Polymerization of Actin by Positively Charged Liposomes

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Abstract. By cosedimentation, spectrofluorimetry, and electron microscopy, we have established that actin is induced to polymerize at low salt concentrations by positively charged liposomes. This polymerization occurs only at the surface of the liposomes, and thus monomers not in direct contact with the liposome remain monomeric. The integrity of the liposome membrane is necessary to maintain actin in its polymerized state since disruption of the liposome depolymerizes actin. Actin polymerized at the surface of the liposome is organized into two filamentous structures: sheets of parallel filaments in register and a netlike organization. Spectrofluorimetric analysis with the probe N-pyrenyl-iodoacetamide shows that actin is in the F conformation, at least in the environment of the probe. However, actin assembly induced by the liposome is not accompanied by full ATP hydrolysis as observed in vitro upon addition of salts.

ACTIN is the major protein of muscle cells and has been found in the cytoplasm of all other eukaryotic cells (6, 18, 31). Actin exists as a monomer, G-actin and as a polymer, F-actin. Polymerization of actin may be induced by millimolar concentrations of divalent cations and/or physiological ionic strength (10, 15, 16, 20, 27, 35, 39, 49). A molecule of ATP is bound to each actin monomer and is hydrolyzed during polymerization (25, 28–30, 36, 50). In an excess of divalent cations, such as Mg^{++}, actin filaments associate into paracrystals (1, 8, 12, 25, 41, 42, 46, 51).

The reversible monomer–polymer transition of cytoplasmic actin in nonmuscle cells is a fundamental phenomenon which is thought to be the basis of many cellular functions such as motility, cytokinesis, phagocytosis. Thus, actin polymerization has been extensively studied in vitro (9, 18, 33).

In a recent paper (38), we have shown that the polymer, F-actin is able to interact directly with the positively charged lipids of artificial membranes. In the present paper, we show that the monomer, G-actin may also interact with positively charged liposomes. However, in these instances, actin polymerizes at the surface of the liposomes even at low salt concentrations.

Materials and Methods

Preparation and Labeling of Actin

Actin was prepared from rabbit muscle by the Spudich and Watt technique (43) as modified by Nonomura et al. (26) using one step polymerization of actin in KCl 0.1 M + $MgCl_2$ 2 mM. In all experiments, actin was dissolved in the G-buffer (Tris-HCl 2 mM; ATP 0.2 mM; $CaCl_2$ 0.2 mM; β-mercaptoethanol 0.5 mM, pH 8.0).

Actin was covalently bound to N-(1-Pyrenyl) iodoacetamide (Molecular Probes, Junction City, OR) according to Cooper et al. (7).

Preparation of Liposomes

Liposomes were prepared in G-buffer by the reverse phase technique of Szoka and Papahadjopoulos (47). Neutral liposomes were made solely from phosphatidyl choline, whereas positively charged liposomes were prepared from phosphatidyl choline and 1–20% stearylamine. Negatively charged liposomes were made from phosphatidyl choline and 10% oleic acid. All lipids were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification.

Measurements of Interaction between Actin and Liposomes by Turbidimetry

G-actin and liposomes were mixed in G-buffer at various final concentrations indicated in the results section. After 10 min of incubation at room temperature, the turbidity of the solution was determined by optical density at 550 nm. Liposomes were then sedimented by centrifugation at 90,000 *g* × 120 min at 4°C. Concentration of actin remaining in the supernatant was measured by optical density at 290 nm.

Measurements of Actin Polymerization by Fluorimetry

Polymerization of actin was monitored by measuring the increase in fluorescence of a pyrene probe-labeled-actin after the protocol of Cooper et al. (7). Fluorescence of polymerized labeled actin is greater than the monomer. Wavelengths were 342 nm for excitation and 407 nm for emission. The increase of fluorescence was recorded with a Shimatzu RF 540 spectrofluorimeter. Usually a ratio of 1:9, (pyrene-actin:unlabeled actin) was used.

Determination of the Actin-bound Nucleotide

G-actin was labeled with 2, 8 [^{3}H]ATP at 27.6 Ci/mM (New England Nuclear, Boston, MA) by exchange with the bound ATP in accordance with the technique used by Magasanik et al. (22). [^{3}H]ATP-labeled G-actin at 0.2 mg/ml was induced to polymerize by liposomes in an amount sufficient to polymerize all the actin (1 μM of lipids/ml) or by 2 mM $MgCl_2$ + 100 mM KCl, and incubated for 1 h at room temperature. Free nucleotides were removed by ion exchange chromatography on Dowex. The nucleotides attached to F-actin were released by denaturation with 10% TCA and identified by paper chromatography as described by Magasanik et al. (22) with the following eluant: isobutyric acid/ammonium hydroxide/EDTA

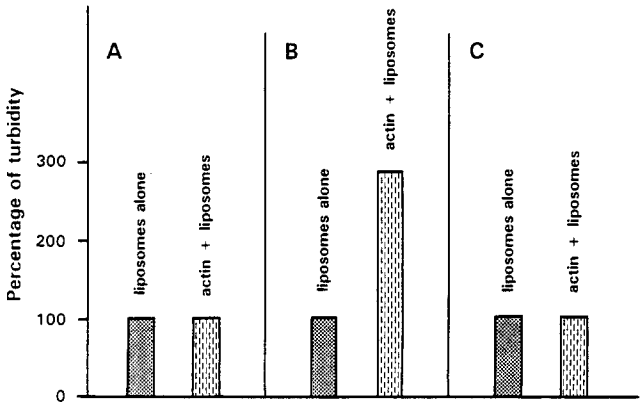

Figure 1. Turbidity at 550 nm of a solution of liposome before and 10 min after addition of G-actin at final concentrations of 1 μM of lipids/ml and 0.25 mg/ml G-actin. 100% of turbidity was defined as the turbidity of the liposome solution alone. Turbidity of G-actin alone = 0. (*A*) neutral liposomes; (*B*) positively charged liposomes; (*C*) negatively charged liposomes.

0.1 M/water, 66:1:1:32, and total radioactivity was measured with a LKB β counter.

Electron Microscopy

One drop of the liposome-actin mixture was put on a formvar-carbon coated grid and left undisturbed for 30–60 s to enhance the adherence of the specimen to the grid. The preparation was then negatively stained with 1% uranyl acetate and observed with a Phillips 201 electron microscope operated at 80 kV.

Results

Turbidimetric Measurements

When G-actin is mixed with positively charged liposomes, a visible precipitate immediately forms, suggesting that an interaction has occurred between the two constituents. This interaction may be quantified by turbidimetry as presented in Fig. 1. As shown in this figure, no increase in turbidity occurs when solutions of G-actin are mixed with negatively charged or neutral liposomes. These results are confirmed by

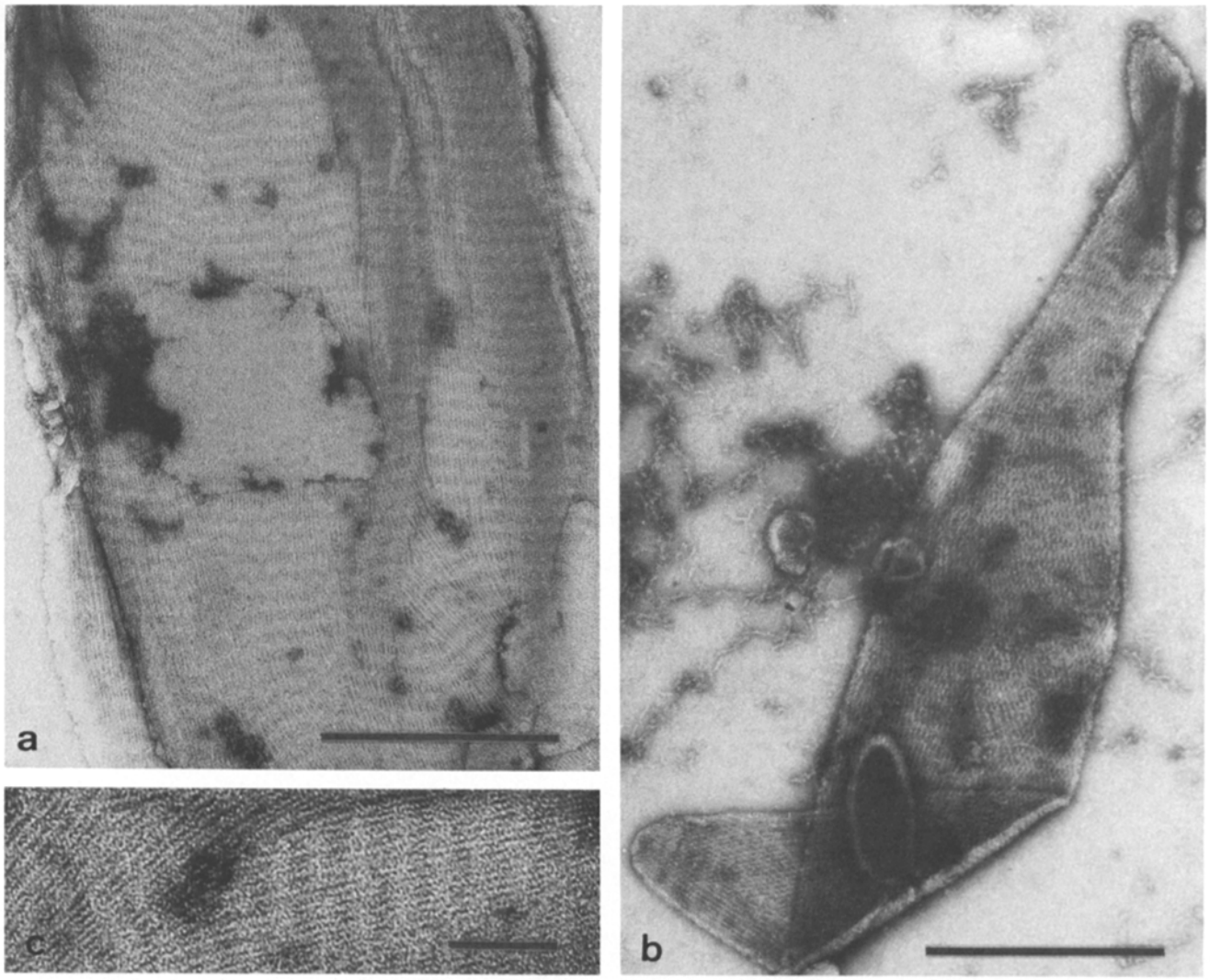

Figure 2. Electron micrograph of actin polymerized at the surface of positively charged liposomes (*a*) actin make a paracrystalline sheet of parallel filaments in register. The liposome is elongated by the sheet of actin. (*b*) Actin is organized in a net like organization. Liposomes are flattened and elongated by the skeleton of actin. (*c*) Detail of a paracrystalline sheet observed at higher magnification. Bars: (*a* and *b*) 0.5 μm; (*c*) 0.1 μm.

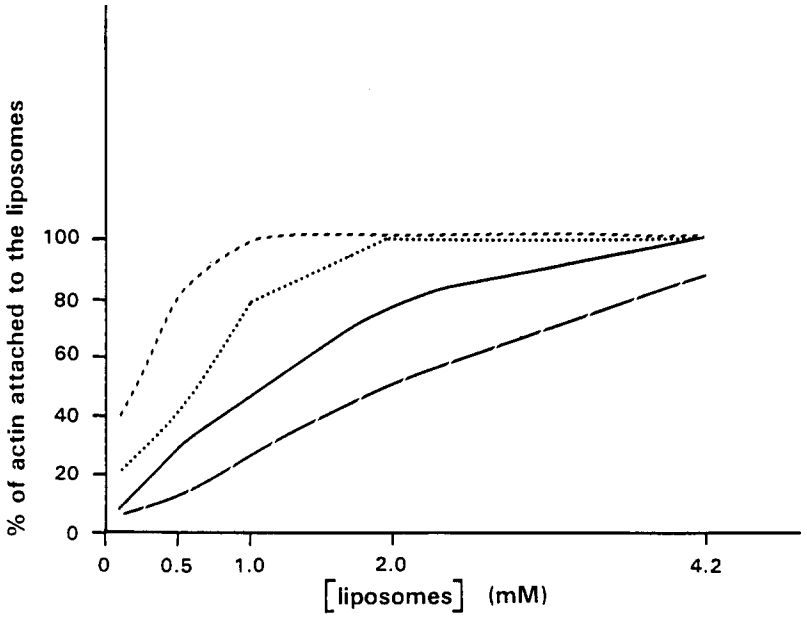

Figure 3. Percentage of actin attached to the liposomes for different concentrations of actin and of liposomes. The amount of actin attached to the liposomes was calculated after sedimentation of positively charged liposomes covered with actin and the determination of actin in the supernatant. Initial concentration of actin: 0.1 mg/ml (- - - -); 0.25 mg/ml (· · · ·); 0.5 mg/ml (▬▬); 1 mg/ml (——).

electron microscopy where aggregates of liposomes covered with actin are observed only with positively charged liposomes. Indeed at higher magnification, a new phenomenon may be observed. The actin which is present at the surface of the positively charged liposomes has polymerized into filaments and crystals even if the incubation medium contains low concentrations of salts (Fig. 2).

Cosedimentation Measurements

Quantitative attachment of G-actin to positively charged liposomes was determined by cosedimentation experiments for different concentrations of G-actin and liposomes. In these experiments, actin was incubated for 1 h with liposomes and the liposomes were then centrifuged to estimate the amount of actin attached to the liposomes. Fig. 3 shows that actin binding increases with the liposome concentration until complete binding is observed. At high actin concentrations, no complete binding is observed due to the saturation of the liposome surface by actin molecules. No binding of actin was detected with neutral or negatively charged liposomes.

Fluorimetric Measurements

Kouyama and Mihashi (19), and Cooper et al. (7) have shown that the fluorescence of the probe N-(1-Pyrenyl) iodoacetamide covalently linked to actin increase when actin is polymerized. This technique was then used to measure the rate and the extent of polymerization of actin in vitro. Fig. 4 presents the increase of fluorescence for different concentrations of labeled G-actin after addition of a constant quantity of positively charged liposomes. There is a rapid increase in fluorescence during the first 20 min followed by a stabilized situation. This shows that actin is polymerized by positively charged liposomes in solutions containing low salt concentration.

However, depending on the conditions, not all of the G-actin present in solution is induced to polymerize since the amplitude of the fluorescence increase is not proportional to actin concentration. Therefore, for high concentrations of actin, some of the actin must remain in the G form. This is confirmed by the observation that addition of KCl may lead to a further increase of fluorescence for concentrations of G-actin above 0.1 mg/ml (Fig. 4). In these instances the amplitude of this second phase of polymerization, induced by KCl, is proportional to the initial concentration of actin added. These results are interpreted as follows: if polymer-

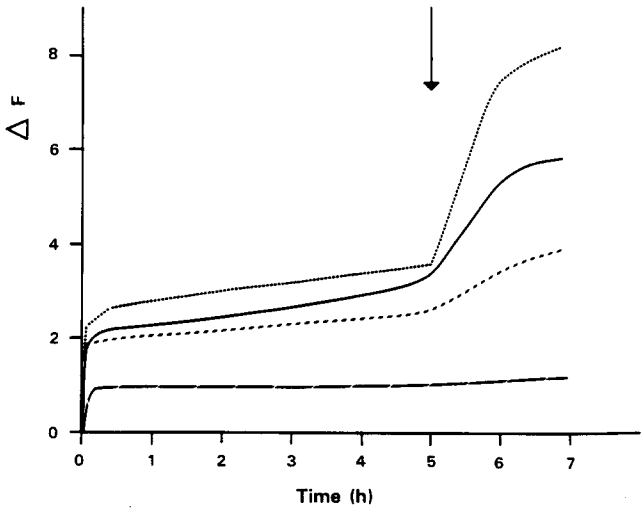

Figure 4. Polymerization of actin at constant concentration of positively charged liposome (1 μM of lipids/ml) for different concentrations of G-actin. 0.1 mg/ml (——); 0.3 mg/ml (- - -); 0.5 mg/ml (▬▬); 0.8 mg/ml (· · · ·). Polymerization was measured by the increase of fluorescence of the pyrene probe linked to actin. Since the various concentrations of pyrene-actin differ in their initial fluorescence intensity, we represent the ΔF as the fluorescence of the sample minus the fluorescence before addition of liposomes. At the arrow, 0.1 M KCl + 2 mM $MgCl_2$ are added to the solution to induce full polymerization of the remaining G-actin.

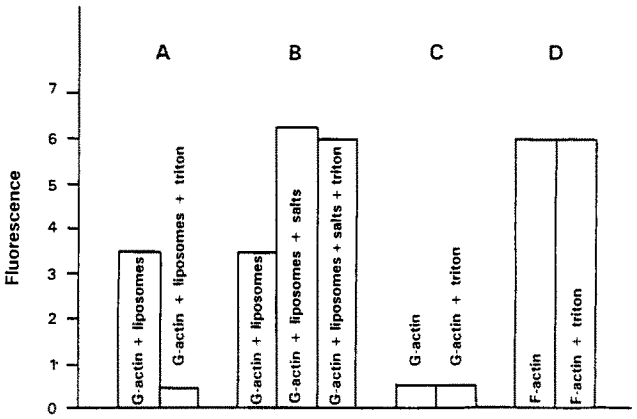

Figure 5. Effect of liposome disruption by Triton X-100 on the polymerization state of actin as measured by fluorescence of the pyrene probe linked to actin. G-actin pyrene is induced to polymerize by positively charged liposomes at final concentration of 0.5 mg/ml and 1 μM/ml, respectively. Then, fluorescence is measured before and after addition of Triton (*A*). Control experiments were made by adding salts (KCl 0.1 M + $MgCl_2$ 2 mM) prior to Triton (*B*), with labeled G-actin alone (*C*), and with labeled F-actin (*D*).

ization of actin only takes place at the surface of the liposomes, this surface must be a limiting factor. At low concentrations of actin, there is enough surface to polymerize all of the actin, but for high concentrations of actin there is not enough surface, and the monomers which cannot make contact with the liposome remain in the G form. These results suggest that the liposomes do not have a catalytic action and that actin polymerized at the surface of the liposome must remain in contact with this surface to remain in the F form.

This is confirmed by the results of an experiment in which actin was first polymerized by liposomes, and the liposomes were then dissolved with a detergent (Fig. 5). An immediate decrease of fluorescence was observed which shows that the integrity of the liposome is necessary to maintain actin in the F conformation. This decrease in fluorescence was not produced by the action of the detergent on the probe since labeled G or F-actin have the same fluorescence with or without detergent (Fig. 5).

Influence of the Density of Positive Charges

As suggested by the results shown in Figs. 1 and 4, actin binding and polymerization depend on the positive charges of the liposomes and also on the amount of liposome surface available. We then studied how the density of positive charges on the liposomes surface influences the actin polymerization, by measuring the increase of fluorescence when pyrene-labeled actin is incubated with liposomes of increasing stearylamine content, thus bearing an increasing proportion of positive charges. Fig. 6 shows that the amount of polymerized actin is proportional to the density of positive charges on the liposome surface, reaching a maximum at 10% stearylamine. At higher proportions of stearylamine, there is a decrease of actin polymerization due to the fact that for more than 10% stearylamine, liposomes are unstable and do not form.

Conformational State of Actin

Actin exists under 2 conformational states: the G and F conformations. Our electron microscopic observations show that actin polymerized at the surface of positively charged liposomes has the morphological aspect of actin filaments even at low salt concentrations where actin is normally in the G state. What is the conformational state of the actin molecule polymerized at the surface of a liposome? To answer this question, we have compared the variations of the fluorescence spectra of the probe N-(1-pyrenyl) iodoacetamide covalently linked to actin before and after its polymerization by liposomes or by salts. Figs. 7 and 8 show that excitation and emission spectra of actin polymerized by liposomes are identical to actin polymerized by salts and very different from the G-actin profile. Therefore, we can conclude that the environment of the pyrene probe in the actin molecule polymerized by liposomes is the same as in conventional F-actin.

Analysis of the Bound Nucleotide

When G-actin solubilized in G-buffer, is polymerized in vitro by salts (KCl 0.1 M + $MgCl_2$ 2 mM), the bound ATP of the monomer is hydrolyzed to ADP. However, when G-actin is polymerized by liposomes made in the same buffer, only half of the ATP bound to the actin molecule is hydrolyzed to ADP (Table I).

Electron Microscopy

When G-actin interacts with the liposome, it polymerizes into filaments which have the same morphological aspect as F-actin showing a double strand with a periodicity of 37.5 nm. These filaments are found only at the surface of the liposome and rarely away from the membrane.

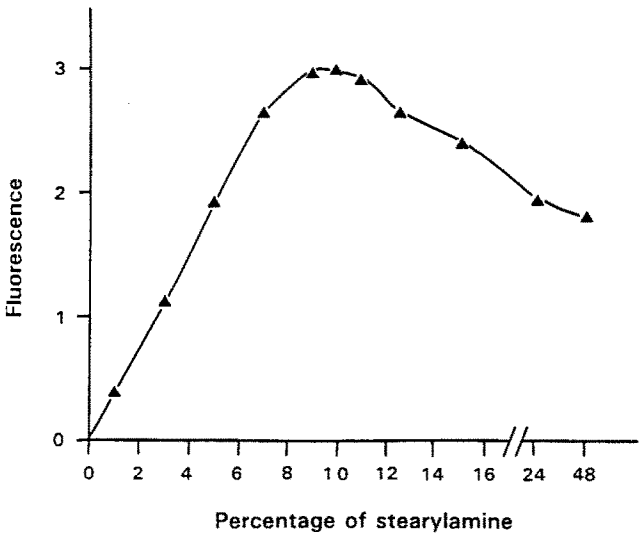

Figure 6. Effect of the density of positive charges on the amount of actin polymerized by the liposomes as measured by the fluorescence of the pyrene probe linked to actin. Fluorescence was noted 1 h after mixing positively charged liposomes to actin, final concentration 1 μM/ml and 0.6 mg/ml, respectively. Positive charges were added to the liposome surface by increasing the percentage of stearylamine forming the liposomes.

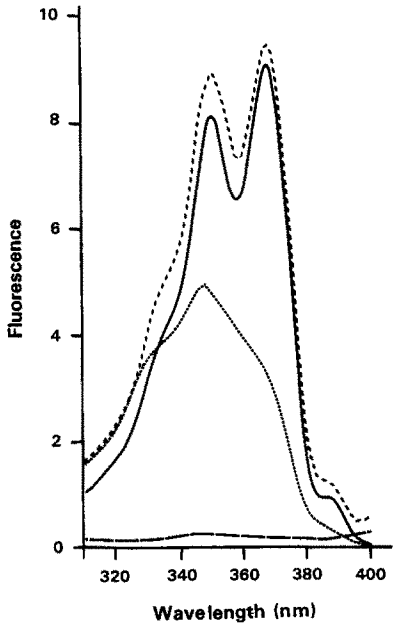

Figure 7. Excitation spectra of G-actin-pyrene alone (· · · ·), polymerized by salts (- - - -) or by positively charged liposomes (▬▬), liposomes alone (— —). Emission wavelength 407 nm.

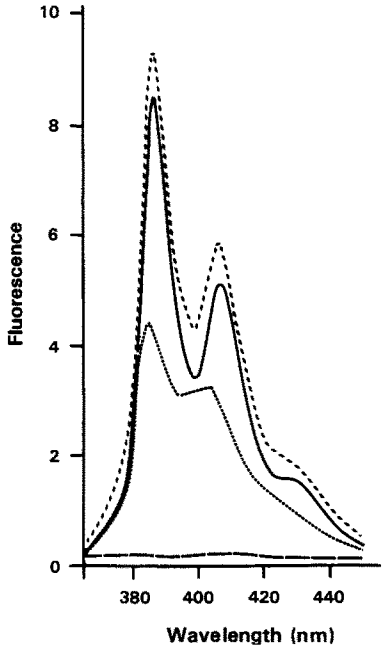

Figure 8. Emission spectra of G-actin-pyrene alone (· · · ·), polymerized by salts (- - - -), or by positively charged liposomes (▬▬), liposomes alone (— —). Excitation wavelength 342 nm.

The filaments which are found at the surface of the liposomes are not randomly distributed but are rather organized into regular structures. Two types of lattices may be found: (*a*) The paracrystalline sheet made with juxtaposed and parallel filaments in register having the same longitudinal periodicity as the filament (Fig. 2, *a* and *c*). (*b*) The net like organization made of two rows of parallel filaments disposed at an angle (Fig. 2 *b*).

Both types of organization exists simultaneously in the same preparation and sometimes on the same liposome.

Discussion

Binding of G-actin and its further polymerization occurs only with positively charged liposomes, and is never observed with negatively charged or neutral liposomes. Therefore, this phenomenon is not a suspension artifact or an unspecific adsorption of actin to a lipid surface. Because actin is an anionic protein, we may conclude that this phenomenon is electrostatic in nature.

Polymerization of actin, induced by salts is produced by the reduction of the net negative charge of the actin monomer (1, 13, 23, 24) which results in a change in molecular conformation (14, 37, 39). Since in our experiments, polymerization of G-actin occurs only with positively charged liposomes, it seems highly probable that the basic phenomenon is of the same nature. However, in our system, the positive charges are fixed on a surface and actin can only polymerize on this surface. This may have important consequences on the kinetics of polymerization: when actin is polymerized in a solution, the monomers are meeting in three dimensional space, but when actin polymerizes on a two dimensional surface, this surface influences the meeting of the monomers in three ways: Firstly, by electrostatic attraction, the membrane may concentrate the monomers on the surface. Secondly, the probability of the monomers meeting depends on the lateral motility of these monomers. Thirdly, the available surface for polymerization is a limiting factor. Monomers which cannot enter into contact with the membrane cannot polymerize.

The kinetics of actin polymerization has been the subject of extensive studies (3, 17, 32, 34, 40, 49). It will be interesting to discover the parameters of polymerization of actin in these unusual conditions.

Actin may polymerize into filaments or paracrystals. Polymerization and formation of paracrystals induced by divalent cations such as Mg^{++} depend on the concentration of these cations (23, 45, 46). At low concentrations, divalent cations saturate first the high affinity sites on the actin monomer which then polymerize into filaments, while at high cation concentrations they saturate the low affinity binding sites, favoring lateral interactions between actin filaments and consequently the formation of paracrystals (12, 13, 44). In our

Table I. Determination of Nucleotides Linked to Actin before and after Polymerization. Actin was Dissolved in G buffer and Polymerization was Induced by 0.1 M KCl + 2 mM $MgCl_2$ or by Liposome (1 μM of lipids/ml) Containing G buffer.

	Percentage of nucleotide linked to actin		
	G-actin	Actin polymerized by salts	Actin polymerized by liposomes
ATP	80	18	44
ADP	14.5	75	43
AMP	2	3	5
Adenosine	3.5	4	8

experiments, we obtained only paracrystals. Attempts to obtain single filaments by decreasing the charge density on the liposome were unsuccessful, and when the charge was decreased too far no attachment of actin to the liposome surface was observed. Two hypotheses may explain the fact that actin made only paracrystals.

(*a*) When a monomer of actin anchors to the membrane, stearylamine molecules bearing the positive charges can be attracted by the negative charges of the actin molecule and may move by lateral diffusion within the liposome membrane. Then, the concentration of positive charges would be always high around the actin molecule, generating only the paracrystalline form. (*b*) The binding sites involved for the paracrystal formation by liposomes may be completely unrelated than the binding sites for divalent cations.

The conformation of polymeric and monomeric actin have been found to be different (14, 19, 21, 37, 39). Judging by the changes of fluorescence spectra of the pyrene probe, G-actin polymerized by the positively charged liposomes is converted to the F conformation and is maintained in this conformation as long as the membrane is intact. If this membrane is destroyed, for example by detergents, actin depolymerizes and returns to the G conformation. Thus, the membrane of the liposome does not have a catalytic effect on the polymerization of actin but is included with the polymerized actin.

However, we found a difference between actin polymerized by salts and by liposomes. When actin is polymerized by salts, the bound nucleotide ATP is hydrolyzed to ADP, but ATP hydrolysis occurs some time after polymerization (4, 28, 29, 30, 36). Then, the growing filament has both ends ATP-capped (2, 5, 11, 30). In our polymerization system, hydrolysis of ATP is incomplete and occurs only in half of the population of monomers. This may be due either to a more rapid exchange with the G–ATP present in the medium or to a specific interaction of lipids with the monomers which limit ATP hydrolysis. Since actin polymerization in paracrystals implies multiple interactions between the monomers, it seems unlikely that actin monomers may be more easily exchangeable with the medium.

Since natural membranes are composed of negatively charged and neutral lipids, this polymerization of actin at the surface of membranes may occur rarely in vivo. However, this new system may offer a new opportunity to study the mechanism of actin polymerization in unusual physical conditions.

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