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Sucrose-permeability Induced by Reconstituted Connexin32 in Liposomes.

Seung Keun Rhee* and Eun Jung Hong

Department of Biochemistry, College of Science, Yeungnam University, Kyongsan 712-749, Korea
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Abstract: Functional study of the gap junction channel has been hindered by its inaccessibility *in situ*. Identification of forms of this channel in artificial membrane has been elusive because of the lack of identifying channel physiology. Connexin32 forms gap junction channels between neighboring cells in rat liver. Connexin32 was affinity-purified using a monoclonal antibody and reconstituted into artificial phospholipid vesicles. The reconstituted connexin32 formed channels through the vesicle membrane that were permeable to sucrose (Stokes radius: 5Å). The permeability to sucrose was reversibly reduced by acidic pH. In addition, the pH effect on the permeability to sucrose fit well with by the Hill's equation (where, n=2.7 and pK=6.7).
Key word: affinity-chromatograpy, connexin 32, liposome, reconstitution, sucrose-permeability.

The gap junction channel is a gated pathway for intercellular diffusion of ions, metabolites and second messengers (Lowenstein, 1981; Spray and Bennett, 1985). The intercellular communication mediated by gap junction channels is thought to play important roles in many cellular processes, including metabolic homeostasis, regulation of cell growth and development (Pitts, 1978; Ginzberg and Gilula, 1979; Bennett *et al.*, 1981; Hertzberg and Johnson, 1988).

A gap junction channel is composed of connexin protein, of which there are several closely related variants that have tissue-specific distributions (Kumar and Gilula; 1986; Beyer *et al.*, 1990). Hexamers of connexin form membrane spanning structures called connexons or hemichannels (Unwin and Zampighi, 1980). A gap junction channel forms when two connexons in the plasma membranes of closely apposed cells interact end to end to create a structure that spans both membranes. The cellular basis for regulation of gap junction channel gating has remained elusive. The physiology of gap junction channels has been described primarily by experiments which observe the effects on junctional conductance in coupled cells exposed to various agents (Spray and Bennett, 1985; Hertzberg and Johnson, 1988). These experiments have documented sensitivity of gap junctional conductance to cytoplasmic calcium ions, hydrogen ions, cyclic nucleotides, protein kinases, and to extracellularly applied octanol and anaesthetics. Unfortunately, it is difficult to show that an effect on junctional conductance is due to a direct effect of the applied agent on the channel itself since most such agents affect many aspects of cell physiology. Different cells show a great range (including the lack there of) of sensitivity to pH, calcium, cyclic nucleotides, and kinases (Wiener and Loewenstein, 1983; Saez *et al.*, 1986; Azarnia *et al.*, 1988; White *et al.*, 1989;).

The situation is made more complex by the fact that each connexin is likely to have distinct modulatory sensitivities, and each tissue seems to express more than one connexin (Lee *et al.*, 1992). Even the same connexin expressed in different cells can have apparently different physiology (Swenson *et al.*, 1989; Werner *et al.*, 1989; Ehgbali *et al.*, 1990). The inaccessibility of the channel *in situ*, and the multiple forms of the channel forming protein are obstacles that do not apply to study of most other channels. To study the gating, permeability and modulation of connexin channels with the rigorous standards routinely applied to other channels, it would be advantageous to study channels formed by a single connexin in a system where there is direct access to the modulatory sites.

Until recently, there was no way to purify a single connexin under non-denaturing conditions. Preparations of gap junction membrane typically contain more than one connexin species (as well as other proteins), are essentially insoluble, and are exposed to denaturing treatments during isolation (Hertzberg, 1984; Nicholson *et al.*, 1987). Recently, however, we were able to affinity purify a single connexin species (connexin32) under non denaturing conditions using a monoclonal anti-

*To whom correspondence should be addressed.
Tel: 82-53-810-2381, Fax: 82-53-812-6759.

body in different detergents (Rhee *et al.*, 1989; Rhee 1994).

A reconstituted system can provide access to the modulatory sites of channels. Study of the double membrane junctional channel is desirable, but its reconstitution in a pair of closely-apposed phospholipid membranes has severe technical impediments. In this report, we combine affinity purification with single-membrane reconstitution. We report here the functional reconstitution of a single connexin species into phospholipid membranes. The connexin32 was affinity purified with a monoclonal antibody under non denaturing conditions. It forms an aqueous pore through single membranes, that has permeability similar to that of gap junction channels *in situ*.

Materials and Methods

Materials

Egg phosphatidylcholine, bovine phosphatidylserine, and lissamine rhodamine B-labeled phosphatidylethanolamine were purchased from Avanti Polar Lipids, Inc. (Birmingham, USA). L-α-phosphatidylcholine (prepared from soybean), Tween 20, nitro blue tetrazolium (NBT), diisopropyl-fluorophosphate (DIFP), and (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propansulfonate) (CHAPS) were obtained from Sigma Chemical Co. (St. Louis, USA). Bio-Gel (A-0.5 m, exclusion limit 500,000 Da) and Silver-staining kit were obtained from Bio-Rad Laboratories (Richmond, USA). Immobilon PVDF mem brane was from Enprotech (Boston, USA). Alkaline phosphatase-conjugated goat anti-mouse IgG and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were purchased from Boehringer Mannheim Biochemicals (Indiana polise, USA). Sprague-Dowley rats (CD strain, 35 days old, female) were obtained from Life Science Inc. (Taegu, Korea).

Buffers

The homogenization buffer (HB) contained 5 mM $NaHCO_3$, 5 mM EDTA, 3 mM NaN_3, 0.5 mM DIFP, at pH 8.2, and phosphate buffer contained 50 mM Na-phosphate, 50 mM NaCl, 5 mM EDTA, 3 mM NaN_3, 0.5 mM DIFP, at pH 7.0. The urea buffer contained 10 mM KCl, 10 mM HEPES, 0.1 mM EDTA, 3 mM sodium azide and 459 mM urea at pH 7.4. In the sucrose buffer, 400 mM sucrose was substituted for the urea. Osmolality for both urea and sucrose buffers were 490 mOsm/kg, and the specific gravities (D^{20}) of the urea and sucrose buffers were calculated to be 1.0056 and 1.0511, respectively.

Purification of connexin32 by immunoaffinity purification using a monoclonal antibody

Connexin32 was affinity-purified from a CHAPS-solubilized plasma membrane fraction of rat liver using a monoclonal antibody, as previously described (Rhee, 1994). A plasma membrane fraction was prepared from livers and solubilized with 1% CHAPS in phosphate buffer. The supernatant following centrifugation at 100,000×g for 1 h was applied to a column containing Sepharose beads to which was attached a monoclonal antibody directed against connexin32 (M12.13) (Goodenough *et al.*, 1988). Connexin32 was eluted from the antibody by brief exposure to urea buffer (adjusted to pH 4.0), and the eluent was rapidly neutralized by dropping it directly into 1 M HEPES (pH 7.5). One mg/ml phospholipid and 1% CHAPS were present throughout the purification procedure.

SDS-polyacrylamide gel electrophoresis and immunoblotting

Samples were dissolved in SDS sample buffer (20% (v/v) glycerol, 125 mM Tris-HCl, 4% (w/v) SDS, 20 mM dithiothreitol, 0.005% (w/v) bromophenol-blue, pH 6.8). A discontinuous polyacrylamide gel electrophoresis was performed with a Bio-Rad minigel apparatus by the method of Laemmli (1972) (4% (w/v) stacking gel, 13% (w/v) separating gel). Protein was electrotransferred to Immobilon PVDF membrane (pore size 0.45 μm) with a semi-dry blotter (American Bionetics; Emeryville, CA) in standard transfer buffer (25 mM Tris, 192 mM glycine, 15% (v/v) methanol, (pH 8.3) at current density of 0.8 mA/cm^2 for 36 min at 4℃. For the immunoblots, the membrane was preincubated with 5% (w/v) non-fat dry milk in PBS-Tween 20 (0.5%, v/v) for 1 hour, and then incubated with primary antibody (M12.13) at 5 μg/ml for 2 h at 37℃. After washing with PBS-Tween 20 for 30 min with changes every 10 min, the membrane was incubated with secondary antibody (alkaline phosphatase-conjugated goat anti-mouse IgG) at 5~10 μg/ml for 1 h at 37℃, and then developed in 0.1 mg/ml NBT and 0.05 mg/ml BCIP in alkaline phosphate buffer (10 mM Tris, 10 mM $MgCl_2$, 100 mM NaCl, pH 9.5). For the visualization of protein bands, the gel was silver-stained following the manufacturer's protocol.

Reconstitution of connexin32 into phospholipid vesicles

Vesicle formation and protein incorporation followed the protocol of Mimms *et al.* (1981). Phosphatidylcholine (PC), phosphatidylserine (PS), and rhodamine-labeled phosphatidylethanolamine (PE) were dissolved in chloroform at a molar ratio of 2 : 1 : 0.25. The lipid mixture was dried to a thin film under a stream of argon and desiccated to remove any residual solvent. The lipid film was suspended at 1 mg/ml in urea buffer

containing 1% CHAPS and immunoaffinity-purified connexin32 at a concentration of 10 μg/ml. The protein-lipid-detergent mixture was incubated on ice for at least 20 min with occasional gentle swirling, and then applied to a 1.5×20 cm Bio-Gel A-0.5 m column pretreated with sonicated phosphatidylcholine vesicles. The column was eluted at a urea buffer flow rate of 9 ml/h at 4℃. The vesicles were collected in the void volume. The size distribution of the vesicles was established by filtration (0.5 ml/min) over a calibrated TSK G6000PW HPLC column (Ollivon *et al.*, 1986) to be highly monodispersed with a mean diameter of approximately 45 nm. In the vesicle-forming mixture, the protein to lipid ratio was 1:300 (w/w), corresponding to an amount of connexin32 equivalent to less than two connexons per vesicle.

Transport-specific density shift technique

The procedure used to fractionate vesicles into two populations based on sucrose-permeability is described and fully characterized by Harris *et al.* (1989). Linear iso-osmolar density gradients were formed from the urea and sucrose buffers in 5-ml ultracentrifuge tubes (Beckman, Palo Alto, CA) using a gradient maker (Hoefer, San Francisco, CA). An aliquot of vesicles mixture (typically 200 μl) was layered on top of each gradient. Gradients were typically spun at 300,000×g for 2 h in a swinging bucket rotor (SW 60, Hitachi, Japan) at 4℃. Gradient fractions of 200 μl were collected. A 50-μl aliquot of each fraction was diluted with 330 μl of PBS (phosphate-buffered saline)-Triton X-100 (0.2%). The distribution of the vesicles in the gradient was determined by measuring the specific intensity of rhodamine fluorescence in each fraction with a Perkin-Elmer 650-10S spectrofluorometer (560 nm excitation; 590 nm emission).

Results

Incorporation of connexin32 into phospholipid liposomes

A non-ionic detergent, CHAPS-solubilized connexin 32 was affinity-purified from rat liver membranes using a monoclonal antibody (Rhee, 1994), as shown in Fig. 1. The immunopurified connexin32 was incorporated into unilamellar phospholipid liposomes by gel-filtrating the protein/lipid/CHAPS mixture, using Bio-Gel column pre-equilibrated with sonicated phosphatidylcholine to avoid non-specific binding (Mimms *et al*, 1981; Ollivon *et al*, 1986). This method yielded liposomes with a monodispersed size distribution, as indicated by the narrow elution spectra (Fig. 2A, 2B). The liposomes prepared in the presence of connexin32 were eluted in earlier fractions than the liposomes prepared without connexin32, indicating that the incorporation of connexin32 contributed to the size of the liposomes. A similar phenomenon was also observed in reconstitution studies of the acetylcholine receptor (Anholt *et al.*, 1982), in which incorporation of the acetylcholine receptor increased the size of the liposomes by 50%.

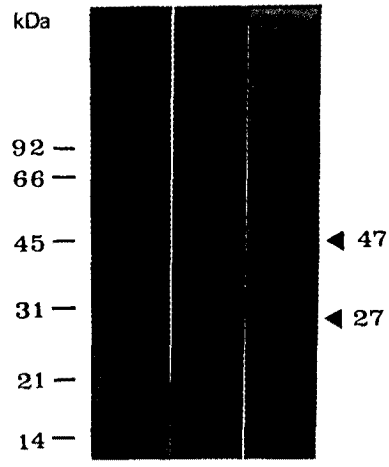

Fig. 1. SDS polyacrylamide gel electrophoresis and Western blotting of connexin32 purified by immunoaffinity chromatography. Lane A: silver-staining of CHAPS-solubilized plasma membrane fraction of rat liver; Lane B: silver-staining of immunoaffinity purified connexin32; Lane C: Western blotting of immunoaffinity purified connexin32.

Sucrose permeability of the reconstituted connexin 32 channel in liposomes

A functional study of the reconstituted connexin32 channel was performed on the basis of its sucrose permeability. Previous studies of gap junction channels reported that the sucrose (Stokes radius 5Å) permeates through the gap junction channel both *in vivo* and *in vitro* (Rhee and Harris, 1994; Harris *et al.*, 1992). Liposomes permeable to sucrose were identified and separated by a transport-specific density gradient technique (Harris *et al.*, 1989), as described in Materials and Methods. Liposomes that are impermeable to sucrose stay at their own densities in urea-to-sucrose iso-osmotic density gradients; however, the liposomes that are permeable to sucrose become more dense as urea molecules are exchanged by sucrose molecules, moving to a lower position in the gradient.

As shown in Fig. 3, liposomes formed in the absence of connexin32 were not sucrose-permeable, banding at high position in the gradient. In contrast, liposomes formed in the presence of immunoaffinity-purified connexin32 separated into two bands; one band of sucrose-impermeable liposomes at the same position as the liposomes formed in the absence of connexin32,

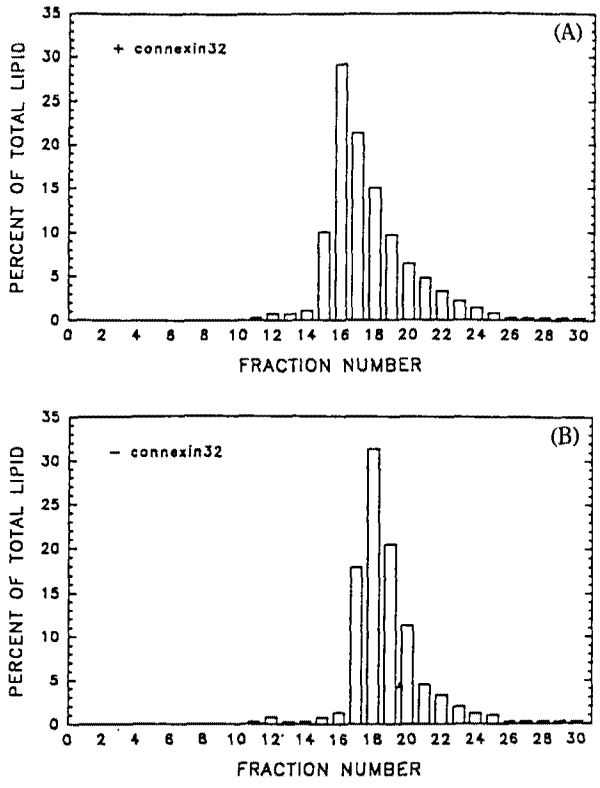

Fig. 2. Elution profile of reconstituted liposomes from gel-filtration chromatography. Liposomes formed by gel-filtration method (Mimms *et al.*, 1981), either in the absence (A) or in the presence of connexin32 (B). Liposomes formed in the presence of connexin32 eluted earlier than liposomes formed without connexin32 (peak fractions are indicated as arrows). Each fraction contained 200 μl of eluent.

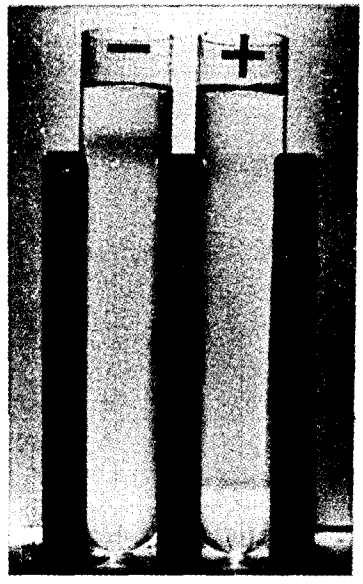

Fig. 3. Sucrose-permeability of liposomes formed in the presense of connexin32. Liposomes formed without connexin32 (−) were found in a single band near the top of the tube, indicating sucrose-impermeability. Liposomes formed in the presence of connexin32 (+) separated into two populations, one at the upper position characteristic of sucrose-impermeability, and the other, near the bottom of the tube, characteristic of sucrose-permeability. Bands of liposomes are colored due to rhodamine-PE incorporated into liposomes.

and a second band of sucrose-permeable liposomes near the bottom of the gradient. To quantify the fraction of the liposomes that were sucrose-permeable, the distribution of liposomes on the gradient was determined by measuring the fluorescence of the rhodamine-labeled PE in the liposomes membranes (Fig. 4A, 4B). Typically, more than one-half of the liposomes formed in the presence of connexin32 (protein-lipid ratio 1 : 300; w/w) were sucrose-permeable.

To determine the distribution of connexin32 in the two populations, protein in the sucrose-impermeable and sucrose-permeable liposomes was examined by immuno-dot blotting. The immuno-dot blots were probed with a monoclonal antibody against connexin32 (M12.13). Typically, most fractions containing detectable connexin32 was in the sucrose-permeable liposomes. Occasionally, a small amount of connexin32 (<5 %) was detected in the sucrose-impermeable liposomes (Fig. 5). We conclude that the immunoaffinity-purified connexin32 forms a sucrose-permeable channel in phospholipid liposomes. The small amount of connexin 32 present in the sucrose-impermeable liposomes may fail to form functional channels, perhaps due to damage during purification or the reconstitution procedure.

Low pH reversibly inhibits sucrose-permeability

To determine whether pH affected the connexin32-induced sucrose-permeability, the transport-specific fractionation was performed at pH's ranging from 6.0 to 8.0. The fraction of liposomes that were sucrose-permeable decreased when the pH was below 7.5 (Fig. 6). Sixty percent of the liposomes formed in the presence of connexin32 were sucrose-permeable at pH 7.5, while only 16% were sucrose-permeable at pH 6.0. The pH-dependent sucrose-permeability of the reconstituted connexin32 was plotted using the Hill equation $\{P=K^n/(K^n+[H^+]^n\}$, where P is the fraction of the vesicles permeable to sucrose, K is the dissociation constant for protonated sites, and n is Hill's coefficient. The curved line is fit to $pK=6.7$ and $n=2.7$. To determine whether the pH inhibition of sucrose-permeability was reversible, liposomes that were sucrose-impermeable at pH 6.0 were recentrifuged at pH 7.5. Approximately 35% of the usual shift occurred, indicating incomplete recovery of sucrose-permeability (data not shown).

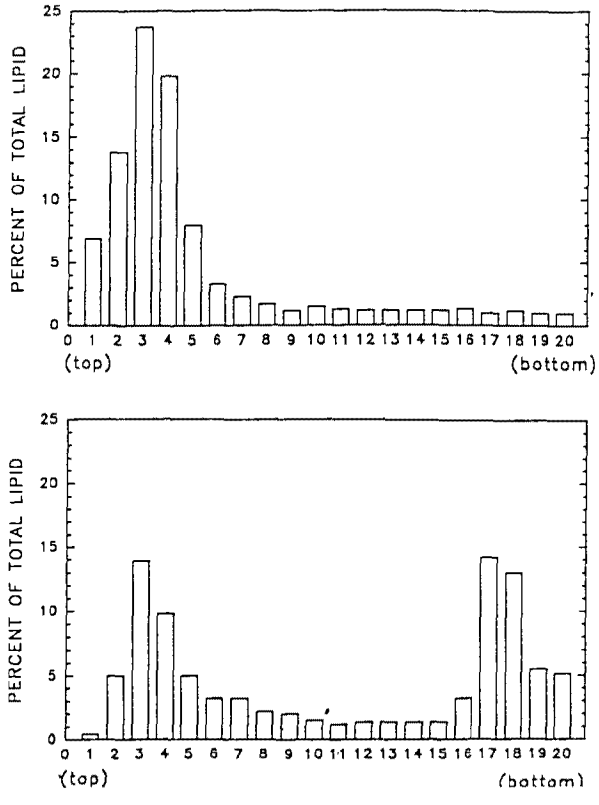

Fig. 4. Distribution of vesicles after transport specific fractionation. The distribution of vesicles was determined by measurement of the fluoresence of rhodamine-PE. (A) Liposomes formed without connexin32 were in a single peak (fractions 3 & 4). (B) Liposomes formed in the presence of connexin32 separated into two peaks; one at fractions 3 & 4 (sucrose impermeable), and the other at fractions 17 & 18 (sucrose permeable).

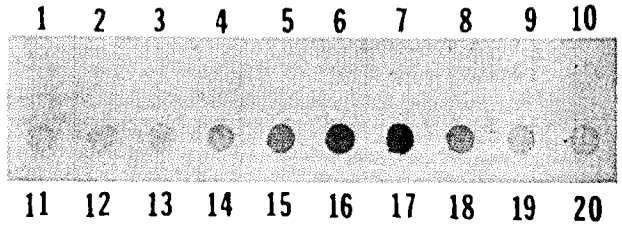

Fig. 5. Distribution of connexin32 in the liposomes populations. Each fraction of density gradient, in which liposomes formed in the presense of connexin32 were loaded, was subjected to immuno-dot blotting. Most of connexin32 appeared at density-shifted fractions (sucrose-permeable).

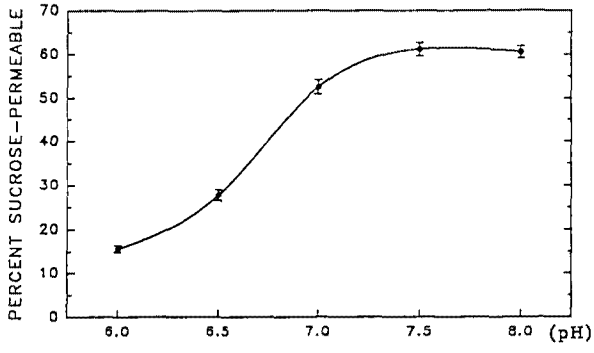

Fig. 6. Sucrose premeability of lipsomes formed in the presense of connexin32 over a range of pH's. The fraction of liposomes that were sucrose permeable decreased when the pH was below 7.5. At pH 6.0, liposomes permeable to sucrose was decreased by 75%, compared to pH 7.5. These data were normalized and results ± S.D. were illustrated.

Discussion

We report here that affinity-purified connexin32s from rat liver form permeable channels in phospholipid liposomes. It had been previously shown that connexin 32 solubilized from gap junctional membranes of rat liver can form open channels in single membranes (Harris *et al.*, 1988; Harris *et al.*, 1990). The isolated junctions used in that study contained non-connexin components, and were exposed to denaturing conditions during isolation. Connexin32 was identified as a channel forming protein by its relative enrichment in liposomes that were permeable to sucrose.

Is it surprising that connexin can form channels in single membranes? On the one hand, if a single connexon exists in a plasma membrane, such large channels would be expected to adversely affect cell health, if they were open for significant periods of time. There is no direct evidence that nonjunctional connexin persists in plasma membrane (Beyer *et al.*, 1989), but this may be due to the difficulty of identifying dispersed connexin. Also, in a macrophage cell line, extracellular ATP can induce a significant permeability to Lucifer Yellow for many minutes or even hours without being lethal to the cells (Buisman *et al.*, 1988). There is evidence that this permeability is, in fact, due to connexin 43 in the plasma membrane (Beyer and Steinberg, 1990).

The fraction of liposomes that are sucrose-permeable decreases when the pH is lowered to between 7.5 and 6.0. A similar effect of cytoplasmic pH on junctional conductance has been observed in a variety of cell types (Turin and Warner, 1977; Spray *et al.*, 1981; Spray *et al.*, 1986). The pH sensitivity of the reconstituted system indicates that pH can act directly on gap junction channels.

In cells, junctional conductance usually recovers fully when intracellular pH is returned to normal. We, however, found only partial recovery of the sucrose permeability. One possible explanation is that the prolonged exposure to a low pH (several hours during the spin) causes irreversible damage to some of the channels, especially since at the low ionic strength used the surface pH may be much lower. Another possibility is that some connexin channels are oriented with a pH-sensitive site inside the vesicle. Such channels would close

during the initial centrifugation at low pH, and remain closed (due to trapped acid) during the second (neutral pH) centrifugation.

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