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Analysis of vertebrate gap junction protein

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A new method for the purification of gap junctions is described which depends on the extraction of cell monolayers or tissue homogenates with Triton X-100. The major band on SDS-polyacrylamide gel electrophoresis (PAGE) of junctional preparations from a variety of vertebrate sources has an apparent mol. wt. of 16 000 (16 K). Further evidence for the junctional origin of the 16 K protein is provided by the results of four different experimental approaches. (i) The junctions form a sharp band in potassium iodide density gradients at 1.195 g/cm^3 and the 16 K protein is the only detectable band in fractions of this bouyant density. (ii) The junctions are progressively solubilised by increasing concentrations of SDS (in the range 0.1–0.5%) and the dissolution of the junctional structure, observed by electron microscopy, parallels the release of the 16 K protein. (iii) Glutaraldehyde fixation of intact junctions cross-links the 16 K protein. (iv) The recoverable amount of the 16 K protein correlates with known changes in gap junctional area in the regenerating weanling rat liver after partial hepatectomy and in V79 cell cultures exposed to 4β-phorbol 12-myristate 13-acetate.

Key words: gap junctions/intercellular communication/cell interactions/membrane channel

Introduction

Gap junctions are cell surface features formed at the points of contact between cells. They are composed of aggregates of trans-membrane particles (connexons) containing channels which join neighbouring cells and allow the intercellular movement of water-soluble substances with a mol. wt. less than 900–1000 (Makowski *et al.*, 1977; Simpson *et al.*, 1977; Flagg-Newton *et al.*, 1979; Unwin and Zampighi, 1980; Pitts and Simms, 1977; Finbow and Pitts, 1981). Intercellular communication through gap junctions is probably a feature of all metazoan animals (see Finbow, 1982).

A number of studies over the past 10 years have attempted to characterize the protein component of gap junctions isolated from mammalian tissues. The junctions have been separated from plasma membrane preparations on the basis of their resistance to detergents. The characteristic morphology of the junctions is retained throughout the isolation procedures and is the only criterion which can be used to monitor purification. However, there has been disagreement between different workers about the junctional origin of the various proteins derived from gap junction preparations (see Robertson, 1981).

Goodenough and his co-workers (Goodenough and Stoeckenius, 1972; Goodenough, 1974, 1976) showed that morphologically pure gap junction fractions could be obtained if plasma membrane preparations from mouse liver were treated with trypsin and collagenase prior to detergent extraction. Such junction fractions contained one or two major protein components with a mol. wt. of 10 000–12 000. More recent studies using denaturing agents to remove contaminants instead of added proteases, suggest these low mol. wt. components to be breakdown products of a protein with a mol. wt. of 26 000–28 000 (Hertzberg and Gilula, 1979; Henderson *et al.*, 1979; Finbow *et al.*, 1980; Nicholson *et al.*, 1981). Although a number of laboratories have reported the 26–28 K protein to be the major constituent of liver gap junctions, recent studies in Goodenough's laboratory failed to detect this as a major component in morphologically pure junction fractions prepared from mouse liver or mouse heart in the absence of added proteases (Kensler and Goodenough, 1980; Fallon and Goodenough, 1981). Several major bands with a mol. wt. between 10 000–21 000 were observed for the mouse liver preparations but there were no significant bands below mol. wt. 34 000 in the mouse heart preparations. There is thus still confusion as to the identity of the gap junction protein.

We have devised a new method for the isolation of gap junctions from various sources which relies on partial solubilization of cellular components in Triton X-100. This method, which is rapid and versatile, allows junction fractions to be prepared from different tissues and cultured cells. The major component of junction fractions prepared in this manner from a wide variety of sources is a protein with a mol. wt. of 16 000 (16 K). Four different experimental approaches have been used to confirm the junctional origin of the 16 K protein.

Results

Purification of gap junctions using Triton: the 16 K protein component

Previous methods for the preparation of gap junctions have started with the isolation of plasma membranes. This has limited the application of these methods to those tissues which give good yields of plasma membranes (e.g., liver and heart). We have therefore devised a method for the preparation of junctions which is suitable for a greater variety of tissues and which can be applied successfully to cultured cells. The method relies on the partial solubilisation of tissue homogenates or cultured cell monolayers in 1% Triton X-100, 0.15 M NaCl. Under these conditions, nuclei and cytoskeletal elements remain intact and can thus be removed by low speed centrifugation. The procedure then uses adaptations of existing techniques to remove contaminating proteins (Finbow *et al.*, 1980) and the gap junctions are recovered by centrifugation through discontinuous sucrose gradients (see Materials and methods). Examination by electron microscopy of negatively stained preparations made by this method from various sources including mouse liver, cultured rat liver epithelial cells and Norway lobster hepatopancreas show the final preparations are enriched in gap junctions (Figures 1 and 2, and see Figures 6–9). The morphological purity of the

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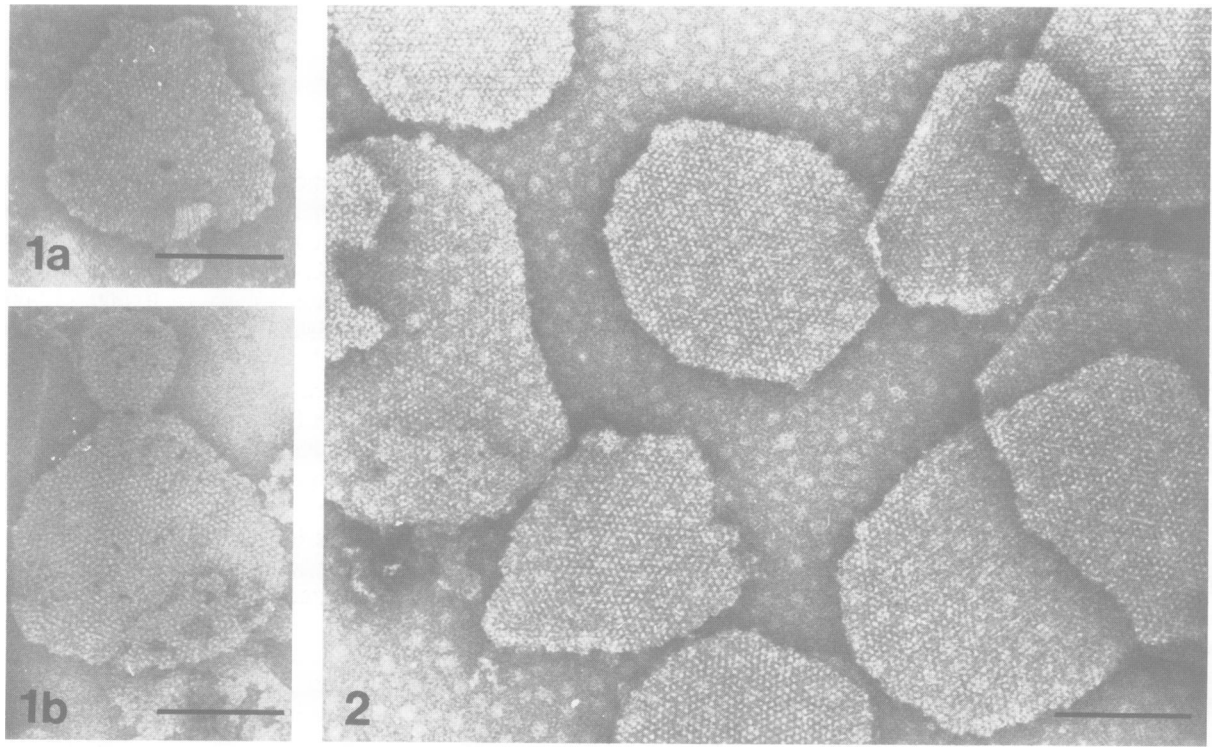


Figs. 1 and 2. Negatively stained gap junctions from BRL cells and Norway lobster hepatopancreas. Gap junctions were prepared from cultured BRL cells (**Fig. 1a** and **1b**) and Norway lobster (*Nephrops norvegicus*) hepatopancreas (**Fig. 2**) as described in Materials and methods. Bars represent 0.15 μm.

preparation is improved by the addition of trypsin during extraction with 6 M urea and, in the case of preparations from liver and hepatopancreas, by purifying the crude tissue homogenate to a post-mitochondrial pellet before Triton extraction (see Materials and methods). The total gap junctional area seen by electron microscopy is less in preparations from cultured cells than from mouse liver or Norway lobster hepatopancreas due to the reduced amount of starting material and the smaller size of the junctional plaques.

Analysis by SDS-polyacrylamide gel electrophoresis (PAGE) of junction fractions prepared from various vertebrate sources consistently show only one major band which has a mol. wt. of 16 K. Preparations from arthropod tissues show an equivalent band but with a mol. wt. of 18 000 (Finbow, Lane, Shuttleworth and Pitts, submitted). The method has been applied to mouse (Figures 3 and 4), rat (Figure 11) and chicken liver, mouse heart, brain and uterus and to cultured BRL cells (Figures 3 and 4), BHK cells (Syrian hamster fibroblasts), V79 cells (Chinese hamster lung cells, Figure 11), HEp-2 cells (human pharyngeal carcinoma cells) and RTG 2 cells (rainbow trout fibroblasts).

The method of solubilisation of the junctional preparations in SDS sample buffer has been found to be important. Solubilisation at room temperature in 1% SDS (standard procedure) produces the 16 K band while solubilisation in 1% SDS at 100°C for 5 min results in a decrease in staining of this band and the appearance of a band with a mol. wt. of 26 K as well as increased staining at the top of the gel (Figures 3 and 4). The 26 K band is most likely a dimeric form of the 16 K protein as elution of either the 16 K or the 26 K band in 0.1% SDS produces a mixture of the 16 K and 26 K bands when rerun on second SDS gels. The dimerisation of the 16 K form is more complete than the dissociation of the 26 K form, presumably reflecting the stability of the dimeric state

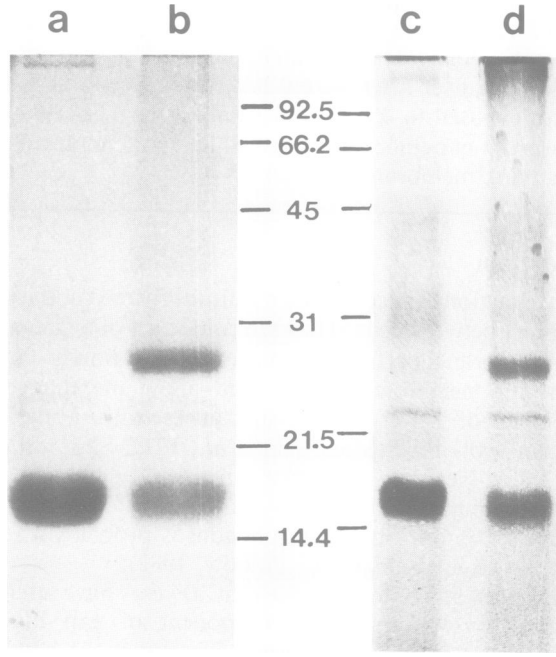


Fig. 3. SDS-gels of gap junction fractions from mouse liver and BRL cells. Gap junctions were prepared from mouse liver (**lanes a** and **b**) and BRL cells (**lanes c** and **d**) as described in Materials and methods. Equal aliquots were solubilized in SDS sample buffer either at room temperature (**lanes a** and **c**) or at 100°C for 5 min (**lanes b** and **d**). Approximately 1/10th and 1/5th of the final preparations from mouse liver and BRL cells, respectively, were loaded into each lane. The positions of the Biorad low mol. wt. standard proteins are shown as mol. wt. x 10^{-3}.

in SDS solutions. The presence of 2-mercaptoethanol during solubilisation has no effect on the migration of the 16 K protein or its dimerisation on heating.

When trypsin is omitted during extraction with 6 M urea

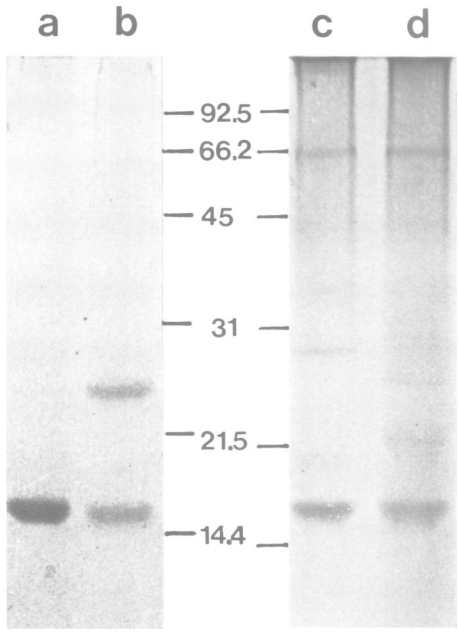


Fig. 4. SDS-gels of gap junction fractions from mouse liver and BRL cells isolated in the presence of protease inhibitors. Gap junctions were prepared from mouse liver (**lanes a** and **b**) and BRL cells (**lanes c** and **d**) as described in Materials and methods with the following modifications. After pelleting from the 1 mM $NaHCO_3$ wash, the junction fractions were suspended in 70 ml (35 ml for BRL cell material) 6 M urea (without trypsin) containing 0.5% N-lauryl sarcosine and centrifuged at 23 000 r.p.m. for 30 min in the 6 x 35 rotor of an International B60 ultracentrifuge. The pellets were dispersed in a small volume of 1 mM $NaHCO_3$ and loaded on to two (one for BRL cell junctions) 32–50% discontinuous sucrose gradients as described in Materials and methods. All buffers and solutions contained 0.1 mM PMSF (Sigma), 2.6 mM Nα-p-tosyl-L-arginine methyl ester. HCl (Sigma) and 0.1 mM 1,10-phenanthroline (Sigma). Equal aliquots were solubilized in SDS sample buffer either at room temperature (**lanes a** and **c**) or at 100°C for 5 min (**lanes b** and **d**).

and steps are taken to limit endogenous protease activity by the addition of 0.1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma), 2.6 mM Nα-*p*-tosyl-L-arginine methyl ester (Sigma) and 0.1 mM 1,10-phenanthroline (Sigma) throughout the isolation, the 16 K protein is still the major band in preparations from either mouse liver or BRL cells (Figure 4). This suggests that the 16 K component is not generated by the action of either added trypsin or endogenous proteases on some higher mol. wt. protein. There is more non-junctional material, judged qualitatively by electron microscopy, in preparations made without added trypsin, but much of this extra material is insoluble in 1% SDS and only a few extra minor bands are seen on SDS-PAGE (Figure 4).

The gap junctional protein – a dilemma

Previous studies have presented evidence (see Introduction) that the gap junctional protein has a mol. wt. of 26–28 K. This presents an anomoly for which there are several possible explanations. (i) The 16 K protein is derived from the 26–28 K protein by endogenous proteolytic activity which is not inactivated by the added inhibitors. (ii) The 26–28 K protein is a dimeric form of the 16 K protein. (iii) The 16 K protein is unrelated to the 26–28 K protein and one (or both) is not derived from gap junctions. The most important question raised by these possible explanations, in the context of this present study, is whether the 16 K protein is or is not of junctional origin. This question is not simple to answer because the only known 'assay' for the presence of junctions during purification is electron microscopy. Although this method can be used with confidence to show that a given fraction contains gap junctions, it cannot be used to exclude the possible presence of other material. To overcome this problem, new methods have now been applied, using different properties of junctions, to correlate the structures, seen by electron microscopy, with their protein composition. These methods show that the 16 K protein is a major junctional component.

Isopycnic centrifugation of gap junctions

Previous work of Goodenough (1976) and Hertzberg and Gilula (1979), using continuous sucrose gradients, showed that gap junctions from mouse and rat liver have an average buoyant density of 1.17 g/cm³ and 1.165 g/cm³, respectively. It has been found that mouse liver gap junctions, purified using Triton, form a much sharper band in continuous potassium iodide density gradients (which are less viscous than sucrose gradients of the same density). The junction preparations produce two visible bands in the gradients, an upper opaque band and a lower band of aggregated material. Measurement of absorbance at 280 nm during collection of the gradients by upward displacement (Figure 5), gives a sharp, smooth peak (buoyant density, 1.195 g/cm³) partly separated from a series of spikes caused by the aggregated material in the second band (buoyant density 1.21 g/cm³) passing through the light path. Both bands occur reproducibly in gradients of junction preparations but the quantity of material in the lower band is variable and is greatest in preparations from older mice. Analysis of fractions collected from the gradients shows that the 16 K protein (quantitated by SDS-PAGE) is most abundant in the fractions containing most gap junctions (measured qualitatively by electron microscopy) and that these fractions, coincide with the 1.195 g/cm³ peak (Figures 5 and 6). The aggregated fibrous material of the lower band is insoluble in SDS sample buffer (and so can be pelleted) and no detectable gel bands can be related to these fractions.

Solubilisation of gap junctions in SDS

It has generally been assumed that gap junctions are solubilised in SDS solutions and that the junctional proteins can therefore be analysed by SDS-PAGE. Goodenough and Stoeckenius (1972) mentioned that mouse liver gap junctions are disrupted between 0.1% and 1.0% SDS but no morphological evidence is presented. The process and conditions of SDS solubilisation of junction preparations was therefore examined to correlate the loss or changes of gap junctions visible by electron microscopy with the appearance of soluble protein. Junctional preparations were suspended in different concentrations of SDS for 10 min at room temperature then centrifuged to pellet insoluble material and the pellets examined by negative staining (Figures 6–9). The pellets and the supernatants were also analysed by SDS-PAGE (Figure 10).

After treatment with 0.1% SDS, gap junctions are still present in the pellet and, apart from an apparent increase in the regularity of connexon packing, appear the same as untreated junctions. Gap junctions are still recognisable in large numbers in the pelleted material after extraction with 0.2% SDS but their appearance has changed (Figure 8). They have become 'patchy' where regions of connexons have been removed. These denuded regions vary in size from small holes where only a few connexons have been solubilised to large

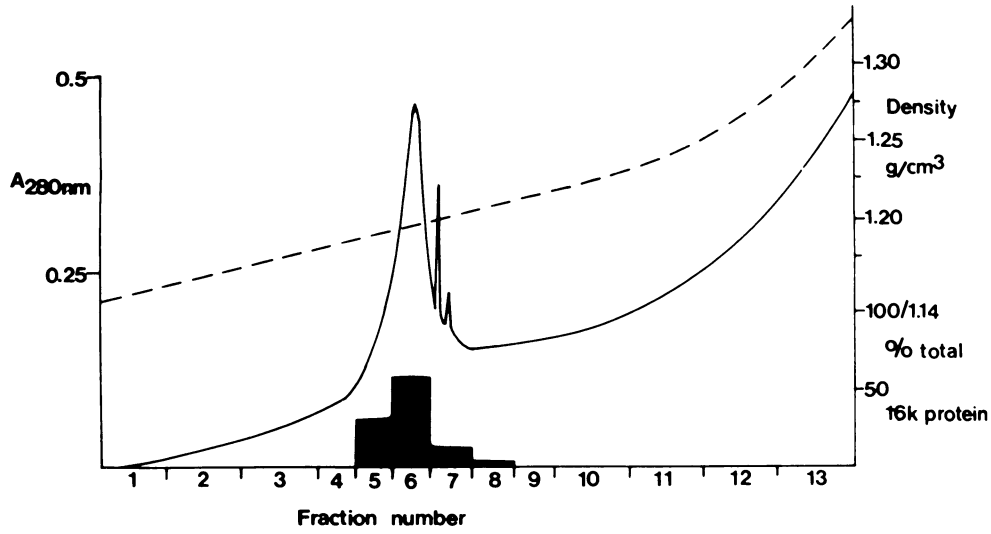


Fig. 5. Isopycnic centrifugation of mouse liver gap junctions in potassium iodide gradients. Gap junctions were prepared from mouse liver as described in Materials and methods. The final junction pellets were suspended in 0.5 ml 40% (w/v) KI and layered underneath a 12 ml linear KI gradient (20–38.3%). The gradient was centrifuged in the 6 x 14 rotor of an International B60 ultracentrifuge at 35 000 r.p.m., 15°C for 20 h and harvested by upward displacement with fluorochemical oil. $A_{280\ nm}$ was monitored using an LKB 'Uvicord' and 0.6 ml fractions were collected. After measuring the refractive index with an Abbe refractometer (calibrated with KI solutions of known density), pairs of fractions above (**1–4**) and below (**10–13**) the main peaks were combined to 1.2 ml fractions. The samples were each diluted to 4 ml with H_2O and centrifuged for 30 min at 40 000 r.p.m. in the 6 x 4.2 rotor of an International B60 ultracentrifuge. The pellets from each fraction were suspended in 6 μl H_2O and 3 μl used for electron microscopy after negatively staining, and 3 μl analysed by SDS-PAGE. The lower right-hand scale shows the percentage total 16 K protein in each fraction determined by microdensitometry of the Coomassie stained SDS-gels.

areas where upwards of a hundred connexons have been lost. The holes are angular which suggests that the solubilisation process runs preferentially along rows of connexons in a manner which might be expected of a quasi-crystalline structure. Similar angularities also occur around the edges of the junctional plaques suggesting that connexons are also lost, in a similar way, from the peripheries of the junctions, or that the junctions have fractured preferentially between connexon lines. After extraction with 0.3% SDS, gap junctions are still found in the pelleted materials but in reduced numbers. The residual junctions are more disrupted and generally much smaller although occasional large junctional plaques have been found (Figure 9). Some of the junctions remaining after treatment with 0.3% SDS may have vesicularized resulting in junction profiles where two similarly shaped junctional plaques appear to overlie each other. Junctions have not been found in the pellets after extraction with 0.5% SDS or with higher concentrations but occasional areas of fibrous and amorphous material, which are seen in all the pellets, are still present. These observations show that gap junctions are solubilized progressively with increasing concentrations of SDS and that the largest changes occur between 0.1% and 0.3%.

SDS-PAGE analysis of the pelleted material and the supernatant material shows a progessive movement of the 16 K protein from the insoluble to the soluble fraction as the SDS concentration increases, particularly between 0.1% and 0.3% (Figure 10) which suggests that the 16 K component is liberated as the junctions dissolve. In some preparations from mouse liver, the junctional fractions contain minor components which band on SDS-PAGE in the 21 K and 9 K regions, but these components are fully solubilised in 0.1% SDS.

The close self-association of the 16 K protein in gap junction preparations

The models of Makowski *et al.* (1977) and of Unwin and Zampighi (1980) show the junctional connexons to be made of six subunits, closely associated and possibly identical. If these models are correct, then short range cross-linking reagents such as glutaraldehyde might be expected to cross-link the subunits in the isolated junctions. Fixation of junction fractions in 1% glutaraldehyde fo 30 min results in the conversion of the 16 K protein to multimeric forms. The presumptive dimer and trimer (mol. wts. 26.5 K and 37 K) are the major cross-linked species although higher mol. wt. forms can sometimes be seen up to a presumptive hexamer. As yet, however, conditions have not been found under which a hexameric (or other) form becomes the predominant species.

Correlation between changes in gap junctional area and the recoverable amount of 16 K protein

Quantitative morphometric analysis of electron micrographs made from freeze-fractured preparations has shown that the total area of gap junctions decreases markedly after: (i) two thirds partial hepatectomy of weanling rats (reduced to 1.7% of control; Yancey *et al.*, 1979) and (ii) treatment of V79 cells with the tumour promoting agent TPA (4β-phorbol 12-myristate 13-acetate) (reduced to 6.7% of control; Yancey *et al.*, 1982). In the former case, the junctional area is minimal between 28 and 36 h post-operation.

The procedures for the purification of junctional protein depend on the isolation of intact junctional plaques, so the amount of protein recovered should be proportional to the total area of junctions seen by electron microscopy. The yield of 16 K protein from samples of liver 30 h after partial hepatectomy of 3–4 week-old rats falls to 90% compared with the yield from control animals (Figure 11). There is a similar 90% reduction in the amount of recoverable 16 K protein, 18 h after treatment of V79 cells with 2 x 10^{-7} M TPA with the amount returning to higher levels (78% control) 27 h after reversal. These changes in TPA-treated V79 cells are paralleled by changes in functional communication (data not given) measured by the extent of [^{3}H]uridine

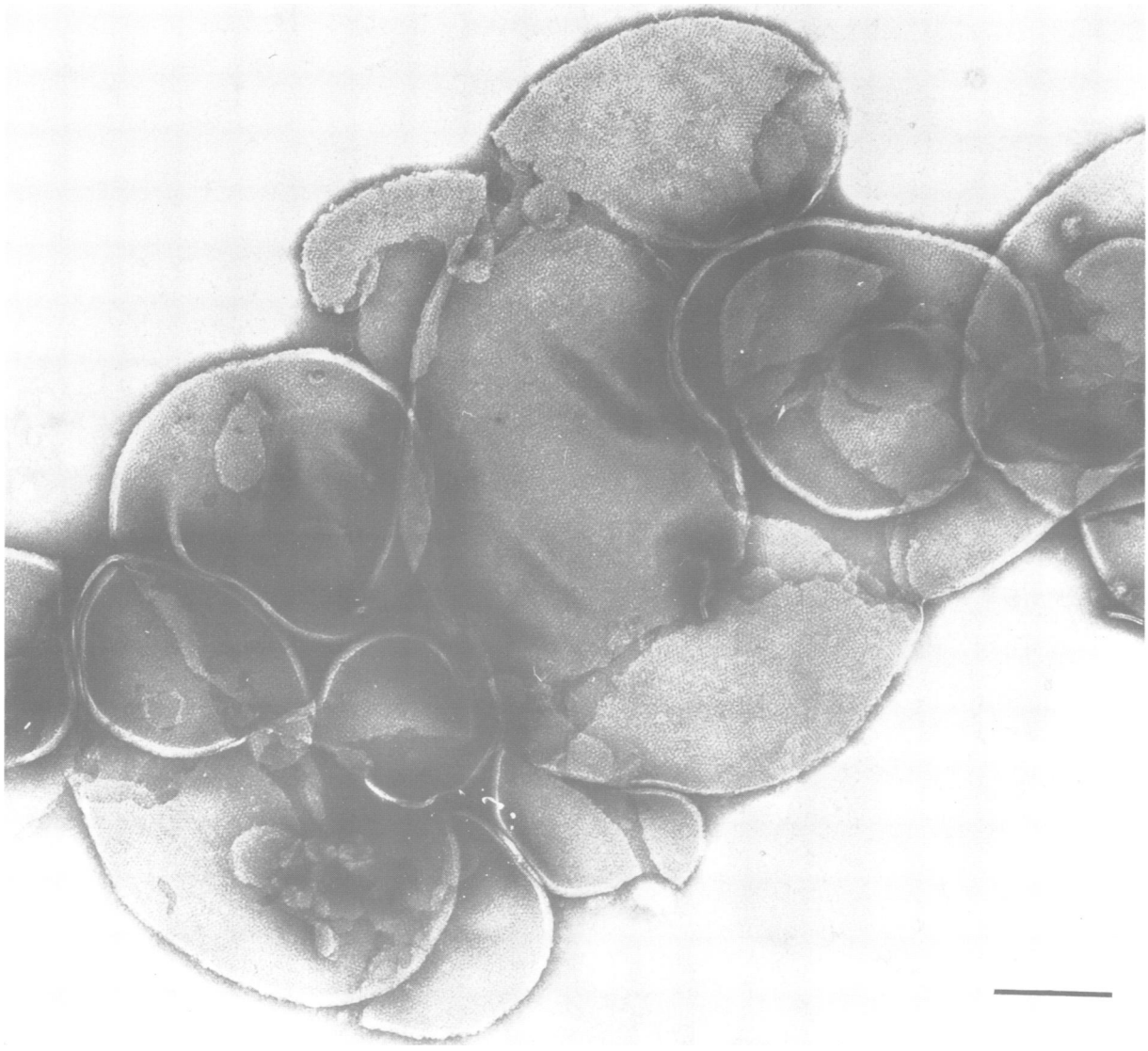


Fig. 6. Negatively stained mouse liver gap junctions from fraction 5 of potassium iodide gradient. For experimental details see legend to Figure 5. Bar represents 0.2 μm.

nucleotide transfer between cells in contact (Pitts and Simms, 1977; Newbold and Amos, 1981; Pitts and Bürk, submitted). In contrast, junctional communication between BRL cells (measured by [^{3}H]uridine nucleotide transfer) is unaffected by TPA treatment (Pitts and Bürk, submitted) which is consistent with the smaller reduction (27%) in the yield of the 16 K protein in such treated cultures compared with control cultures.

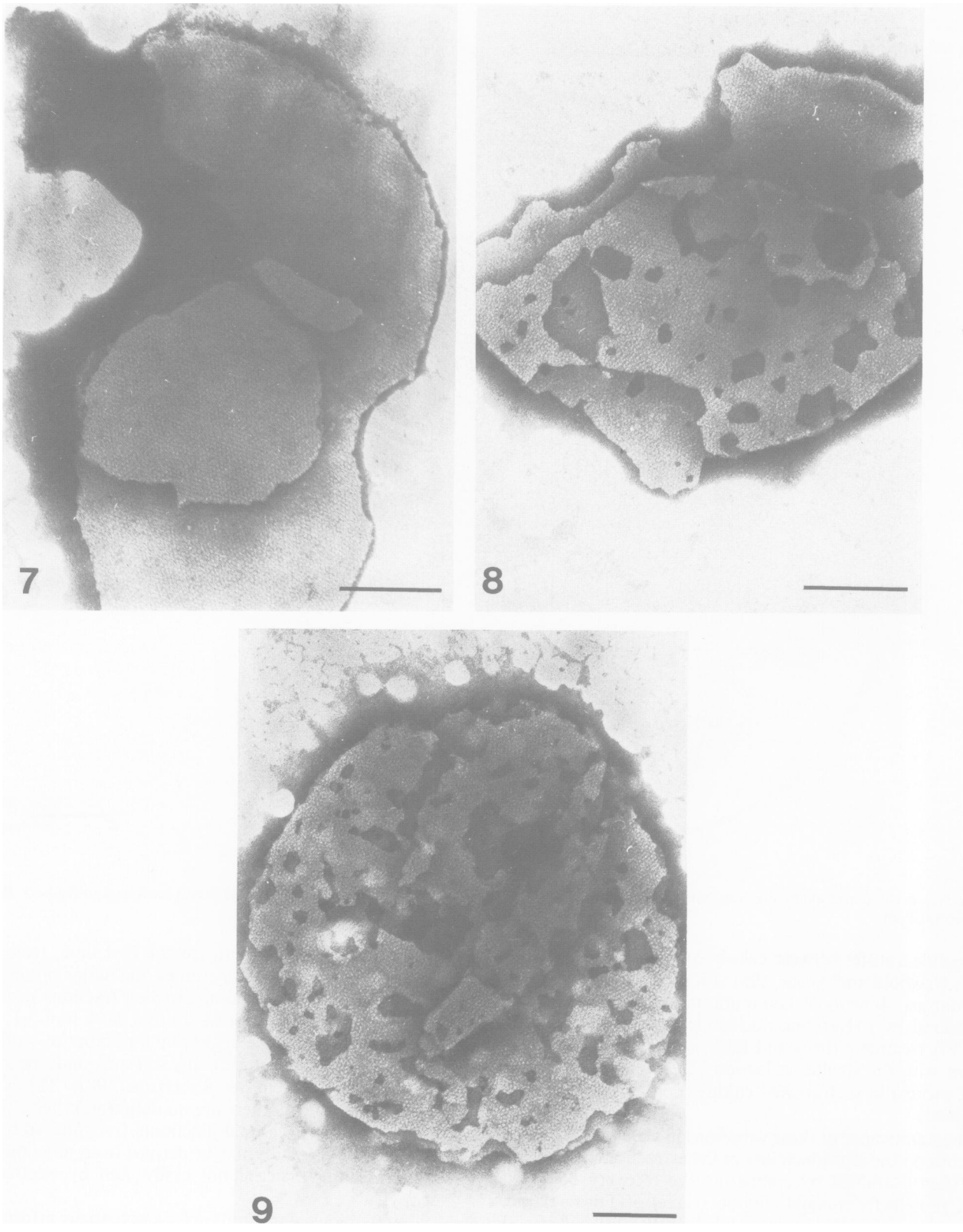
The significance of these variations in yield of 16 K protein depends on the reproducibility of the extraction method. This has been examined by comparing the recovery of labelled 16 K protein from equal aliquots (three) of Triton extracts, prepared from [^{35}S]methionine-labelled BRL cell cultures, the yield in such experiments varies by ±5%. It therefore seems unlikely that the large changes in the recovered amount of the 16 K protein, after partial hepatectomy of rats or TPA treatment of V79 cells, are due to chance variations in yield.

Discussion

The procedure for gap junction isolation described in this paper is simple, fast and versatile. Junctions can be prepared from different tissues and, for the first time, from cultured cells. In contrast to earlier studies, the major protein component found in gap junction enriched fractions produced by this method from vertebrate sources has a mol. wt. of 16 K.

Despite considerable efforts by a number of workers during the last 10 years, uncertainty still surrounds the identity of the junctional protein (see Robertson, 1981). This is because, as explained above, there are no definitive criteria for assessing the purity of the final junctional fractions, so bands produced by SDS-PAGE may be derived from junctions or from contaminating material not easily seen by electron microscopy.

Immunological methods offer a potentially informative approach to the identification of junctional components. An antiserum raised against a 26 K protein in gap junction preparations from mouse liver has been shown to bind to isolated junctional plaques (Janssen-Timmen *et al.* 1983). However, another antiserum raised against the viral transforming protein ppsrc60 shows a similar level of binding to gap junctions *in situ* (Willingham *et al.*, 1979). Furthermore, while Bok *et al.* (1982) found that an antiserum raised against



Figs. 7–9. Solubilization of gap junctions by increasing concentrations of SDS. Mouse liver gap junctions were prepared as described in Materials and methods and the final junction pellet suspended in 25 μl H_2O. Six equal (4 μl) aliquots were each added to an equal volume of a 2x concentrate of SDS in water to give final concentrations between 0% and 1.0% SDS. After 10 min at room temperature, the samples were centrifuged in an Eppendorf microfuge for 10 min. The supernatants (SDS-soluble fractions) were removed and an equal volume of 2x SDS sample buffer added before analysis by SDS-PAGE. The pellets (SDS-insoluble fractions) were washed with 200 μl H_2O, re-centrifuged and suspended in 10 μl H_2O. Half of the suspension was analysed by SDS-PAGE and the other half negatively stained for electron microscopy. The micrographs show gap junctions from the pellet fraction after 0.1% **(Fig. 7)**, 0.2% **(Fig. 8)** and 0.3% **(Fig. 9)** SDS extraction. Bars represent 0.15 μm.

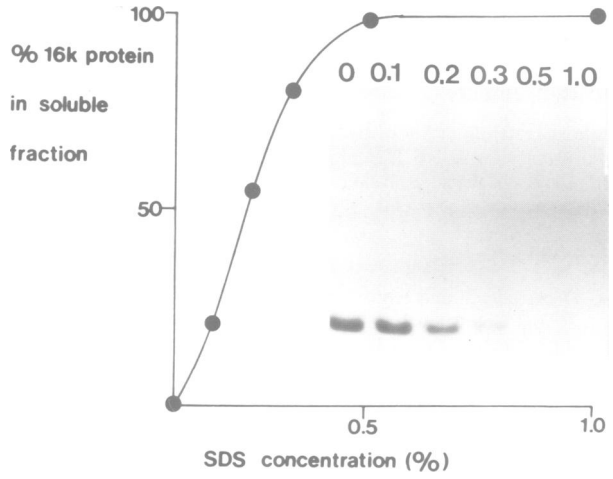


Fig. 10. Solubility of the mouse liver 16 K protein in SDS. For experimental details see legend to Figures 7–9. The amount of the 16 K protein in the SDS-soluble fraction (graph) and the SDS gel of the SDS-insoluble fractions are shown for each SDS concentration. The amount of the 16 K protein in each soluble fraction was measured by microdensitometry of the Coomassie stained gel and is expressed as a percentage of the value obtained after 1.0% SDS extraction.

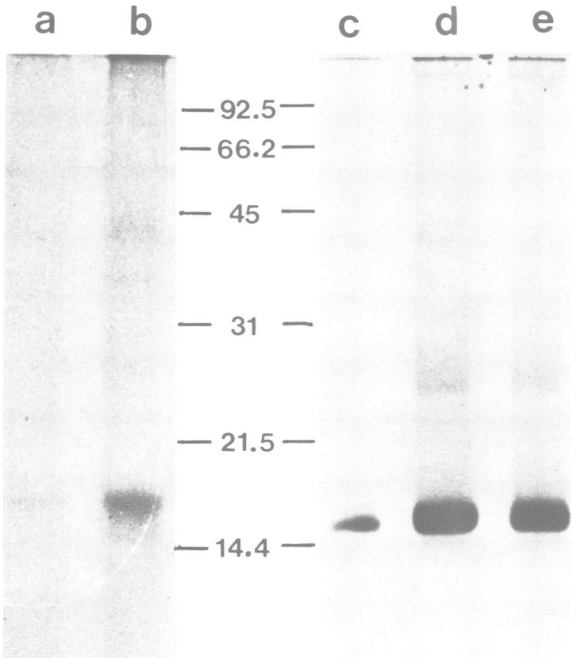


Fig. 11. The effect of partial hepatectomy of rats and TPA treatment of V79 cultures on the yield of the 16 K protein. 3–4 week-old weanling rats were divided into two groups. The livers were partially (2/3) removed (Yancey *et al.*, 1979) from one group (6 animals) and the other group left as controls (3 animals). Gap junctions were isolated as described in Materials and methods (except without the addition of N-lauryl sarcosine in the SP buffer) starting with 10 g wet weight of liver from 30 h post-operative rats (**lane a**) and control rats (**lane b**). One third of each of the final preparations were loaded in each lane. Confluent cultures of V79 cells were treated with 2×10^{-7} M 4β-phorbol 12-myristate 13-acetate for 18 h as described by Yancey *et al.* (1982) and junction fractions prepared as described in Materials and methods. **Lane c** shows fractions from TPA-treated V79 cells, **lane d** from the control and **lane e** shows fractions from cells 27 h after removal of the TPA.

the MIP26 (Main Intrinsic Protein, mol. wt. 26 000) of lens bound to isolated lens junctions, Paul and Goodenough (1983) showed that similar antisera bound only to non-junctional areas of lens fibre plasma membranes. The protein concentration in gap junctions is much higher than in non-junctional membrane and with the presently available antisera and techniques, some caution is necessary in interpreting these binding data.

This paper introduces a number of new approaches designed to use a wide range of different physical (buoyant density), chemical (detergent solubility), structural (cross-linking) and biological (changes induced by partial hepatectomy and TPA) characteristics of gap junctions, to relate the junctions to a specific protein. All these procedures, and the less stringent criteria which have been used previously, provide evidence for the junctional origin of the 16 K protein. Furthermore, the 16 K protein is the only common component of gap junction fractions prepared from different tissues (liver, heart, brain and uterus) and a wide range of cultured cell types. Different contaminants appear in preparations from different sources while the 16 K protein is always a major band and sometimes the only detectable band. Also, the yield of 16 K protein is consistent from a given source (e.g., 1–2 μg/g wet weight mouse liver no matter whether starting from a crude homogenate or a post-mitochondrial pellet) and varies in preparations from different sources, as would be expected from freeze fracture studies; liver >V79 cells >BHK cells (Yancey *et al.*, 1978, 1982; Revel *et al.*, 1971).

While no single line of investigation gives unequivocal evidence for the 16 K protein being a major junctional constituent, all the lines taken together provide the strongest evidence to date for the junctional origin of a particular protein. But this poses the question, 'how does the 16 K protein relate to the other proteins previously thought to be derived from gap junctions?'

As detergents are used for the isolation of gap junctions (in all methods) it is possible that the 16 K protein is derived by endogenous proteolysis (even in the presence of protease inhibitors) from a larger, native polypeptide. This could be the 26–28 K protein isolated by others (Hertzberg and Gilula, 1979; Henderson *et al.*, 1979; Finbow *et al.*, 1980; Nicholson *et al.*, 1981; Traub *et al.*, 1982) or this latter may be a dimeric form of the 16 K protein. This does not appear to be the answer, at least in the case of the 28 K protein isolated by Nicholson *et al.* (1981). Peptide mapping analysis by Nicholson and Revel (personal communication) shows that while there is considerable homology between the rat and mouse liver 16 K proteins (isolated by Finbow), there is no detectable homology between these and the rat liver 27 K protein (isolated by Nicholson). If the 26–28 K protein(s) described by the other groups is the same as the 28 K protein isolated by Nicholson *et al.*, then it appears that either the 26–28 K protein is not of junctional origin or that there are two (or more) classes of gap junction in the same tissue each containing different proteins. If this were so, it is conceivable that different isolation procedures could preferentially enrich for the different junctions. Application of the correlative techniques described in this paper to the 26–28 K component(s) should help to clarify the situation. However, analysis of the junction fractions produced by other published methods for mouse liver (Henderson *et al.*, 1979) and rat liver (Finbow *et al.*, 1980) show, in this laboratory, that while there are major bands in the 26–29 K region after SDS-PAGE, there is also a prominent 16 K band.

The versatility of the method presented in this study has permitted the preparation of gap junction fractions from

arthropod tissues (Figure 2). An 18 K protein, which exhibits the same property of dimerisation in 1% SDS solutions (to a 28 K form), has been found to be a major component of these fractions (Finbow *et al.*, 1983). Arthropod junctions might be expected to have a protein which is different from the vertebrate form because they have slightly larger dimensions (Perrachia, 1973a, 1973b).

Materials and methods

Preparation of gap junctions from mouse liver

A post-mitochondrial pellet was prepared as previously described (Finbow *et al.*, 1980) from mouse liver. The homogenate of 25 mice livers (20–30 g wet weight) was diluted to 2.1 litres in 1 mM $NaHCO_3$, 0.5 mM $CaCl_2$ (IB) and, after standing on ice, filtered through cheesecloth. The filtered homogenate was centrifuged for 15 min at 3000 r.p.m. in the Sorvall RC5 centrifuge (GS-3 rotor) and the supernatant discarded. The pellets were combined in 1 litre IB and the centrifugation repeated. The pellets (post-mitochondrial) were taken up in 150 ml SP buffer (0.15 M NaCl, 5 mM $NaPO_4$ pH 7.4) and 1.5 ml Triton X-100 (Sigma) added. After stirring for 5 min, the suspenson was centrifuged for 10 min at 3000 r.p.m. in a Sorvall GSA rotor. The supernatants were collected and the centrifugation repeated. N-Lauryl sarcosine (0.75 g; Sigma) was added and, when dissolved, the supernatants were centrifuged at 20 000 r.p.m. for 30 min in a Sorvall SS-34 rotor. The pellets were collected and taken up in 35 ml 1 mM $NaHCO_3$ and the centrifugation repeated. The pellets were taken up in 6 ml 6 M urea containing 1.5 mg tyrypsin (Sigma, type XI) and the suspension incubated at 37°C with occasional shaking for 20 min. N-Lauryl sarcosine in 6 M urea was added to a final concentration of 0.5% and the trypsinized material centrifuged again at 20 000 r.p.m. for 30 min. The pellet was taken up in a small volume of 1 mM $NaHCO_3$ and layered on top of a discontinuous gradient containing 4 ml 32% (w/v) sucrose and 3.5 ml 50% (w/v) sucrose and centrifuged at 35 000 r.p.m. for 1.5 h in an International B-60 ultracentrifuge (6 x 14 rotor). The junctions collected at the 32–50% sucrose interface, which after harvesting was diluted with 1 mM $NaHCO_3$ to 13.5 ml and centrifuged at 30 000 r.p.m. for 30–60 min to pellet the junctions. Preparations of junctions from tissues other than liver were carried out using the same method compensating for differences in wet weight of the starting material by appropriately adjusting the volumes of the buffers at each stage.

Preparation of gap junctions from cultured cells

Cells were maintained in Glasgow modification of Eagle's Medium (Flow Laboratories Ltd., Irvine, Scotland) supplemented with 10% foetal calf or calf serum. BRL cells (Pitts and Bürk, 1976) are derived from Buffalo rat liver and V79 cells (Newbold and Amos, 1981) are derived from Chinese hamster lung.

Confluent monolayers of cells (total growing area of 15 000 cm^2) were extracted with 280 ml SP containing 1% Triton for 5 min. The extract was then processed as described above except that N-lauryl sarcosine was not added after the initial two low speed centrifugations. The 20 000 r.p.m. pellet was taken up in 3 ml 6 M urea plus 0.75 mg trypsin and after addition of N-lauryl sarcosine to a final concentration of 0.5%, layered on top of a 32–50% discontinuous sucrose gradient. In experiments to measure total junctional protein (TPA treatment) the 50% sucrose was not included and the junctions centrifuged as above through 32% sucrose.

Electron microsopic analysis by negative staining

Junction pellets were suspended in 10 μl H_2O by vortexing and part (2 μl) of the suspension was mixed by pipetting with a 10 μl droplet of 2% phosphotungstate (pH 7.4) on a plastic weighing boat. The sample was dispersed by pipetting and then placed on to a carbon coated copper grid (300 mesh) and drawn through by blotting onto filter paper. Preparations were viewed in a Phillips 301 electron microscope operated at 60 kV.

SDS-PAGE

Junction pellets were suspended in a small volume of H_2O by vortexing and an equal volume of 2x concentrate of SDS solubilization buffer added (final SDS concentration of 1%; Laemmli, 1970). SDS-PAGE was carried out as described by Laemmli (1970) on 12.5% acrylamide gels measuring ~6.5 cm by 1 mm with a 1 cm stacking gel and stained with Coomassie blue R250. The Biorad low mol. wt. marker series were used for estimation of mol. wts. consisting of phosphorylase b (92.5 K), bovine serum albumin (66.2 K), ovalbumin (45 K), carbonic anhydrase (31 K), soybean trypsin inhibitor (21.5 K) and lysozyme (14.4 K). No change in the gel profile was observed when samples were left for 1 week at room temperature after SDS solubilization, or on storage at −20°C for prolonged periods (6 months) as pellets or after SDS solubilization. Quantitation of gel bands after Coomassie staining was carried out using a Joyce-Loebel densitometer.

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