# Isolation and Purification of Gap Junction Channels

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*Abstract.* This paper reports methods we have developed to solubilize gap junction channels, or connexons, from isolated gap junctions and to purify them in milligram quantities. Two sources of material are used: rat liver gap junctions and gap junctions produced by infecting insect cells with a baculovirus containing the cDNA for human liver $\beta_1$ protein (connexin 32). Complete solubilization is obtained with long chain detergents (lauryl dimethyl amineoxide, dodecyl maltoside) and requires high ionic strength and high pH as well as reducing conditions. The purification involves chromatography on hydroxylapatite and gel filtration on Superose 6. A homogeneous product is indicated by a single band on a silver-stained gel and a homogeneous population of doughnut-shaped particles under the electron microscope. These particles have hexameric symmetry. The purified connexons have a tendency to form aggregates: filaments and sheets. The filaments grow by end-to-end association of connexons and are nonpolar, suggesting that the connexons are paired as in the cell-to-cell channel. The sheets grow by lateral association of the filaments.

G AP junctions are regions of plasma membrane that contain arrays of channels connecting the cytoplasms of adjacent cells. They are found in places where two cells approach each other closely, leaving a gap of ~30 Å between the two plasma membranes. This gap is bridged by the channels. They are water-filled pores with a minimum diameter of ~15 Å and have little or no chemical selectivity. Thus they allow for the passage of ions and small molecules to equilibrate the chemical milieu of neighboring cells and to rapidly propagate chemical and electrical signals. (For recent reviews see Guthrie and Gilula, 1989; Bennett et al., 1991; Kumar, 1991).

Gap junction plaques, composed of two membrane layers, have been isolated from several different tissues (Henderson et al., 1979; Hertzberg, 1984; Manjunath and Page, 1986; Kistler et al., 1985) and examined by EM. The channels in isolated junctional membranes are most easily visualized by negative staining and appear as doughnut-shaped particles ~80 Å in diameter. The stain at the center of the particles delineates the pore, and three-dimensional image analysis shows that it accumulates mostly at the extracellular end of the structure (Unwin and Zampighi, 1980).

Structural and biochemical studies suggest that individual channels are made up of a single species of polypeptide arranged as a hexamer around a central pore (Makowski et al., 1977; Unwin and Zampighi, 1980). These hexamers are called connexons. Two oppositely facing connexons, in register, form a complete water-filled pathway linking neighboring cells.

Various polypeptides have been proposed to form subunits of the connexon (Hertzberg and Gilula, 1979; Finbow et al.,

1983; Nicholson et al., 1987; Gruijters et al., 1987). Most of them appear to belong to a family of membrane proteins that have been called connexins. The nucleotide and derived amino acid sequences of a number of connexins have become available in the past few years (Paul, 1986; Kumar and Gilula, 1986; Beyer et al., 1987; Gimlich et al., 1988; Beyer et al., 1988; Nicholson and Zhang, 1989; Ebihara et al., 1989; Gimlich et al., 1990). Features of these amino acid sequences support the notion that connexins are pore-forming membrane proteins: they are predicted to contain four transmembrane $\alpha$-helices, one of which is amphiphilic and could line the wall of an aqueous pore (Milks et al., 1988). However, solubilization of gap junctions and the handling of connexons in solution has been difficult because of their low abundance and their insolubility properties. Thus detailed biochemical characterization of this channel has not so far been possible.

To facilitate further work on the gap junction channels, we investigate here conditions needed for their solubilization and purification. As a source of material for these studies, we make use of gap junction-like plaques composed of the human liver $\beta_1$ polypeptide (connexin 32; nomenclature of connexins according to Risek et al., 1990) overproduced in a baculovirus/insect cell expression system, as well as gap junctions (composed of $\beta_1$ (connexin 32) and $\beta_2$ (connexin 26) polypeptides) isolated from rat liver. By application of appropriate detergent and salt conditions, we obtain completely solubilized connexons from the isolated plaques. Subsequent chromatographic purification yields homogeneous populations of this molecule in milligram quantities. Our studies of purified connexons confirm that they are com-
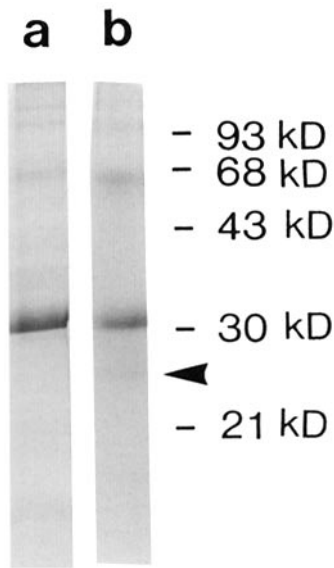
*Figure 1.* SDS-PAGE of gap junction plaques isolated from (*a*) Sf9 cells infected with baculovirus containing the cDNA for the human $\beta_1$ polypeptide, and (*b*) rat liver. Both preparations were obtained by extraction of membranes with sodium hydroxide followed by floating of plaques on a 42%/30% sucrose step gradient. The gel was 15% acrylamide. Protein was visualized with Coomassie blue. The strong bands in either gel correspond to the $\beta_1$ polypeptide. The weaker bands migrating above 60 kD correspond to oligomers of $\beta_1$ polypeptide. The arrow in *b* points to a weak band running at about 26 kD, which corresponds to the $\beta_2$ polypeptide. The relative intensity of this band varied from one preparation to another.

posed of six subunits and provide information on their aggregation behavior. The purified connexons are stable in solution over a period of months.

## Materials and Methods

### Materials

Female Sprague-Dawley rats were obtained from Holtzman Lab (Madison, WI). Powdered Grace's insect tissue culture medium was purchased from JR Scientific (Woodland, CA). TC Yeastolate and Lactalbumin Hydrolysate were from Difco Laboratories (Detroit, MI) and Gentamycin and Amphotericin B from Sigma Chemical Co. (St. Louis, MO). Dodecyl maltoside (DoDM),[1] decyl maltoside, and nonyl glucoside were purchased from Sigma Chemical Co., octyl glucoside from Bachem (Bubendorf, Switzerland), lauryl dimethyl amineoxide (LDAO) from Fluka (Buchs, Switzerland), decyl dimethyl amineoxide from Oxyl (Bobingen, Germany). $C_{12}E_9$ and $C_{12}E_8$ from Calbiochem (La Jolla, CA), and the MEGA compounds from Boehringer Mannheim Biochemicals (Mannheim, Germany). Octyl polyoxyethylene was a gracious gift of Jurg P. Rosenbusch. All other chemicals were of the highest purity available.

### Construction and Isolation of Recombinant Baculovirus

Construction of baculovirus vector containing the human $\beta_1$ cDNA will be described in a separate report. Briefly, the human $\beta_1$ cDNA (Kumar and Gilula, 1986) was inserted into the vector pAC373 (Summers and Smith, 1987) using standard molecular biological techniques. This placed the $\beta_1$ cDNA under control of the baculovirus polyhedrin promoter. The $\beta_1$ cDNA integration into the baculovirus genome was accomplished by cotransfection of the transfer plasmid and wild-type baculovirus DNA into Sf9 cells using a calcium phosphate transfection scheme (Summers and Smith, 1987). Recombinant virus containing the $\beta_1$ cDNA and displaying the characteristic occlusion–negative plaque morphology were plaque purified three times.

### Tissue Culture

Culture of Sf9 cells followed the instructions given by Summers and Smith

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1. *Abbreviations used in this paper*: DoDM, dodecyl maltoside; LDAO, lauryl dimethyl amineoxide.

(1987). The cells were grown in suspension cultures in spinner flasks of up to 1 liter volume. The medium was TNM-FH supplemented with 10% FCS, 2.5 $\mu$g/ml Amphotericin B, and 50 $\mu$g/ml Gentamycin. Batches of 1 liter cells were infected at densities of ~2 million/ml with recombinant virus at multiplicities of infection of at least 10. Cells were harvested typically 65 h postinfection. Batches of 500 ml cells were harvested after 48 h. Harvesting of cells was done by centrifugation at 1,000 $g$ for 10 min. Pelleted cells were washed once in 150 mM NaCl, 1 mM PMSF, 5 mM MES, pH 6.2, and quickly frozen in liquid nitrogen. They were stored at −20°C, generally for several days, before subsequent steps were carried out.

### Isolation of Gap Junctions from Rat Liver

Preparation of plasma membranes and isolation of gap junctions by alkaline extraction (Hertzberg, 1984) were carried out following the procedures in Zimmer et al. (1987) using the livers of 80 female retired breeder rats per batch.

### Isolation of Gap Junctions from Insect Cells

Frozen cells were used to prepare the isolated junctions and all operations were carried out at 4°C. The cells were thawed by suspension in bicarbonate buffer (1 mM sodium bicarbonate, pH 8, 1 mM PMSF). The suspension was made 20 mM in NaOH and sonicated for ~15 s in a Kontes sonicator operated at 5 W using a 3-mm tip. The broken cells were incubated on ice for 50–60 min and pelleted at 35,000 $g$ for 30 min. Pellets were resuspended in bicarbonate buffer, and sucrose was added to a final concentration of 42% (wt/wt). This suspension was overlayered with 30% (wt/wt) sucrose and with bicarbonate buffer, and then centrifuged at 100,000 $g$ for 100 min in a swing-out rotor. Bands at both the 42%/30% and the 30%/bicarbonate interfaces were collected as well as any material in the 30% sucrose bulk. These samples were pooled, diluted with bicarbonate buffer, and pelleted at 35,000 $g$ for 30 min. Pellets were taken up in small amounts of bicarbonate buffer and analyzed for protein composition by SDS-PAGE according to Laemmli (1970) using 15% acrylamide gels and total protein content according to Lowry (1951). Small aliquots were adsorbed onto freshly glow-discharged electron microscope grids coated with collodion and carbon. Samples were left to adsorb for ~1 min and then the grids were briefly washed in 100 mM sodium cacodylate, pH 6.8, and negatively stained with 5% uranyl acetate.

### Solubilization of Connexons

Membranes were pelleted and resuspended in 2 M NaCl, 10 mM EDTA, 100 mM DTT, 1 mM PMSF, 5% DoDM, 100 mM glycine-NaOH, pH 10, at a protein concentration of not more than 2 mg/ml. The solubilized material was briefly sonicated in a bath sonicator and left in the cold for ~2 h before being chromatographed. Samples of 1–2 $\mu$l were used for negative stain EM.

### Purification of Connexons

Solubilized connexons were diluted to ~15 vol with 10 mM DTT, 1 mM PMSF, 0.2% DoDM, 100 mM sodium phosphate, pH 6.8, and the pH was adjusted to 6.8. The sample was applied to a freshly packed column of hydroxylapatite (Calbiochem) equilibrated in the same buffer. After applications of the sample, the column was thoroughly washed and then the phosphate concentration was increased to 700 mM to elute the $\beta_1$ polypeptide. Fractions were collected and analyzed by SDS-PAGE. $\beta_1$ polypeptide-containing fractions were pooled and concentrated in a Centriprep30 microconcentrator (Amicon, Danvers, MA). By repeated concentration and redilution, the sample was equilibrated to 10 mM DTT, 1 mM PMSF, 5 mM Hepes, pH 7.5. This buffer was used as low ionic strength buffer for the subsequent chromatography step on DEAE-Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ). The high ionic strength elution buffer was 1.5 M NaCl, 10 mM EDTA, 10 mM DTT, 0.2% DoDM, 50 mM Hepes, pH 7.5. Eluted protein was collected and analyzed by SDS-PAGE, and then fractions containing $\beta_1$ polypeptide were pooled and concentrated as before. Finally, the concentrated protein was injected into a Superose 6 FPLC-column (Pharmacia Fine Chemicals) and eluted with 500 mM NaCl, 5 mM EDTA, 10 mM DTT, 0.2% DoDM, 50 mM Hepes, pH 8.0. Fractions containing pure $\beta_1$ polypeptide were pooled and concentrated in a Centricon30 microconcentrator. At the end of this procedure, the protein was equilibrated to 200 mM NaCl, 10 mM EGTA, 10 mM $MgCl_2$, 10 mM DTT, 50 mM Hepes, pH 8.0. Since the concentration device used concentrated the detergent along with the protein, no detergent was added to this
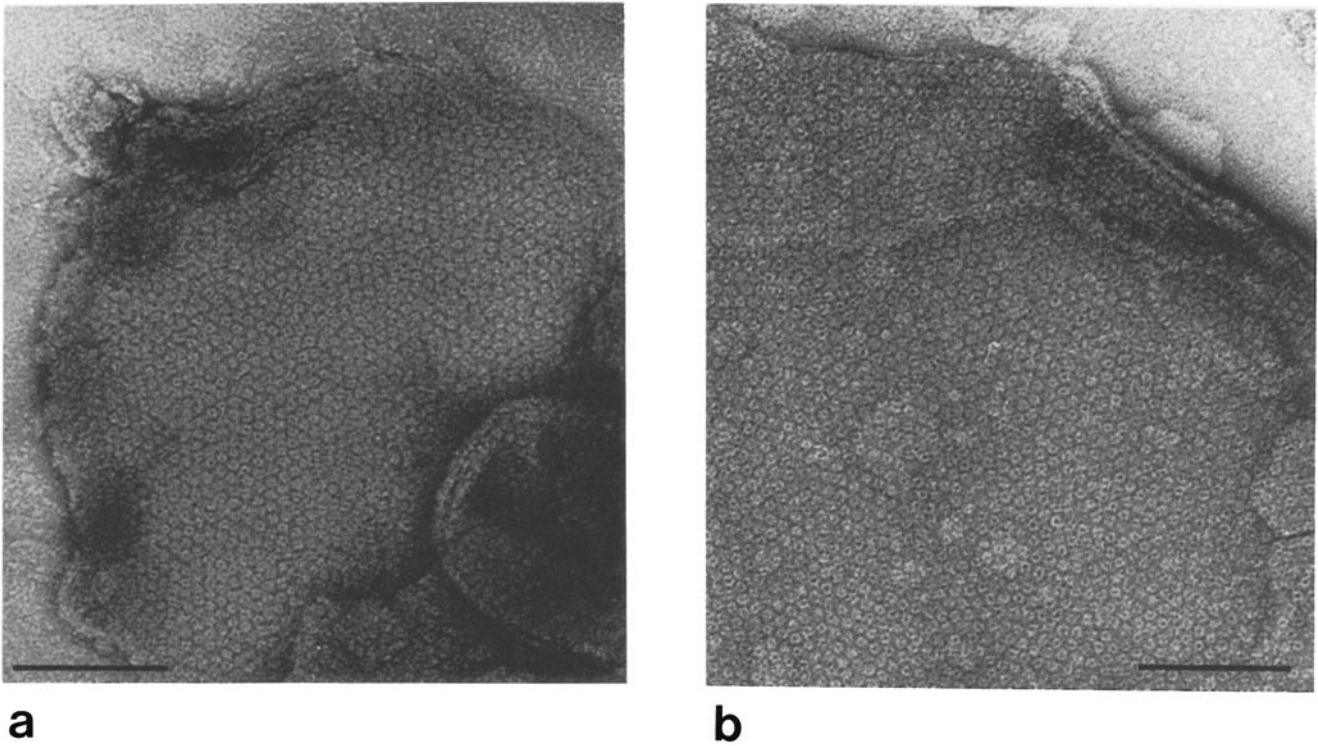
*Figure 2.* Gap junction plaques negatively stained with uranyl acetate. (*a*) Plaque isolated from baculovirus-infected Sf9 cells. (*b*) Plaque isolated from rat liver. Comparison of the two preparations shows no visible differences. As with rat liver gap junctions, the recombinant ones are composed of densely packed connexons of ~80-Å diam. At the edges it can be clearly seen that the plaques consist of two membrane layers. Bars, 0.1 μm.
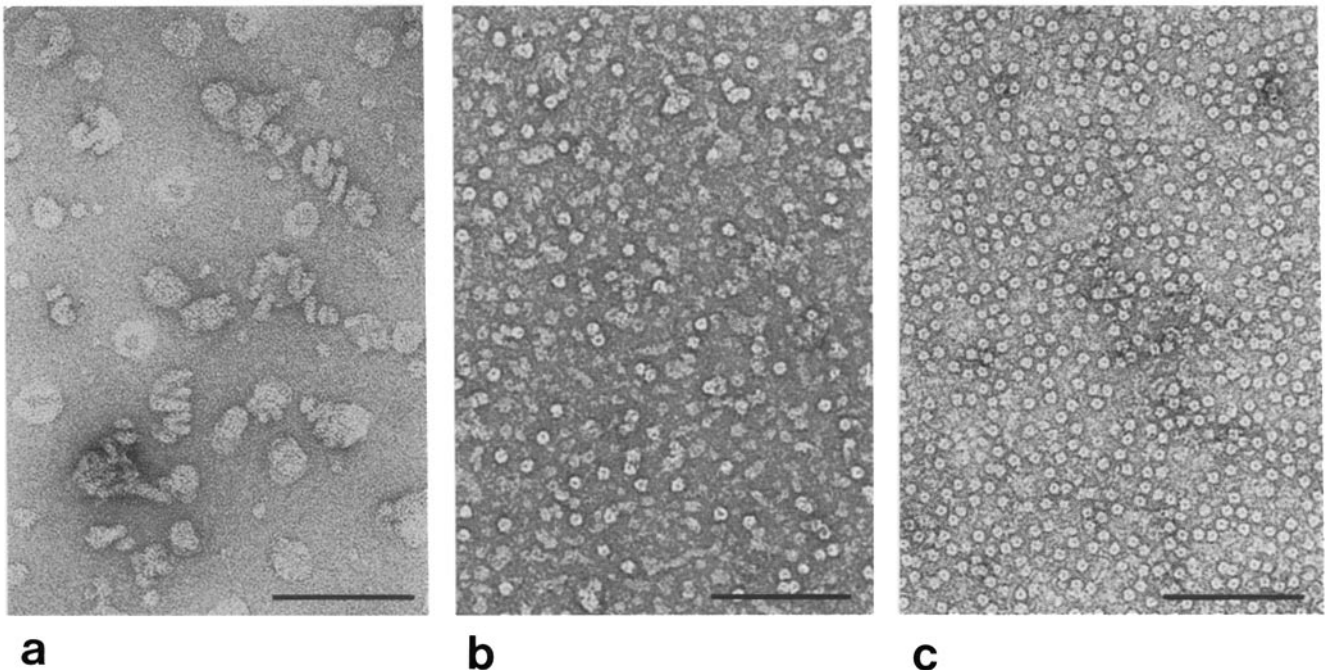


*Figure 3.* Solubilized connexons in negative stain. (*a*) Rat liver gap junction membranes were pelleted and the pellet was dissolved in 2 M NaCl, 10 mM EDTA, 100 mM DTT, 2% octyl glucoside, 1 mM PMSF, 100 mM Na-glycine, pH 10 by sonication in a bath sonicator. The connexons are partly solubilized into molecular aggregates of various sizes. (*b*) Rat liver gap junction membranes were treated in the same way as in *a* but using 2% dodecyl maltoside instead of 2% octyl glucoside. The more efficient detergent led to the appearance of doughnut-shaped connexons with a consistent diameter of ~80 Å. (*c*) Recombinant connexons after chromatographic purification by the procedure outlined in Materials and Methods. The preparation is noticeably more homogeneous than that prior to purification. Bars, 0.1 μm.
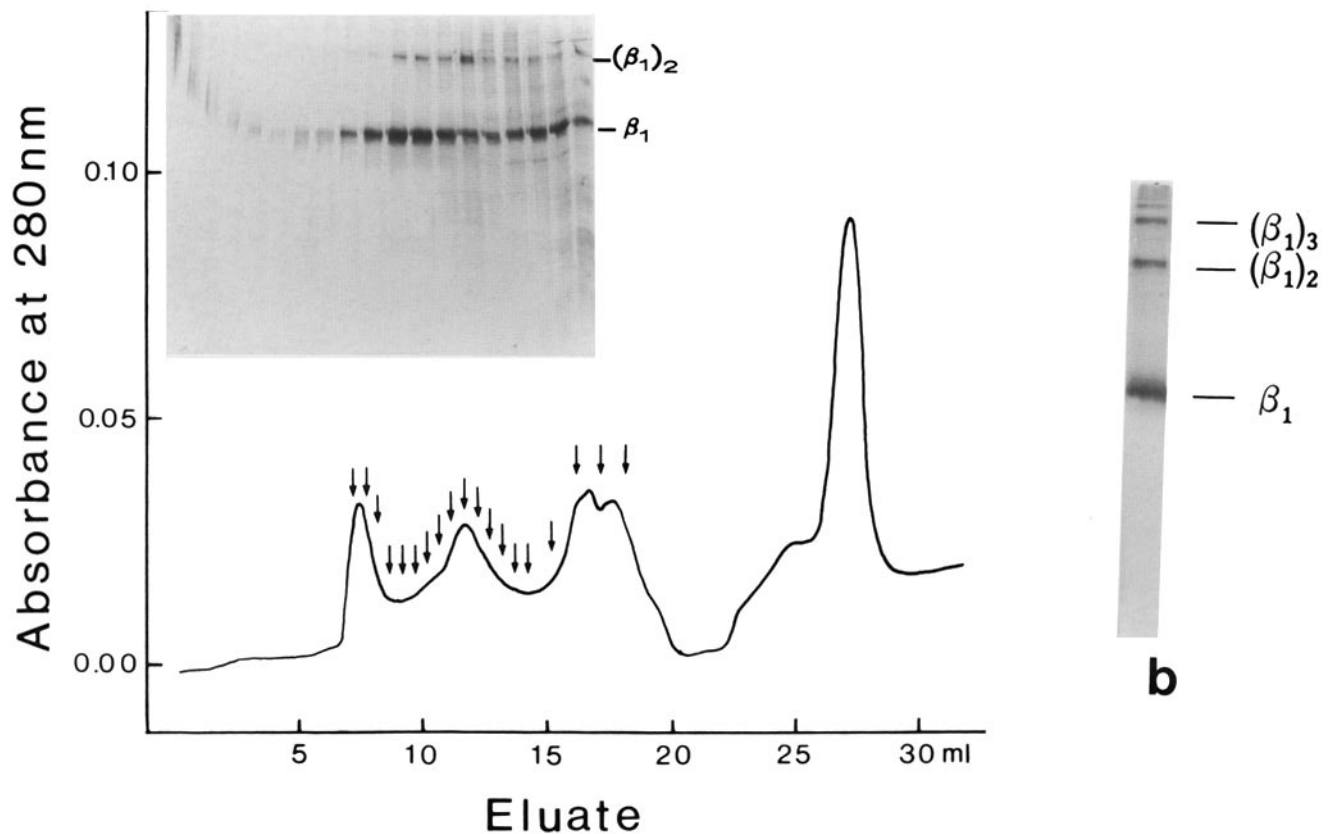
*Figure 4.* (*a*) Elution diagram of recombinant connexon sample chromatographed on a Superose 6 (*HR 10/30*) column and (*inset*) SDS-PAGE of selected fractions. 0.5 ml of sample was injected. The column was developed at a flow rate of 0.5 ml/min with 500 mM NaCl, 5 mM EDTA, 10 mM DTT, 0.2% dodecyl maltoside, 50 mM Hepes, pH 8.0. Fractions of 0.5 ml each were collected. The material in the second peak was pure connexons. Residual lipid, but no protein, was contained in the strong peak at the far right. (*Inset*) SDS-PAGE of this column. Aliquots of fractions indicated by arrows were applied to a 15% acrylamide gel. Protein was visualized by silver stain. (*b*) SDS-PAGE of a sample of connexons after purification by the procedure described in Materials and Methods (fractions between 10 and 15 ml in *a*). No bands other than $\beta_1$ and its oligomers $(\beta_1)_2$ and $(\beta_1)_3$ were detected. Protein was visualized by silver stain.

buffer. A final concentration step was then performed in a Centricon 100 microconcentrator, resulting in detergent concentrations in the order of 1%.

### Lipid Analysis

Samples of 20–50 $\mu$l protein were extracted with 5–10 vol of chloroform. Aliquots of the organic phase were applied to TLC plates (Silica gel 60; Merck Chemical, Rahway, NJ) and developed in chloroform:methanol:water 20/6.7/1 (vol/vol/vol). Lipids were visualized with iodine vapor. Phosphorus compounds were stained with molybdenum blue.

### Electron Microscopy

Grids were either negatively stained with 5% uranyl acetate or frozen by rapid immersion in liquid ethane. Specimens were examined in a microscope (model 420; Philips Electronic Instruments, Inc., Mahwah, NJ) equipped with a low-dose kit, and a cryo-holder (Mark II; Gatan Inc., Warrendale, PA) for observing the frozen specimens. Images were recorded on film (SO163; Eastman Kodak Co., Rochester, NY). Rotational power spectra were computed from digitized images of connexons following the procedures of Crowther and Amos (1971).

## Results

### Preparation of Isolated Plaques

We used an alkaline treatment to solubilize the non-gap junc-

tional membranes and isolate the gap junction plaques. This procedure was originally developed for the extraction of gap junctions from rodent livers, and was found to be equally applicable to the recombinant plaques from insect cells. Thus we obtained membranes that contained ~90% pure $\beta_1$ polypeptide as judged by SDS-PAGE (Fig. 1 *a*) in quantities of typically 2–3 mg and occasionally up to 10 mg protein per liter of cell culture. The gel of a preparation of rat liver gap junctions is shown in Fig. 1 *b* for comparison. The main difference was the presence of the 26-kD $\beta_2$ polypeptide (see Nicholson et al., 1987) in the rat liver sample; the samples were otherwise of similar purity.

Fig. 2 compares the negative stain appearance of isolated gap junctions, prepared from the two sources. There were no discernible differences in size and shape of connexons. In both cases plaques could be found that contained partly ordered hexagonal arrays of connexons.

### Solubilization of Connexons

The treatment of gap junction plaques to obtain individual connexons required the combined application of high salt, high pH, reducing agent, and detergent. DoDM and LDAO
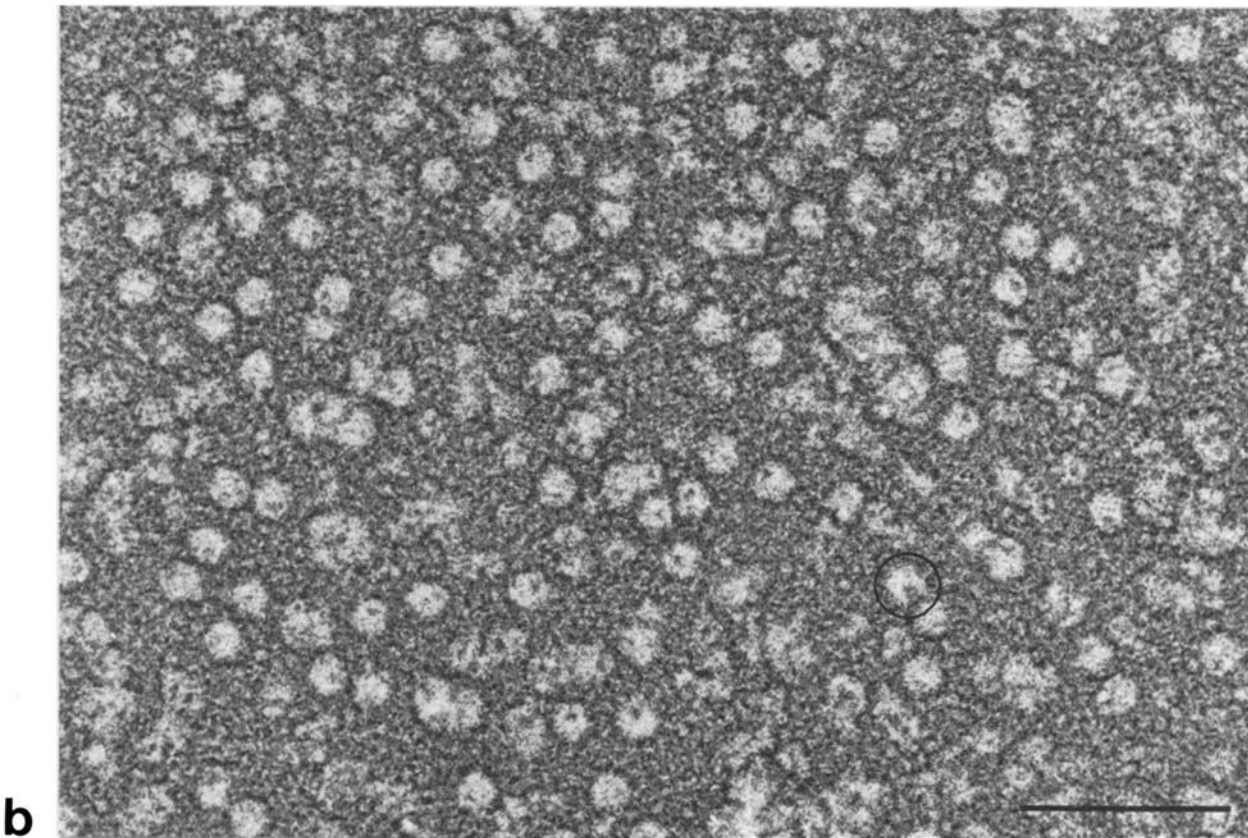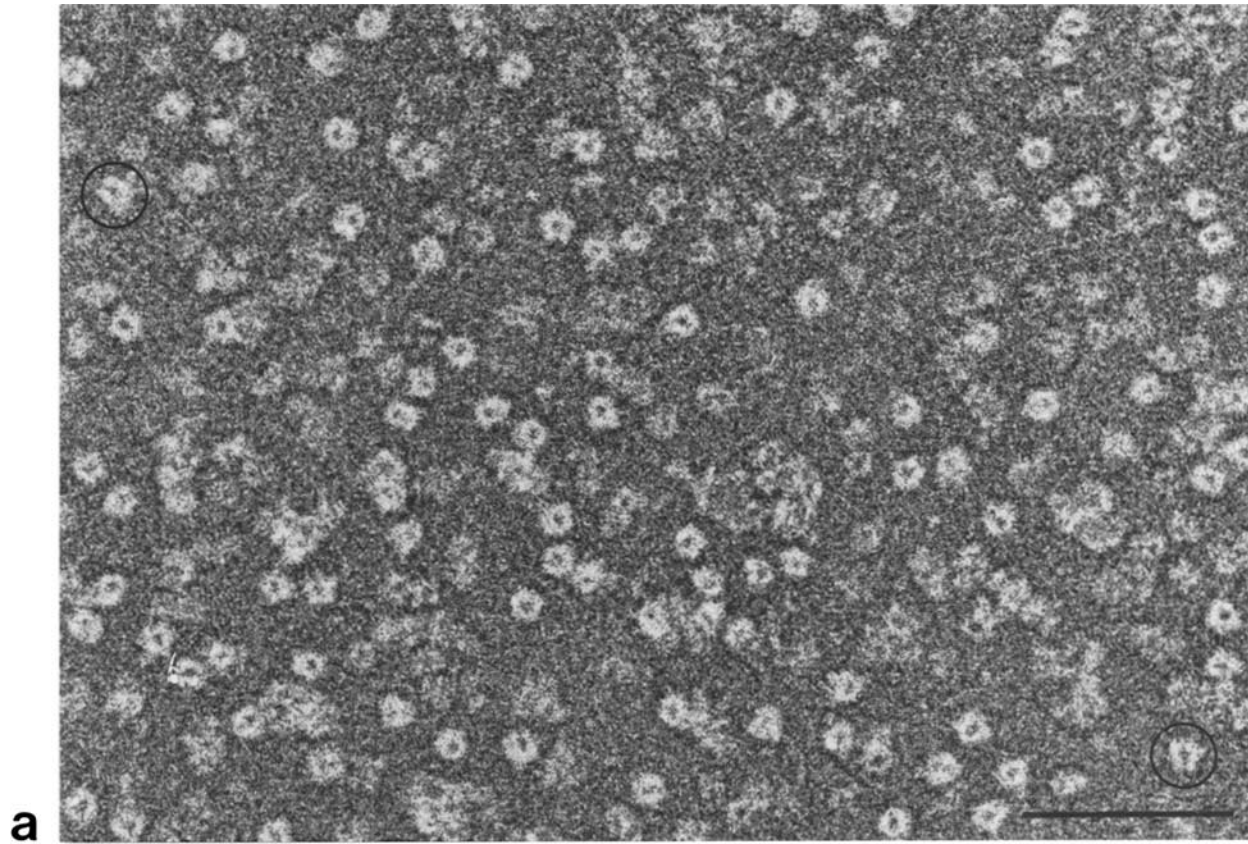
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*Figure 5.* Purified recombinant connexons negatively stained with uranyl acetate. (*a*) Connexons oriented predominantly with the channel axis perpendicular to the support film, resulting in a doughnut-shaped appearance. (*b*) Connexons in thicker stain which are predominantly tilted so that the stain-filled indentation (corresponding to the extracellular entrance of the channel) is no longer in the center of the particle. Some edge-on views (*circled*) are present in both figures. Bars, 50 nm.

a

b

c
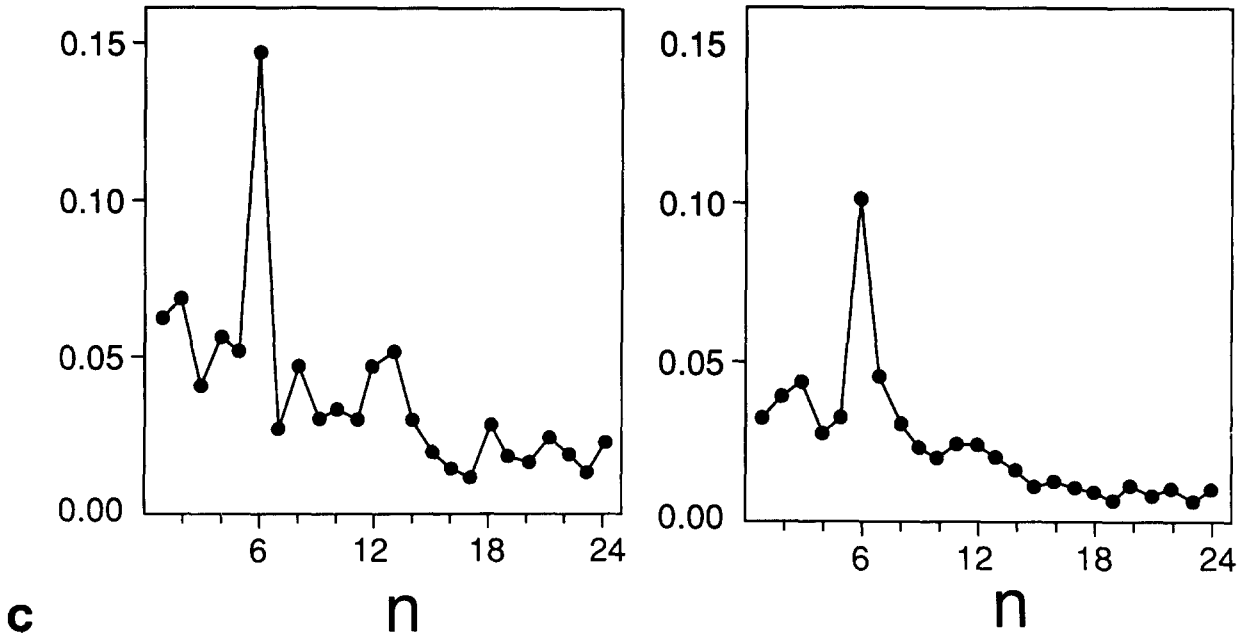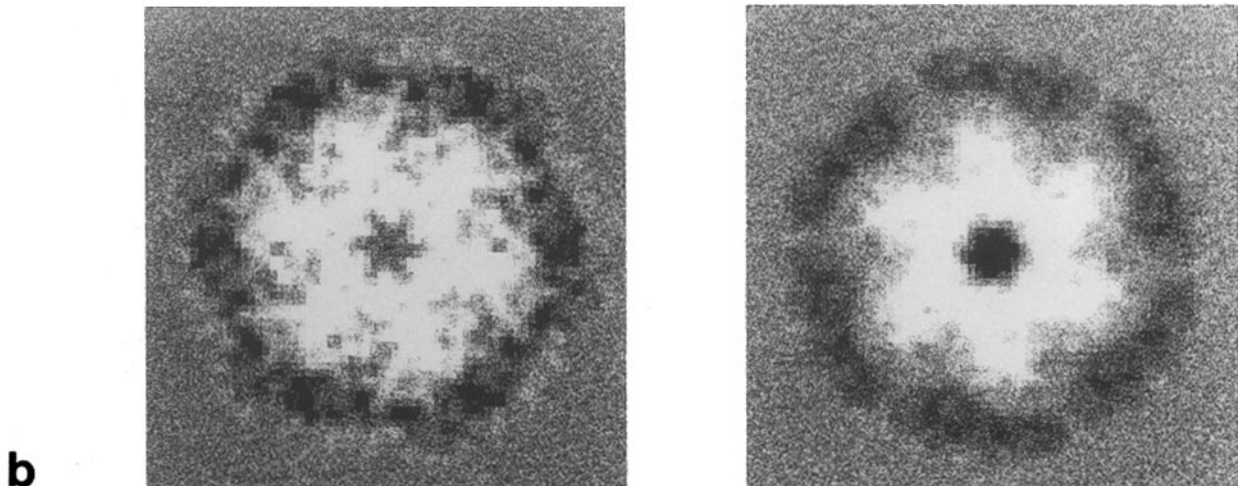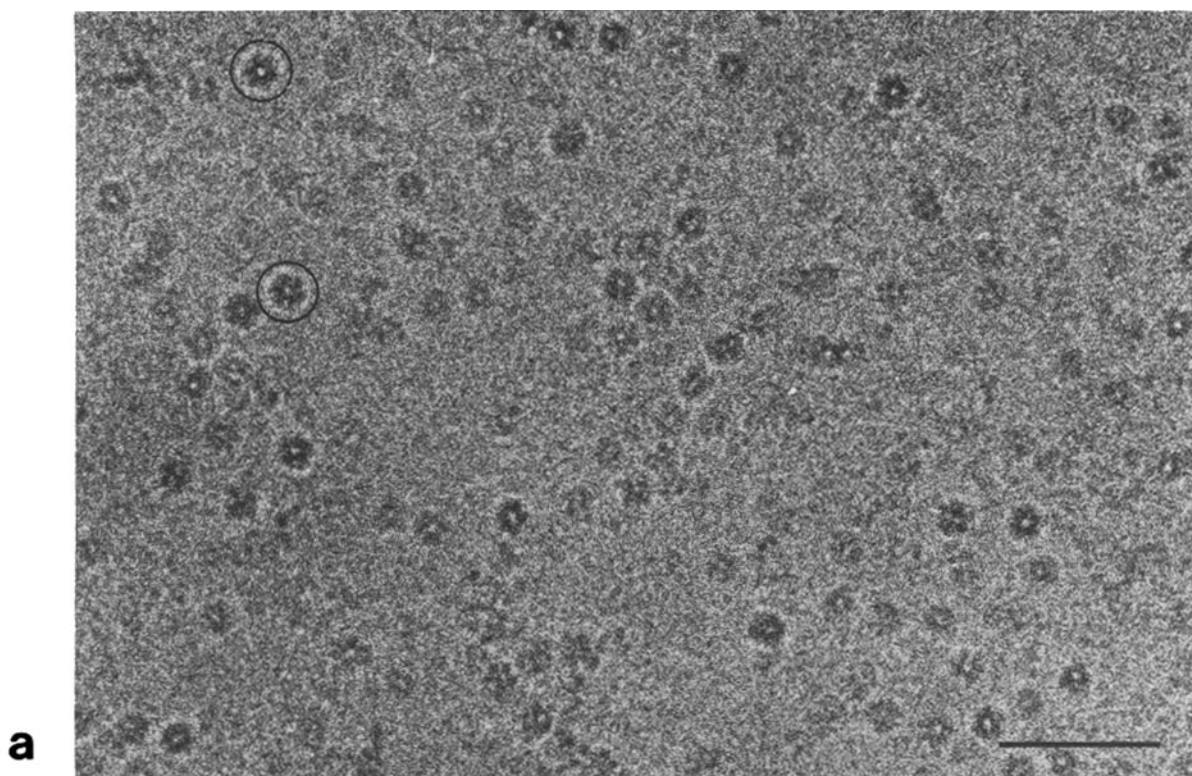
were the most effective detergents in producing complete solubilization. Fig. 3 *b* shows a negatively stained sample of the material obtained after treatment of plaques with 2 M NaCl, 10 mM EDTA, 100 mM DTT, 5% DoDM, 100 mM glycine-NaOH, pH 10. The plaque is now dissolved into single particles of roughly 80-Å diam which display a central stain-filled cavity. This is the appearance of the connexon expected from low-resolution crystallographic studies (Makowski et al., 1977; Unwin and Zampighi, 1980). The breakdown into single channels appeared to be complete in this case. However, milder conditions, using for example lower salt or pH, or less DTT, or different detergents, tended to produce larger aggregates (see Fig. 3 *a*); such samples formed a substantial pellet when centrifuged at 10,000 *g* for 20 min, whereas no pellet formed if the conditions used to obtain Fig. 3 *b* were applied. Once connexons were solubilized, the pH, ionic strength, and concentrations of reducing agent as well as detergent could be lowered considerably. We found that identical protocols could be used for the solubilization of material from rat liver or insect cells.

The solubilization of the connexons took place within minutes after the addition of solubilization buffer to pelleted plaques, providing that the plaques were well dispersed, as for instance by sonication, and providing that the temperature was kept below 4°C. The observation that gap junction proteins have a strong tendency to aggregate if warmed was made previously for material solubilized in SDS (Henderson et al., 1979) and we found the same phenomenon with connexons solubilized under nondenaturing conditions.

We tested a range of different nondenaturing detergents using the appearance of single connexons on electron microscopic grids as a criterion for complete solubilization. We found that at protein concentrations of more than ~0.1 mg/ml, the only really effective detergents were LDAO and DoDM. At very low protein concentrations, octyl glucoside, decyl dimethyl amineoxide, and decyl maltoside could also be used successfully (see also Mazet and Mazet, 1990). Among the detergents found to be ineffective were octyl polyoxyethylene, $C_{12}E_9$, and other $C_nE_m$ compounds, as well as Triton X-100, several compounds from the MEGA series, cholate, deoxycholate, and CHAPS. LDAO, octyl glucoside, and nonyl glucoside tended to break down the connexons into smaller species after a few days (John A. Berriman, personal communication). DoDM seemed to be the most suitable detergent.

## Purification of Connexons

Connexons were purified by adsorption to hydroxylapatite and subsequent elution with phosphate at pH 6.8. Further purification was achieved by anion exchange chromatography on DEAE Sepharose followed by gel filtration on Superose 6, where connexons were not only separated from contaminant proteins and lipids, but also from small amounts of incomplete or disintegrated connexons (see Fig. 4 *a* for elution profile and corresponding SDS-PAGE). This resulted

in pure $\beta_1$ polypeptide as judged by SDS-PAGE (see Fig. 4 *b*). Typically, 1 mg of purified protein was recovered from 10 mg of isolated membranes. EM at this stage (Fig. 3 *c*) showed that the protein had retained its doughnut shape. Indeed, connexons of good purity usually remained stable for several months if stored at 4°C.

The above purification procedure was developed using recombinant connexons from insect cells. It was also applied to rat liver material and yielded connexons which looked pure in the electron microscope. However, the $\beta_2$ polypeptide present in rat liver material was not separated from $\beta_1$ by this procedure.

Examination by TLC of chloroform extracts of purified connexons showed that phospholipids as well as cholesterol were removed during the purification. Membranes that showed especially good purity before solubilization were sometimes processed by the gel filtration step alone. However, most of those samples were found to still be associated with phospholipid.

## Appearance of Purified Connexons and Aggregates in Negative Stain and Amorphous Ice

Purified connexons, when examined in the electron microscope, most frequently presented a view in which the channel was aligned roughly perpendicular to the support film and appeared as doughnut-shaped particles of ~80-Å diam (see Figs. 5 and 6 *a*). Rotational power spectra calculated from such views of connexons, imaged in amorphous ice, showed a clear dominance of the sixfold harmonic (see Fig. 6 *c*). Corresponding sixfold filtered images of these connexons (Fig. 6 *b*) displayed quite strong modulations around the periphery produced by the six subunits.

Edge-on views of connexons (circled in Fig. 5) were more rarely observed. These views showed a characteristic accumulation of stain at one end of the structure, in agreement with the three-dimensional maps (Unwin and Zampighi, 1980). In some images (Fig. 5 *b*) many of the connexons contained accumulations of stain which were not central, but located nearer to the periphery. These connexons presumably were tilted, so presenting an appearance intermediate between face-on and edge-on views. In all situations, the maximum and minimum dimensions of the connexon were similar and close to 80 Å, as expected from the three-dimensional maps. This result confirms that the isolated particles are indeed connexons and not the paired assembly of connexons (the dodecamer) which forms the complete cell-to-cell channel.

In the presence of precipitants such as polyethylene glycol, the connexons aggregated in an ordered fashion forming filaments (Fig. 7 *a*) and sheets (Fig. 7 *b*). The filaments have the same diameter as isolated connexons and are divided by alternate lines of weak and strong stain penetration along their length. These lines are spaced ~75 Å apart and thus repeat every 150 Å, suggesting that the filaments are formed by end-to-end association of connexons. The filaments also

*Figure 6.* (*a*) Electron micrograph of purified connexons from rat liver imaged in amorphous ice (Bar, 50 nm). (*b*) Sixfold rotationally filtered images of the two connexons circled in *a*. (*c*) Rotational power spectra of these connexons, demonstrating dominance of the sixfold harmonic. (*n* = 6).
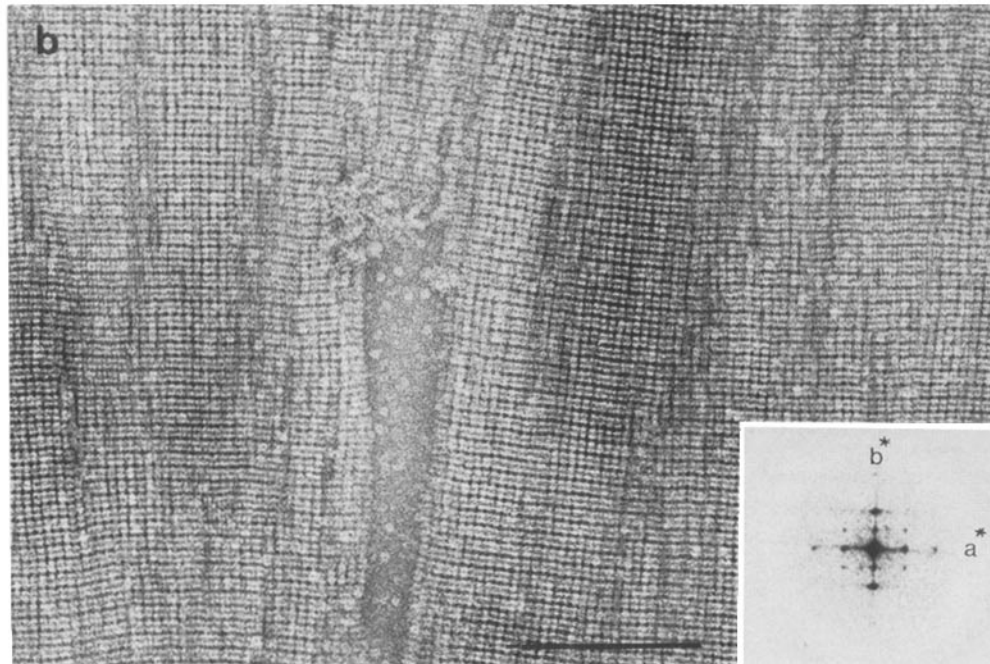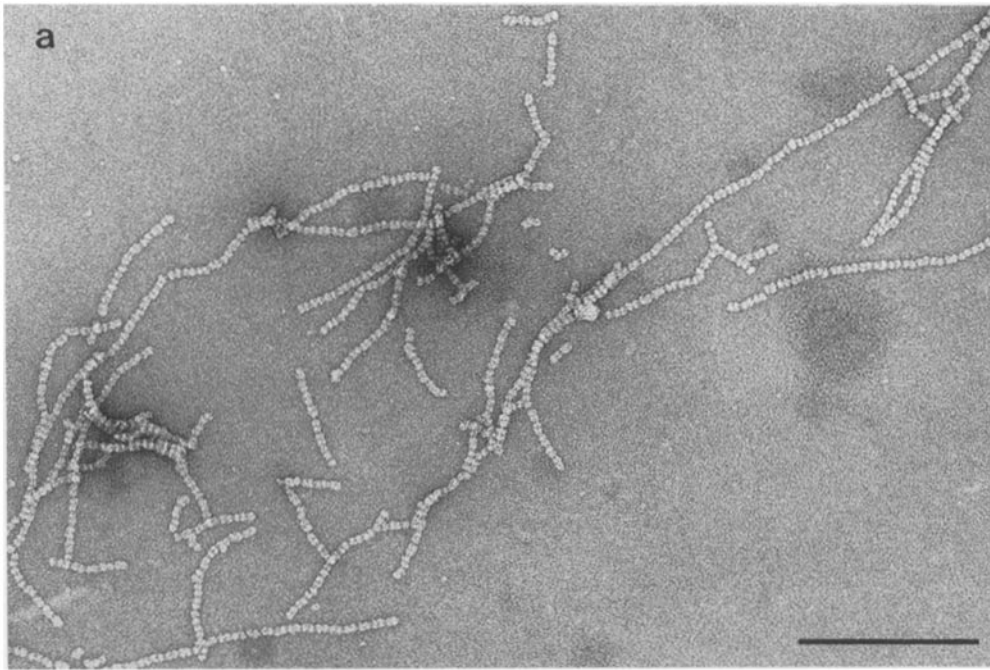
*Figure 7.* Formation of aggregates of purified recombinant connexons. (*a*) 10 μl purified connexons were mixed with 10 μl of 4% polyethylene glycol-2000 and incubated in a closed volume at 4°C in the presence of 1 ml 4% polyethylene glycol-2000. After several days, a small aliquot was withdrawn and examined in negative stain. The connexons have established end-to-end links to form filaments with a uniform width of ~80 Å. The filaments display alternating thicker and thinner striations which repeat at a spacing of ~150 Å. (*b*) Purified connexons were incubated as before but against 10% polyethylene glycol-2000 at 15°C. The sheets found under these conditions can be regarded as filaments packed sideways with the striations in register, thus emphasizing the 150 Å repeat. (*Inset*) Diffraction pattern from the same image, illustrating the crystal packing in a rectangular lattice. The a and b unit cell dimensions are 90 Å and 150 Å, corresponding to the repeat perpendicular to and along the "filament axis," respectively. Bars (*a* and *b*), 0.2 μm.

have no polarity, suggesting that the connexons are paired (as in forming a complete cell-to-cell channel), rather than all facing in the same direction.

We found that filament formation could be induced by the addition of oxidizing agents, which suggests that (intramolecular) disulphide bonds might need to be intact for this type of aggregation to occur. It is of interest that the two regions of the $\beta_1$ sequence which are thought to comprise the extracellular domain each contain three strongly conserved cysteine residues (Kumar, 1991) and these residues may be implicated. The fact that high concentrations of reducing agent are required, in the first place, to obtain individual connexons from the gap junction plaques is consistent with this view.

Observation of samples containing both filaments and sheets indicated that the sheets can grow by sideways association of filaments, and hence that they are related polymorphic structures. The pattern in the sheets consists of alternate weak and strong striations, repeating every 150 Å along one direction (corresponding to the filament axis), and another set of striations repeating every 90 Å in the other direction (see diffraction pattern in Fig. 7 *b*). Sometimes the

sheets were composed of several layers, in which case more complicated patterns were present.

## Discussion

We have developed a procedure to isolate and solubilize liver connexons in milligram amounts under nondenaturing conditions and to purify them. This procedure, when applied to rodent liver gap junctions, leads to two species of polypeptide ($\beta_1$ and $\beta_2$); however, when applied to recombinant junctions overexpressed in insect cells, using a baculovirus vector containing just the $\beta_1$ cDNA, only one species of polypeptide is present and thus is purified to homogeneity.

Overproduction of $\beta_1$ polypeptide in insect cells leads to the formation of extensive gap junction-like plaques (see Fig. 2), allowing essentially the standard gap junction isolation protocol to be used, namely, treatment of cells with sodium hydroxide and subsequent membrane fractionation on a sucrose gradient (Hertzberg, 1984). This simple procedure, applied to insect cells, takes about half a day and yields membranes which are ~90% pure (see Fig. 1). So far, we have optimized the method for quantity rather than quality, processing cells from 500–1,000 ml of culture in one batch, but with smaller amounts of starting material the purity tends to be better.

Solubilization of connexons has presented considerable problems in the past. In our hands, the addition of detergent only to isolated membranes (for example, deoxycholate, octyl glucoside, or digitonin) results in incompletely solubilized material; we found that high ionic strength, high pH, EDTA, and unexpectedly high DTT were necessary additional requirements for complete breakdown into single channels. Once solubilized, the connexons could, however, be maintained in less harsh conditions.

Disruption of gap junction membranes into larger structures than connexons occurred under some conditions (see Fig. 3 a), but did not yield an even approximately monodisperse population of particles. It was not possible, for example, to obtain paired connexons (dodecamers). This finding suggests that at least in the case of liver gap junctions the whole channel, composed of a pair of connexons, is not a stable entity. This statement may not apply to other connexin species, such as protein from heart (Manjunath and Page, 1986) or lens (Kistler and Bullivant, 1988).

The purification of solubilized connexons followed standard biochemical techniques. The first step, chromatography on hydroxylapatite, was originally applied to $\beta_1$ polypeptide in presence of SDS (Hertzberg, 1984). We found it to be equally applicable, with minor changes for optimization, to intact connexons. The second step, anion exchange chromatography, further removed contaminant proteins and most of the lipid. This step was generally omitted with batches of protein that showed reasonable purity after the hydroxylapatite step. The final step was gel filtration, in which the connexons ran clear of most impurities including smaller connexin aggregates. At the same time, the molecular weight range of Superose 6 was sufficient to prevent very high molecular weight contaminants from copurifying. This is of special importance in the purification of connexons from rat liver, which are contaminated by components of connective tissue, typically collagen (Cascio et al., 1990).

Our experiments demonstrate that the connexon, as well

as functioning as a channel, is also a structural complex that remains stable after disruption of the membrane and removal of the lipid. Examination of solubilized and purified connexons by EM confirmed the sixfold symmetry of this oligomer, and also that stain accumulates in the channel predominantly at one end. The orientation of connexons on the support film is usually such that the channel axis lies roughly along the line of view, and only rarely are edge-on views observed. The edge-on and intermediate views demonstrate, however, that we are indeed looking at the hexamer rather than the dodecamer, which would appear as an elongated structure with dimensions of ~80 × 150 Å.

The purified connexons, when incubated under conditions designed to promote crystal growth, form filaments and small crystalline sheets (Fig. 7). The nonpolar appearance of the filaments, and the fact that the repeating unit has dimensions of ~150 × 80 Å suggest that they grow by regular end-to-end association of connexons, one kind of association involving contact between the extracellular domains (as in the cell-to-cell channel), and the other involving contact between the cytoplasmic domains (see Fig. 8 b). The stronger (and more continuous) lines of stain penetration in Fig. 7 a presumably delineate the cytoplasmic contacts and the weaker lines the extracellular contacts, since stain only highlights the pore in the extracellular region (Revel and Karnovsky, 1967; Unwin and Zampighi, 1980).

The crystalline sheets are closely related to the filaments since the repeating pattern is the same along the b cell direction (Fig. 8 c). However, they include additional side-to-side interactions in which the hydrophobic surfaces of the connexons are brought together laterally in an arrangement similar to that which exists in the membrane. The spacing in this direction (the a cell dimension) is ~90 Å, i.e., rather larger than the diameter of a single connexon (~80 Å), suggesting that each molecule is surrounded by an annulus of

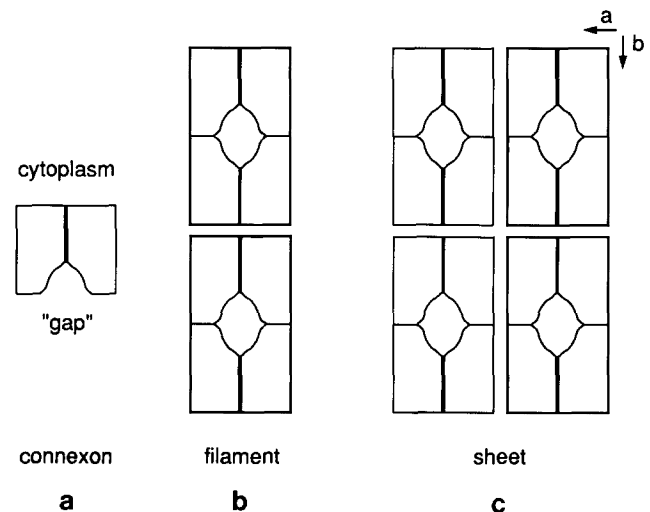

Figure 8. Proposed packing of connexons in filaments and sheets. (a) Single connexon with central pore which is widest at the extracellular end (bottom). (b) Filament formed by pairwise end-to-end association of connexons. (c) Sheet formed by sideways association of filaments leading to a and b cell dimensions of 90 and 150 Å, respectively.

detergent. At present the aggregation behavior of connexons is being studied more closely with a view to proper crystallization for X-ray diffraction analysis.

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