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The effect of rehydration on the thickness of erythrocyte membranes as observed by energy transfer

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SUMMARY

Erythrocyte membranes were embedded between two artificial chromophore layers which function as energy donor and acceptor, respectively. From the rate of energy transfer across the membranes their thickness was derived. In this manner the thickness of human erythrocyte membranes was found to increase from 65 Å in the air-dried state to approximately 100 Å upon rehydration in 7 mM phosphate buffer.

Recently erythrocyte membranes have been incorporated into monolayer assemblies[1,2] Techniques already used to investigate properties of monolayer assemblies[3,4] may thus be applied to study cellular membranes. Studies on energy transfer between membrane chromophores and artificial monomolecular dye layers may be of particular interest, because they should yield new information about the arrangement of chromophores within membranes. Unfortunately, the procedure for incorporating membranes into monolayer assemblies requires the membranes to be dried[1,2]. It is well known, that the structure of erythrocyte membranes is modified upon drying, although membrane proteins and lipids behave in a different way: as judged from the CD spectrum of erythrocyte membranes[5], the structure of proteins does not change significantly on drying whereas the X-ray diffraction pattern[6] of erythrocyte membranes changes abruptly as their water content decreases to 20% and below (with respect to final dry weight); this latter fact is believed to indicate a separation of lipid phases. However, the changes in the X-ray diffraction pattern are reversible[6] as is the hydration isotherm[5]. These observations suggest that changes in membrane structure on drying are at least partly reversible and that the rehydrated erythrocyte membrane therefore is a valuable model for the native membrane. For this reason we have studied whether it is possible to rehydrate erythrocyte membranes, even if they are already incor-

porated in a monolayer assembly.

The method by which air-dried erythrocyte membranes together with a stepwise arrangement of arachidate layers are embedded between a monomolecular dye layer and a thin gold layer are described elsewhere in some detail[2] and therefore will only be summarized briefly. First, a 0.17 mm × 12 mm × 38 mm cover slip on one side is covered with a thin gold layer (transmission, 0.92 at 420 nm) by vacuum evaporation. The gold layer is then partly covered with a stepwise arrangement of arachidate monolayers[2]. Erythrocyte ghosts are prepared in 7 mM sodium phosphate buffer, pH 8.0, according to the procedure of Dodge *et al.*[7]. The ghost suspension is diluted with distilled water to give a final buffer concentration of 0.1 mM. Furthermore, $MgSO_4$ is added to give a final concentration of 0.1 mM to prevent spontaneous vesiculation of the membranes[8]. A small trough[2] is filled with this ghost suspension and a monolayer of bovine serum albumin (Miles Lab., Kankakee, Ill., U.S.A.) is spread on its surface and held under a constant surface pressure of 10 dynes/cm. The cover slip with the gold layer and the arachidate layers is now slowly dipped through the protein monolayer into the ghost suspension. On the way down into the suspension a protein monolayer (possibly together with adsorbed ghosts) is transferred onto the cover slip; upon withdrawal a thin layer of the ghost sus-

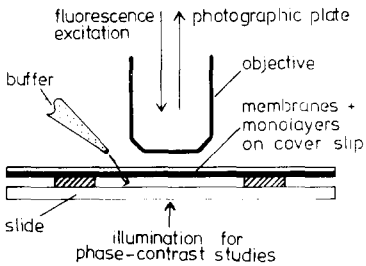
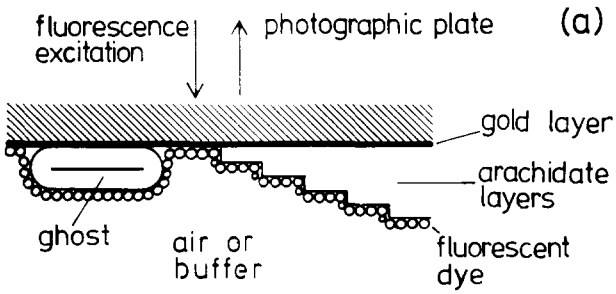
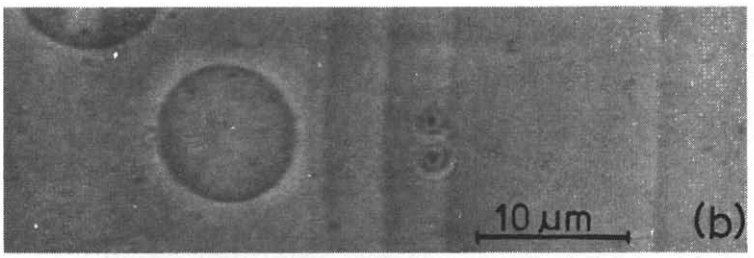
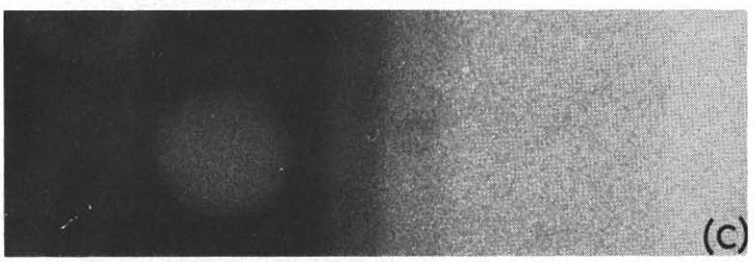


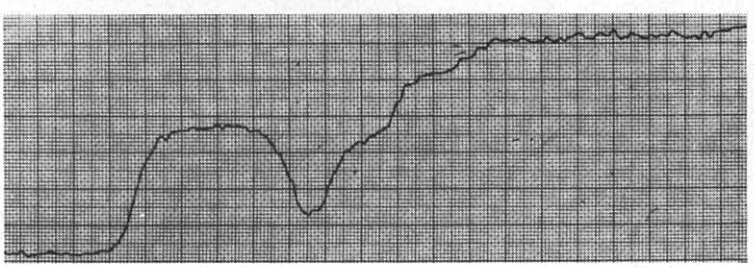
Fig. 1. The arrangement as used for microscopic studies of membranes and monolayers. (The scheme is not drawn according to scale: actually, cover slip, dry ghosts and slide have a thickness of 0.17, 0.000013 and 1.0 mm, respectively.) First the specimen is studied in the dry state where the small space between cover slip and slide is occupied by air. Then this space is filled with buffer. Now the ghosts are immersed in buffer, thus are rehydrated and subsequently are investigated in the rehydrated state.

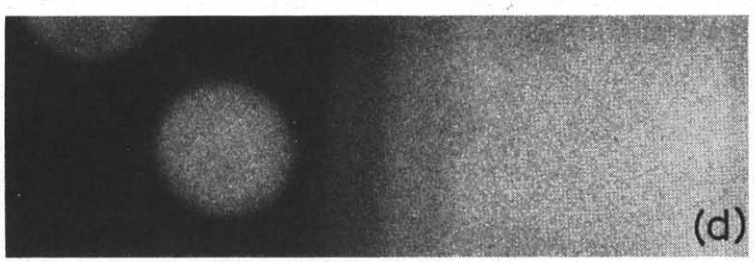
Fig. 2. An estimate of membrane thickness by energy transfer. (a) As indicated schematically, air-dried ghosts together with a stepwise arrangement of arachidate double layers are embedded between a dye and a gold layer. (Extremely different scales have been used for length and width.) (b) Phase-contrast micrograph of such a specimen. A ghost and five monolayer steps, each one representing a double layer of arachidic acid, are seen. The first step is very narrow; it is resolved only in this picture and cannot be recognized in the fluorescence micrographs. (c) Fluorescence micrograph of the specimen in the air-dried state. Since the dye layer functions as energy donor and the gold layer as energy acceptor, the fluorescence intensity is closely related to the thickness of objects separating dye and gold layer. Membrane thickness is estimated by comparison of the fluorescence intensity on ghosts with the fluorescence intensity on arachidate layers. Thus, the ghost is found to be as thick as 2–3 double layers of arachidic acid. The same is evident from the densitometer tracing, taken along a path right through the center of the ghost and through the monolayer steps. (d) Fluorescence micrograph and corresponding densitometer tracing of the same ghost in the rehydrated state. Ghost thickness now amounts to that of 3–4 arachidate double layers.

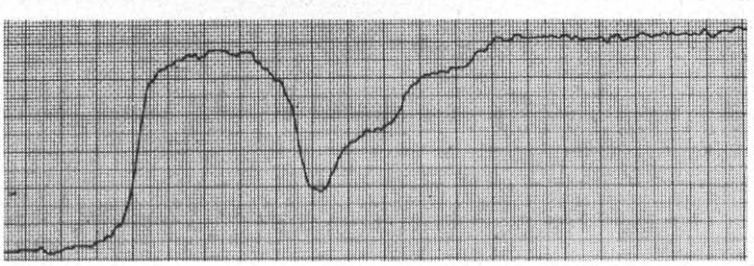

fluorescence excitation
photographic plate
(a)
gold layer
arachidate layers
ghost
air or buffer
fluorescent dye


10 µm
(b)


(c)




(d)



pension together with a second protein monolayer covering the ghosts is transferred onto the cover slip. The ghosts now dry quickly and after a short time they are to be seen in the form of dry flat discs adhering to the cover slip. In an additional step the ghosts are covered with a monolayer of a fluorescent dye. This is done using a conventional Langmuir trough[4]. The cover slip with the adhering ghosts is dipped into the subphase of the trough, and a monolayer of *N,N'*-distearyloxacyanine[9] mixed with arachidic acid at a molar ratio of 1:20 is spread at the air–water interface and is compressed by 30 dynes/cm. The cover slip is then slowly withdrawn and thus becomes covered with a dye layer. The dye layer has an absorption maximum at 384 nm and a fluorescence maximum at 420 nm[4]. The preparation of the specimen is completed by placing the cover slip upside down on a slide in the manner indicated schematically in Fig. 1. A small, approximately 0.5 mm wide, space between cover slip and slide is established by small pieces of scotch tape. Microscopic studies are performed with an equipment of Zeiss (Universalmikroskop, Zeiss, Oberkochen, Germany), which is set up for phase-contrast microscopy with transmitted light and fluorescence microscopy with incident light. A 100 ×, n.a. 1.30 phase-contrast oil-immersion fluorite objective is used.

First the ghosts are studied in the air-dried state, the space between cover slip and slide still being occupied by air. For microphotography ghosts are chosen, which happen to be placed in the immediate neighborhood of the edges of the arachidate layers. Subsequently the space between cover slip and slide is filled with 7 mM sodium phosphate buffer, pH 8.0, so that ghosts and monolayers are immersed in buffer. The same ghosts previously studied in the air-dried state are then relocated and are photographed again.

A typical example out of 17 ghosts studied in the described manner is given in Fig. 2. The architecture of the specimen is indicated in the schematic drawing (Fig.2a). The thin protein double layer covering ghosts as well as arachidate layers has been omitted in the scheme for reasons of clarity. Its thickness is approximately 12–16 Å (ref. 10). In Fig. 2b the phase-contrast micrograph, and in Fig. 2c the fluorescence micrograph of such a specimen in the air-dried state is shown. Using a Joyce–Loebl microdensitometer the density of the photograph has also been registered, the measuring light beam moving in a straight line right through the center of the ghost and through the monolayer steps. Finally Fig. 2d shows the fluorescence micrograph and the corresponding densitometer tracing of the same ghost in the rehydrated state.

Energy transfer of the Perrin–Förster type strongly depends on the distance between energy donor and acceptor. In the present case the dye and the gold layer function as energy donor and acceptor, respectively[12]. Therefore, fluorescence micrographs as shown in Fig. 2 reflect the surface structure of the specimen in a sensitive way: protruding areas are indicated by a strong, depressions by a weak fluorescence; areas of equal height exhibit equal fluorescence intensities. On this basis it is immediately derived from Fig. 2 than in the dry state the ghost is as thick as 2–3, and in the rehydrated state as thick as 3–4 arachidate double layers. A more detailed analysis by microphotometric techniques has shown[2] that the fluorescence intensity on dry ghosts is as high as on 2.3 arachidate

double layers. Taking a value of 28 Å for the thickness of one arachidate monolayer[11] this corresponds to 130 Å. It can be assumed that the thickness of arachidate layers does not change appreciably upon rehydration so that the thickness of the rehydrated ghost is estimated from Fig. 2d to be (3–4) × 2 × 28 Å = 168–224 Å. Since the ghosts, when embedded between monolayers, consist of two adhering erythrocyte membranes, the thickness of a single membrane is half that of the ghost thickness, that is, 65 Å in the air-dried state and approximately 100 Å in the rehydrated state.

It must be pointed out, however, that the determination of membrane thickness as performed above does not take into consideration all the factors, which influence the efficiency of energy transfer (the refractive index of the intermedium, the quantum efficiency of the donor, and the relative orientation of donor and acceptor chromophores). These factors may have a slightly different value depending on whether donor and acceptor layer are separated by an erythrocyte ghost or by arachidate layers, and therefore may be a reason for errors in the determination of membrane thickness. Furthermore, it has been assumed above that the thickness of arachidate multilayers does not change on hydration. Unfortunately, no data about the hydration properties of Cd–arachidate multilayers are available. The uptake of water by related compounds however, is very small. For tristearin, for instance, a water content of 5.6 mg water/g at a relative humidity of 91% (temp., 25 °C) has been determined[13].

From X-ray diffraction studies[14], the thickness of hydrated and of dry erythrocyte membranes has been derived to be 110 Å and 80–85 Å, respectively. With regard to the differences in membrane preparation and measuring principles the similarity with our values is rather astonishing. In the present study, of course, only the distance between donor and acceptor layer is measured so that questions pertaining to the distribution of water in the membrane, for instance, are left open.

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