Turnover of the Plasma Membrane Proteins of Hepatoma Tissue Culture Cells*

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The turnover of the plasma membrane proteins of hepatoma tissue culture cells was examined by three different methods—loss of polypeptides labeled in situ by lactoperoxidase-catalyzed iodination, loss of membrane polypeptides labeled with amino acid precursors, and loss from the membrane of fucose-labeled polypeptides. In both logarithmically growing and density-inhibited cells the proteins of the membrane are degraded with a half-life of about 100 hours. This is longer than the half-life of total cell protein, 50 to 60 hours, and longer than the doubling time of the cells, about 30 hours. Similar values for the rate of degradation of the membrane proteins were obtained by each of the three techniques. The same fucose-labeled polypeptides were present in the microsomal and the plasma membrane fractions of hepatoma tissue culture cells as analyzed by electrophoresis in dodecyl sulfate-acrylamide gels. But the fucose-labeled polypeptides were lost from the microsomal fraction at a faster rate than from the plasma membrane.

 Autoradiographic and double labeling techniques using 125I and 131I, or [3H]leucine and [14C]leucine were used to measure the relative rates of degradation of the proteins in the plasma membrane. All of the leucine-labeled polypeptides and the iodinated polypeptides had similar rates of degradation. These results support a model for the biogenesis of the plasma membrane in which the proteins are incorporated and removed in large structural units.

In mammalian cells essentially all proteins are undergoing turnover (1-3). That is, they are continually being degraded and replaced by new synthesis. There is a marked heterogeneity in degradation rate and a pronounced correlation between degradation rate and molecular size of the polypeptides in the soluble fraction of rat liver (4-6). Further, heterogeneity in degradation rate and a correlation between subunit size and relative rate of degradation have been observed for the proteins of many subcellular organelles (7), including the plasma membrane (8-11). These latter results suggest a model of membrane organization in which polypeptides associate and dissociate from the lipid bilayer with the dissociated form subject to degradation in another compartment of the cell.

However, studies of membrane turnover in intact animals are complicated by the difficulty in obtaining highly purified preparations of plasma membrane. Any contamination of this fraction by proteins from other cell fractions may mask the authentic turnover rates of the plasma membrane proteins. To avoid this problem, we have examined the turnover of the proteins of the plasma membrane of a hepatoma cell in culture, using lactoperoxidase-catalyzed iodination (12, 13) to specifically label the proteins of the membrane (14), and a double iodination technique (15, 16) to measure their rates of degradation. The degradation of the iodinated proteins then was compared to the degradation of total plasma membrane protein labeled with leucine, and membrane glycoproteins labeled with fucose. The results suggest that the major proteins of the plasma membrane of HTC cells are degraded as a large structural unit.

MATERIALS AND METHODS

Most of the chemicals used were reagent grade from commercial suppliers. Acrylamide and N,N'-dimethylacrylamide were obtained from Eastman. Sodium dodecyl sulfate dithiothreitol, and unlabeled amino acids were obtained from Sigma. 125I and 131I as sodium salts were obtained from Amersham Searle and were carrier free. L-[3,5-3H]-Tyrosine (42 Ci/mmol), l-[U-14C]tyrosine (460 mCi/mmol), L-[4,5-3H]-leucine (46 Ci/mmol), and L-[U-14C]leucine (312 mCi/mmol) were obtained from Schwartz/Mann. L-[6-3H]fucose (12.07 Ci/mmol), L-[1-14C]fucose (18.66 mCi/mmol), and Protosol were obtained from New England Nuclear.

Cell Culture—A cloned line of HTC cells originally derived from a rat hepatoma, was provided by Dr. T. D. Gelehrter (University of Michigan), and was grown in Eagle's minimal essential medium modified as described (14). Growth medium contained 5% fetal calf and 5% calf serum. Cells were maintained in spinner culture, and were used as such in all experiments.

Iodination—Cells were iodinated as described previously (14). Briefly, 106 washed cells were suspended in 1.2 ml of Earle's salt solution supplemented with triene (0.1 M, pH 7.6), glucose (7 mM), 60 milliliters of lactoperoxidase (Sigma), 35 milliliters of glucose oxidase, 0.1 milliliters of catalase, and 0.1 milliliters of sodium peroxide. The iodination was carried out for 1 hour at 22°C.

1 The abbreviation used is: HTC cells, hepatoma tissue culture cells, a cloned line originally derived from a rat minimal deviation hepatoma.
was located with silver nitrate (19), cut out, and counted.

Cell Fractionation: A plasma membrane fraction of HTC cells was prepared as described previously (14). In some experiments this fraction as well as a microsomal fraction were prepared from frozen homogenates of cells. In these cases, the cells were suspended in 0.01 M Tris-Cl, pH 7.0, containing 0.005 M KCl and 0.001 M MgCl₂, 1 ml/10⁶ cells. The cells then were homogenized in a Dounce homogenizer with the pestle until more than 90% breakage was achieved, as monitored by phase contrast microscopy. Immediately, an equal volume of 20% sucrose in 0.02 M Tris-Cl, pH 7.0/0.1 M KCl/0.002 M MgCl₂ was added, and this mixture was frozen at -20°. For the preparation of the microsomal fraction, the homogenate was warmed on ice, layered onto a 2.0 ml 30% sucrose cushion. This material was centrifuged in the Sorvall HB-4 rotor for 20 min at 400 × g (6,000 × g·min). A plasma membrane fraction was prepared from the pellet by two consecutive-step sucrose gradients as previously described (14). A microsomal fraction corresponding to the 500,000 × g·min supernatant fraction was centrifugated at 40,000 rpm for 1 hour in the SW 28 rotor. The pellet from this centrifugation was suspended in 10% sucrose and centrifuged again at 40,000 rpm for 45 min. The pellet was washed with 0.10 M KCl followed by 0.01 M Tris-Cl, pH 7.0, and finally with distilled water. The biochemical analysis of the plasma membrane fraction was similar to that described for the microsomes that were not frozen (14), but the yield was considerably less. The light red-colored microsomal fraction was enriched about 4-fold for glucos-6-phosphatase over the homogenate.

Electrophoresis—Sodium dodecyl sulfate-acrylamide gel electrophoresis was performed according to the procedure of Laemmli (17). Gels were cast in 1.5-mm slabs in an apparatus from Hoeffer Scientific Inc. or in glass tubes having an internal diameter of 6 mm. Acrylamide concentration varied and is described in the legends to the figures. Slab gels were analyzed for radioactivity by autoradiography after drying in the SW 28 rotor. Gel slabs were analyzed for radioactivity by autoradiography after drying in a gel-drying apparatus from Hoeffer Scientific Inc. Tubular gels were analyzed by scintillation counting or gamma counting, after slicing in 1-mm sections with an apparatus from Gibson Medical Electronics. Preparation of [¹¹¹]Iodotyrosine—Tyrosine was dissolved in saline (0.9% NaCl solution) (0.5 mg/ml) and iodinated by the procedure described above with carrier free Na¹¹¹I for 1½ hours at 37°. The reaction was terminated by the addition of trichloroacetic acid at a final concentration of 10%. The sample was centrifuged to remove the precipitate, and the supernatant solution was passed over a column of Dowex 50-X2 (Bio-Rad) which had been equilibrated with distilled water. The material which did not bind to the column was eluted with 7 column volumes of distilled water followed by 3 volumes of 0.2 N HCl. The bound amino acids were eluted with 4 M NH₄OH. The NH₄OH was removed by drying in vacuo over P₂O₅. The residue was dissolved in formic acid and chromatographed on Silica Gel G thin-layer plates in butanol-acetone/1 N NH₄OH (1/4/1, v/v/v). Radioactivity was located by autoradiography, and the material which co-chromatographed with authentic monooiodotyrosine was removed from the plate, eluted, and rechromatographed in the same solvent system. Autoradiographic analysis revealed a single species of labeled material, which chromatomgraphed as monooiodotyrosine. This material had a specific radioactivity of 75,000 cpm/mmol.

Identification of Fucose—Labeled fucose in the medium from growing cells and in acid hydrolysates of cells was identified by thin chromatography. Washed cells containing incorporated fucose but no free fucose were homogenized in distilled water. An equal volume of 0.2 N H₂SO₄ was added, and this mixture was heated at 100° for 2 hours under nitrogen (18). Radioactivity in the acid hydrolysate was analyzed by spotting an aliquot directly onto a cellulose thin layer plate, followed by chromatography in two consecutive runs in a solvent system of ethyl acetate/pyridine/water (2/1/2) (19). The fucose spot was located with silver nitrate (19), cut out, and counted.

Labeled fucose in the medium was analyzed in a similar way except the medium was first treated with trichloroacetic acid, 10%, to remove protein. The trichloroacetic acid was extracted into ether, and the resulting solution was applied to a mixed bed Dowex AG 50-X-8 (Bio-Rad) column. The column was eluted with water, and the eluate was concentrated by lyophilization. The lyophilized material was dissolved in water and chromatographed as described.

Radioactivity Determination—[¹¹¹]I and H were visualized by autoradiography or by using a Packard gamma counter or in a liquid scintillation counter (American/Seal Isocap 300) in Scintipre p 2 (Fisher) scintillation fluid made 25% in ethanol. [¹³C] and [³H] were determined by liquid scintillation counting, also in the above scintillation fluid. Gel slices were counted after dissolving the gel in Protosol. Efficiencies were determined by internal standards. In double isotope experiments the amount of each isotope was calculated after determining the efficiency for each isotope in each of two channels. Counting was routinely performed for sufficient time to insure that the values obtained were accurate to ± 1% at 3 S.D.

RESULTS

Effect of Iodination on Protein Turnover

Elsewhere (14), we showed that lactoperoxidase-catalyzed iodination of HTC cells in situ labels specifically polypeptides of the plasma membrane. Light microscopic autoradiography of sectioned cells showed the incorporated label to be localized primarily at the periphery of the cell. Most of this label could be released from the cell by trypsin, but not by collagenase or hyaluronidase. The label was recovered from the cells as either monooiodotyrosine or diiodotyrosine after hydrolysis of cell extracts with a mixture of proteolytic enzymes. The label copurified during cell fractionation with 5'-nucleotidase, an enzyme confined primarily to the plasma membrane of hepatocytes. At least 50 polypeptides in the membrane, as resolved by dodecyl sulfate-polyacrylamide gel electrophoresis, are accessible to iodination. These polypeptides probably represent the bulk of the protein mass of the membrane, and iodinating them does not affect cell viability, growth rate, or cell function as measured by the induction of tyrosine aminotransferase in these cells by corticosteroids. Finally, labeling experiments with fucose and glucosamine showed that at least nine of the iodinated peptides are glycoproteins.

These results suggested that the turnover of the plasma membrane could be studied using iodide as a specific marker for the polypeptides in this membrane, thereby avoiding the problems encountered when labeled amino acids are used to study the turnover of undefined membrane proteins.

However, before examining the turnover of the iodinated membrane polypeptides, it was necessary to determine whether iodination itself affects the turnover of cell protein, and whether the iodinated polypeptides in the membrane turn over in a way similar to that of unmodified plasma membrane proteins. The study presented in Fig. 1 was designed to determine whether iodination affects significantly the turnover of total cell protein. In this experiment, cell protein was labeled by growing HTC cells in the presence of [¹³C] tyrosine for two generations. One aliquot of these cells was iodinated with unlabeled iodide, converting about 1% of the total acid-insoluble radioactivity from [¹³C] tyrosine to [¹³C]iodotyrosine. These cells then were incubated in growth medium at 37°. An identical aliquot of tyrosine-labeled cells (not iodinated) was incubated under the same conditions. As shown in Fig 1, there was no marked difference in the release of total acid-soluble radioactivity between the iodinated culture of cells and the control culture which had not been iodinated. Most of the acid-soluble radioactivity released into the medium from the control culture chromatographed as tyrosine in thin layer chromatography. A constant proportion (0.2%) of the total radioactivity released into the medium from the iodinated cells after 25 or 44 hours in culture migrated as mono- and diiodotyrosine in the thin layer chromatographic systems. The accumulation of labeled amino acids in the medium is due to degradation of the labeled proteins. There is little or no release of radioactivity if the cells are incubated at 4°, and the release can also be inhibited by inhibitors of energy metabolism (20).

J. Tweto and D. Doyle, unpublished observations
From the data of Fig. 1, we conclude that iodination of the cells does not affect appreciably the turnover of total cell protein, at least as measured by release of acid-soluble radioactivity into the medium. However, this way of measuring protein turnover is rather insensitive, since the release of amino acid is a step far removed from the initial step in protein degradation. Therefore, a double isotope procedure was used to assess the effect of iodination on the rate constant of degradation of both total protein and plasma membrane protein of HTC cells (Table I). In this experiment, cells were labeled for 1 hour with \(^3\)H-tyrosine. Then, one aliquot of the \(^3\)H-labeled cells was immediately given a second pulse of tyrosine, but labeled with \(^1\)C. This culture was the zero time control. A second aliquot of the \(^3\)H-labeled cells was incubated at 37° for 24 hours before administration of \(^1\)C-tyrosine. A third aliquot of the \(^3\)H-labeled cells was iodinated with unlabeled iodide before being incubated for 24 hours at 37°. Then, the second \(^1\)C-tyrosine pulse was given. The zero time control cells had \(^3\)H/\(^1\)C ratios of about 3 for each of several cell fractions analyzed. This value is the ratio to be expected with no turnover of protein. The control cells, not iodinated, had isotope ratios of 2.0 and 2.5 for total protein and plasma membrane protein respectively. Thus, some of the \(^3\)H-labeled protein was degraded during the 24-hour incubation. The iodinated cells also had \(^3\)H/\(^1\)C ratios of about 2.0 and 2.5 for total protein and plasma membrane protein, respectively. The higher isotope ratio of the plasma membrane fraction relative to total cell protein suggests that plasma membrane proteins are turning over at a slower rate than total cell protein. The fact that the isotope ratios for the iodinated cells were essentially identical with those for the uniodinated control cells indicates that iodination does not affect significantly the turnover of either total cell protein or plasma membrane protein. This being the case, it is possible to examine the fate of the iodinated polypeptides with some...
assurance that their turnover reflects adequately the normal turnover of unmodified plasma membrane proteins.

Degradation of Iodinated Membrane Proteins—The degradation rate of iodinated membrane proteins was measured by following the loss with time of \(^{125}I\) in trichloroacetic acid-insoluble material (Fig. 2). In exponentially growing cells the iodinated proteins are degraded with a half-life of about 100 hours (Fig. 2A). In nongrowing cells (Fig. 2B), the iodinated proteins are degraded at about the same rate for the first 90 hours in culture. After 60 hours, degradation seems to stop. But this is due to the depletion of some nutrient in the medium, probably glucose (20). If fresh medium is added to these cells, degradation will again continue with a half-life of about 100 hours. In four other experiments, the half-life for the degradation of the iodinated proteins varied from 90 to 120 hours. The label lost from protein was released into the medium at a continuous rate. All of the label in the medium was soluble in trichloroacetic acid, 10%, and was identified by chromatography as either free iodide or mono- and diiodotyrosine.

The value for the rate of degradation of plasma membrane proteins derived from the data of Fig. 2 could be overestimated if iodotyrosine were reutilized for protein synthesis. However, as shown in Table II, this is not the case. There was little incorporation of \(^{125}I\) iodotyrosine into acid-insoluble material, either in the presence or absence of added tyrosine. This was due to inability of the cell to take up the iodotyrosine, since significant label was found in the acid-soluble fraction. The cells were capable of incorporating added label, since \([3H]\) tyrosine was incorporated both in the presence and absence of added \([1^{13}C]\)iodotyrosine.

Degradation of Total HTC Cell Protein—The degradation rate of total protein of HTC cells was measured by monitoring the loss of acid-insoluble radioactivity from cells that were grown in the presence of \([3H]\)leucine for 4 hours (Fig. 3). The degradation rates of labeled protein in growing and density-inhibited cells were measured in the same experiment. Under both growth conditions the half-life for the degradation of total protein was approximately 60 hours. In several such experiments the half-life was found consistently to be between 50 and 60 hours, confirming the results of Table I; the half-life of total cell protein is significantly shorter than that of plasma membrane proteins. The value for the half-life of total cell protein in Fig. 3 is probably overestimated, since the leucine is certainly reutilized to some extent. The doubling time of the cells in this experiment was 40 hours. In other experiments the same 50 to 60-hour half-life for protein was obtained in cells with a more usual doubling time of 30 hours, indicating that the overall turnover of cell protein is not a function of cell density.

Relative Rates of Degradation of Iodinated Plasma Membrane Proteins—We next examined the relative rates of degradation of the iodinated polypeptides in the plasma membrane of HTC cells by two related methods. The first involved an autoradiographic analysis of the loss of radioactivity from iodinated plasma membrane polypeptides separated by electrophoresis in dodecyl sulfate-polyacrylamide gels. The iodinated cells were taken from the experiment described in Fig. 2. The results are presented in Fig. 4. An equal amount of radioactivity was applied to each lane of the gel in Fig. 4. Hence, increasingly more protein was applied as the iodinated cells were longer in culture. This was readily visible in the Coomassie-staining pattern of the gel (not shown). However, the relative labeling intensities of the bands is the same for all the time points. This indicates that there are no large differences in the relative degradation rates of the iodinated plasma membrane proteins. It is clear from Fig. 4 that a large number of proteins are accessible to the lactoperoxidase probe, and since only plasma membrane proteins are labeled, a good sample of these proteins can be analyzed by the autoradiographic technique. The label which does not enter the 7.5% gel is not aggregated material. It represents about 8% of the incorporated label, but does not stain significantly with

### Table I

**Effect of iodination on turnover of total protein and plasma membrane protein of HTC cells**

| Conditions          | Cell Fraction | Homogenate | Plasma membrane |
|---------------------|---------------|------------|-----------------|
|                     |               | \(^{3H}\) | \(^{3C}\) | \(^{3H}\) | \(^{3C}\) |
|                     | dpm \times 10^9/mg of protein |
| Iodinated cells 24 hr in culture | 1.8 | 0.86 | 2.1 | 1.2 | 0.44 | 2.7 |
| Uniodinated control cells 24 hr in culture | 1.7 | 0.92 | 1.9 | 1.7 | 0.66 | 2.6 |

* A value for the half-life can be obtained from the double isotope ratio using the equation describing first order decay, \(P(t) = P(0)e^{-kt}\), where \(t\) is the number of molecules remaining after time \(t\) has elapsed, and \(k\) is the rate constant of degradation. The isotope ratio is equivalent to \(P(0)/P(t)\) (4). The accuracy of the value for the rate constant of degradation is a function of the extent to which the labeled precursor is subject to reutilization. For amino acid precursors reutilization can be significant, while for iodotyrosine, reutilization is not a serious problem.
Reutilization of iodothyrosine by HTC cells

| Label added                          | Trichloroacetic acid-soluble radioactivity | Trichloroacetic acid-insoluble radioactivity |
|--------------------------------------|-------------------------------------------|--------------------------------------------|
|                                      | [I] | [III] | [I] | [III] |
| No. 1 [\(^{125}\)I]iodotyrosine      | 1.21 (0.48\%) | 1.21 (0.48\%) | 580 (0.02\%) | 580 (0.02\%) |
| No. 2 [\(^{125}\)I]iodotyrosine      | 1.65 (0.83\%) | 1.65 (0.83\%) | 322 (0.01\%) | 322 (0.01\%) |
| No. 3 \[^{3}H\]thyrosine             | 0.33 (0.16\%) | 0.33 (0.16\%) |                       |                       |
| No. 4 \[^{3}H\]thyrosine + [\(^{125}\)I]iodotyrosine | 1.88 (0.94\%) | 0.46 (0.18\%) | 1.90 (0.96\%) | 84 (0.03\%) |

* Values in parentheses are the percentages of the added radioactivity found in the various fractions.

Coomassie blue. Fig. 4B shows similar results from a similar study, except that the iodinated membranes were prepared from the density-inhibited cells of Fig. 2B. The results of Fig. 4 suggest that the proteins of the HTC cell plasma membrane are not being degraded with heterogeneous rates. It is difficult to quantitate the autoradiographic evidence in Fig. 4. We therefore devised a double isotope experiment using \(^{125}\)I and \(^{125}\)I to monitor the degradation rates of the proteins in the plasma membrane. We showed previously (14) that both iodine isotopes label the same membrane proteins to the same relative extent. This experiment, presented in Table III and in Figs. 5 and 6, was done with non-growing cells. The details of the design of the experiment are described in the legends to the figures. Essentially, the \(^{125}\)I radioactivity represents the polypeptides in the membrane accessible for iodination, while the \(^{125}\)I radioactivity shows what happens to these polypeptides as the cells are in culture. The ratio of \(^{125}\)I/\(^{125}\)I in the membrane fraction, then, is a measure of protein degradation. As shown in Table III, the ratio of \[^{125}\]I/\[^{125}\]I decreases with time, because the \(^{125}\)I radioactivity is lost from the cells in culture.

The relative degradation rates of the polypeptides in the membrane were examined after dissociation of the membrane with dodecyl sulfate and dithiothreitol and separation of the polypeptides by dodecyl sulfate-polyacrylamide gel electrophoresis. Fig. 5 shows the results of a control experiment in which cells labeled with \[^{125}\]I were mixed at zero time with the \[^{125}\]I-labeled cells. In this experiment there is no turnover of the plasma membrane proteins of the \[^{125}\]I-labeled cells. The mean \(^{125}\)I/\(^{125}\)I ratio for the separated membrane polypeptides was 1.5, with a standard deviation of 0.2. Fig. 6 shows the results from a similar experiment, except that the \[^{125}\]I-labeled cells were in culture for 48 hours before mixing with the \[^{125}\]I-labeled cells. The mean isotope ratio for the separated polypeptides in this membrane preparation was 0.9 with a standard deviation of 0.1. Thus, turnover did occur relative to the control experiment, but there was no significant deviation from the mean ratio of 0.9 among the separated polypeptides, except at the low molecular weight region of the gel. This was also found in the control experiment and, therefore, cannot be due to degradation. In experiments in which the \[^{125}\]I-labeled cells were in culture for shorter (5 hours) or longer (90 hours) periods of time there was no indication of heterogeneity of degradation rates among the dissociated membrane polypeptides.

Degradation of Leucine-labeled Membrane Proteins—Based on the results presented thus far, we conclude that the proteins in the plasma membrane that can be iodinated in situ are degraded at similar rates regardless of the growth rate of the cell, and that these polypeptides turn over as a unit. Previously (14), we presented evidence that those polypeptides that are iodinated in situ constitute a significant portion of the mass of the plasma membrane. However, obviously some membrane proteins do not have tyrosine residues exposed on the outside of the cell, and cannot be labeled by lactoperoxidase-catalyzed iodination. Therefore, we employed a more conventional double isotope procedure using labeled leucine as precursor to examine the degradation of all the proteins associated with the plasma membrane. A problem complicating the use of an amino acid precursor in such studies is that any contamination of the plasma membrane fraction with other cell fractions may mask the authentic rates of degradation of the plasma membrane proteins. It is difficult to obtain a homogeneous preparation of plasma membrane from tissue culture cells. Biochemical analyses of the plasma membrane fraction prepared from HTC cells indicate that it is highly enriched for this membrane (14). The membrane fraction contains negligible contamination from the soluble or mitochondrial fractions of the cell, but it is slightly contaminated with nuclei and microsomes.

The results of a leucine double label analysis of the degradation of the polypeptides in the plasma membrane fraction isolated from HTC cells are presented in Fig. 7. In this experiment the \[^{12}C\]radioactivity represents the leucine-labeled polypeptides in the membrane preparation, while the \[^{3}H\]radioactivity shows what happens to these polypeptides after the cells are in culture for 72 hours, relative to cells that were not in culture. As shown in Fig. 7, all of the polypeptides in this preparation of plasma membrane had similar \[^{3}H\]/\[^{12}C\] ratios, indicating similar rates of degradation. Polypeptides separated from the soluble fraction of HTC cells showed more heterogeneity in \[^{3}H\]/\[^{12}C\] ratio (Fig. 8), indicating that the polypeptides in this fraction are turning over at different rates. In the
The soluble fraction (Fig. 8) are weighted in favor of the more long-lived proteins. This is apparent by comparing the ratios during which turnover of protein occurred. The long labeling period with leucine also would discriminate against the short-lived polypeptides in the plasma membrane. The polyglycrons were separated by slab acrylamide gel electrophoresis. The gels then were fixed, dried, and autoradiographed. The autoradiographic pattern obtained from logarithmically growing cells (Fig. 2A) is shown in A (left), and the pattern from density-inhibited cells (Fig. 2B) in B (right). The approximate migration distance of standard proteins of the indicated molecular weight \( \times 10^3 \) is shown by the numbers (right) and by the arrows (center). Equal amounts of radioactivity were applied to each lane in A and in B. The gels were composed of a layer of 7.5% acrylamide on top of a layer of 10% acrylamide. The interface can be seen as the sharp band approximately at the center of each gel. The amount of acrylamide of each concentration is somewhat different in A and B. The difference in size reflects differential swelling which occurred during processing of the gel.

The long labeling period with leucine also would discriminate against the short-lived polypeptides in the plasma membrane (Fig. 7). But the polypeptides accessible to iodination constitute the bulk of protein in the membrane, and the iodinated polypeptides are degraded at relatively slow rates. Thus, the results in Fig. 7 confirm the studies on the degradation of the iodinated polypeptides and show that much of the protein in the plasma membrane is degraded with similar, and probably identical, rates.

Degradation of Membrane Glycoproteins—The plasma membrane of HTC cells has about 10 major glycoproteins, as detected by growing the cells in labeled fucose or labeled glucosamine (14). The same membrane components, all having molecular weights greater than 50,000, are labeled by both of the sugars. The turnover properties of these glycoproteins were examined using L-fucose as precursor.

Since little is known about the turnover of the carbohydrate moieties of glycoproteins, we first examined the kinetics of loss of fucose from total extracts of HTC cells. HTC cells were grown in the presence of L-[3H]fucose for 24 hours. At least
### Table III

| Incubation time of | $^{125}$I | $^{131}$I | $^{125}$I/$^{131}$I |
|-------------------|----------|----------|------------------|
| hr                | cpm x $10^4$/mg of membrane protein | ratio |
| 0                 | 2.3      | 1.7      | 1.3              |
| 24                | 1.7      | 1.6      | 1.1              |
| 48                | 0.90     | 1.2      | 0.8              |

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**Fig. 5.** $^{125}$I/$^{131}$I Ratios of polypeptides in the plasma membrane fraction of HTC cells as separated on dodecyl sulfate-polyacrylamide gels—zero time control. A suspension of washed HTC cells (10$^6$ cells) was divided into two parts. One-half of the cells were iodinated with $^{125}$I, while the other half were iodinated with $^{131}$I as described under "Materials and Methods." Immediately after iodination, the cells were washed, and a plasma membrane fraction was prepared from a combined frozen homogenate of the two labeled cultures. The polypeptides in the membrane fraction were dissociated in dodecyl sulfate and dithiothreitol and separated on disc gels of 9% acrylamide. Approximate mobility of standard proteins in this system is indicated. $\bullet$, ratio of $^{125}$I/$^{131}$I in the acrylamide fractions; $\circ$, $^{131}$I-labeled polypeptides in the membrane fraction.

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**Fig. 6.** $^{125}$I/$^{131}$I Ratios of polypeptides in the plasma membrane fraction of HTC cells in culture for 48 hours. Details are the same as in Fig. 5, except that the $^{125}$I-labeled cells were incubated in growth medium for 48 hours at 37°C at a density of 1 x 10$^6$/ml. A homogenate of these cells was prepared and frozen with the frozen homogenate of the $^{131}$I-labeled cells which were not in culture. A plasma membrane fraction then was prepared from the combined homogenates.

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65% of the radioactivity incorporated during this interval can be recovered as fucose after acid hydrolysis of cell extracts (Table IV). Thus, most of the precursor is present in the glycoproteins as fucose. The loss of incorporated fucose from the cells followed first order kinetics with a half-life of between 50 and 60 hours (Fig. 9). The radioactivity lost from the cells could be recovered in the medium (Fig. 9, inset). At least 85% of the radioactivity released into the medium was soluble in trichloroacetic acid, and 60% of the acid-soluble material cochromatographed with fucose in thin layer chromatography (Table V).

Since the fucose-labeled glycoproteins turn over with apparent first order kinetics, a double isotope procedure, using fucose as precursor, could be used to measure the relative rates of degradation of these glycoproteins. Details of the design of the experiment are given in the legends to the figures.

After administration of labeled fucose to HTC cells, only the plasma membrane fraction and the microsomal fraction are enriched over the homogenate for acid-insoluble fucose (14). As indicated in Figs. 10 and 11, both fractions appear to contain in common many of the same fucose-labeled glycoproteins, at least as resolved by electrophoresis in dodecyl sulfate-acrylamide gels. Indeed there is an almost exact co-incidence of...
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Fig. 8. $^3$H/$^{14}$C Ratios of leucine labeled polypeptides in the soluble fraction of HTC cells. Experimental details are exactly the same as in Fig. 7, except that the material in the soluble fraction of the cell was subjected to electrophoresis in the dodecyl sulfate-9% polyacrylamide gel system. The soluble fraction was obtained by centrifugation of the homogenate at 240,000 x $g$ for 1 hour.

Table IV
Incorporation of $L$-fucose into HTC cell glycoproteins

Cells at a concentration of 5 x $10^5$ cells/ml were grown in complete growth medium containing $L$-[6-$^3$H]fucose (5.0 µCi/ml, specific activity 12 Ci/mmol). At the end of 24 hours at 37$^\circ$, the cells were collected and washed twice with complete growth medium. The cells then were suspended in growth medium at a concentration of 1 x $10^6$/ml. At this time an aliquot of the cells (1 x $10^7$) was taken for the determination of the amount of incorporated label as fucose. These cells were washed twice with Earle's balanced salt solution and were homogenized in distilled water. An equal volume of 0.2 N H$_2$SO$_4$ was added, and the mixture was heated at 100$^\circ$ for 2 hours under nitrogen. The acid hydrolysate next was centrifuged at 10,000 rpm for 10 min in the Sorvall HB-4 rotor. One aliquot of the resulting clear solution was treated with trichloroacetic acid, final concentration 10%. Another aliquot was mixed with $L$-fucose (0.25 mg) and spotted onto a cellulose thin layer plate. The thin layer plate was chromatographed as described under "Materials and Methods," and the fucose spot was counted.

| Material                              | Total cpm |
|---------------------------------------|-----------|
| Acid hydrolysate of HTC cells         | 24,000    |
| Acid hydrolysate after centrifugation | 26,000    |
| Acid hydrolysate after 10% trichloroacetic acid | 20,800 |
| Radioactivity in hydrolysate migrating with fucose in thin layer chromatography | 15,400    |

Table V
Analysis of radioactivity released into medium from fucose-labeled HTC cells

HTC cells labeled with $^3$H]fucose were suspended in complete growth medium at a density of 1 x $10^6$/ml, as described in the legend to Table IV. The cells were incubated at 37$^\circ$ for 72 hours. During this time acid-soluble radioactivity was released into the medium (Fig. 9). After 72 hours of culture, the medium was collected, and trichloroacetic acid was added to a final concentration of 10%. The precipitated material was removed by centrifugation, and the trichloroacetic acid was extracted into ether. An aliquot of the resulting solution was applied to a mixed bed resin column of Dowex AG 50-X8 (Bio-Rad). All of the applied radioactivity was eluted with water in the void volume and was concentrated by lyophilization. The lyophilized material was dissolved in a minimum volume of water. $L$-Fucose (0.25 mg) was added, and the material migrating with fucose in thin layer chromatography was collected and counted as described in the legend to Table IV.

| Material                              | cpm x 10$^{-9}$/culture |
|---------------------------------------|-------------------------|
| 72 Hour medium                        | 1.8                     |
| 72-Hour medium, acid-soluble          | 1.5                     |
| Radioactivity in acid-soluble fraction migrating with fucose in thin layer chromatography | 0.9                     |

labeled peaks of molecular weight greater than 50,000 when the polypeptides from a $[^3]$C]fucose-labeled microsomal fraction are subjected to coelectrophoresis with a $[^3]$H-labeled plasma membrane fraction in the gel system of Figs. 10 and 11.

There is some cross-contamination of the plasma membrane fraction and the microsomal fraction as prepared from HTC cells. However, both fractions are enriched over the homoge-
Turnover of Plasma Membrane Proteins of HTC Cells

Fig. 10. $^{3}H/^{14}C$ Ratios of fucose-labeled polypeptides in the plasma membrane fraction of HTC cells. A culture of HTC cells was divided into two parts. One part ($5 \times 10^{6}$ cells/ml) was grown in complete growth medium containing L-$[6-{ }^{3}H]$fucose ($5.0$ µCi/ml, $12$ Ci/mmol). The other cells ($5 \times 10^{6}$ cells/ml) were grown in complete growth medium containing L-$[1-{ }^{14}C]$fucose ($1$ µCi/ml, $48$ mCi/mmol). At the end of $24$ hours at $37^\circ$, the cells were collected and washed twice with Earle's balanced salt solution. At this time, aliquots of the $^{14}C$-labeled cells and the $^{3}H$-labeled cells were homogenized and mixed together. This mixture, representing the zero time control, as well as an homogenate of the other $^{14}C$-labeled cells, was immediately frozen.

The other $^{3}H$-labeled cells were suspended in complete growth medium at a concentration of $1.2 \times 10^{6}$/ml. These cells were incubated at $37^\circ$ for $72$ hours. At the end of the incubation, the cell density was still $1.2 \times 10^{6}$/ml. These $^{3}H$-labeled cells next were collected by centrifugation, washed twice with Earle's balanced salt solution, and homogenized.

The homogenate was mixed and frozen with the frozen homogenate of the $^{14}C$-labeled cells. A plasma membrane fraction and a microsomal fraction were prepared from this mixture of cells and from the zero time control cells. These latter cells had $^{3}H/^{14}C$ isotope ratios of $7.8$ for protein in each of the cell fractions analyzed. Arrows enclosed by dotted lines, mean ratio and one standard deviation of the mean for the polypeptides in the plasma membrane fraction of the zero time control cells. The polypeptides were dissociated in $1\%$ dodecyl sulfate and $1\%$ 2-mercaptoethanol, and separated on a $9\%$ polyacrylamide disc gel containing $0.1\%$ dodecyl sulfate. $^{3}H/^{14}C$ ratios for the fucose-labeled polypeptides in the plasma membrane fraction when the $^{3}H$-labeled cells were in culture for $72$ hours.

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$^{3}H/^{14}C$ Ratios of fucose-labeled polypeptides in the microsomal fraction of HTC cells. Experimental details are the same as in Fig. 10, except that the polypeptides in the microsomal fraction of the cell were separated on the $9\%$ acrylamide-dodecyl sulfate disc gel.

Table VI

| Cell fraction    | $^{14}C$ (dpm/mg of protein) | $^{3}H$ | $^{3}H/^{14}C$ |
|------------------|-------------------------------|--------|--------------|
| Homogenate       | 24,000                        | 87,000 | 3.6          |
| Microsomes       | 90,000                        | 204,000| 3.3          |
| Plasma membrane  | 64,800                        | 337,000| 5.2          |

In this paper we have examined the degradation of HTC cell protein by three different methods: the long-term loss of total radioactivity from protein, the concomitant release of label into the medium, and variations of the double isotope technique originally described by Arias et al. (15). Three methods of labeling cell protein were used in each case—labeled amino acids, iodination in situ with lactoperoxidase, and labeled fucose. HTC cells were chosen for these studies because they were derived originally from a rat hepatic tumor. Liver has been the organ of choice for most protein turnover studies, and HTC cells might be expected to use the same or similar degradative mechanisms as the hepatocyte.

The half-life for the degradation of total cell protein in HTC cells as judged by the loss of radioactivity from protein is about $2$ to $2\frac{1}{2}$ days, a value within the range found for the turnover of total protein of rat liver by a variety of different methods (21). In rat liver, the protein in the soluble fraction is degraded...
with heterogeneous rates, and there is a correlation between degradation rate and the molecular size (5, 6) of the protein subunits in this cell fraction. In HTC cells, the proteins in the soluble fraction also are degraded at heterogeneous rates (Fig. 8). However, there is no obvious correlation between degradation rate and subunit size of these proteins. This does not reflect anomalous degradation in HTC cells, but rather experimental design. In Fig. 8, the cells were exposed to labeled leucine for 24 hours, while degradation of the labeled proteins occurred for 3 days. Both of these conditions tend to obscure the degradation of short-lived polypeptides, because (a) these polypeptides are undergoing degradation during the 24-hour labeling period and (b) the 3-day decay period is relatively long compared to the half-life for the degradation of total protein. Therefore, heterogeneity and a size-degradation rate correlation would be difficult to detect under these conditions (4, 15). With a shorter labeling period the soluble proteins of HTC cells show more heterogeneity in their degradation rates and more of a size-rate correlation.

One difference between HTC cells and rat hepatocytes is that the former are actively dividing. However, growth rate has little, if any, effect on the rate of protein degradation. Thus, the results of Fig. 3, with amino acid as precursor, and those of Fig. 2, with iodide labeling, indicate that the rate of degradation of the labeled proteins is not much different in growing or nongrowing cells.

Iodination of HTC cells had little, if any, effect on the degradation of total cell protein as measured by the release of amino acids into the medium (Fig. 1), or, more directly, as measured by the loss of label from both total protein and membrane protein (Table 1). This being the case, the degradation of the HTC cell proteins which are accessible to iodination in situ can be examined. Elsewhere (14) we showed that only plasma membrane proteins are accessible to iodination, and that these proteins represent most of the protein mass of the membrane. The studies of Fig. 2 and Table I show that the membrane proteins accessible to iodination are being degraded exponentially at a rate that is at most one-half that of total cell protein. The value for the half-life of the iodinated proteins obtained in Fig. 2 is somewhat longer than that which can be calculated from the data or Table III (about 3 days). This is because in Fig. 2 a period of about 18 hours elapsed between iodination and the first measurement of incorporated iodide. During this 18-hour period a rapid loss of some labeled material occurs. Similar results have been reported with mouse L cells (22), but the reason for the rapid loss is not known.

1-Fucose also was used to label HTC cell protein. We showed previously (14) that this sugar selectively labels membrane protein of HTC cells. However, the incorporated fucose is present and enriched over the homogenate in both the microsomal fraction and the plasma membrane fraction. Also, the enrichment over the homogenate of incorporated fucose in the plasma membrane fraction is low compared to the enrichment for incorporated iodide. It is not surprising, then, that the value for the half-life (20 hours, Fig. 5) of fucose-labeled protein is different from that found for the iodinated proteins (100 hours, Fig. 2). By calculation, the data in Table VI also yield a half-life of about 100 hours for the degradation of the fucose-labeled glycoproteins in the plasma membrane fraction versus a half-life of about 50 hours for the same glycoproteins (as resolved by dodecyl sulfate-acrylamide gel electrophoresis) in the microsomal fraction. Some of the glycoprotein in the microsomal fraction may be precursors in transit to the plasma membrane. But the difference in degradation rate of the glycoproteins in the two cell fractions suggests that some of these glycoproteins are components of the endoplasmic reticulum.

Most of the incorporated label, regardless of the specific precursor, when lost from protein, appeared in the medium. In every case, the label released into the medium was acid-soluble, indicating that secretion of macromolecules is not a major mechanism of protein removal from HTC cells.

Double isotope techniques using labeled leucine, labeled fucose, and labeled iodide were used to study the degradation of HTC cell protein because these techniques allow the determination of the relative degradation rate of a large number of proteins in a single experiment (15). We used three different precursors for the proteins of the plasma membrane in the double isotope experiments to determine as rigorously as possible whether the proteins of the plasma membrane are being degraded with heterogeneous or homogeneous rates. The importance of this question is that heterogeneous turnover of the membrane proteins implies that these proteins are removed from the membrane independently of each other, and that they exist for some finite period in another compartment of the cell.

However, the results obtained from the double isotope experiments with iodide as label indicate that the membrane proteins turn over at similar, probably identical, rates. These results were confirmed by the double isotope experiment with leucine as precursor for the plasma membrane proteins. The leucine-labeled polypeptides in the plasma membrane also were degraded at almost identical rates. In contrast, there was more heterogeneity in the degradation rates of the fucose-labeled proteins of the plasma membrane. However, the fucose-labeled proteins in the microsomal fraction were degraded with less heterogeneity in rate than those of the plasma membrane. The fact that the protein backbone of the glycoproteins in the plasma membrane are turning over at similar rates, while the carbohydrate moieties are turning over at more heterogeneous rates, may indicate that the sugar side chains can be removed from the proteins by a mechanism independent of protein degradation. Further studies are needed to clarify this point.

It has been reported that the proteins of the plasma membrane of rat liver are degraded at heterogeneous rates (5). This could mean that the HTC cell system is not a good model for studying the mechanism of biogenesis of the liver cell plasma membrane. Another possibility is that the reported heterogeneity in rate of protein turnover in plasma membrane of rat liver reflects contamination of the plasma membrane preparation with proteins from other cell fractions. Still another possibility is that while the major proteins of the membrane turn over at similar rates, minor proteins, such as receptors, are turning over at heterogeneous rates. We have not as yet distinguished among these possibilities. The results presented here favor unit degradation of at least the major proteins of the membrane. Hubbard and Cohn (22) also have concluded recently that in mouse L cells actively phagocytosing polystyrene latex beads, the proteins accessible to iodination turn over as a unit. If all of the proteins of the plasma membrane are degraded as a unit, perhaps by interiorization and fusion with lysosomes, then there must be some sort of coordinate control of the synthesis or insertion of these
polypeptides into the membrane. This aspect of membrane biogenesis is currently being examined.

Acknowledgment—We thank Else Friedman for excellent technical assistance.

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