BIOCHEMISTRY AND DIVERSITY OF THE GAP JUNCTION PROTEIN: A STUDY OF LIVER, HEART AND LENS

Thesis by

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To Mum, Dad and Nanny

who made all of it possible

To my wife, Xochitl

who made these years a joy

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Abstract

Fractions highly enriched for gap junctions by morphological criteria have been isolated from rat liver, heart and eye lens, although some question exists as to the nature of the structures from lens. The junctions from each tissue are comprised of a single major protein of M_r 28,000 in the liver, M_r 30,000 in the heart, and M_r 26,000 (MIP 26) in the lens. The polypeptide profile of the liver fraction is complicated by endogenous proteolysis and aggregation in SDS of the gap junction protein and the presence of about 20% non-junctional material. Heart and lens junction proteins are also found to aggregate in SDS, while endogenous proteolysis typically reduces the cardiac gap junction protein to M_r 28,000 during isolation.

Comparisons of two-dimensional peptide maps of the junctional proteins from these tissues, and the use, where necessary, of a third dimension of resolution (HPLC), demonstrates the three proteins tc be very different in terms of their primary structures. The protein of each tissue, however, seems well conserved between mammalian species. For liver and lens, this finding has been confirmed in amino acid analyses and partial NH_2-terminal sequences (to 58 and 33 residues, respectively). Cleavage products of these two proteins have also been produced to allow further sequence analysis in the future. In spite of the differences in primary structure, some conservation of the tertiary structures of these proteins is suggested by proteolysis of intact junctions (likely restricted to the cytoplasmic surfaces). Liver and heart gap junction proteins are reduced by trypsin to two fragments of $M_r \sim 10{,}000$, while a single M_r 21,000 fragment is produced from lens MIP 26. Sequence analysis (liver and lens only) indicates that most of the protein removed by tryptic hydrolysis is from the carboxy-terminus, although an additional loop of 4,000 daltons is excised from the center of the liver polypeptide and five residues are lost from the NH_2-terminus of the lens protein.

The extent and possible significance of this surprising tissue specificity of the gap junction protein are discussed in the light of these findings.

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INTRODUCTION

With the advent of cell theory, higher organisms were, for the first time, considered to be comprised of millions of individual units, each capable of autonomous, independent activity. Some coordination of these independent activities is, however, provided by the actions of the nervous and hormonal signalling systems of multicellular organisms. The integrity of the individual cellular units is not compromised by these signals, since all messages are "screened" through the cell membrane by either specific surface receptors linked to second messenger systems inside the cell, specific ionic channels within the membrane which mediate carefully balanced exchanges, or selective solubility of the signal molecules in the membranes.

Cell-Cell Coupling: It was not until the discovery of bidirectional electrical coupling between cells in electrically excitable tissues (Bullock, 1945; Wiersma, 1947; Weidman, 1952; Furshpan and Potter, 1959), that it became evident that somewhat less specific although more direct interactions may occur between the unit cells of an organism. The demonstration of similar coupling between the cells of non-excitable tissues which soon followed (Loewenstein and Kanno, 1964) led to the first suggestion that direct communication between the cytoplasms of adjacent cells may be a widespread occurrence. This has indeed proved true, since intercellular coupling is now regarded as the rule rather than the exception in the Metazoa (see Loewenstein, 1981 and Peracchia, 1980 for reviews). In animals, this intercellular coupling seems to display a consistent set of properties. Coupling is bidirectional (with a few rare exceptions) and applies not only to the free passage of small ions, but also to compounds of molecular weights as high as 1000 (Kanno and Loewenstein, 1966; Potter et al., 1966). Recent studies with derivatized fluorescent probes are consistent with the presence, between cells, of non-selective aqueous channels permeable to molecules of $\leq M_r$ 800 (i.e., $\sim$14 Å in diameter)

in mammals and $\leq M_r$ 1000 (i.e., $\sim$ 20 Å in diameter) in arthropods (Flagg-Newton, 1980; Rose, 1980, respectively). Some selectivity of the channels to molecules near the size cut-off suggest that some electrostatic interactions may occur with negative charges in the channel wall or mouth (Brink and Dewey, 1980). While non-specific causes such as cell damage or anoxia may cause cell-cell uncoupling, it is now generally believed that Ca^{++} and H^{+} concentrations are the principle effectors of channel gating (see Loewenstein, 1981 and Bennett, 1978 for reviews) and may serve to mediate uncoupling in even non-specific cases such as those just described.

Gap Junction Structure. Several structures have been proposed as mediators of cell-cell coupling, but the most likely candidate, the gap junction, was first described as we know it today by Revel and Karnovsky in 1967, although it had been observed earlier under different pseudonyms (Sjöstrand et al., 1958; Karrer, 1960; Dewey and Barr, 1962; Robertson, 1963). The plasma membranes of adjacent cells come into close apposition, a likely prerequisite for establishing direct coupling between cells, but remain separated by a uniform $\sim$ 2 nm extracellular space bridged by a hexagonal array of subunits. Similar arrays of intramembrane particles in freeze fracture replicas at sites of close membrane apposition were also detected (Kreutziger, 1968; McNutt and Weinstein, 1970; Goodenough and Revel, 1970; Chalcroft and Bullivant, 1970). Since freeze fracture is believed to split the lipid bilayer and expose the interior of the membrane, these results suggested that the gap junction was comprised of hexagonally arrayed aggregates of proteins (visible as intra-membrane particles in freeze-fracture), presumably forming channels spanning the membranes of both cells and the extracellular gap between them. This view of the gap junction has now been confirmed in X-ray (Caspar et al., 1977; Makowski et al., 1977, 1982) and optical (Unwin and Zampighi, 1980) diffraction studies on isolated liver gap junctions. Each channel is apparently comprised

of two halves (connexons—Goodenough, 1975), one in each cell membrane, which meet head to head in the extracellular space. The connexons are comprised of six, apparently identical, subunits arranged symmetrically around a central aqueous pore 10-15 Å in diameter, although it should be noted that a continuous aqueous passage between cells has yet to be unequivocally demonstrated on structural grounds. Changes in the interrelationships of the subunits of the connexons observed under different conditions have been proposed as possible models for gating of the junctional channel (Unwin and Zampighi, 1980; Makowski et al., 1977), although no evidence exists that these changes occur in vivo.

Correlation Between Gap Junctions and Cellular Coupling. Since the first description of the gap junction, an overwhelming array of circumstantial evidence has accumulated correlating the presence of this structure with the coupling of cells. In addition to the large number of coincident demonstrations of electrical, metabolic or dye coupling of cells and the presence of morphologically recognizable gap junctions (in some cases representing the only close association of coupled cells—J. Hudspeth, personal communication) there have also been several studies where the gap junction contact area between cells has been specifically manipulated. Progressive and reversible disruption of gap junction contacts resulting from shrinkage of the cells following perfusion of a tissue with hypertonic sucrose has been found to also reversibly uncouple the cells (Barr et al., 1965; Dreifuss et al., 1966; Kawamura and Konishi, 1967). An analogous correlation by Azarnia et al. (1974) showed that a cell hybrid formed by fusion of communication incompetent mouse cells and communication competent human cells, was communication competent and formed recognizable gap junctions. However, as human chromosomes were lost, both of these abilities were also lost concurrently. Mouse L-cells similar to those used by Azarnia have been used in studies demonstrating that the absence of gap junctions in some lines is concomitant with an inability to mediate intercellular

coupling (Goshima, 1969; Pitts, 1971; Gilula et al., 1972). Temporal correlations have also been established between gap junction formation and the onset of electrical coupling (Rash and Fambrough, 1973; Johnson et al., 1974).

As more has been understood of gap junction structure, it has become apparent that this structure is compatible with the properties defined for intercellular coupling (see Loewenstein, 1981 and Hooper and Subak-Sharpe, 1981 for reviews). Estimates of the junctional pore size (10-15 Å) would suggest a molecular weight cut-off of about 1000 (see above and Flagg-Newton, 1980), and are consistent with current estimates for the single channel resistance in a gap junction ($10^{10}\Omega$ – Loewenstein, 1975). Furthermore, Ca^{++} and pH, which have been implicated as the mediators of cellular uncoupling (see Loewenstein, 1981 and Bennett, 1978 for reviews), have been demonstrated to cause structural changes in the gap junction (see Peracchia, 1980 for review), albeit on a reportedly longer timescale than the actual uncoupling process (Raviola et al., 1980).

All of these experiments point to gap junctions as sufficient criteria for coupling. However, the demonstration of gap junctions as a necessary criterion for coupling is difficult as long as some systems exist where coupling is demonstrable but gap junctions remain undetected (Daniel et al., 1976; Kurizama and Suzuki, 1976; Meyer et al., 1981; Williams and De Haan, 1981). The problem to be considered here is one of differential thresholds of detectability. It is clear from the study of Meyer et al. (1981) that electrical coupling of neighboring cells can actually appear enchanced when gap junctions are depleted, a phenomenon which can readily be explained through a cable analysis of the tissue. In these same circumstances, the slower diffusion of larger molecules through the junctional channels compared to ions could result in dye transfer between cells falling below detectable limits well before the cells become electrically uncoupled. This could explain several recent results demonstrating compartmentalization in embryos

with respect to dye transfer, while electrical coupling persists (Wier and Lo, 1982; Warner and Lawrence, 1982; Lo and Gilula, 1979).

In addition to the variability in the sensitivities of methods used for detecting coupling, the difficulties associated with structurally defining a gap junction under conditions where they are in low abundance must be considered. There is a limit to how small a cluster of particles can be and still be identified as a gap junction. Therefore, in the limiting case, single connexons, which may mediate coupling in some cases (see Williams and De Haan, 1981; Meyer et al., 1981), would not be included in any calculations of junctional area. These limitations should be considered when evaluating reports of electrical communication in the absence of gap junctions.

Functions of Gap Junction Mediated Coupling. Gap junctions are likely to subserve a multitude of functions throughout the body, but at this stage we can only guess their exact nature until the particular molecules of interest which pass through the junction can be defined. In electrically excitable tissues, it is clear that through the free passage of ions between cells, gap junctions can serve to synchronize electric activity in a tissue, such as that required to maintain synchronous muscular contractions of the heart (Barr et al., 1965; Goshima, 1969) or the uterus during parturition (Garfield et al., 1977). In non-excitable tissues, the demonstration of gap junctional function is more difficult, although a number of possibilities have been proposed. In addition to relatively non-specialized tasks such as the distribution of nutrients in tissues with poor blood supply (e.g., lens—Goodenough et al., 1981) and the coordination of a tissue response to an external signal (e.g., effects of insulin on pancreatic islet cells—Meda et al., 1979), gap junctions have also been implicated in less passive roles. The establishment of developmental compartments in several species has been correlated with the appearance of communication compartments created by selective restrictions in gap junction

communication between cell populations (Warner and Lawrence, 1982; Wier and Lo, 1982; Lo and Gilula, 1980). Such changes in junctional coupling have even been implicated at the stage of ovulation (Gilula et al., 1978). Gap junctions have also been proposed to play a role in control of cell growth. This theory is largely based on various observations which have correlated a reduction in gap junction complement between cells with either expression of a transformed phenotype (see Loewenstein, 1979 for review; also Yancey et al., 1982; Atkinson et al., 1981), the ability of carcinomas to metastasize (McNutt et al., 1971), or the onset of cell division during liver regeneration (Yancey et al., 1979).

Scope of this Thesis. In this thesis the author has approached the study of gap junctions from a biochemical standpoint in the hope that study of the individual junctional components, specifically the protein(s), will lead to a better understanding of the molecular structure of the junctional channels, and hence of their function.

Biochemical analysis of gap junctions has been severely restricted by the small numbers of these structures in any given tissue (frequently less than 1% of the cell membranes are occupied by junctions). The difficulties inherent in isolating such a minor component are increased by the absence of any assay for the gap junction other than its appearance in electron micrographs of the fractions. As a result, gap junctions from only three tissues (liver, heart and lens) have been isolated in any degree of purity, and of these, only the liver gap junctions have been well characterized. The basic approach to isolating gap junctions which has persisted from the earliest preparations (e.g., Goodenough and Stoeckenius, 1972) involves the isolation of a plasma membrane fraction which is treated with detergent to solubilize all but the tightly packed arrays of proteins present in the gap junctional plaques. The junctions are then separated from remaining membranes and fibrous material on a sucrose density gradient. In the earlier isolation protocols, proteases were employed to obtain clean fractions (Goodenough and Stoeckenius,

1972; Goodenough, 1974). Although the structure of the junctions seemed unaffected by these procedures, it was later demonstrated that contaminating proteases in commercial collagenase caused breakdown of the junctional protein (Duguid and Revel, 1976). Since this time, many different polypeptides have been identified as gap junctional in the liver—M_r 35,500 and 25,500 (Duguid and Revel, 1976); M_r 34,000 (Ehrhart and Chauveau, 1977); M_r 40,000 and 38,000 (Culvenor and Evans, 1977); M_r 26,000 and 21,000 (Henderson et al., 1979); M_r 28,000 and 47,000 (Hertzberg and Gilula, 1979); M_r 30,000 (Zampighi and Unwin, 1979); M_r 26,000 (Finbow et al., 1980); M_r 28,000 (Nicholson et al., 1981); M_r 16,000 (Finbow et al., 1983); M_r 46,000 (Henderson, 1982). Although there is now a certain consistency in reports of a component of M_r 26,000-30,000, at the inception of this thesis such was not the case. Indeed, only as this work has progressed have some of the reasons for these widely disparate claims become apparent, as will be detailed in Chapter 2. In any event, the first goal in this project was to define and characterize the protein components of the liver gap junction.

By modifying existing preparative techniques, a fraction highly enriched in gap junctions by morphological criteria was obtained from rat liver (Chapter 1). Despite the apparent purity of the fraction based on morphological criteria, analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) revealed a number of polypeptide components. Analysis of each component by peptide mapping and other methods enabled us to conclude that the isolated liver gap junctions were comprised of a single major protein of M_r 28,000, with all other components being attributable to contaminants ($\sim$20%) or aggregation or partial proteolysis of the "native" junctional protein (Chapter 2). With the nature and properties of the junctional protein in rat liver defined, we proceeded to characterize its primary structure through sequence analysis (Chapter 2) and its higher order structure by proteolytic treatments of the protein while still part of the gap junction

structure (Chapter 3). This latter approach also served to generate fragments useful for future sequence analysis. At this point, one obvious direction for further research lay in additional characterization of the liver protein through sequence analysis. This could be coupled to studies on the disposition of the polypeptide chain within the junctional structure using specific labeling with photoactivatable labels which would be restricted in their access to the protein by solubility (aqueous or lipid) and steric factors.

However, at this time several groups had reported a remarkably high content of junctions in the membranes of eye lens fiber cells (50-70% in the chicken) and had succeeded in isolating highly enriched junctional fractions containing a single protein of M_r 26,000 (MIP 26) (Broekhuyse et al., 1976; Alcalá et al., 1975; Takemoto and Hansen, 1981). Given the similarity in molecular weights of the junctional proteins of liver and lens and the availability of an assay for the two junctional proteins in the form of two dimensional peptide maps, we chose to direct our efforts towards a comparison of the junctional proteins of these tissues, hoping to further characterize each protein in parallel. When comparisons of the proteins revealed them to be very different (Chapter 4), a difficulty arose. Although generally similar in appearance to junctions elsewhere, the lens junctions did have some atypical features which some authors found substantially different from other gap junctions (Zampighi et al., 1982). Since no homology could be demonstrated between the protein components of a recognized gap junction and the lens junction, the possibility existed that the structure in lens was not a gap junction.

To clarify this issue, or at least to settle the question of gap junction tissue specificity, we proceeded to isolate junctions from a third tissue, heart, where the junctions were well characterized and morphologically similar to those in liver. The gap junction fraction isolated from heart by a modification of a previously published protocol (Kensler and Goodenough, 1979) was found, like those of liver

and lens, to contain a single junctional protein (M_r 28,000—apparently derived from an M_r 30,000 "native" protein). However, this protein was also found to share no detectable homology by peptide mapping with the proteins of liver or lens, thus demonstrating the tissue specificity of the junctional protein, and providing a new outlook on the gap junction. Perhaps it is not what it may have seemed at first—a ubiquitous, non-specific channel connecting cells into a syncitium of sorts. Although the gap junction channel may be rather non-specific with respect to the molecules which pass through it, it may well confer on the cell a specificity regarding the other cell types with which it will communicate and the specific signals which will open or close such communication channels. Therefore, the gap junction may prove to represent another means by which a cell may respond specifically to its environment and its neighbors.

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CHAPTER 1

Gap Junctions in Liver.
Isolation, Morphological Analysis and Quantitation

Running title: Isolation of Liver Gap Junctions

Introduction

Gap junctions are arrays of cell-to-cell channels that permit exchanges of cytoplasmic low molecular weight constituents, such as ions, various metabolites, etc. (for reviews, see Revel et al.,[1] Hooper and Subak-Sharpe[2] and Flagg-Newton et al.[3]). Gap junctions allow for metabolic cooperation between the cells of a tissue,[4] for electrical coupling of excitable cells[5] and are believed to play a major role in the control of growth and differentiation.[6,7] Gap junctions are ubiquitous structures, found in every metazoan phylum and nearly all tissues studied.[8] With few exceptions, all these junctions have a characteristic appearance,[9] a feature of major importance since it is one of the only criteria of purity available for following the isolation of gap junctions.

From a biochemical standpoint, the best studied tissues are the liver and eye lens, and this paper focuses attention on the liver gap junctions. Here, the major protein component has been reasonably well characterized,[10-12] and a partial amino acid sequence is available.[12] X-ray diffraction[13] and image reconstruction based on low dose electron microscopy,[14] in combination with the other techniques, indicate that each gap junctional channel is comprised of two halves (connexons[15]), one through the membrane of each adjacent cell. Each connexon is composed of six apparently identical polypeptide chains, probably associated with phospholipid. Gap junctions, as usually visualized, consist of large arrays of closely packed connexon pairs, which appear to retain their integrity under a variety of experimental treatments.[16]

A. A Strategy for the Isolation of the Gap Junction Protein

Virtually all the published procedures for the purification of gap junctions involve the isolation of a plasma membrane fraction and its subsequent treatment with a detergent which solubilizes other membrane components but leaves the gap junctions, clearly recognizable by their characteristic lattices, intact. The junctions are then separated on a sucrose gradient from the other detergent-resistant material of the plasma membrane fraction on the basis of density. Additional treatments with reagents such as 8 M urea,[17,18] and solutions of high pH (e.g., 11[10]) have also been employed in various preparation procedures with little apparent disruption of the gap junction structure. The general protocol outlined above must be specifically modified for each tissue because of differences in membrane or junctional density, the presence of additional components peculiar to that tissue (e.g., uricase in the liver,[10] myosin and actin filaments in cardiac muscle,[19] crystallins of lens[20]), or differences in the sensitivity of gap junctions to detergent treatments.[19] We will review here the isolation of gap junctions as applied to a specific tissue, the rat liver, with emphasis on techniques that we have found to reproducibly provide good yields of highly enriched gap junction fractions.

B. Isolation of Gap Junctions from Rat Liver

I. Isolation of Plasma Membranes

Solutions Needed

Name	Composition	Volume (for 25 livers, i.e., 200g tissue)
1. Isolation buffer (IB)	2 mM $NaHCO_3$ 0.5 mM $CaCl_2$ pH 7.4; (4°C)	20 liters
2. Perfusion buffer (PB)	IB + 0.9% NaCl (37°C)	200 ml

3. Two-phase	80 g Dextran (Sigma: $M_{r(av)}$ 500,000)
[Should be made up a	62 g Polyethyleneglycol 6000 (Baker)
day before the isolation	11.92 g $NaH_2PO_4 \cdot H_2O$
and allowed to separate	16.22 g $Na_2HPO_4 \cdot 7\ H_2O$
in a separatory funnel	0.3 g NaN_3
overnight in a cold room]	1486 ml H_2O (4°C)

(a) Methodology - Available Methods

In the majority of published isolation protocols for gap junctions from rat or mouse liver, the method of Neville,[21] variously modified, has been used to isolate the plasma membrane fraction. The livers are homogenized and the homogenate filtered through cheesecloth to remove coarse fibrous material and precipitated nuclear protein. The homogenate is then centrifuged once or twice at relatively low g to separate the membranes from most of the mitochondria and soluble proteins. The pellets from these spins are then loaded onto a discontinuous sucrose gradient and centrifuged.

This gradient is usually composed of layers of 60, 54, 50 and 43% (w/v) sucrose. The membranes can be collected between the 50% (d = 1.191) and 43% (d = 1.168) (w/v) sucrose layers (for specific details, see ref. 10), or a crude plasma membrane fraction can be collected instead over a 52% (w/v) sucrose cushion.[22] An alternative route is that developed by Lesko et al.[23] in which the sucrose gradient is supplanted by a two-phase polymer system (Fig. 1). This technique produces fractions similar in purity and yield to those obtained from sucrose gradients.[18] Although the ingredients of the two-phase mixture are somewhat expensive, the method avoids the time consuming use of sucrose gradients, and allows one to handle large quantities of material (~120 g wet weight of liver) at one time, even if a zonal rotor is not available. Since the only information available as to the appropriateness of this

method in the preparation of plasma membranes for gap junction isolation applies specifically to rat liver plasma membrane, caution should be used in adapting this technique to other tissues and species.

(b) Methodology - The Two-Phase Method (Fig. 1)

Twenty-five young adult rats (~200 g body weight—if they are much older, contamination with connective tissue becomes a problem) are sacrificed by cervical dislocation. After cutting the vena cava above the liver, each liver (~8 g wet weight) is perfused by injecting 3-5 ml of 37°C PB through the spleen. Perfusion is achieved through the portal system. The perfused liver is excised and placed in ice-cold IB within 30 sec of death. All subsequent steps are performed on ice. Each liver is homogenized in 100 ml of IB with a Tissuemizer (Tekmar Ultra Turrax, SDT-182 EN) at maximum power for 5 sec. This step can also be achieved in a Dounce homogenizer using 25 strokes of a loose fitting pestle after first dicing the liver with scissors. Homogenates of six livers are pooled, diluted to 1800 ml with IB, and placed on ice for 10-15 min to allow nucleoprotein to precipitate. The homogenate is then filtered twice through four layers of cheesecloth and the filtrate centrifuged at 5000 rpm for 30 min (Sorval HG-4L rotor; RC-3 centrifuge: 125,000 g_{av} min). The pooled pellets are resuspended in 3600 ml of IB with vigorous shaking and centrifuged twice at 3000 rpm for 15 min (HG-4L rotor: 22,000 g_{av} min). The supernatant, containing most of the mitochondria and soluble proteins, is discarded each time. After the second centrifugation at this speed, care must be taken to aspirate all of the supernatant, or the subsequent two-phase separation may fail. (If necessary, the pellets obtained after the second spin can be resuspended and compacted at higher g to allow for a more complete aspiration of the supernatant.) These pellets are resuspended by vigorous shaking in 600 ml of each phase of the two-phase system and distributed among four 1-liter bottles (150 ml of each phase/bottle). After shaking, the bottles are allowed to stand for 15 min and then

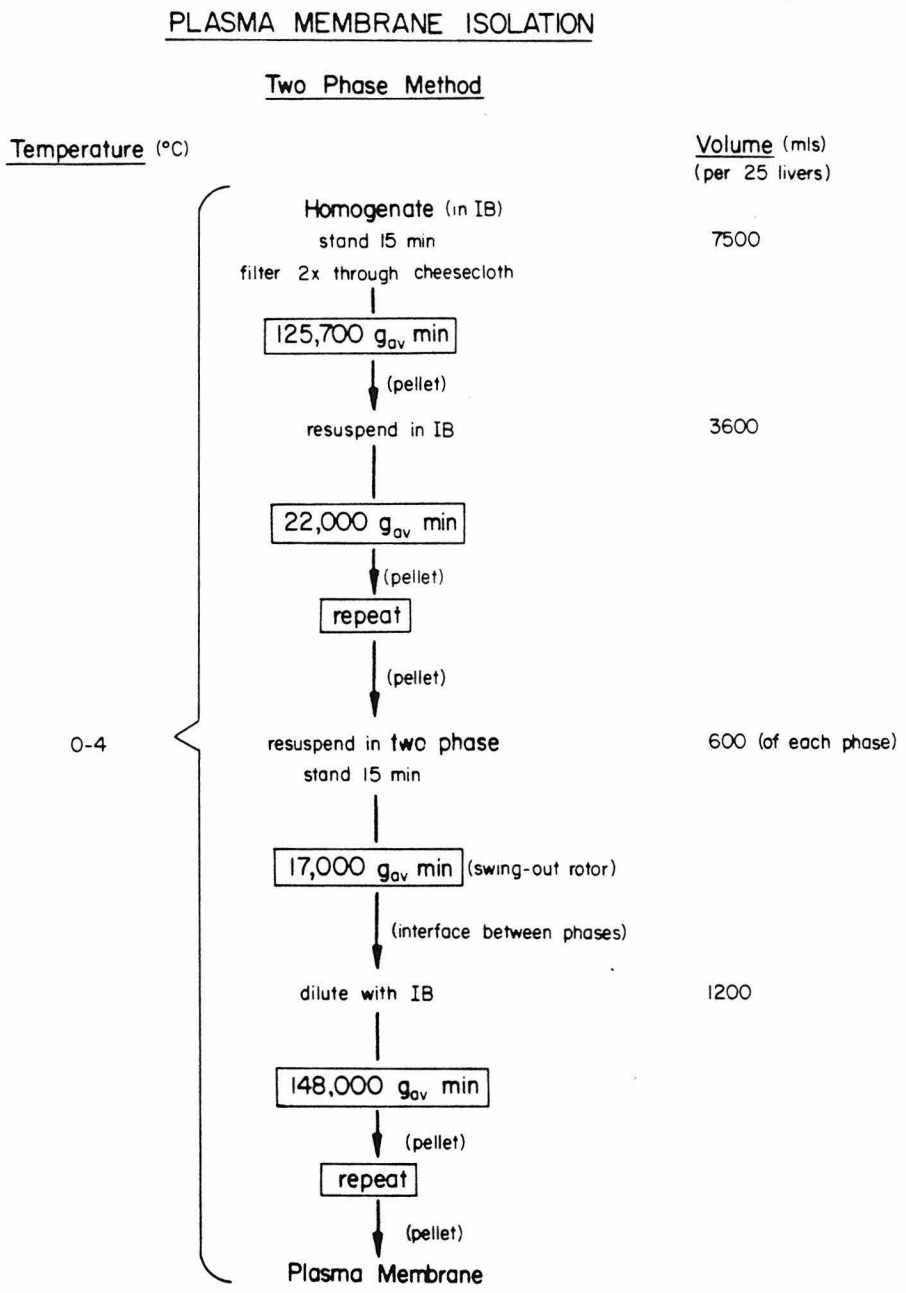

FIGURE 1. Protocol for the isolation of plasma membrane by the two-phase method[23] from rat liver. For details refer to section B I.

centrifuged for 15 min at 2600 rpm (HG-4L rotor: 17,000 g_{av} min) after which most of the nuclei will be in the pellets. The supernatants containing the separated phases and the plasma-membrane rich interfaces are poured into two 1-liter bottles, shaken vigorously, and recentrifuged as above. The "carpet-like" interface is harvested by aspiration, diluted to 1200 ml with ice-cold IB and spun down at 10,000 rpm for 15 min (Sorval GSA rotor; RC-5B centrifuge: 148,000 g_{av} min). This centrifugation is repeated once more out of a volume of 400 ml of IB to yield a plasma membrane fraction. This can be refrigerated overnight for the subsequent isolation of gap junctions.

II. Isolation of "Native" Gap Junctions (Fig. 2)

Solutions Needed

Name	Composition	Volume (for 25 livers, i.e., 200 g tissue)
1. Bicarbonate buffer (BB)	2 mM $NaHCO_3$ pH 7.4 (4°C)	600 ml
2. 0.1 M NaCl	0.1 M NaCl in BB (4°C)	100 ml
3. 1.1% Sarkosyl	1.1% Sarkosyl in BB (R.T.)	50 ml
4. 0.1 M Na_2CO_3	0.1 M Na_2CO_3 (4°C)	5 ml
5. 34% (w/v) sucrose	sucrose in 1 M urea 2 mM BB	5 ml
40% (w/v) sucrose		15 ml
77% (w/v) sucrose		15 ml
81% (w/v) sucrose	sucrose in ~1.5 M urea; + 0.1% Sarkosyl + 2 mM BB	10 ml

Methodology

The first highly purified fractions of gap junctions from liver were obtained by the treatment of plasma membrane fractions with enzymes[24] (e.g., collagenase,

hyaluronidase). It soon became apparent that, while this treatment had little effect on the gap junction's morphological appearance, it did cause partial cleavage of the gap junction protein.[25] While such preparations have their uses (see section B III), most current protocols seek to minimize proteolysis in an attempt to isolate the "native" or undegraded protein. In addition to omitting specific proteolysis treatments from the protocol, the inclusion of protease inhibitors, notably 0.5 mM phenyl methyl sulfonyl fluoride (PMSF), in the original perfusion buffer and all subsequent steps (except the two-phase separations) seems to be useful in preventing cleavage of the junctional protein by endogenous proteases during the isolation procedure.[12]

The procedure we have adopted for the isolation of gap junctions from the plasma membrane fraction (Fig. 2) is very similar to that of Hertzberg and Gilula,[10] but contains modifications which result in an increased yield without an associated loss in purity.[12] Unless otherwise noted, all solutions are at 4°C. The plasma membrane pellets are suspended in 100 ml of 0.1 M NaCl in BB with vigorous shaking or use of a Dounce homogenizer. They are allowed to stand on ice for 15 min to release peripheral proteins adhering to the membranes. The salt washed membranes are pelleted at 12,000 rpm for 15 min (Sorval SS-34 rotor; RC-5B centrifuge: 168,000 g_{av} min), resuspended in BB and the centrifugation repeated. After resuspension in 50 ml of BB (room temperature) by a few strokes of the pestle of a Dounce homogenizer, an equal volume of 1.1% (w/v) Sarkosyl (NL 97) in BB (room temperature) is added. The mixture is stirred at room temperature for 40 min, during which time 15 ml aliquots are removed and sonicated with a micro-tip for 3-4 sec at setting number 4 on a Branson sonicator (S-125). The detergent insoluble material is pelleted at 20,000 rpm for 15 min (SS-34 rotor: 470,000 g_{av} min), washed in ice-cold BB and recentrifuged. The pellets are next resuspended in 4 ml of 0.1 M Na_2CO_3 (pH 11) by brief sonication in order to solubilize the substantial amount

of uricase copurifying with the junctions to this point. After exactly 15 min at 4°C (longer times cause aggregation of gap junctions with copurifying contaminants), the insoluble material, which includes the gap junctions, is collected by centrifugation at 20,000 rpm for 15 min (SS-34 rotor). This pellet is washed once by resuspension in BB and centrifugation as above. For loading on sucrose gradients, the washed pellets are homogenized by brief sonication into 3 ml of BB to which is added 1 ml of 1.1% Sarkosyl, and 8 ml of 81.1% (w/v) sucrose in 1.5 M urea and BB. Three sucrose gradients are then poured, each containing 4 ml of 77.2% (w/v) sucrose, 4 ml of sample (final concentrations of 54% (w/v) sucrose, 1 M urea and 0.09% Sarkosyl), 4 ml of 40% (w/v) sucrose, and 1 ml of 34% (w/v) sucrose. All sucrose solutions are made in 1 M urea in BB. After centrifugation at 35,000 rpm for at least 1.5 hr at 4°C (Beckman SW-41 rotor; L3-50 ultracentrifuge: 13,500,000 g_{av} min), the 40/54% (w/v) sucrose interfaces are harvested, diluted with BB, and the final gap junction fraction collected by centrifugation at 40,000 rpm for 1 hr (Beckman 42.1 rotor: 7,500,000 g_{av} min). The Sarkosyl and urea present in the sucrose gradient seem to reduce the aggregation of gap junctions with themselves and copurifying contaminants such as collagen and other fine fibrous material. Loading the sample in the 54% (w/v) sucrose layer also aids in this respect since the junctions float upwards and the denser contaminants sediment to the lowest interface. This avoids the non-specific trapping of material which occurs when it is spun through the concentrated blanket of material which accumulates at each interface. The interface at which we collect our gap junction fraction (between layers of density 1.16 and 1.121) is consistent with the reported density of gap junctions on continuous sucrose gradients (i.e., 1.165[10]).

The final junctional fraction can be stored as a pellet at -20°C for months without detectable structural or biochemical changes, especially if PMSF is included

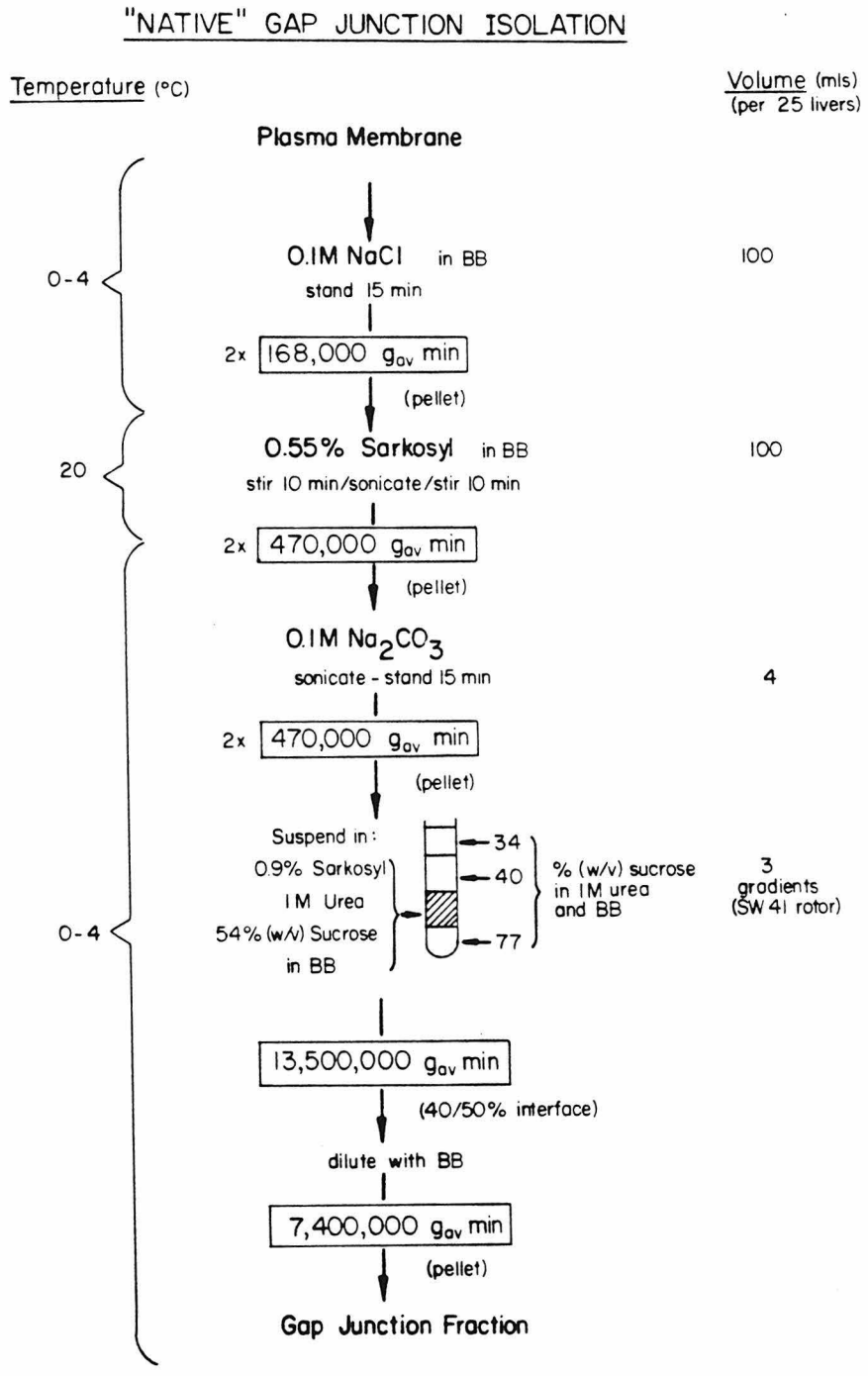

FIGURE 2. Protocol for the isolation of a "native" gap junction fraction from rat liver plasma membrane. For details refer to section B II.

in the final centrifugation. The fraction shows only small amounts of contaminating amorphous material and collagen (see section C for a more detailed evaluation) and contains 150-300 μg of gap junction protein (M_r 28,000), which represents a yield of 1-2 μg of junction protein from every gram wet-weight of liver. The nature of the M_r 28,000 protein and the other components of this fraction will be discussed in section D.

III. Isolation of "Enzyme Treated" Gap Junctions (Fig. 3)

Solutions Needed

Name	Composition	Volume (for 25 livers, i.e., 200 g tissue)
1. Enzyme Buffer (EB)	50 mM Tris/HCl 5 mM $CaCl_2$ pH 7.4; 37°C	100 ml
2. BB	as in previous section	300 ml
3. Sarkosyl	as in previous section	75 ml
4. Sucrose	32% (w/v) sucrose	24 ml
	54% (w/v) sucrose	24 ml
5. Triton X-100 wash (for rapid isolation procedure only)	5% Triton X-100 5 mM Tris/Acetate, pH 7.4	

Methodology

As mentioned above, gap junctions have been isolated from membranes in the past with the aid of proteases, the rationale being that gap junction ultrastructure appears unscathed even after extensive proteolysis. However, it is now recognized that the intactness of the junctional ultrastructure is not reflected in its major constituent protein (M_r 28,000) which is degraded to two fragments of M_r 10,000[12] (representing 20,000 of the original 28,000 daltons upon exposure to proteases

[specifically trypsin]). These polypeptides can be of considerable use in studies on the arrangement of the protein in the lipid bilayer. This method of isolating junctions also has one other major advantage. It produces highly enriched gap junction fractions from a variety of species while the "native" isolation procedure often produces fractions badly contaminated with fibrous material (e.g., collagen) when applied to species other than rat or mouse.

The procedure described here (Fig. 3) is similar to that of Finbow et al.[18] The plasma membrane fraction (section B I) is homogenized using a Dounce homogenizer with a loose-fitting pestle, into 70 ml of EB and then 10 ml of a 1.6 mg/ml collagenase (Worthington) solution is added. After gentle agitation at 37°C for 25 min, 10 ml of 0.6 mg/ml trypsin (Sigma, type XI) is added and the solution shaken gently for an additional 25 min. The enzyme resistant pellet is collected after centrifugation at 10,000 rpm for 15 min (Sorval GSA rotor; RC-5B centrifuge: 150,000 g_{av} min) and then treated with Sarkosyl in a total volume of 150 ml as described in section B I). A spin of 20,000 rpm for 30 min (Sorval SS-34 rotor: 900,000 g_{av} min) is used to collect the detergent-insoluble material which is then resuspended in 30 ml of ice-cold BB and layered on top of six discontinuous gradients containing 4 ml of 32% (w/v) sucrose and 4 ml of 54% (w/v) sucrose in BB. The 32/54% (w/v) sucrose interface is harvested after centrifugation for at least 1.5 hr at 35,000 rpm (Beckman SW-41 rotor; L3-50 ultracentrifuge: 13,500,000 g_{av} min) and collected after dilution with BB by centrifugation at 40,000 rpm for 1 hr (Beckman, 42.1 rotor: 7,500,000 g_{av} min). This gap junctional fraction, which can also be stored for lengthy periods of time at -20°C with no apparent ill effects, looks morphologically very similar to the "native" gap junction preparations, although the yield of junctional protein is usually less (0.5 μg per g wet weight of liver) and the major (and virtually only) band detectable by Coomassie staining after sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has an M_r of 10,000.

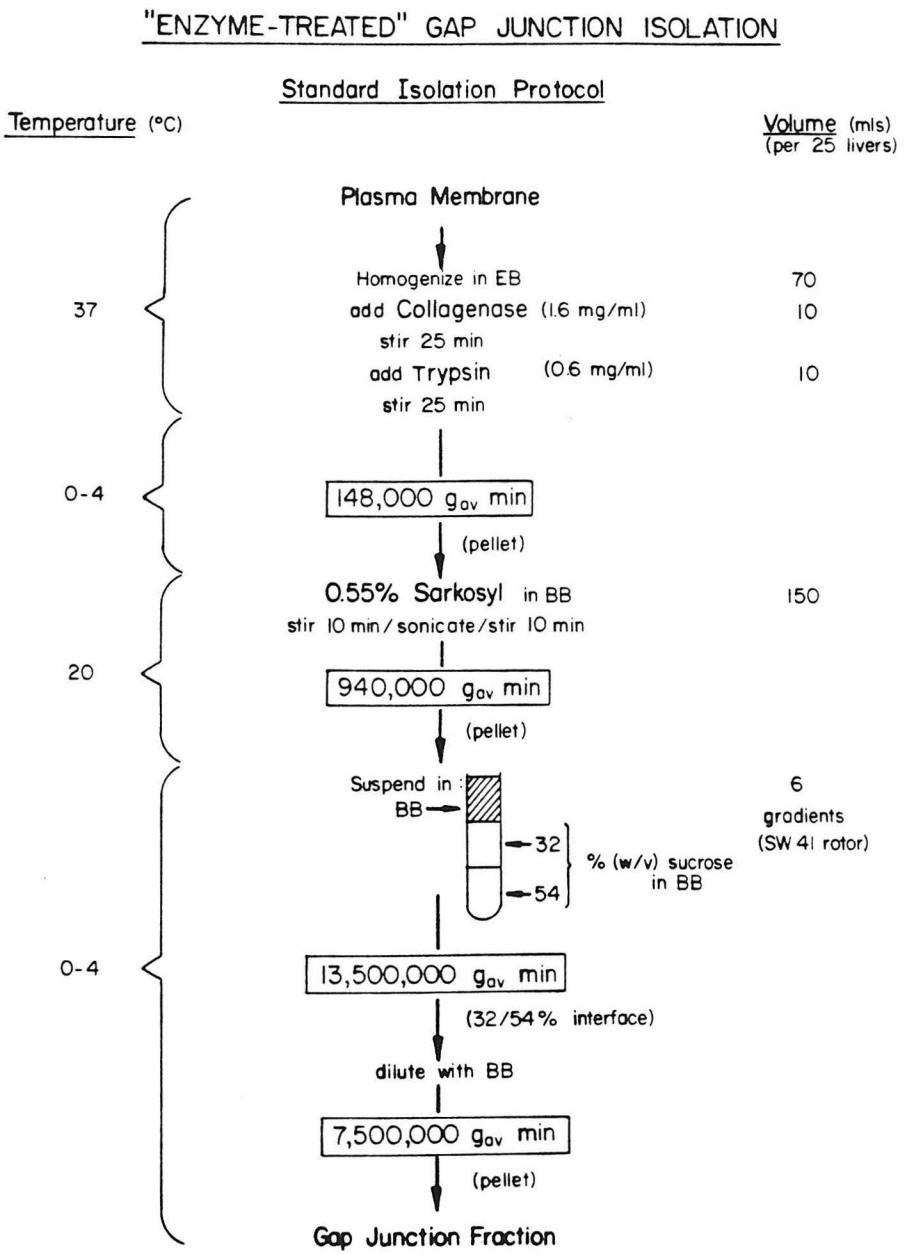

FIGURE 3. Protocol for the isolation of an "enzyme-treated" gap junction fraction from rat liver plasma membrane. For details refer to section B III.

C. Criteria of Purity - Morphological Methods

I. Methodology

Until recently, the only way of estimating the purity of gap junction fractions was to examine them in the electron microscope after negative staining, thin sectioning or occasionally freeze fracture of the final pellet. For thin sectioning and freeze fracture, gap junction fractions are first pelleted and then prepared by the same procedures that are used for blocks of tissue.[26] However, by far the most commonly used and simple technique for examining gap junction fractions is to use negative stain.[27]

After thorough mixing, 1 µl or more of the fraction to be studied is deposited on a dental wax plate and mixed with an equal volume of phosphotungstic acid (PTA) neutralized to pH 7.4. The final concentration of the phosphotungstate is of the order of 0.5%, but should be adjusted up or downwards to give the best results after examination of a sample grid in the electron microscope. A carbon reinforced formvar or celloidin coated grid, held in fine jeweler's tweezers closed by rubber band or paper clip, is touched to the drop. The sample is allowed to stand, drop side up, so that the suspended material can settle on and bind to the surface of the grid. After a few minutes, the excess fluid is removed with a wick (a triangular wedge of Whatman No. 1 filter paper) touched to the edge of the grid. Care must be taken to remove material that might have been aspirated between the tines of the tweezers by capillary action. The grids are allowed to dry thoroughly (5 or 10 min at least) and then examined in an electron microscope operating at 80 kv. The entire grid is surveyed at low magnification and areas where the junctions are well spread chosen for further examination and photography. A magnification of 13 to 15,000 is usually needed to be able to detect connexons. If the material is very poorly distributed on the surface of the grid, heavily clumped in some areas while others are completely bare, fresh grids should be used. Old

ones can be made less hydrophobic again by placing them in the refrigerator over night or by dipping them in 0.1% serum albumin which is then drained off before addition of the material to be negatively stained.

Superior results in terms of clarity and definition of the images have been published with junctions negatively stained with uranyl salts.[28] The use of uranium salts can be more difficult since they precipitate as hydroxides above pH 6. A possible routine for staining with uranyl acetate or formate would be to deposit a drop of the sample to be examined on the grid and allow it to settle as was described for PTA, but in the absence of any negative stain. The freshly dried sample can then be washed with solutions of appropriate pH (a similar routine is used to get rid of excess sucrose). Washing can consist of dipping the grid in a beaker of appropriate solutions (distilled water, or very dilute buffers, or buffers made from volatile mixtures such as ammonium acetate/acetic acid, etc.) draining the excess fluid on the grid and between the tines by touching the edge of the grid to a filter paper and then applying the appropriate negative stain as a droplet. After a few minutes the excess stain is drained off with a filter paper wick and the grid is then ready to be examined in the electron microscope.

II. Appearance of Gap Junction Fractions

In the "native" gap junction fractions, the junctions appear as irregularly shaped flat sheets showing the typical double membrane profile and closely packed, hexagonally arranged particles (connexons) in en face views. Occasionally, small gaps in the array of connexons suggest that some portions of the junction may have been lost, perhaps as a result of partial solubilization during the detergent extraction.[29] Of the non-junctional material present, the most ubiquitous are clumps of fine fibrous material which appear alone and associated with the surfaces of gap junctions. As yet, the origin of this material has not been identified and it cannot be consistently associated with any specific protein present in the gap

junction fraction. Other structures, seen more rarely in the junction fraction, include strands of collagen[12] (these are much more abundant at the 54/77% (w/v) sucrose interface of the final sucrose gradient) and vesicles of non-junctional single membranes[18] which apparently survive the detergent treatments of the isolation procedure and can most readily be detected in thin sectioned material. The "enzyme-treated" gap junctions differ from those in the "native" fraction in that they most frequently appear as closed vesicles or curved sheets.[24] The contaminating material seen in these fractions is similar to that described above with the exception that collagen strands are never seen.

III. Limitations in Estimating Purity

In general, morphological examination of the purified fractions provides some guidelines as to the nature and degree of contamination in junctional fractions and has played an important part in suggesting improved strategies for the isolation of gap junctions. However, as the purity of junctional fractions has steadily improved, the need for a quantitative estimate of the percentage of the final fraction which is composed of gap junctions has become greater. Electron microscopic examination presents problems in maintaining unbiased sampling both in the selection of a portion of the final pellet for examination and in the selection of the region of an electron microscope grid to be used for analysis. Furthermore, if one attempts to quantitate by measuring the surface area of the grid occupied by junctions, compared to that occupied by other material, the density of protein in each structure is ignored. Particularly troublesome has been the association of an M_r 34,000 protein with gap junctional fractions.[25,30] This is likely to have been due to the presence of small crystals of uricase in the preparation which are readily overlooked in negatively stained samples. In addition, not all structures visible in the electron microscope can be correlated with the presence of specific proteins recognizable after SDS-PAGE of the fractions (e.g., the fine fibrous material referred to above).

This could suggest that the structure contains no protein or that it represents a different form (denatured?) of the gap junctions themselves. The possibility also exists that some of the contaminants in a fraction may not be visualized in preparations examined in the electron microscope. For example, in thin sectioned material, the "native" gap junction fractions are found to be comprised of ~81% double membranes (gap junctions) and 19% single membranes (non-junctional). However, as an estimate of purity, these numbers totally disregard non-membranous constituents such as the fibrous material, collagen and uricase crystals which are often seen in negatively stained fractions.

As a result of these difficulties and the lack of an assay for the gap junction protein other than the presence of morphologically intact gap junctions, it has been difficult to reliably determine the purity of junctional fractions. Although this problem still exists in most instances, the recent characterization of the protein components of liver gap junction fractions, specifically those of rat and mouse,[10-12] has enabled us to assess the purity of this fraction biochemically.

D. Criteria of Purity - A Biochemical Approach

I. Identification and Nature of the Gap Junction Protein

The identity of the protein components of the gap junction has been a major point of contention in the field over the last ten years. Polypeptides of M_r 10,000-38,000 have been variously proposed as the major protein of gap junctions from rat or mouse liver.[10,11,24,30,31] The problem was the lack of a direct assay for the gap junction protein and only limited success in the production of antibodies. Only in this past year have preliminary reports of such an antibody appeared.[32] The only course was to isolate fractions highly enriched for gap junctions by morphological criteria. The protein components of this fraction were then examined, usually by separation on an SDS polyacrylamide gel, and the major species identified

as gap junctional proteins. This approach relies heavily on the efficiency of morphological techniques in detecting non-junctional contaminants, the pitfalls of which have been discussed in section C II. Furthermore, the SDS polyacrylamide gel profiles were complex, not only as a consequence of non-junctional contaminants, but also because of the susceptibility of the junctional protein to proteolysis and its tendency to aggregate in SDS, particularly when heated.[11,12]

However, as will be described in Chapter 2,[12] two dimensional peptide mapping,[33,34] along with other techniques, has now enabled the identity and properties of the liver gap junction protein (at least in rat) to be defined. These junctions are comprised of a single major protein of M_r 28,000 $\pm$ 2,000 which has a tendency to aggregate to dimers (M_r 50,000) and higher multimers in the presence of SDS and is susceptible to partial proteolysis (generating polypeptides of M_r 26,000, 24,000 and ultimately M_r 10,000 in "enzyme treated" gap junction fractions). A minor component of M_r 21,000 (~4% of liver gap junction fractions from rat but considerably more abundant in the mouse[11]) seems to be related to the major M_r 28,000 protein, but does not seem to be a direct proteolytic degradation product. Contaminants of collagen, and proteins of M_r 38,000 and 34,000 (uricase) are frequently found to copurify with the gap junctions.

Although the M_r 28,000 protein has now been identified in several laboratories as the sole or major component of purified liver gap junction fractions,[10-12,18,22,32] some contradictory observations have recently led to polypeptides of M_r 46,000[35] and 16,000[36] being identified as the major gap junctional proteins of liver, and in the latter case, of other tissues and cultured cell lines as well. However, since other groups have been unable to find an M_r 16,000 polypeptide in their final junction fractions and since strong evidence exists to implicate components of M_r 45,000-50,000 as dimers of the M_r 28,000 protein,[11,12] the bulk of evidence would still seem to point to a single protein of M_r 28,000 as the structural protein unit of the mammalian liver gap junction.

II. Junctional Proteins as a Criteria for Purity

Since the identity of the gap junction protein and its behaviour under varying conditions has now been defined, it is possible, at least in the case of rat liver, to quantitatively determine both the purity, and consequently, the yield of the various gap junction fractions based solely on a consideration of their protein composition. This can be analyzed by solubilizing a sample of the fraction under consideration in Laemmli solubilization buffer[37] for 30-45 min at room temperature, separating the various protein components by SDS-PAGE and staining the gel with Coomassie blue R-250 (most protocols generally published for staining SDS polyacrylamide gels will suffice). Quantitation of the amount of each polypeptide in the sample can be achieved by scanning the appropriate lane of the gel with a densitometer and integrating the area under each peak (Figure 4). In our laboratory this was achieved by a Joyce Löebel densitometer and a digitizing tablet interfaced to a Tektronix mini-computer (4052). The total area under all the peaks corresponding to junctional polypeptides (as defined in the previous section and in Chapter 2[12]) can then be divided by the total area under the densitometer scan of the gel to obtain an estimate of the purity of the fraction. An estimate of the yield of junctional protein in the fraction requires, in addition, some absolute estimate of protein present. This can be obtained in one of two ways. The protein content of the whole fraction can be determined independently by amino acid analysis or a Lowry assay,[38] although the latter may prove difficult since the junctions are poorly soluble in anything but SDS which is known to interfere with Lowry assays. This quantity can then be adjusted to include only gap junctional protein by correcting for the percent purity determined as described above. Alternatively, the total area of the peaks of gap junctional polypeptides determined from the scan of the stained polyacrylamide gel can be standardized against the areas of the peaks in densitometer scans of adjacent lanes on the same gel in which known amounts of various standard proteins have been loaded.

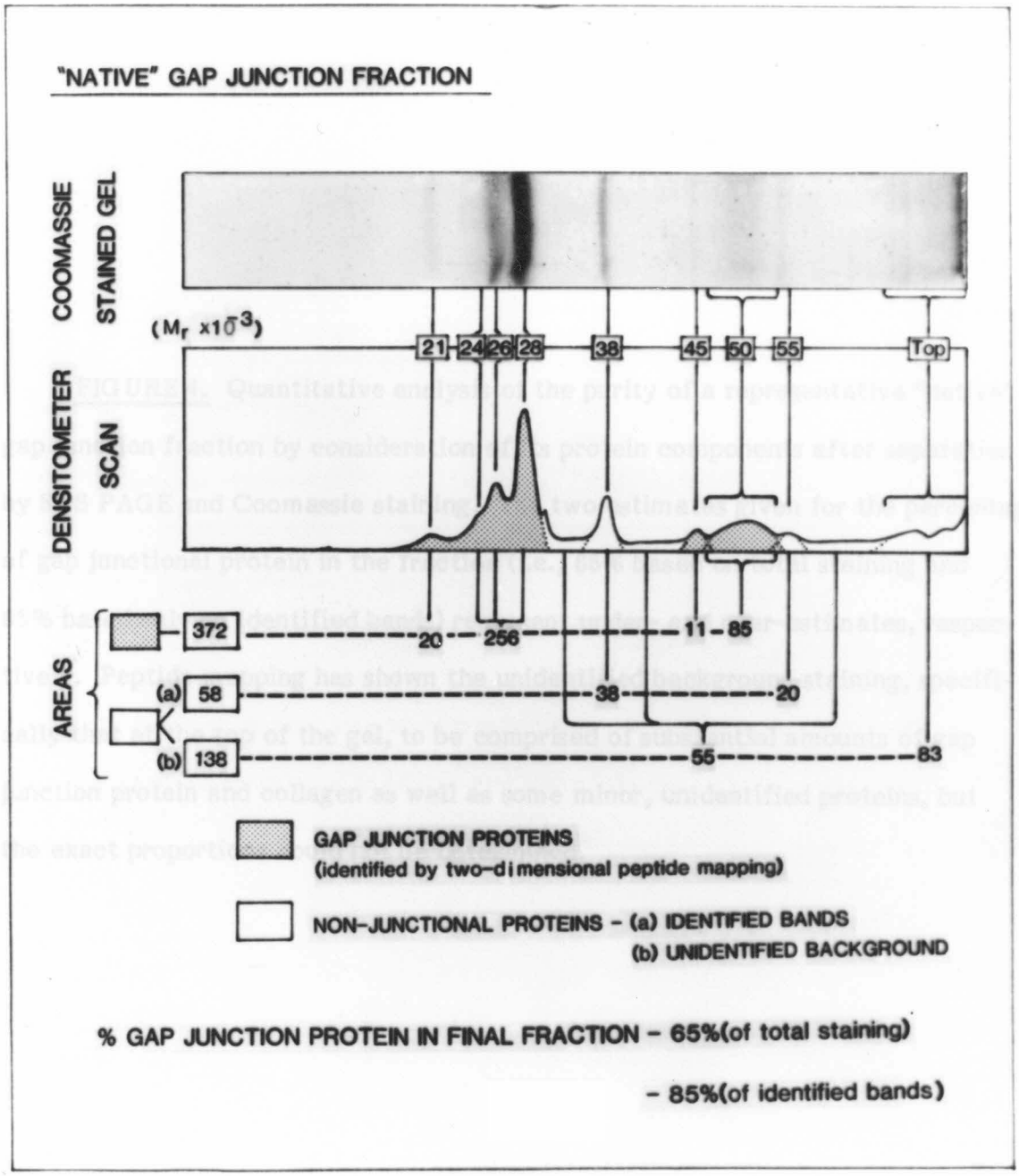
"NATIVE" GAP JUNCTION FRACTION
COOMASSIE STAINED GEL
DENSITOMETER SCAN
(M_r x10^{-3})
21
24
26
28
38
45
50
55
Top
AREAS
372
20
256
11-85
(a)
58
38
20
(b)
138
55
83
GAP JUNCTION PROTEINS
(identified by two-dimensional peptide mapping)
NON-JUNCTIONAL PROTEINS - (a) IDENTIFIED BANDS
(b) UNIDENTIFIED BACKGROUND
% GAP JUNCTION PROTEIN IN FINAL FRACTION - 65%(of total staining)
- 85%(of identified bands)

FIG. 4

FIGURE 4. Quantitative analysis of the purity of a representative "native" gap junction fraction by consideration of its protein components after separation by SDS PAGE and Coomassie staining. The two estimates given for the percentage of gap junctional protein in the fraction (i.e., 65% based on total staining and 85% based only on identified bands) represent under- and over-estimates, respectively. Peptide mapping has shown the unidentified background staining, specifically that at the top of the gel, to be comprised of substantial amounts of gap junction protein and collagen as well as some minor, unidentified proteins, but the exact proportions could not be determined.

TABLE I

Purity and Yield of Various Gap Junction Fractions

Isolation Procedure	Purity[a] (i.e., $\frac{\text{GJ protein}}{\text{Total protein}} \times 100$)	Yield[b] (i.e., $\frac{\mu\text{g GJ protein}}{\text{g wet weight liver}}$)
"Native" Gap Junction Fraction	77 ± 9	0.8-2.0
"Enzyme-treated" Gap Junction Fraction	81 ± 6	0.3-0.7

[a]The mean ± one standard error determined from several (4-9) different isolations are shown.

[b]Based on an average weight of 8 g for a liver from a 200 g rat.

Both the determination of purity and yield are subject to certain inherent errors, based on limitations of Coomassie staining as a quantitative assay for proteins. Firstly, in any given gel system it is imperative to determine over what range and under what conditions the Coomassie staining remains linear with respect to protein concentration. However, even within this range, different proteins bind the dye with different efficiencies, so a determination of absolute protein concentration or even relative amounts of two different, unknown proteins can entail substantial errors, especially in the case of glycoproteins. Fortunately, no carbohydrate has been found to be associated with the gap junctional protein.[10,11] An independent check on the values obtained from Coomassie stained gels can be obtained by radioactively labeling the fraction in vitro with ^{125}I, after solubilization in SDS but before separation by PAGE. An autoradiogram of the gel could then be scanned in a similar way to a Coomassie stained gel. In this instance, differences in the labeling efficiencies of different proteins can be corrected for if their amino acid compositon is known, since iodine is known to specifically label tyrosine residues and, under some conditions, histidine. Figures calculated in our laboratory for the yield of junctional protein in and purity of gap junction fractions prepared from 25 rat livers by each of the methods described in section B are presented in Table I.

III. The Validity of Extrapolating Results to Other Systems

Gap junctions of different origins have many features in common. Only small differences in the size of the connexons or the width of the extracellular gap have been observed,[39] and could often be laid to differences in preparative procedures. There are more differences in terms of packing, but this feature could be under physiological control.[8] Gap junctions can mediate metabolic cooperation or electrotonic coupling between cells in culture.[4] This has proven true even in cell combinations derived from physiologically distinct species,[40] again

suggesting a kinship extending far and wide. The rather uniform molecular weights ascribed to gap junction proteins (M_r 26-34,000[10,11,19,34]) could also suggest a high degree of conservation, but it is only recently that this idea has become testable by detailed analysis of the protein(s).

Peptide mapping of the gap junction proteins from several mammalian species (rat, mouse, calf and rabbit) has shown them to be virtually indistinguishable, while preliminary "fingerprints" of the gap junction protein from chicken liver suggests that there is some conservation even between classes.[41] The main intrinsic protein (M_r 26,000) of eye lens fiber cell junctions, which have a similar appearance and apparent properties to gap junctions in other tissues, is similarly conserved, with homology being demonstrated by both peptide mapping[34] and immunology[42] in species as far distant as man and shark. However, when the gap junction proteins of different tissues are compared, a different story emerges. Major differences in the main intrinsic protein of eye lens and the gap junction protein of liver have now been demonstrated immunologically,[43] by peptide mapping[41] and in partial sequences of the two proteins purified from rat[41] (the N-terminal 18% of the lens protein and 24% of the liver protein). Indeed, if any homology does exist between the two proteins, it is barely detectable by the methods used to date. Recently, we have obtained peptide mapping data which demonstrate that the major protein of gap junction fractions from heart is also very different from those of liver or lens (see Chapter 5). It is, therefore, quite possible that a whole family of tissue-specific junction proteins exists. While there may be homologies between them with respect to both sequence and structure, it is quite clear that results on the properties or biochemistry of gap junctions in one tissue should not, in general, be extrapolated to other systems.

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CHAPTER 2

Rat Liver Gap Junction Protein: Properties and Partial Sequence

Running Title: Sequencing and chemistry of liver gap junction protein

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Rat liver gap junction protein: Properties and partial sequence

(peptide mapping/membrane proteins/micro-sequence-analysis/aggregation/proteolysis)

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ABSTRACT **Gap junctions, strongly implicated as channels for direct cell-to-cell communication, have been isolated from rat liver in high yield and purity. These gap junction fractions contain few morphologically recognizable contaminants, but $NaDodSO_4$/polyacrylamide gel electrophoresis reveals a number of polypeptides. With the exception of a nonjunctional component of M_r 38,000 and some poorly soluble material, including collagen, all the polypeptides have very similar or identical two-dimensional peptide maps and arise from proteolytic cleavage of the COOH-terminus or aggregation of a M_r 28,000 protein. We report the sequence of the NH_2-terminal 52 amino acids of this protein. The polypeptide ($M_r \approx 10{,}000$) characteristic of trypsin-treated gap junction preparations is shown to be two distinct polypeptides, both derived from the M_r 28,000 protein.**

An extensive body of evidence implicates the gap junction as the mediator of exchanges of ions and small molecules between cells. Early attempts at characterization of the junctional proteins were carried out on partially proteolyzed fragments (1–4). Fractions of similar purity can now be obtained without the use of proteases, and junctional proteins of M_r 26,000–28,000 are consistently found (5–9). Finbow *et al.* (6) have also obtained independent lines of evidence associating a protein of M_r 26,000 with gap junctions. Besides identifiable contaminants (e.g., uricase, actin, collagen), other polypeptides found have been shown to result from aggregation of this protein (5), or are related to it, perhaps as proteolytic fragments [e.g., M_r 21,000 protein (5)]. We present here direct evidence as to the nature of all the polypeptides detected on gels of gap junction fractions isolated from rat liver. The evidence is based on analysis by two-dimensional peptide mapping and NH_2-terminal sequence determination. We conclude that rat liver gap junctions are composed of a single major component of M_r 28,000.

MATERIALS AND METHODS

Isolation of Gap Junctions. After perfusion with warm saline, the livers of 50 young adult rats were homogenized, each in 100 ml of cold isolation buffer (2 mM $NaHCO_3$/0.5 mM $CaCl_2$, pH 7.4) in a Tissuemizer (Tekmar Ultra Turrax, SDT-182EN) at maximum power for 4–5 sec. Plasma membranes were then prepared by an adaptation of the two-phase method described by Finbow *et al.* (6). These fractions were treated by a modification of the Hertzberg and Gilula protocol (7). The salt, Sarkosyl, and sodium carbonate treatments were unaltered, except for doubling the volumes. The washed pellet from the carbonate treatment was suspended in 24 ml of 0.09% Sarkosyl and 54.1% (wt/vol) sucrose. This and all other sucrose solutions were made up in 1 M urea/2 mM $NaHCO_3$ (pH 7.4). Six discontinuous gradients were then formed by successively layering 4 ml of 77.2% (wt/vol) sucrose, 4 ml of the sample, 4 ml of 40.3% (wt/vol) sucrose, and 1–2 ml of 33.8% (wt/vol) sucrose. After centrifugation in a Beckman SW 41 rotor at 38,000 rpm for at least 90 min, the gap junction fraction was collected at the 40.3/54.1% sucrose interface, diluted with 2 mM $NaHCO_3$, and pelleted at 40,000 rpm for 60 min in a Beckman 42.1 rotor. Virtually no gap junctions were found at the 33.8/40.3% (wt/vol) sucrose interface, contrary to the original finding by Hertzberg and Gilula (7), but consistent with a recent modification (10). In some cases, 0.5% phenylmethylsulfonyl fluoride ($PhMeSo_2F$) was included in all solutions to reduce endogenous proteolysis. Gap junctions were also isolated by a slight modification of the technique of Finbow *et al.* (6), using increased collagenase and trypsin concentrations of 1.6 and 0.6 mg/ml, respectively, and a Sarkosyl extraction at neutral pH in 35% less volume.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. The Laemmli discontinuous buffer system (11) was used with running and stacking gels of 15% and 4.5% acrylamide (Bio-Rad, electrophoresis grade), respectively (30:0.8 crosslinking ratio). For analytical purposes the microslab system of Amos (12) was used. Samples were solubilized at room temperature for 30–45 min in 2% $NaDodSO_4$ (Bio-Rad, electrophoresis grade) and 5% (vol/vol) 2-mercaptoethanol in 62.5 mM Tris·HCl (pH 6.8)/0.25 mM $MgCl_2$/10% (vol/vol) glycerol. The relative amounts of protein in the bands on a gel after Coomassie blue staining were quantitated by measuring the area under the peaks from a Joyce–Loebl densitometer scan with a digitizing tablet interfaced to a Tektronix minicomputer (4052) and correcting for the width of the lane.

Iodination of Gap Junctions. Gap junction fractions were iodinated in the absence of detergents by the chloramine-T method of Greenwood *et al.* (13). The junctions were separated from free iodine by repeated centrifugations at 40,000 rpm for 30 min in a Beckman 42.1 or type 65 rotor. For peptide mapping, the junctional protein was solubilized in 2% $NaDodSO_4$ before iodination. In this case, separation of protein and free iodine was achieved by $NaDodSO_4$/polyacrylamide gel electrophoresis. Quantitation of the radioactivity of a given band on a gel was determined from an autoradiogram (Kodak XR film exposed at −70°C with a Du Pont Cronex Lightning Plus intensifying screen) as described above for Coomassie blue-stained gels.

Peptide Mapping. The method of Elder *et al.* (14) as modified by Takemoto *et al.* (15) was used to obtain tryptic (Sigma type XI) and α-chymotryptic (Worthington) two-dimensional peptide maps. (Only iodinated peptides generated by complete proteolysis are detected in this system.) In addition to gap junction proteins, we have mapped others, including collagen, actin, glycophorin, and the other erythrocyte ghost proteins, and all have produced unique maps. Results from these proteins showed the recovery of radioactivity from the gel slices to be (70 ± 20)%. The errors represent one standard deviation.

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Abbreviation: $PhMeSO_2F$, phenylmethylsulfonyl fluoride.

Sequence Analysis. Analytical grade reagents, deionized glass-distilled water, and acid-cleaned glassware were used throughout the preparation of polypeptides for sequence or amino acid analysis. The polypeptides present in junctional fractions were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis and identified by brief Coomassie blue staining and destaining (total time 4 hr). After excision of the bands, the polypeptides were removed from the gel by electrophoretic elution (16) (recovery from gel fragments was ≈75%), exhaustively dialyzed against 0.03% $NaDodSO_4$/50 mM NH_4HCO_3 (pH 7.8), lyophilized twice to remove the NH_4HCO_3, and stored at −20°C. NH_2-terminal amino acid sequence analysis using automated Edman degradation was performed by the method of Hunkapiller and Hood (17) and Johnson *et al.* (18). $NaDodSO_4$/polyacrylamide gel electrophoresis of an aliquot of the sample was used to detect possible degradation immediately prior to sequence analysis. The yield of analyzable peptide compared to the total peptide loaded on the sequenator was ≈35%. Total peptide was estimated from Coomassie blue staining on analytical $NaDodSO_4$/polyacrylamide gel electrophoresis or quantitative amino acid analysis of an aliquot.

RESULTS

Characterization of the gap junction fractions and their proteins

Yield and Purity (Morphological Characterization). Aggregation of junctions with denser contaminants causes losses during the preparation of gap junctions in the absence of added proteases (7). These losses were minimized by shortening the exposure of junctions to Na_2CO_3, including Sarkosyl in the loading layer of the sucrose gradient, and loading the junctions between the two interfaces where most of the material was collected. We now isolate 300–600 μg of gap junctional protein from 400 g (wet weight) of liver. This final junctional fraction contains only minor contamination by amorphous material (Fig. 1*a*). Appearing primarily as flat sheets, the junctions show the typical packing of "connexons" (Fig. 1*b*) described previously (5–8). When gap junctions were prepared from trypsin- and collagenase-treated plasma membranes, ≈150 μg of M_r 10,000 junctional protein could be isolated from 400 g (wet weight) of liver. This final fraction also showed minimal contamination in negative stained specimens and the gap junctions seemed to form vesicles or curved sheets (not shown, but see ref. 2).

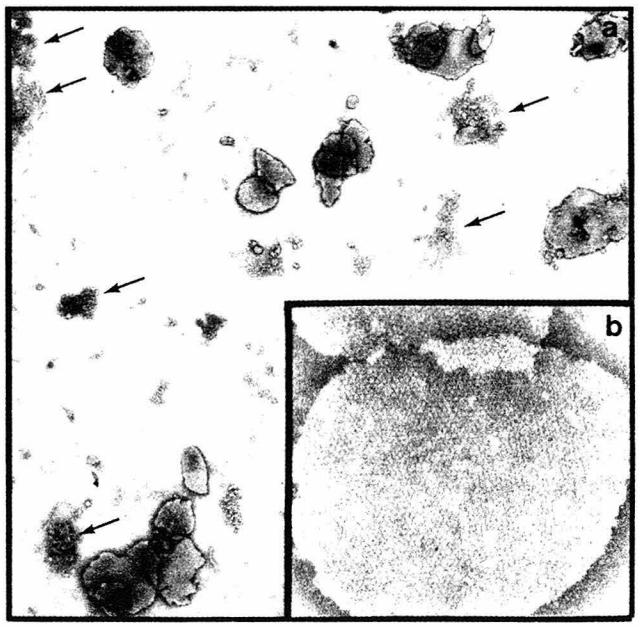

FIG. 1. "Native" gap junction fractions negatively stained with 2% phosphotungstic acid. (*a*) At low magnification (×11,000), the fraction appears highly enriched for gap junctional sheets and shows only minor contamination by amorphous material (arrows). (*b*) At higher magnification (×105,000), the individual gap junction sheets show closely packed connexons in a hexameric array.

Protein Components. When endogenous protease activity is limited by thorough perfusion of the livers before excision and inclusion of $PhMeSO_2F$ in all solutions, the junctional fraction contains a major protein of M_r 28,000 (Fig. 2, lane a). Although not readily identifiable in Fig. 2, lane a, minor and more variable components at M_rs of 50,000, 38,000, 26,000, 24,000, and 21,000 can be clearly seen in fractions loaded at a higher concentration (Fig. 2, lane b). The M_r 38,000 protein is specifically enriched in the lower interface [54.1/77.3% (wt/vol) sucrose] of the final gradient (Fig. 2, lane d), where morphologically and biochemically identifiable gap junctions are sparse. Hence, it is believed to be nonjunctional. The M_r 26,000 and 24,000 polypeptides and the M_r 10,000 polypeptide are likely to be proteolytic degradation products of the M_r 28,000 protein, because they are enriched in preparations in which endogenous proteolysis was more likely (Fig. 2, lanes b and c) or exogenous proteases (trypsin and collagenase) were specifically added (Fig. 2, lane e). *In vitro* trypsin treatment of isolated gap junctions

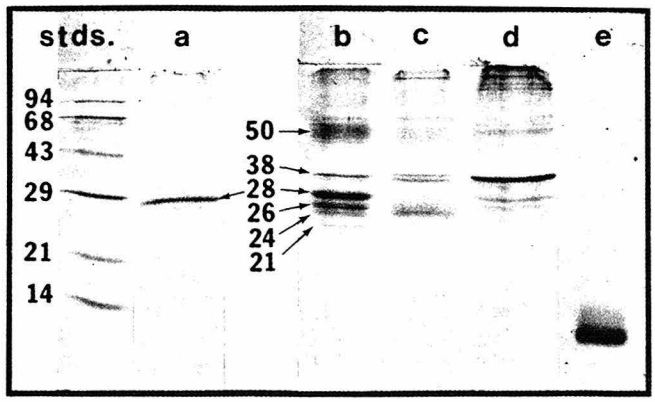

FIG. 2. Coomassie-blue stained $NaDodSO_4$ microslab gel of various gap junction fractions isolated from rat liver. The Bio-Rad low molecular weight standards are shown on the left with their molecular weights in thousands. The estimated molecular weights of the components in lanes a and b are similarly marked. Lane a, a "native" gap junction fraction [i.e., 40.3/54.1% (wt/vol) sucrose interface], prepared in the presence of 0.5% $PhMeSO_2F$ to inhibit proteolysis and run immediately after isolation to reduce aggregation, shows a major band at M_r 28,000 and some very faint minor components. Lane b, a "native" gap junction fraction prepared without specific protease inhibitors contains more obvious components just below the M_r 28,000 protein at M_rs of 26,000 and 24,000. Because the sample was loaded more heavily than in lane a, the minor components are visible (M_r 50,000, 38,000, and 21,000). The relative amount of M_r 50,000 and higher molecular weight (M_r 80,000 and 110,000) material compared to the M_r 28,000 and 26,000 bands was found to increase with concentration of the loaded sample, time in or heating in $NaDodSO_4$, and storage time at −20°C. Lane c, a similar fraction prepared from poorly perfused livers, in which the likelihood of endogenous proteolysis is increased, shows an enrichment for the M_r 24,000 polypeptide compared to lane b. Lane d, the lower interface [54.1/77.3% (wt/vol) sucrose] of the final sucrose gradient of the "native" gap junction isolation also contains junctional bands (see text), but is specifically enriched for the M_r 38,000 protein and contains more insoluble and high molecular weight material contributed in part by the high collagen content of the sample. Lane e, an "enzyme-treated" gap junction fraction prepared from trypsin- and collagenase-treated plasma membranes contains a single diffuse band at M_r 10,000.

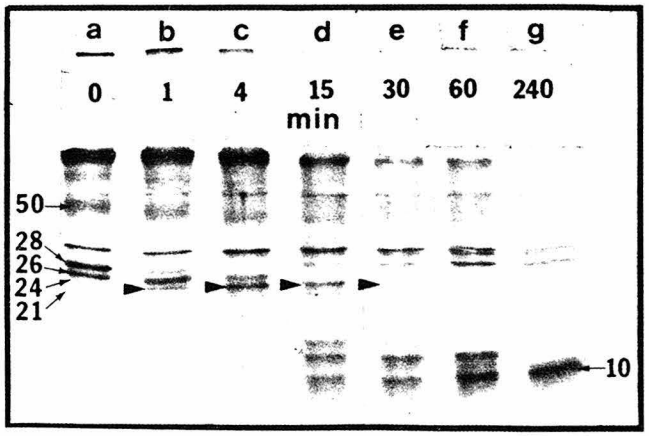

FIG. 3. A purified fraction of "intact" gap junctional sheets (protein ≈ 0.3 $\mu g/\mu l$ in 50 mM $NaHCO_3$, pH 7.8) was subjected to trypsin (Sigma, type XI, 3 $ng/\mu l$) at 37°C in nondenaturing conditions. At various intervals (marked in minutes above each gel lane), samples were removed, added to a 2-fold excess of soybean trypsin inhibitor (Sigma), solubilized for 15 min in regular gel solubilization buffer, and frozen for later analysis by polyacrylamide gel electrophoresis. The M_r 24,000 band is indicated by an arrowhead in each lane where it appears, and the major bands present at 0 time are marked in $M_r \times 10^{-3}$. Lanes a–c and d–g are taken from different gels run on the same experiment.

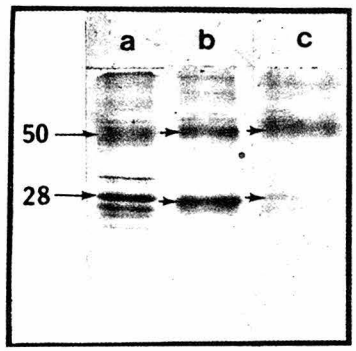

FIG. 4. The protein components of gap junction fractions were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis (lane a), the M_r 28,000 and 50,000 bands were cut out, and the polypeptides were eluted and dialyzed for 3 days against 0.05% $NaDodSO_4$/10 mM sodium thioglycollate/50 mM NH_4HCO_3 (pH 7.8). On reelectrophoresis, the M_r 28,000 protein had apparently aggregated to a dimer (M_r 50,000) (lane b), which comigrates with a band in the original fraction (lane a). Conversely, the isolated M_r 50,000 polypeptide, on reelectrophoresis, had partially dissociated to proteins of M_r 28,000 and 26,000 (lane c). This suggests that this diffuse band of M_r 50,000 is a mixture of dimers of these two proteins, which partially disassociate on standing. Aggregation to higher molecular weight multimers can be seen in all three lanes.

(Fig. 3) supports the idea that these changes in the polypeptide profiles of the various junctional fractions represent progressive stages of proteolysis of the major junctional protein. This protein and its higher molecular weight aggregates (M_r 50,000, 80,000, etc.—see below) seem to be successively broken down to M_r 26,000 and 24,000 polypeptides and their corresponding aggregates (compare Fig. 2, lane c and Fig. 3, lane d) and ultimately, through a series of intermediates (M_r 15,000–12,000) to a M_r 10,000 component (compare Fig. 2, lane e and Fig. 3, lane g). Contaminating proteins of M_r 38,000, 36,000, and 32,000 (the latter two appear in conjunction with the loss of insoluble material at the top of the gel) can also be seen in Fig. 3 to survive trypsin treatment. Although the M_r 21,000 component (≈ 7–15% of the material at M_r 28,000 and 26,000) does not seem to be a product of tryptic digestion of gap junctions (compare lanes b and c in Fig. 2 and see Fig. 3), initial peptide mapping results (not shown) suggest it may also be a degradation product of the M_r 28,000 protein, probably resulting from some non-serine-protease activity (i.e., protease resistant to Ph-$MeSO_2F$; e.g., cathepsins—see ref. 5) in the liver.

As reported previously for mouse (5), the major M_r 28,000 protein of rat liver gap junctions and its degradation products tend to aggregate on heating or prolonged standing in $NaDodSO_4$ or after extended storage at −20°C. Such preparations show an enhancement of diffuse bands at M_r 50,000 and higher molecular weights (Fig. 2, lanes b and c). This aggregation seems partially reversible, because the isolated M_r 50,000 protein partially dissociates to the M_r 28,000 and 26,000 monomers on standing at room temperature (Fig. 4, lane c). Conversely, the M_r 28,000 protein is seen to aggregate, forming the M_r 50,000 and higher molecular weight multimers (Fig. 4, lane b).

Characteristic patterns of all the polypeptides present in gap junctional fractions have been obtained by two-dimensional mapping of iodinated tryptic and chymotryptic fragments. In both cases the conclusions were the same. Only the chymotryptic maps are illustrated (Fig. 5). All of the polypeptides showed closely related patterns with the exception of the M_r 38,000 protein, which shows no homology (Fig. 5*b*). This supports the conclusion (see above) that this protein is nonjunctional. Of the remaining polypeptides, the M_r 28,000, 26,000, and 50,000 components have nearly identical maps (Fig. 5 *a* and *c*). The same pattern is seen in the higher molecular weight proteins at M_r ≈80,000 and ≈110,000 and even in the material failing to enter the running gel (maps not shown). However, these high molecular weight components show additional peptides, including those characteristic of collagen. The lower molecular weight polypeptides of M_r 24,000 (Fig. 5*d*), 21,000 (not shown), and 10,000 (Fig. 5*e*) retain the hydrophobic peptides present in the M_r 28,000 and 26,000 proteins, but lose several of the most basic and hydrophilic peptides while generating a new hydrophobic one. These results are consistent with the stepwise degradation of the M_r 28,000 junctional protein discussed above and show that the portion of the protein protected from proteolysis is hydrophobic.

Quantitative analysis of tryptic digestion of gap junctions

Surprisingly, the complexity (total number of peptides) of the M_r 28,000 and 24,000 proteins is conserved in the map of the M_r 10,000 fragment to a much greater extent than would be expected on the basis of molecular weight. This led us to estimate the recovery of protein after trypsin digestion of gap junctions, using several methods. The recovery of radioactivity in gap junction fractions iodinated in the absence of detergent has been measured both in the total material pelleted before and after trypsin treatment and in the specific junctional proteins of these same fractions after separation by $NaDodSO_4$/polyacrylamide gel electrophoresis. The actual recovery of protein was determined directly in a series of parallel experiments in which protein was measured by Coomassie blue staining of gels or by quantitative amino acid analysis of pellets or of proteins eluted from gels. Despite the different errors inherent in each technique, all estimates indicate that 70 ± 15% (mean 67%) of the total junctional protein is recovered in the M_r 10,000 band after trypsin digestion. That two-thirds of the mass is preserved is completely consistent with the number of peptides shown to be conserved when the maps of the M_r 10,000 and 28,000 polypeptides are compared (Fig. 5 *c* and *e*). This suggests that the M_r 10,000 band is composed of two polypeptides, a conclusion supported by sequence analysis (see below).

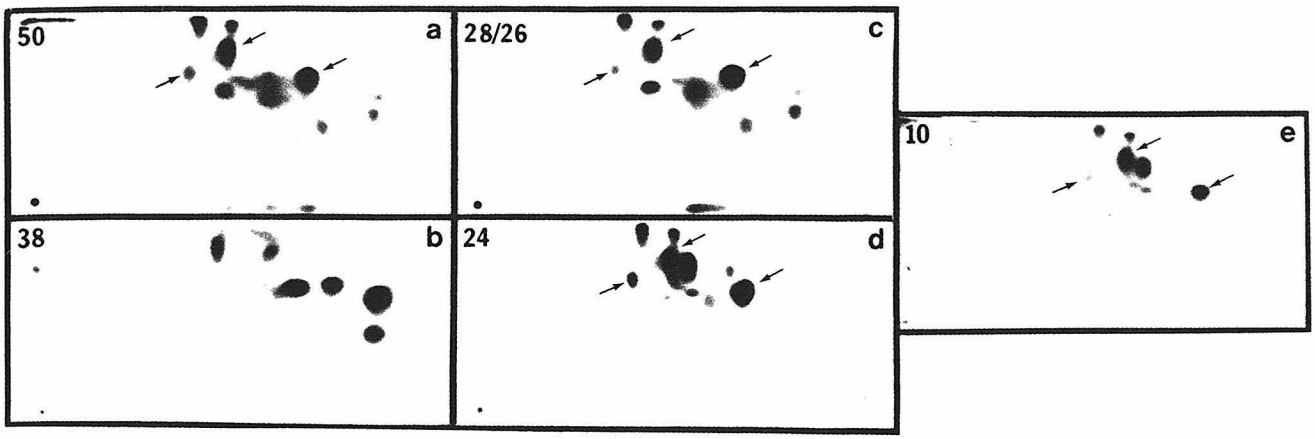

FIG. 5. Characteristic two-dimensional separation of the iodinated chymotryptic peptides of the proteins present in our gap junction fractions. $M_r \times 10^{-3}$ is indicated on each map. In all cases the origin is at the bottom left of the map and separation is achieved on a thin-layer cellulose plate by electrophoresis at pH 1.7 from left to right followed by ascending chromatography in a hydrophobic solvent (see ref. 14 for details). The most basic peptides migrate furthest in the electrophoresis dimension and the more hydrophobic peptides tend to partition to the mobile phase on the chromatography and are found nearest the top of the map. All maps show a great deal of homology amongst the hydrophobic peptides except for that of the M_r 38,000 protein (see text). Three of the peptides conserved in all but the M_r 38,000 protein are marked with arrows to provide a frame of reference, although it should be noted that additional peptides are also conserved. Maps of all polypeptides were repeated at least four times.

Sequence analysis

The sequence of the 52 residues at the NH_2-terminus of the M_r 28,000 gap junction protein (one-fifth of the protein) has been determined (Fig. 6). After 14 uncharged amino acids at the NH_2-terminus, there is a strongly hydrophobic region of 18 residues (nos. 23–40) interrupted by a single charged amino acid (Arg-32) and flanked at the NH_2 terminus by three basic residues (nos. 15, 16, and 22) and at the COOH terminus by a similarly spaced group of three acidic residues (nos. 41, 46, and 47).

The same NH_2-terminal sequence has been found for the M_r 26,000 and 10,000 products of proteolysis, demonstrating that the COOH terminus of the protein is exposed to proteolytic digestion in the intact structure, whereas the NH_2 terminus is protected. In the case of the M_r 10,000 component (sequence determined to 20 residues), one or two major sequences in addition to that seen for the M_r 28,000 protein can be detected, indicating the presence of at least two polypeptide chains. These additional signals could not be resolved into a unique sequence and probably result from tryptic cleavage at several closely spaced and equally susceptible sites on the original M_r 28,000 protein, which could produce a second M_r 10,000 polypeptide with various NH_2-terminal starting points.

DISCUSSION

We have used a modified version of a published procedure (7) to isolate gap junctions from rat liver in high yield [0.75–1.5 μg of junctional protein per g (wet weight) of liver] and with few copurifying contaminants as judged from negatively stained samples. Nevertheless, as found by others, despite this apparent purity several polypeptides can be detected by $NaDodSO_4$/polyacrylamide gel electrophoresis. Examination of these polypeptides leads to the conclusion that gap junctions are composed of a single major protein of M_r 28,000. All other polypeptides can be attributed to demonstrable contaminants [e.g., collagen, a more dense M_r 38,000 protein, and an alkali-sensitive M_r 34,000 protein uricase (19)] or are derived from the M_r 28,000 protein by proteolysis (compare Fig. 3 with lanes b and c of Fig. 2) or aggregation (Fig. 4 and ref. 5). The two-dimensional peptide mapping system of Elder *et al.* (14) used for this analysis was also found to provide an assay for gap junctions, at least those from rat liver. Differences between gap junctional proteins of different species (15, 20) and tissues (20) have been detected by this system.

The quantitative study of the effect of trypsin on gap junctions has provided some insight into the arrangement of the protein in the membrane. Recoveries of 70% of the junctional protein present in junctions not subjected to proteolysis (major protein M_r 28,000) in the M_r 10,000 fragment of junctions after trypsin digestion, and the presence of more than one polypeptide in this fragment, as detected by sequence analysis, leads us to conclude that the M_r 28,000 protein is initially reduced to a M_r 24,000 polypeptide (Fig. 3, lanes a–d), which is in turn cleaved into two pieces of M_r 10,000 by several steps (Fig. 3, lanes c–g). Several lines of evidence suggest that these two polypeptides are protected from further proteolysis by the surrounding membranes. Peptide mapping and amino acid analysis show the M_r 10,000 tryptic polypeptides to be highly hydrophobic. In ad-

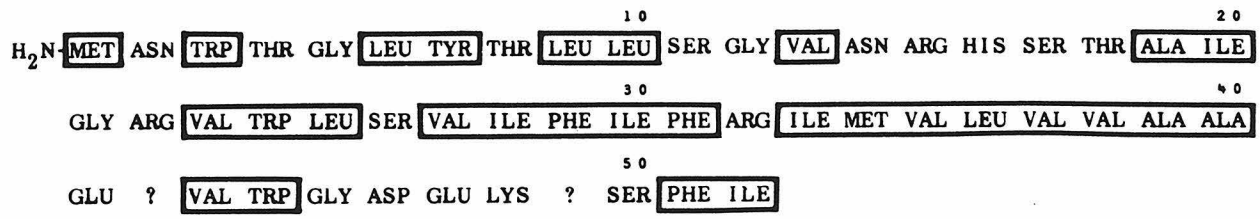

FIG. 6. Sequence of the 52 NH_2-terminal residues of the M_r 28,000 protein of gap junctions. Hydrophobic residues are enclosed in boxes, thus emphasizing the strongly hydrophobic region (residues 23–40) flanked by basic (residues 15, 16, and 22) and acidic (residues 41, 46, and 47) residues. The question marks indicate steps in the sequence at which no unequivocal signal could be distinguished from the background and could indicate a cysteine (undetected by this system) or an amino acid recovered in poor yield.

dition, whereas protein denaturing agents such as 1 M urea have no effect on the trypsin sensitivity of junctions, solubilization of the membrane with low concentrations of $NaDodSO_4$ allows trypsin to digest the junctional protein to small peptides (results not shown). Furthermore, the dimensions of both trypsin and chymotrypsin [greater than 50 Å by x-ray crystallography (21)] make it unlikely [but do not eliminate the possibility (22)] that they can penetrate the junctional pore or the extracellular gap between membranes without prior disruption of the native structure. Because the electron microscopic appearance of isolated junctions remains largely unchanged after trypsin treatment (cf. refs. 2 and 7), it appears that proteolysis is at the cytoplasmic face. The conservation of the NH_2-terminal sequence of the M_r 28,000 protein in its proteolytic degradation products (M_r 26,000 and 10,000) is consistent with a localization of the COOH terminal of the junctional protein at the cytoplasmic face. In addition, the existence of two major portions of the molecule, both apparently protected by the surrounding membrane yet joined by a region accessible to proteases, suggests that the junctional protein crosses the membrane more than once.

This possibility is so far consistent with the sequence of the M_r 28,000 protein. In the NH_2-terminal one-fifth of the molecule, we have identified a highly hydrophobic stretch of 18 residues, bracketed by basic and acidic amino acids (NH_2- and COOH-terminal, respectively), attributes seen in the transmembrane portions of some other proteins (23–27). However, the secondary structure of transmembrane spans of the gap junction protein is open to speculation. In α-helical conformation, ≈23 residues would be required to span the 35 to 40-Å hydrophobic core of the membrane, which in the hydrophobic sequence identified here would require two charged residues (Arg-22 and Arg-32 or Arg-32 and Glu-41) to be buried in a hydrophobic environment. Alternatively, if this hydrophobic region were in β-pleated-sheet conformation, only Arg-32 need be buried in the lipid environment (≈15 residues span the membrane). Both of these alternatives are possible, because ionic bonding between adjacent transmembrane strands could neutralize the charges buried in the lipid (e.g., see ref. 28) or, specifically in the case of the gap junction, the charges could be located in the aqueous channel. Present evidence suggests that the β-sheet conformation is more likely. The Chou and Fasman paradigm (29) applied to the gap junctional protein strongly predicts the hydrophobic region (residues 23–38) to be in a β-pleated-sheet conformation (data not shown). It must be noted, however, that this predictive system, which is based on data from soluble proteins, fails to make consistently correct predictions when applied to at least two membrane proteins of known secondary structure [e.g., *Escherichia coli* protein I (30) and bacteriorhodopsin (our observation)], recent results from x-ray diffraction of liver gap junctions (31) also indicate the presence of β-pleated-sheet structure within the hydrophobic portion of the lipid bilayer. If the protein is in this conformation, it is likely to form a β-barrel structure in order to internally satisfy its hydrogen bonding capacity in a hydrophobic environment. The diameter of such a structure (≈15 Å) is consistent with it representing the wall of the aqueous pore of a gap junction. If such were the case, the polypeptide strands composing the barrel would have to be contributed from separate protein molecules (the six subunits of a connexon), whereas all currently known β-barrels are formed within a single polypeptide chain. As yet, we have insufficient convincing evidence to enable us to distinguish between the possible models, but the complete amino acid sequence should provide an important step towards the final resolution of the three-dimensional structure of this channel.

We thank Dr. Larry Takemoto for his guidance in the use of peptide mapping and Drs. David Meyer and Barbara Yancey for many fruitful discussions. The research was supported by Research Grant GM 06965 and Biomedical Research Support Program RR 07003 from the National Institutes of Health as well as a Ross Foundation Fellowship awarded to B.J.N.

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CHAPTER 3

Structural Studies on the Liver Gap Junction Protein Using Proteolytic Treatments of Isolated Gap Junctions

Running title: Proteolytic analysis of liver gap junctions

Abstract:

In virtually all cases, extensive proteolysis of isolated liver gap junctions has no detectable effect on the junctional ultrastructure, although the M_r 28,000 junction protein is partially degraded. The pattern of this proteolysis is examined for a variety of proteases (trypsin, chymotrypsin, V8 protease and papain). In general, the protein appears to be cleaved into two fragments, with molecular weights between 14,000 and 10,000, which are protected from further proteolysis by the surrounding membrane. Prolonged exposure to papain, however, is found to cause further degradation. The separation of the two M_r 10,000 tryptic polypeptides for possible sequence analysis is reported by both iso-electric focusing and high pressure liquid chromatography. Digestion of these polypeptides by cyanogen bromide provides small fragments of the "native" protein for additional sequence determinations. Analysis of the two-dimensional peptide maps of the two polypeptides produced by partial chymotryptic digestion of gap junctions (M_r 14,000 and 10,000) allows some conclusions to be drawn regarding the disposition of the junction protein within the membrane.

Introduction:

In the preceding two chapters, the isolation of gap junctions from rat liver in high purity and relatively high yield and the subsequent identification and partial characterization (including NH_2-terminal sequence analysis) of the single major protein component of M_r 28,000 was described (Nicholson and Revel, 1983; Nicholson et al., 1981). In this chapter, experiments are reported which provide a basis for further defining the primary (i.e., amino acid sequence) and higher order structure (i.e., folding of the polypeptide chains in the membrane) of the liver gap junction protein.

Primary sequence data beyond that already obtained for the NH_2-terminus will require the generation of fragments of the "native" protein by specific protease or chemical cleavage. The sequences of these fragments can then be pieced together from overlapping sequences of polypeptides produced by different cleavage methods. Since, with the current technology (Hewick et al., 1981), polypeptides up to 6,000 in molecular weight can be sequenced in their entirety, it is most efficient to obtain relatively large fragments for sequence analysis. This can be achieved either through very specific enzymatic or chemical cleavage at residues which occur only infrequently in the gap junction protein (see amino acid analysis in Table 2 of Appendix I) or through proteolysis of intact gap junctions where the membranes should provide some protection against enzymatic hydrolysis for portions of the polypeptide chain (cf. studies of bacteriorhodopsin by Ovchinnikov et al., 1979). It has been demonstrated previously (Henderson et al., 1979; Finbow et al., 1980; Nicholson et al., 1981; Makowski et al., 1982) that extensive treatment of gap junctions with various proteases has virtually no effect on their overall structure in that the membranes remain closely associated, separated by a uniform 2-3 nm gap, and the connexons remain associated in a polygonal (often hexameric) lattice. However, the protein is degraded into smaller polypeptides (of M_r 10,000 in the case of tryptic

hydrolysis; see Figure 3, Nicholson et al., 1981; i.e., Chapter 2). From arguments presented in the previous chapter based on the size and solubility in aqueous solution of trypsin and most other proteases, it seems likely that proteolysis of isolated gap junctions should be restricted to the cytoplasmic faces of the junction, with portions of the polypeptide chain in the membrane, putative transmembrane pore and intermembrane gap being protected. Fragments of the junctional protein produced in this manner are therefore not only useful for sequence analysis, but also provide information on the disposition of the protein in the membrane.

As a result, it is this approach which we have principally used in the production of fragments of the junctional protein for additional sequence analysis. In this chapter, the cleavage patterns of the gap junction protein resulting from various protease treatments of isolated gap junctions are reported in addition to some preliminary results on the chemical cleavage of the solubilized protein. Some aspects of the tertiary structure of the junction protein which can be deduced from peptide maps of the proteolytic fragments just described are also discussed.

Materials and Methods:

Isolation of gap junctions. "Native" and "enzyme treated" gap junction fractions were isolated by the procedures described in Nicholson and Revel 1983 (i.e., Chapter 1). "Enzyme treated" fractions from mice, used in the cyanogen bromide digestion, were prepared in the same way as for rat, with four mouse livers (each 2 g wet weight) being substituted for each rat liver.

Iodination of gap junction fractions by the chloramine T method of Greenwood et al. (1963), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and two-dimensional peptide mapping were performed as described in Nicholson et al., 1981 (i.e., Chapter 2).

IEF and two-dimensional PAGE. After solubilization and iodination, the protein components of an "enzyme treated" gap junction fraction were separated by SDS PAGE, the M_r 10,000 band excised and the protein electroeluted as described by Hunkapiller et al., 1983, as modified by Nicholson et al., 1981. The lyophilized eluate was taken up in 0.4% SDS (Biorad), 8 M urea (Schwartz Mann), 10% mercapthethanol and 1 mM $NaHCO_3$ (pH 7.4) and allowed to stand for 20-30 minutes to ensure complete solubilization. An equal volume of 4% Triton X-100 (Sigma), 8 M urea, 4% ampholines (LKB) and 1 mM $NaHCO_3$ (pH 7.4) was added in order to displace the SDS and replace it with the non-ionic detergent, Triton X-100 (present in 10-fold excess over SDS, see Ames and Nikaido, 1976). Aliquots of the solubilized junctions were then loaded on five 6% acrylamide (9:1 crosslinking ratio) tube gels (0.4 cm diameter) made up in 8 M urea, 2% Triton X-100 and 2% ampholines and polymerized, after degassing, with 0.025% ammonium persulfate and 0.1% TEMED. Ampholines in both the solubilization buffer and the gel were a mixture of LKB 3.5-10.0, 6-8 and 8-9.5 ampholines in ratios of 1:9:4. The samples were overlayed with a 4 M urea, 2% Triton X-100 and 2% ampholine mixture before the 1.0 M NaOH upper reservoir solution was added. The reservoir solution at the anode was 0.2 M H_3PO_4. Isoelectric focusing (IEF) was performed to equilibrium at 400 V for 10 hours (initially the voltage was adjusted to keep the current below 5 mA) and at 550 V for the last hour. Four of the gels were then cut into 2.5 mm slices for pH determination (in 10 mM degassed KCl) and counting in a Beckman 4000 gamma counter. The remaining gel was equilibrated for 2 hours at room temperature in Laemmli solubilization buffer (Laemmli, 1970) and loaded on a Laemmli discontinuous SDS polyacrylamide gel (described in Chapter 2). After running, the second dimension gel was fixed in 7.5% acetic acid, 20% methanol, washed several times in 10% glycerol, dried with heating under vacuum and exposed to Kodak XR film at -70°C with a DuPont Cronex Lightning plus intensifying screen.

High pressure liquid chromatography (HPLC). A sample of an "enzyme-treated" gap junction fraction was iodinated and the gap junctions separated from free iodine by several centrifugations of 4 x 10^5 g_{av} min (15,000 rpm x 30 minutes in an Eppendorf centrifuge). The final pellet was then solubilized in 2% SDS, 50 mM NH_4HCO_3 and 10 mM sodium thioglycollate (pH 7.8) and electrodialyzed (by the same procedure used for electroelution—Hunkapiller et al., 1983) until the SDS concentration was reduced to 0.1%. Fifty µl of the dialysate was loaded on an IBM cyanopropyl silane HPLC column and the polypeptides eluted with a linear gradient of 0-60% acetonitrile in 0.1 M $NaClO_4$, 0.01% H_3PO_4 (pH 2.1) (Meek, 1980) over 90 minutes at a flow rate of 1 ml/minute. Fractions were collected every 30 seconds and counted on a Beckman gamma counter.

Proteolytic treatment of "native" gap junction fractions. Conditions for proteolysis of gap junction fractions were optimized for each enzyme used.

The chymotryptic cleavages shown in Figure 3 were performed in 50 mM NH_4HCO_3, pH 7.8, with 1 mM $CaCl_2$ added during long incubations (lane j) to stabilize the α chymotrypsin (Millipore Corp., Freehold, N.J.). Details are given in the figure legend. The same results are obtained in 80 mM Tris/HCl, 100 mM $CaCl_2$ at pH 8.9 and in the presence of 2 M urea.

Before adding substrate, the active sulphydryl group of papain (Millipore Corp., Freehold, N.J.) was activated by a 30 minute incubation in 1 mM EDTA, 5 mM cysteine HCl and 0.6 mM mercaptoethanol at a concentration of 25% (w/v) enzyme. One µl of the activated enzyme was added to 10 µl of a "native" gap junction fraction (∿18 µg protein) in 0.2 mM EDTA, 0.6 mM cysteine HCl (pH 6.2) with or without 2 M urea and the mixture incubated for 6 hours at room temperature. After removing an aliquot (3 µl) for analysis, 2 µl of a 50% (w/v) activated papain solution was added and incubation resumed for an additional 17 hours. The surviving junctions in both the aliquot and final sample were collected and washed by three successive centrifugations (9 x 10.5 g_{av} min each).

Cleavage of "native" gap junctions by Staphylococcus V8 protease (Miles Lab. Inc., Indiana) was performed at room temperature in 50 mM ammonium acetate, with or without 2 mM urea, at pH 4.0, conditions in which cleavage occurs only at glutamate residues (Houmard and Drapeau, 1972). The concentration of gap junction protein was 180% (w/v), while that of the V8 protease was 6.5% (w/v) for the initial 7 hours of incubation, after which the sample was boosted with an equal amount of V8 protease and incubated for an additional 16 hours. Junctions were then collected in the same manner as for the papain digest.

CNBr cleavage of the gap junction protein. At this time, only the cleavage of the mouse liver M_r 10,000 polypeptides has been examined. After iodination, a mouse liver "enzyme treated" gap junction fraction was run on an SDS polyacrylamide gel, the M_r 10,000 band cut out and the protein eluted and lyophilized. The lyophilizate, containing 3-5 μg of M_r 10,000 polypeptides and some SDS, was taken up in 260 μl of 70% formic acid and 10 mg of CNBr was added. After a 6 hour incubation at room temperature in the dark, an additional 10 mg of CNBr was added and the incubation continued for 20 hours (in the dark). The reaction was terminated by the addition of four times the volume of 5% ammonium acetate followed by lyophilization. Approximately 25% of the radioactivity was lost by this procedure. The resulting polypeptides were separated by urea SDS PAGE (Swank and Munkries, 1971). After Coomassie blue staining, the lane containing the digested junctions was cut out and sliced into 2 mm wide bands for counting on a Beckman gamma counter. This method allows a higher resolution of the distribution of radioactive proteins than is provided by autoradiography.

Results:

Separation of the M_r 10,000 tryptic polypeptides. In the previous chapter it was demonstrated that tryptic hydrolysis of intact gap junctions generates two

polypeptides of M_r 10,000 which could not be separated by SDS PAGE. Two techniques have now been employed in order to isolate each of the two polypeptides. From the sequence data discussed in Nicholson et al. (1981) one of these must represent the NH_2-terminal one-third of the "native" M_r 28,000 junction protein, while the other, must represent a third of the native protein somewhere between the middle and carboxy terminus. NH_2-terminal sequence analysis of this latter polypeptide should provide new data regarding the central portion of the M_r 28,000 junctional polypeptide.

(i) IEF. Gap junction polypeptides have generally proved recalcitrant to attempts at separation by isoelectric focusing, largely because of their insolubility in anything but strong, anionic detergents such as SDS. Although the M_r 10,000 hydrophobic fragments of the junctional protein are particularly difficult in this regard, in one case apparent separation of these two polypeptides was achieved (Figure 1) by first solubilizing M_r 10,000 polypeptides eluted from an SDS polyacrylamide gel in SDS and then displacing this with an excess of the non-ionic detergent Triton X-100. By loading the sample at the basic end of the IEF gel, SDS would be rapidly stripped away and migrate to the opposite end of the gel. Some "streaking" of the polypeptides is evident in the gel (Figure 1a) and could possibly result from precipitation of the protein as SDS is removed. However, this seems unlikely since the distribution of radioactively labeled protein was virtually identical in four separate IEF gels (results of one shown in Figure 1a) and a two-dimensional gel at the level of the M_r 10,000 polypeptides (Figure 1b). Since no radioactivity could be detected corresponding to any other molecular weight on the two-dimensional gel, it would also seem that all of these radioactive peaks represent M_r 10,000 polypeptides with differing pIs. This eliminates the possibility that the multiple peaks seen in IEF might have arisen from free iodine, iodinated lipids or degradation products of the M_r 10,000 polypeptides, since these

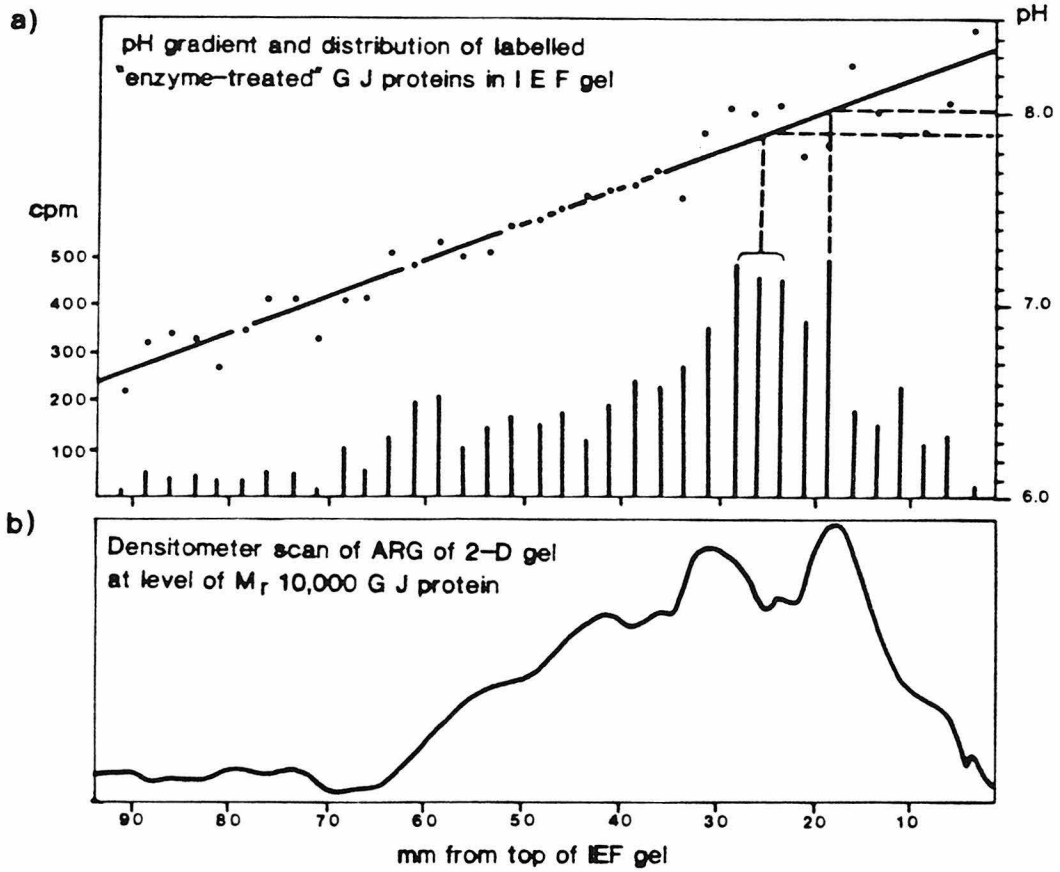


FIGURE 1. Separation of mouse liver M_r 10,000 gap junction polypeptides by iso-electric focusing (IEF).

(a) Iodinated M_r 10,000 polypeptides eluted from an SDS polyacrylamide gel were loaded at the basic end of a 6% acrylamide tube gel and iso-electric focusing conducted to equilibrium (4500 V hrs). The gel was cut into 2.5 mm slices, each of which was then counted on a Beckman gamma counter (histogram results) and then equilibrated in degassed 10 mM KCl for determination of the pH gradient (upper graph). The pattern and pI's of the peaks of radioactivity were reproducible in four gels run in parallel.

(b) After iso-electric focusing as above, a fifth gel was run in a second dimension of SDS PAGE. An autoradiogram of this gel showed all the radioactive material to have a mobility corresponding to M_r 10,000. A densitometer scan of the autoradiogram across its width at the level of this molecular weight is shown. Again, the pattern of peaks corresponds well with those seen in the first dimension—see (a).

would have run at or near the dye front in SDS PAGE. The only possible exception could be the minor peak at pI 7.2 which is visible on the IEF gel (Figure 1a), but not at M_r 10,000 on the second dimension gel (Figure 1b).

Two major peaks were detected at pIs of 8.0 and 7.9. This would be expected from the sequencing and peptide mapping results in Nicholson et al. (1981) and the quantitative estimates of junctional protein recovery after trypsinization (referred to in previous chapter and described in detail in Appendix I), all of which suggest that "enzyme treated" gap junctions are comprised of two different M_r 10,000 polypeptides (representing 20,000 of the "native" protein's 28,000 daltons). The second of these peaks seemed considerably broader than the other. Several minor peaks were also reproducibly found with pIs ranging from 7.2 to 8.1. Studies under similar conditions with standard proteins (Ferritin (pI 4.3), bovine γ globulin (pI 6.5), ribonculease (pI 7.6) and cytochrome c (pI 9.3)) indicated that focusing should have reached equilibrium.

(ii) HPLC. M_r 10,000 polypeptides, especially hydrophobic ones, are somewhat large to be efficiently separated by a cyanopropyl silane HPLC column. Therefore, it is not surprising that most of the radioactivity in an iodinated "enzyme treated" gap junctional fraction was eluted very late in the gradient as two broad peaks (Figure 2), presumably corresponding to the two M_r 10,000 polypeptides alluded to in the previous section. Although both peaks seem to contain similar amounts of protein, as far as can be judged from radioactivity (see Nicholson and Revel, 1983, section D II for a discussion of the problems with such an estimate), the first to be eluted seems to be comprised of a number of components. Other minor peaks are also evident in the HPLC eluate, including some very sharp peaks eluting at 4, 30 and 59 mintues. By analogy with other HPLC separations, the 4 minute peak is likely to be residual free iodine or iodotyrosine produced by partial hydrolysis of the sample. The sharpness of the peaks at 30 and 59 minutes seems inconsistent

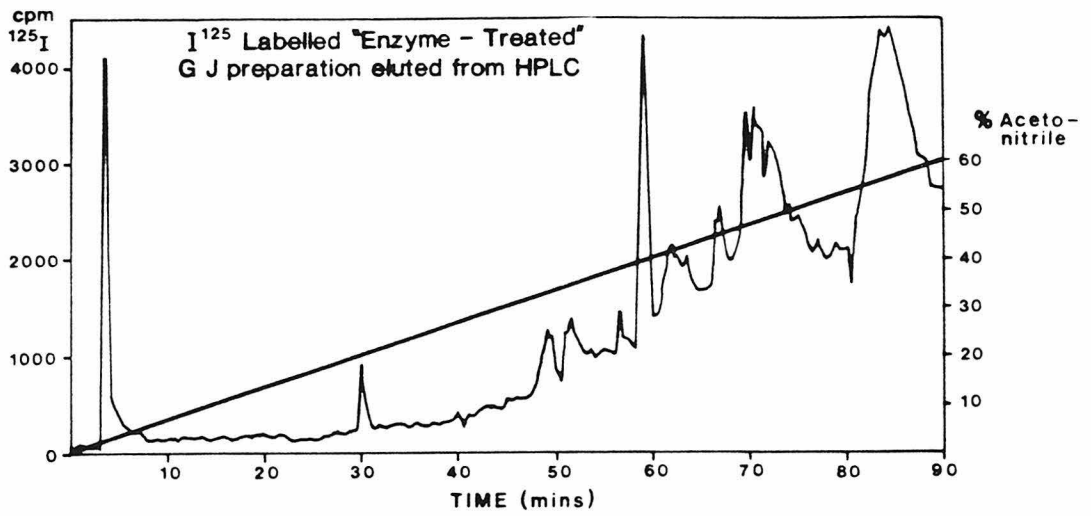


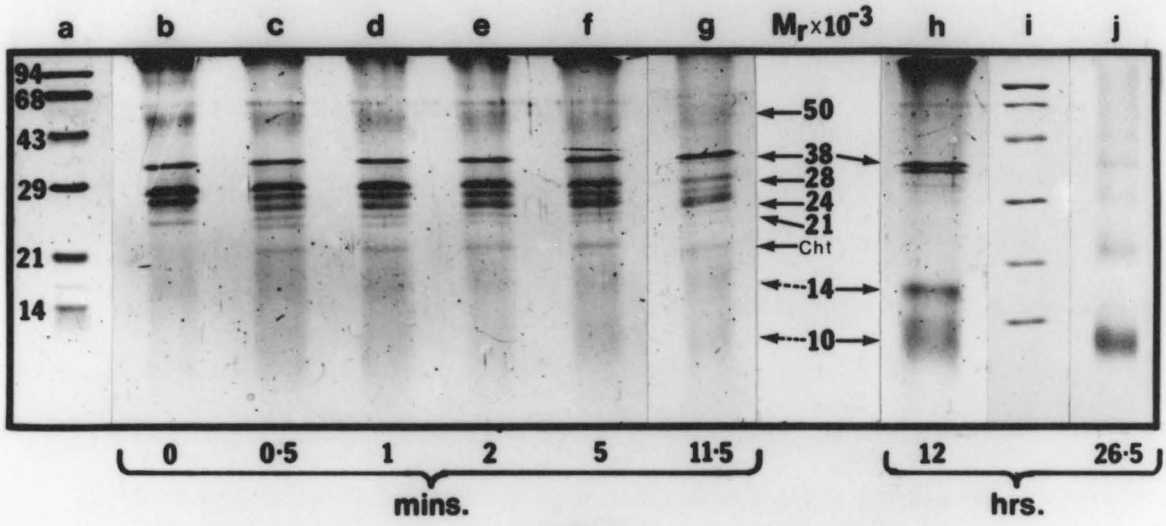
FIGURE 2. Separation of the protein components of a rat liver "enzyme treated" gap junction fraction by high pressure liquid chromatography (HPLC).

An iodinated, "enzyme treated" gap junction fraction from rat liver was solubilized in SDS and the labelled components separated on an IBM cyanopropyl silane HPLC column with a gradient of 0-60% acetonitrile in 0.1 M $NaClO_4$, 0.01% H_3PO_4 (pH 2.1). The two major, broad peaks of radioactive material eluting late in the gradient are likely to represent the M_r 10,000 polypeptides which comprise most of the protein in the fraction. One of these peaks seems to be comprised of multiple components which may represent the tryptic variants of one of the M_r 10,000 polypeptides suggested by sequence data (Nicholson et al., 1981) and discussed in the text. Other peaks must arise from either minor contaminating proteins in the fraction, additional tryptic variants of the M_r 10,000 polypeptides, or labelled phospholipids (see text).

with this material being polypeptides, since polypeptides hydrophobic or large enough to elute this late in the gradient usually form quite broad peaks. Although the nature of this material remains to be determined, it is possible that it could be iodinated phospholipids.

Chymotryptic proteolysis of "native" gap junctions. The time course of chymotryptic digestion of gap junctions (Figure 3) is not grossly different from that of trypsin (described in Nicholson et al., 1981—Figure 3), although some different intermediates can be seen at about M_r 24,000-20,000 (Figure 3, lanes c to g). Even the ultimate end product is a single M_r 10,000 band (Figure 3, lane j) which, by analogy with the tryptic digest,should contain two M_r 10,000 polypeptides. However, there is at least one significant and useful difference. While the transition to the M_r 10,000 polypeptides is rapid in tryptic hydrolysis, with α chymotrypsin there is a relatively stable intermediate with one polypeptide of M_r 10,000 and another of M_r 14,000 (Figure 3, lane h). This latter polypeptide requires several treatments with α chymotrypsin at enzyme: substrate ratios in excess of 1:20 before it is reduced to M_r 10,000 (Figure 3, lane j).

Peptide maps of the M_r 14,000 and 10,000 chymotryptic polypeptides along with a map of the mixture of M_r 10,000 tryptic polypeptides are shown in Figure 4. As might have been predicted, the maps of each of the chymotryptic fragments (Figures 4a and b) contain half of the peptides present in the map of the mixture of tryptic fragments (Figure 4c). Stated another way, the chymotryptic M_r 14,000 and M_r 10,000 polypeptides each contain a mutually exclusive subset of the peptides contained in a mixture of the two M_r 10,000 tryptic fragments. This result has been obtained in five independent mapping experiments and an analogous result is seen in tryptic maps (not shown). Some "cross contamination" of peptides between the maps of the two chymotryptic fragments does occur, resulting often in the two mutually exclusive sets of peptides being less clear-cut. This probably results


a
b
c
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$M_r \times 10^{-3}$
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21
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24
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Cht
14
10
0
0·5
1
2
5
11·5
12
26·5
mins.
hrs.

FIG.3.

FIGURE 3. A timecourse of α chymotryptic hydrolysis of a "native" rat liver gap junction fraction.

(a) and (i) Biorad low molecular weight standards, molecular weights marked in thousands.

(b) - (g) A "native" gap junction fraction (15% (w/v) protein) was treated with 0.2% (w/v) chymotrypsin in 50 mM NH_4HCO_3, pH 7.8, at 37°C. Samples for analysis by SDS PAGE were removed at the times indicated in minutes below each lane and the reaction stopped with phenyl methyl sulphonyl fluoride. Molecular weights of the major bands in thousands are marked to the right of (g), along with the position of residual chymotrypsin (Cht).

(h) Under the conditions described above, half of a "native" gap junction fraction (20% (w/v) protein) was treated with 0.4% (w/v) chymotrypsin for one hour, and for an additional 11 hours following a boost with half as much enzyme again. The junctional proteins are degraded to polypeptides of M_r 14,000 and 10,000, while the M_r 38,000 protein is partially reduced to M_r 36,000.

(j) More extensive chymotryptic hydrolysis of the other half of this "native" gap junction fraction (17 hours at 0.4% (w/v), 2.5 hours following a boost with an equal amount of enzyme, and 7 hours after boosting the enzyme concentration by a factor of five) reduced both of the junctional polypeptides in (h) to M_r 10,000. Chymotryptic autolysis in these longer incubations was reduced by including 1 mM $CaCl_2$ in the buffer.

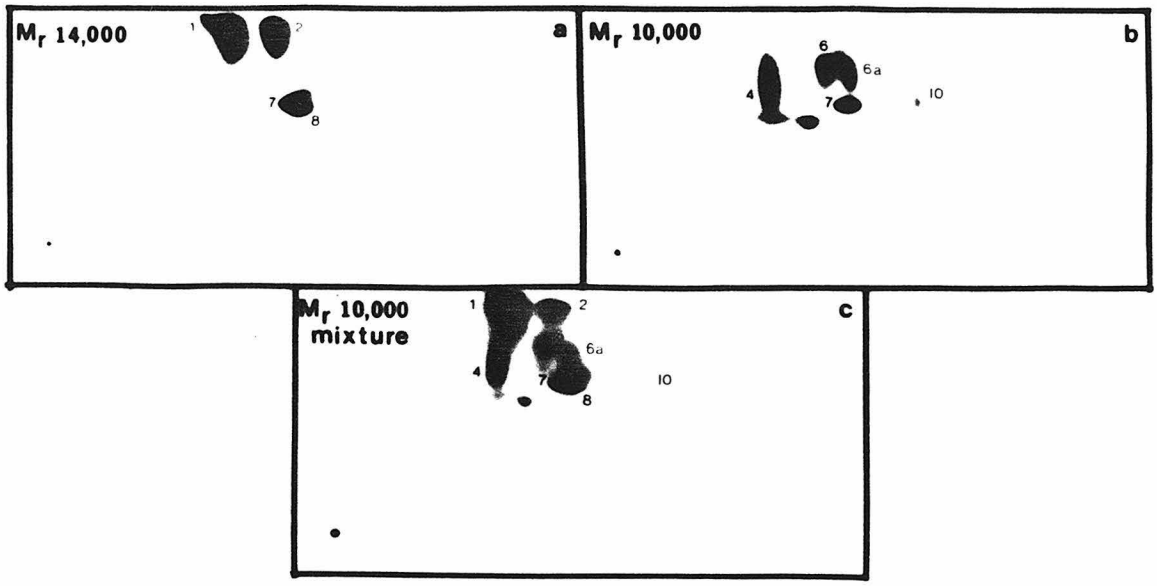

FIGURE 4. Two-dimensional chymotryptic peptide maps of the major polypeptides produced from chymotryptic (a and b) or tryptic (c) hydrolysis of isolated "native" gap junctions.

(a) and (b) Partial chymotryptic hydrolysis of isolated liver gap junctions reduced the "native" M_r 28,000 protein to two polypeptides of M_r 14,000 and 10,000 (Figure 3h). The peptide maps of these polypeptides show that each is composed of a unique set of iodinated peptides, thus demonstrating that they represent different portions of the "native" protein. (Peptide 7 is common to both maps, but this is believed to be iodo-tyrosine and has been found in peptide maps of all proteins studied to date—see text.)

(c) The peptides from both maps (a) and (b) are found in the map of the M_r 10,000 band seen after SDS PAGE of trypsinized (Figure 3g in Chapter 2) or completely chymotrypsinized (Figure 3j in this chapter) gap junction fractions. This further demonstrates that this band is comprised of two polypeptides representing more than two-thirds of the "native" M_r 28,000 molecule.

from the proximity of the bands on the original gel from which they were excised, or some cleavage of the M_r 14,000 polypeptide to its M_r 10,000 fragment (see Figure 3, lanes h and j). In any event, the only consistent exception to the mutually exclusive nature of these two sets of peptides is peptide 7, and this peptide is found in the chymotryptic and tryptic maps of every protein that we have studied. Since it elutes at the very beginning of an HPLC gradient designed to separate small peptides (results not shown), we believe that it is likely to represent free iodotyrosine generated by limited hydrolysis of the sample in acid conditions, and does not, therefore represent a true chymotryptic or tryptic peptide.

One unexpected result also came to light during these experiments. Frequently, polypeptides of M_r 14,000 and 10,000 are present in "native" gap junction fractions stored for protracted periods at -20°C, presumably as a result of some low level of contaminating, unidentified, proteolytic activity. In two cases, peptide maps of these polypeptides have been examined. They are identical to those of the chymotryptic fragments, except that peptide 10, found in the M_r 10,000 chymotryptic fragment, is found in the map of the M_r 14,000 fragment generated by proteolysis in the freezer. This is consistent with the observation that this M_r 14,000 fragment migrates a little slower than the M_r 14,000 chymotryptic fragment on SDS PAGE.

Proteolysis of "native" gap junctions by other proteases. Comparing the chymotryptic or tryptic cleavage of the M_r 28,000 junctional protein after elution from a gel (i.e., peptide mapping) or while part of the intact junction (see Figure 3; in this chapter and Chapter 2), it is clear that the pattern of polypeptides obtained by proteolysis of whole junctions is largely a function of the disposition of the junctional protein in the membrane, and to a lesser extent the presence of available cleavage sites. Therefore, one would expect most proteases to produce similar size fragments. Experiments with Staphylococcus V8 protease (under conditions where cleavage only occurs at glutamate residues) and papain (a much less specific

protease) largely support this conclusion. V8 protease produces several fragments of M_r 14,000 to 10,000 which are poorly resolved by SDS PAGE (Figure 5, lane b). The multiple fragments suggest that proteolysis was not complete by 23 hours (compare Figure 5, lane b with the intermediate stages of chymotryptic (Figure 3, lane g) or tryptic (Chapter 2, Figure 3, lane d) digestion of gap junctions). Papain shows a similar timecourse to chymotrypsin with M_r 14,000 and 10,000 polypeptides evident after 6 hours (Figure 5, lane c). As was the case for chymotrypsin and trypsin, no proteolytic intermediates of the junction are evident between M_r 24,000 and 14,000, despite the non-specificity of this protease.

In some ways, however, these proteases do behave differently from those studied previously and may reveal new aspects of the three-dimensional organization of the gap junction protein with respect to the membrane. While V8 protease cleaves the M_r 26,000 and 24,000 polypeptides of the "native" fraction (Figure 5, lane a) quite efficiently, it fails after 23 hours to affect the M_r 28,000 "native" protein. This result was also obtained in the presence of 2 M urea, which should help to disrupt the secondary structure of the polypeptide chain outside the membrane. Prolonged exposure (23 hours) to papain, on the other hand, reduces the junctional protein to an unresolved smear of polypeptides below M_r 10,000 on SDS PAGE (Figure 5, lane d), a result which is also independent of the presence of protein denaturants (i.e., 2 M urea). Examination of this fraction by negative staining in the electron microscope revealed that junctional plaques with hexagonally arrayed connexons were still intact despite the extensive degradation of the protein components (Figure 6a). Some images, however, did suggest that the membranes of some of the junctions may have been separated (i.e., the gap junctions may have been split; see Figure 6b and c). Further experiments remain to be done to confirm this possibility.

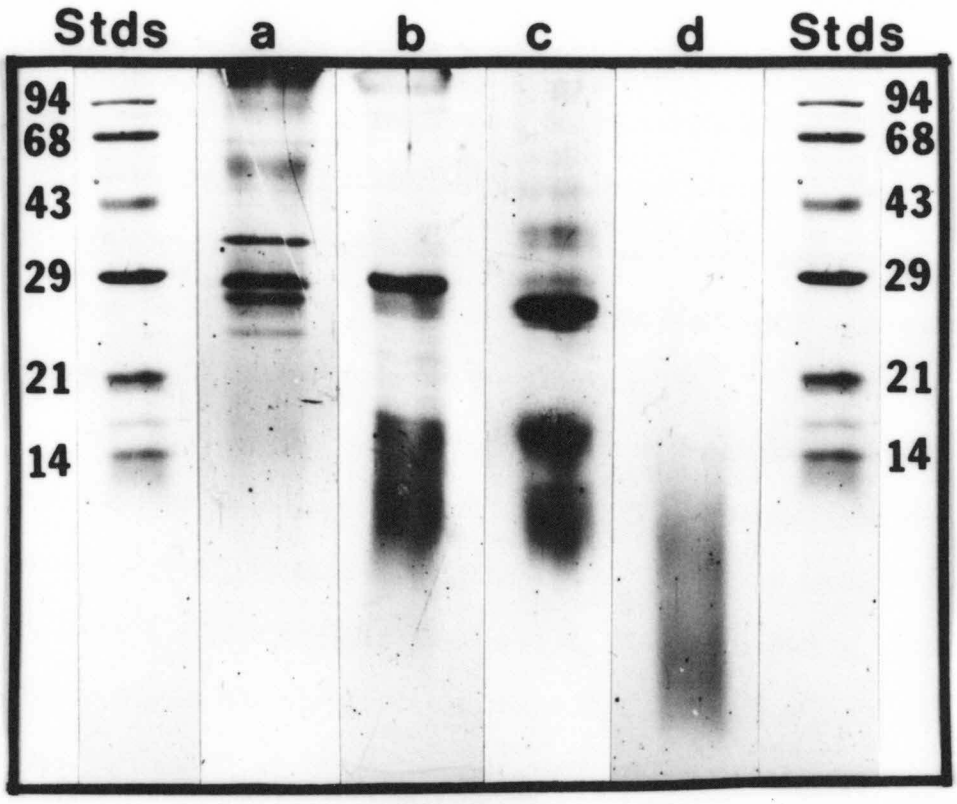

FIG. 5.

FIGURE 5. Analysis by SDS PAGE of the products of hydrolysis of "native" gap junction fractions by Staphylococcus V8 protease and papain.

Biorad low molecular weight standards (molecular weights marked in thousands) are shown in the outer lanes.

(a) "Native" gap junction fraction.

(b) "Native" gap junctions treated for 23 hours with Staphylococcus V8 protease in ammonium acetate at pH 4.0 (cleavage only at glutamate residues). The M_r 28,000 "native" protein is unaffected, but its partial endogenous degradation products (M_r 26,000 and 24,000) are broken down to multiple components of M_r 14,000-10,000. The absence of an M_r 38,000 band would suggest that this contaminant may be susceptible to V8 proteolysis. No multimeric aggregates of the junctional protein are detected in this fraction.

(c) "Native" gap junctions treated for six hours with papain. The junctional proteins have been largely degraded to an M_r 24,000 polypeptide which has in turn been partially cleaved to fragments of M_r 14,000 and 10,000. The M_r 38,000 protein has been partially cleaved to M_r 36,000 (cf. chymotryptic digests—Figure 3h). A dimeric aggregate of the M_r 24,000 polypeptide and some residual "native" M_r 28,000 material are also evident.

(d) "Native" gap junctions treated for 23 hours with papain. Further papain proteolysis reduces the junctional protein to multiple fragments with molecular weights less than 10,000. The M_r 38,000 protein is also apparently degraded to smaller polypeptides. See text for details of proteolysis conditions used.

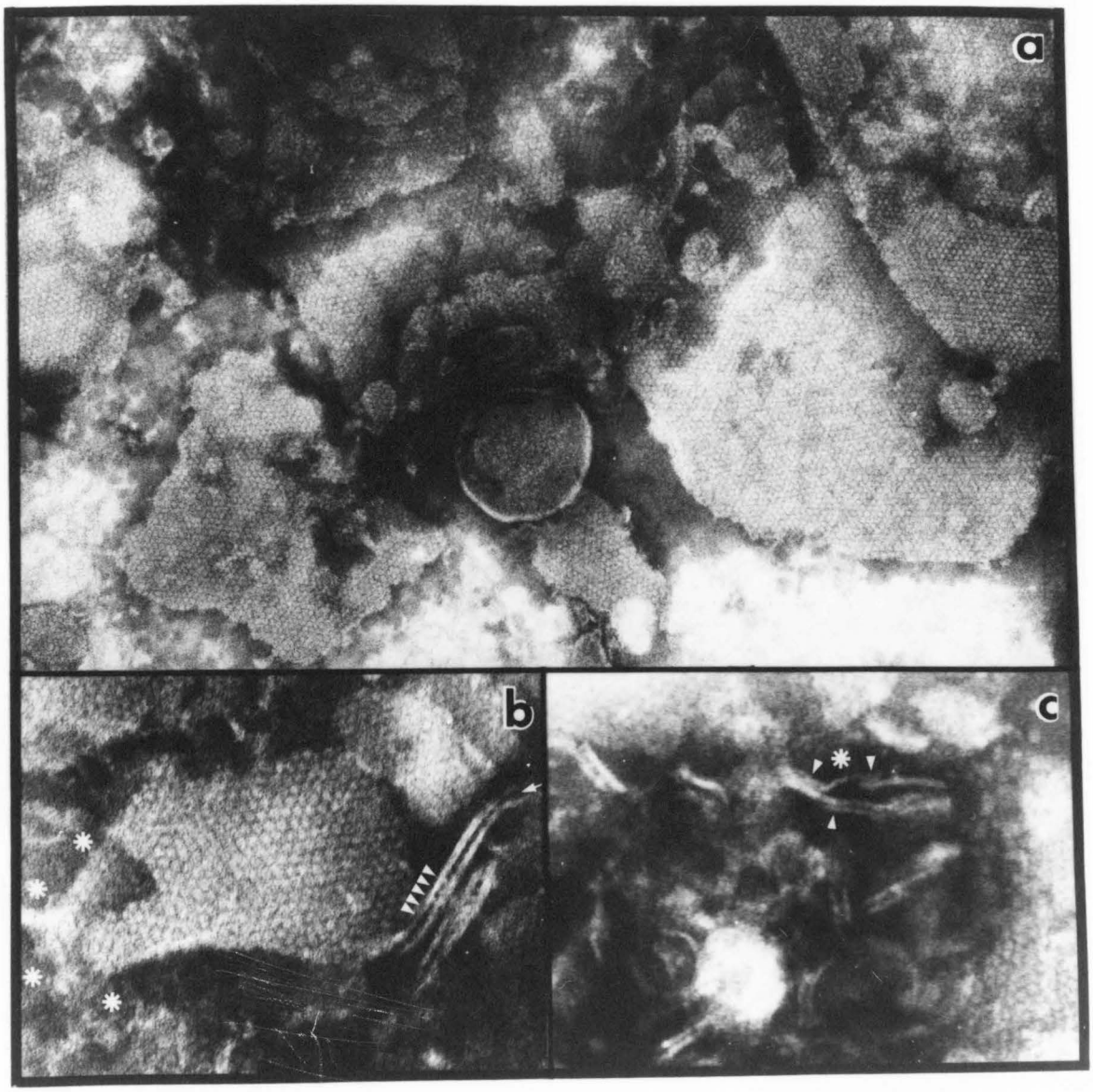

FIGURE 6.

FIGURE 6. Negatively stained "native" gap junction fractions treated with papain for 23 hours (samples of the same fraction examined by SDS PAGE in Figure 5, lane d).

(a) An overview of the fraction (mag - X120,000) shows it to be comprised almost totally of gap junction sheets which display very ordered, hexagonal arrays of connexons (particularly evident to the right of the picture). The lack of discontinuities or holes in the lattice of connexons suggests that there is little disruption of the lipid bilayer. Occasional, non-junctional vesicles are also seen (centre of picture).

(b) Views of the junctional sheets in profile demonstrate, in some cases, the intact nature of the double membrane gap junction structure (note the stain excluding cross-bridges spanning the gap between membranes—white arrowheads). However, at times the two halves of the junction seem to be separating from one end (white arrow). En-face views are suggestive of similar splitting. In the region marked by (*), a portion of the upper half of the junction seems to have been removed, exposing the lower half (mag - X215,000).

(c) More extensive separation of the junctional membranes can sometimes be seen (*). The pairing of one membrane sheet with two other membranes, in one case on opposite sides of the bilayer (white arrowheads), and the reduced gap between membranes might suggest that these junctional membranes have been separated and the halves are now re-adhering to one another non-specifically rather than through specific connexon-connexon interactions (mag - X215,000).

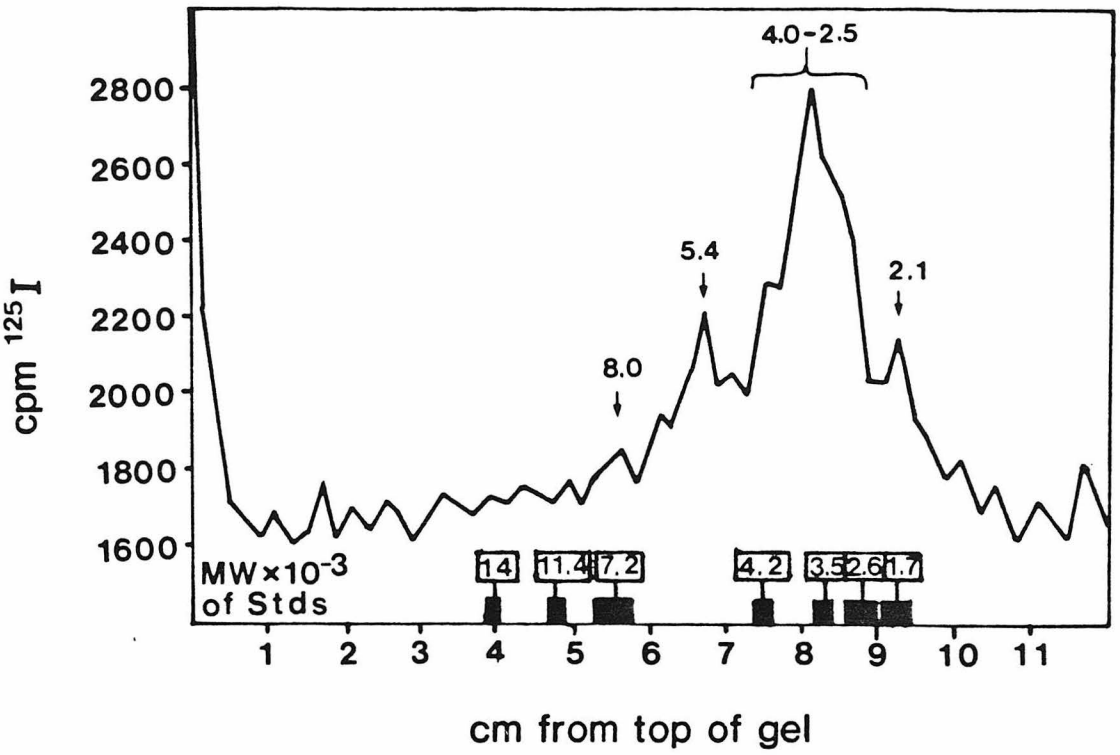

FIGURE 7. Distribution of radioactivity in a urea SDS polyacrylamide gel of a CNBr digest of iodinated mouse liver M_r 10,000 gap junction polypeptides. Loading end of the gel is towards the left of the figure and the migration pattern of the standards run on the gel along with their molecular weights in thousands are marked along the X-axis. Standards are ribonuclease (M_r 14,000) glucagon (M_r 3,500) and a partial CNBr digest of cytochrome c (M_r 11,400; 7,200; 4,200; 2,600; 1,700). Major CNBr fragments of M_r 5,400, 2,100 and several between M_r 4,000 and 2,500 are detected (molecular weights marked in thousands in figure) as well as a minor component of M_r 8,000, which may represent a partial cleavage product. See text for details of cleavage conditions.

CNBr cleavage of the mouse M_r 10,000 tryptic polypeptides. The initial experiment on the cleavage of mouse liver M_r 10,000 gap junction polypeptides with CNBr yielded bands of M_r 8,000 and 5,000, as well as a diffuse band from M_r 3,000 to 2,000 when examined by urea SDS PAGE (results not shown). However, a subsequent and more complete cleavage of iodinated M_r 10,000 polypeptides with CNBr (Figure 7) revealed much reduced levels of the M_r 8,000 polypeptide, relatively sharp peaks at M_r 5,400 and 2,100, and a broad peak containing most of the radioactivity centered around M_r 3,200. The profile of radioactivity in this major peak (Figure 7) suggested that it was composed of multiple components which could not be resolved by urea SDS PAGE and appeared as shoulders on the larger peak (a sharp one at M_r 4,000 and a broad leading edge shoulder around M_r 3,000). The most likely interpretation of these results would seem to be that CNBr treatment of the mixture of M_r 10,000 tryptic polypeptides from mouse liver produces a partial cleavage product of M_r 8,000 and fully cleaved products of M_r 5,400, 2,100 and several of M_r 2,500 to 4,000.

Discussion:

In order to further the determination of the amino acid sequence of the liver gap junction protein, we have produced several fragments of the M_r 28,000 "native" protein. Since proteolysis of intact junctions was largely used for this purpose, certain conclusions as to the tertiary structure of the junctional protein can also be made. As alluded to in the introduction, most proteases are too large (e.g., trypsin and chymotrypsin are both 5 nm across at their smallest diameter) to penetrate the transmembrane junctional pore (1.5 nm in diameter) or the extracellular gap between the two membranes of a gap junction (2-3 nm wide), and, due to their hydrophilic surfaces, are equally unlikely to enter the lipid bilayer. This allows us to deduce the approximate location of the polypeptides which remain as part of the intact gap junction structure after proteolysis.

Tryptic hydrolysis of gap junctions has been studied most extensively (Nicholson et al., 1981) and has been demonstrated to first remove about 4,000 daltons from the COOH-terminus of the native protein before cleaving the remaining M_r 24,000 polypeptide into two fragments of M_r 10,000. One of these polypeptides represents the NH_2-terminal end of the native protein (Nicholson et al., 1981). No unique sequence, however, could be deduced for the second polypeptide. This is likely the result of several, closely-spaced, tryptic cleavage sites (approximately 14,000 daltons from the amino-terminus) exposed at the cytoplasmic surface just before the polypeptide chain enters the bilayer. These would provide a number of potential amino-terminal starting points for the sequence of the second polypeptide. This problem is not made any easier by the inability of SDS PAGE to separate the two major portions of the junctional protein. Using IEF and HPLC, we have attempted to separate the two M_r 10,000 polypeptides, and some of the "tryptic" variants of second M_r 10,000 fragment. Two major components were resolved by both techniques. Furthermore, there is evidence to suggest that at least some of the proposed tryptic variants of the M_r 10,000 polypeptides may have also been resolved. The first of the major peaks to be eluted from HPLC (Figure 2) seems to be composed of multiple components. In IEF, several minor bands in addition to the major species at pIs of 7.9 and 8.0 can be detected. Their mobilities in a second dimension confirm that they represent M_r 10,000 polypeptides, which could have been derived from one of the major species by the loss of a few basic residues. Resolution of this issue will require preparative IEF or HPLC in order to isolate sufficient of these fractions to allow NH_2-terminal sequence anlaysis. The isoelectric points of the major M_r 10,000 polypeptides determined here are consistent with their amino acid composition (Appendix I, Table 2) if one assumes that about half of "glx" and "asx" are present as the amide, an assumption which appears justified on the basis of the available sequence (three amides and three free acids are present in the NH_2-terminal 58 residues).

Chymotryptic proteolysis of gap junctions provides an alternative approach to separating the two protease resistant halves of the gap junction protein. Although eventually chymotrypsin also reduces the junctional protein to two M_r 10,000 fragments (Figure 3, lane j), the final reduction of one of the polypeptides from a stable M_r 14,000 intermediate (Figure 3, lane i) proves to be very slow. Partially chymotrypsinized gap junctions should, then, contain easily resolvable components of M_r 14,000 and 10,000 analogous to the two M_r 10,000 tryptic polypeptides. Given this premise, certain predictions follow which can be tested by peptide mapping. Stated briefly, the peptide map of the mixture of M_r 10,000 tryptic fragments (Figure 4c) should contain two, mutually-exclusive sets of peptides, one of which should comprise the map of the M_r 14,000 chymotryptic fragment and the other the map of the M_r 10,000 chymotryptic fragment (of course, one or two additional peptides might be found in the M_r 14,000 polypeptide considering the additional 4,000 daltons of mass). This indeed proves to be the case, as can be seen in Figure 4 with peptides 1, 2, 5 and 8 in the M_r 14,000 polypeptide and peptides 6, 6a, 4 and 10 in the M_r 10,000 polypeptide (peptide no. 5 was not labeled in the maps shown, but was detected in most other experiments). As alluded to in the Results, peptide no. 7 appears to represent free iodotyrosine. Interestingly, there are apparently no tyrosines or other iodination sites in the protein lost from the M_r 14,000 polypeptide in its conversion to M_r 10,000 since no additional peptides are seen in the map of the M_r 14,000 fragment. A comparison of these peptide maps with those of the M_r 28,000 and 24,000 polypeptides (see Chapter 2, Figure 5) allows us to build a crude model of the gap junction protein in which some of the labeled peptides can be located (Figure 8).

In reducing the M_r 28,000 protein to M_r 24,000 several hydrophilic peptides are lost (peptides 11, 12 and 13 and most of 9) and a new hydrophobic one appears (peptide 6a). Since the M_r 28,000 and 24,000 polypeptides share the same NH_2-terminal sequence (Nicholson et al., 1981), these peptides must be removed

from the COOH-terminus. For a new peptide to appear in the map of the M_r 24,000 polypeptide (i.e., peptide 6a), one of the labeled peptides of the M_r 28,000 protein must have been cleaved, leaving its hydrophobic portion bearing the labeled tyrosine embedded in the membrane and attached to the M_r 24,000 polypeptide. The most likely candidate for such a precursor peptide is no. 9, since residual amounts of it remain in most maps of the M_r 24,000 polypeptide. Since peptide 6a must represent the COOH-terminal peptide of the M_r 24,000 polypeptide, it follows that the M_r 10,000 chymotryptic fragment, which contains this peptide, must represent the COOH-terminal half of the M_r 24,000 protein. The M_r 14,000 polypeptide, therefore, must represent the NH_2-terminal half of the "native" protein. Peptide no. 10 was deduced to be at the NH_2-terminal end of the M_r 10,000 fragment from a chance observation of "native" gap junction fractions stored for prolonged periods at -20°C. Such fractions undergo partial proteolysis by an unidentified enzyme to fragments of M_r 10,000 and 14,000 (actually, slightly higher in molecular weight than the M_r 14,000 chymotryptic fragment). These share identical maps with those of the corresponding chymotryptic fragments, with the one exception that peptide no. 10 is found in the map of the M_r 14,000 polypeptide, and not that of the M_r 10,000 polypeptide. This would suggest the existence of an accessible cleavage site for this unidentified protease to the COOH-terminal side of peptide no. 10, and would argue that this peptide is wholly exposed at the cytoplasmic surface of the junction (not a surprising result considering the hydrophilic nature of the peptide). Since, as yet, no probes have been used to study groups located in the hydrophobic portion of the bilayer, in the lining of the transmembrane channel, or in the extracellular gap, the specific distribution of peptides 1, 2, 5 and 8 of the M_r 14,000 polypeptide and peptides 4 and 6 of the M_r 10,000 polypeptide remains unknown. A similar model to the one shown in Figure 8 can also be constructed using peptides from tryptic maps.

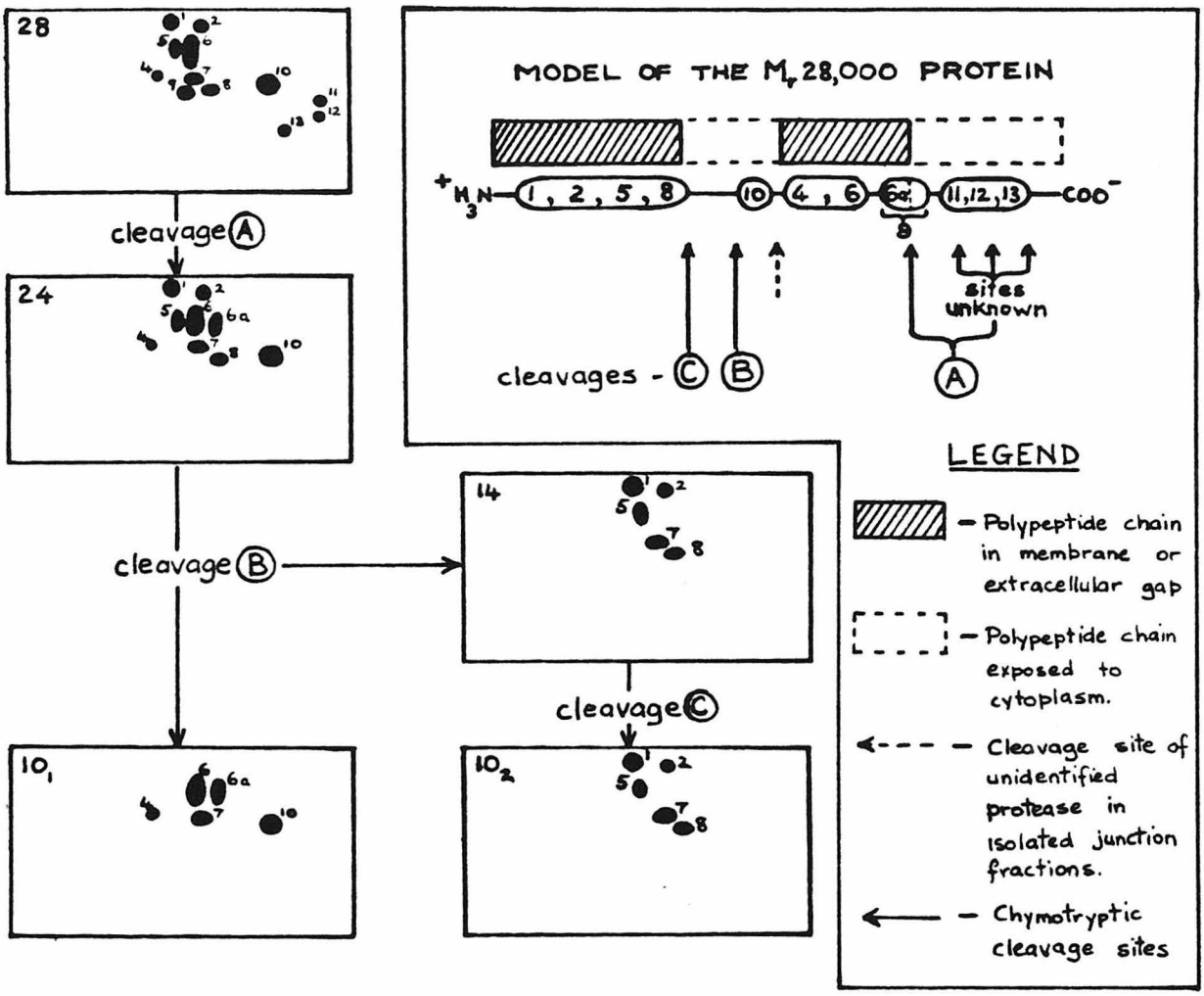
28
cleavage A
24
cleavage B
14
cleavage C
10₁
10₂
MODEL OF THE Mr 28,000 PROTEIN
+H3N
1, 2, 5, 8
10
4, 6
6a
11,12,13
COO⁻
sites unknown
cleavages - C B A
LEGEND
- Polypeptide chain in membrane or extracellular gap
- Polypeptide chain exposed to cytoplasm.
- Cleavage site of unidentified protease in isolated junction fractions.
- Chymotryptic cleavage sites

FIGURE 8.

FIGURE 8. Model of the rat liver M_r 28,000 gap junction protein deduced from chymotryptic cleavage of isolated gap junctions.

Diagrammatic chymotryptic peptide maps of some of the polypeptides produced by chymotryptic hydrolysis of isolated gap junctions (see Figure 3) are shown here with the peptides numbered for ease of identification. These maps are based on data from five independent experiments (one shown in Figure 4). Using available sequence data and these peptide maps, the numbered peptides can be located along the length of the polypeptide chain of the M_r 28,000 "native" junction protein as presented in the model (see text for detailed discussion). The specific order of peptides 1, 2, 5 and 8, peptides 4 and 6, and peptides 11, 12 and 13, along with the orientation of the NH_2-terminus (extracellular or cytoplasmic) remains to be determined. Shaded regions are deduced to represent portions of the protein in the membrane or extracellular gap between membranes since in the intact junction they are inaccessible to proteases which are considered too large to penetrate these areas (see text). The cleavage site of the unidentified protease present in isolated junction fractions (dotted arrow) was deduced from peptide maps of M_r 14,000 and 10,000 polypeptides which appear in fractions stored for prolonged periods (peptide 10 is found in the map of the M_r 14,000 rather than the M_r 10,000 polypeptide).

Preliminary studies with other proteases (Staphylococcus V8 protease and papain) have provided some interesting observations, which may later prove useful in understanding the folding of the junctional protein. In general, both proteases follow the pattern of chymotrypsin and trypsin in that the junctional protein is reduced to a molecular weight of about 24,000 before cleavage into two fragments with molecular weights between 14,000 and 10,000 (Figure 5, lanes b and c). This supports the contention that proteolytic cleavage patterns in the intact junction are mainly a function of junctional structure rather than available cleavage sites. Some unique properties are seen, however. The resistance of the "native" M_r 28,000 junctional protein to V8 proteolysis is in sharp contrast to the susceptibility of its tryptic degradation products (M_r 26,000 and 24,000; see Figure 5, lane b). This seems to suggest that the carboxy-terminal 2,000 daltons of the junctional protein possesses no glutamate residues for V8 protease cleavage, and is folded so as to obscure any V8 proteolytic sites on the cytoplasmic surface. Once this 2,000 dalton segment is removed by some other protease, these V8 cleavage sites are exposed. Although 2 M urea fails to disrupt the secondary or tertiary structure which is responsible for this behavior, it is possible that the folding is stabilized by disulfide bonds or by a portion of this carboxy-terminal 2,000 daltons being buried in the lipid bilayer.

In contrast to V8 protease, papain is a much less specific protease. Perhaps as a result of this, papain is the only protease we have used which cleaves the junctional protein within the structure of the gap junction to fragments smaller than M_r 10,000. Several mechanisms could possibly explain this result. Although papain has a similar molecular weight to trypsin and chymotrypsin, it does not share the overall spherical shape of these proteases (Stroud et al., 1974). X-ray diffraction studies indicate that the papain polypeptide chain folds into a dumbbell shape with dimensions of 36 x 36 x 48 Å(Dreuth et al., 1968). By analogy with

other proteases and from the intact appearance of the junctional sheets after proteolysis (Figure 6a),it seems unlikely that papain would penetrate the lipid bilayer. However, the smaller size of the papain molecule (compared to trypsin and chymotrypsin which are >50 Å in diameter) may allow penetration of the aqueous gap separating the membranes of the junction. Even if penetration is limited, the non-specificity of papain should increase the chances of cleavage sites being exposed in the gap at the edge of the junction, thus allowing proteolysis to proceed inward, much like the undoing of a zipper. This same lack of specificity might also explain the observed results even if papain were excluded from the extracellular gap, since there may be portions of the polypeptide chain exposed at the cytoplasmic surface which do not possess sites for more specific proteases such as trypsin, chymotrypsin or V8 protease, but which do contain sites susceptible to papain hydrolysis. Although inconclusive, negatively stained images of the fraction (Figure 6) are not inconsistent with the "unzipping" model proposed above. If indeed, proteolysis is occurring in the gap, exhaustive papain treatment of junctions followed by isolation of the polypeptides on HPLC should provide some excellent material for the sequencing of the junctional transmembrane sequences.

In addition to proteolysis of intact junctions, we have also begun preliminary studies on specific chemical cleavage of the isolated proteins to produce smaller polypeptides for sequence analysis. CNBr cleavage of mouse M_r 10,000 polypeptides (under conditions where cleavage occurs preferentially at methionine residues; Givol and Porter, 1965) produces final fragments of M_r 5,400 and 2,100 and several around M_r 3,000. Since the amino-terminal 20 residues of mouse liver junctional protein are identical, with one exception, to those of rat liver (Nicholson et al., 1981), and since the peptide maps of the proteins from these two species are essentially identical (Nicholson et al., 1983; Figure 6—i.e., in Chapter 4), it seems reasonable to assume that CNBr cleavage of the mouse liver protein should be very similar

to that of the rat. The M_r 5,400 fragment most likely represents the NH_2-terminal peptide of the protein, since in the amino-terminal 52 residues, no methionines are seen except that at the NH_2-terminus itself. Unfortunately, the remaining methionines are apparently well dispersed throughout the remaining sequence, resulting in the production of a series of M_r 3,000 fragments (which cannot be resolved by SDS PAGE). While it is possible that CNBr cleavage of the "native" M_r 28,000 protein may produce polypeptides which can be resolved by PAGE, it currently seems that HPLC will have to be used in the preparation of CNBr fragments for sequence analysis.

The isolation of these various fragments of the M_r 28,000 junction protein will clearly be useful for obtaining further sequence data on the protein, and has already enabled us to determine the relative positions in the "native" protein of the tryptic and chymotryptic peptides of our two-dimensional maps (Figure 8). It is also tempting to use this data, in conjunction with that available from NH_2-terminal sequence analysis (Nicholson et al., 1981), X-ray (Makowski et al., 1977; 1982) and optical (Unwin and Zamphigi, 1980) diffraction, to speculate as to how the gap junction protein is arranged in the membrane and at the extracellular and intercellular surfaces. The number of possible models which can be constructed, however, makes such speculation meaningless until further information is available regarding the secondary and tertiary structure of the protein within the membrane (e.g., does it form α helices, β barrels or β-jelly rolls, etc?).

The one conclusion that can be drawn from these studies is that the junctional protein is likely to span the membrane at least three times. The COOH-terminal 4,000 daltons (~35 residues) is exposed at the cytoplasmic surface after which the polypeptide chain appears to enter the membrane, since it is no longer subject to proteolysis. Ten thousand daltons or approximately 90 residues later, the chain emerges again at the cytoplasmic surface to form a 4,000 dalton (~35 residue)

protease-labile loop before re-entering the membrane for its final NH_2-terminal 10,000 daltons. Clearly the protected 10,000 dalton portion closest to the COOH-terminus must span the membrane at least twice in order to return to the cytoplasm. This assumes that it is not energetically permissible for a polypeptide chain to reverse direction halfway across a lipid bilayer, because of energy cost of the non-paired H bonds in a reverse turn in a hydrophobic environment. Since the location of the NH_2-terminus on the extra-or intra-cellular side of the membrane has yet to be determined, the minimum number of times the NH_2-terminal M_r 10,000 fragment must span the membrane is unknown. However, since a polypeptide chain can cross the membrane in 20 residues as an α helix or even less as a β sheet, it is possible for each of the M_r 10,000 tryptic fragments of the junctional protein to traverse the membrane as many as four times. This is unlikely to be the case for both fragments since then no protein would protrude into the extracellular gap, an arrangement of the polypeptide chain which is contrary to that suggested by both X-ray and optical diffraction studies of isolated gap junctions (Makowski et al., 1977; Unwin and Zamphigi, 1980).

Failing the generation of more ordered crystals of the gap junction protein and subsequent high resolution X-ray analysis, the only approach to finally answering these issues lies in obtaining more of the sequence of the protein and determining its location in the three-dimensional structure of the junction. The work presented here lays groundwork necessary for producing such data.

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CHAPTER 4

Differences between Liver Gap Junction Protein and Lens MIP 26 from Rat: Implications for Tissue Specificity of Gap Junctions

Running Title: Tissue-specific junctional proteins

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Differences between Liver Gap Junction Protein and Lens MIP 26 from Rat: Implications for Tissue Specificity of Gap Junctions

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Summary

Liver gap junctions and gap-junction-like structures from eye lenses are each comprised of a single major protein (M_r 28,000 and 26,000, respectively). These proteins display different two-dimensional peptide fingerprints, distinct amino acid compositions, nonhomologous N-terminal amino acid sequences and different sensitivities to proteases when part of the intact junction. However, the junctional protein of each tissue is well conserved between species, as demonstrated previously for lens and now for liver in several mammalian species. The possibility of tissue-specific gap junction proteins is discussed in the light of data suggesting that rat heart gap junctions are comprised of yet a third protein.

Introduction

Gap junctions can be defined as patches of closely packed intramembrane particles (termed connexons in Goodenough, 1975; Kreutziger, 1968; Goodenough and Revel, 1970; McNutt and Weinstein, 1970) that span the membranes of adjacent cells and the uniform, ~2 nm gap which separates them. The consistent correlation of electrical coupling and/or the intercellular transfer of low molecular weight (<1000) compounds with the presence of gap junctions (for review see Bennett, 1978) has led to the consensus that these physiological properties also denote the existence of gap junctions (see the recent discussion in Meyer et al., 1981). By the latter criteria or by direct morphological observation, gap junctions have been described in every metazoan phylum and in the vast majority of tissues studied (for reviews see Loewenstein, 1979, and Staehelin, 1974). Gap junctions in most of these systems display generally similar features, and cells from several different tissues and species have been reported to establish direct intercellular communication with one another in tissue culture (Michalke and Loewenstein, 1971; Epstein and Gilula, 1977; Gaunt and Subak-Sharpe, 1979). However, there is some evidence that all gap junctions are not identical. There are a number of instances where communication-competent cells derived from different tissues or species establish only miminal coupling or no coupling with one another (Fentiman et al., 1976; Pitts and Burk, 1976; Gaunt and Subak-Sharpe, 1979). In addition, minor heterogeneities have been observed in the morphology (Staehelin, 1974; Larsen, 1977), physiological properties (see Peracchia, 1980, for review) and molecular exclusion limits (Simpson et al., 1977; Flagg-Newton et al., 1979) of gap junctions from different sources.

The biochemical basis of these heterogeneities is unclear. Analysis has so far been confined to two systems: gap junctions from liver, which contain a single major protein reported as M_r 26,000 or 28,000 (Hertzberg, 1980, and Nicholson et al., 1981, rat; Henderson et al., 1979, mouse; Hertzberg et al., 1978, calf); and junctions of eye lens fiber cells, which also appear to be comprised of a single major protein, called main intrinsic protein (MIP), of M_r ~ 26,000 (Broekhuyse et al., 1976, calf; Alcalá et al., 1978, chicken; Goodenough, 1979, mouse). While the work presented here was in progress (Nicholson et al., 1980), evidence of differences between the junctional proteins of rat liver and bovine lens, based on one-dimensional peptide mapping and lack of immunological cross-reactivity, was published (Hertzberg et al., 1982). This evidence, in conjunction with the morphological (Goodenough, 1979; Zampighi et al., 1982) and physiological differences (Schuetze and Goodenough, 1982) reported between lens fiber junctions and gap junctions in other tissues, could be interpreted to mean that the structures in the lens are not gap junctions. Alternatively, one could argue that these represent differences between gap junctions of different tissues. In this report, we speak of lens junctions without necessarily implying that these structures in the lens are the equivalent of gap junctions elsewhere. We attempt to clarify the nature of these lens junctions by comparing the lens MIP and liver gap junction protein (both from rat) using two-dimensional peptide mapping and partial amino acid sequencing of the two proteins.

Results

Morphological Assay of Junctional Fractions from Liver and Lens

The usual method for assaying the purity of gap junction fractions involves electron microscopic analysis of a sample of the fraction to determine the relative percentages of intact junctions and recognizable contaminants.

Liver

The morphology of isolated rat liver gap junctions has been extensively described previously. In agreement with these observations, our final "native" gap junction fraction (that is, isolated under conditions of minimal proteolysis—see Experimental Procedures) contains predominantly flat sheets of membrane which in profile appear as two closely apposed membranes

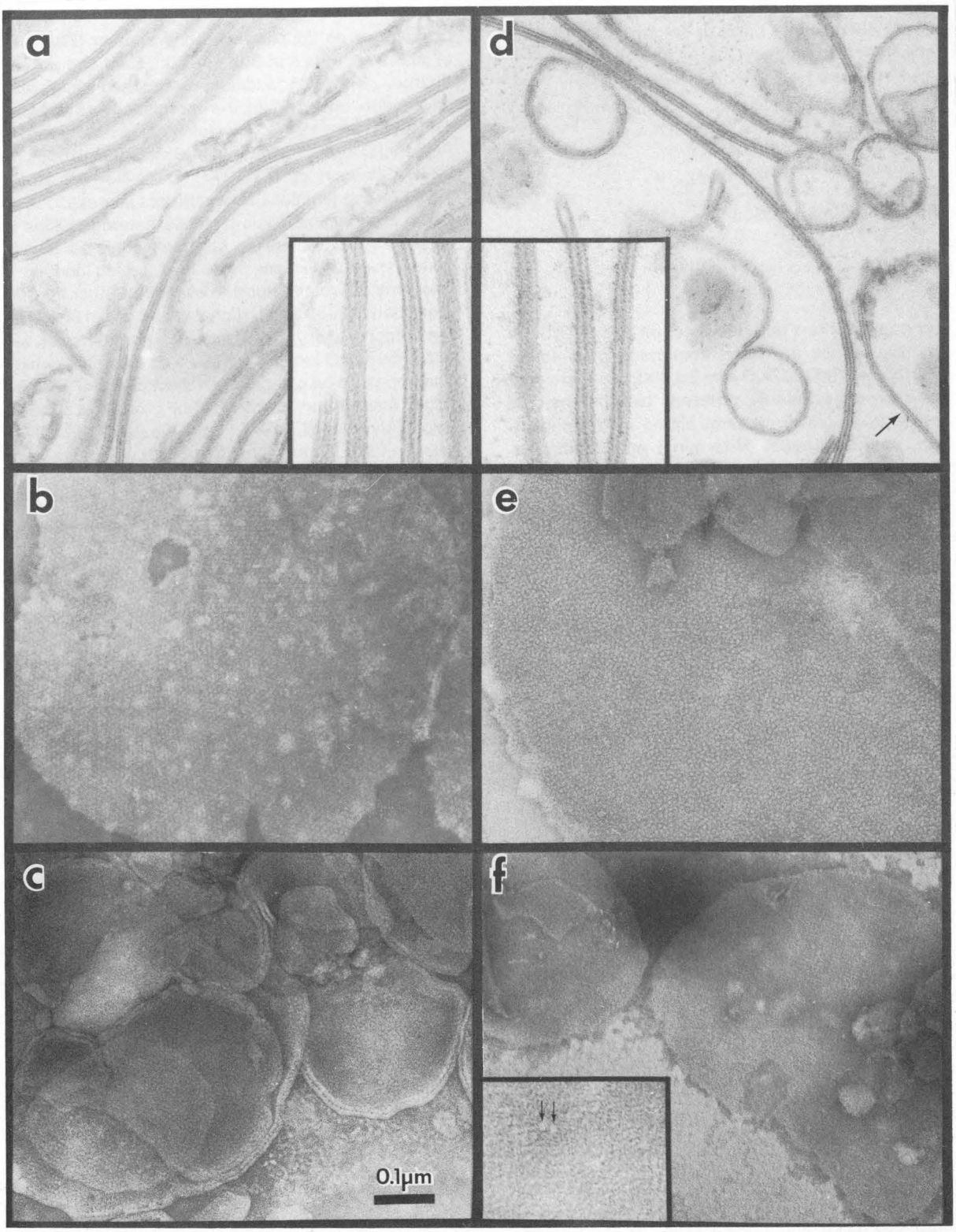

Figure 1. Morphology of Isolated Liver Gap Junctions and Lens Fiber Junctions from Rat

Liver gap junctions. (a) Thin sections of a "native" fraction reveals profiles of paired membranes separated by an approximately 2 nm "gap," a few single membranes and some contaminating fibrous material. Detail of the junctions, magnified 210,000×, is seen in the inset to (a). (b and c) Liver junction fractions negatively stained with 2% phosphotungstic acid: in "native" fractions the gap junctions are seen as flat sheets of connexons arranged in a closely-packed hexagonal lattice (b); the gap junctions of "enzyme-treated" fractions display a similar arrangement of

separated by a gap of approx. 2 nm (Figure 1a) and en face display a closely packed hexagonal array of connexons 7–8 nm in diameter (Figure 1b). Some fibrous material and occasional collagen strands are also seen in this fraction. Negatively stained "enzyme-treated" gap junction fractions (that is, isolated with the aid of trypsin and collagenase treatment—see Experimental Procedures) appear similar except that the gap junctions occur as vesicles or curved sheets and collagen is never seen (Figure 1c).

Lens

Deoxycholate-insoluble fractions of lens fiber cell plasma membranes, isolated by the procedure of Dunia et al. (1974), contain membranes in closely apposed pairs and a small number of single membranes (Figure 1d). Whole plasma membrane fractions isolated by the method of Alcalá et al. (1975) as modified by Takemoto et al. (1981) appear similar, but with a higher percentage of single membranes. Since the membranes of the lens junctions are separated by less than the 2 nm characteristic of liver junctions, their cross-sectional profile is less (see inserts of Figures 1a and 1d), a feature previously reported by Hertzberg et al. (1982) and Zampighi et al. (1982). Even narrower profiles could be seen in some fractions (see arrow, Figure 1d). However, since these regions display a reduced thickness of the lipid bilayer, it seems likely that they result from partial lipid extraction during detergent treatment. Negative staining of lens fractions yielded somewhat variable results (Figures 1e and 1f). Particles on the membrane sheets (referred to here as connexons) were frequently difficult to detect on many of the membrane sheets, perhaps as a result of reduced amounts of negative stain trapped in the narrow extracellular gap. When connexons could be detected, their packing varied from one preparation to another. Hexagonal arrays, indistinguishable from those of liver (compare Peracchia and Peracchia, 1980a); disordered, loosely packed arrays (Figure 1e; also compare Goodenough, 1979), and tetragonal lattices (Figure 1f; also compare Zampighi et al., 1982, and Peracchia and Peracchia, 1980b) were each seen as the predominant arrangement of connexons in different fractions of lens fiber junctions.

In spite of these variations in appearance, each fraction contains the same major protein (M_r 26,000), which always yields the same peptide map (see Figure 3b) and comprises 60%–90% of the protein in the fraction. Although this raises the possibility that these various arrangements of connexons derive from the same structure during the isolation procedure (for example see Peracchia and Peracchia, 1980a and 1980b), problems with sampling, caused by the poor negative staining characteristics of the isolated fractions, leave open the possibility suggested by Zampighi et al. (1982) that more than one type of junction may be isolated from the lens (that is, tetragonal arrays and less prevalent, more "conventional" gap junctions). We varied the Ca^{2+} and H^+ concentrations, pH and buffers used, factors which might affect the arrangement of connexons (see Peracchia and Peracchia, 1980a and 1980b), but could not consistently link them to the differences in the appearance of the final fractions.

Protein Composition of Junctional Fractions from Liver and Lens

Liver

When proteolysis is minimized by the inclusion of 0.5% phenylmethylsulfonyl fluoride in all solutions during the isolation of the "native" gap junction fraction from rat liver, a single major component of M_r 28,000 is seen in sodium dodecylsulfate (SDS) gels of this fraction (Figure 2b). The minor components of this fraction have previously been demonstrated (Hertzberg and Gilula, 1979; Henderson et al., 1979; Nicholson et al., 1981) to be either associated with nonjunctional contaminants (M_r 38,000 and 34,000 proteins and some collagen at the top of the gel), or derived from the M_r 28,000 protein by proteolysis (M_r 26,000 or 24,000 polypeptides) or aggregation (M_r 50,000 and 45,000 proteins).

Lens

A fraction highly enriched for junctions, as judged from thin-sectioned samples, can be isolated from eye lens fiber cells by the rigorous preparative procedure of Dunia et al. (1974). When examined by SDS–polyacrylamide gel electrophoresis, this fraction reveals a single major component of M_r 26,000 (MIP of lens; Figure 2d). The fractions obtained by the method of Alcalá et al. (1975) as modified by Takemoto et al.

connexons, but occur principally as curved sheets or vesicles (c). In both fractions, some amorphous, fibrous material is seen in addition to the gap junctions.

Lens fiber junctions. (d) Thin sections of fractions isolated by the method of Dunia et al. (1974) contain paired membranes separated by a "gap" narrower than that of liver gap junctions. This is evident at a higher magnification (210,000×) seen in the inset to (d). Amorphous material and some single membrane profiles are also evident. The latter form a greater percentage of fractions isolated by the method of Alcalá et al. (1975) as modified by Takemoto et al. (1981) (not shown). Narrower profiles of paired membranes in which the thickness of the lipid bilayer is reduced are also occasionally seen (arrow). (e and f) Lens junction fractions negatively stained with 2% phosphotungstic acid. In general, lens fiber junctions stain poorly, many membrane faces revealing no clear pattern of connexons. When connexons can be identified, their arrangement seems to vary from preparation to preparation: connexons arrayed predominantly in loosely packed, disordered arrays, as reported by Goodenough, 1979 (e); connexons in tightly packed tetragonal arrays similar to those reported by Zampighi et al., 1982 (f). A portion of this array, magnified 240,000× is shown in the inset to (f). Two readily identifiable connexons are indicated with arrows. Junctions displaying hexagonal arrays of connexons indistinguishable from those of liver (Figure 1b) have also been detected in some lens junction preparations (not shown).

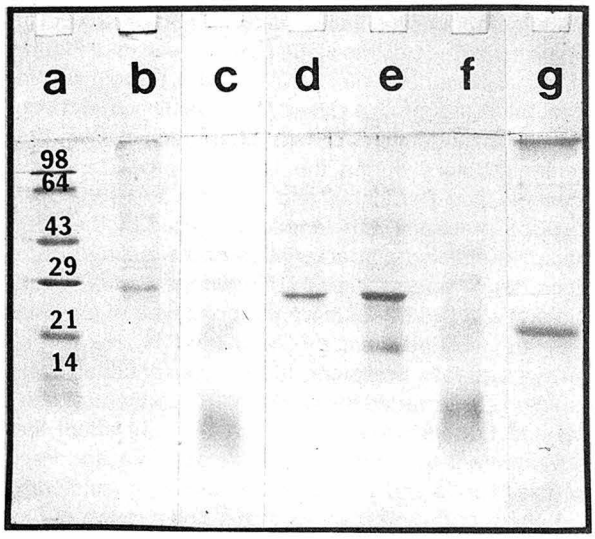

Figure 2. An SDS–Polyacrylamide "Microslab" Gel of Various Liver and Lens Gap Junction Fractions

(Lane a) Bio-Rad low molecular weight standards (marked in kilodaltons): phosphorylase A, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme.

(Lane b) A "native" gap junction fraction from rat liver: a major band of M_r 28,000 is evident. Also visible are faint dimers at M_r 50,000 and 45,000, a partial proteolysis breakdown product at M_r 26,000 and contaminants at M_r 38,000 and 34,000 (uricase) (see Nicholson et al., 1981).

(Lane c) An "enzyme-treated" gap junction fraction from rat liver: in addition to the major, rather diffuse band at M_r 10,000, there are several bands of higher molecular weights, which represent multimers of the M_r 10,000 component resulting from aggregation in SDS.

(Lane d) A gap junction fraction from rat lens isolated by the method of Dunia et al. (1974): the major band has an M_r of 26,000.

(Lane e) A plasma membrane fraction from rat lens isolated by the method of Alcalá et al. (1975) as modified by Takemoto et al. (1981): in addition to the M_r 26,000 protein, several other unrelated polypeptides (as demonstrated by two-dimensional peptide maps) are evident.

(Lanes f and g) Gap junction fractions isolated in the absence of proteases were subjected to trypsin digestion in the absence of detergents or other membrane-denaturing agents. (f) liver; (g) lens.

(1981) also contain a major protein of M_r 26,000 in addition to other components (a major species at M_r 17,000 and several minor bands; Figure 2e) probably associated with the nonjunctional contaminants seen in thin-sectioned or negatively stained samples. Peptide mapping shows these components to be unrelated to the M_r 26,000 protein. Peptide mapping has also been employed to ensure that the bands seen at M_r 26,000 in gels of fractions from both isolation protocols represent the same protein. However, sequence analysis has been based on lens MIP prepared by the method of Takemoto et al. (1981) because of the much higher yield it provides.

Peptide Mapping

Peptide mapping, especially in two or more dimensions, has proven to be a highly sensitive method for detecting differences, of even a single amino acid, between proteins (Bond et al., 1980). When the iodinated peptides from a complete α-chymotryptic digest of the junctional proteins of lens and liver are separated by the two-dimensional mapping technique of Elder et al., 1977 ("fingerprinting"), major differences between the two proteins can be seen (Figure 3). Although both proteins clearly contain a predominance of hydrophobic peptides (migrating farthest toward the top of the map), the overall pattern of migration and labeling is different for lens and liver. Mixtures of the digestion products of the two proteins (Figure 3c) show that of the 11 liver peptides and 13 lens peptides readily detectable by this technique, two to four have the same mobilities in both dimensions (arrows on Figures 3a and 3b). Similar comparisons of tryptic digests (not shown) lead to the conclusion that two to four of a possible 11 tryptic peptides comigrate also. This same percentage of comigrating peptides is seen whether the proteins are labeled with the chloramine T method, which labels tyrosine, (Greenwood et al., 1963) or the Bolton and Hunter method, which labels lysine (Bolton and Hunter, 1973). To determine whether this comigration of peptides really reflects homology or is due to the inability of this two-dimensional system to separate some peptides, we have used high pressure liquid chromatography (HPLC) as a third dimension to attempt to separate those peptides which comigrate in two dimensions. Of these, one to two peptides from the tryptic or α-chymotryptic digests of the lens and liver proteins also coelute in this third dimension. However, when comparisons were made between each of these junctional proteins and an unrelated membrane protein, bacteriorhodopsin, a similar result was obtained, with two to three peptides comigrating in two dimensions and one to two of these coeluting in HPLC. It is clear that there is little or no conservation of either tryptic or α-chymotryptic cleavage sites between the junctional proteins of lens and liver, at least as revealed by peptides containing tyrosine or lysine. Any homologies which might exist between the peptides cannot be distinguished from the nonspecific background inherent in the sytems used.

Amino Acid and N-Terminal Sequence Analysis

So far, the sequences of the N-terminal 18% of the lens protein (39 residues) and 24% of the liver protein (58 residues) have been determined. As can be seen in Figure 4, no homology has yet been detected between these sequences. There are, however, some common features. The liver protein contains an 18 residue stretch of strongly hydrophobic amino acids (residues 23–40) bracketed by three basic residues at the N-terminal end and three acidic residues at the C-terminal end, and interrupted by only one charged residue (Arg 32). A comparison of this region with transmembrane sequences of other proteins (see Ni-

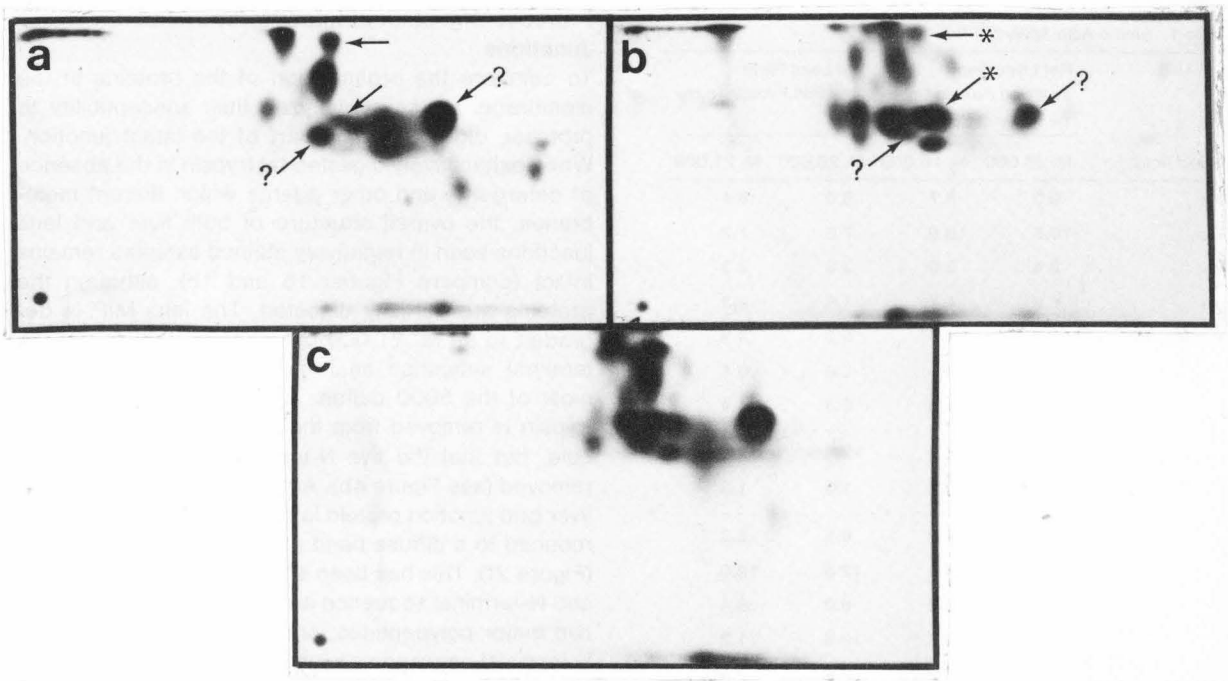

Figure 3. Two-Dimensional Peptide Maps of Iodinated, α-Chymotryptic Peptides

(a) From rat liver, M_r 28,000 gap junction protein. (b) From rat lens, M_r 26,000 junction protein. (c) From a mixture of equal amounts (that is, equal total radioactivity) of (a) and (b). The origin is at the bottom left of each map. Electrophoresis in acidic solvent (pH 1.7) was performed from left to right followed by ascending chromatography in a hydrophobic solvent. The most basic peptides migrate farthest to the right and the most hydrophobic farthest to the top of the maps. This comparison was repeated four times with both α-chymotryptic and tryptic (not shown) digests. The arrows denote peptides that seem, on the basis of all these comparisons, to comigrate in this system. In one case, these peptides were eluted and run on HPLC. Question marks denote peptides that did not appear to coelute in this third dimension. Peptides marked with an asterisk in the map of the lens protein have also been shown by Takemoto et al. (1981) to be conserved between the lens MIP 26 from a wide variety of vertebrate species.

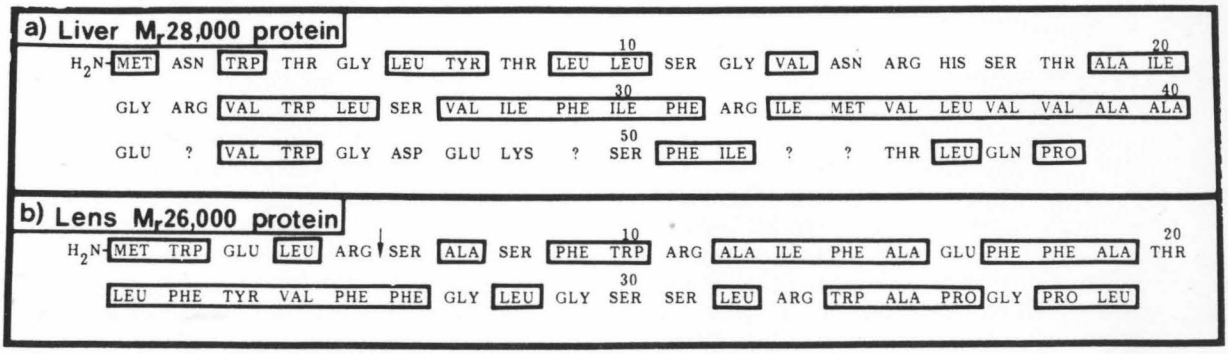

Figure 4. Partial Amino Acid Sequences of the Amino Terminus of the Liver and Lens Junctional Proteins

No primary sequence homology is evident. The hydrophobic residues are enclosed by boxes, emphasizing the highly hydrophobic stretches of 18–21 residues identifiable in both proteins. The sequence of the N-terminal 12 residues of each protein has been confirmed in a minimum of three independent Edman degradations. In the case of the lens, the N-terminal sequences of the M_r 26,000 protein isolated by either protocol (see Experimental Procedures) agree exactly for the 34 residues determined to date. Question marks denote steps in the sequence where no residue could be unambiguously assigned due to weak signals. The arrow between residues 5 and 6 in (b) indicates the tryptic site accessible when the protein is still in the membrane.

cholson et al., 1981), suggests that this portion of the molecule may span the membrane. A similar region of hydrophobic amino acids (exceptionally rich in aromatic residues) of slightly greater length (residues 12–32) is seen in the lens protein. By analogy, this is also a likely candidate for a transmembrane segment of this protein. In this case, however, the sequence is located closer to the N-terminus, bracketed at each end by single basic residues and interrupted by a single acidic residue (Glu 16).

The amino acid analyses of the two proteins also show some differences (Table 1). The lens MIP contains a consistently greater percentage of hydrophobic residues and fewer charged residues (especially

Table 1. Amino Acid Analysis

Amino Acid[a]	Rat Liver Gap Junction Protein (mole %)		Rat Lens Fiber Junction Protein (mole %)	
	M_r 28,000	M_r 10,000	M_r 26,000	M_r 21,000
Asx	9.0	8.7	6.0	6.4
Glx	10.5	10.5	7.5	7.2
His	2.4	2.0	2.3	2.3
Lys	6.2	4.7	1.9	2.3
Arg	6.9	4.4	5.3	3.9
Ser	9.0	9.1	7.4	6.4
Thr	4.6	4.8	5.3	4.4
Gly[b]	16.5	16.6	12.8	17.9
Tyr	3.1	2.3	1.9	1.8
Phe	3.3	4.1	6.5	6.3
Ala	5.8	7.1	12.5	12.0
Val	5.8	6.8	8.0	9.4
Leu	7.7	8.8	13.6	11.2
Ile	3.9	4.2	2.8	2.9
Met	1.4	1.4	0.8	1.8
Pro	3.9	4.5	5.4	3.8
% Polar residues[c]	48.6	44.2	35.7	32.9
% Hydrophobic residues[d]	31.8	36.9	49.6	47.4

[a] Cys and Trp are destroyed during hydrolysis.
[b] Gly is artificially elevated, since the proteins were eluted from a Laemmli gel run in Tris–glycine buffer.
[c] Includes Asx, Glx, His, Lys, Arg, Ser, Thr.
[d] Includes Phe, Ala, Val, Leu, Ile, Met, Pro.

lysine) than the liver gap junction protein. In fact, when compared with other membrane proteins for which amino acid analyses are available, the liver protein is found to contain a relatively low percentage of hydrophobic residues—a property which may reflect its role in forming a hydrophilic channel of large diameter across the membrane. The significance of the higher content of hydrophobic amino acids in the lens MIP is unclear. It could indicate that the lens protein is not involved in the formation of intercellular channels, or it could simply suggest differences in the way in which the channels in liver and lens are constructed. More complete sequence data will be needed to interpret this difference. The M_r 10,000 polypeptides of liver and the M_r 21,000 polypeptide of lens that survive trypsinization of their respective intact junctions contain lower percentages of polar or charged residues than the "native" proteins from which they were derived. This result is consistent with the proposal, discussed below, that they represent the portions of the junctional proteins protected from further proteolysis by the surrounding membrane.

Protease Digestion of Intact Liver and Lens Junctions

To compare the organization of the proteins in the membrane, we have analyzed their susceptibility to protease digestion while part of the intact junction. When exhaustively digested by trypsin in the absence of detergents and other agents which disrupt membranes, the overall structure of both liver and lens junctions seen in negatively stained samples remains intact (compare Figures 1b and 1c), although the proteins are partially digested. The lens MIP is degraded to an M_r 21,000 polypeptide (Figure 2g). N-terminal sequence analysis has demonstrated that most of the 5000 daltons cleaved from the MIP by trypsin is removed from the C-terminus of the molecule, but that the five N-terminal residues are also removed (see Figure 4b). After similar proteolysis, the liver gap junction protein is affected differently, being reduced to a diffuse band of M_r 10,000 on SDS gels (Figure 2f). This has been shown by peptide mapping and N-terminal sequence analysis to be comprised of two major polypeptides, one of which has the same N-terminal sequence as the "parent" M_r 28,000 protein (Nicholson et al., 1981). A consideration of the intermediate products of this tryptic digestion (polypeptides of M_r 26,000 and 24,000—see Figure 3 in Nicholson et al., 1981) reveals that at least 4000 daltons are removed by trypsin from the C-terminus of the "native" liver gap junction protein before it is cleaved into two equal fragments of M_r 10,000.

A Comparison of Liver Gap Junction Proteins from Different Species

The use of proteases during the isolation of gap junctions allows the preparation of "enzyme-treated" gap junction fractions from a variety of mammalian species (rat, mouse, rabbit and calf), all of which reproducibly appear highly enriched for gap junctions in negatively stained samples and show a single, but diffuse, major band at M_r 10,000 after separation by SDS–polyacrylamide gel electrophoresis (Figure 5). In the rat, these M_r 10,000 polypeptides have been demonstrated, by peptide mapping, to be identical with those generated by trypsinization of "native" gap junctions described above. We have now compared the proteins from each of these mammalian species by peptide mapping using complete α-chymotrypic digests (Figure 6). Although there is some variability in the relative labeling of different peptides, it is clear that the patterns are very similar in all four species. Peptide maps of tryptic digests (not shown) have yielded the same conclusion. Preliminary maps of an M_r 12,000 polypeptide of a gap junction fraction isolated from chicken liver according to a method developed by R. Gomer (personal communication) suggest that there is some conservation of several of the peptides, even between different classes of vertebrates.

Discussion

Comparison of Lens Fiber Junctions and Gap Junctions of Liver

Morphological and Physiological Properties

The controversy over the nature of the lens fiber junctions began with the observation of morphological differences between these junctions and the gap junctions of other tissues (Goodenough, 1979; Zampighi et al., 1982). In comparing lens fiber junctions with gap junctions from liver, we have also observed that lens junctions display a reduced extracellular gap (compare Figures 1a and 1d) and connexons arrayed in a variety of forms in addition to the hexagonal arrays typical of liver gap junctions (that is, random arrays, Figure 1e; tetragonal arrays, Figure 1f). However, the significance of these differences is unclear for two reasons: the possibility, as proposed by Zampighi et al. (1982), that these different arrays may represent different junctions which co-isolate; and the observation of similar tetragonal and random arrays in liver gap junctions under some conditions (for example, in freeze-fracture of rapidly frozen liver—Yancey and Heuser, unpublished observations). Lens junctions also differ from those of other tissues in their physiological properties. In most other tissues the cells can be uncoupled by treatments that elevate the internal H^+ and/or Ca^{2+} concentrations. The same manipulations fail to uncouple lens fiber cells, at least beyond embryonic stage 14 (Schuetze and Goodenough, 1982). However, since there is a variation among tissues as to the ease with which uncoupling can be achieved, one could argue that the lens simply represents the extreme of a continuum, rather than a unique structure. Taken together, however, these differences between liver and lens junctions have led to some doubt as to whether these abundant structures of the lens are "true" gap junctions.

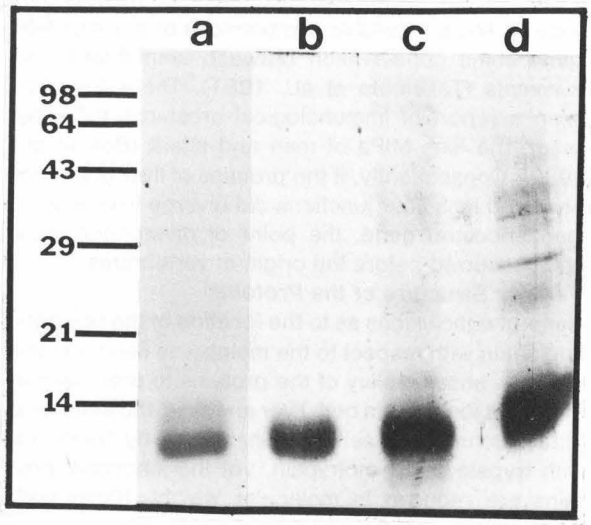

Figure 5. An SDS–Polyacrylamide "Microslab" Gel of "Enzyme-Treated" Liver Gap Junction Fractions from Four Mammalian Species

The positions and molecular weights (in kilodaltons) of the Bio-Rad low molecular weight standards are indicated on the left. (a) rat; (b) mouse; (c) rabbit, and (d) calf.

Primary Structure of the Proteins

We have attempted to clarify the nature of the lens junction by comparing its major protein component with that of the well defined gap junction from liver. A degree of homology between the proteins would allow

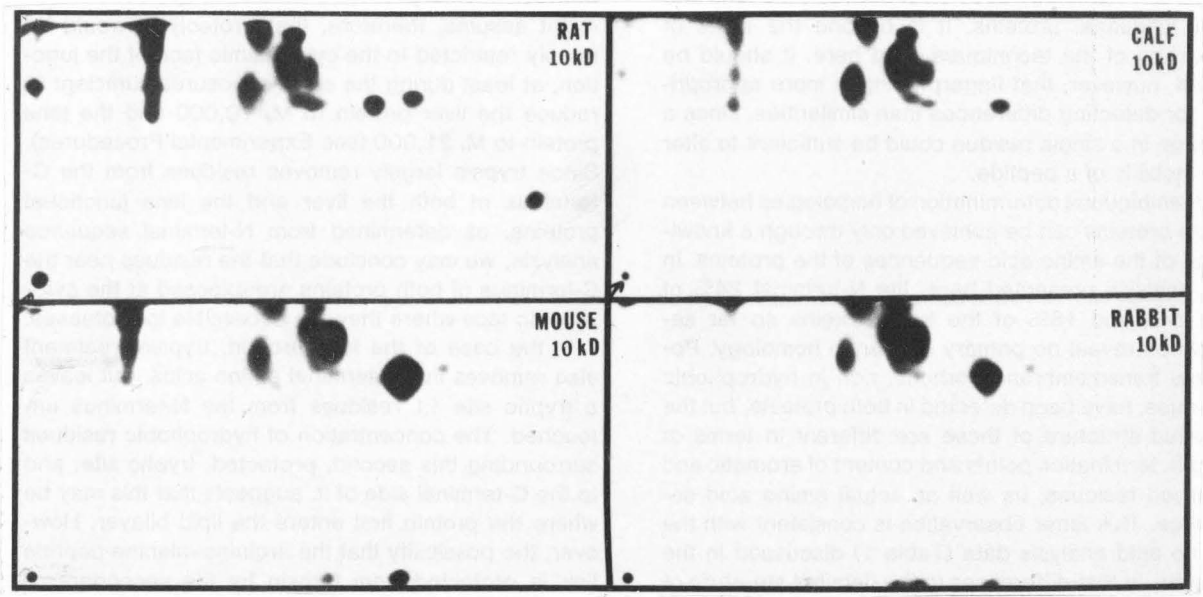

Figure 6. Two-Dimensional Peptide Maps of the M_r 10,000 Components of "Enzyme-Treated" Liver Gap Junction Fractions from Rat, Mouse, Calf and Rabbit

Apart from some variability in the intensity of labeling of the peptides, it is clear that these proteins are essentially identical, indicating that at least the portion of the liver gap junction protein which is protected from proteolysis is highly conserved between mammalian species.

one to conclude that the two junctions are derived from a common "ancestral" gap junction and are likely to be still performing similar functions as adapted to their specific tissue.

There is already evidence that the major proteins of lens and liver junctions (M_r 26,000 and 28,000, respectively) differ. The bovine lens and rat liver proteins display different patterns in one-dimensional peptide maps and show no immunological cross-reactivity (Hertzberg et al., 1982). Although both of these techniques clearly demonstrate differences between the proteins, they are limited in their sensitivity to anything but the closest homologies. It is likely, in an immunological analysis, that many determinants on the protein would not be represented in the immune serum. The inadequacy of one-dimensional peptide maps can be appreciated by examining the number of peptides that comigrate in the electrophoretic dimension of the two-dimensional peptide maps in Figure 3. Obviously, many "peptides" seemingly homologous in one dimension are readily shown to differ in a second dimension.

We have used two-dimensional peptide maps (and in selected cases, three-dimensional maps) and partial N-terminal sequence analysis to achieve a more sensitive and quantitative comparison of these two proteins. Two-dimensional peptide mapping, a sensitive technique for detecting differences between proteins, shows the proteins of liver gap junctions and lens fiber junctions to be quite different. Extension of the system to three dimensions (by analysis of eluted peptides on HPLC), and comparisons with maps of unrelated proteins, like bacteriorhodopsin, led to the conclusion that if any homology does exist between the lens and liver junctional proteins, it is beyond the limits of detection of the techniques used here. It should be noted, however, that fingerprinting is more appropriate for detecting differences than similarities, since a change in a single residue could be sufficient to alter the mobility of a peptide.

Unambiguous determination of homologies between these proteins can be achieved only through a knowledge of the amino acid sequences of the proteins. In the analysis presented here, the N-terminal 24% of the liver and 18% of the lens proteins so far sequenced reveal no primary sequence homology. Potential transmembrane portions, rich in hydrophobic residues, have been detected in both proteins, but the detailed structure of these are different in terms of length, termination points and content of aromatic and charged residues, as well as actual amino acid sequence. This latter observation is consistent with the amino acid analysis data (Table 1) discussed in the Results, in that differences in the detailed structure of the protein inside the membrane would be predicted from the differences in the content of nonpolar residues.

The junctional proteins of liver and lens thus differ from each other by the criteria used to date. This is in marked contrast to the results comparing liver or lens proteins of different species. There are virtually no differences between the four mammalian liver gap junction proteins compared by two-dimensional peptide mapping, and there is considerable homology with the major protein of a fraction from chicken liver enriched for gap junctions. In the case of the lens junction, sequence analyses of lens MIP from calf and rat show only three conservative amino acid differences in the N-terminal 30 residues (results not shown). More extensive comparisons of the lens MIP show some conservation between amphibians and mammals (Takemoto et al., 1981). There has also been a report of immunological cross-reactivity between the lens MIPs of man and shark (Bok et al., 1982). Consequently, if the proteins of liver gap junctions and lens fiber junctions did diverge from a common ancestral gene, the point of divergence must have occurred before the origin of vertebrates.

Tertiary Structure of the Proteins

General conclusions as to the location of the polypeptide chain with respect to the membrane can be made from the susceptibility of the proteins to proteases in the intact junction. In both liver and lens, the junctional ultrastructure is essentially unaffected by treatment with trypsin or chymotrypsin, yet the junctional proteins are reduced in molecular weight. Since both proteases are water soluble and have a minimum diameter of 5 nm (Stroud et al., 1974), it seems likely that they would be excluded from the hydrophobic domains of the membrane, the 1–1.5 nm aqueous pore of the connexons and the gap of 2 nm or less between membranes of lens or liver junctions. One might assume, therefore, that proteolysis would be largely restricted to the cytoplasmic face of the junction, at least during the short exposures sufficient to reduce the liver protein to M_r 10,000 and the lens protein to M_r 21,000 (see Experimental Procedures). Since trypsin largely removes residues from the C-terminus of both the liver and the lens junctional proteins, as determined from N-terminal sequence analysis, we may conclude that the residues near the C-terminus of both proteins are exposed at the cytoplasmic face where they are accessible to proteases.

In the case of the lens protein, trypsin treatment also removes five N-terminal amino acids, but leaves a tryptic site 11 residues from the N-terminus untouched. The concentration of hydrophobic residues surrounding this second, protected, tryptic site, and to the C-terminal side of it, suggests that this may be where the protein first enters the lipid bilayer. However, the possibility that the arginine–alanine peptide link is protected from trypsin by the secondary or tertiary structure of the protein cannot be excluded. Assuming, as outlined above, that trypsin can digest only polypeptides exposed at the cytoplasmic face of the junctions, this result would infer that both the N-

terminus and the C-terminus of the lens MIP are on the cytoplasmic side of the membrane. This would be a similar situation to that suggested for band 3 of erythrocyte membranes (Sabban et al., 1981), and would suggest that the lens MIP is not inserted into the membrane with an N-terminal signal sequence (Blobel and Dobberstein, 1975), but by "hairpin" insertion or by way of an internal signal sequence (von Heijne and Blomberg, 1979; Engelman and Steitz, 1981; Blobel, 1980).

As yet, similar speculations as to the location of the N-terminus of the liver protein are impossible, since no sequence analysis is available on polypeptides generated by a protease with a potential cleavage site near the N-terminus (in liver, the first potential tryptic site is between residues 15 and 16). At first glance, the fact that identical trypsin treatments of the isolated junctions reduce the liver M_r 28,000 protein to M_r 10,000 and the lens M_r 26,000 MIP to M_r 21,000 suggests major differences in the arrangement of these proteins in the membrane. However, we have shown previously (Nicholson et al., 1981) that in liver gap junctions two M_r 10,000 polypeptides, representing 20,000 of the original 28,000 daltons, survive trypsin digestion. Consequently, this difference in susceptibility to trypsin could be attributed to relatively minor changes in the folding of the polypeptide chain, in the distribution of a few basic residues in the primary sequence and consequently the distribution of enzymatic cleavage sites (for example, at the N-terminus of the molecules) or in other factors such as the disposition of lipids in the membranes.

Comparisons with Other Gap Junction Proteins

The evidence for biochemical differences between the lens and liver junctional proteins has been used to support the contention that lens fiber junctions are not gap junctions (Hertzberg et al., 1982). However, before this can be inferred, one must first answer the question of whether the gap junction proteins of other tissues are similar to that of liver or whether the difference between the liver and lens proteins is typical of the variation in the gap junction protein from tissue to tissue.

An argument that has been used to support the idea of conservation of gap junctions between tissues is that, in general, cell lines derived from a wide variety of vertebrate tissues can form functional gap junctions with each other (Michalke and Loewenstein, 1971; Epstein and Gilula, 1977; Gaunt and Subak-Sharpe, 1979), although some exceptions have been reported (Fentiman et al., 1976; Pitts and Burk, 1976; Gaunt and Subak-Sharpe, 1979). The ability to form contacts capable of supporting electrotonic coupling, however, does not provide direct evidence for or against the heterogeneity of gap junctional proteins for the following reasons: different gap junction proteins could be able to interact to form heterojunctions; tissue culture cell lines may no longer express their original tissue-specific gap junctions but some dedifferentiated product; differences in geometry of growth or presence of a basement lamina could prevent junction formation between cells even if the proteins were the same. It would thus be difficult to arrive at any firm conclusion without a biochemical study of junctions derived from different species and organs.

There are now some data on the gap junction protein in a tissue other than liver or lens. Kensler and Goodenough (1980), Colaco and Evans (1981) and Manjunath et al. (1982) have isolated gap-junction-enriched fractions from mammalian hearts, and several polypeptides of molecular weights from 19,000 to 46,000 have been correlated with the presence of gap junctions (most consistently, polypeptides of M_r 28,000 and 35,000). Recent work in our laboratory by D. Gros has led to the isolation of a fraction from rat heart highly enriched for gap junctions and containing no visible contamination by desmosomes, as judged from thin sections and negatively stained fractions (Gros et al., 1982). When examined by SDS–polyacrylamide gel electrophoresis, this fraction reveals a single major protein of M_r 28,000 and some minor polypeptides shown by peptide mapping to be related to this major protein. From two-dimensional peptide maps similar to those described here, this protein appears to be as distinct from the liver and lens proteins as the latter are from each other. One must thus conclude that although there is junctional homology in a given tissue over a number of species (Takemoto et al., 1981; Figure 6, this paper), there is little homology detectable by these methods among the gap junction proteins of the different tissues examined so far.

Conclusions on the Nature of Lens Fiber Junctions and the Tissue Specificity of Gap Junctions

The extent of the data available makes it difficult to draw specific conclusions as to the nature of the lens junction. The electrical coupling (Eisenberg and Rae, 1976) and transfer of dye and radioactive metabolites (Rae, 1974; Goodenough et al., 1980) between lens fiber cells does not provide definitive evidence for the identity of the lens junctions as gap junctions. It is possible that this coupling may not require the extensive distribution of lens fiber junctions observed and could be mediated by a small number of "real" gap junctions scattered on the membrane. However, the low intercellular resistance recorded in the lens (Eisenberg and Rae, 1976) compared with that measured for other tissues (for example, liver; Meyer et al., 1981) suggests that gap junctions in the lens should comprise a high percentage of the membrane (greater than the 3% reported for liver by Yancey et al., 1979). On the other hand, it appears that the major differences seen in the proteins comprising the lens and liver junctions cannot be used to infer that

the structure in the lens is not a gap junction, since similar differences exist between the proteins of well defined gap junctions from other tissues (for example, heart and liver).

Given all these considerations, it is currently impossible to determine whether the MIP 26 is the major component of lens gap junctions, or the major component of another type of junction, or a major component of single membranes that tend to adhere to one another after isolation. However, the results on the heart gap junctions do indicate that there exists a family of tissue-specific gap junction proteins. It also seems possible that the eye lens MIP is one of these. Presumably, each protein has evolved to support the specific functions fulfilled by the gap junctions of that tissue. Analysis of the portions of the gap junction structure which are conserved in a given tissue, but vary between tissues, may provide clues as to the nature of these proposed functions and may serve to identify functional sites on the protein (such as Ca^{2+} or H^+ binding sites controlling gating).

In addition, analysis of the peptides we have identified as comigrating in our peptide mapping systems should allow a test of the interesting possibility that there may be conservation of some regions of these proteins between tissues, possibly those which are fundamental to the formation of a channel with "junctional" properties (that is, permissive to all molecules of sufficiently low molecular weight). Additional sequence analysis will also be necessary to eliminate (or substantiate) the rather unlikely possibility that the various gap junction proteins arose from the convergent evolution of independent proteins to form similar structures with apparently similar properties.

Experimental Procedures

Isolation of Gap Junctions

Liver

"Native" Gap Junction Isolation

Gap junctions were isolated from rat liver by the method described previously (Nicholson et al., 1981) in which proteolysis was minimized (0.05% phenylmethylsulfonyl fluoride was used throughout the isolation). Plasma membranes were isolated by the two-phase method and then washed in 0.1 M NaCl to remove proteins adhering nonspecifically to the membrane. After treatment with 0.55% Sarkosyl to solubilize the nonjunctional membranes, the fraction was exposed to 0.15 M Na_2CO_3 (pH 11) for 15 min to dissolve uricase, and the junctions were separated from fibrous material and lipid vesicles on a discontinuous sucrose gradient containing 1 M urea and 0.09% Sarkosyl (33%, 40%, 54%, 77% [w/v] sucrose layers; gap junctions were collected at the 40%–54% interface).

"Enzyme-Treated" Gap Junction Isolation

For our comparison of gap junctions from the livers of rat, mouse, calf and rabbit, fractions were obtained with the use of proteases by the method described by Finbow et al. (1980) and modified by Nicholson et al. (1981). Plasma membranes were treated with trypsin and collagenase to reduce contamination before treatment with 0.55% Sarkosyl and final separation of the junctions on a discontinuous sucrose gradient (0%, 32%, 54% [w/v] sucrose layers; gap junctions were collected at the 32%–54% interface). As a result, only large fragments (M_r 10,000) of the protein are isolated.

Lens

As for liver isolations, eye lenses were obtained from young adult rats. The method of Alcalá et al. (1975) as modified by Takemoto et al. (1981) which consisted of repeated washes of a decapsulated lens homogenate in 5 mM Tris/HCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (pH 7.9) buffer followed by several washes in 8 M urea in the same buffer yielded a pellet enriched for gap-junction-like structures. A more highly purified fraction was obtained by the protocol of Dunia et al. (1974). Plasma membranes, isolated on a discontinuous sucrose gradient, were treated with 1% deoxycholate, and the insoluble material was separated on a continuous sucrose gradient. The buffer used throughout was 1 mM $NaHCO_3$, 0.5 mM $CaCl_2$ (pH 7.4). Although causing a considerable drop in yield, this detergent treatment resulted in the isolation of a highly purified junctional fraction.

Electron Microscopy

Isolated junctional fractions were prepared for thin sectioning according to the method of Zampighi et al. (1982), although tannic acid was not used in the fixation of liver gap junctions. Negative staining of junctional fractions was achieved with 2% phosphotungstic acid (pH 7.2), and the sample was layered on a carbon-coated grid treated with 0.02% Alcian blue to minimize clumping and stacking of the membranes.

SDS–Polyacrylamide Gel Electrophoresis

The method of Laemmli (1970) as applied by Nicholson et al. (1981) was used throughout. As pointed out previously (Henderson et al., 1979; Nicholson et al., 1981), the solubilization of gap junction fractions, especially from liver, was performed in 2% SDS in the presence of reducing agents for 30–45 min at room temperature in order to minimize aggregation of the junctional proteins.

Peptide Mapping

Gap junction fractions were iodinated by the chloramine T method (Greenwood et al., 1963) after solubilization in SDS, as described by Nicholson et al. (1981). Other studies (Krohn et al., 1977) indicate that tyrosine, and in some cases phenylalanine and histidine, are labeled by this procedure. In one case, the iodination was achieved by the method of Bolton and Hunter (1973), which labels amino groups (lysine and the amino terminus). The method of Elder et al. (1977), as modified by Takemoto et al. (1981), was used to generate peptides by complete tryptic or α-chymotryptic digestion of the junctional proteins and to separate them in two dimensions on thin-layer cellulose plates. The iodinated peptides were then detected by autoradiography. To determine which, if any, of the peptides from any two proteins actually comigrate (thus suggesting homology), samples of the protease digests of each, containing equal amounts of radioactivity, were mixed together and the peptides were separated by the usual procedure. The peptides, especially those that apparently comigrate in the two-dimensional system, could be further analyzed by HPLC to provide a third dimension of resolution. Individual spots identified on the autoradiograph were cut out, the cellulose scraped into a plastic well and the peptide eluted with shaking over 24 hr into 80% acetic acid (aldehyde free; Baker). The eluate was collected, the excess cellulose spun out and the supernatant lyophilized. The sample was loaded onto an IBM cyanopropyl HPLC column in 0.1 M sodium perchlorate, 0.01% phosphoric acid (pH 2.1) (buffer A) with 0.02% SDS. Elution from the HPLC was achieved with buffer A and a 0%–60% acetonitrile gradient (Meek, 1980), and the peptides were detected by counting fractions on a Beckman gamma counter.

Amino Acid or N-Terminal Sequence Analysis

The polypeptides of the various junctional fractions were separated by SDS–polyacrylamide gel electrophoresis, excised and prepared for amino acid or N-terminal sequence analysis as described previously (Nicholson et al., 1981). Following hydrolysis in 6 N HCl under vacuum at 110°C for approximately 18 hr, amino acid analyses were obtained on a Durrum D500 analyzer. Sequence information was

obtained by automated Edman degradation according to the methods of Hunkapiller and Hood (1980) and Johnson et al. (1979), or in some cases by the improved gas phase system of Hewick et al. (1981). In all cases, the yield of sequenceable peptide was compared with the amount loaded on the sequenator. The latter was estimated from an aliquot of the sample either by the intensity of Coomassie staining of the proteins after separation by SDS–polyacrylamide gel electrophoresis on a "microslab" gel (as described in Nicholson et al., 1981) or by quantitative amino acid analysis. These methods estimate that 35% of the liver and 50% of the lens proteins loaded on the sequenator could be sequenced. Under the same conditions, proteins known not to be N-terminal-blocked in vivo typically gave a yield of 40%–70% of sequenceable peptide (Hunkapiller, personal communication). Several factors are responsible for this lack of 100% recovery, the most common of which are incomplete cleavage of the N-terminal residue at each step, resulting in a certain lag (at times substantial), and partial N-terminal blockage of the polypeptide resulting during the manipulations (for example, loading on a polyacrylamide gel) required to prepare the polypeptide for the sequenator.

Proteolysis of Intact Junctions

Isolated junctional fractions from either liver or lens were suspended in 25 mM NH_4HCO_3, 1 mM $CaCl_2$ (pH 7.8) to a concentration of 0.3 mg/ml of protein. Trypsin (Sigma, type XI) was added to a concentration of 10 μg/ml, and the sample was incubated at 37°C for 3 hr. After boosting with an equal amount of trypsin and incubating for an additional 2 hr, the proteolysed junctions could be collected by centrifugation (15,000 rpm × 30 min in an Eppendorf microcentrifuge, model 5412).

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CHAPTER 5

Differences Between the Gap Junction Proteins of Heart and Liver in the Rat: Evidence of Gap Junctional Tissue Specificity

Running title: Tissue Specific Gap Junction Proteins

Abstract:

A fraction, highly enriched for gap junctions by morphological criteria, has been isolated from rat myocardium. The major protein component of this fraction has a molecular weight of 28,000, although a minor component of M_r 30,000 seems to be the "native" form of the protein. Other polypeptides which occur inconsistently in this fraction appear, by peptide mapping, to be either non-junctional or derived from the "native" protein by endogenous proteolysis or aggregation. In vitro trypsin treatment of the isolated junctions cleaves the "native" protein into two M_r 11,000 polypeptides—an analogous behaviour to that of the liver gap junction protein (Nicholson et al., 1981). However, despite the overall similarity in appearance of gap junctions from heart and liver and the similar molecular weights and trypsin sensitivities of their constituent proteins, comparisons by two-dimensional, and in some cases three-dimensional, peptide mapping reveals no homology between the gap junction proteins of these tissues.

Introduction:

Despite the widespread occurrence of gap junctions in different tissues and phyla (see Larsen, 1977 and Loewenstein, 1981 for reviews), biochemical analysis of their components has been hindered by the limited amounts of this membrane specialization present in any given tissue. The only two tissues to be studied in any detail in this respect have been mammalian liver and lens, tissues in which the junctions are relatively abundant [$\sim$3% of the lateral surface of hepatocytes (Yancey et al., 1979) and 20-70% of the surface of lens fiber cells (Bloemendal et al., 1972; Kuszak et al., 1978)]. The junctions in each tissue are comprised of a single protein of similar size (M_r 28,000 in the liver and M_r 26,000 in the lens—Goodenough, 1979; Hertzberg, 1980; Nicholson et al., 1981; Broekhuyse et al., 1976; Alcala et al., 1978; Takemoto and Hansen, 1981). However, several studies using immunological (Traub and Willecke, 1982; Ziegler and Horwitz, 1981; Hertzberg et al., 1982) peptide mapping (Hertzberg et al., 1982; Nicholson et al., 1983) and sequencing techniques (Nicholson et al., 1983) have demonstrated that the primary structures of the two proteins differ, although the protein in each tissue is substantially conserved between species (lens: Bok et al., 1982; Takemoto et al., 1981; liver: Nicholson et al., 1981). The implications about tissue specificity which stem from these results must be tempered by the questions which have arisen regarding the identity of the lens junction. Lens fiber cells are well coupled (Rae, 1974; Eisenberg and Rae, 1976; Goodenough et al., 1980; Schuetze and Goodenough, 1982) and the structures which connect them resemble the gap junctions in other tissues which seem to mediate such coupling (see Figure 1 in Chapter 4; also Kistler and Bullivant, 1980a, b; Bernardini and Peracchia, 1981; Kuszak et al., 1982). However, several differences, both morphological (Zampighi et al., 1982) and physiological (Schuetze and Goodenough, 1982) have been demonstrated between the junctions in lens and other tissues. Furthermore, recent immunological studies (Paul and

Goodenough, 1983) have even questioned whether the M_r 26,000 MIP of lens is actually a component of lens junctions since it can only be detected in single membranes. While these issues remain unresolved, there will be a question as to whether the protein differences betwen liver and lens reflect tissue specificity of the gap junction or merely differences between two types of junction. To gain a better perspective, we have turned to another tissue, heart, where the gap junctions have been thoroughly characterized morphologically and physiologically (McNutt and Weinstein, 1970; Gros et al., 1978 and 1982; Page and Shibata, 1981; de Mello, 1982) and are present in relatively large numbers (~1% of the cell surface: Gros et al., 1979; Page and Shibata, 1981). In collaboration with Dr. Gros, we have developed an isolation protocol for cardiac gap junctions and have identified their major protein component (M_r ~28,000). Despite the similarity in molecular weights, this protein appears very different to the junctional proteins of liver and lens based on comparisons of their two-dimensional peptide maps. Therefore, a tissue specificity of the gap junction protein is conclusively demonstrated. The extent of the protein differences remains to be determined through sequence analysis, but similarities in the tryptic sensitivity of liver and heart gap junction proteins while part of the intact junctional structure may reflect some homology with respect to tertiary structure.

Materials and Methods:

Junction isolation protocols.

1. Heart: See Figures 1a and 1b for flowchart.

Buffers used:	BB	1 mM $NaHCO_3$, pH 8.2
	KI Tris	5 mM Tris/HCl, pH 9
		0.6 M KI
		6 mM $Na_2S_2O_3$
	Tris	5 mM Tris/HCl pH 9

Procedure: The protocol represents an adaption of that published by Kensler and Goodenough (1980). All steps, unless noted otherwise, were performed at 0-4°C. The hearts of 25 adult rats were excised, self-perfused by allowing them to beat in warm saline (in BB) for approximately one minute, and then trimmed of major vessels, connective tissue and atria before mincing and homogenization for 30 seconds at maximum power by a tissuemizer (Tekmar Ultra Turrax, SDT-182 EN). The homogenate was diluted with BB to 1600 ml, allowed to stand for 15 minutes and then filtered through 32 layers of cheesecloth. The filtrate was centrifuged at 4,000 rpm for 20 minutes (Sorvall GSA rotor; 43,000 g_{av} min). The pellet was resuspended in 1500 ml of BB and the spin repeated. Supernatants from both these spins were also collected for centrifugation at 11,500 rpm for 40 minutes (GSA rotor; 704,000 g_{av} min). The pellets of this spin were pooled with the pellets from the second low speed spin above and suspended in 600 ml of 0.6 M KI, 6 mM $Na_2S_2O_3$ in BB. After stirring for at least 12 hours, the insoluble material was collected and washed by successive centrifugations at 12,000 rpm for 30 minutes (GSA rotor: 576,000 g_{av} min), the second in KI-Tris buffer. This KI insoluble pellet was homogenized (15 seconds with the Tissuemizer) into 40 ml of KI-Tris and brought to 50% (w/v) sucrose by the addition of 80 ml of 76% (w/v) sucrose in KI-Tris. This formed the bottom layer of 12 discontinuous sucrose gradients (50/45/35/10% (w/v) sucrose in KI-Tris) which were spun at 25,000 rpm for 2 hours (SW27 rotor; 1×10^7 g_{av} min). Plasma membranes were harvested at the 10/35% interface, diluted to 200 ml with Tris, and collected and washed by successive centrifugations at 25,000 rpm for 30 minutes (SW27 rotor; 2.5×10^6 g_{av} min).

The plasma membrane pellet was then homogenized with three strokes of a loose fitting pestle in a Dounce homogenizer into 600 ml of 0.3% Sarkosyl NL-97 (ICN Pharamaceuticals, Inc., Plainview, New York) in Tris and stirred for 10 minutes at room temperature. Detergent insoluble material was collected by centrifugation

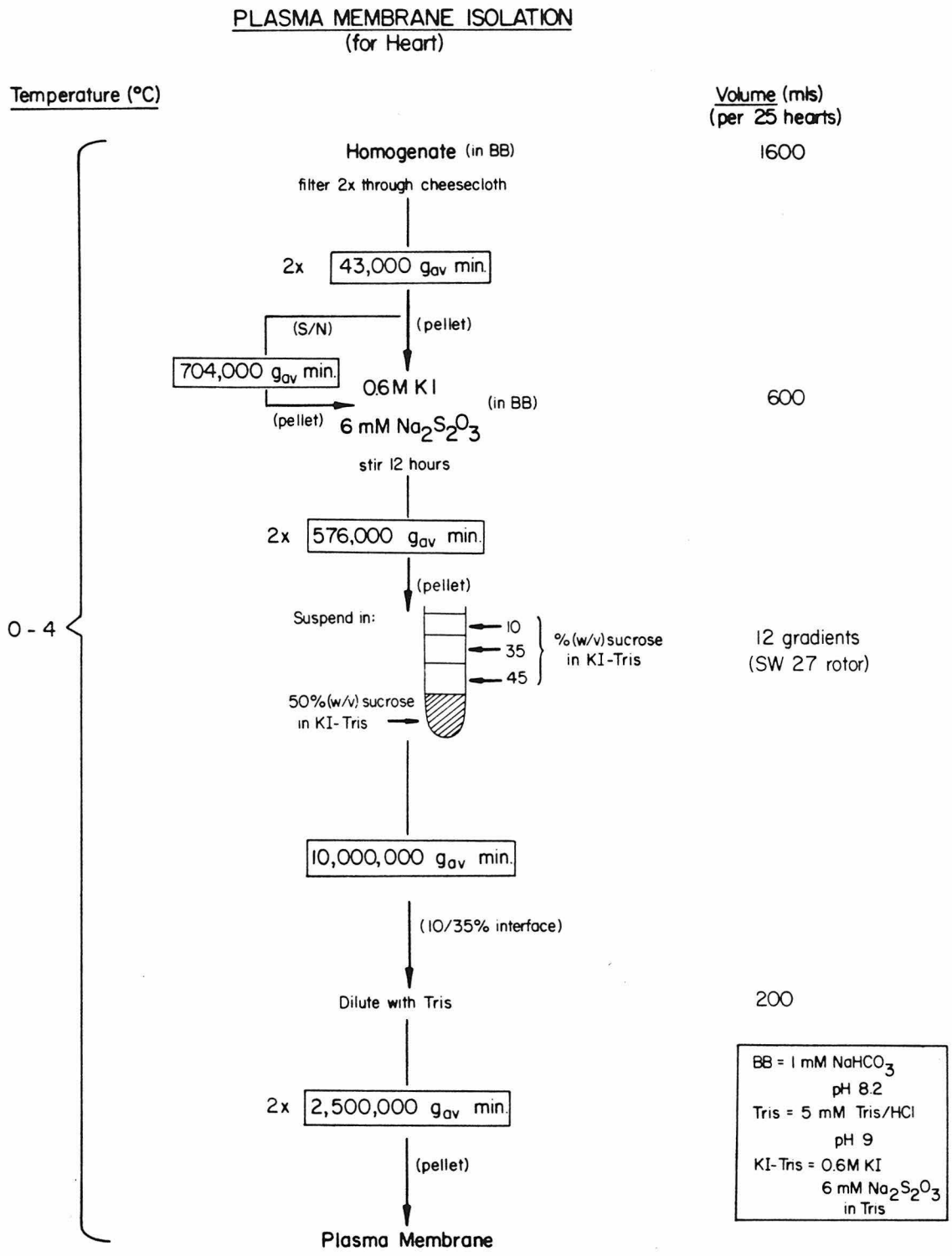


FIGURE 1. (a) Protocol for the isolation of plasma membranes from rat heart, based on the procedure of R. W. Kensler and D. A. Goodenough. J. Cell Biol. **86**, 755-764 (1980).

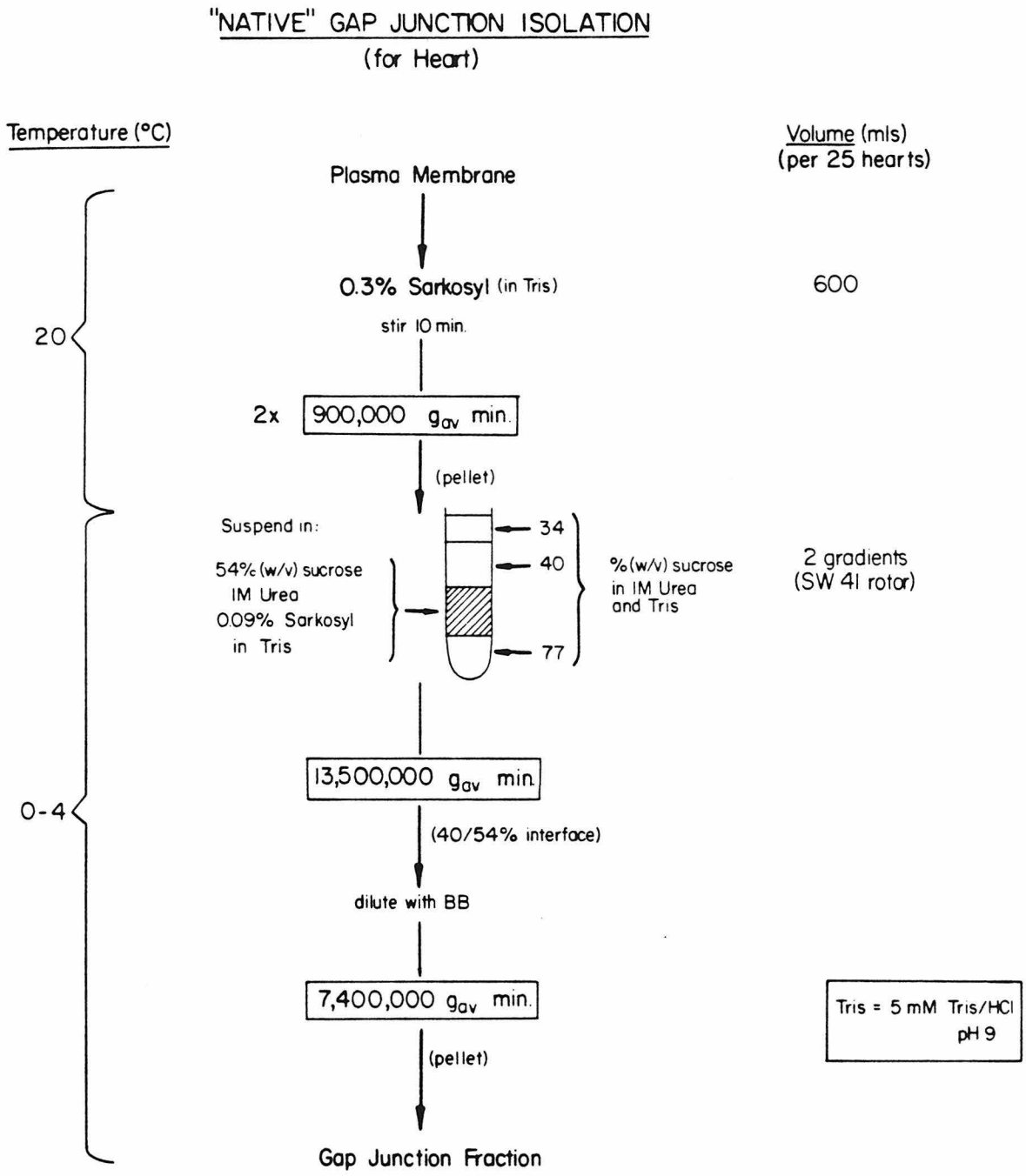


FIGURE 1. (b) Protocol for the isolation of cardiac gap junctions from plasma membrane.

at room temperature for 30 minutes at 18,000 rpm (SS-34 rotor; 900,000 g_{av} min), followed by resuspension in Tris and centrifugation as above. This washed pellet was suspended by brief sonication (setting 3, Branson Sonicator for 2-4 seconds) into 1.5 ml of Tris. To this was added 0.5 ml of 1.1% Sarkosyl and 4 ml of 81% (w/v) sucrose in 1.5 M urea and Tris. Two discontinuous sucrose gradients were then poured (77%/54% and sample/40%/30% (w/v) sucrose in 1 M urea and Tris) and centrifuged for at least 1 1/2 hours at 35,000 rpm (SW41 rotor; 1.35 x 10^7 g_{av} min). The final gap junction fraction was harvested at the 40/54% interface and collected after dilution in BB by centrifugation at 45,000 rpm x 1 hour (Ty65 rotor; 7.4 x 10^6 g_{av} min). All solutions except Sarkosyl were made with either 0.1 mM or 1 mM each of sodium ethylenediamine tetraacetate (EDTA), N-α-p-tosyl-L-arginine-methyl-ester (TAME) and iodoacetamide. In some experiments, 1 mM phenylmethylsulfonyl fluoride (PMSF—from a 4 M stock in dimethylsulfoxide) was present throughout.

2. Liver: "Native" liver gap junctions were isolated as described in Nicholson and Revel, 1983 (Chapter 1).

3. Lens: Lens junctions were isolated by the Dunia et al. (1974) method, described as protocol I in Appendix II.

4. Isolations from other tissues analogous to heart: i) Liver junctions. Due to differences in the contaminants which co-isolate with gap junctions in heart and liver, it was not possible to use absolutely identical procedures to isolate both liver and heart junctions. However, modifications to the usual liver isolation were introduced to make it analogous to that used for heart. After the initial series of low speed spins (2 x 23,000 g_{av} min compared to 2 x 43,000 g_{av} min for heart), the pelleted material was treated overnight with KI and the insoluble material collected as described for heart. Plasma membranes were then collected at the 0/43% (w/v) sucrose interface of a discontinuous sucrose gradient (0, 43, 54, 59

and 69% (w/v) sucrose in BB). The membranes were then treated with 0.1 M NaCl and 0.55% Sarkosyl as previously described for liver (Nicholson and Revel, 1983) and the junctions separated on the same sucrose gradient used for the isolation of heart gap junctions.

ii) Lens junctions. Lens homogenate was treated in an identical manner to that described for heart, with volumes adjusted for the relative wet weights of material available. In separate comparisons with liver, lens plasma membranes were treated with 0.55% Sarkosyl in an analogous way to that for liver. Although the yield was low, the surviving insoluble junctions were comprised of the same M_r 26,000 protein (i.e., MIP 26) present in previously characterized lens junction fractions (Dunia et al., 1974; Takemoto et al., 1981).

Tryptic digestion of heart gap junctions—quantitation of protein recovery. A heart gap junction fraction, after iodination with chloramine T as described for intact junctions in previous chapters, was suspended by sonication in 40 mM Tris/HCl, 50 mM $CaCl_2$, pH 8. (~20% (w/v) junctional protein.) To half of the suspension, trypsin (Sigma, type XI—i.e., treated with diphenylcarbamylchloride to inhibit chymotryptic activity) was added to a concentration of 3% (w/v). After incubation at room temperature for 21 hours, half as much trypsin was added again and the incubation continued for 3 hours, at which time a 1.3 fold excess of soybean trypsin inhibitor (Sigma, type 1-S) was added, and the remaining junctions pelleted and washed by two successive spins at 15,000 rpm x 45 min in an Eppendorf centrifuge model 5412. The second half of the suspension was treated identically except that only buffer was added in place of trypsin or soybean trypsin inhibitor. After counting the pellets in a γ counter, they were resuspended in water and two-thirds taken for examination by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) on a preparative size gel. The remaining one-third was analysed by microslab SDS PAGE. The protein content of each of the bands resolved by

SDS PAGE was determined from densitometer tracings of the Coomassie stained gel, and autoradiograms of the dried gels, as described in Chapter 1. In the case of the microslab, a silver stained gel was similarly analysed.

The following procedures were performed as described in previous chapters: negative staining (Chapter 1), thin sectioning (Chapter 4), SDS PAGE (Chapter 2), peptide mapping (Chapters 2 and 4), HPLC (Chapter 4) and comparative tryptic digestions of intact junctions from different tissues (Chapter 4).

Results:

Morphological assay of gap junction fractions from heart. Most of the contractile system of myosin and actin fibrils in cardiac cells is dissociated and solubilized by overnight incubation in 0.6 M KI and 6 mM $Na_2S_2O_3$ prior to the isolation of plasma membranes on a discontinuous sucrose gradient in the presence of KI. Consequently, following treatment of the plasma membranes with 0.3% Sarkosyl to solubilize non-junctional membrane, the insoluble fraction collected by centrifugation proves to be highly enriched for gap junctions when examined in the electron microscope (Figure 2a). The major contamination appears to be some single membranes which survive the rather mild detergent treatment. These are readily separated from the junctions by a sucrose density gradient virtually identical to that used in the last step of the isolation of "native" liver gap junctions (Nicholson and Revel, 1983).

The final fraction contains some amorphous material similar to that described in liver gap junction fractions (Nicholson et al., 1981) and occasional vesicles which display no connexons on their surfaces. However, the vast majority of the sample is comprised of gap junctions, as flat sheets or vesicles (Figure 2c). In profile, these junctions display the typical paired membranes separated by a 2-3 nm gap (Figure 2b and arrows in Figure 3a). En face, hexagonal arrays of connexons, 5-7 nm in diameter

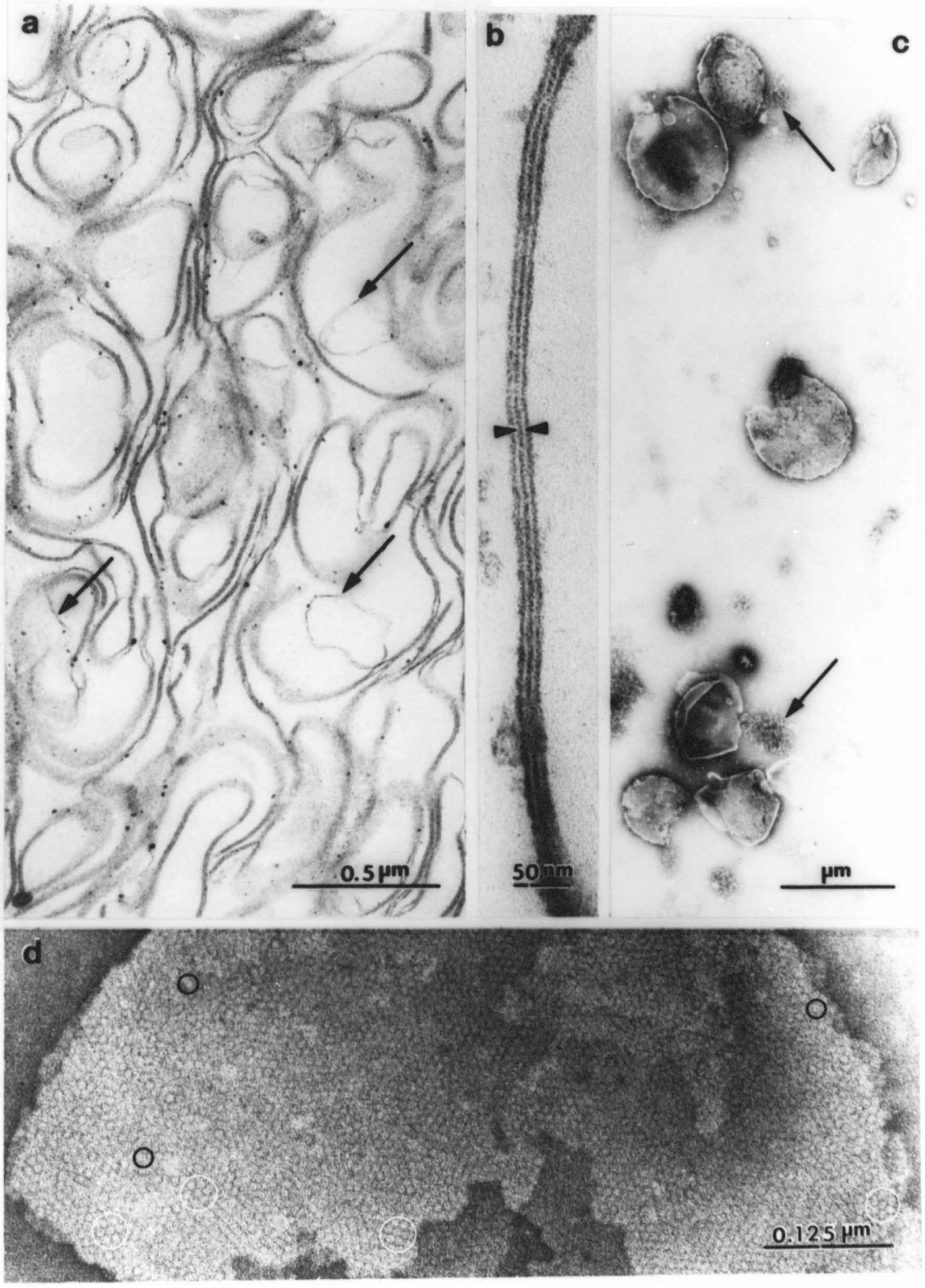


FIGURE 2.

FIGURE 2. (a) Electron micrograph of a thin section through a pellet of 0.3% Sarkosyl-resistant material, stained "en bloc" with uranyl acetate, showing transverse and grazing sections of heart gap junctions. Note that this fraction, enriched with gap junctions, is contaminated by single membranes (arrows). It can be freed of single membranes by using discontinuous sucrose gradients (refer to the isolation procedure). X-51,000.

(b) Electron micrograph of a thin section through an isolated "native" heart gap junction stained both with tannic acid and uranyl acetate. The junction appears heptalaminar with a 2 to 3 nm wide gap (arrow-heads) filled with the stain. Note the periodic appearance of the density in some parts of the gap. X 200,000.

(c) Electron micrograph, at low magnification, of the end-product of the isolation procedure after negative staining with 2% phosphotungstate. The fraction appears highly enriched for gap junctions and shows minor contamination by amorphous material (arrows). X 19,500.

(d) High magnification of an isolated "native" heart gap junction negatively stained with 2% phosphotungstate. The lattice structure is due to closely packed connexons; although hexagonal arrays of connexons can be recognized (white circles), the long-range order of the lattice is usually imperfect. Note the densely stained region in the center of most of the connexons (black circles). X 185,000.

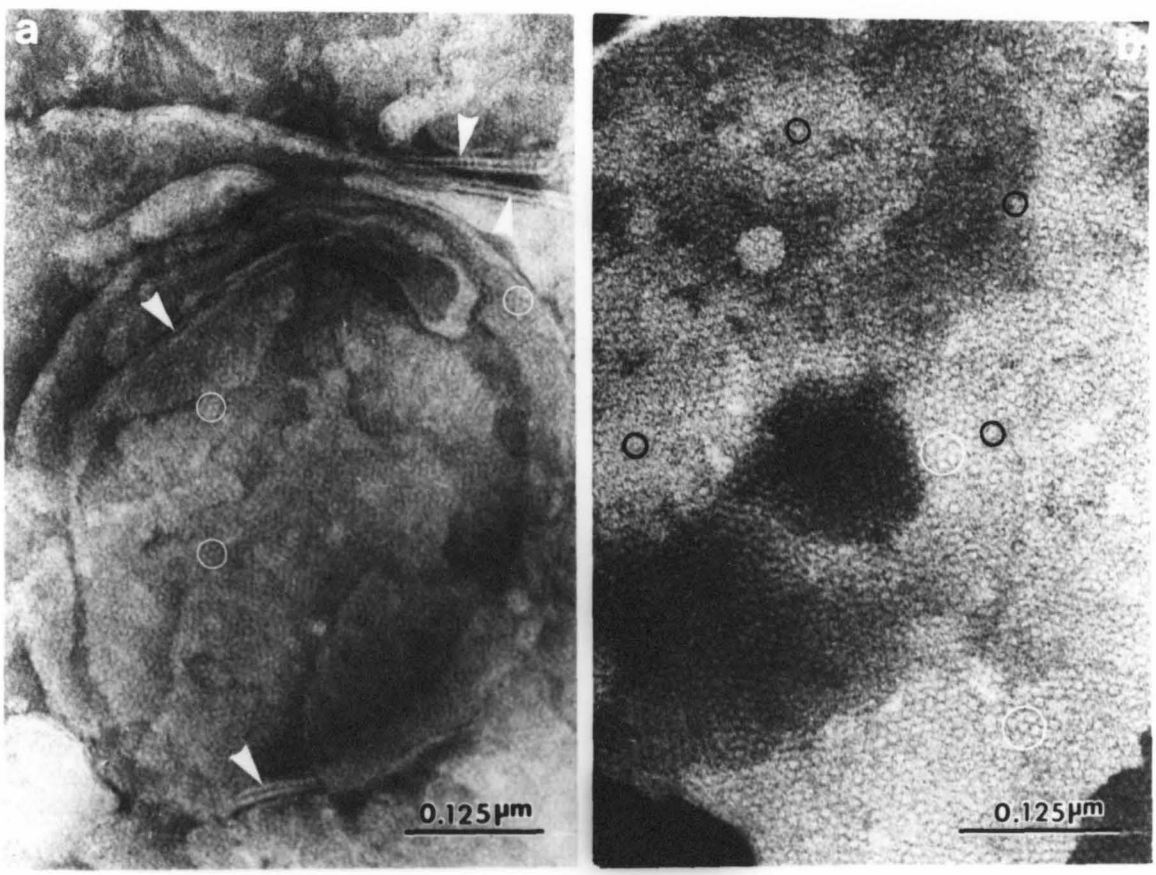


FIGURE 3.

FIGURE 3. (a) Isolated "native" heart gap junctions negatively stained with 2% phosphotungstate. Note the double-membrane profiles (arrows) of broken vesicles as well as the polygonal array of connexons (white dots) in the vesicles. Hexagonally arrayed connexons can also be seen (white circles). Periodic densities can be observed in the double-membrane profile (upper right corner). X 150,000.

(b) Isolated heart gap junctions treated with trypsin and negatively stained with 2% phosphotungstate. The junctions subjected to in vitro trypsin treatment retain their characteristic lattice structure and look ultrastructurally identical to native junctions (compare with Figure 2c). As in native junctions, the central region of the connexons is densely stained (black circles) and hexagonal arrays are recognizable (white circles). X 185,000.

and with a center to center spacing of about 9-10 nm, can be seen with negative staining (Figure 2d). As for liver gap junctions, the hexagonal arrays are usually imperfect and, especially in the heart, are often broken up into small domains.

Protein composition of cardiac gap junction fractions. The plethora of proteins present in the plasma membrane fraction (Figure 4, lane b) are mostly solubilized by Sarkosyl treatment, leaving an insoluble fraction enriched for gap junctions (see above) and for an M_r 28,000 polypeptide (Figure 4, lane c). Further enrichment for gap junctions on a discontinuous sucrose gradient, as documented above, was found to be concomitant with a further enrichment for the M_r 28,000 polypeptide (Figure 4, land d). A faint band at M_r 30,000 is also persistently present, and can be enriched for if 1 mM EDTA, TAME and iodoacetamide are included throughout the isolation to reduce proteolysis (Figure 4, lane e). Additional use of 1 mM PMSF to further control proteolysis produced no change in the pattern of polypeptides in the final fraction. Some minor, and less consistent components of the final fraction of M_r 32,000, 43,000, 50,000 and 55,000 can sometimes be detected in fractions loaded at higher concentration (Figure 4, lane e). Of these, the M_r 32,000 polypeptide was most frequently seen, and in one case, for unknown reasons, was the major protein component of the final fraction. However, examination of this fraction by negative staining revealed a marked absence of gap junctions.

Each of the components of this final fraction has also been examined by two-dimensional peptide mapping ("fingerprinting") using both tryptic (Figure 5) and chymotryptic (results not shown) digests. The M_r 30,000 and 50,000 proteins share virtually identical "fingerprints" with the major M_r 28,000 polypeptide, while all the other components display peptide maps which are unrelated to one another or to that of the M_r 28,000 polypeptide (maps of the M_r 43,000 and 32,000 polypeptides are shown in Figure 5).

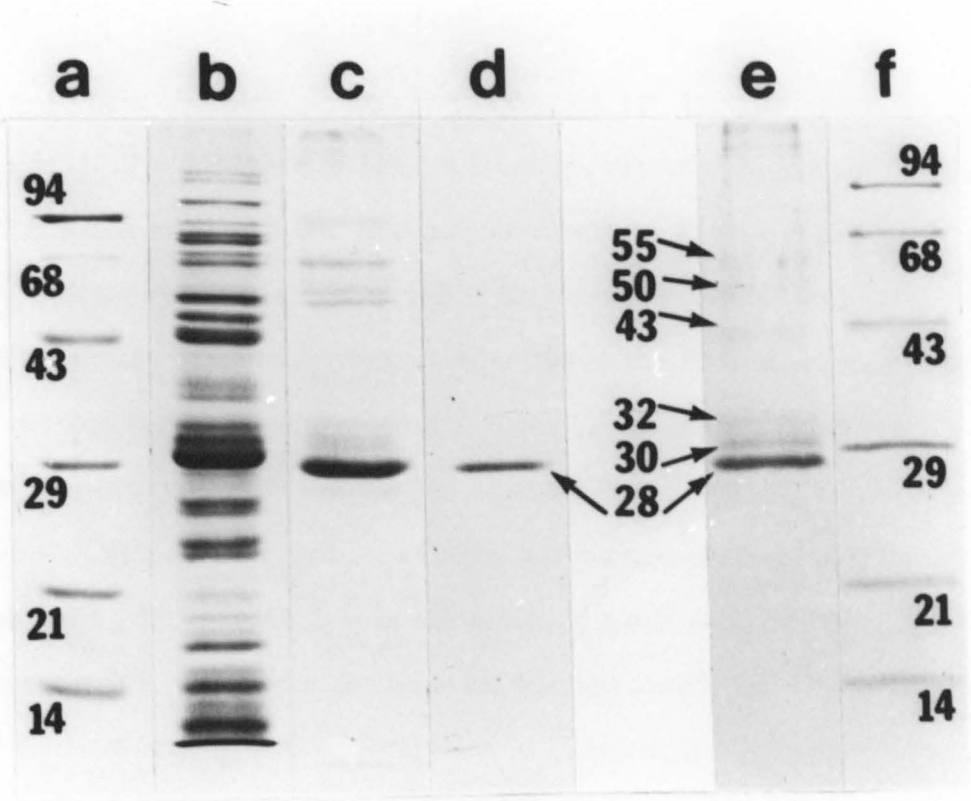


FIGURE 4.

FIGURE 4. Analysis of various fractions obtained during the purification of myocardial gap junctions. SDS polyacrylamide gels were stained with Coomassie blue. Lanes a and f: standard proteins with their molecular weights in kilodaltons. Lane b: plasma membrane fraction collected at the 10/35% sucrose interface of sucrose-potassium iodide gradients. Lane c: the 0.3% Sarkosyl-resistant material shows a prominent band at M_r 28,000. Lane d: the gap junction fraction collected at the 40.3/54% sucrose interface of the last sucrose gradient contains a major protein of M_r 28,000 (solutions made up with 0.1 mM each of EDTA, TAME and iodoacetamide). Lane e: a gap junction fraction isolated with solutions made up with 1 mM each of EDTA, TAME and iodoacetamide. Inhibitors allow preservation of a band of M_r 30,000. Because the sample was loaded more heavily than in lane d, minor components are visible (M_r 32,000, 43,000, 50,000, 55,000 and proteins of higher molecular weights).

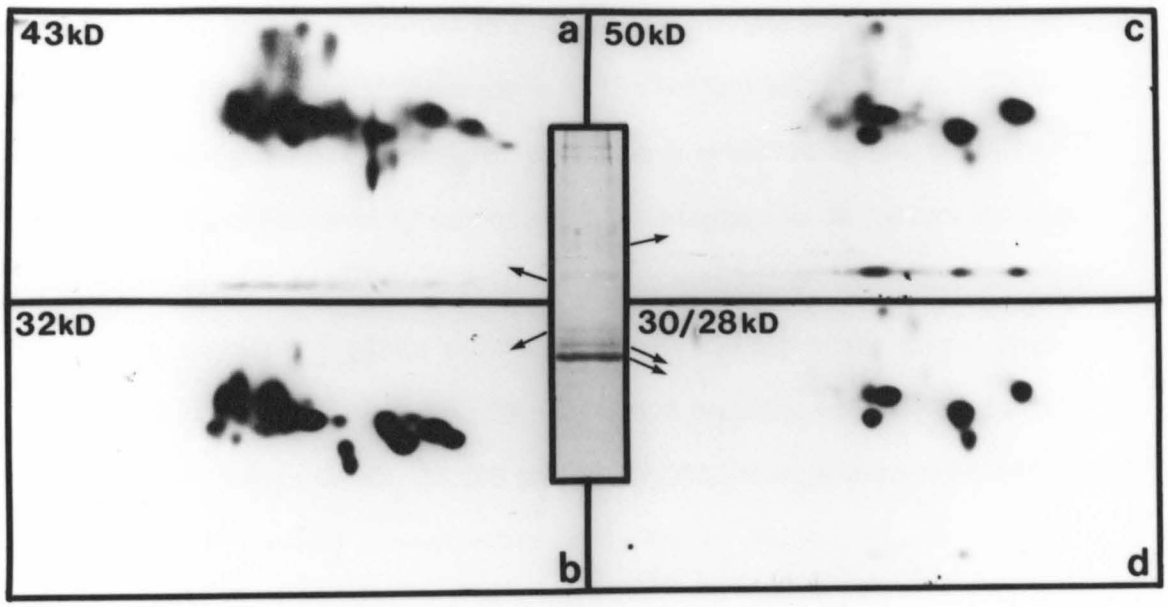


FIGURE 5.

FIGURE 5. Two dimensional maps of the iodinated tryptic peptides of the proteins present in a myocardial gap junction fraction. The molecular weight ($\times 10^{-3}$) of the protein subjected to two-dimensional peptide mapping is indicated on each map. The origin of the maps is at the bottom left (black dot). The separation of peptides generated by tryptic digestion is achieved by electrophoresis from left to right followed by ascending chromatography. In the system used the most basic peptides migrate furthest in the electrophoretic dimension and the more hydrophobic peptides are found nearest the top of the maps. Note that the maps of the M_r 50,000 and M_r 28,000/30,000 proteins are very similar. In contrast, the maps of the M_r 32,000 and M_r 43,000 proteins show that these proteins are neither related to each other nor to the M_r 50,000, 30,000 and 28,000 proteins. Two-dimensional maps of α-chymotryptic peptides of the above mentioned proteins lead to the same conclusions. The map (d) was obtained from the M_r 28,000 protein (see map of the M_r 30,000 protein in Figure 8).

Our purest gap junction fractions yield about 0.1-0.2 μg of M_r 28,000 protein per g wet weight of starting material. Eliminating the final sucrose gradient increases this yield by a factor of almost 10. An even greater increase in yield can be achieved by isolating plasma membranes in the presence of 5 mM Ca^{++}, even when the final sucrose gradient is employed. However, under these conditons contamination increases drastically.

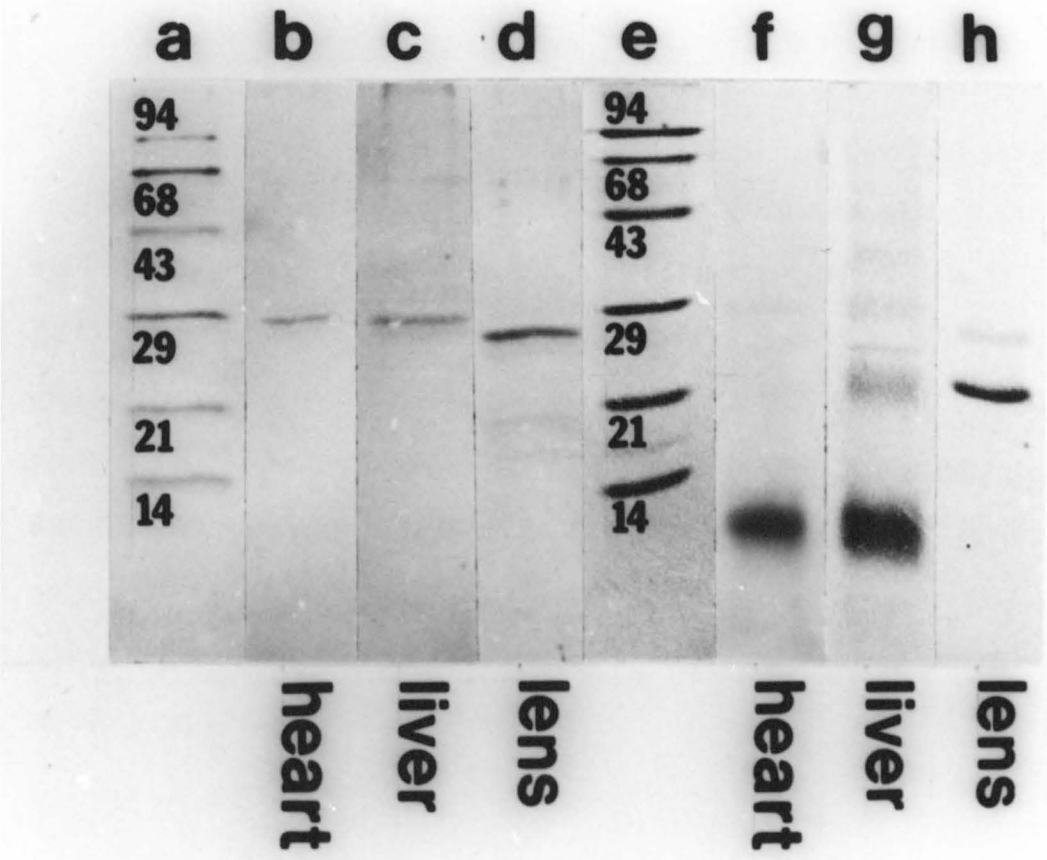
Comparisons of gap junctions from heart, liver (and lens).

(a) Morphology.

As has been observed in vivo (Revel and Karnovsky, 1967; NcNutt and Weinstein, 1970; Gros et al., 1978; Yee and Revel, 1978), heart gap junctions are virtually indistinguishable from liver gap junctions. They have the same appearance in profile (thin sections) and en face (by freeze fracture). The differences between these junctions and those of lens have been discussed thoroughly in Chapter 4.

(b) Protein components—peptide mapping.

The junction rich fractions from heart, liver and lens are each comprised of a single major protein, all of which have rather similar molecular weights [i.e., heart—M_r 28,000 (Figure 6, lane b—probable native protein—M_r 30,000); liver—M_r 28,000 (Figure 6, lane c); lens—M_r 26,000 (Figure 6, lane d)]. However, at this point the similarities seem to end. Comparisons of the peptide fingerprints, both chymotryptic (Figure 7) and tryptic (Figure 8, a-c), of these proteins reveal major differences between all three proteins. This held true whether the proteins were iodinated by the chloramine T method (Greenwood et al., 1963—generally labels tyrosine) or by the Bolton and Hunter method (Bolton and Hunter, 1973—labels amino groups)—compare the peptide maps in Figure 7, a-c and Figure 7, d-f, respectively. Indeed, beyond their generally hydrophobic nature, no homologies could be detected between them. Based on five independent comparisons three peptides from the chloramine T labelled proteins (numbered arrows in Figure 7, a-c) did


a
b
c
d
e
f
g
h
94
68
43
29
21
14
94
68
43
29
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14
heart
liver
lens
heart
liver
lens

FIGURE 6.

FIGURE 6. Comparative analysis by SDS-PAGE of junctional proteins from rat heart, liver and lens. Gels were stained with Coomassie blue. Lanes a and e: standard proteins with their molecular weights in kilodaltons. Lane b: myocardial gap junction fraction with its major polypeptide of M_r 28,000. Lane c: liver native gap junction fraction with a major protein of M_r 28,000. Lane d: lens native junction fraction containing the MIP of M_r 26,000. Lanes f, g and h: exhaustive digestion by trypsin of heart, liver and lens junctional fractions in vitro generates polypeptides of M_r 11,000 (lane f), 10,000 (lane g) and 21,000 (lane e), respectively. Quantitative data show that the bands of M_r 11,000 and 10,000 are comprised of two polypeptides each.

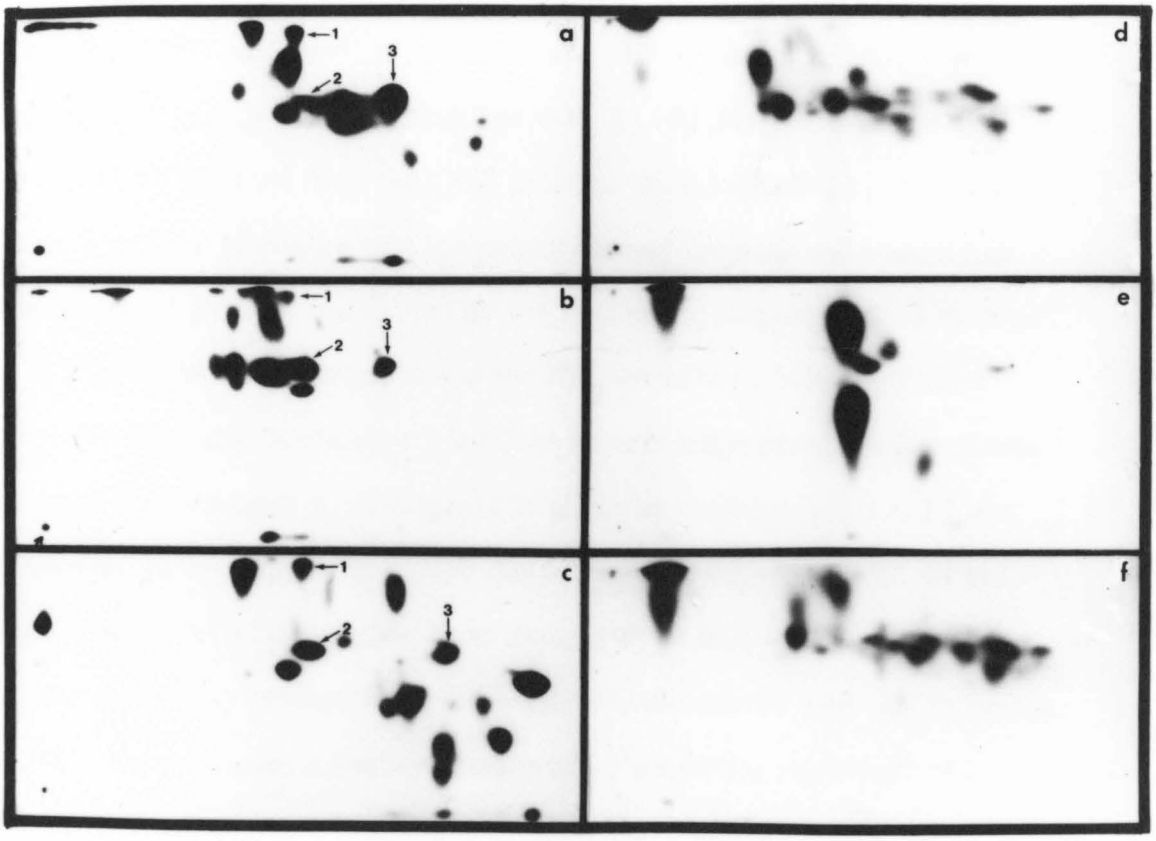

FIGURE 7.

FIGURE 7. Two-dimensional, α-chymotryptic peptide maps of the liver M_r 28,000 gap junction protein (a and d), the lens M_r 26,000 main intrinsic protein (b and e) and the heart M_r 28,000 gap junction protein (c and f), all isolated from rat. The proteins were iodinated by the chloramine T method in maps a to c (principally labels tyrosine and sometimes histidine and phenylalanine), and by the Bolton and Hunter method in maps d to f (principally labels lysine and the NH_2-terminus). Although three peptides from the chloramine T labelled protein maps (numbered peptides in maps a-c) did seem to co-migrate in all three proteins, peptide #3 was shown not to co-migrate in a third dimension of resolution (HPLC of the eluted peptides) while peptide #2 is thought to be free iodo-tyrosine since it was universally present in all maps studied, and eluted very rapidly on HPLC. Furthermore, a similar number of co-migrating peptides could be found in comparisons between peptide maps of any of the junctional proteins and an unrelated integral membrane protein— bacteriorhodpsin. Therefore, it is clear that the junctional proteins from these three tissues are very different, to the extent that no homology can be detected by these mapping techniques.

appear to consistently co-migrate in the maps of all three proteins. However, when these peptides were cut out, eluted and run on HPLC for a third dimension of resolution, one was shown not to coelute in the three proteins (peptide # 3 in Figure 7, a-c), while another was demonstrated to be a very small peptide, most likely free iodo-tyrosine. Furthermore, when the junctional protein maps were compared with that of an unrelated membrane protein, bacteriorhodopsin, the same number of peptides were found to co-migrate in both two and three dimensions.

One unavoidable difficulty with these results is that the most favorable methods for isolating junctions from the three tissues are quite different (see Materials and Methods). Therefore junctional fractions were also obtained from both lens and liver using protocols identical or very similar to that employed in the isolation of gap junctions from heart (see Materials and Methods). These protocols specifically included overnight treatment with KI, isolation of plasma membrane by sucrose gradients, Sarkosyl treatment (0.3 or 0.55%) and a final separation on a sucrose-urea density gradient. The fractions isolated in this manner displayed essentially the same pattern of polypeptides as fractions isolated by the usual procedures for lens and liver (see Material and Methods). Two-dimensional peptide mapping confirmed that the major components of the fractions isolated in this manner from lens or liver were identical to the proteins isolated by the standard procedures.

(c) Protein components—tryptic sensitivity in the junction.

As previously demonstrated for liver (Goodenough, 1976; Henderson et al., 1979; Makowski et al., 1982) and lens (Kistler and Bullivant, 1980b; Nicholson et al., 1983) junctions, heart gap junctions display virtually no morphological changes in response to extensive tryptic hydrolysis (see Figure 3b). Although the junctions seem unaffected, the major protein component in all three cases is reduced in molecular weight to M_r 11,000 in the heart (Figure 6, lane f), M_r 10,000 in the

liver (Figure 6, lane g), and M_r 21,000 in the lens (Figure 6, lane h). In Chapter 2 and Appendix I it was shown that the tryptic degradation products of liver gap junctions are actually two polypeptides of M_r 10,000, representing 20,000 daltons of the original M_r 28,000 protein. By analogy with the liver, we have quantitated the recovery of junctional protein in trypsinized heart gap junctions to determine if two fragments of similar size are also produced in the heart. Based on molecular weights of 28,000 for the major protein present before trypsinization, and 11,000 for the polypeptide(s) remaining after trypsinization (determined from SDS PAGE), one would in theory estimate the recovery of junctional protein after trypsinization to be 78% if two polypeptides are generated, but only 39% if one is produced. The actual recovery was measured by several independent methods (see Materials and Methods) which involved estimating protein from total radioactivity in pelleted material, radio-activity present in specifically identified junctional bands on SDS gels (estimated from an autoradiogram), and Coomassie (two independent estimates) and silver staining of these same bands. Although each of these techniques is subject to considerable errors (discussed in Chapter 1, Section D and Appendix I), the results from all four methods agreed quite well, estimating the recovery of protein to be 71 $\pm$ 8% (mean $\pm$ 1 standard error). Estimates ranged from 58 to 82% recovery. The results clearly suggest that, as in the liver, two polypeptides, each of M_r 11,000, were produced from the "native" M_r 28,000 protein by tryptic hydrolysis of intact junctions.

The peptide maps of these tryptic fragments in all three tissues are compared to the maps of the respective "native" proteins in Figure 8. In all cases, the more hydrophilic and basic peptides (those towards the bottom and right of the maps, respectively) are lost during the tryptic hydrolysis (arrows in Figure 8, a-c). Some new peptides appear in the maps of the fragments of liver and lens which survive trypsin treatment (arrows (*) in Figure 8, d-f). Presumably, during hydrolysis of

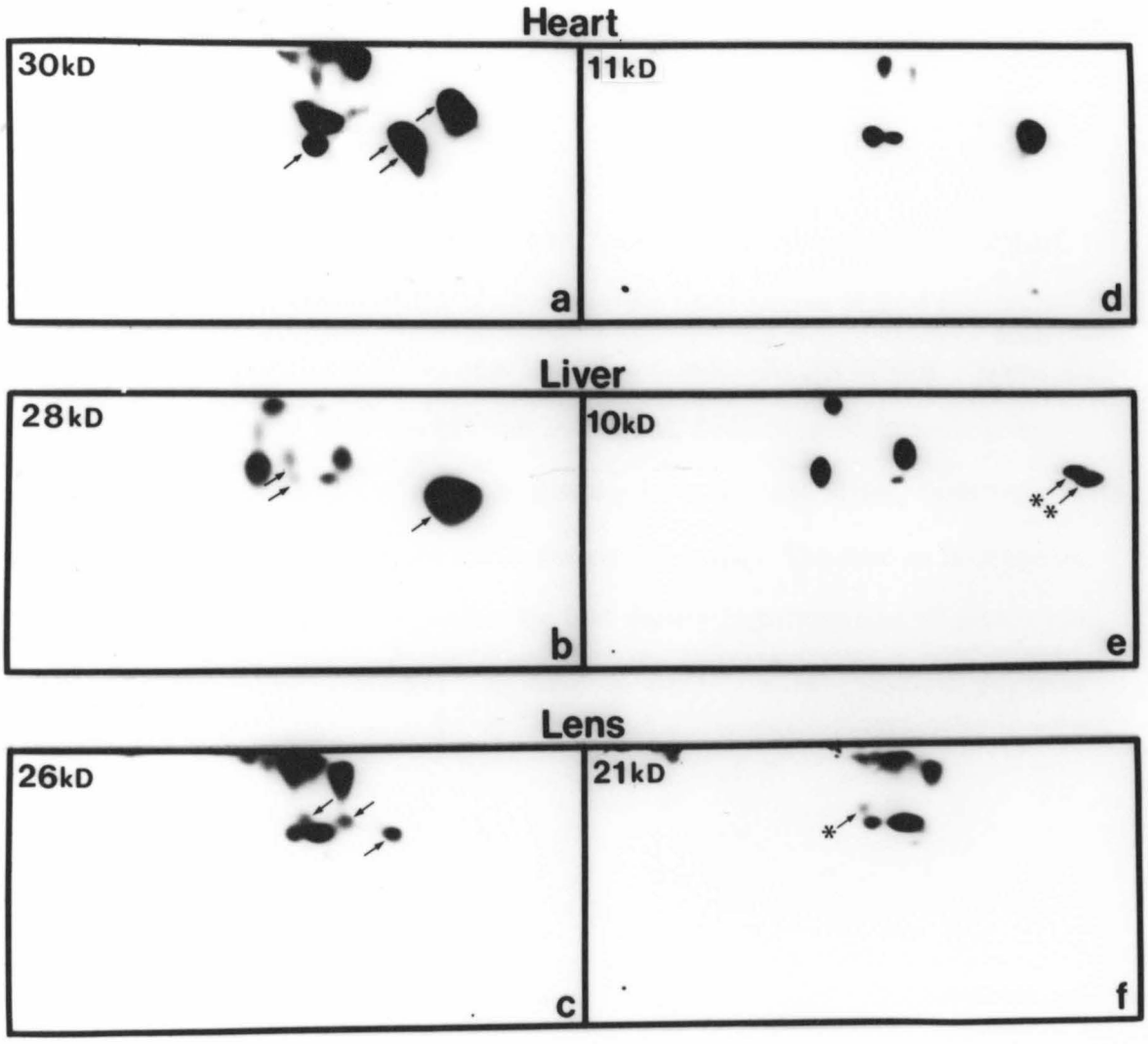

FIGURE 8.

FIGURE 8. Comparative analysis by means of two-dimensional maps of iodinated tryptic peptides, of the major proteins from native (a,b,c) and trypsinized (e,f,g) junctional fractions of rat heart, liver and lens. As previously (Figure 5), the origin of the maps is at the bottom left (small dot); electrophoresis is conducted from left to right and chromatography from bottom to top; molecular weights of analyzed proteins are indicated on the maps. The arrows in maps (a), (b) and (c) denote the peptides which are lost during trypsinization of intact junctions, while the arrows labelled (*) of maps (e) and (f) indicate the new peptides generated by this proteolysis.

the gap junction, one of the labelled peptides in the native protein is cleaved and the labelled portion remains with the surviving protein embedded in the membrane.

Discussion:

Characterization of heart gap junctions. Gap junctions, almost identical in structure to those present in liver fractions (see Nicholson et al., 1981, 1983), have been isolated from rat heart in very high purity, the only copurifying contaminants being some amorphous material and occasional non-junctional membrane vesicles. During the isolation protocol, as the fractions became progressively more enriched for gap junctions by morphological criteria, a concomitant enrichment for an M_r 28,000 protein was also observed (Figure 4, lanes b, c and d), culminating in the final fractions where it was virtually the only component (Figure 4, lane d), along with a faint band at M_r 30,000. This observation contrasts with those of previous authors who found multiple protein components in their final gap junctional fractions from heart (M_r 28,000, 31,000, 33,500 and 47,000 according to Kensler and Goodenough, 1980; M_r 28,500, 30,000, 33,000, 40,000 and 46,000 according to Manjunath et al., 1982a). These additional proteins most likely represent contamination by cytoskeletal material and especially desmosomes (see Kensler and Goodenough, 1980 and Colaco and Evans, 1982), which could arise from differences in the isolation protocol and in the species from which the hearts were obtained.

When loaded heavily on gels, many of our heart fractions are seen to contain small amounts of proteins corresponding approximately to those observed by Kensler and Manjunath, notably polypeptides of M_r 55,000, 50,000, 43,000, 32,000 and 30,000 (Figure 4, lane e). The bands at M_r 55,000 and 43,000 are not consistently found, and are absent from our cleanest fractions. The M_r 32,000 polypeptide is a more consistent component of the final fraction but is also believed to be a contaminant since in rare cases where it is enriched in the final fraction, few gap junctions could be detected morphologically.

Peptide maps of all these components support these conclusions in that the M_r 55,000, 43,000 and 32,000 proteins have "fingerprints" unrelated to one another or to the major M_r 28,000 polypeptide (Figure 5—M_r 55,000 polypeptide not shown). The polypeptides of M_r 50,000, 30,000 and 28,000, on the other hand, have virtually identical "fingerprints." Since the M_r 30,000 protein is enriched in the final fraction obtained when 1 mM protease inhibitors (EDTA, TAME and iodo-acetamide) are used throughout the isolation (as opposed to 0.1 mM), it seems likely that it represents a precurosr of the M_r 28,000 polypeptide, largely broken down by endogenous proteases in vivo or during isolation. The M_r 50,000 protein is unlikely to represent a precursor, since its presence does not correlate with the use of protease inhibitors. It remains to be conclusively demonstrated whether or not the M_r 50,000 protein represents a dimer of the M_r 28,000 polypeptide, as might be surmised by analogy with the liver (Chapter 2). However, preliminary studies on the M_r 28,000 polypeptide eluted from gels do indicate that it undergoes some aggregation in SDS, although to a much lesser degree than demonstrated for the rat liver gap junction protein (Nicholson et al., 1981). From these results, we conclude that isolated heart gap junctions are comprised of a single protein with a "native" molecular weight of at least 30,000, although it is usually reduced to M_r 28,000 after isolation.

The procedure described here produces gap junction fractions of high purity. However, the protein yield ($\sim$0.1-0.2 µg/g wet weight of heart) is rather low, even compared to that obtained for liver. When corrections are made for the gap junction complement in the whole tissue [1.5% of total hepatocyte surface area (Yee and Revel, 1978; Yancey et al., 1979) compared to 1% of myocyte surface area (Gros et al., 1979; Page and Shibata, 1981)], a yield of 0.7-1.0 µg of junctional protein/g wet weight of starting material would be comparable to that obtained in the liver. Currently, this kind of yield can only be obtained, and often exceeded, at the sacrifice of purity by either eliminating the final sucrose gradient from the protocol or including 5 mM Ca^{++} during the isolation of plasma membranes.

Comparison of heart, liver (and lens) junctions.

(a) Morphology:

In virtually all respects, heart gap junctions are very similar to those from liver. Morphologically they are almost indistinguishable and both have even been reported to become more crystalline in response to uncoupling procedures (Peracchia, 1977; Baldwin, 1979; Dahl and Isenberg, 1980), although the significance of these observations are still the subject of debate. The only consistent difference detected so far, other than in the protein components, is that heart junctions are more sensitive to detergents, being destroyed by Sarkosyl concentrations in excess of 0.3% (Kensler and Goodenough, 1980; this chapter), while 0.55% Sarkosyl is consistently used in the isolation of hepatic gap junctions.

Lens junctions, in contrast, show many differences when compared to cardiac or hepatic gap junctions, including the packing of the connexons, separation of the membranes, etc. These were discussed previously (Nicholson et al., 1983—i.e., Chapter 4).

(b) Junctional proteins:

All three tissues have now been demonstrated to be comprised of a single major protein. The small differences in the molecular weights of these components (heart: M_r 30,000; liver: M_r 28,000; lens: M_r 26,000) could result from differences as small as a single amino acid substitution (de Jong et al., 1978). However, rather than this being the case, two-dimensional peptide maps of these proteins have shown them to differ from one another to a much greater extent than previously expected. Peptide maps of tryptic and chymotryptic peptides labelled either at tyrosine residues and possibly phenylalanine and histidine, (Krohn et al., 1977) (chloramine T method of iodination) or lysine residues and the amino terminus (Bolton and Hunter method of iodination) have all been examined, and even a third dimension of HPLC employed in some instances. The end result of these comparisons

is that any apparent homologies between the proteins indicated by any given method is found not to exceed the background level of the method. Although all these techniques fail to demonstrate homology between the proteins of heart, liver and lens junctions, the degree to which the primary sequences of the proteins differ is difficult to estimate, since the techniques are more suited to detecting differences than similarities. An instance of this is a recent comparison of bovine brain calmodulin and rabbit skeletal muscle troponin C, where a 50% sequence homology (Watterson et al., 1980) remained undetected in two-dimensional tryptic peptide maps (Stevens et al., 1976).

Since the optimized preparation protocols for junctions from each tissue differ substantially, it would seem prudent to check if the differences in the proteins isolated was more a function of the protocol than of tissue-specificity in the gap junction protein. In fact, this proved not to be a problem, since isolation procedures closely resembling that used for the heart produced final fractions from liver and lens which were comprised of the same major proteins as found in fractions isolated by the standard procedures.

(c) Tryptic hydrolysis of gap junctions:

As discussed in Chapters 2 and 4, tryptic hydrolysis of the junction protein while it is part of the whole gap junction structure is most likely restricted to the cytoplasmic surfaces. As an aqueous protein, it should not penetrate the hydrophobic core of the membrane and its size (5 nm in diameter—Stroud et al., 1974) should exclude it from the extracellular gap (2-3 nm) and aqueous transmembrane pore (1.5-2 nm diameter). It is probably for this reason that the junction structure in all three tissues is so little affected by trypsin. It has previously been demonstrated that the liver M_r 28,000 junction protein cleaved to produce two polypeptides of M_r 10,000 by such trypsin treatment (Nicholson et al., 1981), while the lens M_r 26,000 protein is reduced to M_r 21,000 (Takemoto et al., 1981; Nicholson et al.,

1983). In both cases COOH-terminal residues are lost, and in the case of the lens, five NH_2-terminal residues also (Nicholson et al., 1983). It has now been shown that the heart gap junction protein (M_r 28,000) is, like that of liver, reduced to two polypeptides of M_r 11,000. Which portions of the polypeptide chain are removed by the hydrolysis remains to be determined. However, the marked similarity of the effects of trypsin on both heart and liver junction proteins may well suggest that, in spite of considerable differences in primary sequence, the overall folding of the proteins in the membrane may be similar.

Tissue-specificity of junctional proteins—implications.

One possible explanation of this truly surprising diversity in the proteins comprising very similar structure in two different tissues is that there is very little evolutionary pressure to conserve the primary sequence of a gap junction protein. In other words, since it forms a rather non-specific intercellular channel, the gap junction protein may only require certain very general features of its structure such as strategically placed hydrophobic and hydrophilic domains to be conserved, to retain its function. This would allow rapid changes to accumulate in the primary sequence. However, despite the differences between different tissues, the gap junction proteins of liver and lens have both been demonstrated to be quite well conserved between a variety of vertebrate species (liver—Nicholson et al., 1983; lens— Takemoto et al., 1981; Ziegler and Horwitz, 1981; Bok et al., 1982). Similar molecular weights (M_r 28-29,000) of the major protein present in gap junction enriched fractions from mouse, rabbit and guinea pig hearts (Kensler and Goodenough, 1980; Manjunath et al., 1982b) might suggest that this same conservation is true in the heart. These observations would suggest that the tissue-specific differences between junctions have specific significance and that evolutionary "pressures" exist to maintain them. Certainly, the finding of such diversity in the proteins comprising structures that are so similar morphologically is surprising

and contrary to previously held concepts of the gap junction as a non-specific channel which was probably the same in different tissues, species and even phyla. It is now possible that gap junctions may join the ranks of the growing number of systems (e.g., intermediate filaments, Osborn, M. and Weber, K., 1982) where the proteins exist as a gene family, all capable of making very similar structures.

What significance could such tissue-specificity of the gap junction have? One possibility is that different gap junction proteins are made in different tissues during development in order to prevent promiscuous coupling and establish compartmental boundaries (note the junctional discontinuities observed in this regard by Weir and Lo, 1982). Arguments against such a function could be made from tissue culture studies in which cells derived from different tissues couple effectively (Michalke and Loewenstein, 1971, Epstein and Gilula, 1977; Gaunt and Subak-Sharpe, 1979). However, in some experiments such coupling failed or was very inefficient (Fentiman et al., 1976; Pitts and Burk, 1976; Gaunt and Subak-Sharpe, 1979). Furthermore, it is quite possible that the tissue-culture cells used, mostly transformed lines, may no longer express their original tissue-specific junctional proteins, as suggested by Pitts (1980), who writes ". . . specificity of junction formation in culture is a property of established cell lines rather than primary cells."

A large number of studies have now demonstrated the gating of gap junctional channels in response to a variety of manipulations, most of which have been linked to changes in pH or $[Ca^{++}]$. (See Loewenstein, 1981, for review.) With such ubiquitous effectors controlling junctional communication, it is likely that the sensitivity of junctions to their action might need to vary from tissue to tissue. Changes in the putative divalent cation binding site on the junctional protein could be a way to achieve such modulation. Schuetze and Goodenough (1982) have already documented a case in the eye lens where junctional sensitivity to pCO_2 as an uncoupler is lost during development. Given the already established differences

between the junctions of lens epithelial cells (fiber cell precursors) and adult fiber cells (Waggoner and Maisel, 1978; Broekhuyse et al., 1979; Peracchia, 1978), it is possible that the change in pCO_2 sensitivity is a function of a wholesale replacement of one junctional protein with another. At present, it is also possible that the change reflects changes in junctional accessory proteins (see Kistler and Bullivant, 1980a). With current isolation protocols, any accessory proteins which might exist would almost certainly be removed and would not be expected to be seen in the final fraction. The existence of such components has already been suggested by the apparent association of avian sarcoma virus (Willingham et al., 1979) and calmodulin (Welsh et al., 1982, Peracchia et al., 1981) with gap junctions.

The significance of tissue-specific differences in the gap junctional protein will become clearer as we better understand the extent of the differences both in terms of the degree of diversity between primary sequences and in terms of the number of different junctional types. Since heart is mesodermal, liver endodermal and lens ectodermal in origin, it is possible at this stage that junctional diversity will be explained in terms of embryological germ layer specificity. In any event, the differences in the protein structure of different gap junctions should not only prove of significance in physiological and embryological terms, but should also provide a source of diversity useful in the study of structure—function correlations of the gap junction protein (see end of discussion in Nicholson et al., 1983—i.e., Chapter 4).

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GENERAL CONCLUSION

When the work presented here was first begun, knowledge of the composition of the gap junction was rather limited. No agreement had been reached on either the number or molecular weights of the gap junction proteins. Various candidates from M_r 10,000 to 40,000 had been proposed as components of liver gap junctions, while polypeptides of M_r 34,000 and/or 26,000 had been associated with isolated junctional fractions from lens, the only other tissue to be studied in this regard (see Introduction for detailed bibliography). The situation has been much clarified in the interim, in some part by the results presented in this thesis. In some respects, the picture that emerges is pleasingly simple, in that each gap junction appears to be comprised of a single polypeptide. In other ways, however, the field has been shown to be far more complex than previously guessed with the demonstration of a remarkable degree of variability in the gap junction proteins from different tissues.

The gap junction from mammalian liver, the model most used for study, has now been shown to be comprised of a single major protein of M_r 26-28,000 (Henderson et al., 1979; Hertzberg, 1980; Nicholson et al., 1981). Several properties of this protein have been established, including its tendency to aggregate in SDS (Henderson et al., 1979; Nicholson et al., 1981), its sensitivity to proteolysis while still part of the junctional structure (Duguid and Revel, 1976; Henderson et al., 1979; Finbow et al., 1979; Nicholson et al., 1981) and its apparent lack of carbohydrates (Hertzberg and Gilula, 1979). Two-dimensional peptide "fingerprints" of this gap junction protein have played an important role in its identification and characterization (Nicholson et al., 1981) and now may also provide the first assay for the junctional protein, enabling it to be identified in a mixture of polypeptides. A partial amino acid sequence for the protein has been obtained (Nicholson et al., 1981) and several fragments of the protein suitable for further sequence analysis have been generated by enzymatic and chemical cleavage methods (Chapter 3).

The studies involving protease treatments of the intact junction, have also cast some light on the tertiary structure of the protein (Chapter 3). Both the COOH-terminal 35-40 residues (~4,000 daltons of the molecule) and a loop of similar length in the center of the molecule (between 10,000 and 14,000 daltons from the NH_2-terminus) are apparently exposed at the cytoplasmic face of the junction, leaving the remaining two, protease-resistant M_r 10,000 fragments disposed within the lipid bilayer and extracellular gap between membranes. X-ray and optical diffraction studies on liver gap junctions (Caspar et al., 1977; Makowski et al., 1977, 1982; Unwin and Zampighi, 1980) have also greatly contributed to our understanding of the relationships of the individual polypeptide subunits to one another as they associate, apparently as hexamers, to form the unit transmembrane channels of the gap junction (connexons) which in turn interact head to head to form the connecting pathway between adjacent cells. Recently, there has even been evidence that β-pleated sheet structures may comprise a significant part of the secondary structure of the gap junction polypeptide within the lipid bilayer (Makowski et al., 1982 - also see discussion in Nicholson et al., 1981).

Despite the extensive characterization of the M_r 28,000 "native" gap junction protein, it is still not certain that it is the only component of liver gap junctions in vivo. Minor components of the structure (e.g., <5%) could easily have been overlooked in our analyses, a problem which is exemplified by the work of D. Luck on flagellar components, which revealed that in addition to the major proteins, more than a hundred minor flagella proteins could be detected on two-dimensional gels (Piperna et al., 1977). A less extensive multiplicity of minor proteins has also been detected in intermediate filaments (Lazarides et al., 1981). In liver gap junctions, the M_r 21,000 polypeptide detected by Henderson et al. (1979) in mouse and by us, in much smaller amounts, in the rat (Nicholson et al., 1981) has already been identified as a possible second, although minor, gap junction component.

It cannot be easily explained as a proteolytic degradation product of the M_r 28,000 protein (see Nicholson et al., 1981; Traub and Willecke, 1982), although it is clearly related to it by peptide mapping. It should also be kept in mind that the final junctional fraction has been isolated under rather stringent washing conditions, including 0.1 M NaCl, 2 M urea, pH 11, and a rigorous treatment with 0.55% sarkosyl which included brief sonication. As a result, accessory proteins, essential to the function of the gap junction in vivo, could have been inadvertently lost, since no assay exists for the functionality of isolated junctions. Recent reports associating calmodulin activity (Peracchia et al., 1981; Welsh et al., 1982) and the sarc gene product (Willingham et al., 1979) with gap junctions may point to the existence of such accessory proteins. However, it does seem to have been demonstrated that the existence of a basic junctional channel requires only a single major protein.

Studies on the composition of gap junctions have now also been extended beyond the liver model to other tissues where gap junctions are relatively abundant—notably eye lens and heart. In these tissues also, the basic junctional structure which can be isolated is comprised of a single major protein—M_r 26,000 in the lens and M_r 30,000 in the heart, although in the latter case only its degradation product of M_r 28,000 can be isolated in quantity. These junctional proteins do seem to share some common properties with that of liver in that they aggregate, to varying degrees, in SDS and display a sensitivity to proteolysis which seems to have no detectable repercussions on the integrity of the gap junctional ultrastructure (Chapter 5). In this latter case, both heart and liver junctional proteins are affected similarly by proteolysis (e.g., by trypsin), being reduced to two M_r 10-11,000 polypeptides, while the lens junction protein is reduced to a single M_r 21,000 polypeptide. A comparison of the two-dimensional peptide maps of the "native" junctional proteins of these tissues, however, reveals that their primary sequences are very different, to the extent that no reliable degree of homology

could be demonstrated by this technique. In the case of lens and liver, this result has been substantiated both immunologically (Hertzberg et al., 1982; Traub et al., 1983) and through direct sequence analysis at the NH_2-terminus (Nicholson et al., 1983). It is still possible that some homologies exist between these proteins, if not at the level of primary sequence, at least in terms of tertiary structure, as might be suggested by their similar protease sensitivities in the intact gap junction. However, given the similar structures and properties of gap junctions from different tissues, the extensive variability in their protein components has proven to be truly surprising. It is tempting to speculate that the gap junction protein may form a gene family analogous to those previously described for actin (Kindle and Firtel, 1978; Fryberg et al., 1980), intermediate filaments (Weber and Osborn, 1982), and others. Of course, after the fact it is not difficult to propose many possible reasons for this variability in the gap junction. These are discussed at length in Chapter 5, and include such roles as establishing non-communicating compartments in the embryo during development (e.g., Wier and Lo, 1982), changing the sensitivity of the gap junction channels in different tissues to gating by common effectors such as Ca^{++} or pH and perhaps even conferring a degree of selectivity on the junctions in different tissues to the passage of specific metabolites of importance.

In some ways, our observations of gap junctional tissue specificity are not straightforward, especially when considering the junctions from eye lenses. As discussed in Nicholson et al. (1983), although lens junctions are generally similar in their features to gap junctions of other tissues (Peracchia and Peracchia, 1980a,b; Goodenough, 1979; Hertzberg et al., 1982), they do display some differences which have been reported by some authors to be substantial (Zampighi et al., 1982). These structural differences, in conjunction with the differences in their protein components, have been used to argue that the lens fiber junctions do not represent gap junctions, notwithstanding the demonstration of coupling between lens fiber

cells (Rae, 1974; Eisenberg and Rae, 1974; Goodenough et al., 1981; Schuetze and Goodenough, 1982). The whole issue is further complicated by the heterogeneity of structures observed in lens junction fractions (see Appendix II for a detailed account). The significance of the different "junctional" profiles seen in thin sections (i.e., paired membranes) is unclear, as is their correlation with the varying connexon arrays observed in negative stain (see chapter 4, Figure 1). Even the association of the major M_r 26,000 protein with one or all of these structures is not easily demonstrated, particularly in the light of recent conflicting immunological reports (Bok et al., 1982; Paul and Goodenough, 1983). It is possible that several or all of the structures observed are variations of a single "junction" caused by factors such as lipid extraction with detergents (e.g., the narrower junction profiles detected by Zampighi et al., 1982; Nicholson et al., 1983; Paul and Goodenough, 1983) or changes in divalent cation concentrations or pH (Peracchia and Peracchia, 1980a,b). Some of the "junctions" observed may even represent single membranes which non-specifically adhere to one another. Alternatively, one could take a very hard line attitude and regard even minor variations in structure as evidence of different junctional types. Unfortunately, in the case of the lens, it is currently impossible to distinguish between this profusion of possible explanations on the basis of the rather confusing data available. It does appear that the most consistent components of lens junction fractions from several laboratories are "junctions" with similar or slightly narrower profiles than liver gap junctions, and the M_r 26,000 MIP. Whether these represent a lens specific gap junction, some other junction or even an artifact created by urea and detergent treatment of lens membranes remains to be established. What is cear from this case is that our current definitions of the gap junction are inadequate, largely because we lack a functional assay. Ultimately, the demonstration of an aqueous channel of appropriate dimensions (i.e., 1-1.5 nm) which spans the membranes of adjacent cells and the extracellular

space, may prove to be the only reliable way of universally defining a gap junction or, more appropriately, a communicating junction (at times referred to as macula communicans). Certainly, the variability which we have detected in the junctional proteins of different tissues seems to forestall any prospect of a universal definition of the gap junction based on its protein components.

Although several issues have been settled by research in this field over the past six years, even more questions have been raised. Arising directly from the work presented here, several directions for future research are indicated. Perhaps foremost among these is understanding the significance of gap junction tissue specificity. Its elucidation is likely to be greatly aided by determining the extent of this tissue variability, both in terms of the degree of difference between any two tissue specific gap junction proteins and in terms of the number of different proteins in any given organism. For the first analysis, it is clearly necessary to first fully understand the structure of one gap junction protein, on which to base further comparisons. Progress has already been made in this regard with the liver protein, although much still remains to be done to define its primary and higher order structure. With respect to the second analysis, the study of gap junction diversity could be approached by applying the techniques used here to other tissues. However, this is likely to prove very laborious given the low abundance of gap junctions in most other tissues. A more promising approach is provided by the current genetic engineering technology, whereby synthetic oligonucleotide probes can be constructed from the known sequence of the liver gap junction protein (Nicholson et al., 1981) and used to select cDNA clones of the junctional protein messenger RNA and ultimately to screen the genome for the liver and related gap junction genes. Some progress has already been achieved along these lines by Dr. Yancey in our laboratory in collaboration with Drs. Horvath (in Dr. Hood's lab) and Gorin (in Dr. Horwitz's lab at UCLA Medical School).

The elucidation of the gap junctional protein structure from various tissues which should stem from such research will enable us to address many issues including the mechanisms of channel gating in the gap junction and the nature of the protein-protein interactions involved in maintaning the integrity of the gap junction structure (between subunits of a connexon; between connexons in the plane of the membrane; between connexons of adjacent cells; etc.). It is even possible that the structure of the junctional channels, especially near the cytoplasmic surface, may confer, in different tissues, some selectivity, thereby providing a clue to one of the central questions of gap junction research—the identity, in any given system, of the metabolites or signals which pass between cells via the gap junctions.

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APPENDIX I

Quantitative Estimates of the Recovery of Junctional Protein after Tryptic Hydrolysis of Isolated "Native" Gap Junctions

[A detailed treatment of Chapter 2—"Results—quantitative analysis of tryptic digestion of gap junctions"]

When "native" gap junction fractions, characterized by a single major protein of M_r 28,000, are subjected to extensive tryptic proteolysis, the gap junctions appear virtually unaffected structurally (see Chapter 4—Figure 1 b and c). However, examination by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) reveals a single band of M_r 10,000 as the only junctional protein to survive this treatment. At first, it might seem surprising that the ultrastructure of the junction is so little affected by this rather drastic reduction in the molecular weight of its constituent protein. The initial clue to an explanation of this phenomenon is provided by comparisons of the two-dimensional peptide maps of the M_r 28,000 and M_r 10,000 polypeptides. These show that two-thirds of the iodinated peptides present in the "native" protein persist in its major tryptic fragment, thereby suggesting that the material at M_r 10,000 may represent more than the one-third of the "native" protein implied by its apparent molecular weight. This contention is supported by sequence analysis of the M_r 10,000 band which reveals the presence of at least two major sequences. Therefore, to unequivocally determine whether or not tryptic hydrolysis of isolated "native" gap junctions cleaves the junctional protein into two M_r 10,000 polypeptides, we have employed several different methods to estimate the yield of junctional protein which remains associated with the gap junction structures after tryptic hydrolysis. A yield of 36% would be expected if one M_r 10,000 fragment is produced (Theoretical model (a) in Table 1B) and a yield of 71% would be predicted if two fragments result (Theoretical model (b) in Table 1B).

An aliquot of "native" gap junctions labeled with ^{125}I using chloramine T oxidation, was added to 400 µl of a "native" gap junction fraction in 5 mM Tris/HCl, 1 mM $CaCl_2$ (pH 7.4) (600 - 700 µg gap junction protein). This junction suspension was homogenized by brief sonication and divided into two equal aliquots. One, the "trypsinized sample", was treated with 10 µg trypsin (Sigma type XI—chymo-

tryptic activity inhibited by diphenylcarbamylchloride) for 10 hr at room temperature, and for an additional 3.5 hr following a boost with a further 5 μg of trypsin. The second aliquot, the "control sample", was treated in an identical manner except that trypsin was eliminated from all solutions. After these incubations, junctions were collected and washed by several centrifugations of 15,000 rpm for 30 min in an Eppendorf centrifuge. As an approximate estimate of junctional recovery, the pellets from the trypsinized and control samples were counted in a Beckman 4000 gamma counter (Experimental estimate (1) - Table 1A).

An analogous estimate was made using one-third of each pellet for amino acid analysis on a Durrum D-500 analyser in order to directly determine the total protein content. Half of the material taken was hydrolyzed in vacuo for 20 hrs at 105°C while the other half was used as an unhydrolyzed blank to correct for background levels of free amino acids. Sufficient material was available to allow determinations to be made using both 6 N HCl hydrolysis in which tryptophan and cysteine are destroyed (Experimental estimate (4a) - Table 1A) and 3 M mercaptoethanasulfonic acid hydrolysis, which preserves tryptophan (Experimental estimate (4b) - Table 1A). The results of these analyses are given in Table 2.

The remaining two-thirds of each pellet was solubilized and the proteins separated by preparative SDS PAGE. The amount of junctional protein present in each sample was determined from a densitometer scan of the appropriate lanes of the Coomassie stained gel, as outlined in section DII of Chapter 1. The recovery of junctional protein after trypsin treatment compared to the control sample was calculated after correcting for the small amount of degradation to M_r 14,000 and 10,000 polypeptides which had occurred in the control (Experimental estimate (6a) - Table 1A). The individual junctional bands in each sample were then cut out of the gel, pooled and counted in a Beckman 4000 gamma counter, thus providing experimental estimate (2) in Table 1A. After counting, the polypeptides were

TABLE 1: RECOVERY OF GAP JUNCTION PROTEIN AFTER TRYPTIC HYDROLYSIS OF INTACT JUNCTIONS

A.

	Method of Estimate	% Recovery of Gap Junction Protein
(1)	Radioactivity[1] in pelleted material - [gamma counter]	61
(2)	Radioactivity[1] in specific junctional bands after separation by SDS PAGE - [gamma counter]	61
(3)	Radioactivity[1] in specific junctional bands after separation by SDS PAGE - [autoradiography][2]	65
(4)	Amino acid analysis of pelleted material[3]	(a) 58 (b)[4] 53
(5)	Amino acid analysis of specific junctional proteins after separation by SDS PAGE and elution[3]	85
(6)	Coomassie staining of specific junctional bands after separation by SDS PAGE	(a) 68 (b)[5] $\geq$55
	MEAN ESTIMATE	67

B.

Theoretical protein recovery, assuming:

(a)	"Native" M_r 28,000 protein hydrolysed to ONE M_r 10,000 polypeptide	36
(b)	"Native" M_r 28,000 protein hydrolysed to TWO M_r 10,000 polypeptides	71

[1]Proteins labeled with ^{125}I using chloramine T oxidation in the absence (estimate No. 1) or presence (estimates 2 and 3) of SDS.

[2]Figure 1a.

[3]Results of amino acid analyses given in Table 2.

[4]Amino acid analysis in mercaptoethanesulfonic acid to preserve tryptophan.

[5]Figure 1b.

TABLE 2: AMINO ACID ANALYSES OF THE GAP JUNCTION PROTEIN

AMINO ACID	MOLE PERCENT			
	"Native" Liver G.J.		"Enzyme-Treated" Liver G.J.	
	Whole Pellet [3][a]	Eluted M_r 28,000 band [2][a]	Whole Pellet [3][a]	Eluted M_r 10,000 band [2][a]
Asx	7.1	9.0	6.1	8.7
Glx	10.2	10.5	7.7	10.5
Lys	5.0	6.2	3.2	4.7
Arg	5.5	6.9	4.6	4.4
His	3.9	2.4	3.8	2.0
Ser	7.6	9.0	8.9	9.1
Thr	5.0	4.6	5.9	4.8
Gly	9.1	16.5[b]	6.8	16.6[c]
Tyr	3.5	3.1	4.1	2.3
Phe	3.9	3.3	5.1	4.1
Trp	4.7[c]	--	5.4[c]	--
Ala	6.6	5.8	7.5	7.1
Val	6.1	5.8	8.1	6.8
Leu	9.3	7.7	10.2	8.8
Ile	3.7	3.9	4.5	4.2
Met	2.3	1.4	2.5	1.4
Cys[d]	--	--	--	--
Pro	6.5	3.9	5.6	4.5
% Polar[e] residues	44.3	48.6	40.2	44.2
% hydrophobic[f] residues	43.1	31.8	48.9	36.9

[a]Number of independent analyses performed.

[b]Glycine values are elevated in analyses of proteins eluted from SDS polyacrylamide gels run in Tris/glycine buffer.

[c]Tryptophan values are based on a single analysis after hydrolysis with 3M mercaptoethanesulfonic acid.

[d]Cystine and cysteine are destroyed during hydrolysis.

[e]Includes Asx, Glx,Lys, Arg, His, Ser and Thr.

[f]Includes Phe, Trp, Ala, Val, Leu, Ile, Met and Pro.

electroeluted from the gel slices for amino acid analysis to directly determine the protein content (Experimental estimate (5) - Table 1A). Corrections were made for yields from electroelution and hydrolysis which were determined by monitoring radioactive recoveries. Amino acid analyses, the results of which are shown in Table 2, were obtained in the same way as for the pelleted material, although only 6 N HCl hydrolysates were examined. It should be noted that a comparison of the analyses of the pellets, or the analyses of the eluted polypeptides, reveals the trypsinized junctions to have a more hydrophobic character than the "native" or control junctions. This is consistent with the proposal, outlined in Chapters 2-4, that the M_r 10,000 polypeptides represent the portion of the junction protein protected from proteolysis by the surrounding membrane.

Additional estimates of the recovery of junctional protein after tryptic hydrolysis could also be obtained from time-courses of the proteolysis. After adding trypsin to a "native" gap junction fraction, samples were taken at various times for analysis by SDS PAGE (see Figure 1 for details). In cases where the "native" junction fraction was iodinated (chloramine T method), densitometer scans of autoradiograms of the analytical gels were used to determine the amount of junctional protein of specific molecular weights at each time point. From this, a time-course for the disappearance of protein of M_r 28,000-24,000 (and multimeric forms) and its appearance at M_r 14,000-10,000 could be established (Figure 1A). Extrapolation would then provide an estimate of the ultimate yield of M_r 10,000 polypeptides from the starting material of M_r 28,000-24,000. An analogous determination could be made using Coomassie stained gels (Figure 1B). One difficulty, aside from those discussed in section DII - Chapter 1, is presented by the material which remains at the top of the running gel and which has been demonstrated by peptide mapping to be comprised of both non-junctional and aggregated junctional protein. Since it is only present in non-trypsinized samples,

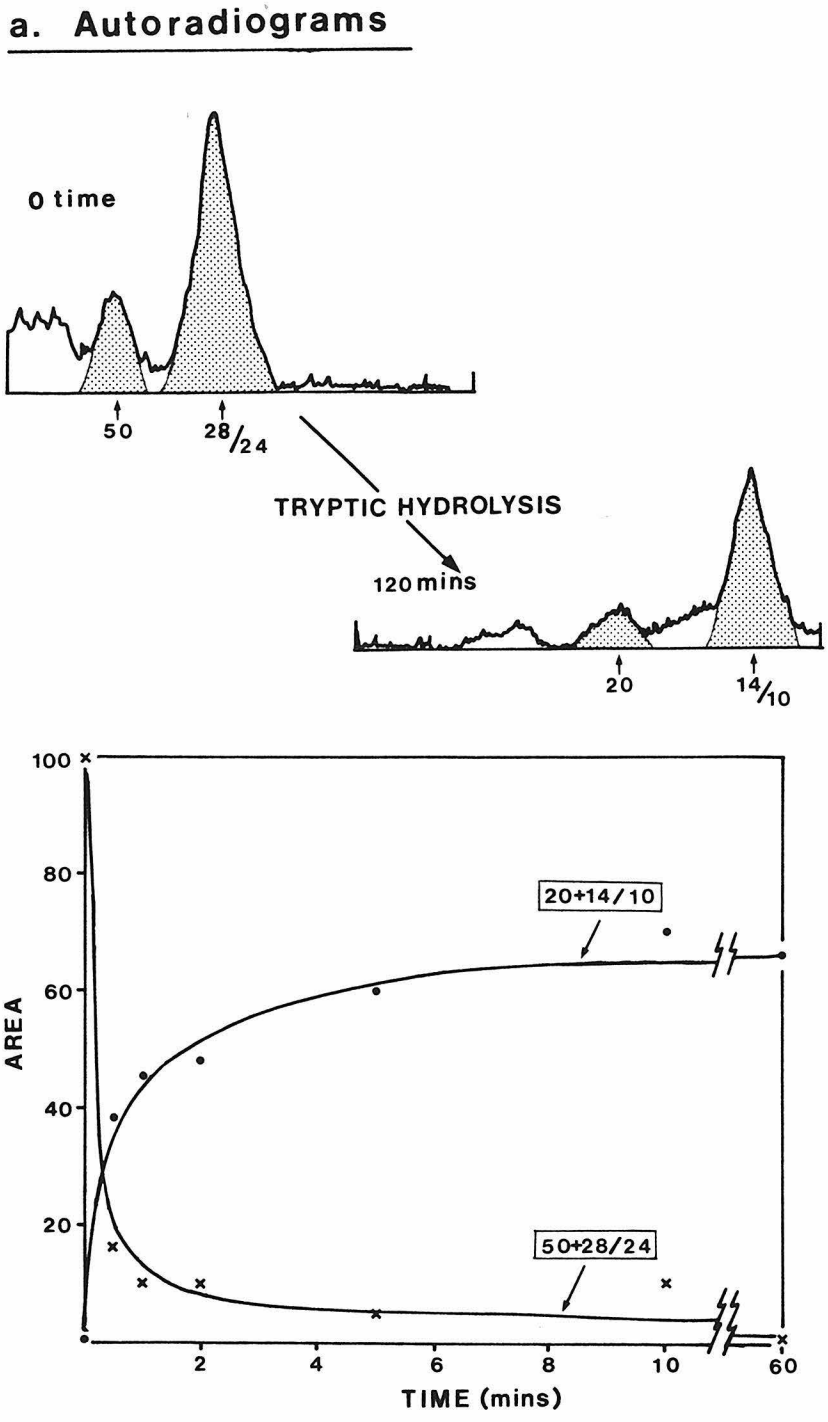
a. Autoradiograms
0 time
50
28/24
TRYPTIC HYDROLYSIS
120 mins
20
14/10
100
80
60
40
20
AREA
20+14/10
50+28/24
2
4
6
8
10
60
TIME (mins)

FIGURE 1.

FIGURE 1.

Time course of tryptic hydrolysis of "native" gap junction fractions followed by radioactivity (a) and Coomassie staining (b) of specific junctional proteins separated by SDS PAGE.

(a) After iodination by chloramine T, a "native" gap junction fraction (0.1 μg/μl junctional protein) in 50 mM NH_4HCO_3, pH 7.8 was treated with 0.01 μg/μl trypsin at 37°C. Samples (15 μl) were removed at various times (see graph), and analyzed by SDS PAGE (see methodology described in Chapter 2) after the reaction had been terminated by the addition of a two-fold excess of soybean trypsin inhibitor. Autoradiograms of the dried gels (using Kodak XR film exposed at -70°C with a Cronex Lightning Plus intensifying screen) were scanned with a Joyce Löebel densitometer. Sample scans before (0 time) and after (120 min) tryptic hydrolysis are shown. Areas of the peaks at M_r 28,000-24,000 and the dimer at M_r 50,000 (e.g., shaded areas in 0 time scan) were used in determining the (x) time points, while those of the peaks at M_r 14,000-10,000 and the dimer at M_r 20,000 (e.g., shaded areas in 120 min scan - the dimer was only evident after 60 and 120 min of hydrolysis) were used to determine the (0) time points. An estimated 66% of the initial junctional protein remained associated with the gap junctions after tryptic hydrolysis (60 min time point).

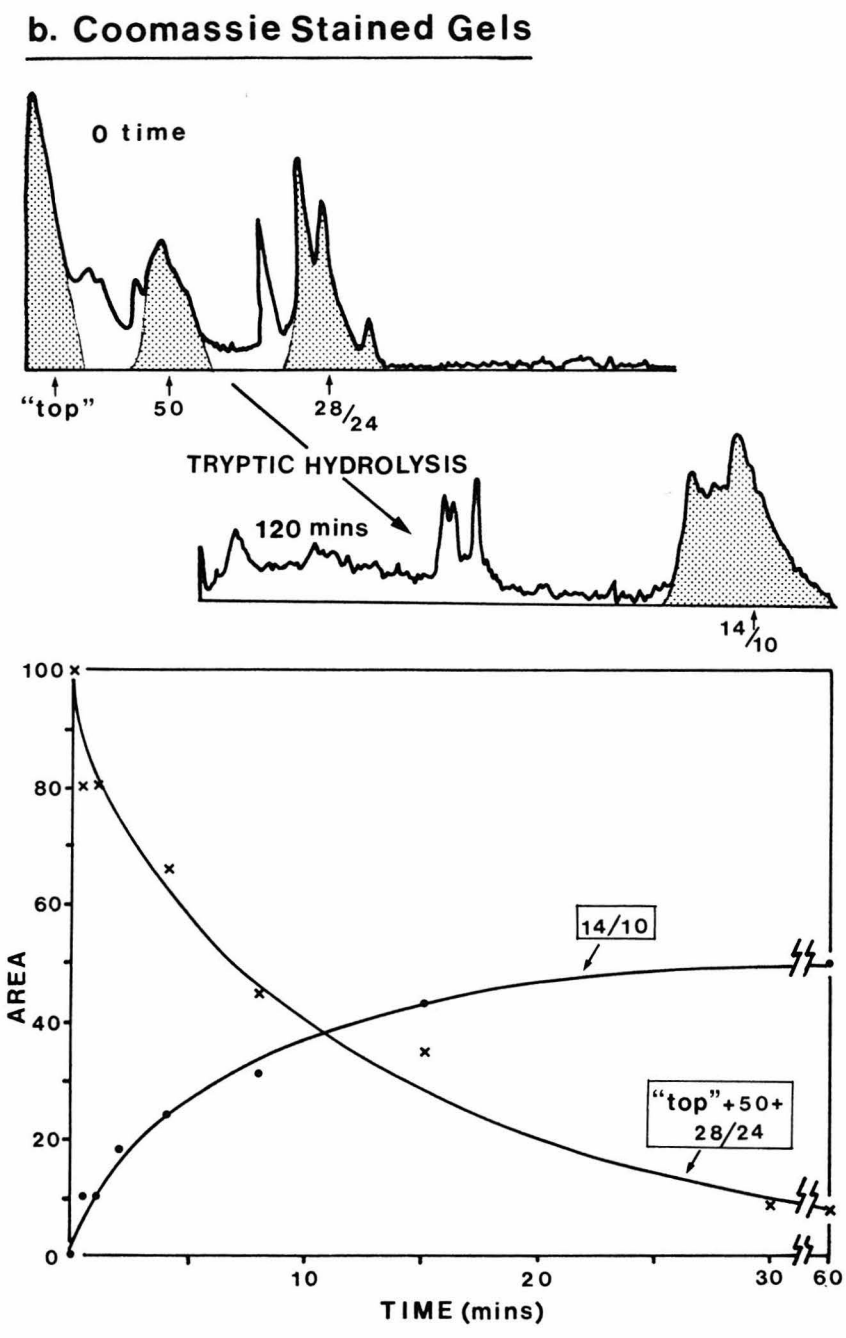

FIGURE 1. (cont.)

FIGURE 1.

(b) A similar analysis to that in (a) was performed using Coomassie staining and unlabeled junctional fractions. Hydrolysis conditions were the same, except that the concentration of junctional protein was 0.3 μg/μl and trypsin 0.003 μg/μl (hence the slower time course). Although the resolution of proteins by SDS PAGE was greater using Coomassie staining than with autoradiography (cf 0 time scans in (a) and (b)), the same peaks were used in determining the time course (areas shaded in 0 time scan for (x) time points, and areas shaded in 120 min scan for (0) time points) with the exception that the material at the top of the running gel was included as junctional in the (x) time points, and no M_r 20,000 dimers were detected after tryptic hydrolysis. In this analysis, 55% of the initial junctional protein was estimated to remain associated with gap junctions after tryptic hydrolysis (60 min time point).

The junctional nature of the polypeptides of the shaded peaks used in these analyses has been established using peptide "fingerprinting" (Chapter 2).

including it as junctional (Experimental estimate (6b) - Table 1; Figure 1B) leads to an underestimate of the yield of M_r 10,000 polypeptides, while omitting it from consideration (Experimental estimate (3) - Table 1; Figure 1A) could lead to an overestimate.

Several techniques have been used in the estimation of protein for this analysis. This was thought to be necessary since any given method suffers from inherent problems, largely stemming from the fact that each method relies on the labeling or reaction of specific functional groups which are unevenly distributed in a given polypeptide chain, or between different proteins. A consideration of each method can be found as an addendum to this appendix.

However, despite the variety of techniques used and the particular problems associated with each, it is clear from Table 1 that all results agreed to within ±15% of the value predicted for theoretical model (b). Therefore, it can be concluded that trypsin treatment of intact junctions digests the "native" M_r 28,000 protein to M_r 26,000 and 24,000 polypeptides, respectively, before ultimately cleaving the molecule into two fragments of M_r 10,000 (see Chapter 2, Figure 3 for analysis of such a time course by SDS PAGE).

Addendum

Specific inaccuracies associated with techniques for estimating protein.

1) Radioactive labeling: Proteins labeled in vitro, in this case with ^{125}I by the chloramine T method, will be labeled on only those residues reactive with, and accessible to the particular modifying reagent used. In the work described here, this would be those tyrosine residues (and possibly some phenylalanine and histidine residues) which are exposed to an aqueous environment.

2) Coomassie blue staining of proteins separated by SDS PAGE: The affinity of Coomassie for proteins is somewhat variable, especially in the case of glycoproteins. While the junction protein is apparently devoid of carbohydrate, it has yet to be

demonstrated that the M_r 10,000 hydrophobic "core" of the protein which survives proteolysis binds Coomassie with the same affinity as the native M_r 28,000 protein.

3) Amino acid analysis of proteins: Although more universally applicable than the other systems, some errors are introduced in this method by the selective destruction of some amino acids during hydrolysis (HCl hydrolysis completely destroys tryptophan and cysteine and partially destroys serine and threonine), the presence of a background of contaminating free amino acids, and the estimation of the percentage of the total sample loaded on the analyzer.

APPENDIX II

Analysis of the Content and Purity of Lens Fiber Junction Fractions

[A supplement to Chapter 4]

Two basic preparative techniques have been used for the isolation of lens junctions (see flow charts in Figures 7 and 8). A relatively crude plasma membrane fraction is obtained by repeated aqueous and 8 M urea washes in Protocol II (Takemoto et al., 1981). Protocol I (Dunia et al., 1974) involves a more rigorous plasma membrane isolation employing a sucrose density gradient, followed by detergent extraction and separation on a second sucrose gradient to specifically enrich for junctions. As for the liver (Chapter 1), two basic methods have been used to assess the purity of the final fractions—morphology and protein composition determined by SDS PAGE. The results, however, prove less straightforward than in liver.

Morphological assays of the final junction fractions from lens are subject to several obstacles above and beyond the more general ones discussed in Chapter 1. These problems all stem from a difficulty in defining what represents a lens junction. In negatively stained samples, "connexons" cannot be detected on most of the membrane sheets, although it is unclear how much of this is due to the genuine absence of junctional protein, and how much results from the poor staining characteristics of lens junctions. When "connexons" are detected, they have been found to occur in varying arrays (from random, to hexagonal (virtually identical to those seen in the liver) and even closely packed tetragonal) from one preparation to another (see Figure 1 in Chapter 4; also Peracchia and Peracchia, 1980a,b; Kistler and Bullivant, 1980a; Zampighi et al., 1982). In our hands we have been unable to determine the specific conditions responsible for this variability, although Peracchia and Peracchia (1980a,b) claim that it results from variations in H^+ or Ca^{++} concentrations. In any event, the various ordered connexon arrays, both hexagonal and tetragonal, would appear to be formed during the isolation protocol, since they have never been observed or induced in the intact lens fiber cells.

Analogous problems to those just described also arise in considering thin sections of lens junctional fractions. Even fractions isolated by Protocol I contain

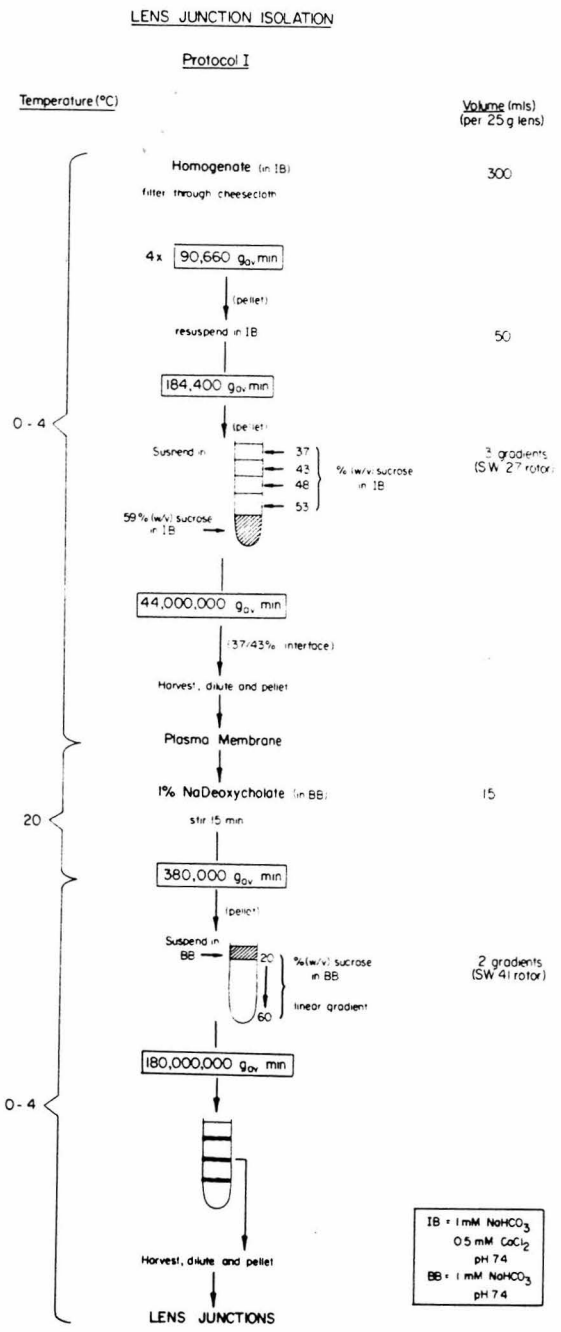

FIGURE 1. Protocol I for the isolation of rat lens fiber junctions based on that of I. Dunia, K. Sen, E. L. Benedetti, A. Zweers and H. Bloemendal, FEBS Lett., **45**, 139-144 (1974).

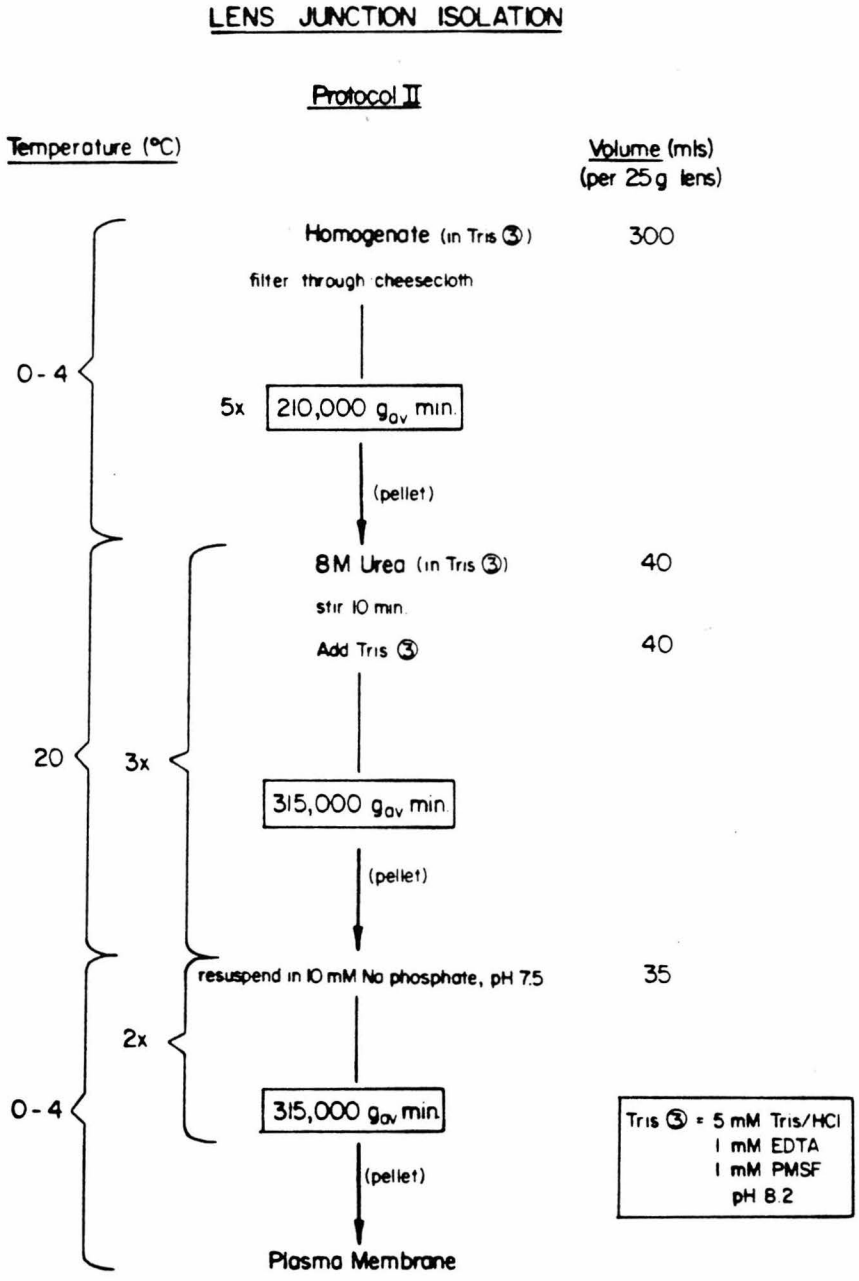

FIGURE 2. Protocol II for the isolaton of rat lens fiber junctions based on the isolation of urea washed plasma membranes in J. Alcalá, N. Lieska and H. Maisel, Exp. Eye Res. **21**, 581-595 (1975) as modified by L. J. Takemoto, J. S. Hansen and J. Horwitz, Comp. Biochem. Physiol. **68B**, 101-106 (1981).

a surprisingly large percentage of single membranes (~45%), although other workers have reported considerably lower levels using similar isolation protocols (Takemoto and Hansen, 1981; $\geq$95% double membrane profiles). In addition, the double membrane profiles, which for brevity will be referred to as "junctions" without any intended inference as to function, seem to form a heterogeneous population. In thin sections of liver gap junction fractions, the junctional profiles range from about 150 to 190 Å in width. "Junctions" with similar dimensions are seen in fractions from lens, although the gap between the two membranes is often less evident (compare Figures 1a and d in Chapter 4). However, much narrower "junctional" profiles, as thin as 100 Å, are detected in the fractions from lens. As yet it has proven impossible to determine whether the widths of the "junctional" profiles in our lens fiber junction fractions fall into two (or more) overlapping categories or if they are better described as a continuous distribution from 100 to 200 Å , skewed slightly towards the wider profiles.

In general, other authors have made no reference to a particularly wide distribution of "junctional" widths in the lens, referring only to junctions with similar or slightly narrower profiles than those of liver gap junctions (Alcala et al., 1975; Dunia et al., 1974; Takemoto and Hansen, 1981; Hertzberg et al., 1982). Recently, however, two reports have differentiated, on the basis of morphological (Zampighi et al., 1982) and immunological (Paul and Goodenough, 1983) criteria, two distinct classes of "junctions" in fractions from the lens—one rather typical of gap junctions elsewhere (termed "wide junctions" in the remainder of this discussion) and one considerably narrower ("narrow junctions"). However, even these authors differ as to the relative abundance of the structures, with Zampighi et al. (1982) claiming an overwhelming predominance of the "narrow junction" and Paul and Goodenough (1983) referring to this as a minor component, undetected in an earlier publication.

The diverse, and often conflicting descriptions of lens junctional fractions arising from different laboratories has made the determination of the interrelationship of these various "junctional" forms very difficult. Several possible explanations exist:

(1) The different structures observed in the fractions represent variations of a single junctional structure induced by the treatments used in the isolation, such as lipid extractions with detergents and protein denaturation in 8 M urea. Such a proposal is supported by the results of Peracchia and Peracchia (1980a,b) who observe changes in connexon arrays in response to fluctuations in Ca^{++} and H^{+} concentrations and by the observation that, irrespective of the junctional forms reported (square, hexagonal or disordered "connexon" arrays; "narrow" or "wide" junctional profiles), the major or only protein component is the M_r 26,000 MIP.

(2) Each structural variant reported represents a different structure or junctional type. Some support for this proposal stems from Kistler and Bullivant (1980b) who associated square arrays of "connexons" with a protein other than M_r 26,000, and claimed that they only existed on single membranes. However, conflicting reports have since been published by Zampighi et al. (1982) who associate the same arrays with double membrane profiles and a protein of M_r 27,000. A difficulty with this theory is that virtually none of the structural variants observed in isolated fractions have been demonstrated in vivo.

(3) The structures observed represent various stages of single membranes adhering to one another non-specifically following urea and/or detergent treatments. In the light of this possibility, the term "junction" should be used advisedly in this instance when referring to the extensive amounts of double membranes present in lens junction fractions.

Without a resolution of these issues, it is impossible to derive any real morphological assay of purity, especially in the light of variable results from different

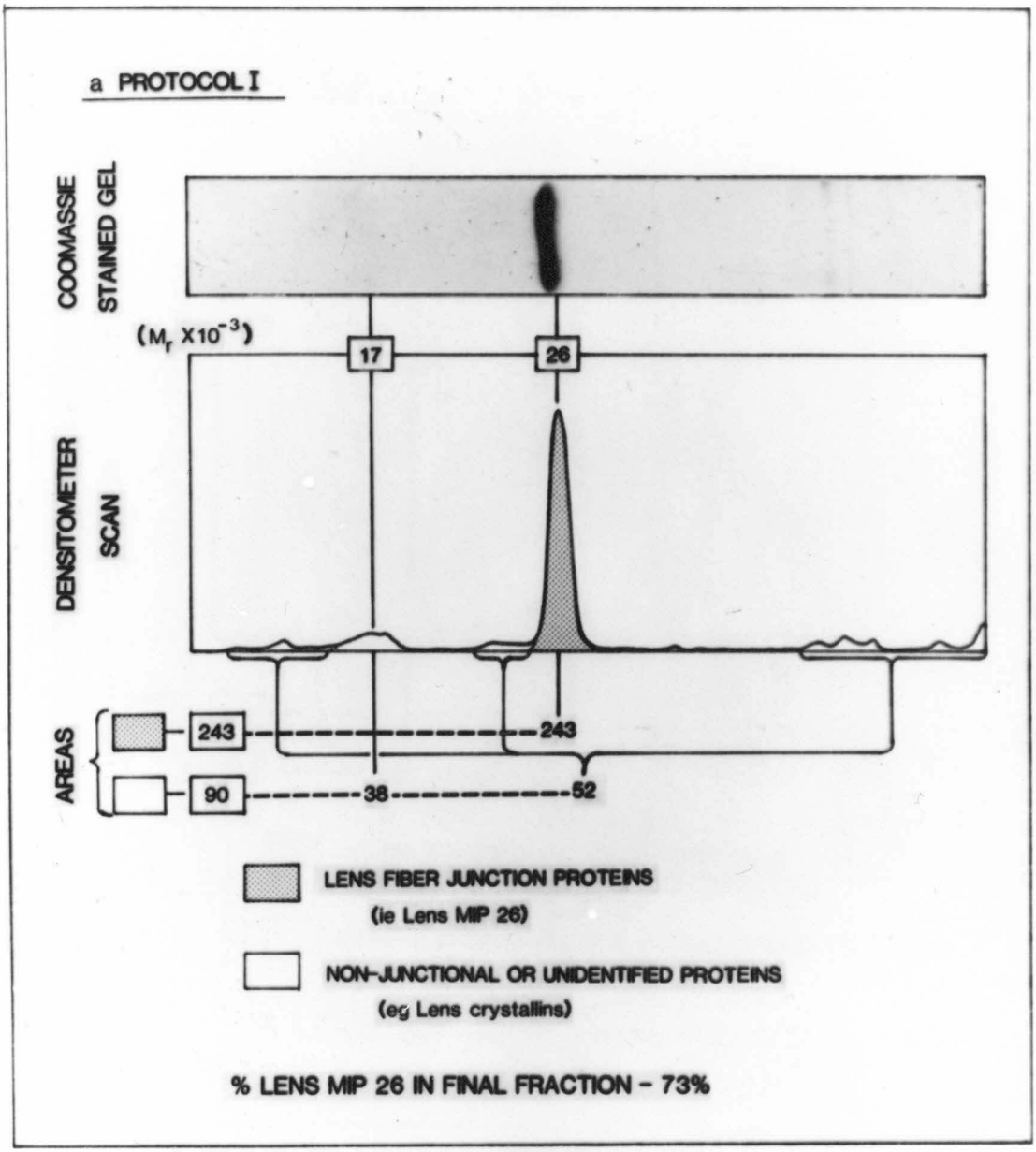

FIG. 3 a

FIG. 3a. Quantitative analysis of the protein content of a lens fiber junction fraction isolated by Protocol I. The protein components of the fraction were separated by SDS PAGE and stained with Coomassie blue. Areas under the peaks in a Joyce Löebel densitiometer scan of the stained gel were determined using a digitizing tablet (Tektronix No. 4956) interfaced to a minicomputer (Tektronix No. 5042).

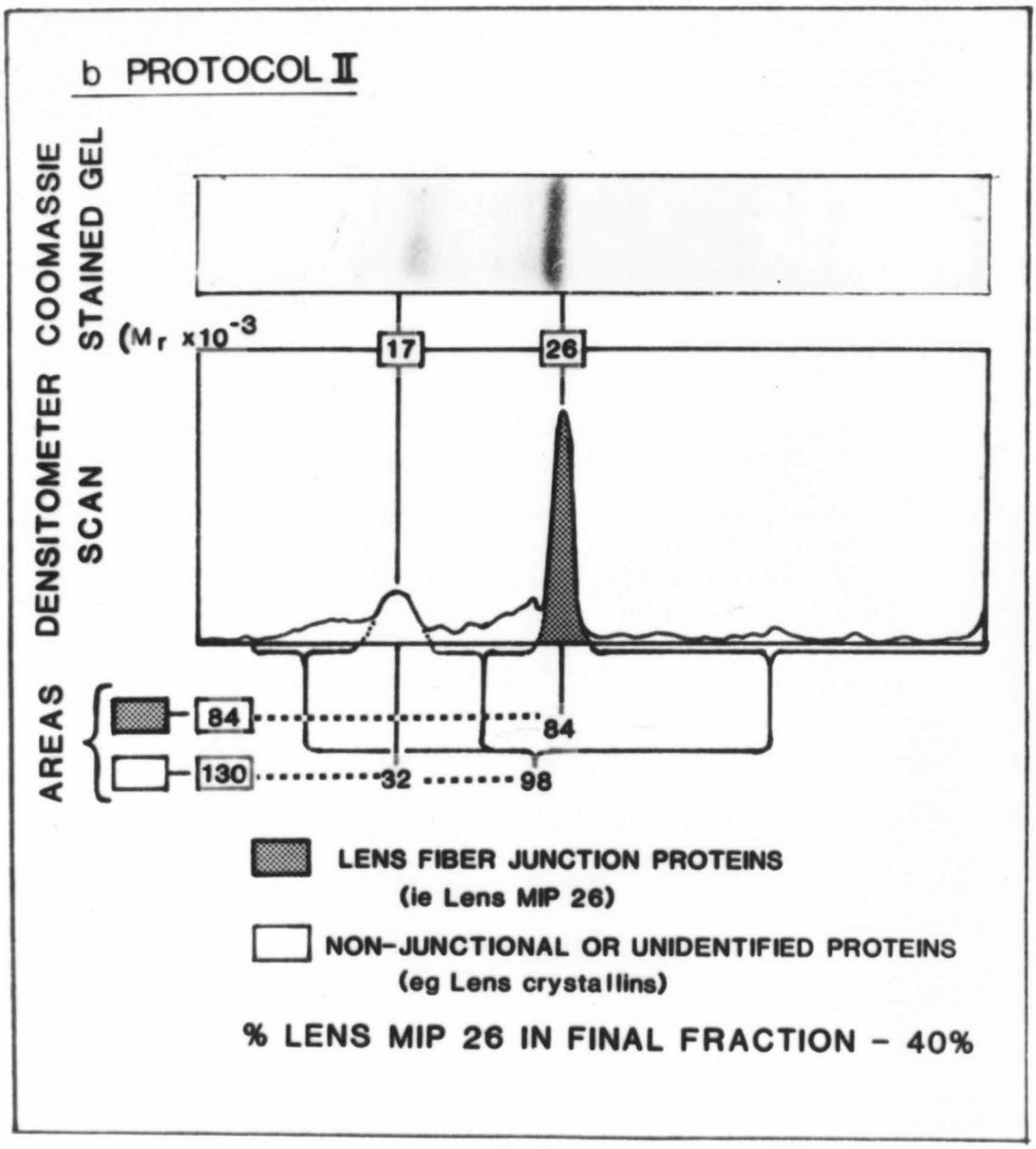

FIG. 3b

FIG. 3b. Quantitative analysis of the protein content of a lens fiber junction fraction isolated by Protocol II. The quantitation procedure was the same as in Figure 3a.

laboratories. Even should such a resolution be at hand, however, the question of whether or not the junctions isolated from lens represent gap junctions will remain, until some universal assay can be developed for gap junctions.

Biochemical assays of the lens fiber junction fractions seem, at first, much more straightforward than the morphological ones. As junctions become a higher and higher percentage of the isolate (i.e., percentage of double membranes seen in thin sections), enrichment is seen for the M_r 26,000 MIP of lens, until in the final fractions isolated by Protocol I, it represents 70-85% of the total protein (Figure 3a). Even in the relatively crude plasma membrane fractions from Protocol II, the M_r 26,000 protein represents 40-65% of the total protein (Figure 3b). Despite this co-enrichment for junctions and lens MIP, the percentage of double membrane profiles present in our final fractions ($\sim$ 55% determined from thin sections including both "wide" and "narrow" junctions) is much less than might be expected from the percentage of M_r 26,000 protein present. This could be explained by recent immunological experiments where antibodies to lens MIP bound to both double and single membranes (Bok et al., 1982), suggesting either that the single membranes represent "split" junctions or that lens MIP or its precursor is present in non-junctional membrane. However, conflicting results have now been reported by Paul and Goodenough (1983) who find that their antibodies to the lens MIP bind to single membranes, and to the "narrow junctions," but not to the "wide junctions" more characteristic of gap junctions in other tissues. Since the M_r 26,000 protein is the only protein detectable by SDS PAGE in their final fractions, and since the majority of their fractions appear to be comprised of "wide junctions" (Goodenough, 1979), there are a limited number of explanations for this observation:

(1) "Wide junctions" are mainly comprised of lipid. In this event, it would be surprising that they would be resistant to detergent treatments. In addition, junctional fractions comprised almost solely of "wide junctions" have been reported to contain large amounts of M_r 26,000 protein (Takemoto and Hansen, 1981).

(2) "Wide junctions" are comprised of a protein which remains undetected on SDS PAGE. If this were true, it would represent a unique case, since we have failed to detect other major protein components in gels of lens fractions (isolated by Protocol I) by Coomassie and silver staining, and autoradiography when the sample was first iodinated.

(3) "Wide junctions" contain the M_r 26,000 protein, but it is present in a different conformation to that in single membranes and cannot be recognized by the antisera.

In the face of this profusion of confusing results, it is difficult to draw any absolute conclusions. In spite of the recent immunological results, circumstantial evidence would suggest that our isolation protocols (and apparently those of others) prepare mostly lens fiber junctions (slightly narrower in profile than the gap junctions of liver), which are comprised of M_r 26,000 protein. Determination of whether these represent a lens specific gap junction, some other junction, or even an artifact created by urea and detergent treatments of lens membranes must await more definitive evidence such as the presence of homologies between other established junctional proteins and the lens MIP.

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