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The Dynamic State of Liver Gap Junctions

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By the use of a simple, rapid method for the isolation of gap junctions from small amounts of rat liver (2-3 g), we have followed the incorporation of the radiolabeled amino acid precursors ^{3}H-leucine and ^{35}S-methionine into the gap junction protein. In timed studies with ^{35}S-methionine as precursor, the specific activity in the protein is maximal by 4 h after a single injection of 300 μCi/100 g body weight. From the decay in the specific activity with time after a single injection, the gap junction protein has an apparent half-life of about 19 h. Because of problems of reutilization of radiolabeled amino acid with ^{35}S-methionine as precursor, this apparent half-life probably overestimates the true half-life and indicates a surprisingly rapid turnover of the gap junction protein. This short half-life suggests that, in rat liver, the gap junctions may be very responsive to alterations in physiological demands.

Key words: gap junctions, protein turnover, junction synthesis, junction degradation, liver, regenerating liver

The transmembrane channels by which cells are ionically and metabolically coupled to each other are believed to form the membrane assemblies we recognize morphologically as gap junction [see Peracchia, reference 1, for recent review]. In excitable tissues, the channels are the route for transmission of electrical signals. Conceivably, in nonexcitable tissues, this pathway of communication provides not only the mechanisms whereby cells may share a common pool of metabolites and present a coordinated response to stimuli but also a mechanism for the regulation of cellular differentiation and growth as the passage of small signal molecules is either restricted or allowed [2-5]. Numerous observations have shown that changes in junctional communication may be at least temporally correlated with morphogenetic events and alterations in patterns of growth [2,4,5].

Gap junctions between cells in adult tissues such as the liver have been believed to be stable structures [see discussion by Gilula, reference 6]. Morphologically, in the normal rat liver, they appear to occupy a quantitatively constant area, on average about 3% of the contact area between hepatocytes [7,8]. Early biochemical studies by Gurd and Evans [9] also seemed to indicate that the gap junctions in liver are relatively stable. In double-labeling experiments, they observed a slow rate of turnover of the proteins in partially purified gap junction fractions isolated from the plasma membrane of mouse liver.

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A reappraisal of this concept of junctional stability is now appropriate. Although the complement of gap junctions in normal liver appears to be unchanging, in regenerating liver there is a rapid, large-scale disappearance of gap junctions occurring as part of the early response to the strong stimulus for growth initiated by partial hepatectomy [7,8]. We have been studying this phenomenon in a variety of ways to determine the underlying physiological and biochemical events leading to and associated with this disappearance, as well as the subsequent reappearance of the gap junctions [8,10–12]. A question that arises is whether this disappearance could result from normal degradation of gap junction protein subsequent to a block in synthesis.

Recent reports have enlarged our understanding of the biochemical organization of the gap junctions in the liver. Several studies have identified a major protein component of gap junction fractions with an M_r in the range of 26,000 to 28,000 daltons [11,13–15]. It now appears that the subunits that form the junctional channels [16,17] are, in rat liver, composed of a single major protein with an M_r, in our hands, of 28,000 daltons [18]. This protein is subject both to aggregation and to proteolytic cleavage, and most of the bands seen after SDS-PAGE analysis of these fractions can be ascribed to these two factors [11,14,18]. Highly purified junctional fractions isolated by procedures that employ proteolytic enzymes are characterized by polypeptides of about 10,000 daltons, that have been given the name "connexin" by Goodenough [19,20]. In controlled proteolysis experiments on gap junction fractions isolated under conditions that minimize proteolysis [11,18]("native" junctions), about 70% of the mass of the junctional proteins present is recovered in the 10,000 dalton band after exhaustive treatment with trypsin. This, along with peptide mapping and sequencing studies [18], suggests that the 10,000 dalton component comprises two polypeptides that represent two-thirds of the original protein.

In the present study, in order to obtain an estimate of the half-life of the gap junctional protein, we have first devised a simple, rapid procedure in which proteolytic enzymes are used to isolate gap junction fractions. We have then followed the incorporation of radioactive label from amino acid precursor into newly synthesized gap junctional protein and the degradation of this labeled protein by monitoring the specific radioactivity in the 10,000 dalton component of the fraction. The apparent half-life we have obtained with ^{35}S-methionine suggests that the gap junction protein has a suprisingly rapid turnover.

MATERIALS AND METHODS

Isolation of Gap Junctional Fractions

Rats (Sprague-Dawley, Simonsen Laboratories, 200–220 g) were killed by cervical dislocation and the livers rapidly removed. Each liver was handled individually throughout the isolation procedure, which was designed to minimize the losses and number of manipulations (Fig. 1). Livers were placed in 200 ml ice-cold (4°C) isolation buffer (1 mM $NaHCO_3$/0.5 mM $CaCl_2$,pH 7.4) in a 250 ml centrifuge bottle and homogenized with a Tissuemizer (Janke and Kunkel, Keka-Werk, Ultra-Turrax) at maximum speed for 6 sec. Homogenates were allowed to stay on ice for 15 min, then sucrose was added to yield a density of 1.16. Homogenates were centrifuged for 5 min at 1,500 rpm (HG-41 rotor, Sorvall RC 3 centrifuge). The pellet, consisting of unbroken cells, nuclei, connective tissue, and coagulated

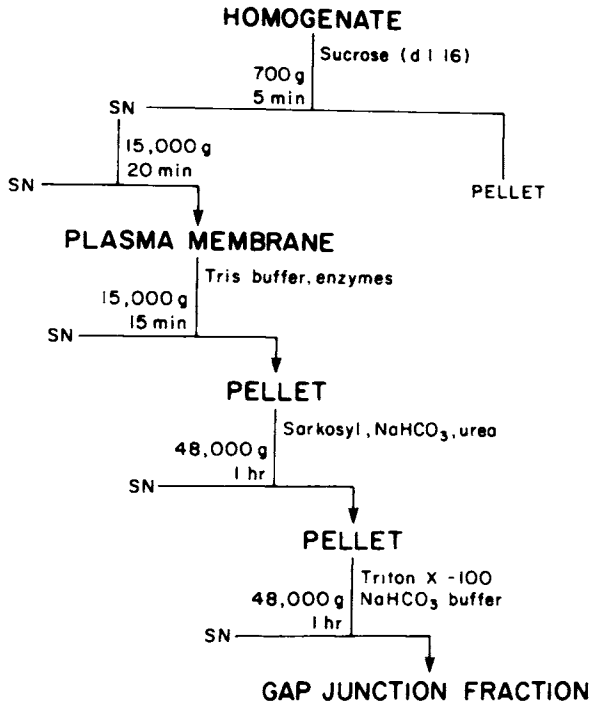

Fig. 1. Flow chart of the preparation technique devised to isolate a gap junction fraction from small amounts of rat liver.

nucleoprotein, was discarded. The supernatant was centrifuged for 20 min at 10,000 rpm (JA-14 rotor, Beckman J-21) to yield a crude plasma membrane fraction. The crude plasma membrane was treated with collagenase (Worthington Type IV) in 40 ml of enzyme buffer (50 mM Tris HCl/5 mM $CaCl_2$/5 mM $MgCl_2$,pH 7.4) at a concentration of 20 mg/250 ml. After 15 min at 37°C with constant stirring, 1 mg of trypsin (Sigma, Type XI) was added for a further 15-min incubation. The digested plasma membrane fraction was diluted to 200 ml with ice-cold isolation buffer and centrifuged at 10,000 rpm for 15 min (JA-14 rotor). The pellet from this centrifugation was treated at room temperature with a solution containing 0.55% sarkosyl, 2 mM $NaHCO_3$, and 10% urea with constant stirring for 15 min. The sarkosyl/urea insoluble material was pelleted by centrifugation at 20,000 rpm for 1 h (JA-20 rotor). The pellet and centrifuge tube were rinsed gently with 5% Triton X-100 in Tris acetate buffer, pH 7.4, to remove lipid contaminants. The pellet was then resuspended in 1 mM $NaHCO_3$ pH 7.4, and centrifuged at 20,000 rpm for 1 h (TY 65 rotor, Beckman L3-50) to yield a gap junction fraction.

Analysis by SDS-Polyacrylamide Gel Electrophoresis

The gap junction fractions were solubilized at room temperature for 30 min in a solution of 2% SDS and 5% mercaptoethanol. Equal aliquots of each sample were loaded onto a 15% polyacrylamide microslab [21,22] , 1.0 mm thick and 6 cm long. Standards were the BioRad low molecular weight series or our own mix-

ture of cytochrome c and RNAase. The gels were stained with 0.025% Coomassie blue in 25% isopropyl alcohol and 10% acetic acid.

Peptide Mapping

The method of Elder et al [23] as modified by Takemoto et al [24] was used to compare the 10,000 dalton polypeptides of our gap junction fraction with the gap junction polypeptides present in highly purified junction fractions [18] and with the 10,000 dalton gap junction polypeptides generated by in vitro trypsinization of the 28,000 dalton gap junction protein.

The fractions were solubilized in SDS and labeled with ^{125}I by the chloramine T method [25]. The free iodine and components of the fraction were separated on 15% polyacrylamide slab gels, 0.75 mm thick by 12 cm long [22]. After staining with 0.25% Coomassie blue (in 50% methanol and 7% acetic acid), the gels were destained (20% methanol, 7% acetic acid), washed (10% methanol), dried, and packaged for autoradiography with Kodak X-Omat R film. Bands, identified by autoradiography or Coomassie staining, were cut from the dried gels and digested at 37°C overnight either with chymotrypsin (Worthington) or trypsin (Sigma Type XI) at 0.05 mg/ml 50 mM NH_4HCO_3 (pH 7.8). Supernatants were lyophilized and the samples analyzed on cellulose-coated TLC plates (Eastman number 13255) as described by Elder et al [23]. The plates were analyzed by autoradiography with Kodak X-Omat R film with a Dupont Cronex "lightning plus" intensifying screen, exposed at −70°C.

Isotope Administration and Estimation of the Half-Life

All rats received a single intraperitoneal injection of 300 μCi/100 g body weight of either ^{3}H-leucine (130 Ci/mmole, New England Nuclear) or ^{35}S-methionine (>800 Ci/mmole, New England Nuclear). For experiments with leucine, rats were injected 4 h before sacrifice and isolation of gap junctions . To determine the time of maximal incorporation of methionine, rats were injected either 1, 3, 4, 5, 6, or 7 h before sacrifice. For estimation of the half-life of the gap junction protein, ^{35}S-methionine was injected 4.5, 24, 48, and 72 h prior to sacrifice. In two experiments cold methionine was injected either in a dose of 10 mg per rat beginning 4 h after the injection of ^{35}S-methionine and then twice daily throughout the experiment or in a dose of 100 μg per rat beginning 2 h after the injection of ^{35}S-methionine and then twice daily.

For measurement of radioactive label in the gap junction protein, the polyacrylamide gels were either treated for autoradiography with En^{3}hance (New England Nuclear), dried, and exposed to preflashed [26] Kodak X-Omat R film at −70°C or serially sliced for scintillation counting into 0.25 cm segments so that a single slice contained the entire band at 10,000 daltons and no other bands. For scintillation counting, the gel slices were incubated in 3% Protosol in Econofluor (New England Nuclear) with constant shaking overnight at 37°C, then counted for two 10-min cycles on a Beckman LS-233 scintillation counter. In order to calculate a relative specific radioactivity for the gap junction protein at the different time points of the experiments, the counts per min above background in the 10,000 dalton band were related to the intensity of the Coomassie staining in the band measured by scanning with a Joyce-Löebl densitometer. The area under the peak in the scan was intergrated by the use of a digitizing tablet interfaced to a

Tektronix computer. The amount of protein in the band was roughly estimated by relating the area under the peak at 10,000 daltons with that under the peak measured for the cytochrome c standard.

Each decay curve was derived from gap junction fractions isolated simultaneously and analyzed on the same slab gel. The minor bands seen on the gels were variable in presence or intensity from preparation to preparation and usually were not labeled significantly above background; therefore no attempt was made to calculate a specific activity for any but the major 10,000 dalton band.

The apparent half-life was calculated from the decay of the protein-bound radioativity in the 10,000 dalton gap junction polypeptide using the following equations:

$$A(t) = A(0)e^{-kt}$$

where A(t) = the specific radioactivity at time (t), A(0) = the original specific activity, and k = the rate constant of decay. The half-life was then calculated from the equation:

$$t_{1/2} = \frac{\ln 2}{k}$$

RESULTS

Analysis of the Gap Junction Fraction

In the isolation protocol outlined in Figure 1, we have minimized the number of transfers of material and other manipulations to simplify handling of the radiolabeled preparation. By this method we are able to isolate gap junctions from as little as 2–3 g of liver so that it is unnecessary to pool the livers from large numbers of rats in order to detect incorporation of radiolabel into the newly synthesized gap junction protein. The yield in gap junction protein is of the order of 0.5–1 μg for 3 g of liver, estimated relative to several of the BioRad standards or either cytochrome c or RNAase. This is of the same order of magnitude as the yield of the best techniques evolved so far. Analysis by SDS-PAGE of the gap junction fraction (Fig. 2) shows a major band at 10,000 daltons. This band comigrates with (not shown) the 10,000 dalton gap junction polypeptides present in the highly purified fractions isolated by the longer, more complex procedures described by Finbow and associates [11]. To establish that this 10,000 dalton component found in our simplified procedure represents gap junction protein, we have compared its peptide map with those of known gap junctional polypeptides [18], including the 10,000 dalton gap junctional polypeptides produced by exhaustive trypsinization of "native" fractions (Fig. 3). The peptide maps from these two 10,000 dalton components are virtually identical. We have also shown clear homologies between the peptide maps of the 10,000 dalton components of our preparation and those of the 28,000 dalton gap junction protein [18,27].

Incorporation of Radiolabeled Amino Acid

Either ^{3}H-leucine or ^{35}S-methionine is incorporated significantly into the gap junction protein by 4 h after a single injection of 300 μCi/100 g body weight

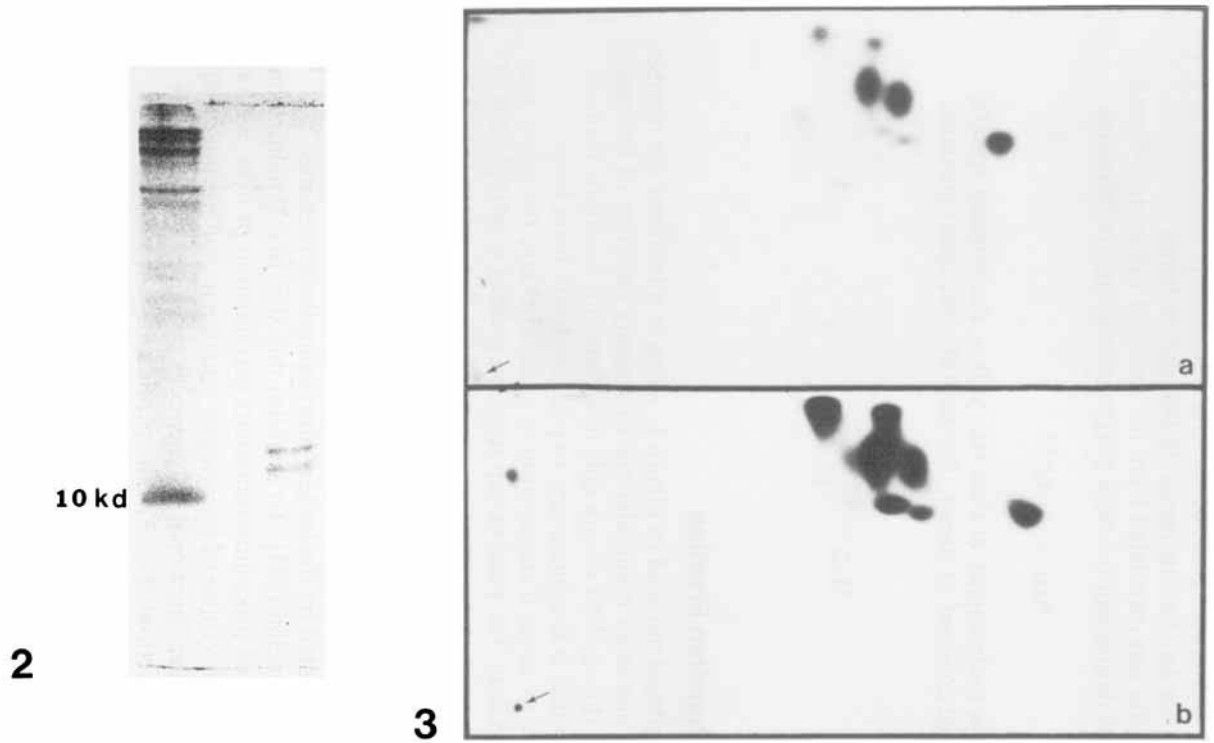

Fig. 2. Analysis of the gap junction fraction by SDS-PAGE. A major band is seen at 10,000 daltons. Standards are cytochrome c (mol wt 12,000) and RNAase (mol wt 14,000).

Fig. 3. a. Peptide map of the chymotryptic, iodinated peptides of the 10,000 dalton component of the gap junction fraction isolated by the procedure outlined in Figure 1. b. Peptide map of the chymotryptic iodinated peptides of the 10,000 dalton gap junction polypeptides obtained by enzyme treatment of intact gap junctions. Comparison of a with b shows that the material we have examined for turnover is authentic gap junction polypeptides. The peptide maps obtained after tryptic digestion (not shown) also support this conclusion. Electrophoresis was in the horizontal dimension and chromatography in the vertical dimension. Arrows indicate the origin.

(Table I). The label is detectable above background in less than 1 μg of gap junction polypeptides analyzed on microslabs. The specific activity in the polypeptides was much greater with ^{35}S-methionine as precursor than with ^{3}H-leucine (Table I). In timed experiments with ^{35}S-methionine, the specific activity was maximal by 4 h as measured either by autoradiography or scintillation counting of the bands cut from gels (Fig. 4). In contrast, the radioactivity in the free amino acids isolated from the liver (data not shown) remains high for as long as 2 days, after which it begins to show a rapid decay.

Estimation of the Half-Life of the Gap Junctional Protein

In four experiments with ^{35}S-methionine as precursor, the decay in specific activity in the gap junctional polypeptides was followed with time after a single injection. The average apparent half-life obtained from these experiments (results of two shown in Fig. 5) is 19 hours. The range in half-life over the four experiments was 16.8 to 21 h (Table II). Administration of nonradioactive methionine after injection of the ^{35}S-methionine in two of the experiments (Exp 3 and 4) as described in Materials and Methods did not result in any detectable shortening of the apparent half-life.

DISCUSSION

By following the decay of radioactive label in the 10,000 dalton gap junctional peptides with time after a single injection of ^{35}S-methionine, we have measured an apparent half-life of the gap junction protein from rat liver of approximately 19 h. The implication from this result is that liver gap junctions are labile, dynamic structures. The gap junctions seen in electron micrographs of the normal rat liver represent structures in flux that seem to be stable because they are in a steady state.

As is also true of other reported procedures for the isolation of gap junctions [11,14,15], our yield in gap junction protein represents only a small percentage of the total one would estimate to be present in the membrane on the basis of the morphological data [7,8]. This small yield would become an important consideration for our study only if there were to be more than one pathway for the biosynthesis, assembly, and degradation of the protein of which the junctions are composed. Under these conditions, the junctions we isolate might not be representative. There is no compelling evidence for the existence of such multiple pathways; however, if this were indeed the case, we would have been measuring the turnover of protein in a particular class of gap junctions that we selectively isolate.

The apparent half-life we have measured with ^{35}S-methionine probably overestimates the true half-life by a factor of 2 or more because of problems of reutil-

TABLE I. Incorporation of Labeled Amino Acid Into Gap Junction Band 4 h After Injection

Amino acid	cpm/μg Protein
^{3}H-leucine 300 μCi/100g	105
^{35}S-methionine 300 μCi/100 g	1300

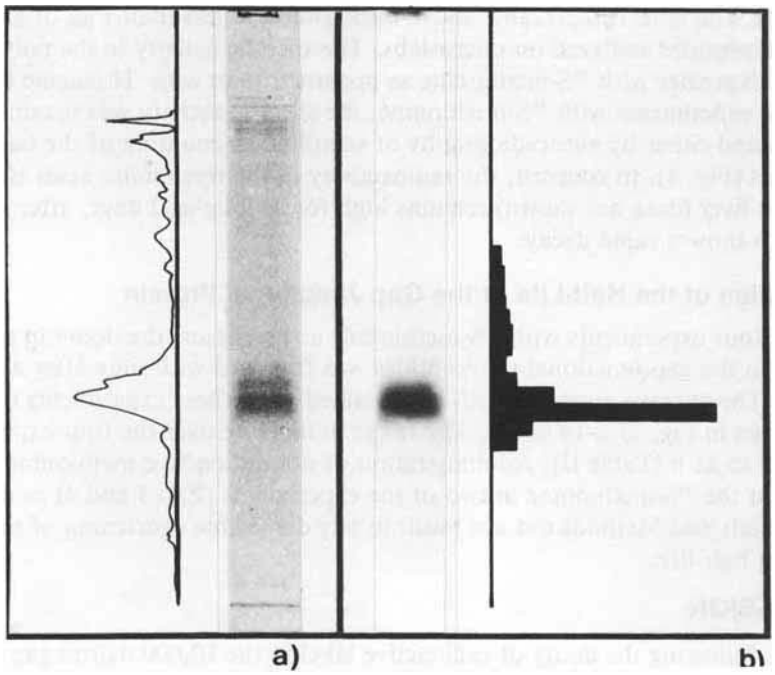

Fig. 4. Typical time point taken 4.5 h after a single injection of ^{35}S-methionine. a)Analysis of the fraction by SDS-PAGE and densitometer scan of the same Coomassie stained gel. The gel shows a main component at 10,000 daltons. The area under the peak at 10,000 daltons in the scan was integrated to estimate the amount of protein in the 10,000 dalton band. b) Autoradiography of the gel and radioactivity in corresponding slices. Autoradiography of the gel shows high specific activity in the 10,000 dalton gap junction polypeptides, and the distribution of counts per minute above background in the gel slices measured by scintillation counting shows that there is a corresponding high peak of radioactivity (720 cpm). The specific activity was calculated from the ratio of the counts per minute to the μg of protein in the 10,000 dalton band. Although it is not present in all cases, the minor 12,000 dalton component seen in this gel (migrating just above the 10,000 dalton band) and the major 10,000 dalton component share the same peptide maps. This minor component is believed to result from incomplete digestion of the gap junctions by trypsin. Radioactivity is essentially the same whether the contribution of the minor component is present in both bands in proportion to the amount of protein, and the relative specific activity is omitted from or included in the calculation.

ization of radioactive amino acid when ^{35}S-methionine is used as the precursor. As proteins that have initially been labeled are degraded, radioactive free amino acids are generated that may then be reutilized in the synthesis of new protein [28,29]. The failure of our attempts to chase radioactive precursor by injections of non-radioactive methionine could have been expected considering the early reports by Loftfield and Harris [30], which indicated that injected unlabeled amino acid may only partially intermix with and, therefore, fail to "wash out" the intracellular pools of radioactive amino acids derived by breakdown of labeled protein. It had already been shown by others [31–33] that reutilization of radioactive amino acid occurred in spite of the administration of large amounts of unlabeled precursor. The problems encountered with this kind of approach have recently been thoroughly discussed by Goldberg and Dice [34]. The extent to which reutilization affects estimates of the turnover can be judged by the fact that the average half-

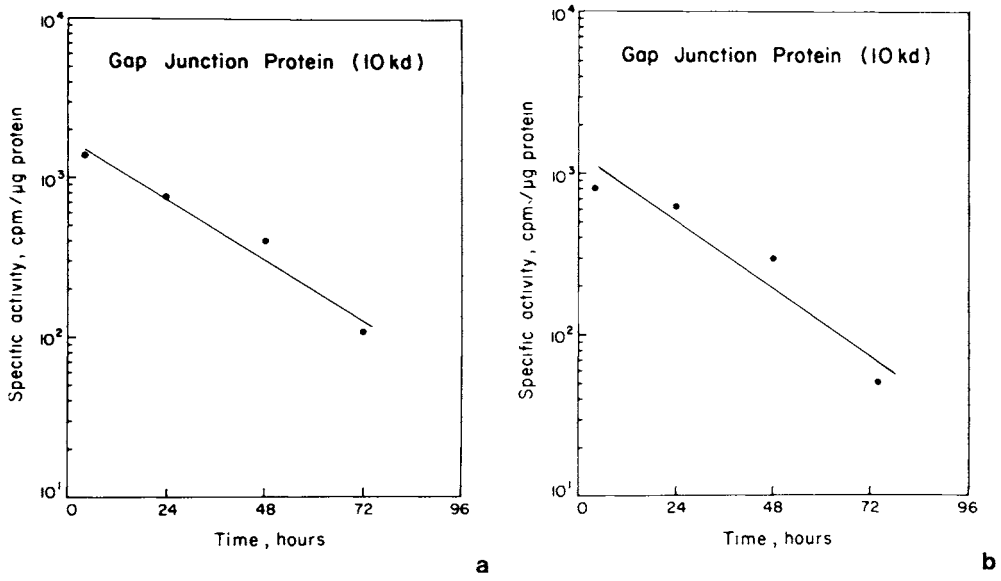

Fig. 5. Decay of specific activity in the 10,000 dalton gap junctional polypeptides after a single injection of ^{35}S-methionine at 300 μCi/100 g body weight. Each point represents the datum from a single rat liver. Counts per minute were determined by scintillation counting of the 10,000 dalton bands cut from gels. The amount of protein in the bands was estimated by measurement of the areas under the peaks obtained by densitometer scans of the Coomassie stained gels on a Joyce-Löebl densitometer. The lines of a and b were drawn by the least-squares method from the determined specific activity points assuming first-order kinetics. Correlation coefficients are, respectively, 0.98 and 0.94. In the example in a, showing the results of a cold chase experiment, the calculated half-life is 19 h. In b, an experiment without cold chase, the calculated half-life is 16.8 h. The average half-life obtained from 4 experiments is 19 h.

TABLE II. Apparent Half-Life of the Gap Junction Protein After a Single Injection of ^{35}S-Methionine

Experiment	Half-life (h)
1	19.6
2	16.8
3	19.0
4	21.0

life of liver cell plasma membrane proteins measured by Tauber and Reutter [35] using ^{35}S-methionine as precursor is of the order of 3.6 days as opposed to the average half-life of 1.7 days measured with ^{14}C-carbonate where reutilization is minimal. ^{35}S-methionine nevertheless has been the most appropriate amino acid for our studies because its high specific activity allows the detection of incorporation of radioactive label in less than 1 μg of junctional protein.

As just mentioned, the average half-life of liver cell plasma membrane proteins using ^{35}S-methionine as precursor is 3.6 days [35]. Other estimates of the mean half-life of the total proteins of the plasma membrane of rat liver range from 30 to 60 h or longer [see Schimke, reference 36, for review]. Thus an apparent half-life of 19 h indicates that the gap junction protein has a compara-

tively rapid turnover. This is also supported by our finding that incorporation of the precursor is maximal by as early as 4 h. In light of our results, it is interesting that Dahl and his associates [37] have recently shown that the communication-deficient Cl-1D mouse cells exhibit electrical coupling within 2–4 h after being loaded with mRNA from rat myometria stimulated to produce gap junctions and that the coupling persists for about 24 h. If this were the result of the introduction of mRNA for gap junctions, rather than coding for another step in junction assembly, it would suggest a similar turnover time for the junction protein.

The rapid turnover of the junction protein implies not only an active synthesis but also swift degradation. The mechanism for degradation of the gap junction protein in rat liver is not known. There is evidence that some other membrane proteins [38] and gap junctions from some other tissues [39] are removed from the cell surface by internalization as whole structural units and then degraded in lysosomes. We have looked for indications that such a process occurs in the rat liver and found none. Our morphological studies [8], however, do not preclude the possibility that the junctions break up into small aggregates or single channels that are then removed from the membrane. The relatively short half-life in comparison with other membrane proteins suggests that the gap junction protein is removed from the membrane independently of other membrane proteins and that it is independently synthesized and inserted into the membrane.

A priori, the rapid rate of turnover of the gap junction protein would appear to be highly "wasteful." Gap junctions are unique among transmembrane channels in that their formation requires the participation of two cells. According to present concepts, the gap junction proteins are assembled in hexameric form (connexons) in the plasma membrane and somehow recognize and align with identical assemblies in the membrane of an adjacent cell. By the end-to-end apposition of the connexons in the adjacent membranes a continuous channel is formed, spanning the extracellular space and the membranes to connect the cytoplasms of the two cells. Considering the complexity of the process, it would appear to be in the interest of cellular economy if the channels, once formed, were removed only slowly from the membrane. However, there could be significant advantages inherent in a rapid rate of turnover for the gap junction protein. The more rapidly a protein is degraded, the more precisely may its concentration be regulated by changes in synthetic rates and the faster may be the response to alterations in physiological demands. A short half-life implies that a block in synthesis of the gap junction protein would result in a rapid disappearance of the junctions, presumably with consequent effects on the cell-to-cell exchange of biologically significant molecules and ions. Rapid turnover could thus provide a mechanism for the regulation of intercellular communication in addition to the mechanism of uncoupling by closure of the channels, as is thought to occur in other cell systems [1,5,40]. This additional, longer term mechanism for regulation could be of particular importance during cellular differentiation and growth. It would be attractive to imagine that the dissappearance of the gap junctions from the regenerating liver could be accounted for by a block in synthesis of the protein followed by normal degradation. However, the situation appears to be more complex than this simple explanation would allow. Our estimates of the rate of junctional degradation, although they indicate a suprisingly rapid turnover, cannot readily account for the rate of disappearance of gap junctions determined morphologically since the

major loss of the gap junctions occurs over only a 4 to 6 h period [7,8]. This does not preclude the possibility, however, that this mechanism plays a role in other events during development and growth when changes in junctional communication are required and when closure of the channels would be either energetically or otherwise metabolically unfavorable.

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NOTE ADDED IN PROOF

The present data were published in abstract form in the J Cell Biol 87:212a, 1980, and also in the J Supramol Struct Cell Biochem Suppl 5:312, 1981, and while in press have been confirmed in mouse liver by Fallon and Goodenough (J Cell Biol 90:521, 1981).