Preparation of Hepatic Gap (Communicating) Junctions

IDENTIFICATION OF THE CONSTITUENT POLYPEPTIDE SUBUNITS

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1. Gap (communicating) junctions are plasma-membrane specializations of characteristic morphology that form transmembrane channels allowing direct communication between cells. Their preparation is described starting from mouse liver plasma membranes and the constituent polypeptides are deduced. 2. Gap junctions co-purify with collagen fibres when the plasma-membrane residues insoluble in *N*-dodecyl sarcosinate are fractionated on sucrose gradients. Sucrose-density perturbation by relipidation of isolated gap junctions or the use of urea to remove non-junctional membranes both failed to diminish the collagen content of fractions. 3. Removal of collagen by treatment with purified collagenase preparations yielded morphologically satisfactory gap-junction fractions. Analysis by polyacrylamide-gel electrophoresis of the polypeptides present in gap junctions prepared by procedures omitting or using collagenases indicated two non-glycosylated polypeptides, a major component of apparent mol.wt. 38000 and a minor 40000-mol.wt. component. These two polypeptides were also present in plasma membranes and the intermediate fractions. 4. Proteolysis of the gap-junction polypeptides yielding components of mol.wt. 34000, 25000 and below 20000 occurred when iodinated gap junctions were subject to prolonged collagenase treatment, thus explaining the variable polypeptide composition of gap junctions reported by others. 5. The morphological properties of the isolated gap junctions prepared by the various procedures are described.

It is becoming increasingly evident that direct communication between cells in tissues and culture is facilitated by transmembrane channels formed when the plasma membranes of co-operating cells are in close apposition to form gap junctions (Loewenstein, 1966; Revel & Karnovsky, 1967; Goodenough & Revel, 1970). The morphological features of gap (communicating) junctions have been studied extensively in a variety of tissues and species, especially by freeze-fracture and negative-staining techniques that show the universal presence of a highly regular polygonal lattice membrane structure. The permeability properties of these junctions have been studied by electrophysiological and metabolic techniques, which show that ions and nucleotides can pass freely between coupled cells (Bennett, 1973; Llinas *et al.*, 1974; Pitts & Sims, 1977; Simpson *et al.*, 1977).

Before defining the molecular organization of gap junctions and the mechanism of their formation, information is required about their composition and especially the nature of the proteins that presumably constitute the lattice structure comprising the transmembrane pores. Gap junctions may be isolated from liver plasma-membrane fractions by taking advantage of their relative insolubility in detergents (Benedetti & Emmelot, 1968; Gurd *et al.*,

1971; Evans & Gurd, 1972; Goodenough & Stoeckenius, 1972; Gilula, 1974). However, despite the preparation of gap junctions judged to be satisfactory by morphological criteria, much confusion has surrounded the number and apparent molecular weight of the constituent polypeptides. This is due to the adoption of collagenase-digestion steps designed to remove collagen fibres and amorphous material that co-purify with gap junctions. The present work is an extension of earlier work (Evans & Gurd, 1972) in which the residues of mouse liver plasma-membrane fractions that are insoluble in *N*-dodecyl-sarcosinate and urea are now subjected to a variety of procedures carried out in the presence or absence of purified collagenases to yield morphologically satisfactory junctions. The results allow the constituent junctional polypeptides to be deduced and explain the production of proteolysis artifacts obtained by others (Goodenough, 1974, 1976).

Experimental

Preparation of plasma membranes

A plasma-membrane fraction was prepared from liver homogenates (100–150 g) obtained from 60–70 Parkes white mice (20–30 g). A low-speed ‘nuclear’ pellet was resolved by a rate-zonal centrifugation

step (MSE A XII rotor), and the plasma membranes were further purified by gradient centrifugation (Evans, 1970). The properties of these plasma membranes have been fully documented (Evans, 1970; Evans & Gurd, 1972). The plasma membranes were kept as pellets covered with 0.25M-sucrose at 4°C up to 3 days.

Preparation of gap-junction fractions

(a) *Without enzymic treatment.* Membranes from three separate preparations made on successive days (approx. 200 mice) were pooled and gap junctions prepared as previously described (Evans & Gurd, 1972) with the following modifications. Plasma membranes (approx. 400mg of protein) were extracted with 117ml of a sarcosyl (*N*-dodecyl-sarcosinate)/Tris buffer, pH7.8, by resuspension with six strokes in a tight Dounce homogenizer at 4°C, and the residue was re-extracted with 51 ml of

sarcosyl/Tris buffer, pH7.8. The final residue (see Scheme 1) was sonicated (microprobe, type 7530A; Dawe Instruments, London W.3, U.K.; set at mark 2) for two 10s periods separated by cooling in ice, and then centrifuged in discontinuous sucrose density gradients. The material at the 54/72% (w/v) sucrose interface was collected, diluted and then washed with 2×5 ml of 5mM-Tris/HCl, pH8.0, by centrifugation (3000g, 10min; MSE Mistral) and resuspension.

(b) *By using collagenase treatment steps.* Since this procedure yielded the best gap junctions morphologically, this is shown in Scheme A. After the second sarcosyl extraction, the pellet was resuspended in 10ml of 50mM-Tris/HCl, pH7.6, and incubated for 45min with 2400 units (defined by Sigma) of collagenase (Sigma type III prepared from *Clostridium histolyticum*; Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.) at 37°C. The preparation

Scheme 1. Preparation of hepatic gap junctions with minimal exposure to purified collagenase

was then washed by dilution in 5 mM-Tris/HCl, pH 8.0, and then fractionated by centrifugation in sucrose gradients (Scheme 1). The material at the 54–72% (w/v) sucrose interface was darker than that obtained in procedure (a). After collection and washing of the material, the pellet was resuspended in 5 ml of 50 mM-Tris/HCl, pH 7.6, and incubated with a further 2400 units of collagenase (see above) for 45 min at 37°C. The final pellet was then washed with 1 × 5 ml of 0.9% NaCl and 3 × 5 ml of 5 mM-Tris/HCl, pH 8.0, by centrifugation at 3000g for 10 min.

(c) *Extraction with urea and sarcosyl/Tris buffer.* Plasma membranes (144 mg of protein) were extracted with 3 × 20 ml of 7 M-urea dissolved in 1 M-Tris/HCl, pH 8.0 at 4°C. The final pellet was resuspended in 10 ml of 0.25 M-sucrose and centrifuged in discontinuous sucrose density gradients constructed of 15 ml of 72% (w/v) sucrose and 10 ml of 33% (w/v) sucrose. Membranes at the 33/72% (w/v) sucrose interface were collected and further extracted with 1% sarcosyl/Tris buffer, pH 7.8. The final residue was recentrifuged in a discontinuous sucrose density gradient (composition as above), and the material at the 33/72% sucrose interface collected and washed by centrifugation at 3000g for 10 min with 3 × 5 ml of 5 mM-Tris/HCl, pH 8.0.

(d) *Density perturbation of gap junctions.* Since gap-junction fragments are lipid-depleted (Evans & Gurd, 1972), relipidation was attempted to separate them on sucrose gradients from collagen fibres. Phosphatidylcholine (1 mg) (egg yolk; Koch-Light, Colnbrook, Bucks., U.K.) was dispersed in 5 mM-Tris/HCl, pH 8.0, by addition of 1 mg of sarcosyl or sodium deoxycholate, and 100 µg of the gap-junction fraction (made by procedure a) added. The sample (2 ml) was resuspended in 60% (w/v) sucrose and sonicated (2 × 10 s, Dawe microprobe) and layered on a 2 ml cushion of 72% (w/v) sucrose. The sample was overlaid with 2 ml each of 55, 45, 35, 25 and 8% (w/v) sucrose. After centrifugation for 2 h at 97000g a fraction containing gap junctions was collected at the 45/55% (w/v) sucrose interface, and washed with 2 × 5 ml of 5 mM-Tris/HCl, pH 8.0.

Iodination of gap-junction fractions

Samples were iodinated with ¹²⁵I by using the Bolton & Hunter reagent (The Radiochemical Centre, Amersham, Bucks., U.K.) as described by Bolton & Hunter (1973). Gap-junction protein (up to 100 µg) was incubated with 5 µCi of reagent in 50 µl of 0.1 M-sodium borate/HCl buffer, pH 8.5, for 16 h at 4°C. Gap junctions were washed twice by centrifugation, at 3000g for 10 min in 5 ml of 0.2 M-glycine dissolved in 0.1 M-borate/HCl buffer, pH 8.5, once in this borate buffer, and twice in 5 mM-Tris/HCl, pH 8.0. About 40% of the ¹²⁵I radioactivity added was incorporated into the sample.

Polyacrylamide-gel electrophoresis

Electrophoresis in slab gels (15 cm × 12 cm × 0.15 cm) was carried out by using the discontinuous buffer system of Laemmli (1970). Membranes were dissolved in a sample buffer containing 2% (w/v) sodium dodecyl sulphate, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 40 mM-Tris/HCl, pH 6.7, by heating at 95°C for 2–5 min. After electrophoresis, polypeptides were stained with Coomassie Blue. Standards used as molecular-weight markers were β-galactosidase (130 000), phosphorylase (94 000), catalase (60 000), aldolase (40 000), pepsinogen (41 000), carbonic anhydrase (29 000), myoglobin (17 000) and cytochrome c (12 000). The following reference proteins were also run: tubulin (pig brain), actin (pig muscle) and collagen (cultured chick cells) given by staff of this Institute. Glycoproteins separated in polyacrylamide gels were identified on the basis of their ability to bind specifically ¹²⁵I-labelled concanavalin A (Gurd & Evans, 1976). Briefly, concanavalin A (3 × crystallized; Miles Laboratories, Slough, Bucks., U.K.) was iodinated with Na¹²⁵I by using the chloramine-T method (Greenwood *et al.*, 1963) in the presence of 10 mM-α-methyl D-mannopyranoside and then dialysed extensively against 0.1 M-sodium phosphate, pH 7.4. Gels, after being washed with several changes in incubation buffer (50 mM-Tris/HCl, pH 7.4, 0.25 mM-MgCl₂, 0.25 mM-CaCl₂), were incubated in ¹²⁵I-labelled concanavalin A (16 µg/ml; 0.4 µCi/µg dissolved in incubation buffer) for 2 h at 24°C. After extensive washing, gels were dried, exposed to Kodak X-ray film and scanned by using a Joyce-Loebl (Gateshead, U.K.) microdensitometer.

Electron microscopy

Gap-junction fractions were routinely examined by negative staining in 2% (w/v) sodium silicotungstate and observed in a Philips EM-300 or JEOL Jem 100C electron microscope. Samples used for thin sectioning were prepared as described by Hirsh & Fedorko (1968), and stained with uranyl acetate and lead citrate.

Other procedures

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Sarcosyl/Tris buffer, pH 7.8, was prepared routinely by dissolving Sarkosyl (Geigy, Simonsway, Manchester, U.K.) and Tris (Sigma Chemical Co.) at a weight ratio of 1:0.545.

Results and Discussion

The objective of the present study was to prepare gap-junction fractions judged as satisfactory on morphological and biochemical grounds, and especi-

ally to ascertain the number and nature of the polypeptide components present in the plasma-membrane region forming the junctional complex. It was found that morphological purity could only be achieved at the expense of varying degrees of proteolytic hydrolysis of the junctional polypeptides ensuing after addition of collagenase. However, by a combined ultrastructural-biochemical analysis of gap-junction fractions prepared by four methods, evidence for the identity of major polypeptides comprising the junctions was obtained.

Preparation of gap junctions without enzymic treatment

The procedure of Evans & Gurd (1972) for the preparation of a gap-junction ('nexus') fraction was modified by decreasing the ratio of sarcosyl/Tris buffer to plasma-membrane protein and by the introduction of sonication steps that helped to lessen the number of lipid-rich vesicles adhering to the gap junctions. In addition to gap junctions, this fraction also contained collagen fibres, some vesicular profiles and amorphous material (Plate 1*a*). Analysis of this fraction by polyacrylamide-gel electrophoresis indicated three groups of polypeptides (Plate 3, gel *g*): two major bands of apparent mol.wt. greater than 130000 that co-electrophoresed with collagen, and occasionally a minor amount of a 125000-mol.wt. component, shown to be a contaminant present in the lipid-rich vesicles by analysis of the sarcosyl/Tris-insoluble light fraction (Scheme 1), varying but minor amounts of several components of mol.wt. near 56000 (co-electrophoresing with tubulin), and two polypeptides, a minor 40000- and a major 38000-mol.wt. component. Very little material lower in molecular weight than these components was detected. The polypeptide profiles were unaltered when mercaptoethanol was omitted from the solution used to solubilize membranes. The polypeptides of mol.wt. 38000 and 40000 were present in all the intermediate steps leading from the liver plasma-membrane fraction to the gap-junction

fraction (Plate 3, gels *c-e*). Analysis of pig mesenteric-node lymphocyte plasma membranes showed a major polypeptide identified as actin (Barber & Crumpton, 1976) (a similar component, presumably actin, was also present in liver plasma membranes; Brandon, 1976), but the 38000- and 40000-mol.wt. components were not seen (Plate 3, gel *b*). There is little evidence for communication across gap junctions between lymphoid cells (Sanderson *et al.*, 1977).

Gap-junction preparations of similar morphology, polypeptide composition and yield (Table 1), were also obtained when liver plasma membranes were extracted successively with 7M-urea/1M-Tris/HCl, pH 8.0, and sarcosyl/Tris buffer, followed by a further density-gradient-centrifugation step (see the Experimental section). In this procedure, the copurification of gap junctions and collagen fibres also occurred. The results confirmed the presence of the three groups of polypeptides obtained in the absence of a urea-solubilization step.

In a further attempt to remove collagen without resorting to enzymic digestion, the gap-junction fraction was relipidated by resuspension in detergents containing dispersed lipids. Uptake of lipids by the gap junctions decreased their density, but morphological examination of the relipidated fraction indicated that collagen fibres remained. Negative staining also indicated a decrease in lattice order and increased subunit centre-to-centre spacing (Plate 2). Examination of the polypeptide components present in the relipidated gap-junction fraction also yielded similar results, the densitometric tracings (Fig. 1) again indicating the presence of three groups of components of molecular weights corresponding to the position of collagen, the 56000-mol.wt. and 38000-40000-mol.wt. positions.

Preparation of gap junctions by using a collagenase-digestion step

Extensive electron microscopy of gap-junction fractions indicated that collagen fibres were not

Table 1. *Distribution of protein during preparation of gap junctions from plasma membranes*

Calculations are normalized for 100g wet wt. of liver. The starting weight for three pooled membrane preparations was approx. 320g of liver. Where appropriate, values in mg of protein represent the means $\pm$ S.D., with the numbers of preparations in parentheses.

Preparative procedure	Plasma membranes	Sarcosyl-insoluble	Sarcosyl-insoluble light	Sarcosyl-insoluble heavy (gap-junction fraction)
Without enzymic treatment	157 $\pm$ 25 (5)	2.26 $\pm$ 0.76 (5)	0.29 $\pm$ 0.12 (4)	0.40 $\pm$ 0.16 (4)
Using collagenase treatment	144 $\pm$ 9.4 (6)	1.44 $\pm$ 0.39 (6)*	0.14 $\pm$ 0.067 (6)†	0.18 $\pm$ 0.54 (5)†
Urea and sarcosyl extraction	131	—	0.016	0.41
	Urea-insol.: 71			
	Urea-insol. heavy:‡ 23.5			

* Determination after one collagenase treatment.

† Determination after two collagenase treatments.

‡ Membranes collected at 33/72% (w/v) sucrose interface (see the Experimental section).

EXPLANATION OF PLATE I

Electron micrographs of sections through gap-junction preparations

Views (a)(1) and (2) show sections through a gap-junction fraction prepared without enzymic treatment. Collagen, showing a characteristic banded pattern and gap junctions in longitudinal section showing a heptalaminar structure at higher magnification [(a)(3)] are seen. Small vesicles and amorphous material are also evident. Gap-junction fractions prepared by using two collagenase treatments (Scheme 1) [(b)(1)] were relatively free of amorphous material and collagen, and the heptalaminar structure is preserved [(b)(2)]. The bars represent $0.1\mu\text{m}$.

EXPLANATION OF PLATE 2

Electron micrographs of negatively stained gap-junction fractions

View (a) shows the hexagonal lattice structure (front view) of gap junctions prepared without enzymic treatment. View (b) shows gap junctions prepared by using urea and sarcosyl extractions (see the Experimental section). Collagen strands and bundles are evident, and there was no change in lattice appearance or spacing of the gap junctions. View (c) shows gap junctions prepared after relipidation (see the Experimental section); the lattice order was decreased and the subunit centre-to-centre spacing increased. The edges of the junctional sheet tended to curl up (arrow) to show the double-membrane structure in cross-section. The bar represents $0.1\ \mu\text{m}$.

EXPLANATION OF PLATE 3

Polyacrylamide-gel electrophoresis of fractions isolated during gap-junction preparation without enzymic treatment

The samples applied to a 10–15% (w/v) gradient polyacrylamide gel were (a) marker proteins: tubulin, actin and pepsinogen; (b) lymphocyte plasma membranes prepared from pig mesenteric lymph nodes (Walsh *et al.*, 1976); (c) liver plasma membranes; (d) sarcosyl-soluble fraction; (e) sarcosyl-insoluble fraction; (f) sarcosyl-insoluble light fraction; (g) sarcosyl-insoluble heavy fraction (gap-junction fraction); 5–10 μg of standard proteins (see the Experimental section for mol.wts.) and 50–100 μg of membrane protein were applied to each gel. The three major groups of polypeptides in the gap-junction protein (see the text) are indicated; group 1, apparent mol.wt. 130 000 (corresponding to the position of collagen); group 2, apparent mol.wt. 54 000–56 000 (corresponding to the position of tubulin); group 3, apparent mol.wt. 38 000 and 40 000 (assigned as the gap-junction polypeptides).

EXPLANATION OF PLATE 4

Electrophoresis of a gap-junction fraction showing the effects of treatment with enzymes

Electrophoresis was carried out in a 10–15% polyacrylamide gradient gel. Gap junctions prepared without enzymic treatment (a) were compared with: (b) gap junctions exposed after the density-gradient step (Scheme 1) to 40 μ g of collagenase; (c) junctions exposed to 400 μ g of collagenase; (d) 10 μ g of collagenase; (e) gap junctions exposed to 40 μ g of trypsin; (f) junctions exposed to 400 μ g of trypsin; (g) 10 μ g of trypsin. Gap junctions (50 μ g of protein) suspended in 0.5 ml of 50 mM-Tris/HCl, pH 7.6, were incubated for 45 min at 37°C with Sigma type III collagenase or Sigma type III trypsin. The gap junctions were washed by resuspension in 10 ml of water, centrifuged at 3000g for 10 min, and then resuspended in electrophoresis sample buffer (see the Experimental section). After collagenase treatment the major contaminants were bands indicated by the small arrowheads. The major gap-junction protein band that decreased in intensity is indicated by large arrowheads. Treatment of gap junctions with trypsin also resulted in the appearance of additional bands of low molecular weight, and the gap-junction protein disappeared.

EXPLANATION OF PLATE 5

Polyacrylamide-gel electrophoresis of gap junctions treated with collagenase after iodination

Samples were run on a 10–15% grading gel; (a) collagen (two bands), tubulin (T), and actin (A) stained with Coomassie Blue; (b) gap junctions stained with Coomassie Blue; (c) radioautograph of enzymically untreated gap junctions; (d) radioautograph of gap junctions treated with collagenase after iodination (1 mg of Sigma type III, in 0.5 ml of 50 mM-Tris/HCl, pH 7.6, 45 min, 37°C). The major 38 000-mol.wt. component of the gap junction samples is indicated (arrowheads).

Fig. 1. Polyacrylamide-gel electrophoresis of relipidated gap junctions

The samples were electrophoresed in a 10–15% polyacrylamide gradient gel. After staining with Coomassie Blue the gels were scanned at 595 nm: (a) plasma membranes; (b) gap junctions prepared without enzymic treatment; (c) junctions relipidated with phosphatidylcholine dispersed in deoxycholate; (d) gap junctions relipidated with phosphatidylcholine dispersed in sarcosyl. Reference standards (see the Experimental section), collagen (C); tubulin (T) and actin (A), are indicated on the scale. The gels of the gap junctions (b–d) were scanned at a 10-fold higher sensitivity than the plasma membranes (a).

directly associated with gap junctions (Plates 1a, 2a, 2b). Thus, to remove collagen contamination, additional steps involving the controlled addition of collagenase were included in the preparative procedure (Scheme 1). Because of the molecular heterogeneity and proteolytic activity associated with various collagenases, enzyme substantially free of proteinase and peptidase activities was used. The procedure shown in Scheme 1 yielded a gap-junction fraction that was largely free of collagen, vesicles and amorphous material (Plate 1b), and the yield of protein in this gap-junction fraction was much decreased (Table 1). Polyacrylamide-gel electrophoresis of these gap junctions gave variable results (Plate 4). This variation in polypeptide composition was found to be caused by the adhesion of added enzyme to the gap junctions and ensuing proteolysis of the junction-associated polypeptides. Thus, although treatment of gap junctions with collagenase decreased the collagen contamination (shown also by morphological examination), electrophoresis showed a decrease in the components of mol.wt. 56000 and 38000–40000, and variable amounts of additional bands appeared (Plate 4, gels a–d). Two of these additional polypeptides were of mol.wt. 130000 and 42000 and were shown to be contributed by the Sigma type III collagenase (Plate 4, gel d); other commercial collagenase preparations yielded different profiles. A number of other components of mol.wt. below 38000 were also produced after exposure to collagenase. Hence the polypeptides present in the gap-junction complex are the 56000-, 38000- and 40000-mol.wt. components. The bands of mol.wt. approx. 55000 varied between various preparations, and were always of lower staining intensity than was the 38000-mol.wt. band. The bands in the 55000-mol.wt. region and the minor 40000-mol.wt. band were also highly susceptible to degradation after enzymic treatment. Thus the 38000-mol.wt. band emerges from these studies as the most likely major junctional protein. Treatment of gap junctions with trypsin also modified the polyacrylamide-gel-electrophoretic profiles by producing low-molecular-weight polypeptides (Plate 4, gels e–g). Morphologically recognizable junctions apparently remain after trypsin treatment (Goodenough, 1976).

To investigate further the nature of the proteolytic products, and to confirm the molecular weight of the junctional polypeptides, a gap-junction fraction initially prepared in the absence of an enzymic step was iodinated. Subsequent treatment of these iodinated junctions with collagenase indicated by radioautography that exposure to collagenase decreased the 56000- and 40000-mol.wt. peak regions and the 38000-mol.wt. peak to a lesser extent and that radioactive peaks of mol.wt. 34000, 32000, 25000 and others below 20000 appeared (Plate 5). This demonstration by radioautography showed more directly

than by protein staining (Plate 4) that the gap-junction polypeptides were indeed readily susceptible to proteolysis by added enzymes, and allowed the nature of the proteolytic products to be more clearly identified. The variable components of low molecular weight produced after crude collagenases were used to prepare gap junctions are now explained (Goodenough, 1974; Gilula, 1974). Under mild conditions of collagenase treatment the 38 000-mol.wt. component was more resistant to proteolysis. The results suggest that three major populations of polypeptides are found on exposure to proteolytic enzymes: those of 34 000 and 25 000 mol.wt. and others below 20 000 mol.wt.; these proteolytic products may correspond to 'connexins' described by Goodenough (1974).

Identifying the junctional polypeptides

On the basis of the correlation of ultrastructural and polypeptide analysis in gap junctions prepared as described, the above information narrows down the junctional polypeptides to the 38 000- and 40 000-mol.wt. components, and all polypeptides of a lower molecular weight must now be considered as proteolysis products. Since these two polypeptides were also present in plasma membranes and the intermediate fractions prepared, they are unlikely to have arisen themselves by degradation. Furthermore, since the 38 000-mol.wt. component was always present in large excess over the 40 000-mol.wt. component, it appears to be the most likely candidate for forming the hexagonal array network evident in negatively stained and freeze-fractured membranes. In the plasma-membrane fraction and the sarcosyl-soluble fraction (Plate 3, gels *c* and *d*) these two polypeptides were present in similar amounts. It is possible that the 40 000-mol.wt. polypeptide is loosely attached to the junctional complex and is partially lost during the preparation of gap junctions.

On the basis of their function in forming communication channels allowing ions and small molecules (up to 1200 mol.wt.; Simpson *et al.*, 1977) to move between contiguous cells, the gap-junction proteins are likely candidates for traversing the lipid bilayer. Thus in this context, and also in considering the molecular basis of the specificity of junctional formation between the cells, it is important to determine the properties of the components constituting the junctional channel, especially whether they are glycoproteins. This was investigated by exposing the polypeptides of plasma membranes and gap junctions, separated by electrophoresis on polyacrylamide gels, to an iodinated lectin, a sensitive method for identifying glycosylated polypeptides (Gurd & Evans, 1976). Fig. 2 shows that the junctional polypeptides of mol.wt. 38 000 and 40 000 did not bind 125 I-labelled concanavalin A, although some other components

Fig. 2. Binding of 125 I-labelled concanavalin A to polypeptides of plasma-membrane and gap-junction fractions. Gel electrophoresis was carried out in a 10–15% (w/v) polyacrylamide gradient. After staining with Coomassie Blue, densitometric scans of the radioautographic film of the gels exposed to 125 I-labelled concanavalin A (see the Experimental section) were made: (a) plasma-membrane fraction; (b) gap-junction fraction; (c) plasma-membrane fraction incubated with lectin in the presence of 2.5% (w/v) α -methyl D-mannopyranoside (control). Arrows on the molecular-weight scale indicate the position of the gap-junction polypeptides of mol.wt. 38 000 and 40 000.

in plasma membranes, and the components of mol.wts. 130 000 and 56 000 in the gap-junction fraction, bound the lectin.

The present results also suggest a possible association or interaction of the gap-junction part of the plasma membrane with submembranous or cytoplasmic components. A major polypeptide in the liver plasma-membrane fraction corresponding to the position of actin was absent from the gap-junction fraction (Plate 3; Fig. 1). The bands of mol.wt. 56 000 present in the gap-junction fraction that co-electrophoresed with tubulin also bound 125 I-labelled concanavalin A.

Molecular heterogeneity of microtubular components, including the possible presence of a glycosylated tubulin, has been demonstrated (Truding *et al.*, 1975). Although further verification of the authenticity of tubulin and the association of microtubular

element with the gap junction is required, the present results, when taken with demonstrations by immunofluorescence microscopy that microtubules interact with the plasma membrane of a cultured cell line at points of intercellular contact (Weber, 1975), suggest that rapid intercytoplasmic communication mediated by microtubules and gap junctions acting in concert may occur.

Freeze-fracture studies indicate that the formation of gap junctions can be seen as the aggregation of a population of intramembranous protein particles to form the crystalline lattice network in which there is not only strong protein-protein interaction between laterally adjacent particles, but also strong and specific interaction between corresponding particles of the contributing plasma membranes. The properties of the component of mol.wt. 38000 (i.e. the protein is very insoluble in aqueous solvents and has a high ratio of hydrophobic amino acids and is cross-linked by bifunctional reagents; J. C. Carreira & W. H. Evans, unpublished work) also indicate that this polypeptide is probably the major junctional component. Correlative ultrastructural and biochemical approaches cannot provide definitive answers, and further extension of this work by the reconstitution of the junctional polypeptide of mol.wt. 38000 into membranes, and the labelling of animal cell membranes by antisera raised against the junctional polypeptide, will help to support the present identification.

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References

- Barber, B. H. & Crumpton, M. J. (1976) *FEBS Lett.* **66**, 215-220
- Benedetti, E. L. & Emmelot, P. (1968) *J. Cell Biol.* **38**, 15-24
- Bennett, M. V. L. (1973) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **32**, 65-75
- Brandon, D. L. (1976) *Eur. J. Biochem.* **65**, 139-146
- Bolton, A. E. & Hunter, W. M. (1973) *Biochem. J.* **133**, 529-539
- Evans, W. H. (1970) *Biochem. J.* **116**, 833-842
- Evans, W. H. & Gurd, J. W. (1972) *Biochem. J.* **128**, 691-700
- Gilula, N. B. (1974) *J. Cell Biol.* **63**, 111a
- Goodenough, D. A. (1974) *J. Cell Biol.* **61**, 557-563
- Goodenough, D. A. (1976) *J. Cell Biol.* **68**, 220-231
- Goodenough, D. A. & Revel, J. P. (1970) *J. Cell Biol.* **45**, 272-290
- Goodenough, D. A. & Stoebenius, W. (1972) *J. Cell Biol.* **54**, 646-656
- Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.* **89**, 114-123
- Gurd, J. W. & Evans, W. H. (1976) *Can. J. Biochem.* **54**, 477-480
- Gurd, J. W., Evans, W. H. & Perkins, H. R. (1971) *Biochem. J.* **122**, 43p-44p
- Hirsch, J. G. & Fedorko, M. E. (1968) *J. Cell Biol.* **38**, 615-627
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685
- Llinas, R., Baker, R. & Sotelo, C. (1974) *J. Neurophysiol.* **37**, 560-571
- Loewenstein, W. R. (1966) *Ann. N.Y. Acad. Sci.* **137**, 441-472
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Pitts, J. D. & Simms, J. W. (1977) *Exp. Cell Res.* **104**, 153-164
- Revel, J. P. & Karnovsky, M. J. (1967) *J. Cell Biol.* **33**, C7-C12
- Sanderson, C. J., Hall, P. J. & Thomas, J. A. (1977) *Proc. R. Soc. London Ser. B* **196**, 73-84
- Simpson, I., Rose, B. & Loewenstein, W. R. (1977) *Science* **195**, 294-296
- Truding, R., Shelanski, M. L. & Morell, P. (1975) *J. Biol. Chem.* **250**, 9348-9354
- Walsh, F. S., Barber, B. H. & Crumpton, M. J. (1976) *Biochemistry* **15**, 3557-3563
- Weber, K. (1975) in *Microtubules and Microtubule Inhibitors* (Borgers, M. & De Brabander, M., eds.), pp. 313-325, North-Holland, Amsterdam