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Comparative Liberation of Bound Phosphatides from Red Cells of Man, Ox, and Camel.* (24415)

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It has recently been reported that lecithin is lacking from stromata of red blood cells of the ox, sheep, and goat(1). On the other hand, cells of 8 species of non-ruminants all contained substantial amounts of lecithin. The findings could be correlated with susceptibility to direct hemolysin of cobra venom. The cells of most animals are lysed by venom but those of true ruminants (ox, sheep, goat) are completely resistant. The venom toxin appeared, therefore, to be a lecithinase, and susceptibility to its action dependent upon presence of lecithin in the cell membrane. It was also noted that the erythrocytes of the *Pecora* are distinguished from those of other groups of mammals by additional peculiarities including their remarkably small size(2). From a morphological point of view it became of particular interest to examine the phosphatides of red cells of the camel, since they are unique among mammalian types in being oval, rather than circular, discs. When washed red cells of a dromedary and of a guanaco were tested for susceptibility to direct hemolysin of cobra venom they were quite resistant to lysis. The finding suggested that camel red cells might lack lecithin. However, when lipid extracts of both dromedary and guanaco cells were made and subjected to paper chromatography, large spots corresponding to lecithin were found(1), as well as other spots indicating presence of appreciable amounts of cephalins and of sphingomyelin. Enough material has not yet been obtained for a properly detailed analysis of the lipids of camel cells. Some additional evidence was secured nonetheless by allowing venom to act on lipid extracts according to the method of Hanahan (3). When chromatography was subsequently carried out, it was found that most of the lecithin and the cephalins had disappeared, to be replaced by spots of slower mobility corresponding to lysophosphatides. These preliminary data indicated that lecithin is probably present in the camel red cell and that resistance to venom in this case may be due to a binding of lecithin in such a way that it is "unavailable" to venom lecithinase(4). The experiments now reported represent an attempt to examine comparative stability of li-

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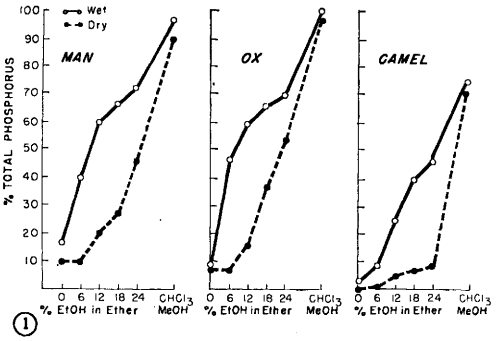
pid complexes of red blood cells. It will be shown, with a system of extraction by ether-alcohol mixtures, that the stromal phosphatides are extracted much less readily from camel cells than from either human or bovine cells. The method of extraction employed derives from that of Macheboeuf as applied to plasma lipoproteins(5), and was adopted on the suggestion that similar studies might be made of various tissues(6).

Methods. The red cells of citrated or oxalated fresh whole blood were washed 3 times in 0.9% NaCl and the buffy coat removed. Lysis was carried out with 10 to 20 volumes of water and the ghosts collected by centrifugation. These were now washed several times in 20 volumes of water, until the supernatant was phosphorus-free. The washed ghosts were then lyophilized and stored at −20°C until used. Extractions with ether-ethanol were carried out at 4°C on 100 mg lyophilized ghosts containing a total of 200 to 300 μg P. Separate studies were made of the influence of water by adding 0.3 to 0.4 ml H_2O to 100 mg lyophilized ghosts. In both cases the material was extracted by mixing in 50 ml centrifuge tube with 25 ml solvent. This was centrifuged and the supernatant collected after filtering. An additional extraction with 25 ml of the same solvent was carried out in the same way and the supernatants were pooled. The sedimented ghosts were then extracted with the next solvent. The first solvent was anhydrous diethyl ether, and this was followed by a series of increasing amounts of absolute ethanol in ether, *viz.*, 6%, 12%, 18%, 24%. Finally, a last extraction was performed with $CH\,Cl_3$: Me OH (2:1) at room temperature. Studies were also made using a series of ether-methanol mixtures at room temperature. Phosphorus was determined by the method of Beveridge and Johnson(7) after appropriate concentration of the extracts. The effectiveness of the lyophilization process was examined several times by further drying over P_2O_5 and the additional loss of water was never more than 3% of weight. Chromatography on silicated papers of concentrates of many of the extracts was carried out as previously described(1). It should be emphasized that extractions were carried out as quickly as possible, *i.e.*, time elapsing between first exposure of ghosts to a solvent and their transfer to the next solvent was about 30 minutes. This procedure was adopted on the view that the shorter the time the less the degree of denaturation. Some clumping of ghosts occurred, particularly in the solvents containing little or no alcohol. In separate experiments with human ghosts macroscopic clumping was appreciably reduced by mixing and grinding in a mortar the dry ghosts with twice their weight of Celite, before extraction. This step did not result in increased removal of phosphatides by the ether-alcohol mixtures. Furthermore, the Celite remained with the final residue of ghosts and interfered with P determinations on those residues. It was concluded that use of inert dispersing agents was likely to aid little in increasing the solvents' accessibility to the cells and might well introduce new errors. Moreover, the simple method of direct extraction of the ghosts was validated by the reproducibility of the results and the magnitude of the differences that could be shown between camel cells and those of other species.

Results. Fig. 1 exhibits the principal data. It can be seen that for lyophilized human and bovine cells very little phosphatide is removed until the percentage of ethanol reaches 12%. Thereafter appears a steady rise in the curve, so that with completion of the last (24%) ethanol extraction some 45 to 55% of the total P has been removed from the cells. On the other hand, the corresponding point on the curve for camel cells gives a value of less than 10%.

When the cells were moistened with water before extraction, liberation of phosphatides was enhanced in all cases. Again, however, the curve in the case of the camel is less steep than for man and ox. Thus, for example, at the 12% ethanol level, 24% of the P has been extracted from camel cells, whereas 60% has been removed from human and bovine cells. When lyophilized cells were extracted at room temperature with ether-methanol mixtures, similar results were obtained (Fig. 2). Rate of liberation of phosphatides from camel cells once more lags behind those of the other animals.



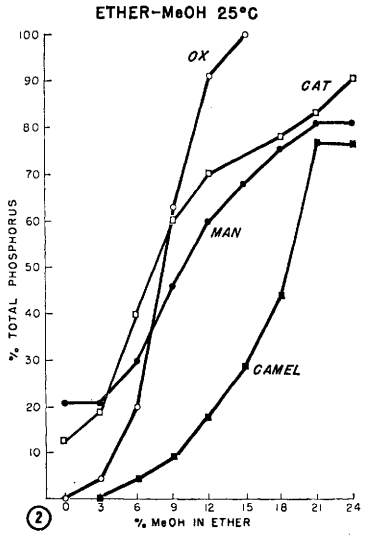


FIG. 1. Comparative rates of liberation of phosphatides from red cell ghosts. Ordinate: % of total P extracted. Abscissae: Progressive series of extractions with solvents as noted. Ether-ethanol at 4°C; CH Cl_3: methanol (2:1) at 25°C. Dotted lines: Lyophilized ghosts. Solid lines: Wet ghosts.

FIG. 2. Liberation of phosphatides from lyophilized red-cell stromata by ether-methanol mixtures at 25°C.

In view of Chargaff's studies of complexes of acidic phospholipides, *e.g.*, phosphatidyl serine(8) it became of interest to see whether the neutral choline-containing phosphatides were liberated from red cells more rapidly than the cephalins. The paper chromatograms made of the various extracts of human and bovine cells were all that was attempted in this respect and no differences in rates of release of the individual phosphatide components were observed. No attempt was made, however, to distinguish phosphatidyl serine from phosphatidyl ethanolamine. In separate experiments with human stromata 0.1 M acetic acid was incorporated in one set of solvents and 0.1 M pyridine in another; the curves of removal of P were much the same as those of Fig. 1, and the paper chromatographs of the extracts showed no preferential removal of choline-containing as opposed to amino-containing lipides.

As for the effect of water in accelerating release of lipid into organic solvents (Fig. 1) it is worth noting that this was accompanied by appearance of "proteolipid" which was not encountered in extracts of dry ghosts. In separate experiments direct extractions of wet and of dry stromata by chloroform-methanol (2:1) were compared. When extracts of the wet material were evaporated *in vacuo* to dryness and the residue then taken up in chloroform-methanol an appreciable amount of insoluble brownish-red material was encountered. This corresponds to the protein of Folch's "proteolipids," seen with great regularity in extractions of wet tissues(9). Extracts of dry ghosts were free of such protein even though the removal of total phosphorus of the cell was virtually complete, and it would appear that at least for red cells "proteolipid" may represent under these experimental conditions an artefact of denaturation rather than a biochemical entity.

Discussion. The findings could be explained either in terms of a firmer binding of the lipids of the camel ghost, or as a manifestation of some fundamental difference in orientation at the surface. Thus, recent studies of the human red cell ghost lead to the view that the lipid is disposed at or near the outside of the membrane(10). Perhaps in the camel this is not true, and, if further, the structural arrangement were such as to restrict the penetration of organic solvents, the data could thus be explained essentially in mechanical terms. But the lyophilized material appears microscopically to be largely amorphous, and the shape of the native cell is not seen after addition of saline. Also, the curious effect of water in accelerating liberation of lipid (Fig. 1) applies to the camel as well as to human and bovine cells. This could conceivably involve opening of pores even in the denatured membrane, but such a mechanical operation is probably not the whole story. It has been

pointed out, for instance, that "proteolipid" appears in extracts of wet ghosts, and this would be difficult to account for on a completely mechanical view. It seems most probable that the experimental extraction procedures employed do give an indication of relative stability of certain undefined lipid complexes in the red cell membrane. The data may therefore be regarded as evidence for the view that insusceptibility of camel cells to cobra hemolysin is due to a peculiar binding of lecithin which makes it inaccessible to venom enzymes. The idea has been advanced many times in connection with the old observation that the red cells of some species, notably the ruminants, are resistant to venom(4), but never examined experimentally. In any case, either the binding or the spatial orientation of red cell phosphatides, or both, would appear to be different in the camel. At the same time it is clear that detailed analysis of these lipids is desirable; the possibility exists, however unlikely, that the findings may be further related to a novel sort of phosphatide.

It is impossible to say whether phosphatide composition or structure may be related to the morphological features of size and shape. But it can be noted (a) that appreciable amounts of lecithin cannot be found in the red cells of the 3 species of true ruminants thus far examined, while the cells of this group as a whole are relatively small in size, and (b) that a peculiarity of lipid constitution appears to exist in the camel cell, which has an oval shape.

The method of extraction may perhaps, with suitable modifications, be applied to pathological tissues, *e.g.*, in human diseases involving the erythrocyte it is possible that lesions of the red cell membrane could exist that might be manifested in terms of the ease or difficulty with which bound lipids are liberated.

Summary. The red cells of the camel are like those of true ruminants in being resistant to lysis by cobra venom but dissimilar in appearing to contain lecithin. The idea that the resistance of camel cells to venom is due to the binding of lecithin so that it is inaccessible to venom enzymes has been tested in terms of comparative extractability into ether-ethanol mixtures of phosphatides from cells of man, ox, and camel. The lipids were liberated much less readily in the case of the camel, indicating that the lipid complexes are different and more stable.

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Effect of Reserpine* on Mammary Gland Involution, and on Other Organs in the Rat. (24416)

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A number of workers have reported recently on lactogenic effects of reserpine in several species, *i.e.* rabbits(1,2), rats(3,4) and man(5,6). However, in this laboratory, Tindal(7) has investigated mammogenic or

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