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Effects of solutes on the formation of crystalline sheets of the Ca^{2+}-ATPase in detergent-solubilized sarcoplasmic reticulum

Sandor Varga [1,*], Kenneth A. Taylor [2] and Anthony Martonosi [1]

[1] *Department of Biochemistry and Molecular Biology, State University of New York, Health Science Center at Syracuse, Syracuse, NY (U.S.A.) and* [2] *Department of Cell Biology, Duke University Medical Center, Durham, NC (U.S.A.)*

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The Ca^{2+}-ATPase crystals formed in detergent solubilized sarcoplasmic reticulum (SR) at 2 °C in a crystallization medium of 0.1 M KCl, 10 mM K-Mops (pH 6.0), 3 mM $MgCl_2$, 3 mM NaN_3, 5 mM DTT, 25 IU/ml Trasylol, 2 μg/ml 1,6-di-*tert*-butyl-*p*-cresol, 20% glycerol and 20 mM $CaCl_2$ (J. Biol. Chem. 263, 5277 and 5287 (1988)) contain highly ordered sheets of ATPase molecules, that associate into large multilamellar stacks (> 100 layers). When the crystallization is performed in the same medium but in the presence of 40% glycerol at low temperature the stacking is reduced to 4–5 layers and the average diameter of the crystalline sheets is increased from less than 1 μm to 2–3 μm. Glycerol and low temperature presumably reduce stacking by interfering with the interactions between the hydrophilic headgroups of Ca^{2+}-ATPase molecules in adjacent lamellae, while not affecting or promoting the ordering of ATPase molecules within the individual sheets. Electron diffraction patterns could be regularly obtained at 8 Å and occasionally at 7 Å resolution on crystals formed in 40% glycerol, either at 2 °C or at −70 °C. In the same media but in the absence of glycerol, polyethyleneglycol 1450, 3000 and 8000 (1–8%) induced the formation of ordered crystalline arrays containing 10–12 layers that were similar to those obtained in 40% glycerol. Replacement of 40% glycerol with 10–50% glucose or supplementation of the standard crystallization medium with polyethyleneglycol (PEG 3000 or 8000; 1, 2, 5 and 3%) had no beneficial effect on the order of crystalline arrays compared with media containing 40% glycerol.

Introduction

Three-dimensional microcrystals of Ca^{2+}-ATPase develop in sarcoplasmic reticulum membranes solubilized with $C_{12}E_8$ or Brij 36T at 2 °C in a solution of 0.1 M KCl, 10 mM K-Mops (pH 6.0), 3 mM $MgCl_2$, 20 mM $CaCl_2$, 20% glycerol, 3 mM NaN_3, 5 mM dithiothreitol, 25 IU/ml of Trasylol and 2 μg/ml 1,6-di-*tert*-butyl-*p*-cresol [1,2]. The microcrystals consist of stacked lamellar arrays of ATPase molecules with periodicities of ≅ 170 Å, 55.5 Å and 164.2 Å [3]. The presence of a stain-excluding region in the center of the lamellae suggests that their core contains a continuous lipid-detergent phase into which the hydrophobic tail portions of the Ca^{2+}-ATPase molecules are symmetrically inserted from both sides. The surfaces of the lamellae are covered by the exposed cytoplasmic domains of the Ca^{2+}-ATPase. Interactions between specific ATPase molecules *within* the lamellae gives rise to the highly ordered two-dimensional arrays seen in projected images of the crystalline sheets. The two-sided plane group C12 derived from the diffraction patterns is consistent with ATPase dimers as structural units, that are related by a 2-fold rotation axis within the membrane plane in unit cells containing four ATPase molecules [3].

The association of the crystalline sheets into three-dimensional stacks involves interactions between the exposed headgroups of ATPase molecules in adjacent lamellae. The high Ca^{2+} concentration (20 mM) and

* On leave from the Central Research Laboratory, University Medical School, Debrecen, H-4012 Hungary.

Abbreviations were used: Mops, 3-(*N*-morpholino)propanesulfonic acid; SR, sarcoplasmic reticulum; $C_{12}E_8$, octaethylene glycol dodecyl ether; DTT, dithiothreitol; PEG, polyethylene glycol.

Correspondence: A. Martonosi, Department of Biochemistry, State University of New York, Health Science Center at Syracuse, Syracuse, NY 13210, U.S.A.

the low pH (pH 6.0) required for crystallization are assumed to promote these interactions. Since the length of the ATPase molecules in the native sarcoplasmic reticulum is ≅ 100 Å [4–6], the interlamellar distance of ≅ 170 Å implies limited interdigitation of the headgroups of ATPase molecules projecting from the apposed lamellae. These observations were confirmed and extended recently by Stokes and Green [7,8].

Ca^{2+}-ATPase molecules within the 2-D sheets appear to be very highly ordered based upon electron diffraction of frozen hydrated crystals [8], but highly ordered packing of the lamellar stacks is not so clearly indicated. In our earlier work with crystals preserved in negative stain [3], moire fringes in the crystals suggested the presence of a slight rotational disorder in the stacking of crystalline lamellae. This type of lattice defect, known as a coincident lattice defect, has been found in multilamellar crystals of ribosomes [9], bacteriorhodopsin [10], and the light harvesting chlorophyll *a/b* protein complex [11].

For electron crystallographic analysis, large single layered 2-D crystalline sheets are ideal for reasons of image resolution, regularity of crystal thickness, and because they require the least amount of image and diffraction data to determine the structure. By contrast, X-ray diffraction analysis requires highly ordered, stable 3-D cyrstals that are greater than ≅ 50 μm in diameter.

The diameter of the Ca^{2+}-ATPase microcrystals in our earlier studies [1–3] was only 2000–10000 Å, based on electron microscopy of fixed and sectioned material, and they contained as many as 50–100 superimposed layers. Therefore these crystals [1–3] were too small and probably too fragile for X-ray diffraction analysis, and too large for electron microscopy. Stokes and Green [7,8] slightly increased the size of the crystals and obtained X-ray powder diffraction patterns to 4.1 Å resolution.

The purpose of our studies was to improve the crystallization technique developed by Pikuła et al. [2], aimed at the production of large crystalline sheets of the Ca^{2+}-ATPase that consist of a singly layer or of a few layers with sufficient order in all three dimensions to permit the reconstruction of the ATPase structure by electron microscopy. The observations presented in this report indicate that the stacking is drastically reduced by increasing the glycerol concentration of the standard crystallization medium from 20 to 40% and by lowering the temperature during the initial phase of crystallization to −70 °C. Under these conditions crystalline disks of 5–10 μm diameter containing 4–5 layers can be regularly obtained. The molecular packing in these crystals can be related to the arrangement of ATPase molcules in the E_1 type crystals induced in native sarcoplasmic reticulum by calcium or by lanthanides [1,12].

Experimental procedures

Materials

Glycerol was supplied by Aldrich Chemical Company, Inc., Milwaukee. WI 53233; uranyl acetae by Alfa Products, Thiokol/Ventron Divsion, Danvers, MA 01923; $CaCl_2$ by J.T. Baker Chemical Co., Phillipsburg, NJ 08865; propylene oxide and Araldite by Ernest F. Fullam, Inc., Latham, NY 12110; Trasyloi by Mobay Chemical Corporation, FBA Pharmaceuticals, New York, NY 10022; KCl and NaN_3 by Fisher Scientific Company, Fair Lawn, NJ 07410; glutaraldehyde by Polysciences, Inc., Washington, PA 18976; and Brij 36T, Mops, $MgCl_2$, DTT, PEG and 1,6-di-*tert*-butyl-*p*-cresol by Sigma Chemical Company, St. Louis, MO 63178.

Methods

Sarcoplasmic reticulum was isolated from rabbit skeletal muscle as described earlier [13]. The preparations were rapidly frozen in liquid nitrogen and stored before use in a medium of 0.3 M sucrose, 10 mM Tris-maleate buffer (pH 7.0) at a protein concentration of 30–40 mg/ml at −70 °C. The enzymatic activity and the protein composition were assayed as described by Varga et al. [14].

For crystallization, the solubilized sarcoplasmic reticulum (2 mg protein/ml) was incubated in a crystallization medium of 0.1 M KCl, 10 mM K-Mops (pH 6.0), 3 mM $MgCl_2$, 3 mM NaN_3, 5 mM dithiothreitol, 25 IU/ml Trasylol, 2 μg/ml, 1,6-di-*tert*-butyl-*p*-cresol, 20 mM $CaCl_2$, 20–50% glycerol or glucose, and 8 mg/ml Brij 36T, representing a detergent/protein ratio of 4. The composition of the crystallization medium is similar to that reported earlier [1–3], except for the changes in the concentration of glycerol, and for the occasional use of glucose instead of glycerol.

Immediately after solubilization at 2 °C the samples were divided into small aliquots, sealed under nitrogen, and incubated at temperatures of 2 °C, −19 °C, −70 °C or −196 °C for times ranging from a few hours to 15 weeks.

The primary purpose of the low temperature treatment was to slow the initial phase of crystallization and to reduce the number of nucleation sites. After this preliminary low temperature phase, the samples were transferred to 2 °C for times ranging between a few minutes to several weeks and the progress of crystallization was monitored by electron microscopy of negatively stained or sectioned material. For some samples the temperature was maintained at 2 °C both during the initial and the final phase of crystallization.

For negative staining 1% uranyl acetate (pH 4.3) was used [15]. For thin sectioning the crystalline suspensions were fixed with 1% glutaraldehyde for 24 h at 2 °C, postfixed in 1% OsO_4 at room temperature for 1

h. After dehydration in a graded ethanol series, the samples were embedded in Araldite and sectioned. The specimens were stained with uranyl acetate and lead citrate and viewed in a Siemens Elmiskop I electron microscope at an accelerating voltage of 60 kV at instrumental magnifications ranging between 4000 and 59400.

Crystals were prepared for electron diffraction by first reducing the glycerol concentration by dilution or by dialysis. We were able to obtain diffraction patterns to 8 Å resolution by freezing either in 20% or in 2% glycerol; the fraction of good diffraction patterns from untilted crystals was somewhat greater when the glycerol concentration was reduced to low levels. Specimens for electron diffraction were prepared using the back blotting method of Toyoshima [16]. Diffraction patterns were recorded as described in Ref. 8.

Results and Discussion

The formation of multilamellar stacks of Ca^{2+}-ATPase crystals at 2 °C, in the presence of 20% glycerol

The three-dimensional Ca^{2+}-ATPase crystals produced by the standard crystallization procedure of Pikuła et al. [2] consist of large stacks of two-dimensional crystalline sheets that can be seen in edge-on views either by negative staining (Fig. 1A) or by thin sectioning (Fig. 1C). The distance of separation between lamellae varies between 103 and 147 Å in sectioned specimens, between 130 and 170 Å in negatively stained material, and between 170 and 180 Å in frozen-hydrated specimens [3]. The smaller spacings obtained in sectioned material are presumably due to shrinkage of the specimens during dehydration, embedding and sectioning. The stain-excluding core of the layers seen in the side views of the stacks represents the lipid-detergent phase of the membrane, and the periodic projections that bridge the stain-filled gap are the hydrophilic domains of the Ca^{2+}-ATPase molecules. The 160–170 Å thickness of the layers is consistent with the dimensions of the ATPase molecule in the native membrane [5,6], if the cytoplasmic domains of ATPase molecules project by ≅ 60 Å out of both sides of a lipid-detergent phase of ≅ 40 Å thickness.

Occasionally the crystalline sheets are deposited flat on the support film; the projected image obtained with the electron beam normal to the plane of the lamellae shows ordered arrays of stain-excluding particles that represent the cytoplasmic domain of the Ca^{2+}-ATPase (Fig. 1B). The computed diffraction patterns obtained from stacks of frozen-hydrated two-dimensional sheets gave unit cell dimensions of 164.4 × 55.5 Å, with an included angle of 90°, consistent with a centered lattice in the two-sided plane group of C12 [3,7,8]. The diameter of the crystalline particles was usually 1 μm or less. Superimposition of the layers gave rise to superlattice periodicities and moire fringes. The type of image shown in Fig. 1B was less frequent, compared with side views of the stacks (Figs. 1A and C), suggesting that the conditions of these experiments favored the formation of long, cylindrical, multilayered aggregates, at the expense of in-plane lateral crystal growth.

The effect of glycerol concentration on the crystallization of Ca^{2+}-ATPase

By increasing the concentration of glycerol in the crystallization medium from 20 to 40% at 2 °C, the stacking was dramatically suppressed and large flat sheets of 2–3 μm average diameter were regularly obtained (Fig. 2). The edge-on views of negatively stained crystalline stacks that represented the dominant crystal form in the presence of 20% glycerol (Fig. 1A), were absent at 40% glycerol concentration. We assume that the increase in glycerol concentration reduced the number of nucleation sites and promoted the lateral growth of crystals within the lamellae. As a result, fewer but larger crystalline sheets developed, and the chance for the formation of multilayered stacks was reduced.

The frequency of large stacks containing 10 or more layers was also reduced by raising the glycerol concentration to 40% in crystals formed at 20% glycerol concentration. These observations suggest that glycerol also promotes the dissociation of preformed stacks by weakening the interlamellar interaction between ATPase molecules.

The effect of subzero temperature on the crystallization of Ca^{2+}-ATPase in media containing 40% glycerol

Lowering the temperature of the crystallization mixture to −70 °C during the initial phase of crystallization (1–10 weeks), followed by continued incubation at 2 °C for 5–10 weeks, further reduced stacking and increased the diameter of the crystalline sheets to 3–10 μm. In addition to the flat two-dimensional arrays, the preincubation at −70 °C induced the formation of crystalline tubules of ≅ 2000 Å diameter and 6000 Å length that appeared intermixed with the crystalline sheets of the Ca^{2+}-ATPase (Fig. 3). The walls of the tubules are composed of 2–3 membrane layers of the same thickness (≅ 170 Å) as seen earlier in the multilamellar stacks produced by incubation at 20% glycerol concentration at 2 °C (Fig. 1A). The tubules are frequently seen adhering to the crystalline sheets and they are sufficiently stable to retain their tubular shapes after negative staining. Single-walled tubules were not seen, perhaps because they are not sufficiently stable to withstand negative staining. Tubules with walls containing more than three layers did not form, probably because multilayered sheets are too rigid to be bent readily into a cylindrical shape.

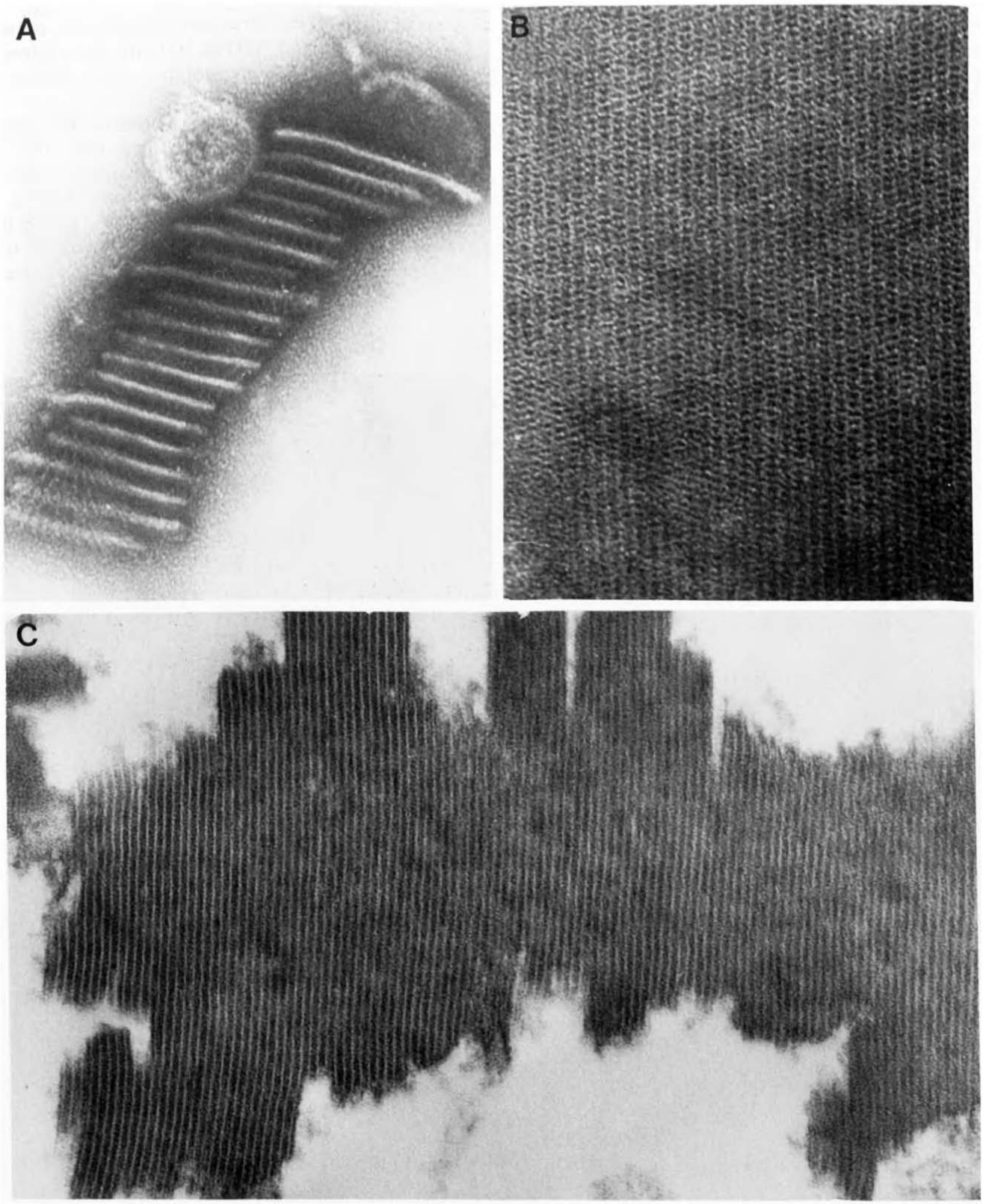


Fig. 1. Crystallization of Ca^{2+}-ATPase at 2 °C in a medium containing 20% glycerol. The crystals were formed in the standard crystallization medium containing 0.1 M KCl, 10 mM K-Mops (pH 6.0), 3 mM $MgCl_2$, 20 mM $CaCl_2$, 20% glycerol, 3 mM NaN_3, 5 mM dithiothreitol, 25 IU/μl Trasylol, 2 μg/ml 1,6-di-*tert*-butyl-*p*-cresol, 2 mg/ml sarcoplasmic reticulum protein and 4 mg Brij 36-T/mg protein, at 2 °C under nitrogen atmosphere. Incubation time was about 15 days. (A) Edge-on view of a crystalline stack of Ca^{2+}-ATPase negatively stained with 1% uranyl acetate. Magnification: 312000×. (B) Projection view of a crystalline stack negatively stained with 1% uranyl acetate. Magnification: 308000×. (C) Thin cross section of a crystalline stack after fixation with glutaraldehyde and embedding. Magnification: 150000×.

The crystalline tubules may represent either an alternative crystalform or could be due to asymmetric insertion of ATPase molecules, lipids or detergents into the layers, causing them to assume a cylindrical curvature. The prior exposure of the solutions to −70 °C was essential for the appearance of the tubules, and they were not seen under similar conditions in lipid-detergent systems containing extracted sarcoplasmic reticulum lipids without Ca^{2+}-ATPase.

Thin sections of glutaraldehyde-fixed Ca^{2+}-ATPase crystals

The combined effect of exposure to −70 °C and 40% glycerol on the stacking characteristics of the Ca^{2+}-ATPase is best illustrated by comparing thin sections of Ca^{2+}-ATPase crystals obtained in the presence of 20% glycerol at 2 °C (Fig. 1C) with those formed in the presence of 40% glycerol at −70 °C and +2 °C (Fig. 4).

The stacks formed in the presence of 20% glycerol usually contain more than 50 layers (Fig. 1C). The stacking in the presence of 40% glycerol is limited to about 6–10 layers in crystals grown at 2 °C (Fig. 4C) with an average layer number of 9.3 (Fig. 5A, filled circles); in crystals grown at −70 °C followed by transfer to 2 °C (Figs. 4A and B) the average number of

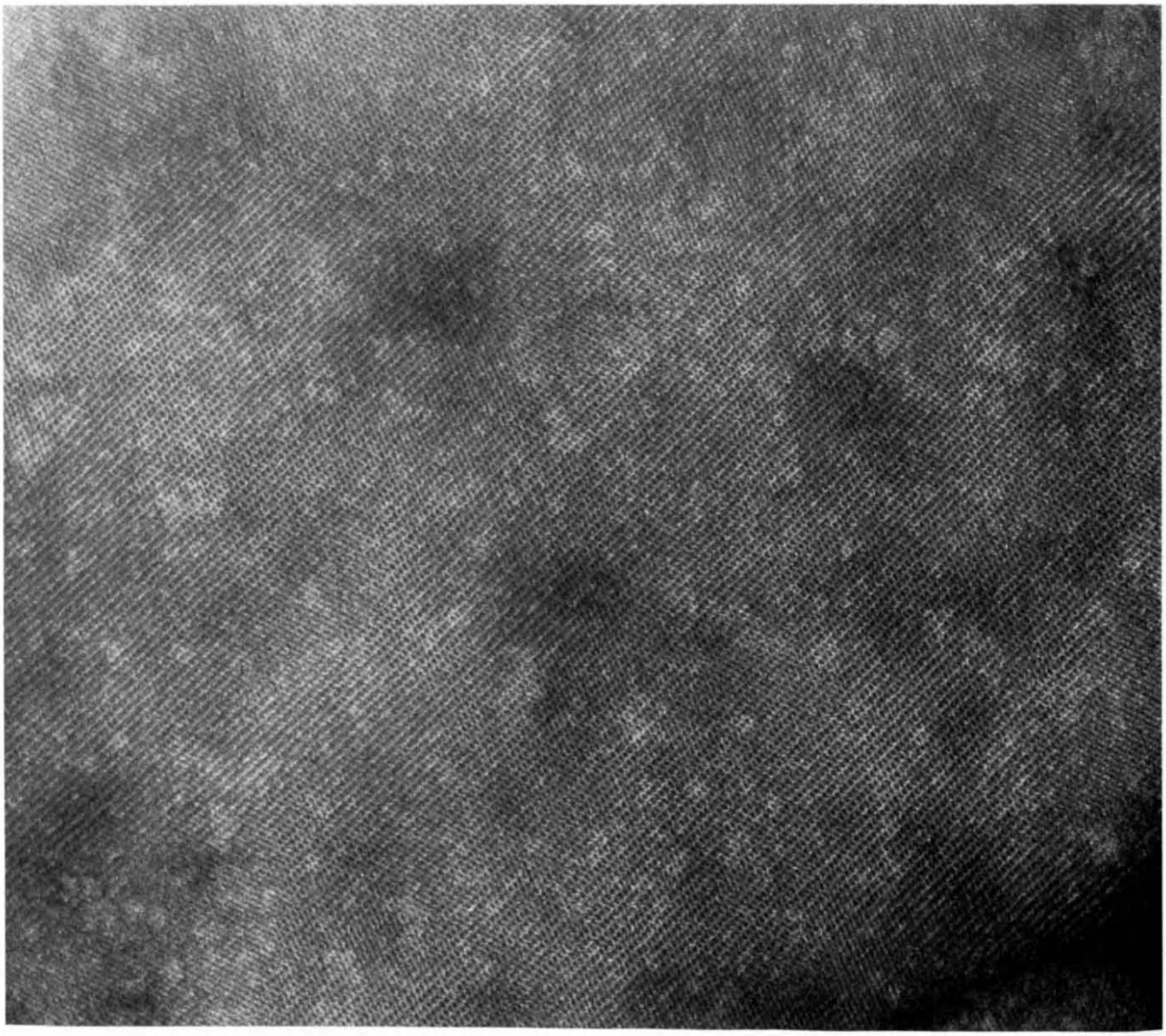


Fig. 2. Crystallization of Ca^{2+}-ATPase at 2 °C in media containing 40% glycerol. Projection view of a crystalline sheet of Ca^{2+}-ATPase formed at 2 °C in the standard crystallization medium containing 40% glycerol. Incubation time was 10 days, followed by negative staining with 1% uranyl acetate. The average area of the sheets regularly exceeded 1 μm^2. Magnification: 161 250×.

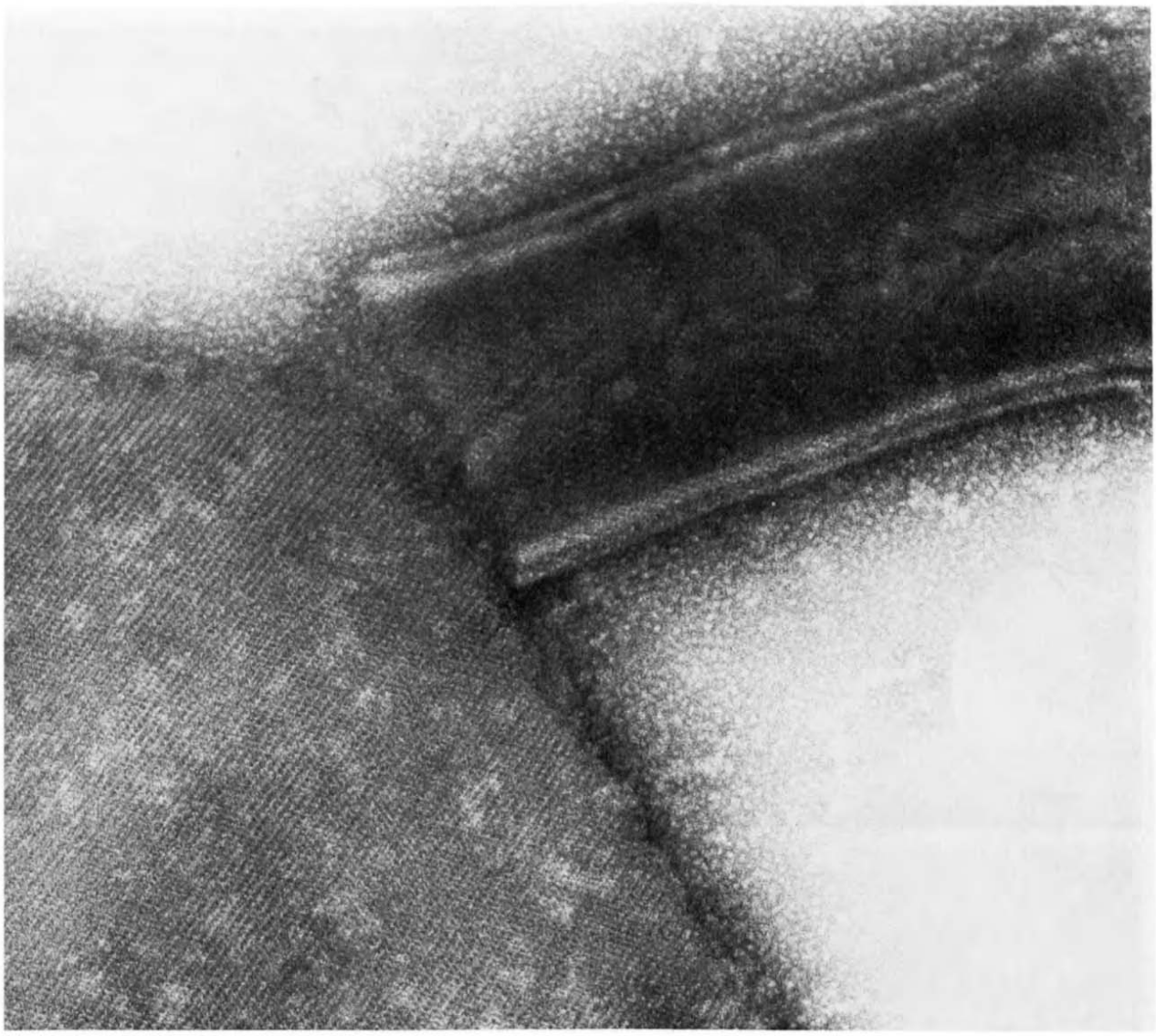


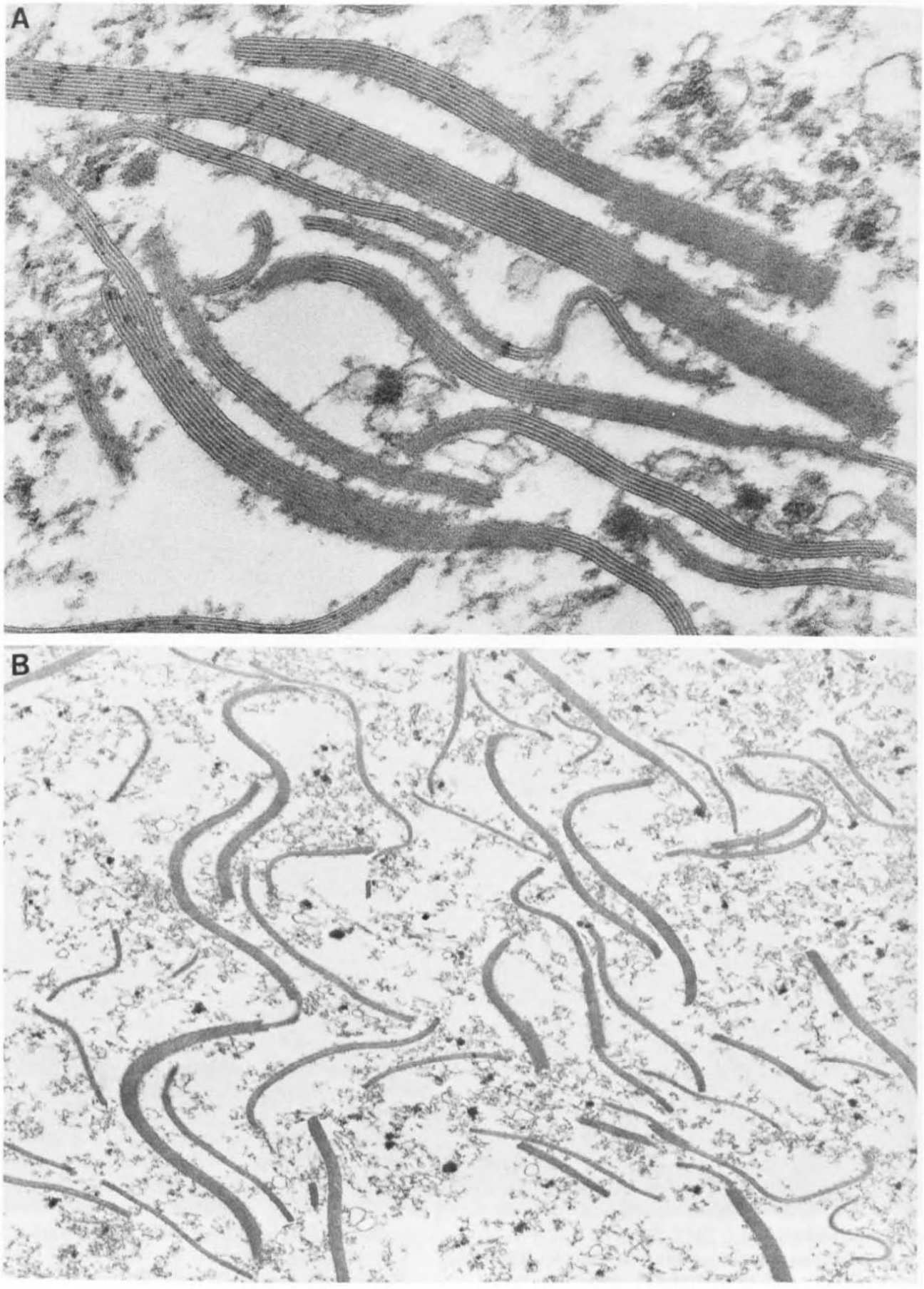
Fig. 3. Crystallization of Ca^{2+}-ATPase in a medium containing 40% glycerol (−70 °C and 2 °C). The crystals were formed in the standard crystallization medium containing 40% glycerol. Immediately after solubilization the samples were cooled to −70 °C for 1 week, followed by incubation at 2 °C for 2 weeks. Negative staining with 1% uranyl acetate. Large flat sheets with an average diameter in excess of 3 μm represent the dominant crystal form. The preincubation at −70 °C also induced the formation of a large number of crystalline tubules of ≅ 1700–2600 Å diameter and 5000–7000 Å length. Magnification: 161 250×.

sheets per ribbon was 5.4 (Fig. 5A, open circles), and the distribution was less heterogeneous. Single sheets were rare (Fig. 4) perhaps due to their mechanical instability under the conditions of fixation and embedding; the preservation of single sheets in frozen-hydrated specimens may be better.

The length of the ribbon-like structures (Fig. 5B) is representative of the average diameter of the flat sheets, seen by negative staining (Fig. 6). The average length of the ribbons was 1.67 μm in specimens crystallized at 2 °C (Fig. 5B, filled circles) and 2.89 μm in specimens incubated at −70 °C followed by transfer to 2 °C (Fig. 5B, open circles); there was a greater heterogeneity of the length distribution in the latter group.

The average diameter of the crystalline sheets evaluated from the low magnification images of negatively stained material (Fig. 6) is consistent with the measurements made on fixed and sectioned material (Fig. 4A, B, C). These dimensions provide sufficient surface area for three-dimensional reconstruction of the Ca^{2+}-ATPase.

Although the low-speed centrifugation used to collect the crystals into a tight pellet is likely to produce some bending and folding of the sheets, a surprisingly large number of the sheets produced reasonably straight cross sections over fairly large distances (Figs. 4A and C), suggesting that the insertion of the Ca^{2+}-ATPase molecules on the two sides of the lipid-deter-


A
B

gent layer is symmetrical and it is not significantly distorted by defects (if any) in the interaction between the sheets.

Electron diffraction patterns of Ca^{2+}-ATPase crystals formed in 40% glycerol

The crystals formed in standard crystallization medium could not be used directly for electron diffraction studies in the frozen-hydrated state due to the high (40%) glycerol concentration. Therefore, after crystallization at 2 °C in 40% glycerol for 2 weeks the crystals were diluted with glycerol-free crystallization medium to 20, 13.3, 10, 5, and 2.5% final glycerol concentration and tested by negative staining electron microscopy 0.5, 1, 2, 3, 4, and 24 h later. There was no significant effect of dilution either on the frequency or on the visible order of crystalline arrays. The same crystals were also subjected to dialysis against glycerol-free crystallization medium without loss in crystallinity.

Electron diffraction patterns could be regularly obtained at 8 Å and occasionally at 7 Å resolutions (Fig. 7) on crystals formed in 40% glycerol either at 2 °C or

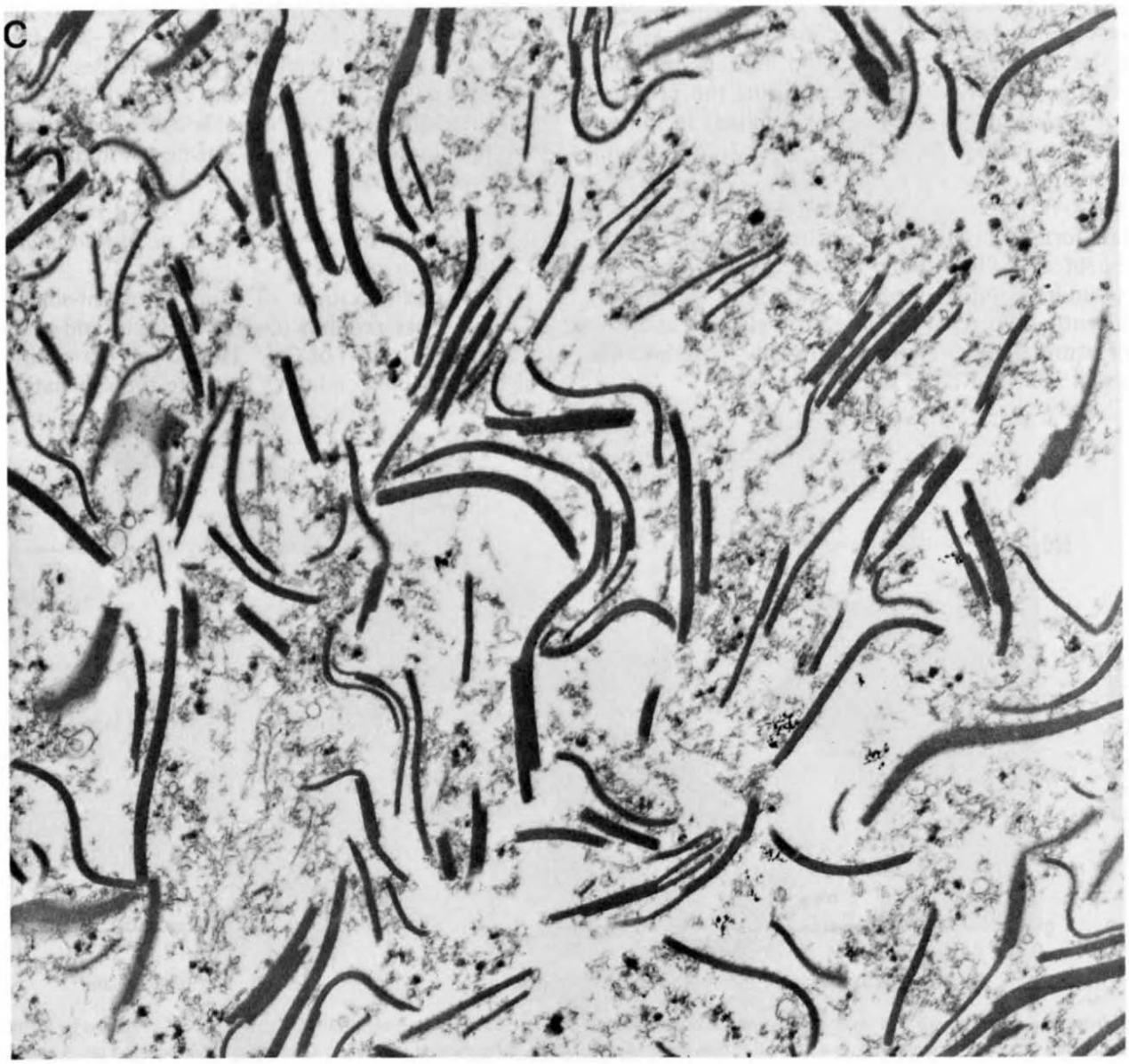


Fig. 4. Thin-sections of Ca^{2+}-ATPase crystals formed in crystallization media containing 40% glycerol. After incubation in a standard crystallization medium containing 40% glycerol for 6 weeks at −70 °C and 9 weeks at 2 °C (A), 9 weeks at −70 °C and 6 weeks at 2 °C (B) and for 15 weeks at 2 °C (C) the crystals were fixed with glutaraldehyde, embedded and sectioned as described under Methods. Magnifications: A = 66750×; B = 12930×; C = 12930×.

at −70 °C. To obtain these diffraction patterns it was necessary to reduce the glycerol concentration from 40% to at least 20% in order to make thin specimens. Comparing the best diffraction patterns obtained from untilted crystals in 20% glycerol (Fig. 7A) with those from crystals in 2% glycerol, the removal of glycerol either by dialysis or by dilution had no significant effect on the crystalline order. However, in specimens tilted to 30° or higher, glycerol concentration had a more significant effect. In 20% glycerol reasonably symmetric diffraction patterns could be obtained (Figs. 7C and D), whereas in 2% glycerol the resolution perpendicular to the tilt axis was significantly less. Two effects contribute to the resolution perpendicular to the tilt axis, specimen flatness and registration between the layers, neither of which can be easily distinguished. However, higher glycerol concentrations probably reduce the rate of water evaporation, thereby reducing any change in solvent conditions during the preparation of frozen specimens. Rapid alterations in solvent conditions may exert forces on the crystals that disrupt the regularity of the stacking.

The 7–8 Å resolution regularly achieved on the crystals formed in 40% glycerol indicates that a high degree of crystalline order is maintained within the sheets under conditions where stacking of sheets was significantly reduced. Therefore 40% glycerol and low temperature appear to weaken primarily those interactions that are involved in stack formation.

The mechanism of the effect of glycerol and temperature on the crystallization of Ca^{2+}-ATPase

The effect of glycerol and temperature on the different types of interactions leading to the formation of the crystalline sheets and their stacking probably involves several mechanisms. The immobilization of the Ca^{2+}-ATPase in the viscous environment created by the high glycerol concentration at low temperatures [17,18] probably reduces the number of nucleation sites, permitting the formation of a smaller number but larger crystalline sheets. The binding of glycerol to the protein surface [19,20] may interfere with the ATPase–ATPase interaction involved in the adhesion of the lamellae, inhibiting stack formation and causing their dissociation. Increased structuring of the water by sugars strengthens hydrophobic interactions in the nonpolar regions of the membrane [21–23], favoring the association between ATPase molecules within the bilayer and promoting in-plane growth of the crystals. All these effects are presumably enhanced by lowering the temperature to −70 °C during the initial phase of the crystallization.

The effect of cations and anions on the formation of Ca^{2+}-ATPase crystals

The crystallization of the detergent-solubilized Ca^{2+}-ATPase requires low pH (pH 6.0) and relatively high concentration of Ca^{2+} (10–20 mM) in addition to glycerol. These conditions promote the association be-

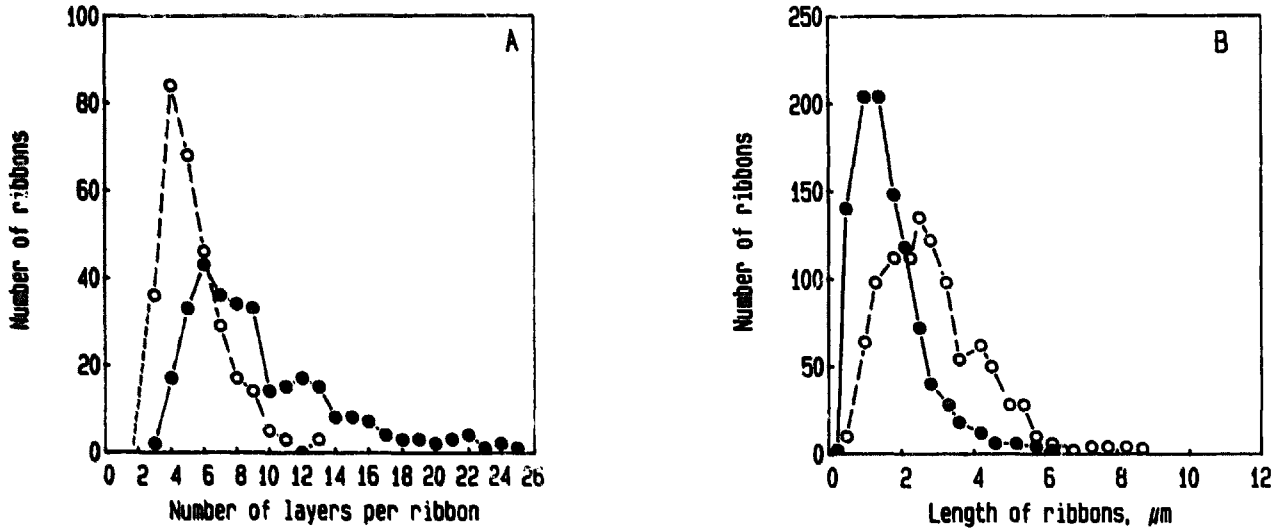


Fig. 5. Histogram of the length and layer number of the crystalline ribbons seen in sectioned material. The crystals were prepared by incubation of sarcoplasmic reticulum membranes in a crystallization medium containing 40% glycerol under the conditions described in Figs. 4 and 6. The number of layers per ribbon (A) is related to the number of superimposed sheets within crystalline stacks. The length of the ribbons seen in sectioned material (B) is related to the diameter of the sheets seen after negative staining (Fig. 6). Symbols: •, preparations incubated at 2 °C for 15 weeks; ○, preparation incubated at −70 °C for 6–9 weeks followed by incubation at 2 °C for a total of 15 weeks. A total of 2242 crystalline arrays were counted. In samples incubated at 2 °C 30% of all crystalline arrays had greater than 2 μm diameter; the corresponding fraction of large arrays after exposure to −70 °C was 71.2%.

tween ATPase molecules [2,24]. Decrease in Ca^{2+} concentration to 0.2 mM or increase in pH to pH 7–8 prevented crystallization, although the high-affinity Ca^{2+} binding sites of the Ca^{2+}-ATPase still remained saturated by Ca^{2+} under these conditions. The simplest explanation is that cation binding to low-affinity anionic sites on the Ca^{2+}-ATPase promotes interactions between ATPase molecules, by neutralizing negative charge repulsions.

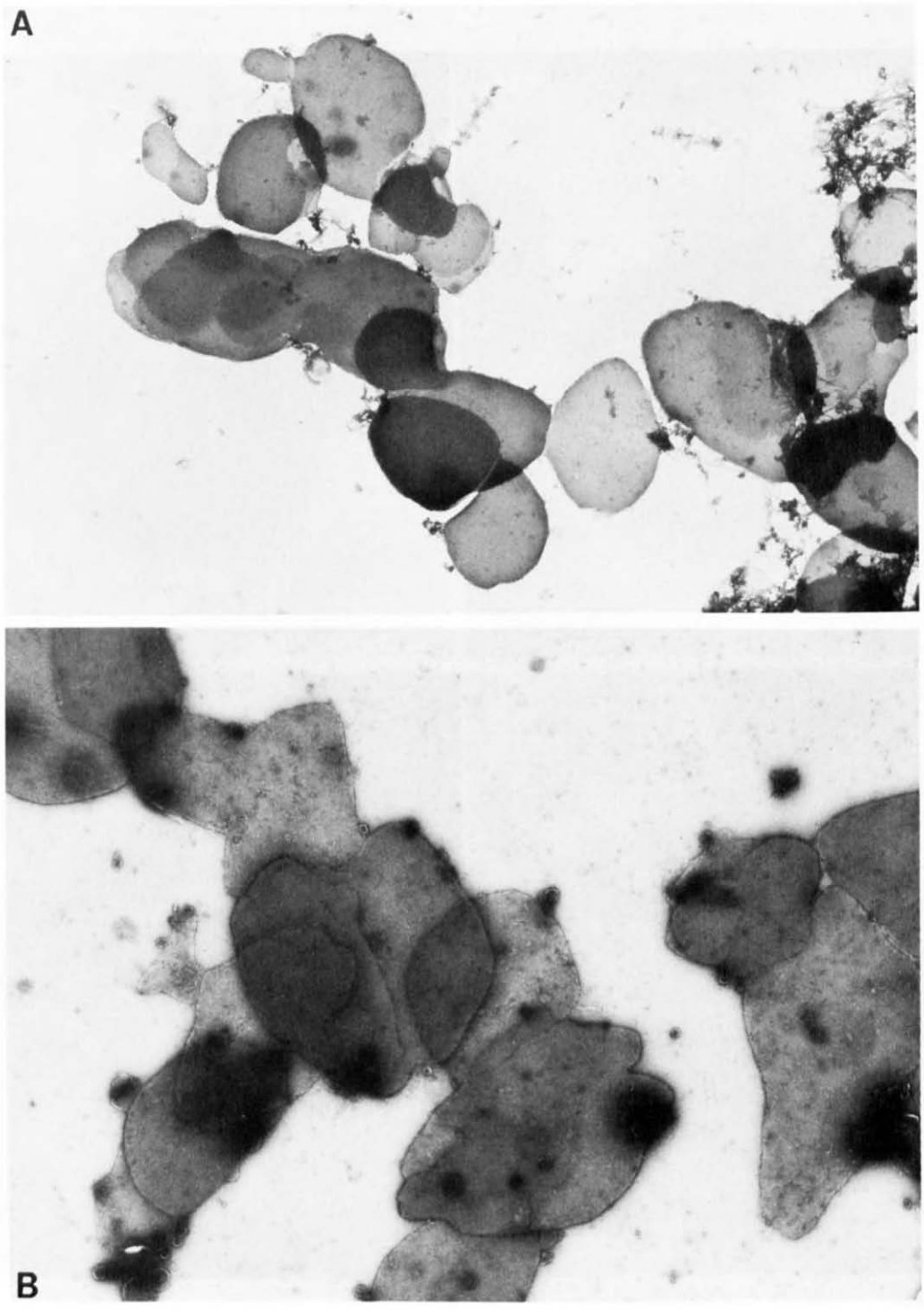


Fig. 6. Representative low magnification images of negatively stained crystalline disks formed in 40% glycerol. The solubilized sarcoplasmic reticulum was incubated in a crystallization medium containing 40% glycerol for 6 weeks at 2 °C (A) or for 1 week at −70 °C and an additional 4 weeks at 2 °C (B). Negative staining with 1% uranyl acetate. Magnification: 8400×. The average diameter of the disks is 2.38 μm in A and 4.27 μm in B.

The effect of polyethylene glycol on crystallization

The usefulness of polyethylene glycol in the crystallization of proteins [25–27] and some recent observations that polyethylene glycol improves the stability of Ca^{2+}-ATPase in detergent solutions [28] prompted us to investigate the effect of polyethylene glycol on the stacking characteristics of the Ca^{2+}-ATPase crystals.

Crystallization of Ca^{2+}-ATPase was induced by polyethylene glycol 1450, 3000 or 8000 at concentrations ranging between 1 and 8% in standard crystalliza-

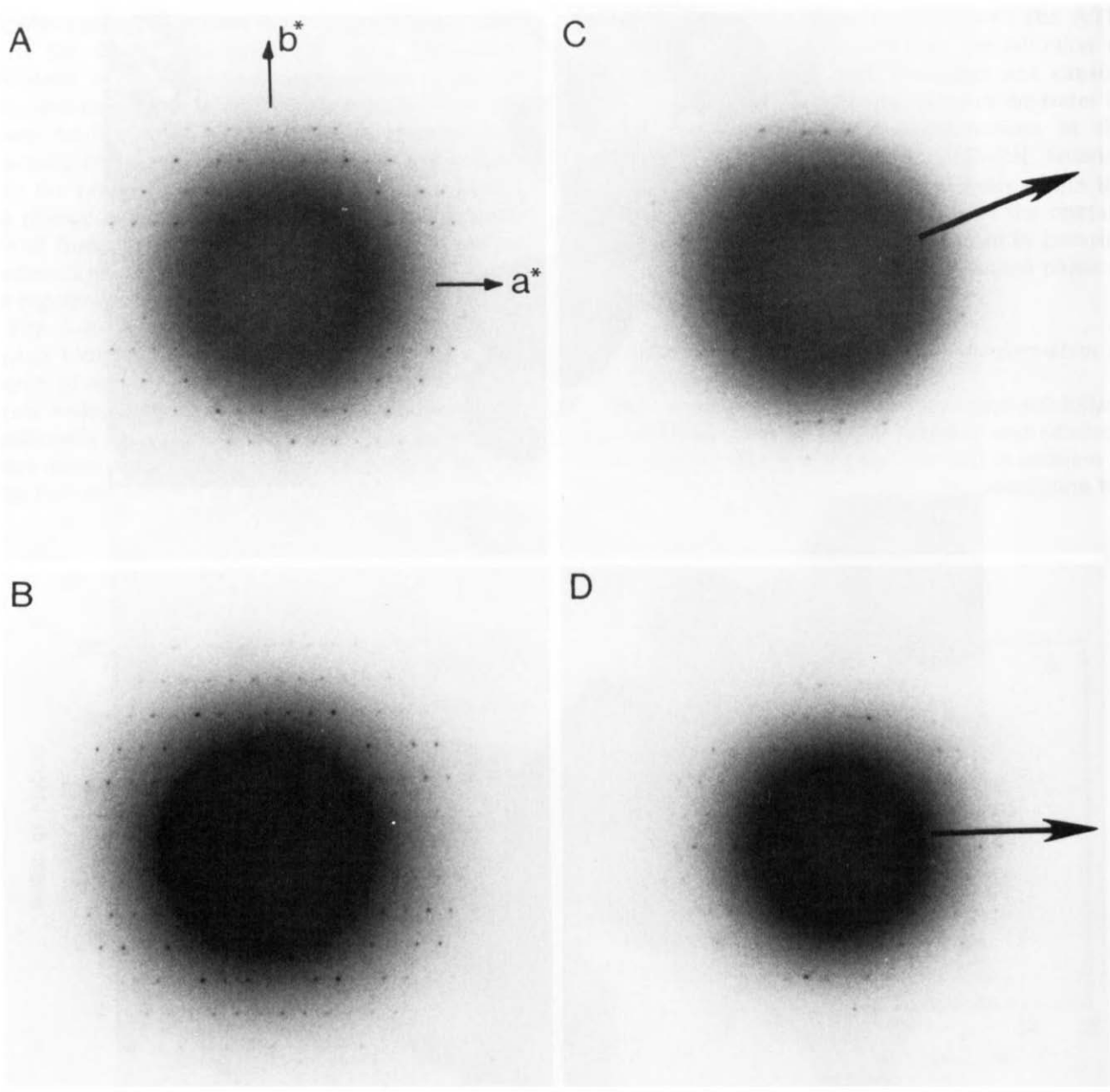


Fig. 7. Electron diffraction patterns of Ca^{2+}-ATPase crystals formed in the presence of 40% glycerol under crystallization conditions similar to those reported by Pikuła et al. [2]. The specimens were preserved for electron diffraction in the frozen-hydrated state using the back blotting method of Toyoshima [16]. (A) Nominally untilted crystal frozen after dilution in crystallization medium containing 20% glycerol. (B) Another untilted crystal after overnight dialysis against a crystallization medium containing 2% glycerol. (C) and (D) Crystals tilted nominally to 30° and frozen in crystallization media containing 20% glycerol. Arrows indicate the approximate direction of the tilt axis.

tion medium containing 20 mM $CaCl_2$ but no glycerol (Fig. 8). The regularity of the crystalline arrays increased with PEG concentration up to about 2–5%, producing stacks that appear rectangular in cross-section with a thickness of 10–12 layers and an average length of 0.3 μm. The crystals induced by polyethylene glycol 3000 and 8000 in the absence of glycerol are similar in stacking characteristics to the crystals obtained in 40% glycerol, but the diameter of the crystalline sheets is smaller, as reflected by the shorter length of the ribbons seen in cross-sections (Figs. 8C and D).

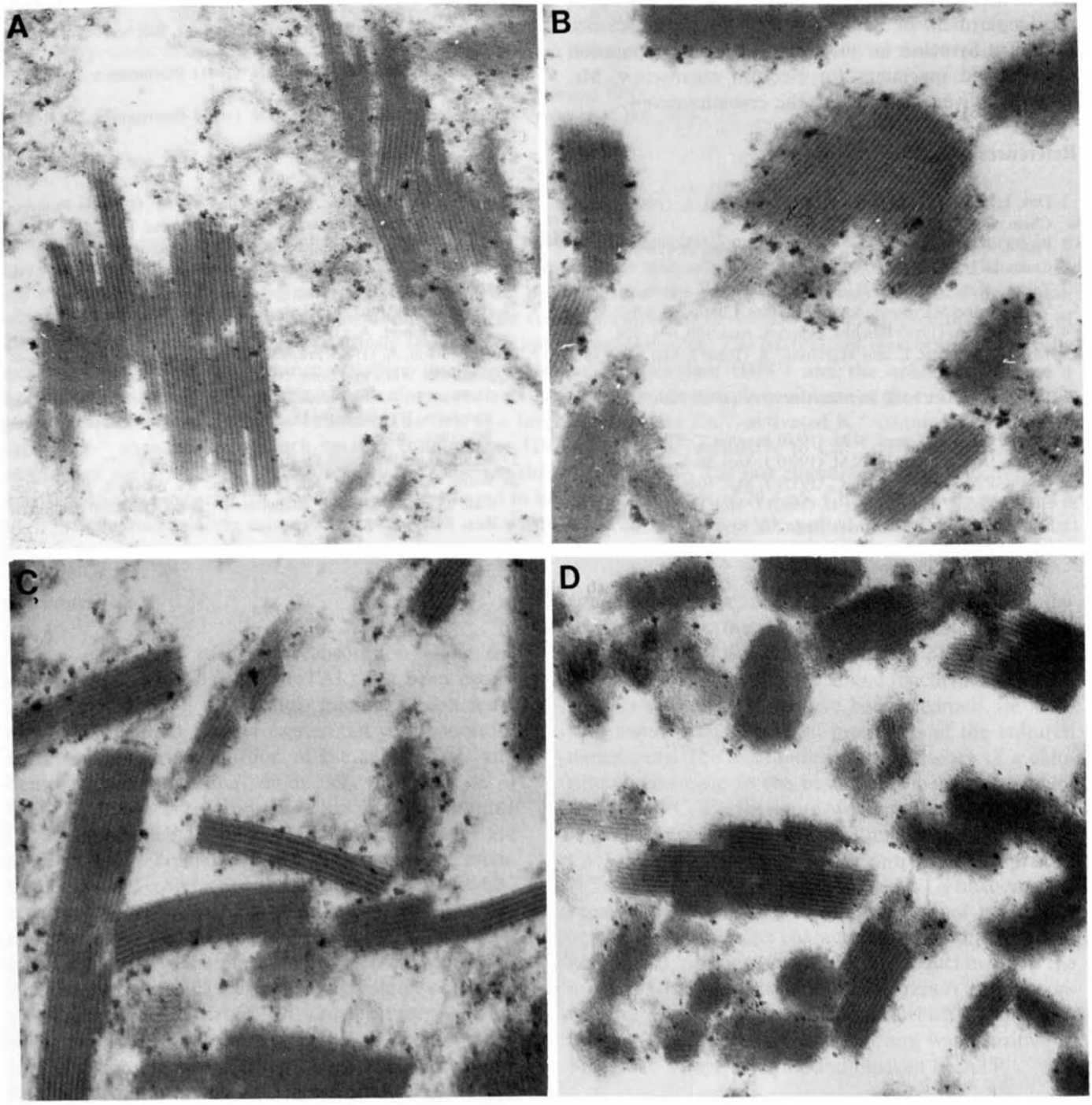


Fig. 8. Crystallization of Ca^{2+}-ATPase by polyethylene glycol in glycerol-free media containing 20 mM $CaCl_2$. The crystals were formed in a medium of 0.1 M KCl, 10 mM K-Mops (pH 6.0), 3 mM $MgCl_2$, 5 mM DTT, 3 mM NaN_3, 2 μg/ml 1,6-di-*tert*-butyl-*p*-cresol, 25 IU/ml Trasylol, 2 mg/ml sarcoplasmic reticulum protein, 8 mg/ml Brij 36T, and 20 mM $CaCl_2$, at 2 °C with the following additions: (A) 20% glycerol; (B) polyethylene glycol 1450, 2%; (C) polyethylene glycol 3000, 0.5%; (D) polyethylene glycol 8000, 1.5%. After incubation under nitrogen for 2 weeks the samples were fixed with 1% glutaraldehyde, embedded and sectioned as described under Methods.

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