Studies on a Protein Component of Guinea Pig Erythrocyte Membranes

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Recent investigations of the properties of structural proteins of various cell organelles such as cilia (1), microtubules (2–4), and surface membranes (5, 6) indicate that these organelles are formed in part by proteins which have certain properties in common. These properties are also shared by actin—a principal structural protein of muscle tissue. As a result of these observations it has been postulated (6) that such proteins represent a class of actin-like proteins which function as structural units of their respective organelles.

In a previous study (7) it was shown that tryptic digestion of red cell membranes resulted in the formation of long polymers which closely resemble F-actin when examined by negative staining and electron microscopy. The appearance of such a fibrous structure is shown in Fig. 1.

As a result of this observation, a method for the extraction of this fibrous component from native membranes was developed (5) based on the assumption that the components which formed these actin-like structures also showed the solubility properties of actin. When this procedure was applied to the extracts of intact membrane ghosts of guinea pig erythrocytes, approximately 25 % of the total membrane protein was isolated in low ionic strength media. The extract was found to be made up of a single protein species—named spectrin. Under appropriate conditions spectrin polymerized into fibrous structures identical in appearance to those formed by fibrous actin (Fig. 2).

Actin is a well-characterized structural protein of muscle which has a number of distinctive chemical and physical properties (8). The isolated protein is a 48,000 mol wt unit (9), is soluble in low ionic strength media, is stabilized in monomer form by bound nucleotide, and has the capacity to polymerize in the presence of salt or divalent cations. Actin also interacts specifically with muscle myosin to form acto-myosin complexes, and this

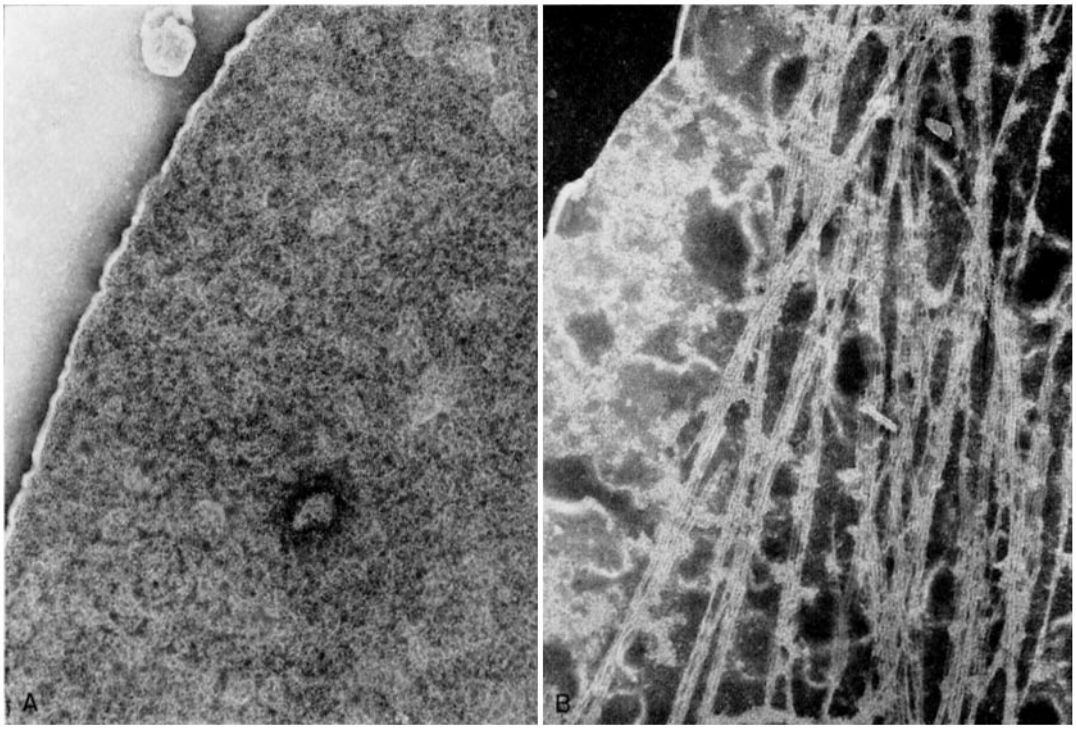

FIGURE 1 Surface appearances of red cell ghost membranes as visualized by negative staining and electron microscopy. As a result of digestion by trypsin (7), bands of filaments 50–60 A in diameter are seen on the surface of the ghost membrane (*B*). These filaments are not seen in control ghost membranes (*A*).

interaction results in a marked stimulation of magnesium-activated myosin ATPase (10).

Since spectrin has certain actin-like properties, a comparative study was carried out to determine how closely this protein resembled muscle actin. Various physical, chemical, and biological properties of spectrin were compared with those of actin extracted from muscles of the same animals.

Actin was isolated from acetone-treated back and leg muscles of Hartley strain guinea pigs according to published procedures (11) and was further purified on a Sephadex G-200 column (9). Spectrin was isolated from red

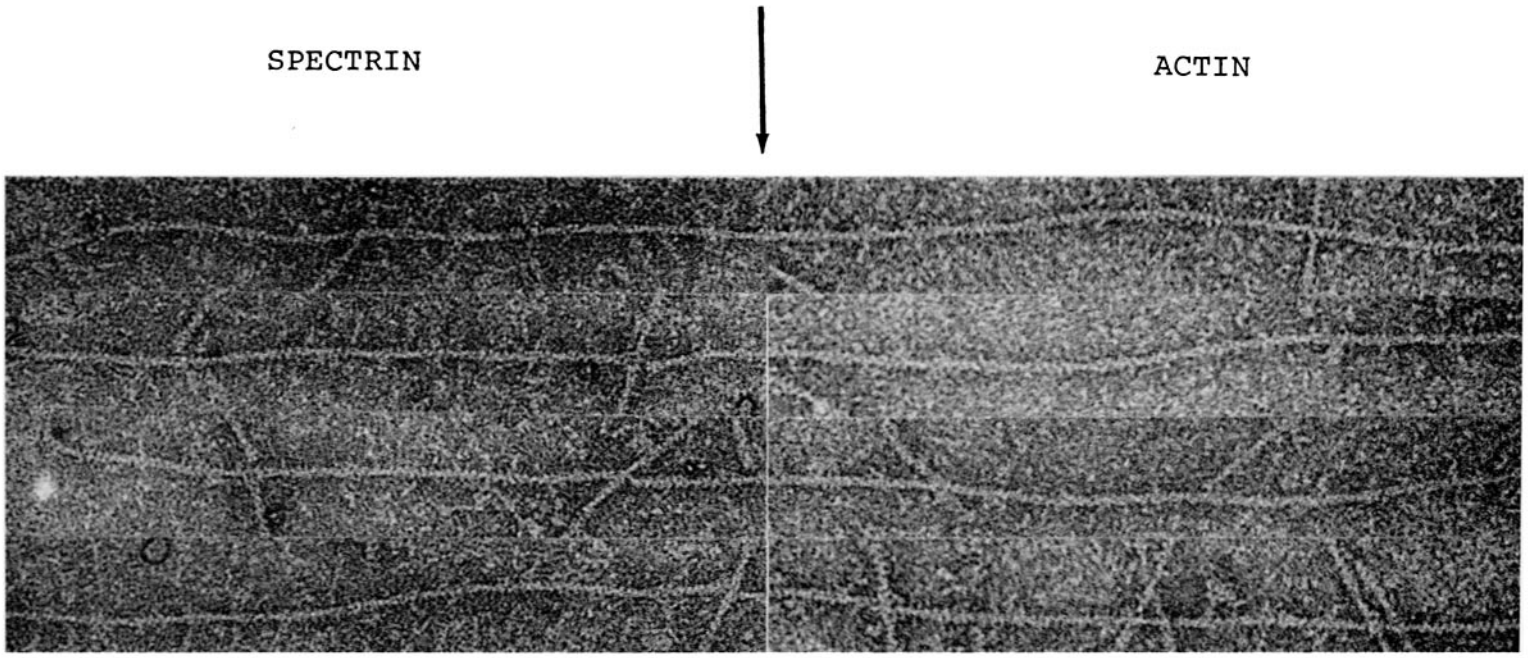

FIGURE 2 A protein extracted from red cell membranes (called spectrin) can be induced to form coiled filaments which are similar in appearance to the filaments formed by F-actin. Electron micrographs of spectrin and actin were cut and mounted in this photograph to emphasize their likeness.

blood cell membranes from the same animals by the method described earlier (5). Filaments of actin were produced by incubating purified actin with 0.1 M KCl and 0.001 M $MgCl_2$ at 37°C for 15 min. Filaments of spectrin were produced by dialyzing isolated spectrin against 0.04 mM $MgCl_2$ overnight at 4°C, and then incubating aliquots of the dialyzed protein in 0.1 M KCl and 0.001 M $MgCl_2$ for 15 min. Samples of both proteins were mounted on carbon-coated grids and negatively stained with 2% phosphotungstic acid (neutralized to pH 7.0 with KOH) and examined and photographed in a Siemens Elmiskop 1A electron microscope.

Aliquots of protein (approximately 2.0 mg) were hydrolyzed in constant boiling (5.7 N HCl) at 110°C for 22 hr *in vacuo* and analyzed on a Beckman model 120B amino acid analyzer by procedures described elsewhere (12). Sedimentation velocity studies were carried out on samples containing 4–6 mg protein/ml in a Spinco model E analytical ultracentrifuge. Sedimentation coefficients were calculated according to Schachman (13). Samples of both proteins were run in 5% polyacrylamide disc gels containing 8 M urea according to procedures outlined in the manual supplied by the Canal Industrial Corp., Bethesda, Md.

Rabbits were immunized with samples of spectrin by subcutaneous injection in Freund's complete adjuvant. Reactions of the antiserum to spectrin and actin were studied by double diffusion in agar.

Myosin was extracted from muscles of the same animals used for the isolation of spectrin and actin by previously described methods (14), and its ATPase activity in the presence of magnesium was determined by using the incubation conditions described elsewhere (15).

Actin and spectrin are soluble in low ionic strength media and both form polymers when incubated with salt and divalent cations. Actin extracted from guinea pig muscle forms the same characteristic helically coiled filaments described earlier by Hanson and Lowy (16) for rabbit actin. Spectrin also forms coiled filaments of the same dimensions (approximately 50–60 A in diameter) and when compared directly (Fig. 2) filaments formed from the two proteins are indistinguishable. These two observations suggested that the proteins might be homologous and prompted the further comparative studies described below.

The amino acid composition of the two proteins is also similar, although there are notable differences in the content of glutamic acid, glycine, leucine, and threonine (Fig. 3). Spectrin has also been found to lack 3-methyl histidine which is specifically found in actin. Fig. 3 also shows the similarity between actin, spectrin, and the protein isolated recently from the outer fibers of cilia by Renaud, Rowe, and Gibbons (1). The protein extracted from cilia appears to be more similar in composition to actin than does spectrin. However, spectrin has more residues in common with actin and the outer fiber

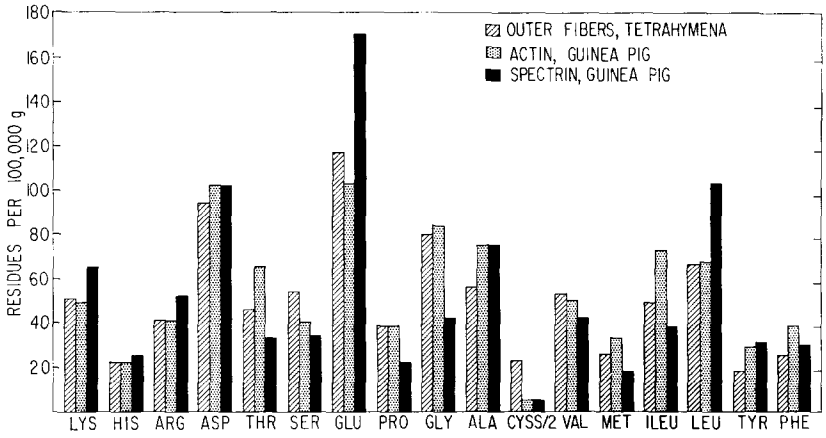

FIGURE 3 The amino acid composition of guinea pig spectrin, rabbit actin, and the outer fiber protein from the cilia of tetrahymena (1). Values are expressed as number of residues per 100,000 mol wt.

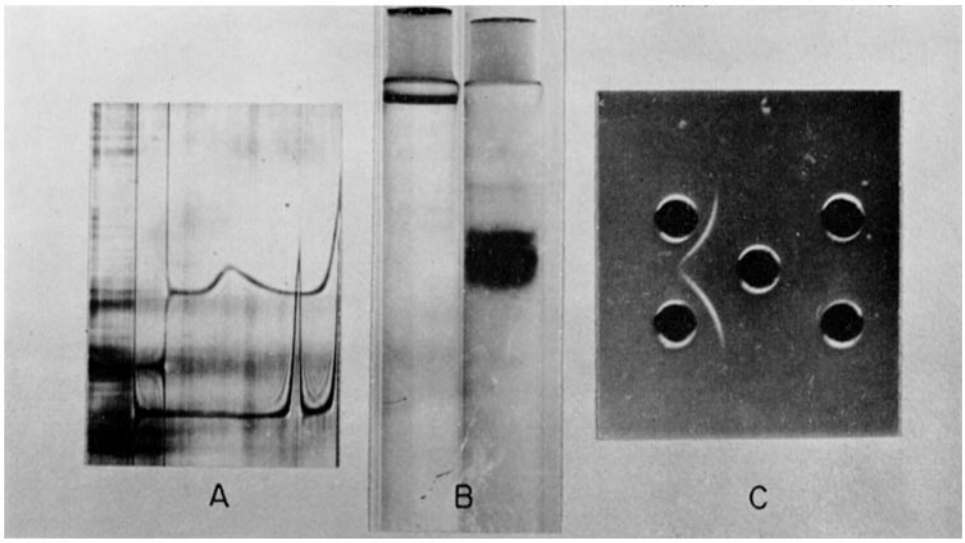

FIGURE 4 (*A*) Ultracentrifugal pattern of muscle actin (upper pattern) and membrane spectrin (lower pattern). Protein concentration is 6 mg/ml in 10^{-3} M ATP, 5×10^{-3} M β-mercaptoethanol pH 7.5. (*B*) Polyacrylamide gel electrophoresis in 8 M urea; tris-glycine buffer pH 8.4. Spectrin (left) and actin (right) were run under identical conditions. (*C*) Immunodiffusion pattern obtained by reacting rabbit antispectrin serum (center wall) against spectrin (left upper and lower walls) and muscle actin (right upper and lower walls).

protein of cilia than it has with other randomly selected proteins when analyzed statistically (17).

When other physical and biological parameters are compared, however, actin and spectrin show strikingly different properties. Actin sediments as a

broad boundary with a sedimentation value of approximately 3S, while spectrin sediments as a hypersharp boundary with a sedimentation value of approximately 13S (Fig. 4 *A*) when both are run under the same conditions of neutral pH, low ionic strength medium. The electrophoretic mobility of the two proteins is also markedly different when they are run in polyacrylamide gels in the presence of 8 M urea (Fig. 4 *B*).

Antiserum prepared in rabbits immunized with spectrin form a single precipitin zone with spectrin, but do not react with actin (Fig. 4 *C*).

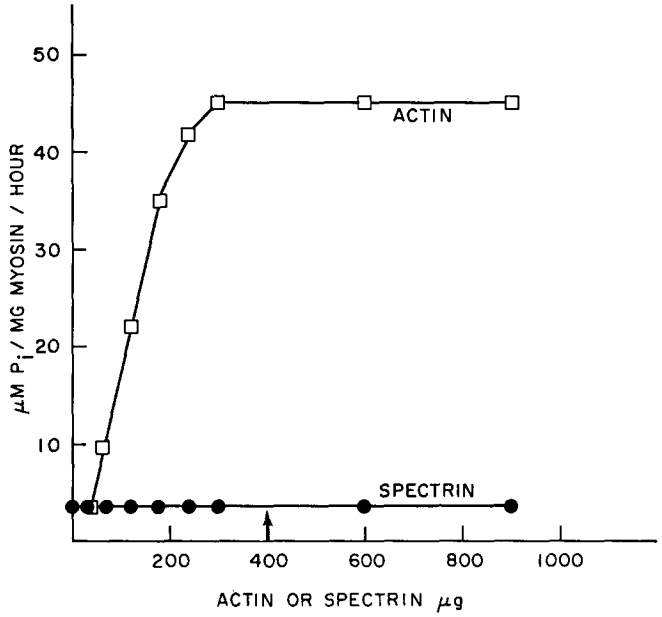

FIGURE 5 Aliquots of actin or spectrin were incubated with myosin extracted from guinea pig muscle and tris-HCl buffer pH 7.5, $MgCl_2$·003 M and ATP 0.003 M. ATPase activity of myosin was assayed by measuring production of inorganic phosphate as described previously (15).

One of the most characteristic biologic properties of actin is its capacity to interact with myosin. When the conditions are appropriate, actin stimulates the Mg-ATPase of myosin approximately 15-fold. Fig. 5 shows the effect of muscle actin preparation on myosin and the lack of any stimulating effect by comparable amounts of spectrin.

Although spectrin, extracted from red cell membranes, is remarkably similar to muscle actin in terms of its solubility properties, amino acid composition, and the capacity to form morphologically identical filaments under similar conditions, it is clear that the two proteins are not identical molecules. When studied by ultracentrifugation, acrylamide gel electrophoresis, and immunodiffusion, spectrin and actin show no similarities, and spectrin

does not activate myosin ATPase or inhibit the activation of myosin ATPase by actin.

Previous reports of actin-like proteins extracted from other nonmuscular cells must also be investigated further before the functional properties of actin can be assumed to apply to them. In particular, the ability to form filaments and interact with myosin must be demonstrated.

If membrane spectrin functions as a supporting unit of the cell membrane, it might then be considered homologous to muscle actin and also related to proteins extracted from cilia, microtubules, and mitotic spindles.

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Discussion from the Floor

Dr. Henry Tedeschi (State University of New York at Albany): I would like to comment on Dr. Racker's table giving the scoreboard of the chemical intermediate hypothesis versus the chemiosmotic hypothesis. In order to put all those plus signs on the chemiosmotic side, Dr. Racker was leaning over backwards. Perhaps Dr. Racker should put a plus sign for the chemiosmotic hypothesis for prediting that electrons are transferred during oxidative phosphorylation! In connection with the proposal that a membrane potential may be coupled to phosphorylation, I would also like to report an experiment carried out by one of my students, Joseph Tupper. He has succeeded in inserting microelectrodes in giant mitochondria from insects. He finds potentials of the order of 20 mv, inside positive in state 3. This is rather low to have a role in oxidative phosphorylation. The potential is osmotically sensitive and therefore is likely to correspond to a potential across the mitochondrial semipermeable membrane. He finds a decrease (about 10 mv) from state 3 to state 4. These potentials are insensitive to KCN.

I would also like to comment on Dr. Schatz's presentation. It seems to me that Dr. Hewson Swift presented a paper at the Cell Biology meetings last year (1967. *J. Cell Biol.* **35**:131 (Abstr.)) indicating that he has evidence for the presence of particles corresponding to mitochondria in yeast grown anaerobically. As I remember, he used gluteraldahyde fixation. This would be in agreement with Dr. Schatz's idea that conventional fixation may not work because of the lack of unsaturated bonds in the premitochondrial granules.

Dr. Racker: I am somewhat concerned about the meaning of experiments of this kind. I wonder how certain one can be what changes have occurred when a microelectrode is placed into a giant mitochondrion.

Dr. Tedeschi: This is always a problem with data of this kind since the possibility of damage having occurred can never be entirely discarded.[1]

However, the measurements are highly reproducible from experiment to experiment.

Dr. Racker: Even if the measurements are reproducible, couldn't we say that the damage is reproducible?

Dr. Tedeschi: I agree. However, it is interesting to see that upon withdrawal of the electrode the potential and resistance difference measured disappears.

Dr. Schatz: Regarding the findings of Swift, I would like to add that there have been several reports of mitochondria-like structures in anaerobically grown yeast cells. To

[1] In later experiments we found that when the same mitochondrion is impaled twice, the potential remain approximately the same. This is an indication that the initial damage is not likely to be extensive enough to affect the measurements.

the best of my knowledge, however, all these observations were restricted to cells that had been grown in a medium supplemented with Tween 80 and ergosterol. This has led to the wide-spread view that anaerobic yeast cells are mitochondria-free provided they are grown in the absence of added lipid cofactors. In contrast, our findings indicate that mitochondrial structures are invariably present in anaerobic yeast cells regardless of the composition of the growth medium.

Dr. Peter Marfey (State University of New York at Albany): I would like to ask Dr. Wallach about possible contribution of membrane lipids and carbohydrates to the observed ORD and CD spectra obtained on intact membranes. These classes of membrane constituents contain asymmetric atoms and chromophores that may contribute significantly to the observed ORD and CD spectra. For example, an ester function of phospholipids contains one oxygen atom which is directly bound to the asymmetric carbon atom of a glycerol moiety. This may induce asymmetric electronic transitions in the ester chromophore, and thus contribute to the observed CD spectrum.

Dr. Racker: Dr. Wallach, may I ask you whether you determined the extent to which the phospholipids were degraded by phospholipase C and A, and whether there was in this respect a significant difference between the action of the two lipases? Also, could you tell us to what extent were the enzyme preparations purified?

Dr. Wallach: There are a number of questions here. Dr. Marfey's question concerns the optical activity of ester n-transitions. This possibility was raised by Urry and coworkers to explain some of the peculiarities of the CD and ORD of membranes. We have looked for such contributions and have examined a number of phosphatides in solution and in dispersion, as have Lenard and Singer and several other people, and find these transitions too weak in themselves to contribute to the spectrum.

But there's always the possibility that in the membrane the lipid is ordered by its interaction with the protein so that the contribution of the ester transitions becomes significant. This is possible but not probable. For one thing there are bacterial membranes, those of the halophile bacteria, which don't have ester phosphatides, but their optical activity characteristics are identical to the membranes which I've mentioned. Also the proposed ester $n - \pi^*$ bands cannot account for the distortions of the $\pi^0 - \pi^*$ regions of the CD spectrum.

Now as to the lipases, purity is a problem, particularly with phospholipase C. We have tested whether the preparations which we employed exhibited any peptidase, amidase, or glycosidase activities; they did not. Secondly one cannot simulate the lipase effects with peptidases.

The third question concerned the extent of lipase action. For the phospholipase A, I gave data at two levels of enzymes. At the high concentration, where we have complete inactivation of ion-sensitive ATPase, for example, the glycerol phosphatide of the membrane was completely cleaved.

As far as phospholipase C action is concerned, this depends on the membrane. In the case of the Ehrlich ascites carcinoma membranes, about 45 % of the phosphorylcholine, phosphorylethanolamine is split off, and this is the limit under our conditions for this particular membrane. Sphingomyelin, lecithin, and phosphatidylethanolamine are all attacked. In the case of red cells, the action is more complete and reaches a level of about 65 %.

I think this is also what Lenard and Singer found.

Dr. Racker: May I emphasize the importance of using highly purified preparations of phospholipase for such experiments. Many crude preparations are contaminated with proteolytic enzymes and the results are difficult to interpret. Phospholipase A has been crystallized (de Haas, G. H., N. M. Postema, W. Nieuwenhuizen, and L. L. M. van Deenen. 1968. Purification properties of phospholipase A from porcine pancreas. *Biochim. Biophys. Acta.* **159:**103) and a partial purification of phospholipase C has also been achieved (Partick Kemp, personal communication). Since both these enzymes require Ca^{++} for activity, we perform control experiments in the presence of the enzyme and excess EDTA. With crude preparations we have observed at times that EDTA had no effect on the results and we did not know what we had. With the purer preparations these controls are quite clear-cut.

My second comment refers to the effect of phospholipase C. I believe the data indicated that there was considerable residual enzyme activity in your preparation after the phospholipase C action was completed. Could you comment on this?

Dr. Wallach: All our controls were calcium-free and under those conditions we did not observe membrane perturbations. I'm aware of the pure enzymes and we're switching to those that are becoming available.

The effect of phospholipase C on the enzyme activities we have measured is rather small, despite the dramatic structural changes.

Dr. Thomas Haines (The City College of The City University of New York): I have three questions: After phospholipase C digestion of the membrane preparation did either Singer or you look at the transport properties of the ghosts or the tumor cells? The second question is: Phospholipase C, if I remember correctly, requires a fairly high calcium concentration, with the phospholipase A digestion I notice you didn't use any calcium. I wonder if the calcium concentration would have any effect on the electron micrograms you obtained of either preparation? The third question is: Following Dr. Rothfield's fine example, perhaps you could theorize or state hou you envision the moving about of the "bumps" in the electron micrographs you obtained after phospholipase C interaction with the membrane.

Dr. Wallach: We have no transport data and I don't known about Lenard and Singer. The tumor membranes are rather small vesicles and there are major technical problems in measuring transport with such particles.

The erythrocyte ghosts vesiculate with time, giving the same problem. We've made no effort to get at this.

As far as calcium and phospholipase A are concerned, we did not add calcium; this is correct. We have found that there is some very tightly bound calcium associated with membranes, even if they had been isolated with an EDTA wash. This is enough to activate the enzyme. If one adds EDTA to the incubation medium or passes the membrane through a chelating resin just prior to use, you don't get lipase A activity.

In the case of lipase C, we did not use the very high calcium concentrations which are often employed but worked at 2 mM calcium. Within the period of observation which I've described here, the controls for electron microscopy showed no alteration under these conditions.

I might add though, that if one simply stores erythrocyte ghosts for several days in the presence of calcium, one gets some rearrangement of the particles. I don't believe

that the particle rearrangement occurs exclusively with lipase C, but we see it within 2 min after initiation of enzyme action. It is a very rapid event unlike vesiculation.

Dr. Samuel Silverstein (Rockefeller University): Dr. Schatz, are there outer membranes on the submitochondial particles or promitochondria that you have isolated from yeast? And secondly, did I understand you to say that antibody against F_1 inhibits the ATPase activity of the submitochondrial particles? Are they inside-out particles within the cell or do they become so during the process of isolation?

Dr. Schatz: These are two very pertinent questions. Fortunately, I think I can give answers to both of them. As for the first question, our electron micrographs of the anaerobic yeast cells indicate that the promitochondria *in situ* are surrounded by a distinctly double-layered envelope. Moreover, the isolated promitochondria contain a pigment whose spectral properties are identical with those of mammalian cytochrome b_5. We therefore suspect that the mitochondrial outer membrane, too, is still formed by the anaerobic cells.

Regarding your second question I should add that our promitochondrial preparations are almost certainly damaged because they were isolated from mechanically disintegrated yeast cells. Nevertheless, we found it necessary to sonicate the isolated promitochondria in order to render their ATPase activity *completely* sensitive to the F_1 antibody. It thus looks as if the ATPase is "inside" in the native particles but becomes partially exposed to the surrounding medium during the isolation procedure.

Dr. Racker: May I emphasize the last point Dr. Schatz has made. We believe that antibodies are very useful tools for orientation studies with respect to the two sides of the membrane. Heavy layer mitochondria are not inhibited by an antibody against F_1, but submitochondrial particles which are "inside-out" are over 90% inhibited. In contrast, heavy layer mitochondria are inhibited by an antibody against cytochrome *c* but submitochondrial particles are not (diJeso, F., R. O. Christiansen, H. Steensland, and A. Loyter 1969. Localization of inner mitochondrial membrane components. *Fed. Proc.* **28**:663). Finally, light layer mitochondria are about 50% inhibited by antibody against F_1, so we conclude that they are considerably damaged and Dr. Schatz is using the same argument with respect to his promitochondria.

Dr. Peter L. Pedersen (Johns Hopkins University): I would like to address a question to Dr. Racker. Intact rat liver mitochondria and intact inner membrane–matrix fractions prepared from rat liver mitochondria catalyze an oligomycin-sensitive ADP-ATP exchange reaction; have acceptor control of respiration; and undergo gross morphological changes. Moreover, they still have matrix proteins present.

In your reconstitution studies where you have demonstrated a reconstitution of oxidative phosphoryalation, do you have any of these three properties of the intact structure? And finally, do you have any matrix proteins such as malate dehydrogenase or glutamate dehydrogenase present?

Dr. Racker: We seem to have removed the activities you mention. We are particularly puzzled by the fact that we cannot detect an oligomycin-sensitive ADP-ATP exchange. We have reported these findings (Zalkin, H., M. E. Pullman, and E. Racker. 1965. *J. Biol. Chem.* **240**:4011) and have attempted to explain them. We rather hope that one day we shall detect an oligomycin-sensitive ADP-ATP exchange in our phosphorylating particles and it would make good sense to us if we did. We

also cannot observe the classical phenomenon of respiratory control in our reconstituted particles; i.e., a stimulation of respiration on addition of P_i and ADP. But we can see stimulation of respiration by uncoupling agents provided we add a compound such as dicyclohexylcarbodiimide. You will find data on this point and appropriate references in a recent publication (Racker, E. 1967. *Fed. Proc.* **26:**1335). What were your other questions?

Dr. Pedersen: Well, two other questions. One was, do these particles of yours undergo any gross morphological changes such as seen by Hackenbrock? And also do you have any matrix enzymes still present in your submitochondrial particles?

Dr. Racker: The reconstituted particles cannot oxidize Krebs cycle intermediates except for succinate. I discussed the resolution of succinate dehydrogenase in particles treated with silicotungstate. The particles do have DPNH dehydrogenase. We have not been able to detect the type of large conformation changes which can be seen in intact mitochondria. We have looked for them in the presence of ATP and of uncouplers. I personally think that these large structural changes are not primarily linked to oxidative phosphorylation but are secondary; e.g., perhaps due to movement of water. May I ask whether you have seen large conformational changes in digitonin particles?

Dr. Pedersen: I don't think anyone has looked for them in digitonin particles.

Dr. Racker: Why not?

Dr. Pedersen: I am sure the mitochondrial morphologists are thinking about it.