Lipids of Rabbit Blood Cells. Data for Red Cells and Polymorphonuclear Leucocytes

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For a number of years chemists have been interested in the lipids of both red and white blood cells. Many of these early observations have been reviewed by Bloor (1943). Recent publications from this laboratory have been concerned with the chemistry of white blood cells, particularly the enzymes of the polymorphonuclear leucocyte of the rabbit (Cram & Rossiter, 1949; Rossiter & Wong, 1949, 1950; Haight & Rossiter, 1950). It seemed of interest to investigate the lipids in these cells by the micro methods already in use (Johnson, McNabb & Rossiter, 1948*a*, *b*; 1949*a*, *b*, *c*). At the same time observations were made on the lipids of the rabbit red cells.

Many workers have investigated the lipids of blood cells, but few have attempted to distinguish between the different phospholipins. Exceptions are the reports of Thannhauser & Setz (1936*b*) on ox red cells, Erickson, Williams, Bernstein, Avrin, Jones & Macy (1938) on red-cell stroma, and Kirk (1938), Williams, Erickson, Avrin, Bernstein & Macy (1938), Erickson, Avrin, Teague & Williams (1940) and Hack (1947) on human red cells. As far as we are aware, no attempt has been made to determine the distribution of phospholipins in white cells.

METHODS

Red cells. Blood was withdrawn from the marginal ear vein of the rabbit. Heparin was used as an anticoagulant as recommended by Boyd & Murray (1937). The blood was centrifuged at 3000 rev./min. and the plasma and the buffy-coat layer aspirated off. The red cells were pipetted from the bottom of the packed cell layer to avoid contamination with white cells, which are considerably richer in lipids. The complete analysis in duplicate could be done on 7–8 g. packed cells.

Polymorphonuclear leucocytes. Polymorphonuclear leucocytes were obtained from the peritoneal cavity of the rabbit by the method of de Haan (1918), the details of which have already been described by Cram & Rossiter (1949). Usually 12 rabbits were injected at the same time and the resulting white cells pooled. These cells, 95% of which were polymorphonuclear leucocytes, were concentrated by centrifuging, washed with 0·9% (w/v) NaCl, packed at 3000 rev./min. in a tared centrifuge tube and allowed to drain for 30 min. The tube was then re-weighed. By this means 2–5 g. packed cells could be obtained, sufficient for the complete analysis in duplicate. No attempt was made to control the age, sex, weight or the dietary condition of the animals.

Analytical methods. The lipids were extracted from the cells as described by Johnson *et al.* (1948*a*). The concentration of cerebroside (or glycosphingoside), total and free cholesterol, total phospholipin, monoaminophospholipin (or phosphoglyceride), lecithin (phosphatidyl choline) and total fatty acid was determined for each sample. From these figures the concentration of ester cholesterol, sphingomyelin (phosphosphingoside), kephalin, neutral fat and total lipid was calculated. Details of the analytical procedures, together with a discussion of their specificity and accuracy are given in papers by Johnson *et al.* (1948*a*, 1949*c*). In the absence of information concerning the mean molecular weights of the fatty acids in the various lipid components of blood cells, the assumptions made previously for the lipids of the nervous system were used as a basis for the calculations. It is known that the fatty acids of the sphingomyelin from both red blood cells (Thannhauser, Setz & Benotti, 1938) and spleen (Thannhauser & Boncoddo, 1948) are different from those of brain sphingomyelin. The same is probably true for the fatty acids of the other lipid components of both red and white cells. The general nature of the results would not, however, be altered appreciably if the mean molecular weights of either the sphingolipins or the phosphoglycerides of blood cells were slightly different from those that have been arbitrarily assumed.

RESULTS

Table 1 gives the mean value and the standard error of the mean for the cerebroside, free, total and ester cholesterol, total phospholipin, total fatty acid, neutral fat and total lipid of both the red cells and the polymorphonuclear leucocytes of the rabbit. Table 1 also gives the value for each lipid component expressed as a percentage of the total lipid, and the ratio of the value found for the polymorphonuclear leucocytes to that for the red cells.

The concentration of total lipid in the polymorphonuclear leucocytes (1764 ± 110 mg./100 g.) was much greater than that in the red cells (484 ± 19 mg./100 g.). The polymorphonuclear leucocytes contained more of each of the lipid components investigated. The greatest difference was in the figures for neutral fat. Whereas the polymorphonuclear leucocytes contained 530 ± 130 mg./100 g., the red cells contained negligible quantities (41 ± 18 mg./100 g.). The polymorphonuclear leucocytes also contained over three times as much total phospholipin

Table 1. *Lipids of rabbit blood cells*

(Results as mg./100 g. fresh tissue.)

	Red cells				Polymorphonuclear leucocytes				
	No. of observations	Mean	S.E. of mean	Percentage of total lipid	No. of observations	Mean	S.E. of mean	Percentage of total lipid	Ratio white cells/ red cells
Cerebroside	6	33	8	6·8	6	41	30	2·3	1·24:1
Free cholesterol	6	146	3	30·2	7	234	10	13·3	1·60:1
Total cholesterol	6	146	3	30·2	7	243	10	13·8	1·66:1
Ester cholesterol	6	0	0	0	7	9	2	0·5	—
Total phospholipin	6	264	8	54·5	7	950	40	53·9	3·60:1
Total fatty acid	6	230	18	47·6	7	1140	100	64·6	4·96:1
Neutral fat	6	41	18	8·5	7	530	130	30·2	12·9:1
Total lipid	6	484	19	100·0	7	1764	110	100·0	3·65:1

Table 2. *Phospholipins of rabbit blood cells*

(Results as mg./100 g. fresh tissue.)

	Red cells				Polymorphonuclear leucocytes				
	No. of observations	Mean	S.E. of mean	Percentage of total phospho-lipin	No. of observations	Mean	S.E. of mean	Percentage of total phospho-lipin	Ratio white cells/ red cells
Total phospholipin	6	264	8	100·0	7	950	40	100·0	3·60:1
Monoaminophospholipin	6	212	9	80·4	7	670	30	70·6	3·16:1
Lecithin	6	94	5	35·6	7	300	30	31·6	3·19:1
Sphingomyelin	6	52	2	19·6	7	280	10	29·4	5·29:1
Kephalin	6	118	7	44·8	7	370	20	39·0	3·14:1

(950 ± 40 mg./100 g.) as did the red cells (264 ± 8 mg./100 g.). The concentration of cerebroside was similar in each type of cell, but the polymorphonuclear leucocytes had slightly more total cholesterol. Although there was no measurable ester cholesterol in the red cells, there was a small, but definite, concentration of ester cholesterol in the polymorphonuclear leucocytes.

The relative distribution of the various lipids in each of the cell types was also different. Neutral fat accounted for only 8·5 % of the total lipid of the red cells, whereas for the polymorphonuclear leucocytes the figure was 30·2 %. The percentage of the total phospholipin was similar for each cell type, but the percentage of cholesterol and cerebroside was somewhat greater in the red cells.

Table 2 gives the mean value and the standard error of the mean for each of the individual phospholipins, together with the value for each individual phospholipin expressed as a percentage of the total phospholipin. The ratio of the value found for the polymorphonuclear leucocytes to that for the red cells is also given in Table 2. As seen previously, there was over three times the concentration of total phospholipin in the polymorphonuclear leucocytes as there was in the red cells. Each of the three individual phospholipins was increased, but the greatest difference was for sphingomyelin. Whereas the white cells contained 280 ± 10 mg./100 g., the concentration in the red cells was only 52 ± 2 mg./100 g. This difference is also seen when the individual phospholipins are expressed as a percentage of the total phospholipin. The percentage of sphingomyelin in the polymorphonuclear leucocytes was much greater, while for the red cells the percentage of kephalin was increased.

DISCUSSION

The rabbit polymorphonuclear leucocytes were obtained from sterile peritoneal exudates by the method of de Haan (1918). Although microscopic examination showed that these cells appeared similar morphologically to those circulating in normal rabbit blood, the possibility that they may differ from normal cells, either physiologically or chemically, cannot be excluded. An outstanding feature of the results is that these cells contained much more lipid than rabbit red cells. The greatest difference was in the concentration of neutral fat, but they contained more of each of the lipid constituents studied. In the red cells, kephalin was the phospholipin present in greatest concentration, while in the polymorphonuclear leucocytes there was relatively less kephalin and more sphingomyelin.

The figures obtained for cholesterol, total phospholipin, total fatty acid and total lipid of the red cell are essentially similar to those reported in the literature. When the diversity of the methods used by previous workers is considered, it is remarkable that the agreement is so good. Iscovesco (1912), Mayer & Schaeffer (1913), Horiuchi (1920) and Bloor (1921) have given figures for the red cells of the rabbit, while similar figures for the red cells of man have been given by Mayer & Schaeffer (1913), Bloor (1916), Iwatsuru (1924), Boyd (1936*a*) and Erickson, Williams, Hummel & Macy (1937).

Bürger & Beumer (1913) reported that the stroma of red cells contained sphingomyelin, an observation confirmed by Thannhauser & Setz (1936*b*), using the reineckate precipitation method developed by Thannhauser and his colleagues (Thannhauser & Setz, 1936*a*; Thannhauser & Benotti, 1938). Subsequently sphingomyelin was isolated from the stroma of ox red cells by Thannhauser *et al.* (1938). It is now known that the reineckate precipitation procedure for the determination of sphingomyelin is unreliable (Hack, 1946; Schmidt, Benotti, Hershman & Thannhauser, 1946), so the finding of Thannhauser & Setz (1936*b*), that up to 50% of phospholipin of red cells is sphingomyelin, is probably an overestimate. However, Erickson *et al.* (1940), using a micro modification of Thannhauser's reineckate method, reported that sphingomyelin accounted for 16% of the phospholipins of the red cells of man. Hunter (1942), also using the reineckate method, reported a figure of 26% for the red cells of the cat. Kirk (1938), determining sphingomyelin as ether-insoluble phospholipin, found that 24% of the total phospholipin of the red cells of man was sphingomyelin, while Hack (1947), employing the method Schmidt *et al.* (1946) used in our study on the rabbit, reported a value of 25%. For the rabbit the figure was 20%.

The relatively high concentration of kephalin in red cells was commented upon by Bürger & Beumer (1913) and Haurowitz & Sládek (1928) who failed to detect any choline after the saponification of the ether-soluble phospholipins of red cells. The high content of kephalin has been reported for the red cells of man by Kirk (1938), Williams *et al.* (1938), Erickson *et al.* (1940) and Hack (1947). Similar figures are now reported for the red cells of the rabbit.

Few workers have reported on the concentration of cerebroside in red cells. We constantly found a substance in the red cells of the rabbit which was estimated as cerebroside, but the limitations of the method, discussed previously (Johnson *et al.* 1949*c*), must not be forgotten. Our figures for rabbit red cells are similar to those reported by Kirk (1938) for man. It is interesting to note that most of the lipid of the red cell is attached to the cell stroma and that the lipid distribution in the insoluble material after the cells have been haemolysed is similar to that of the intact cells (Bürger & Beumer, 1913; Erickson *et al.* 1938; Erickson *et al.* 1940).

The results obtained for the polymorphonuclear leucocytes are in good agreement with those of Boyd & Stevenson (1937), who studied cholesterol, total phospholipin, total fatty acid and total lipid of rabbit white cells. Our figures for cholesterol are somewhat lower, but it should be remembered that Boyd & Stevenson (1937) were estimating the lipids of suspensions of the mixed white cells obtained by centrifuging rabbit blood. Boyd & Stephens (1936) suggested that lymphocytes may have a different lipid content from that of polymorphonuclear leucocytes. Also it is possible that the white cells studied by Boyd & Stevenson (1937) were contaminated with blood platelets, which are known to contain much lipid material (Erickson, Williams, Avrin & Lee, 1939). In addition to his results on the white cells of the rabbit, Boyd (1933, 1936*b*) obtained essentially similar figures for the lipids of mixed white cells obtained from the blood of man.

Boyd did not attempt to measure either cerebroside or the individual phospholipins of white cells. Our figures for the concentration of cerebroside in rabbit polymorphonuclear leucocytes were low and somewhat variable, but those for sphingomyelin were high and very consistent. We are not aware of any previous suggestion that white cells contain sphingomyelin. We have shown that the polymorphonuclear leucocytes of the rabbit contain a phospholipin that, on hydrolysis, behaves as a diaminophospholipin or phosphosphingoside. Limitation of material has precluded any attempt to isolate this substance from the white cells, but the isolation of sphingomyelin from the spleen (Thannhauser & Setz, 1936*a*; Thannhauser & Boncoddo, 1948) makes it probable that this phospholipin of the rabbit polymorphonuclear leucocytes is indeed sphingomyelin.

SUMMARY

1. The concentration of cerebroside, total and free cholesterol, total phospholipin, monoaminophospholipin, lecithin and total fatty acid has been determined in both the red cells and the polymorphonuclear leucocytes of the rabbit. From these figures has been calculated the concentration of ester cholesterol, sphingomyelin, kephalin, neutral fat and total lipid. The red cells were obtained from the peripheral blood and the polymorphonuclear leucocytes from sterile peritoneal exudates.

2. The polymorphonuclear leucocytes contained more total lipid than the red cells. This was true for every lipid constituent measured, but the greatest difference was in the concentration of neutral fat. The polymorphonuclear leucocytes contained over twelve times as much neutral fat as did the red cells.

3. The relative amount of the phospholipin in the two cell types was similar, but the distribution of the individual phospholipins was different. The red cells contained more kephalin and the polymorphonuclear leucocytes contained more sphingomyelin.

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Observations on a Growth Factor for *Leuconostoc citrovorum*

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Sauberlich & Baumann (1948) first reported the presence in liver extracts of a growth factor for *Leuconostoc citrovorum*. Such a factor has been found both in liver extracts and in concentrates of vitamin B_{12} prepared from the fermentation liquors of *Streptomyces griseus* (Lees & Emery, 1949). Our aim has been to produce a concentrate of the factor, free from vitamin B_{12}, in order that it might be tested clinically for activity against pernicious anaemia. To help achieve this, a microbiological tube assay was developed.

EXPERIMENTAL

The microbiological tube assay

The assay differs from the assay of vitamin B_{12} with *Lactobacillus leichmanii*, to be reported elsewhere (Lees, Tootill & Emery, 1950), only in the nature of the test organism and its maintenance and in the omission of tomato juice from the medium.

The test organism is strain ATCC 8081 of *L. citrovorum* (Sauberlich & Baumann, 1948), maintained by fortnightly transfer in stab culture in the medium: Difco yeast extract,