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ELECTRON DIFFRACTION STUDIES OF HUMAN ERYTHROCYTE MEMBRANE AND ITS LIPID EXTRACTS

EFFECTS OF HYDRATION, TEMPERATURE AND HYDROLYSIS

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Summary

The organization of lipid molecules in individual human erythrocyte ghost membranes and single bilayers of their total lipid extracts were studied by low-dose electron diffraction in a controlled environment. The highest onset temperature (T_s) at which diffraction rings corresponding to a gel state appeared, were found to be in the range of −2 to −4°C for both the whole ghost membrane and bilayers of its total lipid extracts. The onsets were abolished by dehydration before separated crystallizations of cholesterol and phospholipid occurred. T_s increased as a result of free fatty acids accumulation in membranes after phospholipase A_2 treatment or storage.

Introduction

Although lipid phase separation has been known in membranes of microorganisms [1,2], rat mitochondria membrane [3] and microsomes [4], its role in human erythrocyte membrane is not clear. A break in the glucose transport rates was found at 19°C [5]. Viscosity measurement also showed a discontinuity at the same temperature range [6]. Hemolysis was observed when the temperature of hypertonically suspended cells was lowered through this range [7]. Some of these abrupt changes in membrane properties were attributed to the membrane lipid phase transition. Recently, pH-dependent lipid phase transitions in erythrocyte membranes at about 0 and 20°C were observed by Raman spectroscopy [8,9]. Discontinuities in the order parameter were also observed by spin label electron paramagnetic resonance (EPR) [10]. On the

other hand, X-ray diffractions of erythrocyte ghost membrane [11–13] and its lipid extract [13,14] showed no evidence of lipid packing at the gel (solidus) state down to −5°C, in spite of an earlier claim that an X-ray diffraction ring corresponding to the solidus state of the membrane lipid exist at room temperature [15]. Calorimetry measurements of individual lipid components did show definite phase transition peaks [16,17], yet the results from total lipid extracts, including cholesterol, remain uncertain [17,18]. Possibly, the temperature-sensitive functions of the membrane are related only to the organization of those lipids immediately surrounding the functional protein, while the rest of the lipid (bulk phase) remain in the fluidus phase and have less effect on the function of the protein [19]. However, a recent experiment on ether-extracted ghost seems to indicate the contrary [20]. Pertinent questions to ask are that if some portions of membrane lipids do undergo phase transition as detected by spectroscopic experiments [8–10], do these lipids present in sufficient percentage and have enough long range interaction (crystalline domain size) to be detected by diffraction methods. Could the product of hydrolysis reaction in the membrane affect the onsets of the phase separation in a similar manner to the effect of fatty acids in phospholipid vesicles [21,22]. In an attempt to answer these questions, we studied the electron diffraction of human erythrocyte ghost membrane and its lipid extracts under controlled environment [23, 24]. The strong interaction between electrons and the specimen, and the ability to focus the incident beam enables us to examine a small area in 1–2 membrane layers over short time intervals (several seconds), at extremely low electron beam dose. The results are related to freeze fracture [25,26] and spin label EPR [27] studies.

Methods and Materials

Ghost membrane and lipid preparation. Fresh human blood was drawn in a Vacutainer (Beckman-Dickison, NJ) containing citrate and EDTA. Ghost membranes were prepared according to the procedure of Dodge et al. [28] as modified by Jung et al. [29], and finally suspended in 1/15 strength of Dodge [28] buffer under a nitrogen atmosphere. The entire preparation takes 4 h to complete. Diffraction experiments followed immediately after the completion of the preparation process. Phospholipase A_2 (bee venom from Sigma) treatment followed that of Verkleij et al. [26]. Suspensions of 100 mg of ghosts/ml buffer containing 10 mM $CaCl_2$ were incubated at 37°C for 1 h with 100, 200 and 1000 I.U. of enzyme. After incubation, ghosts were pelleted at 15 000 rev./min for 20 min, washed and resuspended in Dodge buffer.

Lipid was extracted from 1 ml of ghost membrane using the extraction technique of Folch et al. [30] as modified by Dawson et al. [31]. The extracted lipids were sealed in nitrogen and, if not used immediately, stored at −70°C. Two dimensional thin-layer chromatography (TLC) was performed on silica gel H plates (Applied Science), the first solvent phase being $CHCl_3/CH_3OH/NH_4OH$ (3 : 3 : 1) and the second phase $CHCl_3$/acetone/$CH_3OH/CH_3COOH/H_2O$ (5 : 2 : 1 : 1 : 5). Spots were visualized by spraying ninhydrin, phosphomolybdate, sulfuric acid and heating. Phosphotidylcholine, phosphotidylserine, oleic and stearic acids were used as standards in the thin-layer chromatography.

Electron diffraction. In membrane ghost experiments, 5 μl of freshly prepared ghost suspension was pipetted onto a carbon-Formvar-coated electron microscope grid and was allowed to settle for 1 min. Excess fluid was removed under saturated water vapor in a nitrogen atmosphere. The wet grid was transferred to the environmental stage of the electron microscope via a transfer chamber. In extracted lipid bilayer experiments, 5 μl of the chloroform/methanol lipid extract was spread on the surface of the buffer solution in a Langmuir trough in an atmosphere of water-saturated nitrogen. The subphase is either a Dodge buffer solution or a buffer solution containing 5 mM Tris and 0.5 mM $CaCl_2$ at pH 7.4. An unsupported bilayer of the lipid was formed on a 1000 mesh electron microscope grid by the dipping method previously described [32,33]. The hydrated bilayer was transferred to the environmental stage as described above. As control experiments: dried ghost, dried lipid extracts deposited on carbon film, and microcrystals of extracted lipid grown from organic solvent [34] were used.

Methods for low-dose electron diffraction of wet specimen has been previously described [32]. A differentially pumped environmental stage build into a Siemens 1A Elmiskop was used for this experiment [24]. The specimen on the grid was always normal to the incident electron beam. Selective area electron diffraction was achieved by limiting the electron beam to about 2—5 μm in diameter at the specimen level. The specimen was exposed to no more than 10^{-4} C/cm^2 of dose in the whole process. This dosage is within the threshold of tolerable damage for our experiment [23]. The intensities of the electron diffraction patterns were measured from densitometer tracings of the film, using a Joyce-Loebl Mark II densitometer.

Results

The electron diffraction of the unsupported bilayers of total lipid extracts from the ghost membranes at room temperature show typically a diffuse ring at a spacing centered at 4.6 Å (Fig. 1A), in agreement with the X-ray diffraction results [14]. When cooled slowly down to a temperature just below zero degrees, the bilayers give a faint diffraction ring at 4.12 Å besides the 4.6 Å band, indicating a portion of the lipid has phase separated into gel (solidus) domains (Fig. 1B). Densitometer tracings of this pattern are shown in Fig. 1C. The integral intensity ratio between the 4.12 Å ring and the 4.6 Å band, after correcting for the scattering angles [35] and the incoherently scattered background intensity, remains 1 : 4 in the temperature range of —2 to —10°C. This onset temperature increases with increasing freeze storage time at —70°C and with the number of times of thawing from the frozen storage. With prolonged storage of several months, the value of T_s may reach room temperature. Table I presents the highest temperature (T_s) at which the sharp ring at 4.12 Å is detectable. To locate a T_s value for each experiment, we took ten patterns from different areas of the same specimen grid. The experiments were repeated many times using extracts from different ghost preparations. The error in Table I is the standard deviation obtained from the number of diffraction patterns recorded.

The electron diffraction of whole ghosts is very similar to that of their total

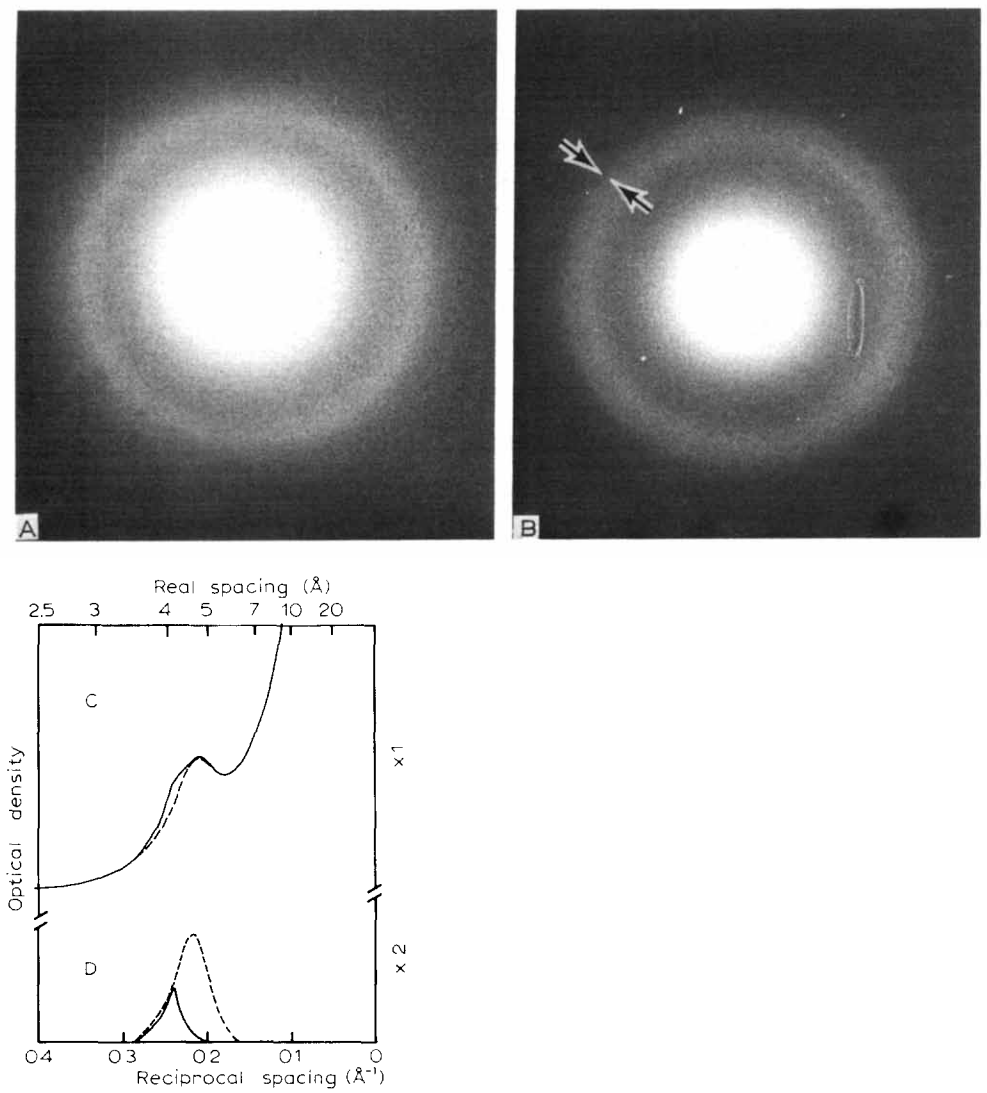

Fig. 1. Electron diffraction pattern of a single unsupported bilayer of lipid extracted from erythrocyte membrane. (A) The pattern, obtained at 4.2°C in a fully hydrated state, shows a diffuse band at 4.6 Å. (B) The pattern, obtained at −5.5°C and otherwise the same condition, shows a faint ring at 4.12 Å (arrow) beside the diffuse band. (C) Densitometer tracings of patterns (A) (- - - - -) and (B) (——). (D) Intensities of the peak in (A) and the additional peak in (B) after background subtraction.

lipid extract, i.e. a 4.6 Å band at all temperatures, plus a 4.12 Å sharp ring at temperatures below −4°C. When the ghost membrane has been frozen and thawed, or when it was stored at 4°C for a prolonged (5 days) period, T_s increased slightly but never reached room temperature. The values of T_s are listed in Table I. In some cases, an additional ring at spacings of 3.74 Å was observed from the stored specimens.

The freshly dried ghost membranes and bilayer deposits of their lipid extracts give a diffuse diffraction band at 4.6 Å at all temperatures. The inten-

TABLE I

T_s OF ERYTHROCYTE GHOST MEMBRANES AND TOTAL LIPID EXTRACTS

Specimen	Treatment	T_s (°C)	No. of experiments
Ghost	Fresh	−4 ± 3	10
	Stored 5 days at 4°C	12 ± 4	3
	Frozen and thawed	5 ± 4	1
	Phospholipase A_2	18 ± 4	6
Extracted lipid	Fresh	−2 ± 3	3
	Stored, frozen and rethawed	17 ± 2	10
	Rethawed three times in 2 months	25 ± 4	4
	From phospholipase A_2-treated ghost	20 ± 4	2

sity and the width of the band are comparable to that of the wet membrane at room temperature. When the grids containing the dried membrane were stored at room temperature over a period of 10 days or more, the diffraction patterns showed many striking rings (Fig. 2). The ring patterns indicated that submicron size microcrystals had formed during the storage period. Most of the spacings of the recrystallized membrane fit the reported spacings for cholesterol monohydrate [36,37], and the cholesterol microcrystal spacings we obtained. The electron diffraction of thin microcrystals of lipid extracts crystallized from solvent showed, in addition to the cholesterol spacing, two rings of 4.14 Å and 3.75 Å spacings. Spot patterns of single orthorhombic crystals with the (200) and (110) spacings at 4.14 Å and 3.75 Å, respectively, could be distinguished by selective area diffraction.

Fully hydrated ghost membranes treated with phospholipase A_2 showed a marked increase of T_s over the control sample (which was incubated in a buffer

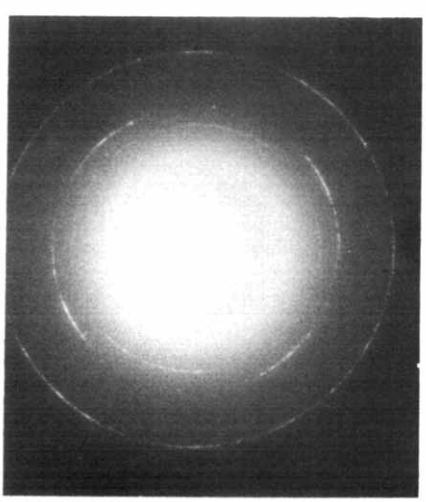

Fig. 2. Electron diffraction pattern of a dried erythrocyte ghost membrane showing extensive lipid recrystallization during a period of 10 days. The diffraction spacings coincide with those from cholesterol microcrystals. No sharp ring is detectable from freshly dried ghost membrane.

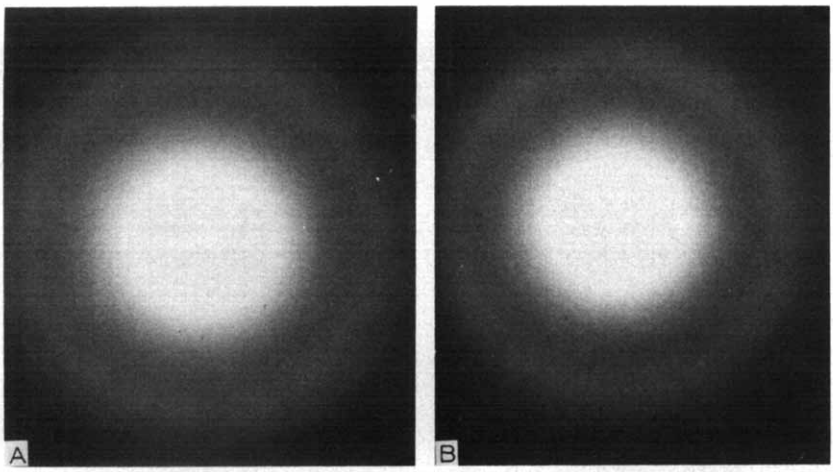

Fig. 3. Electron diffraction patterns of hydrated erythrocyte ghost membranes taken at 15°C. (A) From a ghost membrane treated with 100 I.U. of phospholipase A_2/100 mg of membrane. (B) From a control ghost membrane. The diffraction ring in (A) has a spacing of 4.02 Å. The diffuse bands in both patterns are caused by the membrane lipid as well as by the supporting carbon film.

solution containing 10 mM of Ca^{2+} without the enzyme). The increase of T_s does not depend on the concentration of enzyme at this range (100—1000 I.U./100 mg of membrane). An electron diffraction pattern is shown in Fig. 3A for an enzyme-treated ghost as compared to that from an untreated ghost (Fig. 3B) at the same temperature. The 4.6 Å band is superimposed on the background

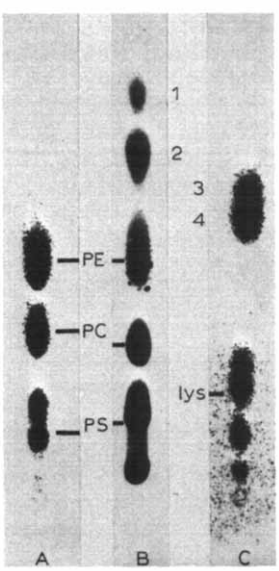

Fig. 4. Thin-layer chromatographs of total lipid extracts. (A) Immediately after extracting from fresh ghost membranes. (B) After frozen and thawed three times from −70°C storage in two months. (C) Immediately after extraction from phospholipase A_2-treated ghost. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; lys, lysophosphatidylcholine. Spots 1—4, free fatty acids of different chain lengths.

diffraction band produced by the supporting carbon substrate. Subtracting the combined pattern from that of a wet, empty carbon film reveals the existence of this diffraction band. T_s of bilayers of lipids freshly extracted from phospholipase A_2-treated ghost membranes was raised to 20°C, as compared to −2°C for lipid extracts from untreated ghost membranes (Table I).

TLC of the fresh, stored, as well as from the lipid extracts from phospholipase A_2-treated ghosts, are shown in Fig. 4. The consequence of freezing and thawing of the extracted lipid is very similar to that caused by the enzyme-induced hydrolysis of the membranes, i.e the dissociation of phosphatidylcholine into lysophosphatidylcholine and free fatty acids (spots 1—4). These spots contain no phosphate detectable by phosphomolybdate spray. The position of these spots lie between oleic acid and stearic acid standards. The relative amount of free fatty acids corresponded qualitatively to the increase in T_s in the electron diffraction data.

Discussion

Although electron diffraction has been applied to study the structure of materials for sometime [35], its application in membrane structure research has only recently been demonstrated [23,38].

In order to compare electron diffraction data with the rich collection of X-ray diffraction data, the correspondence between electron and X-ray diffraction needs to be established. Our finding in this paper confirms all [11—14] but one [15] X-ray diffraction result reported earlier. Contrary to popular belief, drying the ghost membranes or their lipid extracts on the grid does not immediately result in lipid crystallizations, which takes place slowly during prolonged drying periods of days, as mentioned in a previous X-ray diffraction study [11]. The absence of the diffraction rings at 4.14 Å and 3.75 Å in the recrystallized membrane and their presence in hydrolyzed bilayers in place of cholesterol lines suggest that acyl chains and cholesterol crystallize independently under different conditions, as if microcrystals growing from solvents. The suggested separated crystallizations of lipids in dried ghost membrane [11] is thus proven by selective area electron diffraction. The dehydration effect is so pronounced that it precludes any attempt to study lipids in membranes by electron diffraction without adequate specimen protection, despite certain claims [39]. Based on these agreements and previously established equivalence between X-ray and electron diffraction results [32], we felt justified to extend previously reported X-ray structural studies with our electron diffraction data.

The onset temperature of the fresh, hydrated ghost membrane and the extracted lipid overlap within the standard deviations of the experiments. The similarity of T_s for these two samples shows that the transition is due to membrane lipid alone. This is the first diffraction evidence of such a transition, which has previously been observed by Raman spectroscopy [8,9]. The broadening of a thermal transition peak in calorimetric measurements [17,18], possibly as a result of complex phase mixing, does not preclude the detection of minute solid domains by diffraction techniques. The weakness and smoothness of the detected diffraction ring indicated that at the onset the solidus crystallines are few and much smaller than the the selected diffraction area of 2 μm. The low

intensity of the solidus diffraction ring did not permit mapping of the solidus domain by diffraction contrast microscopy [40]. So far, no X-ray diffraction evidence for this onset has been reported. It should be noted, however, that when the onset of phase separation of a complex system is defined by the highest temperature at which solidus diffraction patterns become detectable, the onset values are rather arbitrary, depending on the sensitivity of the detection methods. This could explain the difference between the onset temperature as measured by X-ray diffraction experiments [13,14] and by our electron diffraction experiment. Any artifacts possibly introduced by membrane attachment to the supporting film or by radiation damage from the electron beam are unlikely to cause an increase of T_s, since similar T_s was also observed from unsupported bilayers, and an overdose radiation is known to destroy rather than to facilitate crystalline formation. An even weaker phase transition onset at the temeprature range of 15—20°C, as suggested by Raman spectroscopy [8, 9], NMR and fluorescence [20] and sping label EPR [10] measurements, was not observed in our experiment. The relative amount of lipid involved in this transition may be too small even for the sensitivity of electron diffraction measurement. It is also possible that the erythrocyte phosphatidylcholine, which shows a transition point at 18°C in the fluidity measurement by spin label EPR [10], has been dispersed during hemolysis [10], thereby disrupting long range (crystalline) interactions between molecules.

Our results also supported the proposition that some products of hydrolysis remain in the membrane [27] and may diffuse to form clusters or domains which presumably show in the freeze-fractured plane as smooth areas [25,26]. The remaining fatty acid has been proposed to form a more ordered array, based on spin label EPR evidence of increasing immobility of the fatty acyl chains after phospholipase A_2 treatment [27]. Hydrolysis and subsequent patching of fatty acids could also be responsible for the increase in T_s in stored, as well as in repeatedly frozen and thawed specimen, as judged by thin-layer chromatography. These remnant fatty acids may act as fusogens in ghost membrane [41]. A previous wide-angle X-ray diffraction study on phospholipase A_2-treated ghost [42] resulted in the appearance of two extra lines at 3.65 Å and 8.9 Å. The 3.65 Å line was also detected in our experiment and we attribute it to the spacings of an orthorhombic packing of acyl chains. Unfortunately, the experimental temperature of their experiment was not given, thus proper comparison with our data cannot be made.

In conclusion, by employing the electron diffraction technique at controlled environment, we have detected an onset of phase separation in erythrocyte ghost membrane and its total lipid extracts at −2 to −4°C. We also observed the increase of this onset as a result of hydrolysis products remaining in the membrane. This result would serve as a word of caution in membrane storage procedure. Within the sensitivity of the experiment, we found no evidence of collective phase change in fresh ghost membrane and its total lipid extract in the temperature range of 15—20°C, as suggested by other experiments [8—10].

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