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The Phospholipids of the Erythrocyte 'Ghosts' of Various Species

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There is abundant biological evidence of an active transport of cations across cell membranes (e.g. Hodgkin, 1951). Active transport is most easily explained by supposing a carrier molecule within the membrane, and an important task in biochemistry is the identification of such a carrier. A number of authors have suggested that one or more of the kephalins would fit the role (e.g. Davies & Krebs, 1952). Solomon, Lionetti & Curran (1956) found that chloroform-methanol extracts of human blood contained a much higher potassium/sodium ratio than was to be expected from the concentration of these ions in the original blood; on shaking the extract with water the ions were released. With purified phospholipid prepara-

tions, phosphatidylserine was particularly effective in taking up potassium relative to sodium. Kirschner (1959) attempted a crude fractionation of the phospholipids of pig erythrocytes. One fraction, which he tentatively identified as phosphatidylserine, contained sodium/potassium in the ratio 1.6/1, although the internal cation of pig erythrocytes is almost entirely potassium. Recently Hoffman, Schulman & Eden (1959) have described a model system in which phosphatidylethanolamine could specifically transport sodium across an artificial membrane.

Vogt (1957) has suggested phosphatidic acid as a possible cation carrier, and this view is supported by Hokin & Hokin (1959), who showed that slices

of the salt gland of the albatross (which is controlled by cholinergic nerves), when treated with acetylcholine, showed an increase over controls in the incorporation of ^{32}P into phosphatidic acid, whereas the incorporation into other phospholipids was unchanged.

In order to function as a carrier, a suitable phospholipid need be present in cell membranes in small quantities only, if the cation-exchange rate is sufficient. For most tissues it is impossible to prepare membranes freed from other cellular material, but erythrocyte stromata may approximate to this (Davson, 1959). For this reason we examined the composition of stromata and found the ratio of phosphatidylserine/phosphatidylcholine was high in ruminant stromata (noted by Dawson, 1957). While this work was in progress Turner (1957) published a preliminary account of semi-quantitative studies on the phospholipid composition of erythrocyte stromata and noted the absence of lecithin in ruminants, in contrast with non-ruminants. Similar results have recently been reported by Hanahan, Papahadjopoulos & Fennella (1959).

We have therefore extended our studies over a number of species, with a recently developed method (Dawson, 1960) for the analysis of individual phospholipids. This method allows greater discrimination between the acidic cephalins, which might be involved in cation transport.

EXPERIMENTAL

Preparation of stromata. Blood was obtained by venepuncture, except for pig blood, which was obtained immediately *post mortem*, and human blood, for which outdated transfusion blood was used. Heparin was used as anti-coagulant.

In the preparation of stromata, the erythrocytes were separated from plasma by centrifuging for 15 min. at 1200 g. The erythrocytes were washed three times in aq. 0.9% sodium chloride, and then haemolysed with an equal volume of water. The stromata were separated in a Sharples centrifuge at 12 000 g and washed thoroughly with water. On examination under the microscope they gave the typical appearance of erythrocyte 'ghosts'. When whole erythrocytes were used, these were washed only once in aq. 0.9% sodium chloride.

Preparation and analysis of lipid extracts. If the suspension of erythrocyte 'ghosts' or whole cells was directly treated with a chloroform-methanol mixture (2:1, v/v) according to the extraction procedure of Folch, Lees & Sloane-Stanley (1957), it congealed into a plastic putty-like lump. Consequently the suspension was first blended mechanically with 7 vol. of methanol, and then 14 vol. of chloroform was added. This resulted in a fine suspension which, after standing for 10–15 min., was either filtered through a coarse paper (Whatman no. 531) or centrifuged. The extract was shaken with 0.2 vol. of aq. 0.85% sodium chloride solution to remove non-lipid contaminants and allowed to stand at 2° until the solvent layers separated.

The lower layer was withdrawn, filtered and concentrated to about one-fifth on a rotary vacuum dryer. Lipid extracts of plasma were obtained in a similar manner.

The individual phospholipids in the complex lipid mixture extracted were estimated by the successive hydrolysis procedure of Dawson (1960), each determination being done in duplicate. Total phospholipid phosphorus was determined by the procedure of Fiske & Subbarow (1925) after the lipid residue had been digested with perchloric acid.

Phosphatidic acid. Sodium phosphatide was prepared enzymically from ovoidlecithin. The phospholipase D employed was prepared by homogenizing the yellow hearts of Brussels sprouts with 0.1 M-sodium acetate-acetic acid buffer, pH 5.6, and incubating this with lecithin according to the method of Davidson & Long (1958). After incubation the phosphatidic acid was extracted from the reaction mixture with ether, washed with 0.1 N-HCl and then purified by the method of Kates (1955). However, for removing traces of pigment it was found better to precipitate the sodium phosphatide from ether with ethanol rather than with acetone.

On deacylation by mild alkaline hydrolysis (Dawson, 1960), 98% of the phosphorus was recovered as glycerophosphoric acid; no phosphodiester were produced.

RESULTS

The results of the analysis of the phospholipids of erythrocyte 'ghosts' are shown in Table 1. Values for the recoveries show that approximately 91% of the lipid phosphorus was accounted for in the fractions examined, with individual total recoveries varying from 83 to 102%. The reason for this is unknown and it is in contrast with the more quantitative recoveries (94–104%) obtained for the plasma phospholipids (Table 2) and also of the phospholipids of sheep tissues found in a previous investigation (Dawson, 1960). There is a striking difference between the percentages of lecithin in the erythrocyte 'ghosts' of ruminants (mean $\pm$ S.E.M. = 9.1 ± 1.6) and those of non-ruminants (31.7 ± 2.2), a difference which is highly significant ($P < 0.001$). This lack of choline in the form of lecithin in ruminant-erythrocyte 'ghosts' is almost exactly compensated for by larger percentages of the other choline-containing phospholipids, i.e. sphingomyelin and choline plasmalogen. [Mean percentages: sphingomyelin (ruminants) 37.7 ± 2.5 , (non-ruminants) 19.8 ± 2.9 , $P < 0.001$; choline plasmalogen (ruminants) 7.6 ± 0.6 , (non-ruminants) 4.9 ± 0.8 , $P = 0.01$.]

The percentage of phosphatidylethanolamine appears to be much higher in the omnivores (man and pig) than in the herbivores (horse, cow, sheep and goat), the difference being statistically significant (herbivores $7.1\% \pm 0.8$; omnivores 20.4 ± 3.5 , $P < 0.01$). On the other hand, phosphatidylserine shows no significant variation between ruminants (13.6 ± 0.5) and non-ruminants (12.4 ± 1.5), and

this also applies to the minor phospholipid components.

The species variations seen in the phospholipids of erythrocyte 'ghosts' are in no way reflected in the distribution of individual phospholipids in the blood plasma (Table 2). Choline-containing phospholipids accounted for 94–98 % of the lipid phosphorus recovered.

In three cases where whole erythrocytes were examined (Table 1), there was no obvious difference from the distribution found in the 'ghosts'. That this is not simply because all the phospholipids of erythrocytes sediment on the centrifuging was shown by an experiment with haemolysed pig erythrocytes, where 41 % of the lipid phosphorus was found not to sediment on centrifuging at 36 000–90 000 g for 90 min.

During the washing procedure used for purifying lipid extracts there is a tendency for small amounts of acidic phospholipids to be lost in the washing media. Consequently phosphatidic acid (7 %) was added to a chloroform-methanol extract of the lipids of sheep erythrocytes, and after washing with aq. 0.85 % sodium chloride the chloroform layer was analysed for phospholipids. About 80 % of the added phosphatidic acid was recovered, which suggests that any losses of phosphatidic acid occurring are small and unlikely to affect the analytical conclusions reached in this investigation.

DISCUSSION

Although the osmotically disrupted blood cells used in the present work were not entirely erythrocytes, it is unlikely that their analyses would be substantially different from the corresponding values for pure erythrocytes, because of the overwhelming preponderance of these cells in normal blood. It cannot, however, be assumed that the erythrocyte-'ghost' preparation represents only the membrane of the cell for it is possible that it contains other structures which in the original cell carried out functions apart from ion and metabolite transport.

Any theory of cation transport involving the hypothesis of a carrier does not necessarily require that the latter should be present in high concentrations. Nevertheless, it is interesting to look at the present results in the light of past suggestions about the kephalin nature of such a carrier. There is no indication that phosphatidic acid is present as more than a very minor part of the phospholipid fraction. On the other hand, phosphatidylserine is present in the phospholipids of erythrocyte 'ghosts' at a concentration which is probably higher than that in most other tissues apart from the nervous system. Its concentration is, moreover, generally constant in the erythrocytes of all

Table 1. *Phospholipids in whole erythrocytes and 'ghosts'*

	Human		Pig		Horse		Cow		Sheep		Goat	
	'Ghosts'	Whole cells	'Ghosts'	Whole cells	'Ghosts'	Whole cells	'Ghosts'	Whole cells	'Ghosts'	Whole cells	'Ghosts'	Whole cells
Lecithin (+lysolecithin)	28.2	30.3	25.1	24.5	37.1	35.8	14.1	5.5	11.0	4.4	7.5	12.0
Phosphatidylethanolamine	13.6	15.2	27.7	25.2	9.4	4.1	11.4	6.7	6.1	4.8	6.3	7.4
Phosphatidylserine	10.4	13.0	18.6	14.5	9.5	8.2	12.8	15.2	12.4	12.9	14.8	13.6
Sphingomyelin	15.8	19.3	15.4	12.7	23.8	3.9	31.2	48.2	37.0	48.0	33.1	35.8
Choline plasmalogen	5.3	2.3	5.3	5.5	7.9	3.2	7.8	6.2	10.2	7.8	6.7	6.8
Ethanolamine plasmalogen	2.1	6.0	1.5	2.7	4.2	2.8	3.3	2.1	3.4	0.8	3.2	3.7
Serine plasmalogen	0.8	0.8	1.5	1.7	0	1.4	1.2	0	1.7	0.5	2.3	1.7
Monophosphoinositide	1.8	1.4	3.5	2.9	4.8	2.4	3.8	3.2	3.5	3.8	6.4	5.2
Phosphatidic acid	1.2	1.7	0.9	0.8	1.6	0.2	0.7	0.5	0.5	0.3	0	0.5
Polyglycerolphospholipid	0	0	0	0	0.9	0.2	1.0	0	0.8	0	1.9	0.6
Unknown	3.5	3.0	0.9	1.1	2.5	2.0	2.8	2.4	3.5	1.6	1.8	2.7
Recovery	82.7	93.0	100.4	91.6	101.7	92.2	90.1	90.0	90.1	84.9	84.0	90.0

Results express the phosphorus of phospholipids as a percentage of the total lipid phosphorus. Each value represents the mean of a duplicate determination on a different preparation of 'ghosts' or of whole cells.

Table 2. *Phospholipid in plasma of various species*

Results express the phosphorus of phospholipid as a percentage of the total lipid phosphorus.

	Human	Pig	Horse	Cow	Sheep	Goat
Lecithin (+lysolecithin)	74.5	79.0	83.2	82.0	70.3	88.0
Phosphatidylethanolamine	1.8	2.2	0.5	0	2.4	0.5
Phosphatidylserine	0	0	0	0	0	0
Sphingomyelin	12.4	10.3	10.4	10.8	15.8	9.0
Choline plasmalogen	3.8	2.7	1.5	6.4	2.1	2.0
Ethanolamine plasmalogen	0	0	1.0	0	0	0
Serine plasmalogen	0	0	0	0	0	0
Monophosphoinositide	1.4	2.2	0	2.4	0	1.7
Phosphatidic acid	0	0	0	0	0.9	0.4
Polyglycerolphospholipid	0	0.5	0	0.7	1.6	0
Unknown	1.3	0.6	0.7	2.0	0.6	0.8
Recovery	95.2	97.5	97.3	104.3	93.7	102.4

species, in contrast with the large species variations found in the concentrations of lecithin and phosphatidylethanolamine.

Another acidic phosphoglyceride which could conceivably function as a cation carrier, namely monophosphoinositide, was present in small but definite amounts. It was present in highest concentration in the erythrocyte 'ghosts' of the goat and was lowest in those of man. Bangham, Pethica & Seaman (1958) have described the charged groups at the surface of a sheep erythrocyte as having properties consistent with those of a phosphate and it seems likely that these could result from the presence of monophosphoinositide and, to a much lesser extent, polyglycerolphospholipid and phosphatidic acid.

The present results on the phospholipid analysis of whole erythrocytes and their 'ghosts' confirm and extend previous reports of the high phosphatidylserine content of the phosphoglyceride fraction of ruminant erythrocytes (Dawson, 1957), and the very small amounts of lecithin in ruminant erythrocytes compared with those in erythrocytes of the non-ruminant (Turner, 1957; Turner, Anderson & Gandal, 1958; Hanahan *et al.* 1959). They also reveal other species differences, namely an increase in the percentage of sphingomyelin in the ruminant-erythrocyte 'ghosts' compared with those of the non-ruminant. Moreover it is apparent that in the omnivores (pig and man) the concentration of phosphatidylethanolamine in the 'ghosts' is many times greater than the concentration in those of the strict herbivores, i.e. horse, sheep, cow and goat. In the pig erythrocyte 'ghosts' its concentration is as high as that of the lecithin fraction, which is an unusual finding for mammalian tissues.

The reason why the erythrocyte 'ghosts' of ruminants possess so much less lecithin and phosphatidylethanolamine and more sphingomyelin than those of non-ruminants is not obvious. It is pertinent perhaps to mention here that ruminant erythrocytes are much smaller than those of the

non-ruminant and that they are, in contrast with those of the non-ruminant, almost devoid of diphosphoglycerate (Rapaport & Guest, 1941).

The species differences of erythrocyte phospholipids are not in any way reflected in the relative concentrations found in the plasma. In all species, over 94% of the phospholipid fraction can be accounted for as the choline-containing phospholipids, lecithin + lysolecithin, choline plasmalogen and sphingomyelin. This is in agreement with a number of previous reports of the complete dominance of the choline-containing phospholipids in the plasma of various species (e.g. Sinclair, 1948; Axelrod, Reichenthal & Brodie, 1953), and extends these findings to the horse, sheep and cow. Even the small amounts of cephalin present may possibly arise from chylomicrons, which are difficult to remove from the plasma by centrifuging and which are known to contain cephalin (Robinson & Poole, 1956).

Turner *et al.* (1958) have suggested that the lack of lecithin in washed ruminant erythrocytes may explain their relative resistance to haemolysis by snake venoms and the α toxin of *Clostridium welchii*. The present findings, in confirming the lower concentration of lecithin in ruminant-erythrocyte 'ghosts', would agree with this viewpoint, although, in contrast with the results of Turner *et al.* (1958), some lecithin was always found. It is unlikely that this represents trapped plasma lecithin, since ruminant whole cells, which were less efficiently washed than the 'ghosts', contain no more lecithin. Nevertheless, the amount of haemolytic lysolecithin formed by cobra venom and the disruptive effect of α toxin are likely to be much less with ruminant erythrocytes than in the non-ruminant erythrocytes.

SUMMARY

1. The composition of the phospholipid of erythrocyte 'ghosts' and plasma from six species (man, pig, horse, cow, sheep and goat) has been measured.

2. The percentage of lecithin in the non-ruminant 'ghosts' was over three times as high as that found in ruminant 'ghosts'.

3. In contrast, the concentrations of sphingomyelin and choline plasmalogen were higher in ruminant 'ghosts' and consequently there were no significant differences between the concentrations of total choline-containing phospholipid in the various species.

4. Phosphatidylethanolamine was markedly higher in the 'ghosts' of omnivores than in 'ghosts' of herbivores.

5. Choline-containing phospholipids (mainly lecithin) accounted for over 94% of the plasma phospholipid of all species.

6. Of the possible acidic phospholipids in erythrocyte 'ghosts' which could function as cation carriers, phosphatidylserine was present at a relatively high concentration (8.9–16%) in all species, phosphatidylinositol varied from 1.6% in man to 5.8% in the goat and phosphatidic acid was below 1.5% in all species.

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Evidence for the Chemical Interaction of Urease in Solution

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Urease prepared by the method of Sumner (1926) and recrystallized by the modified procedure of Dounce (1941) has in all cases reported shown at least two peaks in the ultracentrifuge (e.g. Sumner, Gralen & Eriksson-Quensel, 1938; McLaren, Sheppard & Wagman, 1948). Enzymic-activity studies (Hofstee, 1949), sedimentation and activity studies (Kuff, Hogeboom & Striebig, 1955), molecular-weight estimations (Setlow, 1952) and kinetic experiments (Kistiakowsky & Lumry, 1949; Ambrose, Kistiakowsky & Kridl, 1950) all provided results which could be interpreted to some degree by assuming that the components observed in the ultracentrifuge are aggregates of the same species. In this connexion it is pertinent

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that a reaction involving the readily detectable sulphydryl groups in the undenatured enzyme (Hellerman, Chinard & Dietz, 1943) to form disulphide linkages has been discussed (Sizer & Tytell, 1941; Kistiakowsky & Lumry, 1949); this disulphide-bond formation may occur intermolecularly, as such a cross-linkage has been postulated with other protein systems where sulphydryl groups are readily 'available' (e.g., Huggins, Tapley & Jensen, 1951; Steinrauf & Dandliker, 1958). Since an understanding of the nature of this aggregation reaction, if it occurs, is essential to proper formulation of the kinetics, and hence to any interpretation of the mechanism of urease action, a detailed study has been made of the ultracentrifugal characteristics of the substance, and is reported here.