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ERYTHROCYTE MEMBRANES: EFFECTS OF SONICATION

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SUMMARY

Chromatographic, ultracentrifugal and electron-microscopic studies show that sonication of red blood cell membranes breaks these membranes into small vesicles and linear fragments having intact unit membrane structures. These fragments are from 100 to 600 Å in size and do not sediment under conditions usually used to define solubility.

INTRODUCTION

Study of the structure and function of proteins from plasma membranes has been hampered by the very low solubility of these molecules in aqueous solution. Recently, several workers have reported the use of sonic energy (sonication) to "solubilize" the components of lipoproteins systems[1–5]. In the course of a study of the general chemistry of membrane proteins we used sonication in an attempt to solubilize intact erythrocyte membranes. The proteins of the sonicated membranes do not sediment in a strong centrifugal field, and thus by definition are in solution. It is not clear, however, whether this solubilization results from deaggregation of the molecular components of the membrane or from breakage of the intact membrane into smaller pieces, each of which still maintains the gross structure of the membrane as a whole. The relative homogeneity which has been reported in sonicated membrane preparations is deceiving, for when the lipid is removed the total protein is seen to be composed of many different conponents[6]. This report is offered in an attempt to clarify the nature of sonicated membrane material.

EXPERIMENTAL

Red blood cell ghosts were prepared by the method of DODGE, MITCHELL AND HANAHAN[7]. The cells were lysed in hypotonic buffer at slightly alkaline pH, removing more than 99 % of the total red blood cell hemoglobin. A turbid suspension containing a volume of about 5 % ghosts was sonicated (Ultrasonic Disintegrator, Measuring and Scientific Equipment Ltd., London; ultrasonic output 60 W, frequency 60 cycles/sec) for 30 min at 4°. Aliquots taken after different sonication times were centrifuged at 100000 × *g* for 1 h and the composition of the supernatant was examined. Protein

was measured by the method of LOWRY *et al.*[8], phosphorus by the method of BARTLETT[9], and cholesterol by the method of ZLATKIS, ZAK AND BOYLE[10].

RESULTS AND DISCUSSION

The turbid ghost suspension became clear and opalescent during sonication. The amount of protein remaining in the supernatant solution after centrifugation is a function of sonication time as shown in Fig. 1. Since the amount of protein in the supernatant solution was constant after 6 min of sonication, this time was used in all subsequent experiments. The total amount of protein "solubilized" varied from 45 to 92 % in different experiments depending on the temperature and the criteria used to define solubility. More protein is solubilized when sonication is performed at higher temperatures and when a lower centrifugal field is used to define solubility*. Over 70 % of the total ghost lipid also remains in the supernatant after this procedure.

The properties of the sonicated protein-containing material were studied by several methods. The supernatant solution after centrifugation was chromatographed on Sephadex G-200 gel-filtration resin (column dimensions 1.5 cm × 30 cm) in a dilute Tris–HCl buffer (Fig. 2). The protein eluted in a single peak in the void volume of the column, accompanied by more than 90 % of the total phospholipid in the supernatant. The remaining phospholipid eluted in a single peak near the bed volume of the column. Similar results were obtained when the sample was made 8 M in urea and chromatographed on resin equilibrated with 8 M urea. Thus urea does not cause further dissociation of the membrane sonicate.

Because the protein appeared to be in solution yet was large and could not be broken down by urea we studied the appearance of the "soluble" sonicate in the

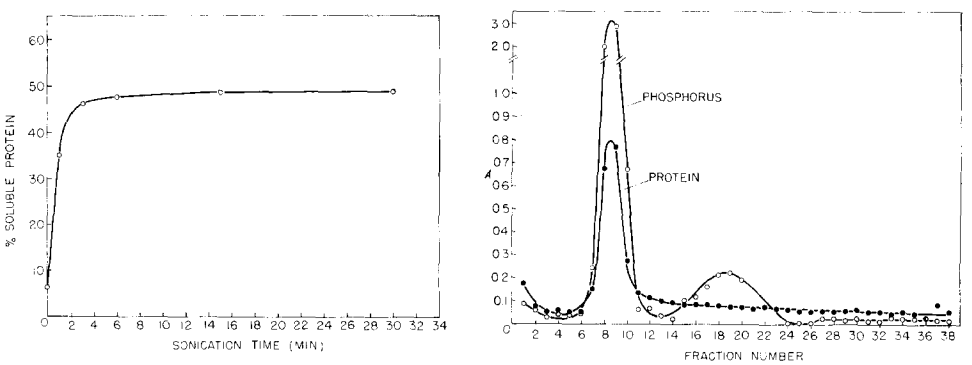

Fig. 1. A 5% suspension of ghosts was sonicated in 15 mosM sodium phosphate buffer at 4°. Aliquots were removed at varying times and centrifuged at 100000 × *g* for 1 h. Protein that remained in the supernatant was considered soluble.

Fig. 2. Following centrifugation of the sonicated ghost preparation the supernatant was chromatographed on Sephadex G-200 gel-filtration resin in 15 mosM buffer. Fractions were assayed for protein and phosphorus. All of the protein and over 90% of the phospholipid eluted in the void volume of the column.

* The criteria for solubility used here (100000 × *g* for 1 h) is stricter than that of most other workers who generally define protein to be soluble if it does not sediment in a field of around 20000 × *g* for about 20 min (see for example refs. 1–4). If this criteria was used the limiting value of solubilized protein was 92%.

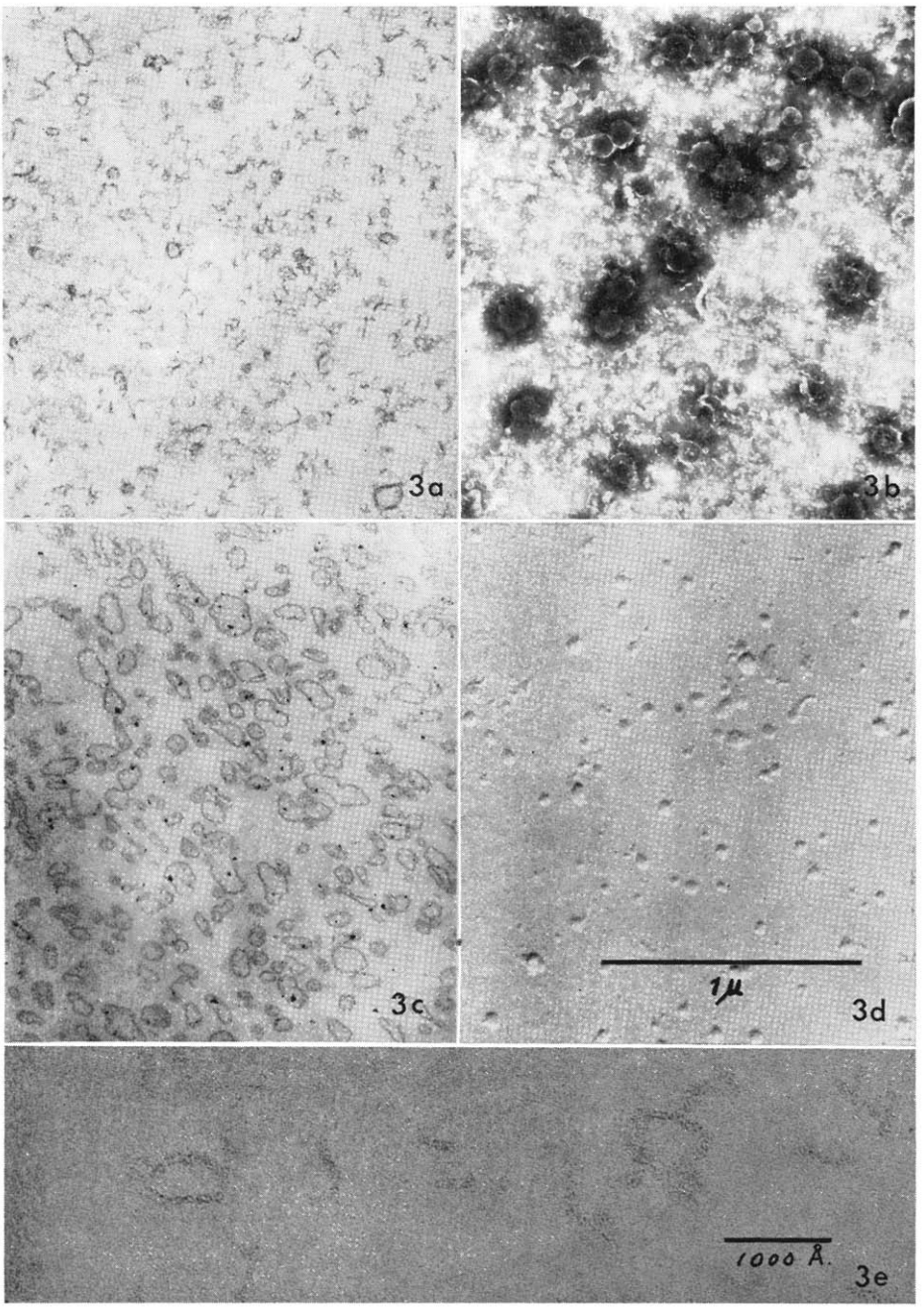

Fig. 3. Electron micrographs of sonicated ghosts. (a)–(d) are × 35 000; (e) is × 140 000. The appearance of the sonicate when negatively stained with uranyl acetate is seen in (b), the air-dry shadow image is shown in (d). (a) and (c) show two different pellets sectioned and stained with lead citrate. Vesicular material predominates in one image, the membrane-like fragments in the other. (e) is a black section of the same pellet shown in (a), stained with uranyl acetate and lead citrate. Both the vesicles and the fragments show a unit membrane appearance.

electron microscope. Several techniques were used to prepare the samples for microscopy and all gave similar results. Some preparations were directly sprayed onto freshly cleaved mica at room temperature or at −20°, dried, and shadowed with platinum after the method of FRIST AND MASER[11]. Other samples were negatively stained with 1 % sodium phosphotungstate at pH 7.0. Still others were fixed for 1 h in 1 % OsO_4. Small pieces of the pellet were then embedded in Epon and sectioned with a diamond knife.

The electron-microscope studies revealed that the sonicate was composed of thin membrane-like fragments and round globular structures varying in diameter from 200 to 600 Å (Figs. 3a–d). In one experiment most of the material was in the globular form, but in two others the fragments predominated (Figs. 3c and a, respectively). Relatively more fragments and fewer vesicles were seen when larger sonication times were used. Stain is bound at the surface of either type of particle, suggesting that the unit membrane structure may survive the sonication treatment.

To examine this possibility, we cut sections thin in comparison with the diameter of the spheroids. These sections showed black as an interference color. Small holes in the plastic were used to focus the microscope to the smallest underfocus Fresnel fringe which could be seen at an instrument magnification of 40000. The resulting plate contained enough image detail to be used directly for measurements. The particles retain the trilaminar appearance of the unit membrane. The typical dark-light-dark structure is seen both in the wall of the round particles and in the edge view of the membrane-like fragments. The mean band widths and standard deviations for twenty membranes are 27 ± 6 Å for the dark bands and 30 ± 5 Å for the light region. When the particles are separated into two sets, one containing only vesicular structures, the other membrane fragments, the dark band from ten vesicle membranes average 27 ± 7 Å; the light band mean is 29 ± 4 Å. Corresponding means for membrane fragments are 27 ± 5 Å for the dark bands and 30 ± 5 Å for the light. The spacing from outer-edge to outer-edge of the dark bands in both structures is thus about 84 ± 10 Å. While this value is larger than the 71 Å reported for the thickness of red cell ghost membranes[12], the difference may not represent a significant difference in structure, considering the difficulty of accurate measurement with the electron microscope and the intrinsic variability of the biological structures. We propose, therefore, that the molecular architecture of the membranes is unchanged by sonication and that the apparent solubilization is due to the fragmentation of the ghost membrane into small pieces not dense enough to pellet under the conditions used to define solubility*.

Sonication has been reported by different workers to solubilize proteins from beef heart mitochondria[1], plasma membranes from bacteria[3], erythrocytes[4], and rat liver cells[5], and transplant antigens from mouse spleen cells[2]. Large molecular complexes have thus been obtained from these membranes. Electron microscopy of very thin sections indicates, however, that in the case of human red cell membranes, the sonic energy serves only to break the membrane into small pieces, not easily broken by dissociating agents. Recent studies by ROSENBERG AND GUIDOTTI[6] on lipid- ex-

* During the preparation of this manuscript MARCHESI AND PALADE[13] reported that the unit membrane structure was visible in ghost membranes sonicated hard enough to make the resulting vesicles smaller than the resolution of the light microscope. Only very rare free edges of the membrane were noted. No measurements on the dimensions of the membrane were given.

tracted erythrocyte membranes have shown that many proteins are present in erythrocyte membranes, the majority of which have a sedimentation coefficient around 2 S and a molecular weight of 50000. Clearly the "soluble" material from the supernatant of the sonicate is a complex aggregate of these proteins with lipid probably retaining the unit membrane form. Thus sonication would seem to offer little advantage as a first step in the separation of the individual components of the plasma membrane.

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