 (Sha'afi and Lieb, 1967) with the addition of 0.2 mg/mL streptomycin sulfate as an antibacterial agent. Each experiment was carried out using cells and plasma from a single donor.

Preincubation of Plasma. Plasma cholesterol was lowered by the action of the enzyme normally present in plasma, lecithin:cholesterol acyltransferase (LCAT), using the technique described by others (Murphy, 1962; Gottlieb, 1976). Plasma, with the addition of streptomycin sulfate was incubated for 72 h at 37 °C and then heated at 56 °C for 50 min to destroy any remaining LCAT activity (Lichtman et al., 1974). Five milliliters of 5% glucose was added per 100 mL of preincubated (depleted) plasma prior to incubation with erythrocytes.

Labeling of Plasma and Erythrocytes. An aliquot of fresh plasma was preheated as above to destroy LCAT activity and then labeled with [^{3}H]cholesterol (New England Nuclear, Boston, Mass.) as described by Murphy (1962). In most experiments, approximately 6 mL of erythrocytes was labeled with [^{3}H]cholesterol by incubation at a hematocrit of 30% for 8–16 h at 37 °C in buffer A containing approximately 4% labeled plasma. The cells then were washed five times in 10 volumes of buffer.

Removal of Cholesterol from Erythrocytes. Cells labeled with [^{3}H]cholesterol were incubated at a hematocrit of 10% in depleted plasma for up to 24 h in a closed flask which was shaken at 37 °C. Aliquots were removed at various times and the erythrocytes separated from the plasma and washed three times in buffer prior to extraction of lipids for analytical determinations. Hemolysis, as determined from absorbance at 540 nm, was less than 1%, except in those experiments in which erythrocytes were incubated in depleted plasma for 24 h, at which time there was a maximum of 5% hemolysis.

Comparison of Cholesterol Exchange and Removal. Erythrocytes labeled with [^{3}H]cholesterol were incubated at 37 °C at a hematocrit of 10% in either (a) depleted plasma or (b) buffer containing preheated control plasma. The volume of control plasma used was chosen so that the amount of unesterified cholesterol in incubations a and b was the same. The radioactivity in the cells at subsequent times of incubation was determined as follows. A volume of 0.3 mL of the suspension was removed and the cells were separated by centrifugation for 3 min at 12 000g. The cells were washed once in 50 volumes of buffer, 0.3 mL of buffer was added to the cells, and duplicate 50-μL volumes of this suspension were placed directly in the scintillation solvent for radioactivity determination.

Analytical Determinations. Lipids were extracted from plasma by the technique of Folch et al. (1957). Unesterified cholesterol was isolated as the digitonide following the procedure of Courchaine et al. (1959). Red cell lipids were extracted by the technique of Dodge et al. (1963). Cholesterol was determined according to Parekh and Jung (1970) and expressed relative to the phospholipid content in the extracted lipids, assayed by phosphorus determination (Gomori, 1942). The phospholipid content of the cells remained constant in all experiments. The radioactivity in the extracted lipids was determined using Aquasol (New England Nuclear, Boston, Mass.) as scintillation solvent.

Results

Model. We propose that cholesterol movement between erythrocytes and plasma, whether exchange or net movement, is governed by the same process. The unidirectional flow of cholesterol from compartment A to compartment B is determined by the cholesterol to phospholipid ratio in compartment A and vice versa.

On the basis of this model one would expect that when erythrocytes are incubated in an excess of depleted plasma, a net movement of cholesterol out of the cells will occur until the gradient is dissipated and the unidirectional flows in both directions are equal. Cholesterol depletion from the red cells will cease when there is sufficient cholesterol in the plasma to generate this plasma to red-cell flow. When this occurs, there still will be cholesterol in the erythrocytes. The time course of the loss of [^{3}H]cholesterol from erythrocytes incubated in depleted plasma also can be predicted from the model. It will be described by the same rate constant as that describing cholesterol mass loss. However, loss of radioactivity from the cells will continue until the [^{3}H]cholesterol is distributed to equalize the specific activities of exchangeable cholesterol in the cells and in the plasma. As the plasma initially contains no labeled cholesterol, equilibrium will be attained after proportionately more [^{3}H]cholesterol than cholesterol mass is lost from the cells.

Cholesterol Depletion Measurements. Erythrocytes incubated in depleted plasma lost cholesterol at a rate which is shown for a typical experiment in Figure 1 (upper curve). The time course of cholesterol loss shown here is similar to that obtained by Gottlieb (1976). We obtained similar results in five experiments (Table I). In the experiment illustrated in Figure 1, cell cholesterol reached a minimum value after a loss of about 40%. The lower curve in the figure shows the loss of [^{3}H]cholesterol from the same erythrocytes. It can be seen that the cell radioactivity reached equilibrium after a loss of about 80%.

The data can be fit precisely on the terms of the model we

TABLE I: Composition of Cells and Plasma in Cholesterol-Depletion Experiments.

Expt	Incubation time (h)	Cells: Cholesterol M/M phospholipid C/P	Cells: [^{3}H]Cholesterol 10^{-5} dpm/μM phospholipid	Plasma unesterified cholesterol (mg/mL of plasma): Control	Plasma unesterified cholesterol (mg/mL of plasma): Depleted
1	0	0.83	7.69	0.41	0.16
	24	0.50	1.59		
2	0	0.86	8.54	0.72	0.28
	13	0.58	2.79		
3	0	0.93	10.28	0.40	0.10
	11	0.43	2.07		
4	0	0.96	6.85	0.47	0.12
	24	0.44	1.73		
5	0	0.88	4.48	0.52	0.19
	11	0.50	1.20		

TABLE II: Cholesterol Depletion Experiments Fit of Single Exponential to Data.

Expt	Cholesterol mass loss: B(%)	τ(h)	σ_τ	[^{3}H]Cholesterol loss: B(%)	τ(h)	σ_τ
1	61	4.76	0.79	21	5.00	0.30
2	60	5.40	1.50	30	4.17	0.78
3	34	2.27	0.28	22	2.00	0.12
4	48	2.22	0.33	29	2.00	0.22
5	57	2.78	0.69	24	3.03	0.60

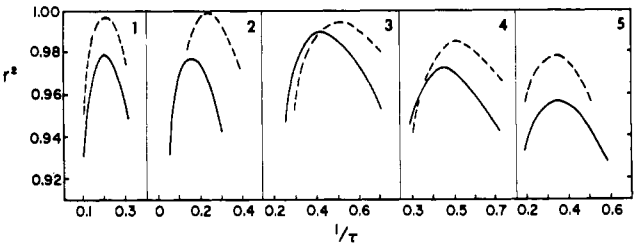

FIGURE 2: The dependence of the coefficient of determination, $r^2(b)$, on b for the five pairs of experiments given in Table I. Solid line refers to cholesterol mass loss data and dashed line refers to [^{3}H]cholesterol loss data.

are proposing. Namely, both curves of Figure 1 can be described by an exponential of the form

$$y = a \exp(-t/\tau) + B \tag{1}$$

with the same values of τ and different values of B, with the values of B reflecting the equilibrium distribution of cholesterol between erythrocytes and plasma.

Statistical Treatment. The determination of the parameters a, B, and τ to provide the best fit of eq 1 to each of the ten experiments and the determination of σ_τ, the variance giving the uncertainty in the determinations of τ, were performed in the following manner.

Choosing a value for b one can find analytically the $a(b)$ and $B(b)$ which minimize the residue

$$\sum_i (y_i - a(b)e^{-bt_i} - B(b))^2$$

where y_i is the data point for time t_i. The resulting minimum residue, $R(b)$, was determined over a range of b. All of the dependence of $R(b)$ on b lies in the coefficient of determination, $r^2(b)$. For each b,

$$R(b) = C(1 - r^2(b))$$

where C is independent of b. The most likely value of b, b_0, is that b for which $r^2(b)$ is a maximum. In Figure 2 the plot of $r^2(b)$ against b is given for the ten curves to be fit. The values of τ shown in Table II are the inverses of the values of b_0 at which the maxima of these functions lie.

To find the variance on the τ so determined, the following procedure was used. The likelihood that, of all possible values of b, the one which underlies the data is a specific one, b' is proportional to the function $L(b')$ given by

$$L(b') = \exp\left(-\left(\frac{1}{2\sigma^2}\right) R(b')\right)$$

Here σ is the variance per data point. σ is estimated from the residue of the best fit:

$$\sigma^2 = \left(\frac{1}{N-3}\right) R(b_0) \tag{2}$$

We found that, in all cases, this statistical estimate is comparable to what we would have estimated as the experimental uncertainty on a data point. This is an indication that the best fits obtained with single exponential curves are as good as is warranted by the data and more elaborate hypotheses as to functional form of the time dependence would not give more significant fits. With this estimate on σ^2, one obtains

$$L(b') = \exp\left[\frac{-(N-3)\,R(b')}{2R(b_0)}\right]$$

Expressed in terms of the coefficients of determination and dropping factors independent of b one obtains

$$L(b') = \exp\left[\frac{-(N-3)\,(r^2(b_0) - r^2(b'))}{2(1 - r^2(b_0))}\right]$$

Multiplying and dividing in the exponent by $(b' - b_0)^2$, we can rewrite this as

$$L(b') = \exp\left[-\frac{1}{2\sigma_b^2}(b' - b_0)^2\right]$$

where

$$\sigma_b^2 = \left(\frac{1}{N-3}\right)\left(\frac{(b' - b_0)^2(1 - r^2(b_0))}{r^2(b_0) - r^2(b')}\right)$$

If the $r^2(b)$ curves were perfectly parabolic, the distribution $L(b')$ would be purely Gaussian and σ^2 would be independent of b'. The slight nonparabolic asymmetry is averaged out in the following way. We find those b' values, b'_+ and b'_-, to the right and left of b_0, respectively, such that

$$r^2(b_0) - r^2(b') = 1 - r^2(b_0)$$

and choose

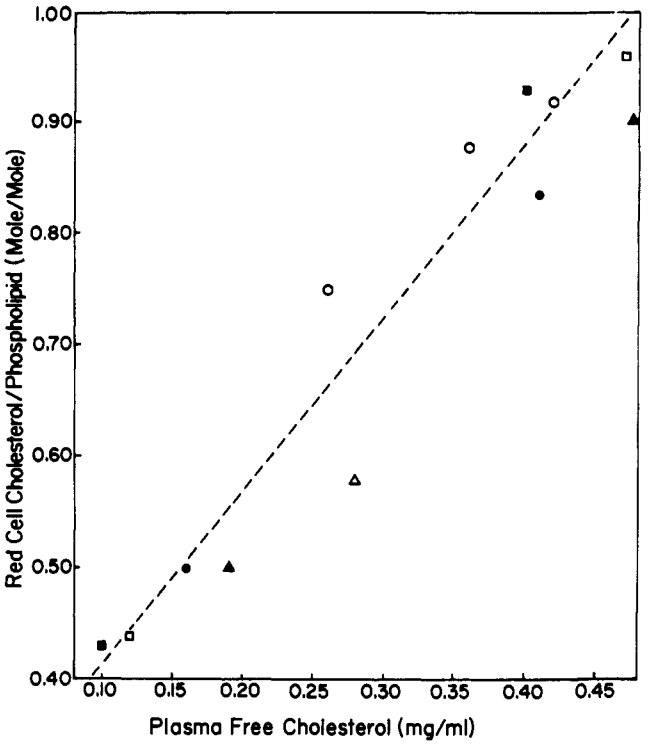

FIGURE 3: Relationship between erythrocyte cholesterol content and plasma free cholesterol at equilibrium. Data were taken from Table I: (●, △, ■, □, ▲) experiments 1–5, respectively. Data from an additional experiment (○) also are given (see text).

$$\sigma_b = \frac{1}{\sqrt{N-3}}\left(\frac{(b'_+ - b_0) + (b_0 - b'_-)}{2}\right)$$

Finally,

$$\sigma_\tau = \frac{\sigma_b}{b_0^2}$$

gives the variance on the determination of τ. The values of B, τ, and σ_τ for the ten curves for the data given in Table I are given in Table II.

For each of the five paired experiments, the value of τ which fits cholesterol mass loss and that which fits [^{3}H]cholesterol loss differ by less than at least one of the σ_τ values for the two experiments. This, together with the fact that the variance per data point given by eq 2 is comparable to the experimental uncertainty implies that the values of τ for cholesterol mass loss and [^{3}H]cholesterol loss are indistinguishable in these experiments. Since the parameter τ is quite well determined, the equality of the time constants for these processes is established.

The other crucial aspect of our present model is that the limitation on cholesterol loss from erythrocytes is due to the attainment of equilibrium with plasma. This is confirmed firstly by the fact that the curve-fitting procedure used yields values of the limiting erythrocyte cholesterol content, B, which are extremely close to those found experimentally. That is, the exponentials which best fit the data fit well at the extreme long time points as well as in other regions of the curve (see Figure 1 which illustrates the best fit to the data of experiment 1).

More directly, and as further confirmation of the hypothesis as to the source of cholesterol flow limitation, it is shown in Figure 3 that the limiting value of erythrocyte cholesterol content is approximately linearly related to plasma free cholesterol content. The figure gives the equilibrium values of cell cholesterol (expressed as mol of cholesterol/mol of phospholipid) from the cholesterol mass loss experiments of Table I. Values both for control and for depleted erythrocytes are plotted. In an additional experiment, erythrocytes were equilibrated with plasma which had been partially depleted of free cholesterol by preincubation for 6 and 24 h, instead of 72 h as was the case in the other experiments. The equilibrium cholesterol content of the erythrocytes in these experiments also is shown in Figure 3 (open circles). Figure 3 demonstrates that the equilibrium amount of cholesterol in the erythrocyte depends on plasma free cholesterol content; the amount of cholesterol removed from erythrocytes into depleted plasma is greater the lower the plasma free cholesterol.

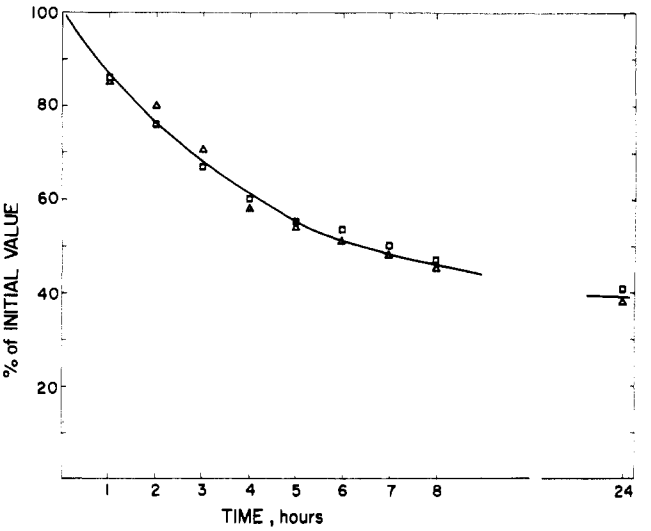

FIGURE 4: Comparison of cholesterol exchange (△) and depletion (□) from [^{3}H]cholesterol-labeled erythrocytes. Cells were incubated in cholesterol depleted plasma (□) or in control plasma (△), the volume of plasma used being chosen so that the amount of unesterified cholesterol in the plasma was the same in both incubations. The amount of [^{3}H]cholesterol in the cells expressed in terms of initial value is plotted against time. The curve gives the fit to eq 1 with τ = 3.8 h and B = 36%.

The experiments described thus far have shown that the transfer of cholesterol out of erythrocytes into plasma is characterized by the same rate constant whether exchange or net movement of cholesterol occurs. Further evidence of this comes from the experiment shown in Figure 4. In this experiment, the loss of [^{3}H]cholesterol from erythrocytes incubated in depleted plasma was compared to the loss of [^{3}H]cholesterol from erythrocytes incubated in normal plasma. The volume of plasma was chosen so that the amount of unesterified cholesterol in the plasma was the same in both cases. These conditions would ensure that the limiting values for the two processes are the same if depletion and exchange are the same process. The data shown in Figure 4 are completely consistent with the hypothesis that they are the same process, since the cell [^{3}H]cholesterol content changes with the same time constant and heads toward the same limiting value. The values of τ and σ_τ for this experiment, determined exactly as described above, were 4.07 h (σ_τ = 0.30) and 3.65 h (σ_τ = 0.20) for [^{3}H]cholesterol loss into normal and depleted plasma, respectively.

Discussion

The experiments described above demonstrate that erythrocyte cholesterol exchange and depletion are characterized by the same rate constant. We propose that both processes occur by the same mechanism and are governed by unidirectional flows. The limitation on cholesterol depletion of the erythrocyte does not imply the existence of a class of strongly

bound cholesterol molecules, as has been suggested previously (Gottlieb, 1976). Rather, it is due to the dissipation of the gradient leading to the net movement of cholesterol out of the cells into the plasma. However, as shown by the lower curve in Figure 1, cholesterol flows still occur between cells and plasma, leading to the equilibration of radioactive cholesterol between cells and plasma.

In a previous study of the extent to which erythrocytes can be depleted of cholesterol it was shown that, after the cells were maximally depleted by incubation with plasma of lowered cholesterol content, no further depletion occurred if the cells were incubated in a fresh aliquot of this plasma (Gottlieb, 1976). This result in no way contradicts our interpretation that cholesterol loss from erythrocytes ceases due to the fact that equilibration with plasma has occurred. Since in the experiment cited the incubations were done at low hematocrit, the plasma cholesterol content did not increase significantly during incubation and hence fresh plasma was identical to that it replaced.

Our results do not exclude the possibility that a membrane structural change could occur if sufficient cholesterol is extracted from the membrane. It can be seen from Table I that in the depletion experiments the erythrocyte cholesterol content at equilibrium varied in the different experiments. The maximum cholesterol removal from the cells was 55% in experiment 3. The removal of this amount of cholesterol resulted in some hemolysis, but did not lead to any anomaly in the time course of cholesterol exchange.

It is possible that the technique used to introduce [^{3}H]-cholesterol into the erythrocytes did not label all of the cell cholesterol, in other words, that all of erythrocyte cholesterol is not exchangeable with plasma free cholesterol. Such a possibility has been suggested by others (Bell and Schwartz, 1971; d'Hollander and Chevallier, 1972). Regardless of the existence of a pool not accessible by exchange, we have shown that those cholesterol molecules which are exchangeable with plasma are the same molecules as those which can be removed from the membrane in depletion experiments, since we have demonstrated that exchange and depletion phenomena are two manifestations of the same process. Thus, cholesterol molecules which participate in exchange and cholesterol molecules which can be removed from the membrane can be thought of as being in the same pool. This in no way precludes the existence of cholesterol in other pools which take part in neither process.

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