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PROTEINS OF THE HEPATOMA TISSUE CULTURE CELL PLASMA MEMBRANE

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The specificity of lactoperoxidase-catalyzed iodination for the proteins of the hepatoma tissue culture cell plasma membrane was examined by histochemical, biochemical, and cell fractionation techniques. Light microscope autoradiography of sectioned cells shows the incorporated label to be localized primarily at the periphery of the cell. Most of this label can be released from the cell by trypsin but not by collagenase or hyaluronidase. The label is recovered from the cells as either monoiodotyrosine or diiodotyrosine after hydrolysis of cell extracts with a mixture of proteolytic enzymes. The label co-purifies during cell fractionation with an authentic liver cell plasma membrane marker enzyme, 5′-nucleotidase. Thus, the incorporated iodide is itself a valid marker for those membrane polypeptides having tyrosine residues accessible to the lactoperoxidase. The polypeptide complexity of the purified plasma membrane was examined by high resolution dodecyl sulfate-polyacrylamide gel electrophoresis. At least 50 polypeptides in the membrane 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. Labeling experiments with fucose and glucosamine show that at least nine of the iodinated peptides may be glycoproteins.

INTRODUCTION

Despite the important role played by the mammalian plasma membrane in many normal cell phenomena and the role proposed for this cell organelle in abnormal cell growth (1), little is known about the biogenesis or organization of the proteins in this membrane. One reason for this has been the difficulty of isolating a homogeneous plasma membrane fraction such that the polypeptides in this membrane can be identified and characterized.

However, the introduction in recent years of chemical reagents that cannot cross the limiting membrane of intact cells but which react with polypeptides exposed on the surface of these cells offers the possibility of easily and unequivocably identifying the polypeptides of the surface membrane. Lactoperoxidase-catalyzed iodination (2, 3) is one

Abbreviations: HTC cells, hepatoma tissue culture cells originally derived from a minimal deviation rat hepatoma; TLCK, N-α-p-tosyl-L-lysine chloromethyl ketone HCl; TPCK, L-1-tosylamide-2-phenylethylchloromethyl ketone.

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of the better procedures for labeling surface proteins. Because the reaction is mild, iodination can be used not only to identify membrane polypeptides but also to study their turnover. One problem with the lactoperoxidase reaction, however, is that proteins other than those of the plasma membrane can also be labeled (4–6). In the present study we establish optimal conditions for surface iodination of HTC cells and show that under these conditions most of the incorporated label is confined to the proteins of the plasma membrane. Further, most of the protein mass of this membrane is accessible to iodination.

EXPERIMENTAL PROCEDURES

Materials

Most chemicals used were of analytical grade. Sources of supplies were as follows: lactoperoxidase (E.C. 1.11.1.7); glucose oxidase (E.C. 1.1.3.4) (type II); sodium lauryl sulfate; dithiothreitol; phenylmethyl-sulfonylfluoride; TLCK; TPCK; 3, 5-diiodo-L-tyrosine; thyroglobulin (bovine type 1); 3-iodo-L-tyrosine; pancreatin (porcine pancrease); protease (type VI, S Griseas); glucose-6-phosphate; adenosine-5′-monophosphoric acid; succinic acid; cytochrome c; and bovine serum albumin (2 × crystalized) were from Sigma. Phosphorylase a (E.C. 2.4.1.1) was purchased from Boeringer Mannheim, β-galactosidase (E.C. 3.2.1.23); ovalbumin, human heavy chain; ribonuclease (E.C. 2.7.7.16); and trypsin (E.C. 3.4.4.4) (2 × crystalized, TPCK treated) were from Worthington Biochemicals. Carrier free ^{125}I and carrier free ^{131}I were obtained either from Amersham/Searle or from New England Nuclear. [6-^{3}H] thymidine (26.7 Ci/m mole, d-[U-^{14}C]-glucosamine (318 m Ci/m mole), and L-[1-^{14}C] fucose were purchased from Amersham/Searle. L-[4, 5-^{3}H] leucine (46 Ci/m mole) was from Schwartz-Mann. X-ray films and NTB-3 liquid emulsion were purchased from Eastman Kodak.

Methods

Cell culture. HTC cells were kindly provided by Dr. Thomas Gelehrter. They were grown as suspension cultures in minimal essential medium with Earle's salts, prepared without calcium and with ten times the concentration of phosphate. The medium was supplemented with 5% fetal calf serum, 5% bovine serum, 0.5% $NaHCO_3$, 50 units/ml of neomycin, and 50 mM tricine, pH 7.6. Media and sera were obtained from Grand Island Biologicals. Cells were maintained in logarithimic growth in loosely capped spinner bottles. Under these conditions, they had a doubling time of about 30 hr. When examined for PPLO contamination the cultures were negative. In some experiments radioactive precursors were added to the cultures. Since the precursor and the conditions varied depending on the experiments, details are presented in the legends to the appropriate table or figure.

Iodination with lactoperoxidase. Cells were iodinated using a procedure based on that of Phillips and Morrison (2) as modified by Hubbard and Cohn (3). Cells were collected by centrifugation and washed twice with Earle's balanced salt solution, without phenol red, calcium, or magnesium. After the second wash, the cells were suspended in this salt solution (henceforth referred to as modified Earle's salt solution) at an approximate density of 2×10^{6}/ml and were stirred for 30 min at room temperature. Cell number and viability, as assayed by exclusion of trypan blue were determined at the end of the 30 min incubation. The cells then were collected by centrifugation and suspended in modified Earle's salt solution (1×10^{7}/ml) which was 0.1 M in tricine, pH 7.6 and 7 mM in glucose. To this mixture (1.2 ml final volume) was added 60 munit of lactoperoxidase and either ^{125}I or ^{131}I. The amount and specific activity of the labeled iodide

depended upon the experiment. However, after preliminary experiments (see text) an optimal specific activity of 170 mCi/m mole was routinely used. The reaction was begun by the addition of 35 munit of glucose oxidase. Iodination was conducted for 30 min in 25-ml capped-culture tubes in a gyrotory shaker at 25°C. The reaction was stopped by adding 10 ml of modified Earle's salt solution followed by centrifugation of the cells. The cells were then washed two times with modified Earle's salt solution.

Assays of lactoperoxidase and glucose oxidase were performed using o-dianisidine as a donor according to the procedures described for horseradish peroxidase and glucose oxidase (7).

Identification of Labeled Amino Acids

The molecular form of the incorporated iodide was identified after precipitating iodinated HTC cells with 10% trichloroacetic acid. The acid insoluble pellet was washed once with 10% trichloroacetic acid and suspended in 0.05 M tricine buffer, pH 7.6. To this suspension was added 25 μg of protease type VI (S. Griseus), 25 μg of pancreatin, and a trace of toluene. After 16 hr incubation at 37°C proteolysis was stopped by the addition of 10% trichloroacetic acid. The small amount of precipitated material was removed by centrifugation. The acid soluble material was diluted with 10 vol 5 mM sodium metabisulphite, and this material was applied to a Dowex 50 X 8 column which had been washed with HCl and equilibrated with H_2O. The column was washed with 3 bed vol 0.2 N HCl, followed by 3 vol water. After this washing procedure no free iodide remained on the column. Bound radioactivity was eluted by washing the column with 4 M NH_4OH, and the eluate was dried over P_2O_5 in a vacuum. The dried sample was dissolved in formic acid. An aliquot was applied to a thin-layer plate coated with silica gel G (Analtech Inc.) and chromatographed in n-butanol/acetone/1 M NH_4OH (1:4:1) or in phenol/water (75:25) to which a trace of concentrated NH_4OH had been added. Authentic mono- and di-iodotyrosine were chromatographed on the same plates. Iodinated material was localized by autoradiography of the thin-layer plate.

Autoradiography of iodinated HTC cells. Iodinated HTC cells were suspended in saline at a concentration of 1×10^7 cells/ml in an Eppendorf micro test tube. An equal volume of cold 4% glutaraldehyde in 0.2 M potassium phosphate buffer, pH 7.4, was added, and the cells were fixed in this solution for 2 hr at 4°C. The cells then were collected by centrifugation. The packed cell pellet in the Eppendorf tube was dehydrated in ethanol, cleared in benzene, and imbedded in paraffin. Sections of 4–6 μ were cut. The sections were coated with Kodak NTB-3 liquid emulsion according to the procedure of Kopriwa and Leblond (8). Exposure time varied from 1 to 3 days. The sections were stained through the emulsion with hemotoxylin and eosin.

Isolation of the HTC cell plasma membrane. Two procedures were used to obtain a plasma membrane fraction of HTC cells – an aqueous two-phase polymer procedure modified from that described by Brunette and Till (9) and a procedure based on sucrose gradient separation. To prepare membranes by the aqueous two-phase polymer system or by the sucrose gradient method, cells were first suspended in 0.01 M tris-Cl, pH 7.0 containing 0.005 M KCl and 0.001 M $MgCl_2$ (1 ml/10^7 cells) and allowed to swell for 10 min at 4°C. The cells then were homogenized in a Dounce homogenizer (Kontes Glass Co.) with the B-pestle until 90% cell breakage was achieved, as determined by phase-contrast microscopy. For the preparation of membranes by the aqueous two-phase polymer system, the "homogenate" was centrifuged in the Sorvall HB-4 rotor for 15 min at 16,000 X g.

The supernatant fraction was discarded and the pellet was suspended by homogenization in upper phase (5 ml). Lower phase (5 ml) was added, and the phases were separated by centrifugation in the HB-4 rotor for 10 min at 1,000 × g. The material at the interface was removed, diluted with water, and collected by centrifugation. The pellet again was suspended in 5.0 ml of upper phase which was mixed with 5 ml of lower phase. The phases were separated by centrifugation for 10 min at 1,000 × g. The material at the interface was removed, diluted with water, and collected by centrifugation. This material was then washed several times with saline.

For the preparation of membranes by the sucrose gradient method, the "homogenate" was mixed with an equal volume of 20% sucrose in homogenizing buffer. This material was layered onto a 2.3 M sucrose cushion and centrifuged for 20 min at 400 × g in the HB-4 rotor. The material sedimented onto the sucrose cushion (8,000 g-min pellet) was collected and layered onto a discontinuous sucrose gradient composed of 2.5 ml of 2.2 M sucrose, overlayered with 1.0 ml of 1.8 M sucrose and 1.0 ml of 1.6 M sucrose. The gradients were centrifuged at 234,000 × g (av) for 60 min in the Beckman SW 50.1 rotor. The material at the 1.6–1.8 M sucrose interface was removed, diluted with 0.01 M tris buffer pH 8.0 and centrifuged through a second discontinuous sucrose gradient at 234,000 × g for 1 hr. The material at the 1.6–1.8 M sucrose interface was again collected and washed several times with saline. This material is referred to as purified membrane.

Membrane purification was monitored by assaying the marker enzymes 5′nucleotidase (10), succinic dehydrogenase (11), glucose-6-phosphatase (12), and tyrosine aminotransferase (13) by established procedures. Contamination by RNA and DNA was determined by the amount of radioactivity in membrane fractions prepared from cells grown in the presence of either [^{3}H] thymidine or [^{3}H] uridine. Protein was determined using the method of Lowry et al. (14) with bovine serum albumin as standard.

Acrylamide gel electrophoresis. Discontinuous gel electrophoresis in buffers containing sodium dodecyl sulfate was performed as described by Laemmli (15). Acrylamide, obtained from Eastman Kodak, was twice recrystallized from methanol. The gels were cast in 1.5-mm-thick slabs in an electrophoresis apparatus from Hoeffer Scientific Instruments. The concentration of acrylamide in the separating gel was 9% unless otherwise indicated. Following electrophoresis, the gels were fixed and stained for protein with Coomassie brilliant blue R250 in 45% methanol, 10% acetic acid. Gel slabs were prepared for radioautography by first swelling the gel in a solution of 20% methanol, 5% glycerol, and 10% acetic acid and then drying the gel onto filter paper by heating under vacuum for 3 hr in a Hoeffer drying apparatus. Cytochrome C, ribonuclease, ovalbumin, bovine serum albumin, phosphorylase a, thyroglobulin, and glyceraldehyde-3-phosphate dehydrogenase were used as molecular weight standards. These proteins were iodinated with lactoperoxidase to locate them on radioautographs.

Counting procedures. Cell fractions containing ^{125}I or ^{131}I radioactivity were prepared for counting by plating an aliquot onto 2.4 cm Whatman 3 filter paper discs. The discs were then swirled in 10% trichloroacetic acid which was made 25 mM in sodium metabisulfate and 5 mM in sodium iodide. After 10 min, the trichloroacetic acid solution was changed, and the discs were washed for another 10 min. The discs were then washed with hot trichloroacetic acid followed by a brief wash with ethanol-ether (1:1). A blank filter paper routinely was included in the washing procedures. No radioactivity was transferred to it. Cell fractions containing ^{14}C or ^{3}H labels were washed via a similar procedure except sodium metabisulfite and sodium iodide were omitted, and if the radioactivity was in nucleic acids, the hot trichloroacetic acid wash was omitted.

An alternative washing procedure was sometimes employed. Iodinated cell fractions were precipitated with 10% trichloroacetic acid. The pellet obtained was suspended (by sonication in a Heat System, Inc. sonicating bath) in 10% trichloroacetic acid containing 25 mM sodium metabisulfite and 5 mM NaI. The precipitate then was collected by centrifugation and washed a second time. The precipitate was dissolved in 0.5 ml of 1 N NaOH and an aliquot removed for counting. All samples were counted in toluene-based scintillation fluid in a Searle Isocap 300 liquid scintillation spectrometer. Efficiencies were determined using internal standards.

RESULTS

Lactoperoxidase-catalyzed Iodination of HTC Cells

The basic protocol for iodination of HTC cells was patterned after that of Hubbard and Cohn (3) as described in the sections "Materials" and "Methods." Several properties of the reaction, however, were examined in detail. David (16) has reported that the addition of a small amount of unlabeled iodide to carrier-free iodide results in an increased amount of radioactivity incorporated into protein. We also have found that dilution of carrier-free iodide with unlabeled iodide gives an increase in the amount of label incorporated into protein. As shown in Fig. 1 addition of increasing amounts of unlabeled iodide to the reaction mixture results in a gradual increase in the amount of incorporated label,

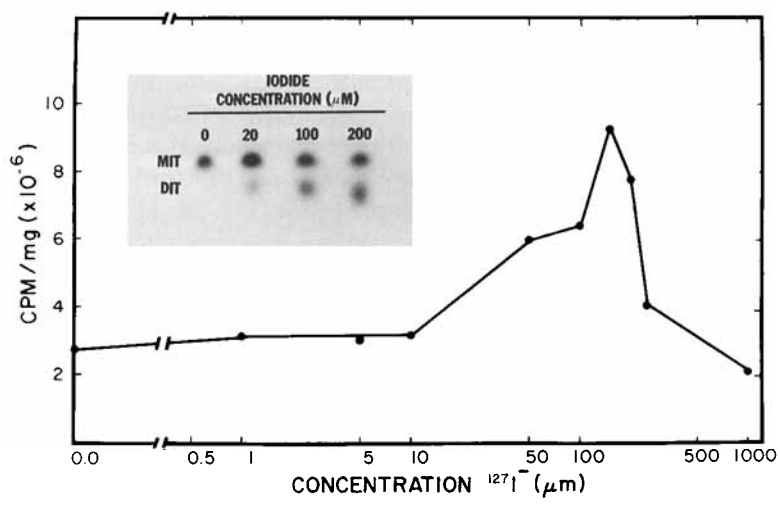

Fig. 1. Effect of unlabeled iodide on the incorporation of ^{125}I in HTC cells. Ten tubes each containing 10^7 washed HTC cells were iodinated with 50 μCi ^{125}I as described in Methods, except the amount of unlabeled iodide varied as indicated. The cells were then washed, centrifuged, and frozen. A total membrane fraction was prepared by thawing the frozen cells in 1 ml of water followed by centrifugation at 13,000 × g for 10 min. The supernatant solution was discarded and the pellet resuspended in water by sonication for counting and protein determinations. Inset. Four tubes each containing 10^7 washed HTC cells were iodinated in the presence of the indicated amount of unlabeled iodide. The cells were washed, the protein hydrolyzed, and the hydrolysate analyzed for amino acid bound iodide by chromatography as described in Methods. A portion of the autoradiogram obtained from the thin-layer plate is shown. The only radioactive spots present were MIT: monoiodotyrosine, and DIT: di-iodotyrosine. Free iodide migrated with the solvent front, and no ninhydrin positive material moved further than monoiodotyrosine (R_f = 0.53).

until a sharp maximum is reached at a concentration of 200 μmolar iodide. Beyond this, the amount of incorporated label decreases as would be expected from simple isotopic dilution. Similar results were obtained when ^{131}I was used in the reaction.

The molecular form of the label incorporated into cells in the presence of 0, 20, 100, or 200 μM unlabeled iodide was determined by proteolytic hydrolysis followed by thin-layer chromatography as described in Methods. An autoradiogram of the separated, labeled amino acids is shown in the inset of Fig. 1. In all cases when unlabeled iodide was added to the reaction mixture, only two spots were found. These spots corresponded exactly with the migration of authentic mono- and di-iodo tyrosine. When unlabeled iodide was omitted only one major spot was found, and this corresponded to mono-iodotyrosine. Free iodide is found at the solvent front in this sytem and is present in only trace amounts. The bulk of the free iodide was removed from the samples in the HCl wash of the Dowex column. None of the radioactivity in the HCl wash was extractable into chloroform, and therefore presumably was not associated with lipid. At least 98% of the incorporated radioactivity was recovered as mono- or di-iodotyrosine, indicating the specificity of the reaction for protein. The increase in extent of labeling in the presence of unlabeled iodide is due to the increased amount of label which becomes bound to protein tyrosines. The distribution of label between the two tyrosine species was examined by elution of the label from the thin-layer plate in the regions of iodotyrosine and di-iodotyrosine. Table I shows this distribution. In the presence of increasing amounts of unlabeled carrier, the amount of di-iodotyrosine is increased until the label is evenly distributed between the two species. Thus, meaningful estimates of the maximum number of sites available for iodination are difficult to make because new sites appear as the extent of iodination is increased. This is further illustrated by results presented in Fig. 2. This figure shows the time course of iodination at 37°C with carrier free iodide or with labeled iodide diluted with unlabeled to a final concentration of 200 μM. Much more radioactivity is incorporated in the presence of unlabeled iodide. However, after 60 min the label is no longer confined to tyrosines (not shown), and cell death during iodination is marked. As also shown in Fig. 2 in the absence of added unlabeled iodide, the extent of iodination increases rapidly in the first 25 min and then at a slower, linear rate for 60 min. Cell viability in this latter case was unchanged during iodination. Based on these and other similar studies, standard conditions for iodination were chosen. Cells (10^7) were iodinated at 25°C for 30 min in a final volume of 1.2 ml with 100 μCi of carrier-free labeled iodide and a final iodide concentration of 100–200 μM. Under these conditions incorporation is dependent on the presence of lactoperoxidase (65 munit per reaction) and glucose oxidase (35 munit per reaction) (Table II). Cell viability, as judged by trypan-blue exclusion, was unaffected, and cultures used for the experiments to be described were always greater than 90% and usually in the range of 95–100% viable. Cell growth rate also was unaffected by iodination in the presence of 200 μM I; both iodinated and untreated cultures had a doubling time of 28–32 hr and showed density inhibition of growth at $1.0–1.3 \times 10^6$ cells/ml.

We also examined the effect of iodination on a complex cell response – the induction of tyrosine aminotransferase by corticosteroids. Figure 3 shows an induction experiment for an iodinated culture, a culture that had been subjected to the routine washing procedure but which had not been iodinated, and an untreated culture. The kinetics of induction are similar in all three cultures. The difference in the initial level of the activity of the iodinated culture from that of the control culture probably reflects a lower cell density since this difference is maintained at the plateau. It is difficult to recover 100% of

TABLE I. Recovery of Acid Insoluble ^{125}I as Iodotyrosine

	Concentration of Iodide (μM)							
	0		20		100		200	
Labeled species	CPM ($\times 10^{-4}$)	Relative %	CPM ($\times 10^{-4}$)	Relative %	CPM ($\times 10^{-4}$)	Relative %	CPM ($\times 10^{-4}$)	Relative %
Monoiodotyrosine	1.71	97	2.94	81	1.11	56	1.24	55
Di-iodotyrosine	0.06	3	0.69	19	0.87	44	1.00	45

TABLE II. Requirements for Iodination of HTC Cells

Conditions	Protein (mg)	Acid insoluble radioactivity CPM ($\times 10^{-3}$)	Specific radioactivity CPM/mg ($\times 10^{-3}$)
Complete	0.66	2,100	3,200
Minus unlabeled iodide	0.98	180	180
Minus lactoperoxidase	0.89	1.6	1.80
Minus glucose oxidase	0.99	4.5	4.5

Washed HTC cells were placed in 4 tubes at a concentration of 9×10^6/ml in modified Earle's salt solution. The complete reaction mixture contained 100 μmoles tricine pH 7.6, 12.5 μmoles D-glucose and 100 μCi of ^{125}I, 65 munits of lactoperoxidase, 35 munits of glucose oxidase, and 200 μmoles of unlabeled KI.

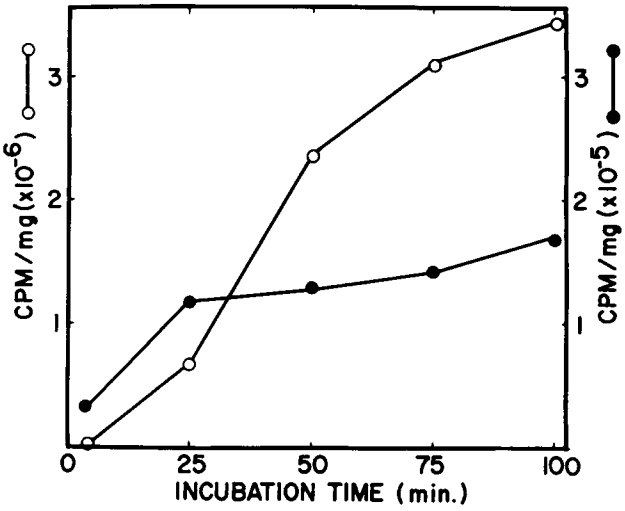

Fig. 2. Time course of iodination of HTC cells in the presence of unlabeled iodide. Closed circles: Five tubes each containing 10^7 washed HTC cells were iodinated with 25μCi ^{125}I for the indicated times at 37°C. No unlabeled iodide was added. Total membrane preparations were prepared and analyzed as described in the legend to Fig. 1. Open circles: Cells were iodinated as described above except 200 μM unlabeled iodide was present in each tube.

the cells from the tubes in which the iodination is performed as some cells stick to the walls of the tubes and pipets. Viability of all three cultures was greater than 90% over the course of the induction experiment. The results presented in Fig. 3 indicate that iodinated cells are responsive to the hormone, and, therefore, indicate that the processes involved in induction, such as the transfer of the hormone into the cell and into the nucleus, the production of tyrosine aminotransferase message and the synthesis of the protein, are not seriously affected by iodination. Since the time course of induction of tyrosine amino transferase is a function of the degradation rate of the enzyme (17), the processes involved in the degradation of the enzyme also are unaffected by iodination.

Cell Localization of the Incorporated Iodide

The cellular localization of incorporated label was examined by histochemical and biochemical techniques. Figure 4 is an autoradiogram of thin sections of iodinated HTC cells. Most of the grains are localized at the cell periphery, and no obvious internal structures, such as the nucleus, have significantly more grains than background associated with them.

Controls in which lactoperoxidase was omitted from the iodination mixture showed no radioactivity associated with the cells. Since no unlabeled cells could be found in the hundreds of fields observed, it was concluded that as a consequence of iodination, virtually all the cells become labeled.

Further evidence for the plasma membrane localization of the incorporated label is presented in Table III. When cells are treated with trypsin after iodination, 70% of the label is removed. Similarly, treatment of cells with trypsin before iodination removed about 70% of the sites available for iodination. The label remaining bound to the cells after trypsin treatment probably is due to the limited bond specificity of this protease.

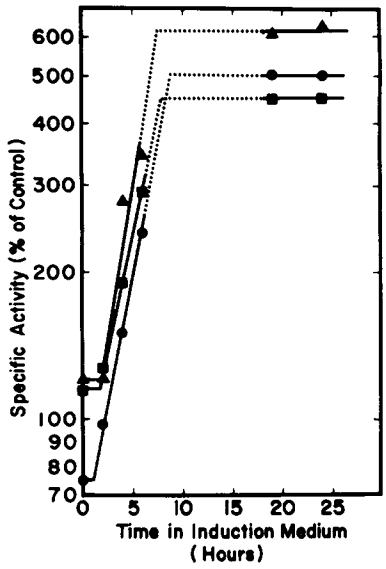

Fig. 3. The effect of iodination on the induction of tyrosine aminotransferase. The induction of tyrosine aminotransferase by dexamethasone was monitored in three cultures each containing 10^8 HTC cells. The cells in one culture (triangles) were removed from growth medium and resuspended at 10^6 cells/ml in medium without serum (induction medium). The culture was divided into two equal parts, and to one was added dexamethasone phosphate, (10^{-6} M). The other half of the culture served as an uninduced control. Aliquots were removed at the indicated times and were assayed for tyrosine aminotransferase as described in Methods. The specific activity at each time point of the induced culture was divided by the specific activity of the uninduced culture. The induced values then were expressed as a percentage of the control specific activity at each time point. For the culture indicated by the trinagles the induced specific activity at 0 hr was 18 units and at 24 hr was 68 units. The dotted line represents the extrapolated portion of the curves. The closed circles represent the relative specific activity of a culture induced with dexamethasone phosphate as described above, except 10^8 cells were iodinated as described in Methods with 50 μCi of ^{125}I in the presence of 200 μM unlabeled iodide before resuspension in induction medium. The specific activity of the induced culture at 0 hr was 8 units and at 24 hr was 54 units. The squares represent a culture of 10^8 cells which underwent the same washing procedures as the iodinated culture but which was not iodinated. The specific activity of the induced culture of this series at 0 hr was 11 units and at 24 hr was 55 units.

Treatment of the cells with pronase was unsuccessful since cell death could not be prevented. Treatment of iodinated cells with hyaluronidase and collagenase removed less than 20% of the bound label, and since these enzymes were crude preparations, this loss of label may be due to contamination with proteolytic enzymes. The data in Table III indicate that most of the label is bound on the exterior surface of the cell and is not found within the lipid matrix of the membrane or on the cytoplasmic surface.

The most convincing evidence for the plasma membrane localization of the incorporated iodide was obtained from cell fractionation studies presented in Table IV. 5′-nucleotidase is an authentic marker enzyme for the plasma membrane of hepatocytes (18). After fractionation of HTC cells this enzyme activity was enriched in the plasma membrane fraction 11.6 fold over the activity in the homogenate with a yield of 1%; the ^{125}I radioactivity in the same preparation of the membrane was enriched 11.8 fold with a similar yield of 1%. The yield of membrane is low in this experiment, probably because no agents were used to stabilize the membrane during isolation (19). The ^{125}I radioactivity and 5′-nucleotidase activity co-purified throughout the membrane isolation procedure.

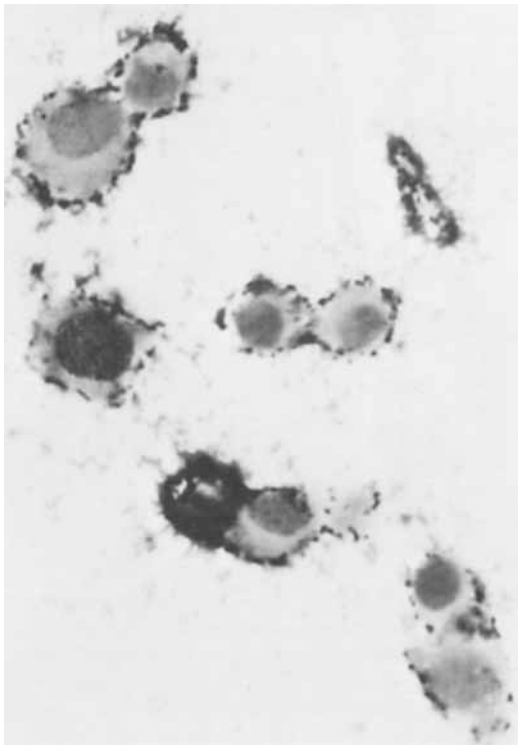

Fig. 4. Autoradiograms of sectioned, iodinated HTC cells. Cells were prepared as described in Methods. (× 1,020).

The biochemical analyses of the plasma membrane preparation indicate that it is relatively free of contamination from soluble proteins as measured by tyrosine aminotransferase activity, 116% of this marker being recovered in the 8,000 g-min supernatant fraction. Glucose-6-phosphatase activity, a marker for the endoplasmic reticulum also was absent in the membrane preparation. However, the presence of [^{3}H] uridine in this fraction suggests that some microsomal contamination is present. The bulk of the labeled RNA and glucose-6-phosphatase activity was recovered in the 8,000 g-min supernatant fraction. Nuclei are not broken extensively during homogenization, and most of the nuclei cosedimented with the plasma membrane in the 8,000 g-min pellet. However, these were separated during the step sucrose gradient sedimentation, and the purified membrane fraction contained little of the [^{3}H] thymidine radioactivity.

By these criteria the plasma membrane fraction appears to be relatively pure. However, neither the purity of the membrane nor the yield is as critical for the objective of this study as is the demonstration that the plasma membrane marker, 5′-nucleotidase, copurifies with the iodine label. This is the case for the membrane preparation of Table IV purified by a sucrose gradient technique. We also have prepared membrane fractions using the aqueous two-phase polymer system of Brunette and Till (9). This method of membrane isolation separates membranes based on their differential partitioning in the two aqueous polymer phases – a principle of separation which is different from the density separations in sucrose gradients. Results presented in Table V show that membranes prepared by the two-phase technique also show an equal enrichment for both the 5′-nucleotidase-specific

activity and the specific radioactivity of the iodine label. While the purification of membranes by this method was quite variable, as judged by enrichment of the two marker activities, the radioactivity and the 5′-nucleotidase activity invariably copurified. The variability in purity of the membrane fraction by the sucrose method was less than that of the two-phase method, but even in preparations of quite different "purity" the 5′-nucleotidase and the incorporated label showed equal enrichment.

TABLE III. Effect of Trypsin on Iodination of HTC Cells

Conditions	^{125}I-acid insoluble radioactivity (CPM × 10^{-5}/10^7 cells)
Iodinated HTC cells	19.0
Iodinated HTC cells + trypsin	5.4
Iodinated HTC cells + collagenase-hyaluronidase	15.4
HTC cells treated with trypsin then iodinated	5.8

HTC cells, 1 × 10^7 cells/ml in modified Earle's balanced salt solution, were incubated with trypsin, 25 μg/ml, either before or after iodination for 15 min at 37°C. Similarly, cells after iodination were incubated with a mixture of hyaluronidase and collagenase, 25 μg/ml. The cells then were washed three times with modified Earle's salt solution, and acid insoluble ^{125}I was determined as described in Methods and Materials.

TABLE IV. Analysis of Hepatoma Cell Fractions

	Homogenate	8,000 g-min supernatant	8,000 g-min pellet	1.6/1.8 M sucrose interface membrane
Protein (mg)	100	68	10	0.1
DNA (CPM × 10^{-6}/mg)	1.64	0.04	–	0.30
RNA (CPM × 10^{-6}/mg)	2.10	1.90	–	0.50
^{125}I-acid insoluble radioactivity (CPM × 10^{-6}/mg)	1.35	0.23	6.50	16.0
5′-nucleotidase (units/mg)	3.9	1.3	16.0	45.6
Glucose-6-phosphatase (units/mg)	5.4	7.5	2.7	0
Succinic dehydrogenase (units/mg)	0.017	0.020	0.055	0
Tyrosine amino transferase (units/mg)	0.50	0.85	0.09	0.08

HTC cells (2 × 10^7) were iodinated with ^{125}I and mixed with about 5 × 10^8 unlabeled cells. A plasma membrane fraction was prepared by the step sucrose gradient method. Protein, ^{125}I-acid insoluble radioactivity, 5′-nucleotidase, glucose-6-phosphatase, succinic dehydrogenase, and tyrosine aminotransferase were monitored during fractionation of these cells. One unit of 5′-nucleotidase and 1 unit of glucose-6-phosphatase activity is equivalent to 1 nm of phosphate formed per minute. The activities of succinic dehydrogenase and tyrosine amino transferase are expressed in arbitrary units based on the decrease in optical density at 600 nm for succinic dehydrogenase and the increase in optical density at 332 nm for tyrosine aminotransferase. Two other cultures of HTC cells were grown for 48 hr in the presence of either [5-^{3}H]uridine (1μCi/ml) or [6-^{3}H]thymidine (2μCi/ml) prior to preparation of a plasma membrane fraction. Acid insoluble radioactivity in each fraction except the 8,000 g-min pellet was determined by standard procedures (see Methods).

TABLE V. ^{125}I-acid Insoluble Radioactivity and 5′-nucleotidase Activity of Hepatoma Cell Membrane Preparations

Experiment	Method of membrane isolation	5′-nucleotidase (units/mg) membrane protein	Specific activity relative to homogenate	^{125}-I acid insoluble specific radioactivity relative to homogenate
1	Aqueous 2-phase polymer	19	2	2.5
2	Aqueous 2-phase polymer	39	7	8.0
3	Aqueous 2-phase polymer	62	13	12.4
4	Sucrose gradient	27	6	6
5	Sucrose gradient	48	11.6	11.0

It is possible that the plasma membrane preparation of HTC cells is contaminated with either lysosomes or lysosomal membranes. This contamination would not be a serious problem unless the proteins of the lysosome were labeled during iodination. It is conceivable that lactoperoxidase is stable in the lysosomes and that such labeling could occur as a result of pinocytosis during the iodination reaction. If pinocytosis were an important means of internal iodination, it should be a time-dependent process (20). The longer the cells are incubated in the presence of the iodinating enzymes the more these enzymes should be interiorized. HTC cells, therefore, were incubated for varying periods of time in the presence of lactoperoxidase at the level used during iodination. Table VI shows the results of this experiment. Addition of lactoperoxidase to the washed cells prior to homogenization resulted in a fully active iodination system. In the absence of added lactoperoxidase little iodination occurred, indicating the absence of significant iodinating capacity endogenous to the HTC cell. The cultures incubated with the enzyme prior to washing showed somewhat more incorporation of the iodine label but showed no time dependency of association, and in fact the 60 min incubation resulted in somewhat lower radioactivity incorporated than the 2 min incubation. This absence of a time dependent correlation argues against pinocytosis as a potential source of internal labeling. The presence of lactoperoxidase activity after 2 min of incubation with the enzyme probably indicates that the washing procedure is not completely effective in removing all the lactoperoxidase adhering to the cell.

In the analysis of the membrane fraction in Table IV only 1% of the total iodine label and the 5′-nucleotidase activity was recovered in the purified plasma membrane. It is possible then that some of the labeled material in the homogenate was selectively lost during purification of the membrane. To test for this possibility the iodinated polypeptides in each cell fraction were analyzed by dodecyl sulfate–polyacrylamide gel electrophoresis. The results are presented in Fig. 5. Lane A shows the Coomassie staining pattern of the polypeptides in the homogenate. Despite the extreme complexity of this pattern, clear differences can be seen between it and the autoradiogram of the homogenate displayed in lane B. Some of the Coomassie bands are not labeled, and the intensity of labeling of different bands does not correlate well with the staining intensity. Lane C is an autoradiogram of the purified membrane. Comparison of this pattern with the autoradiogram of the homogenate in B shows that all of the labeled proteins in the homogenate are present in the purified membrane. Thus, none of these proteins was selectively lost during cell fractionation. Lane D in Fig. 5 is a Coomassie-stained pattern of the plasma membrane preparation. In C and D, there is a remarkable similarity between the patterns,

TABLE VI. Association of Lactoperoxidase with Washed Cells

Addition of lactoperoxidase	Protein (mg/ml)	CPM/ml ($\times 10^{-3}$)	Specific radioactivity CPM/mg ($\times 10^{-4}$)
No addition	2.1	89	4.2
Added after washing	2.0	16,000	800
Added 60 min prior to washing	1.6	320	20
Added 30 min prior to washing	1.8	590	33
Added 15 min prior to washing	2.1	740	35
Added 2 min prior to washing	2.0	680	34

Four tubes each containing 10^7 washed HTC cells in modified Earle's salt solution (1 ml) were incubated for the specified intervals with 60 munits of lactoperoxidase. Two control tubes containing 10^7 cells each in modified Earle's salt solution (1 ml) were incubated without lactoperoxidase. The temperature of incubation was 30°C, and during the incubation the cells were 95% viable. Following this initial incubation the cells from each tube were washed twice with normal saline and suspended in 2 ml of 0.05 M tris HCl pH 7.6, containing 2 mM $CaCl_2$. Each culture was then homogenized as described in Materials and Methods and to each homogenate was added 117 μmoles tricine, pH 7.6, 12 μmoles D-glucose, and 25 μCi ^{125}I. One control received 60 munits of lactoperoxidase prior to homogenization. The iodination reaction was begun with the addition of 35 munits of glucose oxidase to each homogenate and was terminated after 30 min at 37°C by precipitation with 10% trichloroacetic acid. The precipitates were washed by the tube method described in Materials and Methods, and the precipitates were dissolved in 0.5 M NaOH for radioactivity and protein determinations.

with many of the labeled bands corresponding in a roughly quantitative fashion with the Coomassie staining bands. This should be contrasted with the homogenate staining and labeling patterns in lanes A and B. Several stained bands in D do not have a corresponding labeled band, suggesting that these polypeptides are not accessible to the lactoperoxidase probe, either because they represent contamination of the plasma membrane preparation or because they are proteins which are located on the cytoplasmic side of the membrane. One of the major polypeptides associated with the membrane has an apparent molecular weight of about 45,000 daltons. This polypeptide is not iodinated and probably is the actin-like protein which is associated with the interior surface of many eukaryotic cells (21). The fact that it is not labeled with iodine is additional evidence that only externally disposed membrane proteins are labeled. The degree of coincidence between the Coomassie-stained pattern and the autoradiographic pattern of the membrane polypeptides suggests that polypeptides containing tyrosines accessible to iodination comprise a signficant proportion of the protein mass of the membrane. Rigorous proof of this, however, must await the development of separatory systems that can resolve individual proteins of the membrane as homogeneous species. An indication of the polypeptide complexity of the isolated plasma membrane is shown in Fig. 5, lane E. In this experiment, a membrane fraction was purified by the sucrose gradient method and iodinated with lactoperoxidase. A large number of polypeptides are labeled, including all of those which are iodinated in

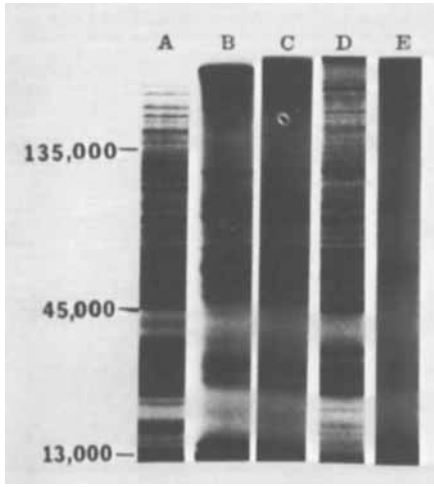

Fig. 5. Dodecyl sulfate-polyacrylamide gel electrophoresis of the polypeptides in HTC cell fractions. Iodinated HTC cells, 5×10^8, were homogenized in 0.01 M tris-Cl buffer, pH 7.0, containing 0.005 M KCl and 0.001 M Mg Cl_2. A plasma membrane fraction was prepared by the step sucrose gradient method. An aliquot, 100 μg, of the homogenate and the plasma membrane fraction was dissociated in 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol by heating for 5 min at 100°C. Polypeptides were separated by electrophoresis in slab gels of 9% acrylamide. A, homogenate stained for protein; B, autoradiogram of the homogenate; C, autoradiogram of the purified plasma membrane; D, plasma membrane stained for protein; E, autoradiogram of a plasma membrane fraction iodinated after isolation by the step sucrose gradient method.

intact cells. The 45,000 mol wt actin-like protein is also labeled. However, the background of labeling is so dense that it is impossible to resolve most of the bands. Thus, the isolated membrane has many more proteins accessible to iodination, but many of these proteins are present in the membrane in very low concentration.

We were concerned that the large amount of iodinated material at the low molecular weight region of the acrylamide gels might be due to proteolytic cleavage of high molecular weight membrane proteins during isolation. However, neither direct homogenization of the cells in sodium dodecyl sulfate nor preparation of membranes by the usual method but in the presence of 50 μg/ml of phenylmethylsulfonylfluoride, TPCK, TLCK, and iodoacetamide affected the pattern of labeled polypeptides.

Another possible source of the low molecular weight material could be breakage of peptide bonds by ionizing radiation. We have found, however, that when a number of high molecular weight standard proteins including β-galactosidase (133,000 mol wt), thyroglobulin (330,000 mol wt), and phosphorylase a (98,000 mol wt) were iodinated under the same conditions as the HTC cells with 50–100 μCi of either ^{131}I or ^{125}I, no new, low molecular weight fragments were formed either immediately after iodination or after a week at −20°C. Unless there are unusually labile peptide bonds in some membrane proteins it would seem unlikely that any extensive cleavage results from ionizing radiation.

High Resolution Dodecyl Sulfate Electrophoresis of Iodinated Membrane Polypeptide

HTC cells were labeled with ^{125}I or ^{131}I in the presence of varying amounts of unlabeled iodide. A plasma membrane fraction was prepared from each sample and approximately equal amounts of radioactive material from each sample were applied to a

dodecyl sulfate gel composed of 7.5% acrylamide on top of 10% acrylamide. This gel was used to resolve some of the high molecular weight polypeptides and some of the low molecular weight polypeptides which were not resolved in Fig. 5. Autoradiography of this gel (Fig. 6) shows that iodination with different amounts of carrier iodide does not affect significantly the pattern of labeled polypeptides. Differences exist between the ^{131}I-labeling pattern and the ^{125}I pattern, but these are due to the greater sensitivity and resolution when ^{131}I is used. At least 54 distinct bands are evident in the ^{131}I autoradiogram.

Analysis of Carbohydrate Content of the Plasma Membrane

Staining of the plasma membrane polypeptides of HTC cells, separated on SDS gels, with periodic acid-Schiff reagent, failed to reveal any protein-bound sugar. However, this technique is quite insensitive. A more sensitive means of detecting surface carbohydrates is to grow the cells in the presence of labeled L-fucose or D-glucosamine. These molecules presumably are incorporated fairly specifically into the carbohydrate chains of membrane glycoproteins, with little of the label incorporated into other macromolecules. When HTC cells were grown in the presence of L-[^{14}C] fucose, acid insoluble label was enriched in both microsomal and plasma membrane fractions (Table VII). Most of the incorporated

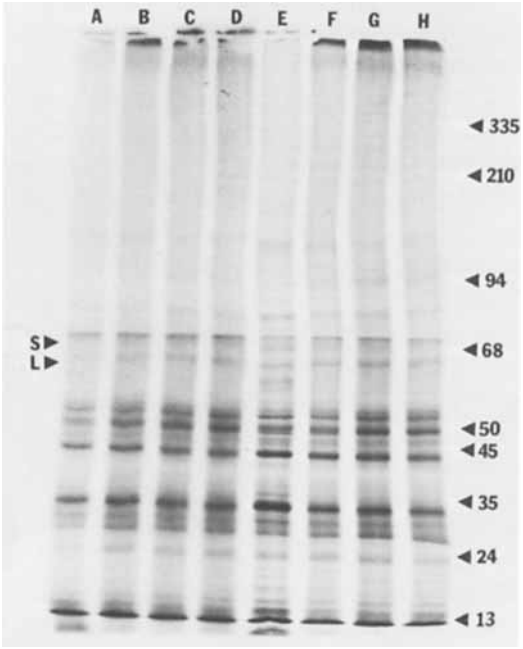

Fig. 6. Autoradiogram of plasma membrane polypeptides from iodinated HTC cells. HTC cells were iodinated with ^{125}I (A–D) or ^{131}I (F–H) in the presence of varying amounts of unlabeled iodide, A and E, no unlabeled iodide; B and F, 50 μM unlabeled iodide; C and G, 200 μM unlabeled iodide; D and H 250 μM unlabeled iodide. Crude membranes were prepared from each sample as described in the legend to Fig. 1. A constant amount of radioactivity was applied to each slot of the slab gel. The gel was composed of 7.5% acrylamide on top of 10% acrylamide. The numbers indicate the approximate mobilities of several standard proteins of known molecular weight (expressed as mol wt $\times 10^{-3}$). L: the mobility of the major component in the preparation of lactoperoxidase used for iodination. S: the mobility of the major protein component in the medium in which the cells are grown. The marker proteins were iodinated and localized by autoradiography. Each marker was run in the presence of unlabeled, crude HTC cell membranes.

TABLE VII. Analysis of HTC Cell Fractions for [^{14}C] Fucose Acid Insoluble Radioactivity

Fraction	Protein (mg)	^{3}H (DPM × 10^{-6}/mg)	^{14}C (DPM × 10^{-5}/mg)	Ratio ^{14}C:^{3}H
Homogenate	43	0.94	0.60	0.064
Supernatant, 8,000 g-min	29	0.93	0.47	0.051
Supernatant, 230,000 × g	25	1.33	0.20	0.015
Pellet 230,000 × g	3.3	0.68	1.1	0.162
Pellet, 2.2 M sucrose	2.7	0.59	0.34	0.058
Plasma membrane	0.4	1.35	2.15	0.160

HTC cells were grown for 24 hr in complete growth medium containing L-[1-^{14}C] fucose, 1 μCi/ml, and L-[4, 5-^{3}H] leucine, 1 μCi/ml. The cells were washed three times with modifed Earle's balanced salt solution and homogenized in 0.01 M tris-Cl, pH 7.0, containing 0.005 M KCl and 0.001 M mg Cl_2. A plasma membrane fraction was prepared by the step sucrose gradient method. The 8,000 g-min supernatant fraction was centrifuged at 230,000 × g for 1 hr to obtain a fraction enriched for microsomes (230,000 × g pellet). A fraction enriched for nuclei was obtained from the step sucrose gradient (2.2 M sucrose pellet). The ratio of ^{14}C:^{3}H radioactivity indicates the relative enrichment of glycoprotein to leucine containing proteins.

fucose was confined to nine or ten polypeptides in the purified plasma membrane fraction as resolved by dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography (Fig. 7). The same polypeptides are labeled when cells are grown in the presence of D-[^{14}C] glucosamine. In addition, the Coomassie staining bands which contain the ^{14}C sugars appear to be iodinated. This is consistent with the surface location of membrane glycoproteins. The labeled glycoproteins have apparent molecular weights greater than 50,000 but due to the aberrant migration of glycoproteins in SDS gels (22), these estimates should be considered as tentative.

DISCUSSION

We have taken some care in these studies to demonstrate that the label incorporated into HTC cells by lactoperoxidase-catalyzed iodination is confined specifically to the proteins of the plasma membrane. To reiterate, all of the incorporated label can be recovered from the cells as either monoiodotyrosine or di-iodotyrosine and therefore must be confined to protein. The incorporated label is localized primarily at the cell periphery as shown by autoradiography of sectioned cells. Much of the label can be released from the cell by hydrolysis with trypsin, but it is relatively resistant to hydrolysis by collagenase and hyaluronidase. Finally, the incorporated iodide co-purifies during cell fractionation with an enzyme, 5′-nucleotidase, which is an authentic marker for the plasma membrane of liver cells (18). From these results, we conclude that the incorporated radioactive iodide is a valid marker for the plasma membrane and for those polypeptides in this membrane accessible to the lactoperoxidase probe. Recenly, Hubbard and Cohn (23) have examined the specificity of the iodination reaction for the proteins of the mouse-L cell membrane. In these cells the label was confined primarily and probably exclusively to tyrosine residues in polypeptides of the plasma membrane. Thus, our results on the plasma membrane localization of the incorporated iodide in HTC cells agree with the results of Hubbard and Cohn on the localization in mouse-L cells. This being the case, there can be little doubt that the iodinated species separated on dodecyl sulfate-polyacrylamide gels are authentic membrane polypeptides. The complexity of the membrane is indicated by the fact that there are at least 50 polypeptides in HTC cells,

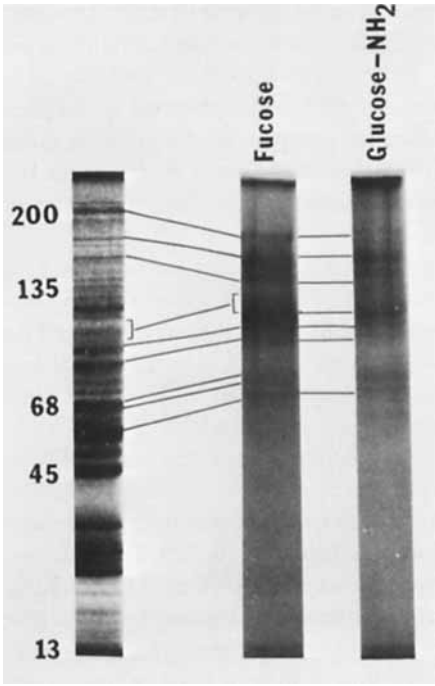

Fig. 7. Autoradiogram of HTC cell plasma membrane fractions labeled with [^{14}C] fucose and [^{14}C] glucosamine as separated on dodecyl sulfate-polyacrylamide gels. The details for labeling of cells and isolation of the membrane are the same as presented in the legends to Table VII except that the cells were not exposed to [^{3}H] leucine. Separating gel concentration was 9%. The lines indicate the relationship of the carbohydrate containing polypeptides to each other and to the Coomassie-stained polypeptides of the membrane. Each of the Coomassie-stained bands which corresponds to a carbohydrate labeled band also corresponds to an iodinated band (by reference to Fig. 5).

varying in apparent molecular weight from around 10,000 to over 300,000, that have tyrosine residues accessible for iodination. These polypeptides are probably not minor components of the membrane. Rather, their intensity of labeling and the coincidence between the autoradiographs of the separated iodinated species and the Coomassie-stained species suggest that much of the protein in the membrane has residues exposed on the outside of the cell. Furthermore, the polypeptides that are iodinated fulfill the criteria for integral membrane proteins as defined by Singer (24). With the exception of the very low molecular weight species, they cannot be dissociated from the membrane except by hydrophobic bond-breaking reagents.*

We have also iodinated HeLa and mouse-L cells using the same reaction conditions as for HTC cells. HTC cells are not unusual in having most of their membrane protein accessible to labeling from outside the cell. The three different cell lines have a similar set of membrane polypeptides, at least as resolved by the one dimensional dodecyl sulfate-acrylamide gel system.*

It is possible that the complex pattern of iodinated polypeptides from HTC cells is due to either incomplete dissociation of the membrane or reaggregation of polypeptides after dissociation. However, we did not observe any appreciable change in the pattern of

*Tweto, J., and Doyle, D., unpublished observations.

iodinated polypeptides when different sulfhydryl reductants and blocking agents were used during dissociation of the membrane or when varying concentrations of membranes were dissociated. Further, there was no shift in the mobility of iodinated standard proteins when they were dissociated and electrophoresed in the presence of unlabeled membranes. It is unlikely that the complex labeling pattern is due to noncovalently bound iodide since membrane preparations which were washed with 10% trichloroacetic acid containing 25 mM metabisulfite gave the same electrophoretic pattern as preparations that were not acid treated.

Carbohydrate precursors of glycoproteins, particularly L-fucose, have been proposed as specific labels for the plasma membrane of some cells (25). Neither L-fucose nor D-glucosamine become incorporated uniquely into any HTC cell fraction, at least after long-term labeling. However, fucose is enriched in the microsomal and plasma membrane fractions. There are at least nine major species in the plasma membrane which become labeled after administration of these sugars to HTC cells.

Finally, under the conditions used for iodination of HTC cells, viability is not affected noticeably nor is there a pronounced effect on the induction of tyrosine aminotransferase. Further, there is no difference in the turnover of either total cell protein or membrane protein after iodination (26, 27). Thus, the fate of the iodinated membrane polypeptides can be studied. In HTC cells (27), as also probably in mouse-L cells (28), all of the iodinated proteins are removed from the membrane at similar rates. Since these proteins constitute the bulk of the membrane protein, there must then be some sort of coordinate regulation of the synthesis and/or insertion into the membrane of a large number of different polypeptides.

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