The Structure and Composition of Rat Reticulocytes. II. Phospholipid and Total Cholesterol in Reticulocytes

By T. Hallinan and E. Eden

MAMMALIAN RETICULOCYTES contain membranous intracellular structures, including mitochondria and an endoplasmic reticulum, which are not demonstrable in erythrocytes. Therefore, a quantitative investigation of the amounts of certain membrane components in the two cells was considered of interest. Phospholipid and cholesterol, both important components of red blood cell membranes, were chosen for this investigation. Reticulocytes of several species have been shown previously to contain more phospholipid per unit volume of packed cells than erythrocytes. However, all of these studies were made on a few samples of mixtures of reticulocytes and erythrocytes and give no estimate of the absolute quantity of phospholipid in the reticulocytes themselves. Similarly, it has been claimed that reticulocytes contain more total cholesterol per unit volume of packed cells than erythrocytes, but here again no estimate of the amount of cholesterol in reticulocytes was arrived at.

In this investigation, therefore, the concentrations of phospholipid and total cholesterol in rat reticulocytes are determined and compared with the concentrations of these lipids in erythrocytes. Findings are discussed in relation to the amounts and localization of lipids in normal reticulocytes and erythrocytes and the way in which these change in reticulocytes during maturation.

MATERIALS AND METHODS

Male white rats (Rattus norvegicus) of an inbred strain, weighing 120–300 Gm., were used in experiments. Anemia was induced by repeated hemorrhage as described by Pritchard or by injecting phenylhydrazine hydrochloride as described previously. Twice washed red blood cells were prepared from anemic and normal animals as described. In some experiments, concentrated samples of reticulocytes were prepared by a method utilizing their low specific gravity compared with erythrocytes. Suspensions of cells in saline were centrifuged at low speed (approximately 500 g) and then at higher speed (2000–3000 g), as recommended by Ferrebee and Geiman. This concentrates the low specific gravity reticulocytes in the upper layers of the packed cell column which were separated off and kept for analysis. Four repetitions of this procedure led to a 4–6 fold concentration of reticulocytes from normal blood. This method concentrates leukocytes as well as reticulocytes, and it is important that the former be removed from samples before analysis. This was done by suspending portions of the "buffy coat" formed or centrifugation in small volumes of saline with a glass rod and aspirating it off. This was repeated 3–5 times after each centrifugation (9–15 times in all), removing almost all of the leukocytes with a minimum loss of reticulocytes which are concentrated below the "buffy coat." The hematocrits of suspensions of cells in 0.15 M sodium chloride

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*For the sake of brevity, the word "erythrocyte" is used in this paper to mean the mature red cell.
were determined by the micromethod of Natelson.\textsuperscript{18} Capillaries of cell suspension were centrifuged at 3,600 g for 30 minutes, receiving an average impulse of 6.35 x 10\textsuperscript{6} dyne seconds, which Hlad et al.\textsuperscript{14} have shown is sufficient to ensure maximal packing of erythrocytes. The volume of packed cells in measured volumes of cell suspension analyzed, was calculated from the hematocrit. The percentage of reticulocytes in the samples of red blood cells analyzed was determined in smears stained with brilliant cresyl blue, fixed with methanol and counterstained with Wright's stain as recommended by Darmady and Davenport,\textsuperscript{15} 500–1000 cells being counted.

Lipids were extracted as recommended by Reed et al.\textsuperscript{16} with 1:1 chloroform-methanol from samples of 30–50 per cent cell suspension in saline containing 0.6–2 ml of packed cells. These were extracted once with 15 volumes of solvent and twice with 5–10 volumes at room temperature and the extract was evaporated to dryness \textit{in vacuo} on a rotary film evaporator at temperatures less than 0 C. The dried residue of the extract was extracted with three 20 ml aliquots of chloroform and the solution filtered through sintered glass and evaporated to dryness. The lipids were then dissolved in 20 ml of chloroform and the solution was termed the \textit{total lipid extract}. The phosphorus content of this solution was unaffected by washing with dilute calcium chloride as described by Folch et al.\textsuperscript{17} indicating that all the phosphorus present was lipid phosphorus.

Lipid phosphorus was estimated by the method of Allen\textsuperscript{18} and was multiplied by a factor of 25 to convert it to phospholipid. Total cholesterol (free cholesterol + any esterified cholesterol present) was estimated by the Liebermann-Burchard reaction by a modification of the method of Marinetti et al.\textsuperscript{19} Color was developed in a solution of chloroform, acetic anhydride and 36 N sulphuric acid, added to the sample in that order, in the ratios of 15:10:1 (v/v). Absorbency was read at 640 nm, 10 minutes after the addition of the sulphuric acid. Nucleic acids were fractionated by the method of Schmidt\textsuperscript{2} and RNA was determined by phosphorus\textsuperscript{18} and ribose\textsuperscript{21} estimation. DNA was determined by phosphorus estimation.\textsuperscript{18}

**Results**

Reticulocytes constituted 2–3 per cent of the red blood cells from normal rats, while in anemic animals they ranged from 7 per cent to 50 per cent. Concentration yielded samples containing up to 90 per cent reticulocytes from anemic animals and up to 12 per cent from normal ones, which were substantially freed of leukocytes as described above. The efficacy of this procedure for removing nucleated cells is demonstrated by the large RNA:DNA ratio of nucleic acids from reticulocyte-rich samples of cells prepared in this way. This ratio averaged 19 for seven samples of cells containing 20–87 per cent reticulocytes, a figure considerably greater than any previously reported for reticulocytes,\textsuperscript{2,22,23} which range from 5 to 10. This excludes the possibility that the samples analyzed contain significant numbers of nucleated red cells or leukocytes, since the latter have RNA:DNA ratios less than 1.\textsuperscript{2,22}

Figures for total phospholipid, total cholesterol and reticulocyte percentage are given in table 1 for samples of red blood cells containing varying percentages of reticulocytes. Bled animals were used in experiments 11 and 21; the remaining experiments were performed with phenylhydrazine-treated animals. Table 1 also shows the P/C ratio in the lipids of 12 samples of cells enriched in reticulocytes. Figures for lipids in reticulocyte-enriched samples are compared with average figures for total phospholipid, total cholesterol and P/C ratio in six samples of erythrocytes from normal blood, containing less than 3 per cent reticulocytes.

The concentration of phospholipid in rat erythrocytes, found in this in-
Table 1.—Phospholipid and Cholesterol in Reticulocytes and Erythrocytes

<table>
<thead>
<tr>
<th>Exp.</th>
<th>P-Lipid (mg./ml. P.C.)</th>
<th>Cholesterol (mg./ml. P.C.)</th>
<th>P-Lipid Cholesterol Reticulocyte%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.7</td>
<td></td>
<td>91</td>
</tr>
<tr>
<td>2</td>
<td>6.9</td>
<td></td>
<td>86</td>
</tr>
<tr>
<td>3</td>
<td>6.7</td>
<td></td>
<td>81</td>
</tr>
<tr>
<td>4</td>
<td>6.3</td>
<td></td>
<td>72</td>
</tr>
<tr>
<td>5</td>
<td>6.1</td>
<td>1.6</td>
<td>3.8</td>
</tr>
<tr>
<td>6</td>
<td>6.0</td>
<td>1.4</td>
<td>4.3</td>
</tr>
<tr>
<td>7</td>
<td>6.1</td>
<td>1.7</td>
<td>3.7</td>
</tr>
<tr>
<td>8</td>
<td>6.1</td>
<td></td>
<td>64</td>
</tr>
<tr>
<td>9</td>
<td>5.7</td>
<td>1.5</td>
<td>3.8</td>
</tr>
<tr>
<td>10</td>
<td>5.6</td>
<td></td>
<td>52</td>
</tr>
<tr>
<td>11</td>
<td>5.8</td>
<td>1.7</td>
<td>3.4</td>
</tr>
<tr>
<td>12</td>
<td>5.4</td>
<td>1.6</td>
<td>3.4</td>
</tr>
<tr>
<td>13</td>
<td>5.3</td>
<td>2.0</td>
<td>2.7</td>
</tr>
<tr>
<td>14</td>
<td>5.5</td>
<td>1.3</td>
<td>4.3</td>
</tr>
<tr>
<td>15</td>
<td>5.8</td>
<td>1.8</td>
<td>3.2</td>
</tr>
<tr>
<td>16</td>
<td>5.5</td>
<td>1.5</td>
<td>3.7</td>
</tr>
<tr>
<td>17</td>
<td>5.4</td>
<td></td>
<td>22</td>
</tr>
<tr>
<td>18</td>
<td>5.0</td>
<td></td>
<td>22</td>
</tr>
<tr>
<td>19</td>
<td>5.3</td>
<td></td>
<td>21</td>
</tr>
<tr>
<td>20</td>
<td>5.0</td>
<td>1.8</td>
<td>2.8</td>
</tr>
<tr>
<td>21</td>
<td>5.0</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>Mean</td>
<td>4.5 ± 0.17</td>
<td>1.6 ± 0.15</td>
<td>3.6 ± 0.4</td>
</tr>
</tbody>
</table>

Figures represent the concentration of lipids in samples of red blood cells enriched in reticulocytes; 11 and 21 are from bled animals, the remainder are from phenylhydrazine-treated animals. Where applicable, standard deviations are reported.

*The erythrocyte figures are averages for six samples of cells from normal blood.

Investigation, agrees well with that reported by Dzienian. In agreement with observations on other mammals, it was found that populations of rat red blood cells, enriched in reticulocytes, contain a higher concentration of phospholipid than do erythrocytes. Further, a highly significant correlation exists between the percentage of reticulocytes in a population and its phospholipid content. (r = 0.93; P < 0.001). However, no significant difference is observed between the concentration of cholesterol in populations of cells enriched in reticulocytes and that in erythrocytes, while the cholesterol concentration and reticulocyte percentage are wholly uncorrelated.

Computation of the Concentration of Phospholipid in Reticulocytes

Since it is not possible to obtain populations of pure reticulocytes, an estimate of the concentration of phospholipid in reticulocytes can only be obtained by extrapolation from data on mixed populations (table 1). The discussion below indicates how this extrapolation was performed, using the following symbols:

\[ C_e = \text{concentration of phospholipid in erythrocytes} \]
\[ C_r = \text{concentration of phospholipid in reticulocytes} \]
\[ Y = \text{concentration of phospholipid in mixed populations of erythrocytes and reticulocytes} \]
Fig. 1.—Curves of best fit relating phospholipid concentration to reticulocyte percentage.

\[ b = \text{ratio of the mean cell volume (MCV) of reticulocytes to that of erythrocytes;} \]
\[ X = \text{reticulocyte percentage.} \]

For any population of erythrocytes and reticulocytes,

\[ Y = \frac{(100 - X)C_e + bX C_r}{(100 - X) + bX} \] \hspace{1cm} (1)

In this equation, the values for \( X \) and \( Y \) can be taken from the data in table 1; the value for \( b \) for rat reticulocytes is not known, but for other mammalian reticulocytes the value of \( b \) varies from 1.75–2.14\cite{25-27}. Consequently, in figure 1 the experimentally determined values of \( X \) and \( Y \) have been plotted using values of \( b \) of 1 and 2.

For \( b = 1 \), the line of best fit is given by the equation

\[ Y = 0.0195X + 4.85 \] \hspace{1cm} (2)

For \( b = 2 \), the line of best fit is given by the hyperbola

\[ Y = \frac{8.84X + 448}{X + 100} \] \hspace{1cm} (3)

Equations 2 and 3 are represented in figure 1 by curves A and B respectively. Equation 2 gives values of 4.85 mg. phospholipid/ml. for \( C_e \) and 6.8 mg./ml. for \( C_r \), while equation 3 gives values of 4.44 mg./ml. and 6.66 mg./ml. respectively. Thus equation 3 gives a value for \( C_e \) which is closer to the ex-
Table 2.—Comparison of the Concentration of Phospholipid in Reticulocytes from Anemic and Normal Rats

<table>
<thead>
<tr>
<th>Normal Rats (mg./ml. of P.C.)</th>
<th>Anemic Rats (mg./ml. of P.C.)</th>
<th>Retic. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>5.1</td>
<td>12</td>
</tr>
<tr>
<td>5.0</td>
<td>5.0</td>
<td>9</td>
</tr>
</tbody>
</table>

experimentally determined concentration of phospholipid in erythrocytes, namely 4.5 mg./ml. In order to test whether the values of \( \bar{C} \), obtained from equations 2 and 3 differed significantly, an estimate of error was computed by summatng the squares of deviations of the experimental values of \( Y \) from the fitted curves. This gave values of 1.06 and 0.82 respectively for equations 2 and 3, from which the standard deviation of \( \pm 0.2 \) was obtained for each curve. Consequently, the two estimates of \( \bar{C} \) do not differ significantly, and have been combined to provide a mean estimate of 6.7 \( \pm 0.2 \) mg. phospholipid per ml. of packed cells.

**Influence of Experimental Anemia on the Phospholipid Content of Reticulocytes**

It has been frequently suggested that reticulocytes produced in experimental anemia, in response to drugs or even hemorrhage\(^2^\) might differ from those normally found in the circulation, though to date this has neither been proven nor disproven. Therefore, efforts were made to establish whether or not short-term bleeding or phenylhydrazine treatment affects the phospholipid content of rat reticulocytes, by comparing reticulocytes from anemic animals with those from normal ones. Table 2 shows that the concentration of phospholipid in two samples of reticulocytes from normal rats is no different from the concentrations calculated for corresponding samples from anemic animals. Therefore, short-term bleeding or phenylhydrazine treatment has no detectible effect on the phospholipid content of rat reticulocytes, so the value of 6.7 mg. per ml. is taken as the phospholipid content of normal reticulocytes.

**Reticulocyte Cholesterol**

There is no significant difference between the concentration of total cholesterol in erythrocytes and in samples of red blood cells containing up to 67 per cent reticulocytes as reported in table 1. In addition, the cholesterol content of these samples and their reticulocyte percentage are completely uncorrelated. This strongly suggests that rat reticulocytes do not differ significantly from erythrocytes in the amount of total cholesterol they contain. This is not in agreement with reports of Bodansky\(^9\) and Ponder,\(^5\) both of whom claimed that reticulocytes contained a higher concentration of total cholesterol than erythrocytes. It is noteworthy that Munn\(^3\) reported that human reticulocytes contain a significantly lower concentration of free cholesterol than erythrocytes. If reticulocytes contain more total cholesterol than erythrocytes, as Bodansky\(^9\) and Ponder\(^5\) suggest, but less free cholesterol, they must contain substantial amounts of cholesterol esters, which have not been conclusively demonstrated to be present in red blood cells to date.\(^3\)
**Phospholipid:Cholesterol Ratios**

The increase in the concentration of phospholipid in samples of red blood cells enriched in reticulocytes is not accompanied by an increase in the total cholesterol concentration. Therefore, the samples enriched in reticulocytes in table 1 have a significantly greater P/C ratio than erythrocytes \( (t = 6.1; P < 0.001) \). Reticulocytes contain 6.7 mg. of phospholipid per ml. of packed cells and taking the mean figure of 1.6 mg. per ml. of packed cells from table 1 as their concentration of total cholesterol, the P/C ratio of a pure sample of reticulocytes would be 4.2. This is 62 per cent greater than the mean P/C ratio for erythrocytes of 2.6.

**Discussion**

The rat reticulocytes investigated here contain 6.7 mg. of phospholipid per ml. of packed cells, 49 per cent more phospholipid than rat erythrocytes. Reticulocytes at all stages of maturity were present in the red blood cell samples analyzed, so this figure must be regarded as the average phospholipid content of a heterogeneous population of reticulocytes. The concentration of ATP in reticulocytes appears to decrease gradually as the cells mature to erythrocytes, so that reticulocytes at an early stage of maturity contain more ATP than those at a later stage. \(^{27}\) Since reticulocytes contain more phospholipid than the erythrocytes into which they mature, maturation must be accompanied by a net loss of phospholipid. Nothing definite is known of the kinetics or mechanism of this loss but it may be caused by activities within the immature cells themselves. Alternatively it may occur as a result of the action of their extracellular environment upon the immature cells. Crosby invoked a mechanism of the latter type when he postulated that the decrease in the concentration of lipids in reticulocytes might be mediated by the spleen. \(^{33}\) On the other hand, however, the decrease in the concentration of phospholipid in reticulocytes during maturation may be due to the action of intracellular phospholipases, similar to the ribonucleases \(^{30}\) and proteases \(^{34}\) which degrade other cellular components over this period.

It has been frequently suggested that reticulocytes from animals with induced experimental anemia might be abnormal. However, comparison of the concentration of phospholipid in reticulocytes from anemic animals with the concentration in reticulocytes from normal animals revealed no significant difference between the two. Reticulocytes from anemic animals contain high concentrations of ATP \(^{27}\) and are able to synthesize hemoglobin from free amino acids, \(^{35}\) which is evidence that they have not suffered serious functional alterations. Definite experimental evidence that they are abnormal is lacking.

It is generally agreed as a result of studies by Erickson et al. \(^{36}\) and Tishkoff et al. \(^{37}\) that most of the lipid of the erythrocyte is associated with the cell membrane. However, no previous experimental studies of the localization of lipid in the reticulocytes have been made, so no evidence exists to support Dziemian's assumption that most of the lipid in this cell is associated with the cell membrane also. \(^{3}\) Ponder worked on this assumption when he attributed the high concentration of lipid in reticulocyte-rich red blood cell populations to the increased quantity of cell membrane material contributed by the large
Reticulocyte phospholipid and cholesterol

reticulocytes. However, reticulocytes are more spherical than erythrocytes—hence a unit volume of them should contain not more but less cell membrane material than the same volume of smaller and less spherical erythrocytes. Therefore, if their membranes contain the same amount of lipid per unit area as those of erythrocytes, as Ponder implies, and most of their lipid is localized in the membrane, reticulocytes should contain less lipid per ml. of packed cells than erythrocytes.

This investigation shows that reticulocytes may indeed contain a slightly lower concentration of total cholesterol than erythrocytes, but their phospholipid concentration is 49 per cent greater than that of erythrocytes. This high concentration of phospholipid in reticulocytes can be accounted for without abandoning Ponder’s assumption of a similar lipid composition for the cell membranes of reticulocytes and erythrocytes, if it is postulated that a substantial proportion of the phospholipid of reticulocytes is localized in phospholipid-rich cholesterol-poor structures other than their cell membranes. Electron micrographs of fixed, sectioned reticulocytes show that they contain mitochondria and an endoplasmic reticulum, which are not demonstrable in erythrocytes. These structures have been shown to contain a high concentration of lipid with a large P/C ratio in other mammalian cells, so the hypothesis that a substantial proportion of the phospholipid of reticulocytes is associated with them would account for the high concentration of phospholipid and the large P/C ratio in these cells compared with erythrocytes.

Summary

1. The rat reticulocytes examined contain 6.7 ± 0.2 mg of phospholipid per ml. of packed cells, 49 per cent more than rat erythrocytes.
2. Reticulocytes from anemic animals appear to contain the same concentration of phospholipid as those from normal animals.
3. The concentration of total cholesterol in the reticulocytes examined does not differ significantly from that in erythrocytes.
4. The phospholipid:cholesterol ratio in lipid from reticulocytes is significantly greater (P < 0.001) than the corresponding ratio in lipid from erythrocytes. This finding, together with previous observations on the ultrastructure of reticulocytes, leads to the hypothesis that a substantial proportion of the lipid of these cells may be associated with phospholipid-rich, cholesterol-poor, intracellular structures such as mitochondria and endoplasmic reticulum.

Summario in Interlingua

1. Le reticulocytos de ratto examinate in le presente studio contineva 6,7 ± 0,2 mg de phospholipido per ml de cellulas paccate, i.e. 49 pro cento plus que erythrocytos de ratto.
2. Reticulocytos ab animales anemic contine apparentemente le mesme concentration de phospholipido como reticulocytos ab animales normal.
3. Le concentration de cholesterol total in le reticulocytos examine non differe significativemente ab illo in erythrocytos.
4. Le proportion phospholipido a cholesterol in lipido ab reticulocytos es significativemente plus grande (P < 0,001) que le correspondent pro-
portion in lipido ab erythrocytos. Iste constatation—insimul con previe ob-
servationes in re le ultrastructura del reticulocytos—supporta le hypothese
que un proportion substantial del lipido in iste cellulas es posibibemente
associate con structuras intracellular que es ric in phospholipido e povre in
cholesterol, i.e. per exemplo le mitochondrios e le reticulo endoplasmic.

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REFERENCES

1. Hallinan, T., Eden, E., and North, R.: The structure and composition of rat
3. Dzieman, A. J.: The permeability and the lipid content of immature red
5. Ponder, E.: Hemolysis and Related Phenomena. New York, Grune & Stratton,
7. Ts'o, P. O. P., and Lubell, A.: Concentration and collision-frequency of
8. Williams, H. H., Erickson, B. N., Beach, E. F., and Macy, I. G.: Biochemical
studies of the blood of dogs with n-
propyl disulphide anemia. J. Lab. &
and cholesterol esters in experimental
158:72, 1949.
12. Ferree be, J. W., and Geiman, Q. M.: Studies on malarial parasites: A pro-
cedure for preparing concentrates of
Plasmodium vivax. J. Infect. Dis. 78:
173, 1946.
13. Natelson, S.: Routine use of ultramicro
methods in the clinical laboratory. Es-
timation of sodium, potassium, chlor-
ide, protein, hemocrit value, sugar,
urea and nonprotein nitrogen in finger-
tip blood. Construction of ultra-
micro pipets. A practical microgasom-
eter for estimation of carbon diox-
affecting hematocrit determinations:
trapped plasma, its amount and dis-
tribution. J. Appl. Physiol. 5:457,
1952.
ical Laboratory Technicians and Medical
Students. 2nd ed. London, J. &
16. Reed, C. F., Swisher, S. N., Marinetti,
G. V., and Eden, E. G.: Studies of
the lipids of the erythrocyte. J. Lab.
17. Folch, J., Lees, M., and Sloane Stanley,
G. H.: A simple method for the iso-
lation and purification of total lipids
from animal tissues. J. Biol. Chem.
18. Allen, R. J. L.: The estimation of phos-
19. Marinetti, G. V., Scaramuzzino, D. J.,
and Stots, E.: Lipides of cytochrome
20. Schmidt, G.: Determination of nucleic
acids by phosphorus analysis. In
Colowick, S. P., and Kaplan, N. O.,
RETICULOCYTE PHOSPHOLIPID AND CHOLESTEROL


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