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THE THICKNESS OF AIR-DRIED HUMAN ERYTHROCYTE MEMBRANES AS DETERMINED BY ENERGY TRANSFER

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SUMMARY

Air-dried human erythrocyte membranes were embedded between a monolayer of a fluorescent dye and a thin gold layer, which function as energy donor and acceptor, respectively. By microscopic measurement of the fluorescence intensity of the donor layer on individual ghosts the efficiency of energy transfer across the membranes has been determined. From this efficiency membrane thickness was found to be 65 Å.

In the past, energy transfer has been used to study monolayer assemblies[1,2]. Some problems which have been investigated so far are the measurement of distances between chromophore layers in monolayer assemblies[1,2], the detection of chromophore clusters in mixed monolayers[3] and the adsorption of proteins to lipid layers[4]. In order to apply techniques of this type to the study of biological membranes, a method has recently been developed by which erythrocyte membranes can be incorporated into monolayer assemblies[5]. The procedure offers the possibility to embed membranes between two chromophore layers, so that membrane thickness can be derived from the rate of energy transfer across the membrane. A first estimate of membrane thickness obtained in this way has been given previously[5]. In the work to be discussed now, membrane thickness is investigated on the same basis. The methods, however, have been improved.

METHODS

(1) *Preparation of membranes*

Human erythrocyte membranes were prepared by hypotonic hemolysis in 7 mM sodium phosphate buffer, pH 8.0, according to the procedure of Dodge *et al.*[6]. The ghost suspension obtained was diluted with distilled water to give a final buffer concentration of 0.1 mM. In order to prevent spontaneous vesiculation of the membranes, which may occur in dilute buffer[7], 0.1 mM $MgSO_4$ was added.

In an early study Waugh and Schmidt[8] found that cellular membranes can be dried without being damaged if they are protected by a monomolecular layer of

protein during drying. These results were utilized and modified in the following manner. The small trough described below was filled with the ghost suspension. On its surface, a monolayer of bovine serum albumin (Miles Lab., Kankakee, Ill.) was prepared and compressed with a movable barrier to establish a constant surface pressure of approximately 10 dynes/cm. A cover slip coated with a thin gold layer and bearing a stepwise arrangement of fatty acid monolayers on part of the gold layer (details are given below), was then slowly dipped through the protein monolayer into the ghost suspension. On the way down into the suspension the protein monolayer (possibly together with adsorbed ghosts) was transferred onto the cover slip, whereas upon withdrawal of the slip a thin layer of the ghost suspension, together with the protein layer covering it, was transferred onto the slip. As it emerged from the ghost suspension the cover slip was therefore wet. However, the layer of the ghost suspension, which was included between two protein monolayers in this manner, dried quickly, so that after a few seconds the ghosts were seen on the cover slip in the form of dry, flat discs. Their microscopic appearance has been shown in Fig. 2 of ref. 18.

The simple trough used for the work with membrane suspensions is shown in Fig. 1. A circular tray of 4 mm depth and a diameter of 86 mm was milled from a compact cylindrical teflon block leaving a rim of 3 mm. Furthermore, a 6-mm wide partition going from the rim to the center of the tray was left. On this partition, right in the center of the tray, a steel pin was attached, which served as the axis of a movable barrier. This barrier was supplied by a 5 mm × 55 mm × 0.03 mm piece of teflon foil and mounted on the steel pin. In another location of the tray (in Fig. 1 directly beneath

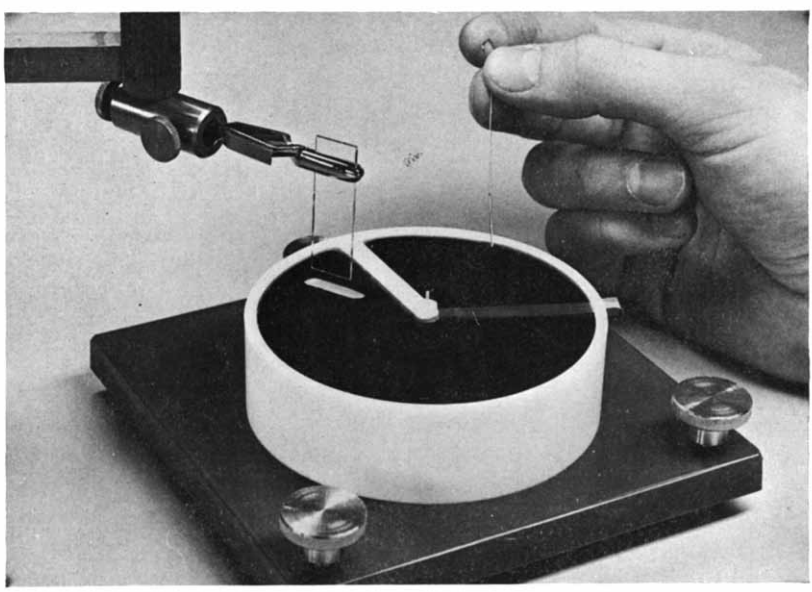

Fig. 1. A trough with a small volume as used in this study for work with membrane suspensions. A circular tray of 1.7 mm depth is filled with 10 ml of water (or any other subphase). The air/water interface is divided into two parts by a fixed partition and a barrier, which can easily be turned around an axis in the center of the tray. A film, spread on one side of the barrier, may be kept under constant pressure by applying a piston oil on the other side of the barrier.

the small slide) a cavity was milled from the teflon block, which allows the dipping of slides up to a depth of 25 mm. A black piece of glass of 2.3 mm thickness had been cut so that it covered the bottom of the tray. The bottom being hydrophilic, the study of very thin liquid films is possible, which in a completely hydrophobic tray would form drops. The use of a black glass plate in addition facilitates the observation of the liquid surface, which is harder to judge above the white teflon material. The trough may be used in two different ways. In the case described above it was filled with a membrane suspension, the plate of a Wilhelmy balance[2] was placed between cavity and partition, and a film spread on the suspension between barrier and partition. The film pressure was established and held constant by manual adjustment of the barrier. Alternatively, a film spread at the air–water interface on one side of the barrier may be kept under constant pressure by applying a piston oil to the film-free part of the interface on the other side of the barrier. The spreading pressure of oleic acid (30 dynes/cm), for instance, is able to drive the barrier in such a manner that an arachidate film is kept under a pressure of 20–25 dynes/cm.

(2) Monolayers

First, a 0.17 mm × 12 mm × 38 mm cover slip was coated on one side with a thin gold layer by vacuum evaporation. In a Langmuir trough, as described in ref. 2, a monolayer of arachidic acid was spread at the air–water interface and compressed by 30 dynes/cm. Part of the cover slip was dipped through this monolayer into the subphase of the trough and then withdrawn so that a fatty acid double layer is deposited. This procedure was repeated several times, varying, however, the degree of immersion of the cover slips by a micrometer screw. For the microphotometric measurements shown in Figs 3 and 4 arachidate steps were prepared having a width of 0.5 mm. Subsequently erythrocyte ghosts were applied to these cover slips as described above and, in a further step, the dried ghosts in turn were covered with a monolayer of a fluorescent dye. This was done as follows: the cover slip already bearing a gold layer, arachidate monolayers and ghosts was dipped into the subphase of the trough. A monolayer of *N,N'*-distearyloxacyanine (dye S in Fig. 3)[14], mixed with arachidic acid at a molar ratio of 1:20, was then spread at the air–water interface and compressed by 30 dynes/cm. The cover slip was then slowly withdrawn and thus covered with the dye layer, which has an absorption maximum at 384 nm and a fluorescence maximum at 420 nm (refs 2, 3).

(3) Microscopy

The cover slips with ghosts and monolayers were placed upside down on slides as shown in Fig. 1 of ref. 18. Microscopic work was performed with Zeiss equipment (Mikroskop-Photometer 01, Zeiss, Oberkochen, Germany). The instrument was set up for phase contrast microscopy with transmitted light and fluorescence microscopy with incident light. For microphotometric studies the fluorescence of dye S was excited by a stabilized 100 W high pressure mercury lamp. The light beam passed through a neutral grey filter (NG 4; 4 mm) reducing the light intensity to approximately 1%, and a coloured glass filter (UG 1; 1.5 mm) isolating the 366 nm emission peak (all filters were obtained from Schott, Mainz, Germany). It then entered a bright-field vertical illuminator and reached the specimen through a 40x, n.a. 0.75

phase-contrast fluorite objective. Fluorescence from the specimen first passed through the objective and then through a filter (GG 400; 2 mm). By means of a diaphragm the light outside the measuring field, which on the object has a diameter of 5 μm, was eliminated, so that only light from the measuring field hits the cathode of a photomultiplier (EMI 6256 B). The multiplier is operated with a stabilized high voltage supply (Nucletron, München, Germany) and the photocurrent was measured directly with a sensitive galvanometer (Hewlett Packard). Finally, the signal was displayed on a strip chart recorder.

RESULTS

(1) *Microfluorometric measurements on monolayers*

A typical registration curve as obtained by the device described above is shown in Fig. 2. A double layer of dye S mixed with arachidic acid at a molar ratio of 1:20 was deposited on a cover slip and its fluorescence intensity recorded. The measuring field has a diameter of 5 μm. In the case of Fig. 2a the intensity of the exciting radiation as supplied by a mercury lamp has been lowered to approximately 1% by a neutral grey filter, so that there is little bleaching. For comparison Fig. 2b shows a registration curve where the dye layer was excited by the full intensity of the mercury

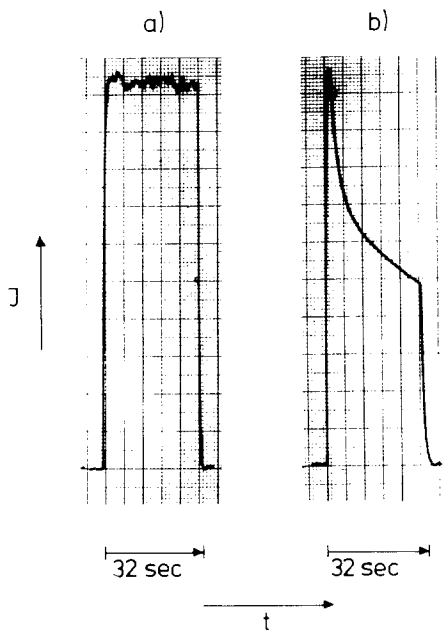

Fig. 2. Registration of the fluorescence intensity, I, of a monomolecular dye layer by a microfluorometric device. The measuring field has a diameter of 5 μm. (a) With the low intensity of the exciting radiation as used in the studies shown in Figs 3 and 4 also, there is little bleaching of the dye. (b) At a high intensity of the exciting radiation (as usually necessary in visual microscopic and microphotographic studies) bleaching of the dye is very pronounced. (The sensitivity of the device has been reduced in this case as corresponding to the higher fluorescence intensity).

lamp (the high-voltage at the photomultiplier had been reduced accordingly). Under these conditions, which are usually given in visual microscopic and microphotographic studies, the bleaching of the dye is of a considerable degree.

A microscopic study of energy transfer between dye S and a gold layer (transmission at 420 nm, 0.92) is shown in fig. 3. As described above, a cover slip with a thin gold layer was partly covered with a stepwise arrangement of arachidate monolayers, and upon this a double layer of dye S, mixed with arachidic acid at a molar ratio of 1:20, was deposited. In this arrangement the dye layer functions as energy donor, the gold layer as energy acceptor, and by means of the interposed arachidate layers the distance between donor and acceptor layer is varied. The fluorescence of the donor layer was measured in areas of the specimen where the dye layer is in direct contact with the gold layer, and where two, four, six, eight and ten arachidate layers respectively, are interposed between donor and acceptor. Donor fluorescence furthermore was measured where 20 arachidate layers have been deposited. At this large donor–acceptor distance energy transfer is negligible. The measured value is denoted as I_∞. In Fig. 3, the circles represent mean values of six measurements. The thickness of one arachidate monolayer has been taken into account with 28 Å (ref. 9). It is seen that the efficiency of energy transfer strongly depends on the distance between donor and acceptor. At small distances energy transfer is highly effective, so that donor fluorescence is heavily quenched. At larger distances energy transfer becomes less effective, and donor fluorescence is stronger. Theoretically[2], donor fluorescence should depend on the donor–acceptor distance in the following way:

$$\left(\frac{I_d}{I_\infty}\right)_S = \left(1 + \left(\frac{d_0}{d}\right)^4\right)^{-1} \tag{1}$$

where I_d is the fluorescence intensity of the donor at the distance d of the acceptor layer, I_∞ is the donor fluorescence at a large distance, d_0 is the distance, where energy

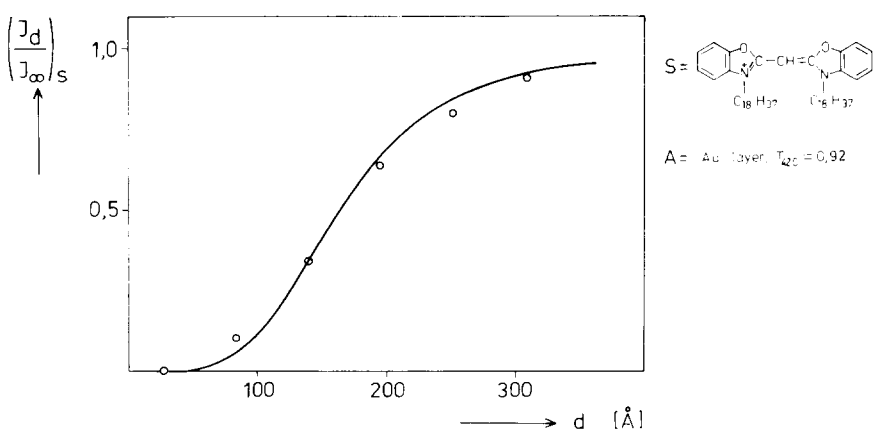

Fig. 3. A microfluorometric study of energy transfer between a monolayer of dye S and a thin gold layer (transmission, 0.92). The relative fluorescence intensity of the donor layer is plotted against the distance between donor and acceptor layer. The circles represent experimental data, whereas the solid line satisfies Eqn 1 with $d_0 = 165$ Å.

transfer is 50% effective. ($I_d/I_r = 0.5$). The solid line in Fig. 3 satisfies Eqn 1. It matches the experimental data best when a Förster distance of $d_0 = 165$ Å is used. Very similar results have been obtained by macroscopic measurements on the same system[10].

(2) *Studies on membrane thickness*

The principle used to determine membrane thickness has been demonstrated previously[5,18]. Dry erythrocyte ghosts were deposited on a thin gold layer together with a stepwise arrangement of arachidate layers. A monolayer of dye S covers the whole assembly. Since the efficiency of energy transfer strongly depends upon the distance between dye and gold layer, donor fluorescence reflects the surface structure of the specimen in a sensitive way: protruding areas are indicated by a strong, depressions on the other hand by a weak fluorescence; and, at a first approximation, areas of equal height exhibit equal fluorescence intensities (assumptions leading to this conclusion will be discussed below). Therefore, membrane thickness is derived by a comparison of the donor fluorescence on ghosts with that on arachidate layers, the height of which is known.

A microfluorimetric study of membrane thickness following this principle is shown in Fig. 4. It was checked, that without the dye layer, ghosts show no detectable fluorescence, and that there is no difference in fluorescence intensities beween areas, where 20 arachidate layers, and those where 20 arachidate layers *plus* an erythrocyte ghost separate donor and acceptor layer. Thus the value measured for a 20 layer area is taken to be I_r. On the left-hand side of the diagram the donor fluorescence on ghosts is shown. Each dot represents a measurement on one individual ghost. The mean relative fluorescence intensity of measurements on 29 ghosts is $(I_d/I_r)_s = 0.38 \pm 0.03$ (S.E.). On the right-hand side of Fig. 4 donor fluorescence is plotted against the thickness d_{AL} of the arachidate layers separating donor and acceptor layer. In this case each point represents mean value and standard error of nine measurements. Membrane thickness may be derived from Fig. 4 by a direct comparison of the

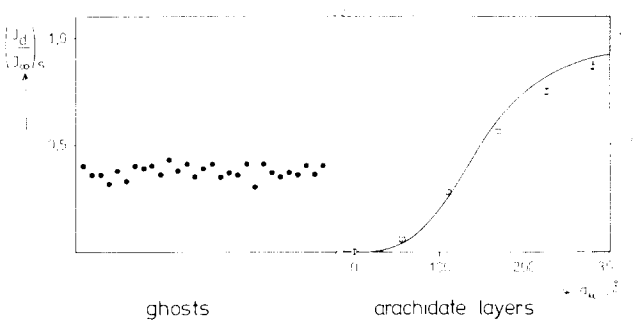

Fig. 4. Determination of membrane thickness by energy transfer. Air-dried ghosts together with a stepwise arrangement of arachidate monolayers are embedded between a monolayer of a fluorescent dye and a thin gold layer. On the left-hand side the fluorescence intensity of the donor layer on ghosts is given. Each dot represents a measurement on one ghost. On the right-hand side, donor fluorescence is plotted against the thickness of the arachidate layers separating donor and acceptor layer. In order to derive membrane thickness the curve on the right-hand side of the diagram is used as a standard to convert fluorescence intensities, which are measured on ghosts, into distances.

fluorescence intensity measured on ghosts with the intensity measured on arachidate layers. This already shows that the ghosts are a little bit thicker than two arachidate double layers or 112 Å. A higher accuracy, however, is obtained by application of Eqn 1 which was used to compute the solid line on the right-hand side of Fig. 4. Since, for this purpose, the real distance d between donor and acceptor layer has to be known, the thin protein double layer which embeds the ghosts and covers the arachidate layers, as described in the experimental section, is taken into account with a thickness of 15 Å (ref. 11): $d = d_{AL} + 15$ Å. Thus, the theoretical curve satisfying Eqn 1 with a Förster distance of $d_0 = 165$ Å is found to match the experimental data best. Consequently, the thickness of ghosts is calculated by inserting the relative fluorescence intensity measured on ghosts into Eqn 1 with $d_0 = 165$ Å. The value obtained for d is then reduced by 15 Å accounting for the protein layer. In this manner ghost thickness is derived to be 130 ± 5 Å. Since the dried ghosts consist of two adhering erythrocyte membranes, the thickness of a single membrane is then half of this value, *i.e.* 65 Å.

DISCUSSION

In a preceding study[5] energy transfer had been used for the first time to determine membrane thickness. We have now continued this work using the same principle, but modifying a number of technical details. In our first study[5], for instance, whole erythrocytes were adsorbed to a slide, the adsorbed cells were then hemolysed and further processed. In the present study the erythrocyte membranes are first prepared according to a standard procedure and subsequently deposited on a slide and dried. This modification should allow the application of the described energy transfer techniques to other membranes besides erythrocyte ghosts. In the present study, furthermore, the fluorescence intensity of the donor layer has been determined by photometric techniques, whereas in the preceding study[5] donor fluorescence was registered on a micrograph, which then was judged by eye. From those experiments the membranes were roughly estimated to be as thick as 3 arachidate monolayers or 80 Å approximately. However, the photometric measurements, which are far more accurate and also avoid bleaching effects, have shown that the membranes are as thick as 2.3 arachidate layers, which corresponds to 65 Å.

A prerequisite for a determination of membrane thickness as performed above is that the parameter d_0 is the same, regardless of whether donor and acceptor layer are separated by arachidate layers or by erythrocyte ghosts. Since d_0 depends on various factors (the refractive index of the intermedium, the quantum efficiency of the donor, and the relative orientation of donor and acceptor) all these factors should be identical in both cases. With regard to the refractive index this condition is approximately fulfilled: the refractive index of arachidate monolayers has been given to be 1.522 (ref. 12), whereas that of ghosts is reported to be 1.504 (ref. 8). About the other factors mentioned, however, little is known so far. In particular, it may be questioned whether under experimental conditions as used in this study the surface of dry erythrocyte membranes is as smooth and regular as expected from the surface of fatty acid monolayers, and whether differences in the ultrastructure of the surfaces will influence orientation and arrangement of the donor chromophores.

Although the thickness of dry erythrocyte membranes has frequently been studied in the past by a number of independent methods including light and electron microscopy and low-angle X-ray diffraction, the values reported in the literature range from 50 Å to 500 Å, approximately, or even more. Reviews on this topic have been given for instance by Ponder[15], Whittam[16] and Weinstein[17]. Recent electron microscopic investigations, however, indicate that membrane thickness amounts to 60–100 Å (ref. 17). This has also been confirmed by X-ray diffraction experiments which yielded a value of 80–85 Å (ref. 13). The value of 65 Å reported in this article for the thickness of air-dried human erythrocyte membranes therefore fits into the current concept about membrane thickness. Whether the difference between our value and that derived by X-ray diffraction must be attributed to differences in membrane preparation or to the parameters which already have been discussed above, cannot be decided at the moment.

In this study energy transfer has been observed across a cellular membrane and this was used to derive membrane thickness. In a similar manner, however, it should be possible to study energy transfer between chromophores, which are located within a membrane, and an artificial monomolecular dye layer deposited on the surface of the membrane. Such experiments hopefully will provide new information about arrangement and properties of membrane chromophores which frequently are of great physiological importance. In this connection it may be relevant that energy transfer experiments of the described type cannot only be performed with air-dried, but also with rehydrated membranes[18].

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