BULK ISOLATION OF MOUSE HEPATOCYTE GAP JUNCTIONS

Characterization of the Principal Protein, Connexin

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INTRODUCTION

The gap junction is a specialized contact between adjacent cells in a wide variety of tissues (1, 2) which is thought to mediate electrotonic coupling (3). This junction is characterized by a hexagonal lattice of subunits which may be seen in lanthanum-impregnated, negative-stained, and freeze-fractured preparations (1). A method for isolation of morphologically intact gap junctions from mouse liver has been published (4), which takes advantage of the gap junction's unique insolubility in the detergent n-lauroyl sarcosine. Analysis of gap junction biochemistry and structure has been limited by the small amounts of material produced by this isolation method. This communication reports a method for bulk isolation of hepatocyte gap junctions, resulting in milligram quantities of pure junctions suitable for structural studies. In addition, new data are presented on the chemistry of the principal protein of the gap junction.

MATERIALS AND METHODS

Retired male and female breeders from Charles River Breeding Laboratories, Inc., Wilmington, Mass., were used throughout. Livers from 100 mice were collected from decapitated, bled animals and immersed in cold buffer (1 mM sodium bicarbonate, pH 8.0). This buffer was used throughout the procedure, except where noted. Usually 250 g of liver were obtained. These were homogenized, three livers at a time, in a 40-ml Dounce homogenizer (Vitro) with a loose-fitting pestle in 5 ml buffer/g tissue. Each batch of livers was homogenized with six forceful, full strokes of the pestle. The homogenate was then diluted with 25 ml/g additional buffer, yielding approximately 7 liters of homogenate. This was stirred briefly and allowed to stand 10 min in the cold to precipitate the released DNA. The homogenate was then filtered through four layers of cheesecloth sus-

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pended in a large Büchner funnel. The filtrate was centrifuged by passing through a Beckman JCF-Z continuous-flow rotor spinning at 5,000 rpm in the Beckman J-21 preparative centrifuge (both instruments from Beckman Instruments; Inc., Spinco Div., Palo Alto, Calif.). The flow rate was increased to 100 ml/min with a pump. The collected pellet was resuspended (vortex) to a final volume of 2 liters and sedimented at 3,500 rpm for 10 min in the JA-10 rotor (approximately 2,000 *g* at R_{max}). The pellet was resuspended to 2 liters and sedimented again as in the previous step. These two low-speed spins serve to dilute the mitochondria and small organelles from the large membrane sheets, nuclei, and unlysed cells comprising the pellet. The pellet was resuspended again, diluted to 1 liter, and pelleted at 10,000 rpm in the JA-10 rotor (17,680 *g* at R_{max}). This last spin serves to concentrate the specimen for the zonal run.

The final pellet, usually about 150 ml in volume, was diluted slowly, with continuous stirring, with 2 vol of 67% sucrose. All sucrose solutions used were buffered with 1 mM $NaHCO_3$. A Beckman Ti-15 zonal rotor was then loaded in the following sequence: 300 ml of 37% sucrose, 800 ml of 45% sucrose, 450 ml (approximately) of 50% sucrose containing the sample, and the rotor filled to capacity with 55% sucrose (about 100 cc). The resulting discontinuous gradient was then centrifuged at 32,000 rpm (100,000 *g*) for 2 h.

The following simple modification of the centrifuge was found extremely useful for the zonal runs. The hose connecting the diffusion pump to the mechanical pump was removed and replaced with a much longer hose which exited the side of the centrifuge through an 8-inch diameter circular hole. A liquid mitrogen cold trap was put on this hose and used during the zonal run. The inevitable sucrose and condensation buildup in the rotor chamber during loading was effectively trapped with this device and prevented from getting into the mechanical pump oil, greatly decreasing pumpdown time and increasing the life of the pump.

After the zonal run, a crude membrane preparation was collected from the 45–37% interface, diluted with 1 mM bicarbonate buffer, and pelleted (17,680 *g* for 10 min, JA-10 rotor). The pellet was diluted to 100 ml of bicarbonate-buffered 0.9% saline containing 0.1% hyaluronidase (Sigma Type I, Sigma Chemical Co., St. Louis, Mo.), and 0.1% collagenase (Worthington Type CLS, Worthington Biochemical Corp., Freehold, N.J.). This mixture was stirred overnight at 4°C and for an additional hour at room temperature. The membranes were pelleted (10,000 *g* for 15 min, JA-20 rotor) and resuspended in 100 cc of 0.5% Sarkosyl NL-97 (Geigy Chemical Corp., Ardsley, N.Y.) buffered with 1 mM $NaHCO_3$. This solution was stirred at room temperature for 1 h, after sonication for three 10-s intervals with a Branson ultrasonicator operating at 0.7 maximum output (Branson Instruments Co., Stamford, Conn.). The standard sonicator horn was used. The sonicate was centrifuged at 13,000 rpm for 1 h in the JS-13 rotor (26,800 *g* at R_{max}). The pellet from this spin was brought to a volume of 10 ml with bicarbonate buffer, sonicated lightly to resuspend, and layered over a one-step discontinuous gradient, 41-30% sucrose. This final gradient was centrifuged at 100,000 *g* for 2 h in the Beckman SW 27 rotor. The purified gap junctions were collected from the 41–30% interface.

Isolated, washed preparations were dissolved in 1% sodium dodecyl sulfate (SDS), boiled immediately for 10 min, then divided into two parts. One part was left as control. To the other was added 50 mM dithiothreitol (DTT) or 50 mM mercaptoethanol. The samples were then heated at 50°C for 30 min before electrophoresis, care being taken not to evaporate the disulfide reducing agents. The samples were then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Fairbanks, et al. (5), using 8% gels with 0.3% *N,N'*-methylenebisacrylamide.

RESULTS AND DISCUSSION

The bulk isolation procedure results in 1–5 mg (dry weight) of gap junctions. There is variability in purity from run to run which is apparent in the different levels of background staining in the electrophoresis gels (Figs. 1–5), and in the variable amount of material which fails to enter the gels. The purity of the preparation may also be assessed by electron microscopy. Fig. 6 and 7 show thin-sectioned and negative-stained preparations of the final junction pellet. In Fig. 6, the 2-nm "gap" characteristic of gap junctions stained with uranyl acetate in the block is clearly seen (arrows). Fig. 7 shows the negatively stained hexagonal lattice characteristic of the junction. In both preparations, nonjunctional contamination (C) may be seen. SDS-PAGE of the junction preparation without DTT shows essentially the same results as published earlier (Fig. 1-3): single bands are seen at 34,000 daltons and at 18,000 daltons, with a closely migrating pair of bands seen at 10,000 daltons (labeled connexin A and B). Quantitatively, the majority of the protein (95%) migrates in either the 34,000- or the 18,000-dalton positions (Figs. 2 and 3). The quantitative distribution between these two bands shows variability from run to run. In one experiment (Fig. 2), the 18,000-dalton band contains approximately 68% of the total protein, while 27% migrates in the

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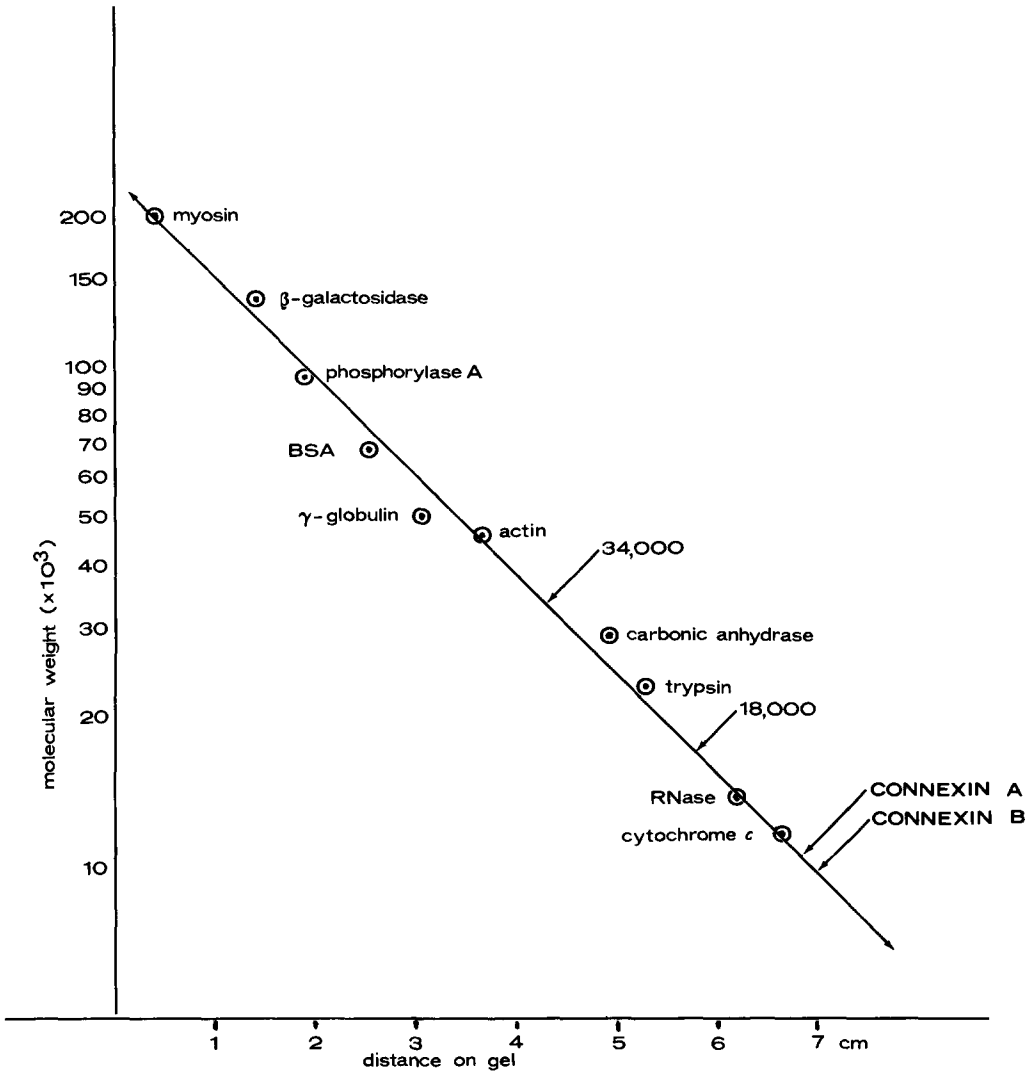

FIGURE 1 The distances migrated by junctional polypeptides are indicated on a standard curve. The reduced, alkylated standards were run under conditions identical to those for the experimental gels. In the presence of disulfide reducing agents the junctional polypeptides (connexin A and B) consistently ran ahead of cytochrome *c* with an apparent molecular weight of 10,000 daltons.

34,000-dalton position. In another experiment (Fig. 3), 50% of the protein runs in the 34,000-dalton band, while 45% migrates in the 18,000-dalton position.

Addition of DTT or mercaptoethanol to the SDS-dissolving mixtures produces a dramatic change in the SDS-PAGE profile (Figs. 4 and 5). The 34,000-dalton band and most of the 18,000-dalton band disappear, and the bulk of the protein appears in the rapidly migrating doublet at 10,000 daltons. Removal of the disulfide reducing agent by dialysis against 1% SDS does not result in reassociation back into the more slowly migrating components.

These findings are interpreted as evidence that the gap junction is composed of two small polypeptides with apparent molecular weights of about 10,000 daltons, which may be linked covalently either by disulfide bonds or by intrapeptide disulfide bonds. The resulting aggregates may be heterogeneous mixtures to the two low molecular weight components.

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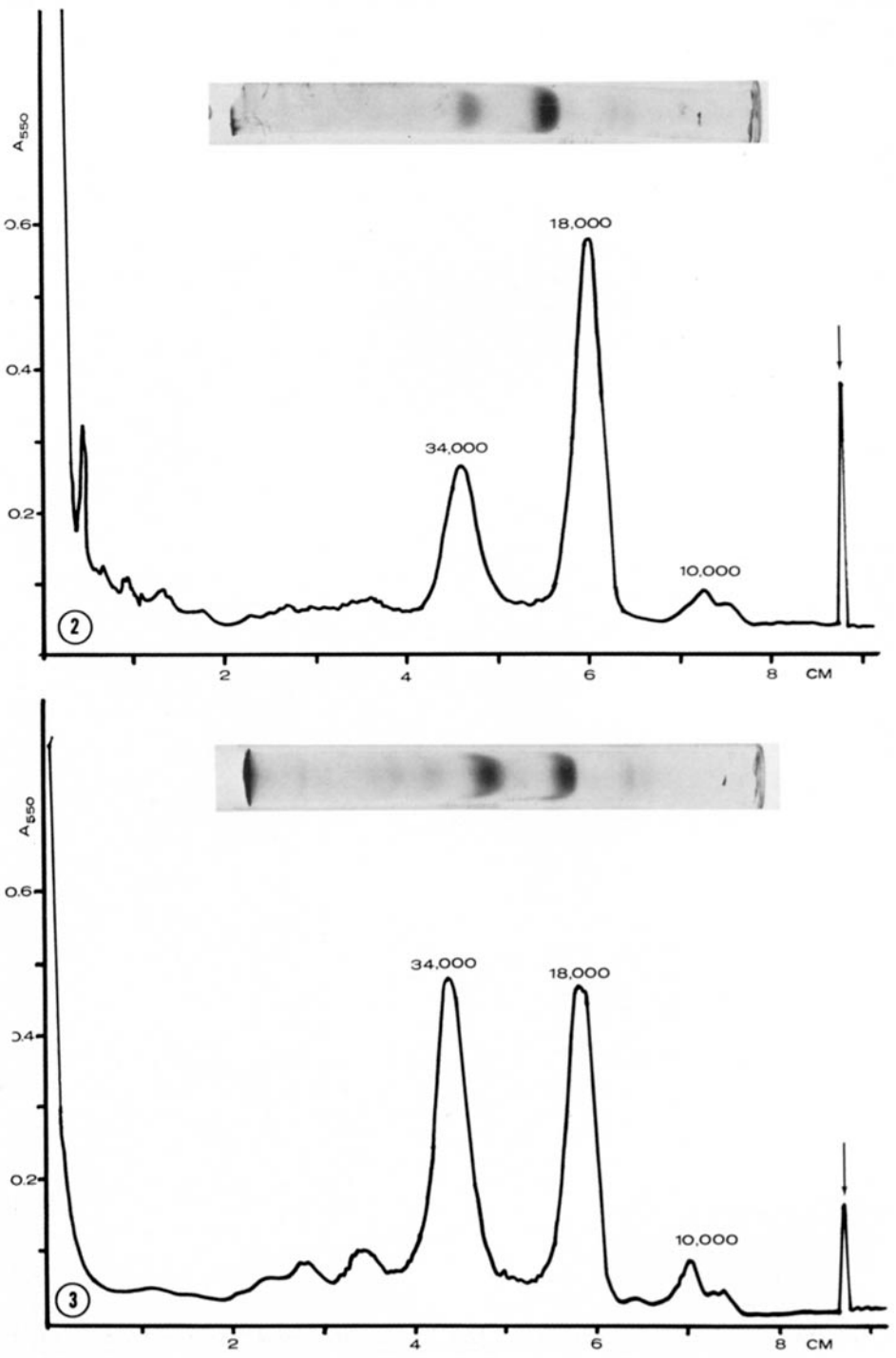

FIGURES 2-5 These figures show scans at 550 nm and photographs of polyacrylamide gels prepared as outlined in the Materials and Methods section. Fig. 2 and 3 are polyacrylamide gels of SDS-solubilized gap junctions without disulfide reducing agents which reveal two major bands at 18,000 and 34,000 daltons. The distribution of protein between these two bands is variable from run to run. There is also a small amount of protein in two closely migrating bands at about 10,000 Daltons. Fig. 4 and 5 are identical to Fig. 2 and 3 except for the addition of disulfide reducing agents to the SDS-solubilized junctions. This results in a shift of most of the gap junction polypeptides into the rapidly migrating doublet at 10,000 daltons. The arrows indicate the position of the tracking dye at the end of the run.

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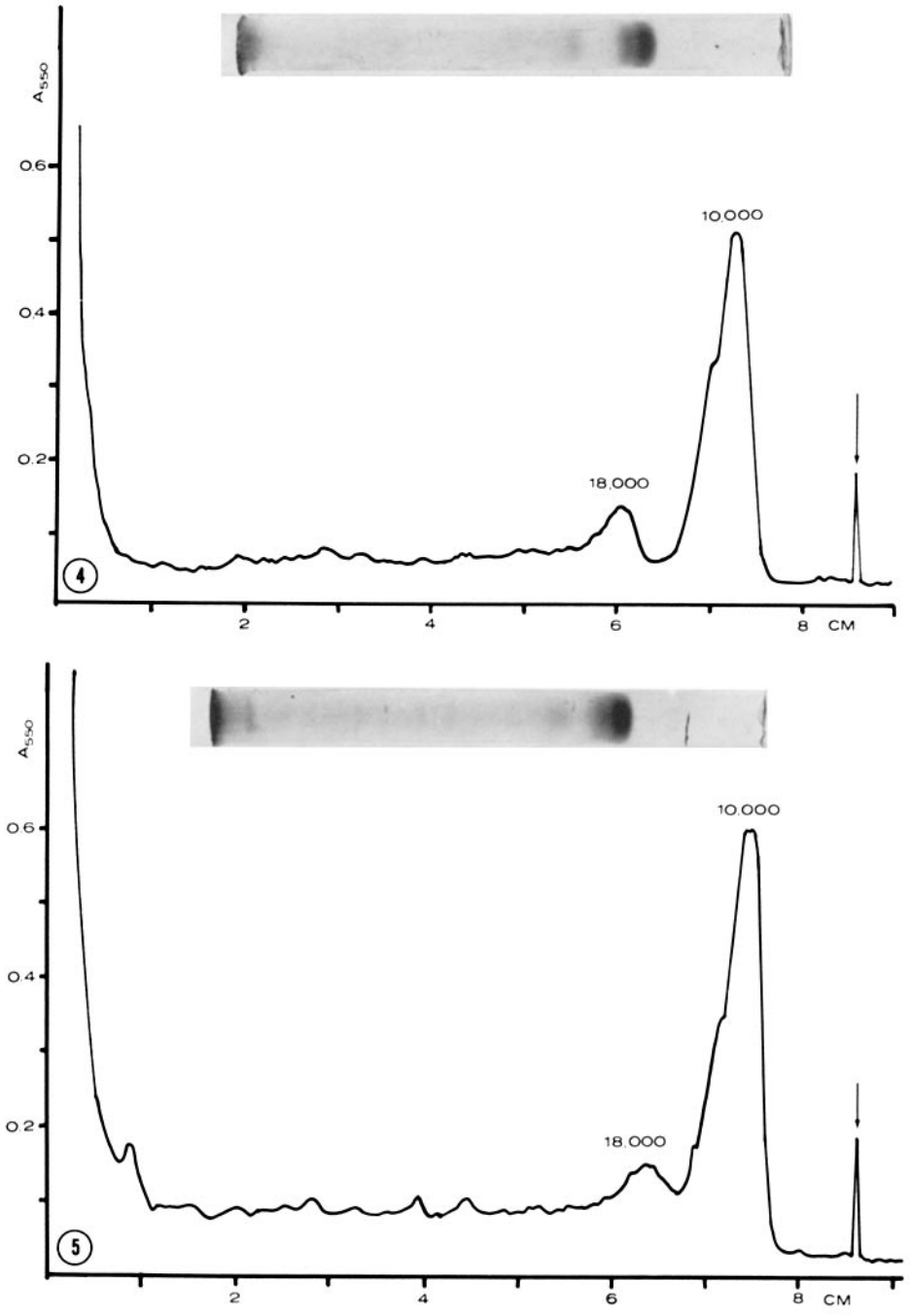
The names connexin A and B are proposed for the two 10,000-dalton components, since this protein presumably forms the connection between adjacent cells in the gap junction. The name is also appropriate since the junction was first described as a nexus by Dewey and Barr in 1962 (6).

It must be emphasized that there are several sources of error accompanying the assignment of molecular weights to the electrophoretic peaks in this study. First, the molecular weights of the connexins are estimated by extrapolation from the low end of a standard curve; second, the amount of sugar attached to the polypeptide is not known; and third, the molecular weights of the 18,000-



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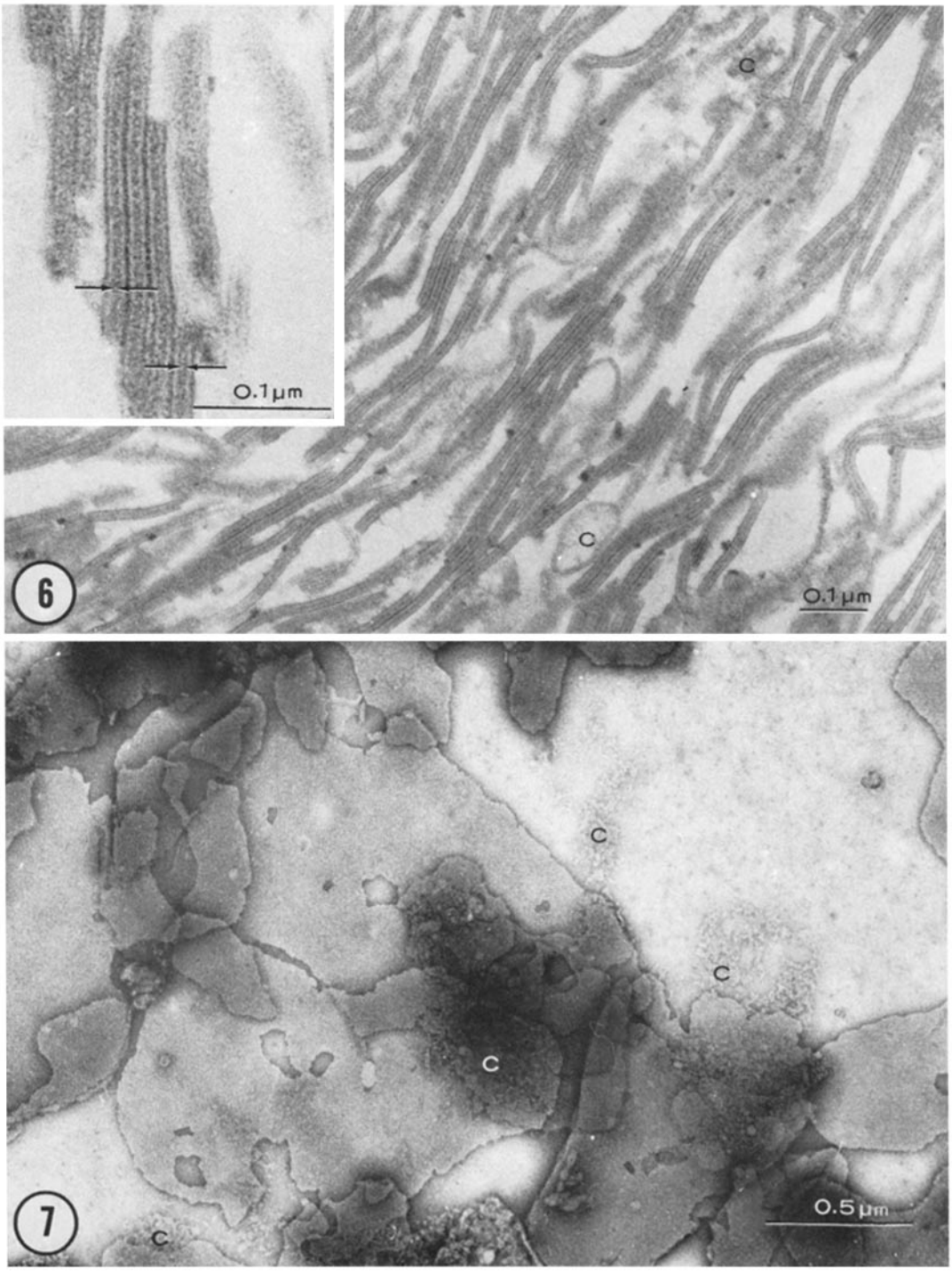

FIGURE 6 An electron micrograph of a thin section through a high-speed pellet of the isolated junctions. Numerous junctional profiles are seen showing the characteristic 2-nm gap (arrows). Varying amounts of contamination (C) accompany the isolated junctions. × 100,000. Inset: × 200,000.

FIGURE 7 This electron micrograph of a negatively stained (1% uranyl acetate) field of isolated junctions reveals that the hexagonal lattice is undisturbed by the isolation procedure. Nonjunctional contamniation (C) may be seen. Estimation of purity from negative-stained specimens alone may produce spurious results due to differential adhesivity of various components to the electron microscope grid. × 44,000.

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and 34,000-dalton peaks are estimated with unreduced samples. Unreduced peptides generally show greater mobilities on the acrylamide gels, so that the apparent molecular weights of the 18,000- and 34,000-dalton peaks are probably too low. All of these factors may significantly affect the apparent molecular weights on the gels.

The possibility exists that such low molecular weight polypeptides represent a proteolytic breakdown product of a larger molecular weight subunit. This possibility is thought to be unlikely for two reasons. First, the junctions are morphologically intact up until the moment of addition of SDS, so that if there is any proteolytic splitting during isolation, it does not structurally affect the junction. Gels may be run immediately, or after 2 days, at room temperature in SDS with no apparent increase or decrease in the quantity of any of the electrophoretic components. If there were proteolysis occurring, one would expect this to be a time-dependent process. Second, the preparations were boiled routinely for 10 min, sometimes for 20 min, which should destroy any proteolytic activity.

It is possible that the disulfide reducing agents are either splitting interpeptide disulfide bonds or intrapeptide bonds. The resultant measured change in molecular weight may reflect a separation of the individual polypeptides or a change in the protein shape and hydrodynamic properties. This latter explanation would be possible only if the peptides remained folded after exposure to SDS and unfolded only in response to disulfide reducing agents and SDS. Experiments with gel filtration may help to distinguish between these possibilities in the future.

SUMMARY

A method is described for the bulk isolation of gap junctions from mouse liver. The method uses continuous flow centrifugation and a discontinuous sucrose gradient in a zonal rotor to prepare large quantities of crude membranes. The purified gap junctions are separated from the crude membrane fraction on a one-step discontinuous gradient after collagenase digestion and differential solubilization with 0.5% Sarkosyl NL-97. The proteins are analyzed with polyacrylamide gel electrophoresis of purified junctions dissociated in SDS. Runs without disulfide reducing agents produce protein peaks at 34,000 and 18,000 daltons, with two minor bands at 10,000 daltons. In the presence of disulfide reducing agents, most of the protein migrates in the doublet at the 10,000-dalton position. The name connexin is proposed for the junction protein.

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