

EXTERNALLY DISPOSED PLASMA MEMBRANE PROTEINS

I. Enzymatic Iodination of Mouse L Cells

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ABSTRACT

The enzymatic iodination technique has been utilized in a study of the externally disposed membrane proteins of the mouse L cell. Iodination of cells in suspension results in lactoperoxidase-specific iodide incorporation with no loss of cell viability under the conditions employed, less than 3% lipid labeling, and more than 90% of the labeled species identifiable as monoiodotyrosine. 90% of the incorporated label is localized to the cell surface by electron microscope autoradiography, with 5–10% in the centrosphere region and postulated to represent pinocytic vesicles. Sodium dodecylsulfate-polyacrylamide gels of solubilized L-cell proteins reveals five to six labeled peaks ranging from 50,000 to 200,000 daltons. Increased resolution by use of gradient slab gels reveals 15–20 radioactive bands. Over 60% of the label resides in approximately nine polypeptides of 80,000 to 150,000 daltons. Various controls indicate that the labeling pattern reflects endogenous membrane proteins, not serum components. The incorporated ^{125}I , cholesterol, and one plasma membrane enzyme marker, alkaline phosphodiesterase I, are purified in parallel when plasma membranes are isolated from intact, iodinated L cells. The labeled components present in a plasma membrane-rich fraction from iodinated cells are identical to those of the total cell, with a 10- to 20-fold enrichment in specific activity of each radioactive peak in the membrane.

The introduction of reagents which specifically react with externally disposed constituents of the plasma membrane provides a method for probing an interesting domain of the cell's surface and for following membrane flow and degradation (2, 5, 8, 9, 27, 36, 48, 53, 59). We previously reported the successful use of a glucose oxidase-lactoperoxidase iodination system in a study of the human red blood cell membrane (21). The use of this technique has now been extended to an examination of the plasma membrane of the L cell, a murine fibroblast derived from a C3H/An mouse (14).

The L cell was chosen because it is a relatively homogeneous cell population and it can be grown easily in either monolayer or single cell suspension

culture (30). The L cell divides rapidly so large numbers can be easily obtained (10^8 in 500 ml), and it will phagocytose polystyrene latex particles, thus internalizing a large proportion of its plasma membrane in a short time (17). In addition, there are published reports on the isolation of L-cell plasma membrane (63, 64).

A brief account of this work has been given elsewhere (22).

MATERIALS AND METHODS

Cell Cultivation

LM cell stocks were obtained from Dr. G. Acs of the Muscle Institute and the American Type Culture Collec-

tion, Rockville, Md. (designated LM26). L929 cells were obtained from Dr. R. Hand of the Rockefeller University. The growth medium was 5–10% fetal calf serum (FCS)¹ (Grand Island Biological Co., Grand Island, New York) which had been stored 2 wk at 4°C, heat inactivated for 30 min at 56°C, and then filtered (0.45 μ m, Millipore Co., Bedford, Mass.) into minimum essential medium (MEM) (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.) to which 1,000 U/ml penicillin were added routinely and 0.60 mg/ml Tylocine (Grand Island Biological Co.) added occasionally. 20 mM *N*-2-hydroxyethyl piperazine-*N*-2-ethanesulfonic acid (HEPES) (Schwarz/Mann) at pH 7.4 was added to maintain a more constant pH than was possible with NaHCO₃. However, examination of cells grown in this organic buffer revealed the presence of vacuoles absent in cells buffered only with CO₂, and subsequently this buffer was omitted. Monolayer stocks were maintained on 100 mm petri dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.), but cells were grown primarily as suspension cultures at $2-7 \times 10^5$ /ml in 50 ml tubes to which no more than 30 ml of cell suspension were added. Cells were kept in suspension by inserting the tubes into a wheel revolving at 60 rpm and growth medium was replenished daily without CO₂ gassing. Large numbers of cells were grown in spinner flasks (150–1,000 ml, Bellco Glass Inc., Vineland, N. J.) in the same media. Growth rates were measured throughout the course of this work and the doubling time ranged from 16 to 20 h. Cells were also grown in 7% horse serum (Grand Island Biological Co.) which was heat inactivated but not filtered, in 0.5% Bacto-peptone (Difco Laboratories, Detroit, Mich.), in Medium 199 (Microbiological Associates, Bethesda, Md.), or in 0.6% bovine serum albumin (BSA) (Armour Pharmaceutical Co., Chicago, Ill.). The last two media did not support growth in a manner comparable to that of the horse or fetal calf sera; however, cells were viable for up to 1 mo, the longest either media was used.

Both the L929 (14) and LM (41) cells used in this study display similar morphology in suspension culture. Cells contain rough endoplasmic reticulum which is often dilated and filled with amorphous material. Multiple stacks of Golgi are evident in the centrosphere region, as are numerous small vesicles.

¹ *Abbreviations used in this paper:* A, acrylamide; AP, ammonium persulfate; BB, bromphenol blue; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; FMA, fluorescein mercuric acetate; GO, glucose oxidase; HEPES, *N*-2-hydroxyethyl piperazine-*N*-2-ethanesulfonic acid; LPO, lactoperoxidase; MBA, *N,N'*-bis-methylene acrylamide; 2-ME, 2-mercaptoethanol; MEM, minimum essential medium; MIT, mono-iodotyrosine; PAS, periodic acid-Schiff; SDS, sodium dodecyl sulfate; SP, phosphate-buffered saline; TCA, trichloroacetic acid; TEMED, *N,N,N',N'*, tetra-methylethylenediamine.

General Labeling Procedures

IODINATION CONDITIONS: L cells were iodinated in suspension at 4°C, 10°–12°C, 23°C, and 37°C from 0 to 30 min. The following conditions were chosen for routine iodination of L cells to achieve reasonable incorporation efficiency in a short time and to minimize membrane activity such as pinocytosis or diffusion of proteins in the membrane plane. Cells were rinsed twice in cold phosphate-buffered saline (SP) at $\sim 2 \times 10^6$ cells/ml and resuspended in 20 mM glucose in SP at a cell concentration of $4-6 \times 10^6$ cells/ml. Lactoperoxidase (LPO, EC no. 1.11.1.7, Calbiochem, and the generous gift of S. J. Klebanoff), glucose oxidase (GO, EC no. 1.1.3.4, Sigma Chemical Corp., St. Louis, Mo., Type V), and Na¹²⁵I (New England Nuclear Corp., Boston, Mass., carrier-free) were added to final concentrations per milliliter of 3–6 mU,² 4–8 mU,² and 100 μ Ci, respectively. The reaction mixture was rotated at 5 rpm for 10 min at 4°C and diluted into 20 vol cold MEM, and the cells were sedimented away from the soluble LPO and GO. The cells were then rinsed at least twice in large volumes of ice-cold SP. The entire procedure took from 1.5 to 2 h, and cell viability was maintained at 95% throughout as assessed by trypan blue exclusion (0.16%) and the ability of iodinated cells to attach to a surface and spread. Controls were performed which consisted of incubation of cells with all reagents except LPO (to assess nonenzymatic iodination) or prior incubation of cells with LPO followed by two washes and addition of the remaining reagents (to assess adsorption of LPO to cells).

RADIOACTIVITY MEASUREMENTS: In early L-cell experiments incorporated radioactivity was measured by trichloroacetic acid (TCA) precipitation of a suitable aliquot followed by centrifugation and numerous rinses of the pellet. In experiments having many samples this technique proved cumbersome and free ¹²⁵I was always present in the TCA-pelleted material. The "disk-batch" method (38) was found to be an excellent solution to these problems. A number of rinse procedures were tested to assess adsorption of free ¹²⁵I or transfer of incorporated label from one disk to another. The following procedure was routinely used. An aliquot (≤ 50 μ l) of a labeled sample was pipetted onto a glass fiber paper disk (2.4-cm diam, Whatman, GF/C, W. & R. Balston, Ltd., Maidstone, Kent, England), and the disk was dropped into 500 ml cold 10% TCA, 50 mM K¹²⁷I for over 2 h, rinsed three times in 400 ml 10% TCA, once in 250 ml 90% acetone at –20°C, air dried, rolled into a cylinder, and counted directly in a gamma spectrometer (Packard Instrument Co., Downers Grove, Ill.) with $\sim 50\%$ efficiency. As many as 30 disks could be processed in one beaker and a blank disk always contained

² One unit of GO or LPO activity is the amount of enzyme necessary to produce (GO) or consume (LPO) 1 μ mol H₂O₂ per min under the assay conditions previously outlined (21). The very low activities used ~ 5 nmol/ml/min, represented approximately 5 μ g LPO and 0.5 μ g GO.

less than 3% of the radioactivity of the lowest sample disk. There was no significant adsorption of free ^{125}I as indicated by removal of greater than 99.8% of $1\ \mu\text{Ci}$ free Na^{125}I applied to a disk in the presence of unlabeled cell protein.

CHARACTERIZATION OF THE IODINATED SPECIES: Procedures similar to those described for the red cell (21) were also performed on iodinated L cells to ascertain the nature of the iodinated species. Filter disks were used in enzyme digestion studies. Iodinated cell material was precipitated with TCA onto disks and rinsed as described above, dried, and placed in a glass scintillation vial. Crude trypsin or pancreatin at 2.5 mg/ml in Tris-HCl, pH 8.0, 0.05% CaCl_2 , and one drop toluene were added to the vial, and the mixture was incubated for 24 h at 37°C with agitation. Additional trypsin was added for another 24 h, the digest was precipitated with TCA, and the supernates were pooled and quantitatively analyzed on a Sephadex G-25 Fine column as described previously (21). The elution profiles of known standards, ^{125}I , M^{125}IT , and diiodotyrosine (OD_{280}), were compared to that of the unknown. The disk-batch method was also used to assess the lipid labeling of L cells. After a suitable number of rinses in TCA, the filter disks were either air dried immediately or rinsed in 90% acetone or 3:1 EtOH/Et $_2\text{O}$, at -20°C for 1 h, dried, and counted.

Isolation of L Cell Plasma Membrane

Methods published by Warren and colleagues (63, 64) employing Zn^{+2} , fluorescein mercuric acetate (FMA), and Tris-HCl at pH 7.4 and 8.0 as membrane-"stabilizing" agents were followed and found to be time consuming and quite variable in yield and purification, as assessed by TCA-insoluble ^{125}I per milligram protein. The more rapid Tris-HCl method of Atkinson and Summers (4), with modifications, was satisfactory for L cells and the use of FMA in the homogenization step of this membrane isolation procedure was found to increase the yield. Three membrane isolation procedures were finally used, two involving FMA, and the third using Tris-HCl.

FMA 1: The FMA membrane isolation procedure of Klenk and Choppin (28) for monolayer cells as modified by Lazarowitz et al. (31) was used with the following changes. The initial steps which removed the cells from their substratum were omitted for the iodinated L cells, already in suspension. Soluble proteins were separated from the membrane ghosts in the second centrifugation step by layering the postnuclear supernate in 30% wt/wt sucrose (30-ml aliquots) over 40% sucrose (20 ml) and centrifuging for 15 min at 27,000g (15,000 rpm, SW 25.2, Beckman L2, Beckman Instruments, Inc., Palo Alto, Calif.). An additional sucrose layer of 35% was included in the final discontinuous gradient. The membranes found at the 55%/60% interface, the 60% layer, and the 60%/65% interface after centrifugation were pooled and

rinsed, and constituted the final membrane-enriched fraction.

TRIS-HCl: The Tris-HCl membrane isolation procedure of Atkinson and Summers (4) was followed with several modifications for L cells. Homogenization of the cells in 0.01 M Tris-HCl, pH 7.4 or pH 8.0, yielded rather unstable ghosts which exhibited membrane vesiculation and reduction in size during the isolation. Neither azide nor iodoacetate improved the yield, so both were omitted. Final concentrations of 1 mM MgCl_2 and 1.5 mM CaCl_2 were added to the homogenate to stabilize nuclei, but no NaCl was added. Finally, the composition of the discontinuous gradient used to obtain a membrane-enriched fraction was the postnuclear supernate in 0.01 M Tris-HCl (8 ml) over 30% wt/wt sucrose (15 ml) and 50% wt/wt sucrose (7 ml). After centrifugation for 20 min at 6,600 g (8,000 rpm, SW 25.1, Beckman L2) membrane ghosts were found predominantly at the 30%/50% interface, were rinsed as described by Atkinson and Summers, and were recycled through the discontinuous gradient as described above.

FMA 11: Iodinated L cells were homogenized just as in the FMA I procedure (31) but sucrose was not added. The nuclei and whole cells were removed by rapid centrifugation (step I, 30 s, 1,500 g, 2,500 rpm, International, PR-2) as in the Atkinson and Summers procedure (4), the pellets were washed twice in 0.079 M NaCl, supernates pooled, and 7-10-ml aliquots layered over 30% (28 ml), 55% (10 ml), and 60% (10 ml) sucrose. The gradient was centrifuged for 15 min at 8,700 g (step II, 8,500 rpm, SW 25.2, L2). The 30%/55% and 55%/60% interfaces, and the 55% layer contained the majority of membrane ghosts. These fractions were pooled, diluted with H_2O , and sedimented (step III) for 5 min at 9,750 g (9,000 rpm, SW 25.2 L2). The pellet was resuspended in 10 mM Tris-HCl, pH 8.0, layered over 30% and 50% sucrose, and centrifuged as in the Tris-HCl procedure described above (steps IV and V).

Gel Electrophoresis

SLAB GELS AND SAMPLE SOLUBILIZATION: The basic gel system used was that of Maizel's SDS-discontinuous pH system with modifications (37). Gels were prepared from stocks of 30% by weight acrylamide (A) and 0.86% *N,N'*-bis-methylene acrylamide (MBA). The final concentrations in the separation gel were 0.375 M Tris-HCl, pH 8.9, and 0.1% SDS. Gels were polymerized chemically by the addition of 0.05% by volume *N,N,N',N'*-tetraethylmethylenediamine (TEMED) and 0.025% ammonium persulfate (AP). Separation gels consisted of either homogeneous 7.5% acrylamide or a continuous gradient of 7.5-15% acrylamide, the 15% solution containing 10-15% sucrose. The slabs were 1.1 mm or 2.5 mm thick and 13-14 cm or 25 cm in length. The stacking gels of 3% acrylamide and 1.5-2.0 cm length contained 0.060 M Tris-0.032 M phosphate,

pH 6.8, and 0.1% SDS, and were polymerized with 0.05% AP-0.05% TEMED. The electrode buffer, pH 8.3, contained 0.050 M Tris, 0.192 M glycine, and 0.1% SDS. Samples contained final concentrations of 0.060 M Tris-0.032 M phosphate, pH 6.8, 2.5% SDS, 10% glycerol, 5% 2-mercaptoethanol (2-ME), and 0.001% bromphenol blue (BB) as tracking dye. The samples were heated at 100°C for 3 min. Electrophoresis was carried out at a total current of 10–20 mA until the tracking dye reached the bottom of the gel (~7 h for 13 cm and 14 h for 25 cm slabs of 1 mm thickness).

TUBE GELS AND SAMPLE SOLUBILIZATION: 0.1% SDS-polyacrylamide gels (6 mm in diameter, 100 mm in length) contained final concentrations of 0.1% SDS, 0.1 M Na phosphate, pH 7.2, 0.02 M ethylene diamine tetraacetic acid (EDTA), 0.1% AP, 0.05% TEMED, 5%, 7.5%, or 10% acrylamide by weight, and varying amounts of MBA by weight to give an A/MBA ratio of 20:1–37:1 depending on the resolution desired. Samples contained final concentrations of 3% SDS, 3% 2-ME, 10 mM Na phosphate, pH 7.2, 8% sucrose, and 0.005% BB. The suspension was mixed vigorously and heated at 100°C for 3 min.

SAMPLE PREPARATION OF L CELLS AND SUBCELLULAR FRACTIONS BEFORE SOLUBILIZATION: Two procedures were used in the preparation of whole iodinated L cells for solubilization and electrophoresis in the SDS systems described above. In the first, whole cells were rinsed well, centrifuged into a pellet, and solubilized directly in the appropriate SDS-buffer system or lysed in 10% sucrose, and aliquots were solubilized. In the second, 1 vol L cells in SP was precipitated with 1 vol cold 10% TCA, rinsed once with 10% TCA, twice in 90% acetone at –20°C, and solubilized. In either preparation, *all* of the radioactivity incorporated into cellular constituents was analyzed as compared to a variable 75–90% when postnuclear supernates were used for gel analysis.

Subcellular fractions were solubilized directly in the appropriate dissociating system. Sucrose or glycerol was omitted from those samples already containing sucrose.

POSTELECTROPHORESIS: After electrophoresis, tube and slab gels were stained for protein with 0.2% Coomassie blue (Schwarz/Mann) in 50% methanol-7% acetic acid (37), or for carbohydrate (67) with the periodic acid-Schiff reagent (PAS) after prolonged washing in 12% TCA to remove excess SDS (15). After destaining, slab gels were either dehydrated on a drying plate (Canalco Inc., Rockville, Md.) and autoradiographed (RP54 X-ray film, Eastman Kodak Co., Rochester, N. Y.), or sliced into 1.8 mm sections and counted. Tube gels were sectioned and counted. Molecular weight markers glucagon (3,600 mol wt), cytochrome *c* (12,700 mol wt) (both from Sigma Chemical Corp.), chymotrypsinogen (25,700 mol wt), ovalbumin (43,000 mol wt), bovine serum albumin (68,000 mol wt, all from Schwarz/Mann), and β -galactosidase (130,000 mol wt, Sigma) were prepared and run in parallel with sample preparations.

Microscopy and Autoradiography

IODINATION AND FIXATION: L cells were iodinated as described above except that 350–1,000 μ Ci/ml Na¹²⁵I were used. After extensive rinsing at 4°C or 20°C, the cells were suspended in 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.4, for 5 min at room temperature, followed by 10 min on ice. 2 vol cold 1% OsO₄ in 0.1 M cacodylate were added for 15 min. An alternative procedure was to suspend the cells in a “mixed fix” of 1 vol 2.5% glutaraldehyde in SP and 2 vol 1% OsO₄ in SP at 4°C for 15 min. The cells were pelleted, rinsed twice in cold saline, postfixed in 0.25% uranyl acetate in 0.1 M acetate, pH 6.3, for 15 min, rinsed twice in saline, resuspended in 1% agar at 60°C, and pelleted. The agar-cell pellet was cut into 1 mm blocks, dehydrated, and embedded in Epon (35) according to Hirsch and Fedorko (18).

LIGHT MICROSCOPE AUTORADIOGRAPHY: Thick sections (~0.4 μ m) were cut, dried onto clean glass slides, dipped into Ilford L4 emulsion (Ilford Ltd., Ilford, Essex, England) prepared as described by Caro and van Tubergen (10), and exposed for up to 2 wk at room temperature. The slides were developed for 2 min (D-19, Eastman Kodak), fixed, rinsed, and stained with 0.2% Azure A in 1.0% Na borate. The sections were mounted in Permunt (Fisher Scientific Co., Fairlawn, N.J.) and viewed at $\times 40$ and $\times 100$ under oil with a Zeiss photomicroscope. Thick sections exposed for 3–8 days averaged 40–60 grains per cell.

ELECTRON MICROSCOPE AUTORADIOGRAPHY: Thin sections (~1,000 Å) were cut (Porter-Blum Ultramicrotome, Sorvall MT-2, Newtown, Conn.), picked up on carbon-coated Formvar grids, and attached to glass slides. Ilford L4 emulsion was applied with a copper wire loop, according to Caro and van Tubergen (10). The coated grids were exposed at room temperature for from 12 days to 1 mo, developed (Microdol X, Eastman Kodak), fixed, rinsed, and while still wet, were inverted over a drop of fresh 0.1 N NaOH for 20 min to remove the gelatin. The grids were then stained with uranyl acetate and lead citrate (60). To assess grain movement, duplicate grids were stained directly without prior removal of the gelatin. Grids were examined in the Siemens Model 1 microscope (70 kV) at a magnification of 10,000. Exposures of 12–25 days were sufficient to give from 15 to 85 grains per cell depending on the amount of isotope used. Grain counts were usually made at the microscope but low magnification pictures were also taken and counts made on prints, with similar results. Individual cells were analyzed for the total number of grains, and the percentage at the periphery, over the centrosphere region, nucleus, and mitochondria was calculated. This was done for 20–25 cells on at least two grids in each preparation. The total number of grains counted per grid was over 1,200.

CONTROLS: A number of controls were performed to examine the possibility that free isotope was artifactu-

ally removed during fixation or that incorporated label was lost during dehydration. Cells incubated with all reagents except LPO, fixed, and processed for autoradiography were negative. Unlabeled cells were mixed with the last rinse from iodinated L cells, processed, and found to be negative. Cells incubated with the complete iodination system in the presence of 10^{-5} M $\text{Na}_2\text{S}_2\text{O}_8$ and processed for autoradiography contained 5% of the label associated with cells iodinated in the usual manner. It was possible that the concentration of $\text{Na}_2\text{S}_2\text{O}_8$ used was insufficient to inhibit completely the iodination reaction. One aliquot of iodinated cells was fixed with glutaraldehyde and osmium tetroxide, and a second aliquot precipitated with TCA. The same percentage of total ^{125}I was retained by both procedures. In addition, only ~0.5% of the incorporated label was extracted in the dehydration steps. It was concluded that more than 95% of the grains seen by autoradiography represented specifically incorporated ^{125}I .

ELECTRON MICROSCOPY OF SUBCELLULAR FRACTIONS: In experiments involving the isolation of plasma membrane, a suitable aliquot of material was pelleted in an SW 39 cellulose nitrate tube, the pellet fixed overnight in 2.5% glutaraldehyde in 0.1 M Na cacodylate at 4°C, postfixes in 1% OsO_4 in cacodylate, and processed as a pellet through uranyl acetate and graded alcohols into 1:1 ethanol-Epon where the pellet was loosened, cut into 1 mm sections, and embedded in full strength Epon.

Enzyme Assays

ALKALINE PHOSPHODIESTERASE I: Alkaline phosphodiesterase I was assayed according to Touster et al. (57); however, the *p*-nitrophenyl-5'-thymidylate (Sigma) concentration was increased to 2 mM. The liberation of *p*-nitrophenol was measured at 400 nm after alkalization and compared to a standard curve. Enzyme kinetics were linear over 2 h of incubation in a range of $8\text{--}40 \times 10^8$ cells.

ARYL SULFATASE: This enzyme activity was measured according to Bowers et al. (7) at 37°C for a maximum of 30 min.

Analytical Procedures

Protein was measured according to Lowry et al. (34) with lysozyme as a standard. Cholesterol was determined by gas liquid chromatography of its trimethylsilyl ether derivative (Bendix 2500, 1% SE 30 on Gas Chrom P column 6 ft \times 0.25 in) and comparison to an internal standard, 5- α -cholestane, added in the first extraction step. Extraction and silylation were performed according to Werb and Cohn (65).

Other Materials

Other materials used in these experiments were purchased from the following sources: bromphenol blue, *p*-nitrophenol, Tris (hydroxyethyl) amino methane, mo-

noiodotyrosine, from Sigma; SDS from Fisher Scientific; acrylamide, *N,N'*-bismethylene acrylamide, TEMED, ammonium persulfate from BioRad Laboratories, Richmond, Calif.; sucrose from Matheson, Coleman & Bell, East Rutherford, N.J. and from Mallinckrodt Chemical, St. Louis, Mo.; FMA from Nutritional Biochemicals Corp., Cleveland, Ohio; 2-mercaptoethanol from Calbiochem, Los Angeles, Calif.; TCA from Mallinckrodt. All other reagents were analytical grade.

RESULTS

General Technique

IODINATION CONDITIONS: The conditions for LPO-specific iodination of red blood cells were used as a starting point in the L-cell study. It was considered important to iodinate at a lower temperature to avoid membrane internalization during the reaction and possible alteration of labeled proteins. A time course of iodination at 4°C and 37°C is presented in Fig. 1. Cell viability was maintained at >95% in all cases for as long as 60 min of incubation. Cells incubated at 4°C without LPO incorporated less than 1% the radioactivity of those which were exposed to the complete labeling

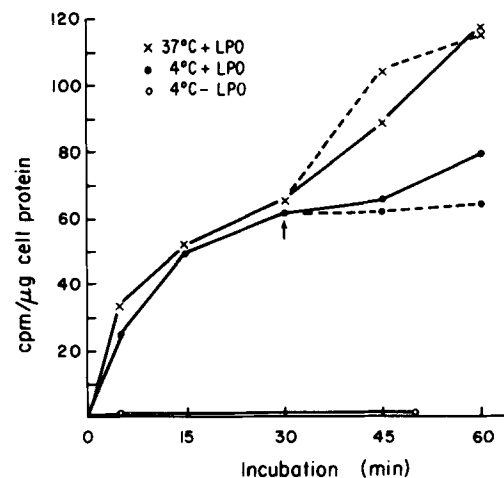


FIGURE 1 Effect of temperature on the iodination of L cells. Cells and reagents preincubated separately for 15 min at the appropriate temperature then mixed. 1 ml of the reaction mixture contained 9×10^8 cells in SP, 5 μmol glucose, 6 mU GO, 3 mU LPO, 50 μCi Na^{125}I . At indicated times 1 ml was removed, added to $10 \text{ ml } 10^{-5}$ M $\text{Na}_2\text{S}_2\text{O}_8$ in SP, and the cells were pelleted and rinsed five times, lysed in 0.5 ml H_2O and aliquots analyzed for TCA-insoluble radioactivity. Arrow indicates the addition of 3 mU more LPO: (—) additional LPO added; (---) no additional LPO added. 1 μg L-cell protein $\approx 6 \times 10^8$ cells.

system, confirming the specificity of the reaction for LPO obtained with red blood cells. The reaction in the presence of LPO was most rapid in the first 5 min but continued to 30 min, at which time the reaction at 4°C appeared to reach a plateau. The addition of more LPO did increase incorporation somewhat. The reaction proceeded in almost parallel fashion at 4°C or 37°C except that there was no plateau at 37°C. Additional LPO did not enhance incorporation. The lack of a temperature effect on the iodination reaction was reproducible. Incubation of L cells and the LPO system at 4°, 10°, or 23°C resulted in identical levels of incorporation, which were 80–90% of the ^{125}I incorporated at 37°C. Carrier-free isotope was used in these experiments at a concentration of 50 $\mu\text{Ci/ml}$ or 0.02 nmol/ml (2×10^{-8} M) of iodide. H_2O_2 was generated at 6 nmol/ml/min (GO activity \approx LPO activity). When increasing amounts of K^{127}I were added to the system, the effect of temperature on the reaction became apparent (Fig. 2). A Q_{10} of > 3 was obtained, in agreement with other enzymatic reactions. The incorporation of ^{125}I increased with increasing amounts of ^{127}I added, from an efficiency of $\sim 0.1\%$ to 2%. This was also found in the red blood cell system (21) and possibly reflects adsorption of iodide to the test tube walls. At the highest iodide concentration used, 12.5 μM , approximately $5\text{--}10 \times 10^6$ iodide atoms were incorporated per cell at 37°C with no effect on cell viability ($>90\%$ excluded dye). At 4°C, 1.0×10^5 atoms per cell were incorporated at 12.5 μM iodide and $\sim 2,000$ per cell at the carrier-free isotope concentrations (2×10^{-8} M) used throughout these studies. In later experiments incorporation at 4°C was increased to 10,000–50,000 atoms per cell by increasing the ^{125}I concentration to 100–150 $\mu\text{Ci/ml}$ and doubling the LPO concentration. We chose to continue to use carrier-free iodide, thereby labeling less than 0.5% of the available sites per cell.

The influence of cell concentration on iodination is shown in Table I. The incorporation was found to be proportional to cell density over a sixfold range (Table I). Varying the LPO activity also affected incorporation; however, varying GO activity did not significantly alter the extent of iodination.

CHARACTERIZATION OF THE IODINATED SPECIES: Approximately 3% of the acid-insoluble radioactivity could be extracted with organic solvents in which either neutral or polar lipids were soluble (Table II). This indicated that very

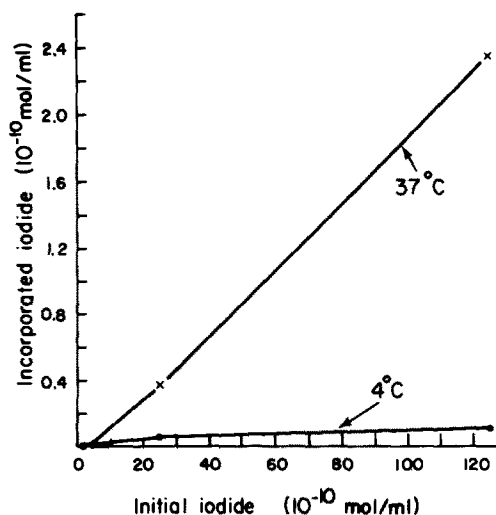


FIGURE 2 The effects of temperature and iodide concentration on the iodination of L cells. 1 ml of reaction mixture contained 4×10^6 cells in SP, 5 μmol glucose, 5 mU GO, 3 mU LPO, 50 μCi Na^{125}I , and varying amounts of K^{127}I . Cells were iodinated 30 min at either 4° or 37°C, rinsed five times in SP, lysed in 0.5 ml H_2O , and aliquots analyzed for TCA-insoluble radioactivity.

TABLE I
Effect of Cell Concentration, LPO, and GO
Activities on the Iodination Reaction*

Experiment	Cell Concentration	Enzyme Activities		Incorporation cpm/ml cells	Change
		LPO†	GO†		
	no. $\times 10^6/\text{ml}$				%
1	1.2	4.8	8	13,000	0
	3.7	4.8	8	36,500	+180%
	7.4	4.8	8	67,000	+415%
	3.7	19.2	8	118,000	+320%
2	4.0	9.6	8	107,500	0
	4.0	9.6	32	133,000	+120%

* L cells iodinated at 4°C for 10 min (experiment 1) or 15 min (experiment 2) in 20 mM glucose, 100 $\mu\text{Ci/ml}$ ^{125}I , and LPO, GO, and cell concentration as indicated. Acid-insoluble radioactivity measured.

† Nmol H_2O_2 produced (GO) or utilized (LPO) per milliliter per minute.

little or no ^{125}I was incorporated into the lipids of the L cell. The nature of the extractable radioactivity was not further investigated. After 48 h of exposure to a crude trypsin preparation, 91–95% of the initially acid-insoluble radioactivity was soluble in TCA (Table III). Previous extraction with lipid solvents did not alter the results. The success of crude trypsin presumably depended upon the

presence of other proteases and peptidases which in concert degraded proteins to the level of amino acids. Greater than 85% of the soluble radioactivity was monoiodotyrosine (MIT), as measured by Sephadex G-25 (Fine) column chromatography. The remainder was either ^{125}I (~10%) or material eluting at the void volume. Diiodotyrosine was never detected although columns were routinely run past the elution volume of this amino acid. Greater than 97% of the applied radioactivity was recovered in the three regions mentioned. As seen in the controls of Table III the presence

of proteolytic enzymes was essential to the liberation of M^{125}IT from acid-insoluble material but did not deiodinate the MIT drastically.

Autoradiography of Iodinated L Cells

The subcellular localization of the label in iodinated L cells was studied initially by autoradiography since all of the incorporated label in each cell could be visualized without resorting to fractionation procedures. Various control experiments (see Materials and Methods) established that the grains seen in autoradiography represented specifically incorporated ^{125}I .

LOCALIZATION: L cells were iodinated with 350–1,000 $\mu\text{Ci/ml}$ of Na^{125}I at 22°C for 20–30 min. Cells remained viable under these conditions. Reproducible results were obtained in four cell preparations. 85–90% of the grains were located at the cell periphery, 5–14% in the centrosphere region, and 0–4% over the nucleus (Fig. 3). Grain distribution appeared to be random over the periphery, occurring at both the tips and the depressions of surface projections. A tangential section through the surface projections of an iodinated cell (Fig. 3d) demonstrated the random labeling obtained. Labeling over the centrosphere was somewhat variable between cells in the same preparation and grains appeared to be localized primarily over vesicles (pinocytic?). It is possible that cells warmed to 22°C for iodination and rinsed with 10°C solutions might have interiorized

TABLE II
Organic Solvent Extraction of Acid-Insoluble Radioactivity from Iodinated L Cells*

L-cell sample	Organic Solvent Used				
	90% acetone		3:1 ethanol/ether		
	None	Change	Change	Change	Change
	<i>cpm</i>	<i>cpm</i>	%	<i>cpm</i>	%
A	28,280†	27,660	–2.5	27,675	–2.5
B	24,700	23,100	–6.4	24,000	–2.8
C	14,950	14,500	–3.0	14,700	–1.7

* 50 μl samples were spotted on Whatman GF/C glass fiber disks, the disks washed as described (Methods and Materials), air dried without further treatment (none), or exposed for 30 min at -20°C to the indicated solvents, air dried, and counted.

† Average of duplicates which were within 1%.

TABLE III
Enzymatic Digestion and Identification of Iodinated Species from L Cells

Sample	+ Trypsin		– Trypsin		M^{125}IT + Trypsin	
	<i>cpm</i>	%	<i>cpm</i>	%	<i>cpm</i>	%
Enzymatic digest*						
TCA soluble	22,563	90.4	1,220	6	48,406	99
TCA insoluble	2,425	9.6	21,045	94	510	1
TCA soluble label†						
MIT	—	85	—	ND	—	90
I [–]	—	10	—	—	—	10
Void volume	—	5	—	—	—	0

* Enzymatic digest was prepared as follows: 1 ml 0.25% crude trypsin in 0.05 M Tris HCl, pH 8.0, 2 mM CaCl_2 was added at 0 h to ^{125}I -labeled L cells previously precipitated with TCA onto filter disks. An additional 1 ml of trypsin was added at 24 h. The material was precipitated with TCA at 48 h, the pellet rinsed, and the TCA supernates and pellets were counted. Controls consisted of the omission of trypsin during the 48-h incubation (third column) or the addition of M^{125}IT only plus trypsin (fourth column).

† Suitable aliquot of TCA supernate chromatographed on Sephadex G-25 Fine Column as described (21). MIT was identified by comparison on the column with authentic MIT.

ND = not determined.

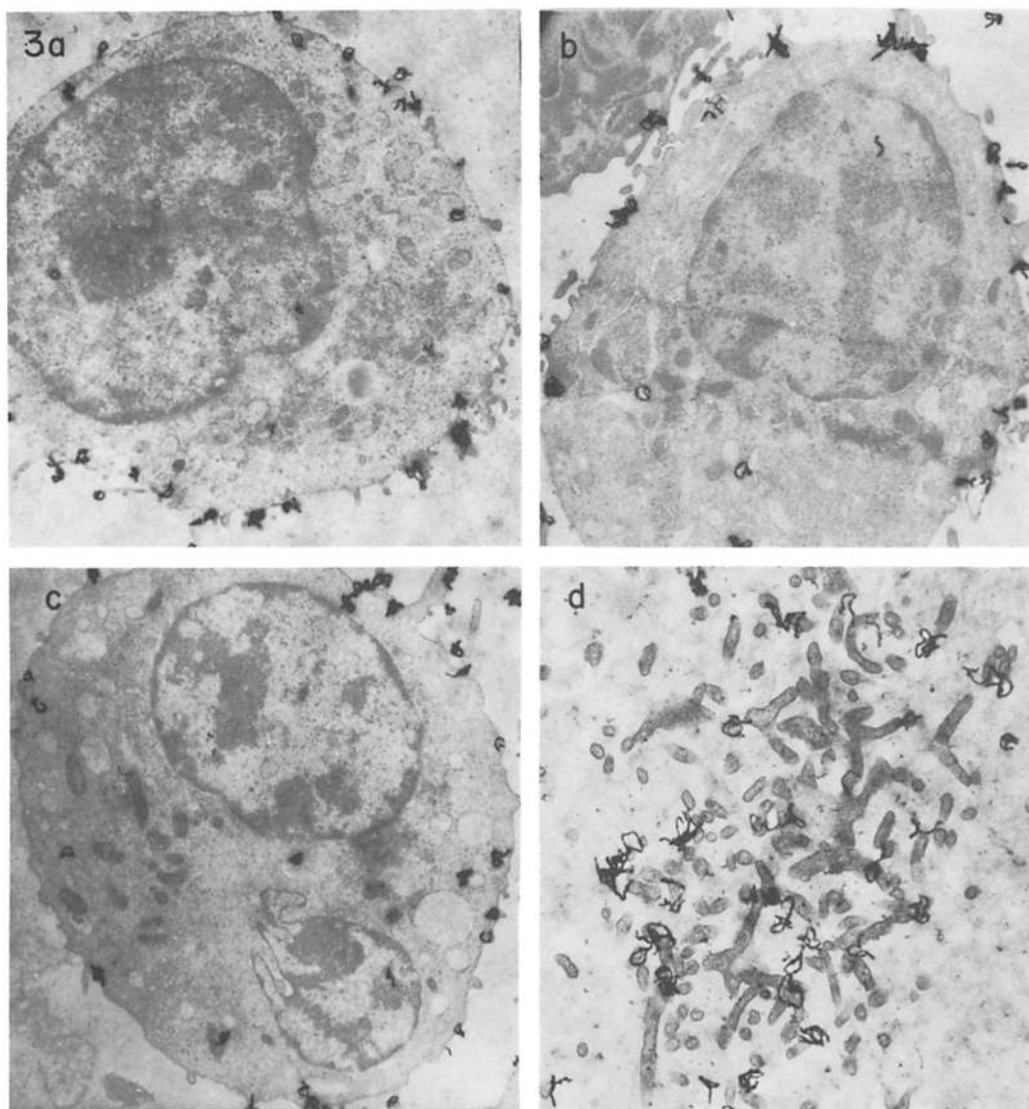


FIGURE 3 EM autoradiographs of three L cells (*a,b,c*) iodinated on three different occasions. Grains represent ^{125}I atoms covalently linked to tyrosine residues in proteins. Grains largely localized to cell periphery. ($\times 7,800$) 4 *d*: Tangential section of an iodinated cell ($\times 27,000$).

membrane before fixation. No grains were found over mitochondria and background counts were negligible. It was concluded that 90% of the label was localized to the cell periphery and presumably to the plasma membrane.

LOCALIZATION OF LABEL IN MONOLAYER CELLS: Early iodination experiments were performed on cells attached to a plastic surface. Light microscope autoradiography revealed the principal location of the incorporated label to be a protein

coat on which L cells were spread. This method of cell iodination was immediately abandoned and suspension cells were subsequently employed.

Isolation of Plasma Membranes from Iodinated Cells

Electron microscope (EM) autoradiography had established that 85–90% of the incorporated iodide was localized to the cell periphery after LPO

iodination. It was of interest to prepare a plasma membrane-rich fraction from iodinated cells and to compare the enrichment and yield of acid-insoluble ^{125}I to that of several membrane markers, such as cholesterol and alkaline phosphodiesterase I, an enzyme in rat liver reported to be associated with membranes containing cholesterol (56, 57).

BIOCHEMICAL ANALYSIS OF MEMBRANE PREPARATIONS: The fractions obtained from each step of the three membrane isolation procedures used were analyzed for protein and acid-insoluble ^{125}I . In addition, the membrane ghosts were monitored throughout the FMA isolation by phase microscopy. A complete analysis of one FMA II membrane isolation is presented in Table IV. In this experiment approximately 60% of the ghosts and radioactivity remained in the supernate after the first centrifugation. In step II

the majority of the unlabeled proteins were well separated from the label and ghosts, with a resulting increase in relative specific activity in the 30%/55% interface fraction. A rapid centrifugation (step III) further separated the sedimentable whole ghosts from smaller vesicular material and trapped soluble protein. Recycling the membrane fraction through a second discontinuous gradient (step IV) and wash (step V) increased the relative specific activity of the membrane fraction only 8% over step III. 23% of the label and 27% of the ghosts were recovered in the final membrane fraction. Correcting for only 71% cell breakage in this experiment, 38% of the membrane ghosts present in the original homogenate were recovered in the membrane fraction.

An analysis of the final membrane-enriched fractions from representative experiments with the three isolation procedures is presented in Table V.

TABLE IV
*Analysis of Fractions from FMA II Membrane Isolation**

Step†	Fraction	Protein		Acid-insoluble radio-activity		RSA§
		mg	%	cpm $\times 10^4$	%	
—	Homogenate	37.2	100	17.2	100	1
I	Supernate	22.0	60	10.5	61	1
	Pellet	8.1	23	5.2	30	1.35
II	Sample layer (soluble proteins, microsomes and membrane vesicles)	20.6	54	2.1	12.2	.22
	30%	1.6	4.3	0.4	2.3	.55
	30%/55% interface	1.65	4.5	6.5	38.0	8.7
	55%	—	—	0.9	5.2	—
	60%	—	—	—	—	—
III	Supernate	—	—	1.0	—	—
	Pellet	0.88	3.25	5.0	29	12.5
IV	30%/55% interface	0.475	1.3	4.4	25.5	19.5
	55%	—	—	—	—	—
V	Supernate	—	—	0.1	—	—
	Membrane fraction	0.63	1.65	3.9	23	13.5

* 14.4×10^7 iodinated cells yielded 10.3×10^7 (71%) whole membrane ghosts in the starting homogenate. 0.46×10^7 whole cells (3%) and 7.95×10^7 (55%) ghosts were present in the supernate of step I. The remainder of cells and membranes were found in the fraction designated pellet. No whole cells and 3.9×10^7 (27%) ghosts were recovered in the final membrane fraction (step V).

† See Materials and Methods for description of isolation.

§ RSA, relative specific activity. Percent acid-insoluble ^{125}I per percent protein in the fraction. Homogenate RSA = 1.

|| Not determined.

Comparable percentages of membrane ghosts, acid-insoluble label, and cholesterol were recovered in the membrane fractions from the FMA isolations, and each membrane marker was enriched 11–15-fold over the initial homogenate. An 11-fold enrichment of label in the Tris-HCl procedure was comparable to that found with FMA; however, less protein and label were recovered in the final Tris preparation. This may have been due to membrane vesiculation during the isolation. Enzyme activities were retained in the Tris procedure and 6% of the alkaline phosphodiesterase I activity (PDase) was found in the final membrane fraction with an eight-fold enrichment. The specific activity of aryl sulfatase was not increased and the low activity present suggested that lysosomal proteins represented less than 20% of the protein in the membrane fraction. A 21-fold purification of label in one Tris-HCl membrane preparation (Table V) was the highest achieved in twelve membrane isolation experiments.

MORPHOLOGICAL ANALYSIS OF MEMBRANE PREPARATIONS: Phase microscopy of the membrane-enriched fractions from FMA I or FMA II isolations revealed the only identifiable structures to be whole membrane ghosts with a characteristic "shower cap" appearance (Fig. 4 *a*). Electron microscope examination revealed primarily whole membranes with rolled edges (Fig. 4). Nuclei or mitochondria were not evident but many ribosome-like particles were attached to the cytoplasmic face of the isolated membranes. The trilaminar appearance of the membrane was seen (Fig. 4 *b*).

The morphology of the Tris membranes is presented in Fig. 5. Tris membranes were vacuolated under phase microscopy (Fig. 5 *a*) and smaller than the FMA ghosts. This was also apparent at the EM level where few whole ghosts could be seen.

The parallel enrichment of incorporated ^{125}I with membrane ghosts, cholesterol, and alkaline phosphodiesterase I in the plasma membrane fraction from iodinated L cells confirmed the membrane localization of label observed using EM autoradiography.

Gel Electrophoresis of Iodinated L Cells

GENERAL LABELING PATTERN—TUBE GELS: The number, size, and relative amount of iodinated L-cell membrane proteins were examined using SDS gel electrophoresis. Iodinated,

TABLE V
Analysis of Membrane Preparations

Isolation	Protein	Membrane ghosts	Acid-insoluble ^{125}I	RSA*
	%	%	%	
FMA I†	1.60	14	15.5	11
FMA II	1.70	27.0	23	13.5
Tris-HCl‡	0.74	ND	8	11
Tris-HCl	0.38	ND	8.2	21

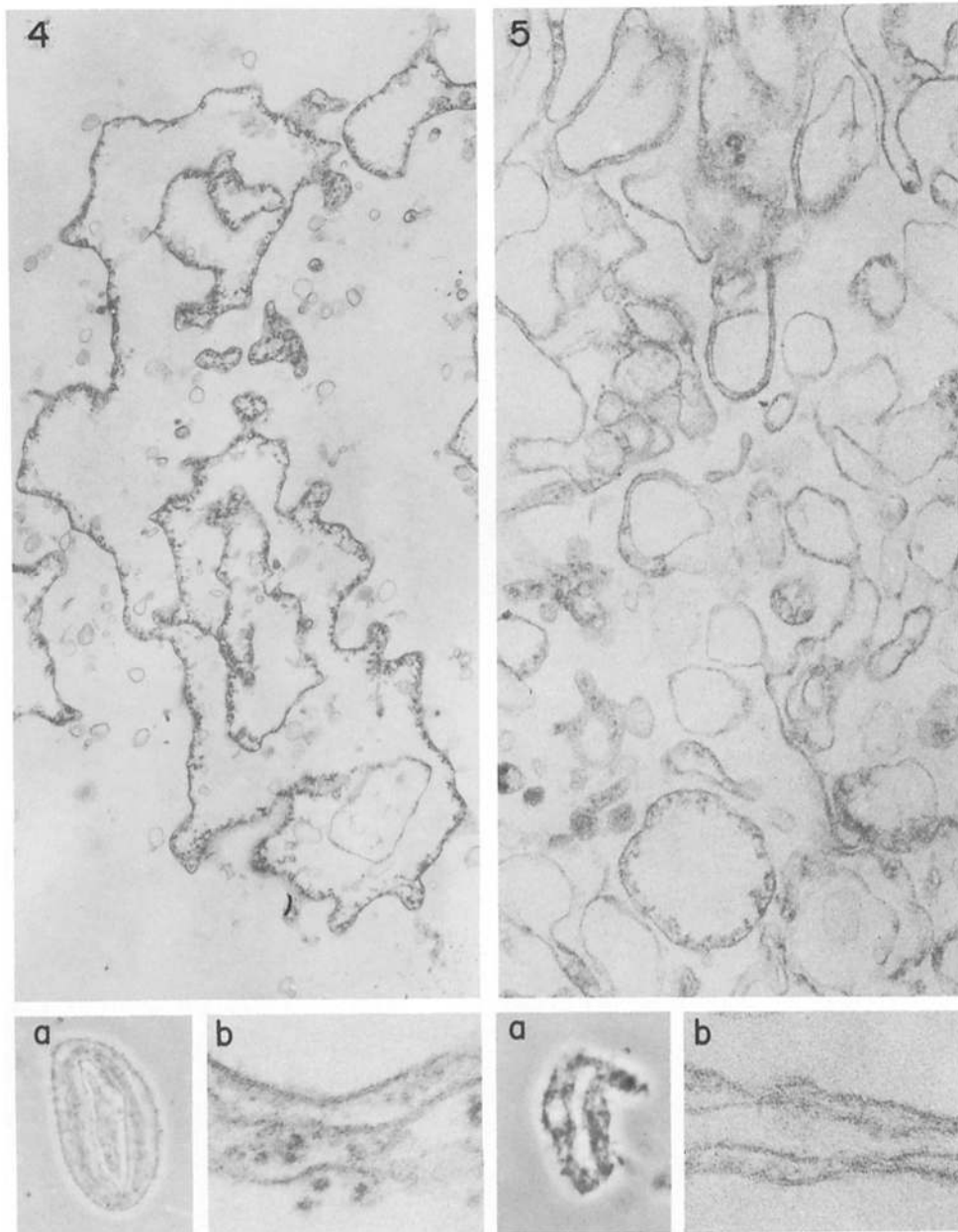
* RSA, Relative specific activity, homogenate = 1.

† Cholesterol determination of fraction: 19.4% yield with RSA of 12.1.

‡ Alkaline phosphodiesterase I determination of fraction: 6% yield with RSA of 8. Aryl sulfatase determination of fraction: 0.25% yield with RSA of 0.3.

ND = not determined.

rinsed, intact L cells were solubilized in SDS-2ME and immediately heated to 100°C, thus avoiding exposure and possible modification of the iodinated proteins by cellular proteases. The total cellular constituents were electrophoresed to insure that all of the incorporated label was analyzed. The radioactivity distribution of iodinated proteins is presented in Fig. 6. The gel patterns were identical, whether cells were iodinated at 4°, 10°, or 23°C. Five peaks were clearly distinguished, ranging from ~50,000 to over 150,000 daltons in size, with one major peak at 80,000–90,000 daltons. Gels were divided into the six regions indicated in Fig. 6 and the relative amount of label in each was calculated (see legend, Fig. 6). Over 63% of the label was found in three peaks (B,C,D) from ~80,000 to 200,000 daltons in size. The presence of excess free over incorporated iodide in these solubilized cell preparations (two to five times more despite 98% of the unincorporated label having been removed) raised the possibility of artifactual adsorption of ^{125}I to proteins. To resolve this question a comparison was made of gels from iodinated L cells solubilized directly in SDS-2ME (free ^{125}I still present, as in Fig. 6), or first precipitated in 10% TCA or 0.5% phosphotungstic acid-0.5 N perchloric acid, and the pellets rinsed in acetone (–20°C) before solubilization (free ^{125}I removed). The gel patterns were identical. This indicated that the radioactivity distribution patterns reflected the presence of iodinated tyrosine residues in polypeptides of a particular size and not artifactual ^{125}I adsorp-



FIGURES 4 and 5 Membranes isolated from iodinated L cells. Fig. 4: FMA II membranes. Fig. 4 ($\times 10,000$), EM appearance of the preparation which consists of whole membrane ghosts with one hole and rolled edges. Ribosome-like particles are attached to the cytoplasmic side of the membrane; external surface is smooth and numerous vesicles are evident. 4 a ($\times 920$), phase-contrast micrograph of a single membrane ghost. 4 b ($\times 97,500$), EM appearance of membranes at high magnification; typical trilaminar structure is evident as well as ribosomes and amorphous material attached to the cytoplasmic surface. Fig. 5 Tris-HCl membranes. 5 ($\times 30,000$), EM appearance of the preparation where no whole ghosts are evident. 5 a ($\times 920$), phase-contrast micrograph of typical whole membrane ghosts, which are vacuolated in appearance. 5 b ($\times 97,500$), trilaminar structure of membranes is evident, as is fibrillar material, primarily on one face.

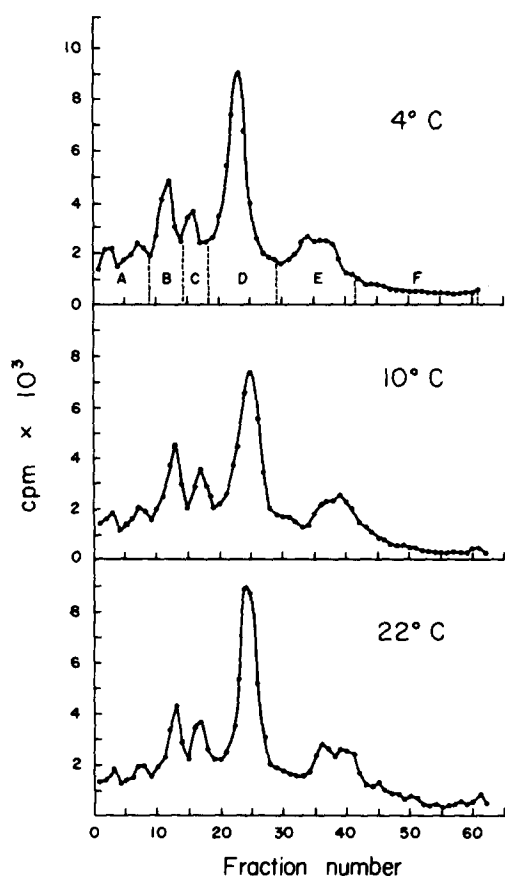


FIGURE 6 SDS polyacrylamide tube gel electrophoresis patterns of L cells iodinated at 4°, 10°, or 22°C. 1 ml of the reaction mixture contained 4×10^6 cells in SP, 5 μ mol glucose, 6 mU GO, 6 mU LPO, 150 μ Ci Na^{125}I . Cells were iodinated for 10 min, rinsed four times in MEM containing 1 mM NaF, and the cell pellet was solubilized in 2% SDS, 2% 2-ME, 10 mM Na phosphate, pH 7.2, and heated for 3 min at 100°C. Tube gel composition 7.5% acrylamide, 35:1 A/MBA, 0.1% SDS. Electrophoresis for 15 h at 5 mA per gel. Graphs represent distribution of ^{125}I . The sum of radioactivity in each of the indicated regions (top gel) was expressed as percent of total recovered label. A, 12%; B, 15%; C, 10.7%; D, 37%; E, 19.9%; F, 5%.

tion. Free ^{125}I was found to electrophorese in front of the dye marker.

SLAB GELS: Increased resolution of the labeled peaks found in SDS tube gels was achieved with SDS discontinuous pH slab gels. A comparison of the number of polypeptides found from tube and slab gel electrophoresis is presented in Fig. 7, where two slab gel variations were also compared.

Molecular weight markers from 12,700 to 130,000 were included in every slab gel and migration was found to be a log-linear function of size, even in 7.5–15% gradients (20). The increased complexity of the Coomassie blue staining pattern (total cell samples) was apparent from gel I (16 bands) to gel III (55 bands). In gel II, a 7.5% slab gel, peptides smaller than 15,000 daltons were not separated from the dye marker, whereas peptides smaller than 10,000 daltons were easily resolved in gradient slab gels (III). The Coomassie blue staining patterns of total L cells revealed those intracellular polypeptides of high concentration and did not reflect the membrane polypeptide pattern. The increased resolution of slab gels was also apparent from a comparison of radioactivity distribution patterns of gels I through III. The general labeling pattern remained the same, that is most of the radioactivity resided in polypeptides of 70,000–150,000 daltons, but the number of radioactive bands increased with each advance in gel technology. Table VI presents the number of labeled polypeptides in different size classes found by the use of tube and slab gels. 6–8 peaks were distinguishable in tube gels but 19–20 could be resolved in gradient slab gels of 20 cm in length. The size range of L-cell membrane proteins accessible to LPO iodination extends from 12,000 to greater than 200,000 daltons.

Tube gels were used for analysis of iodinated proteins during most of the work reported here but it should be understood that the tube gel patterns seen are simple because of the low resolution of tube gels and the counting methods used.

GEL LABELING PATTERNS FROM L CELLS GROWN IN DIFFERENT MEDIA: A number of experiments were conducted to confirm the cell-derived nature of the iodinated proteins seen in SDS gels. Fetal calf serum was iodinated with the LPO system and added to L cells for 30 min at 4°. Less than 0.05% of the iodinated FCS was cell associated after two rinses, and less than 0.001% (background) remained by the sixth rinse. A comparison of gel labeling patterns from an iodinated FCS preparation and iodinated L cells revealed no similarity (Fig. 8). The FCS labeling pattern reflected the Coomassie blue pattern with the major iodinated protein being albumin (BSA, 68,000 daltons). This did not eliminate the possibility of a minor serum component preferentially adsorbing to L cells and exchanging or desorbing with a half-life longer than 30 min. Consequently,

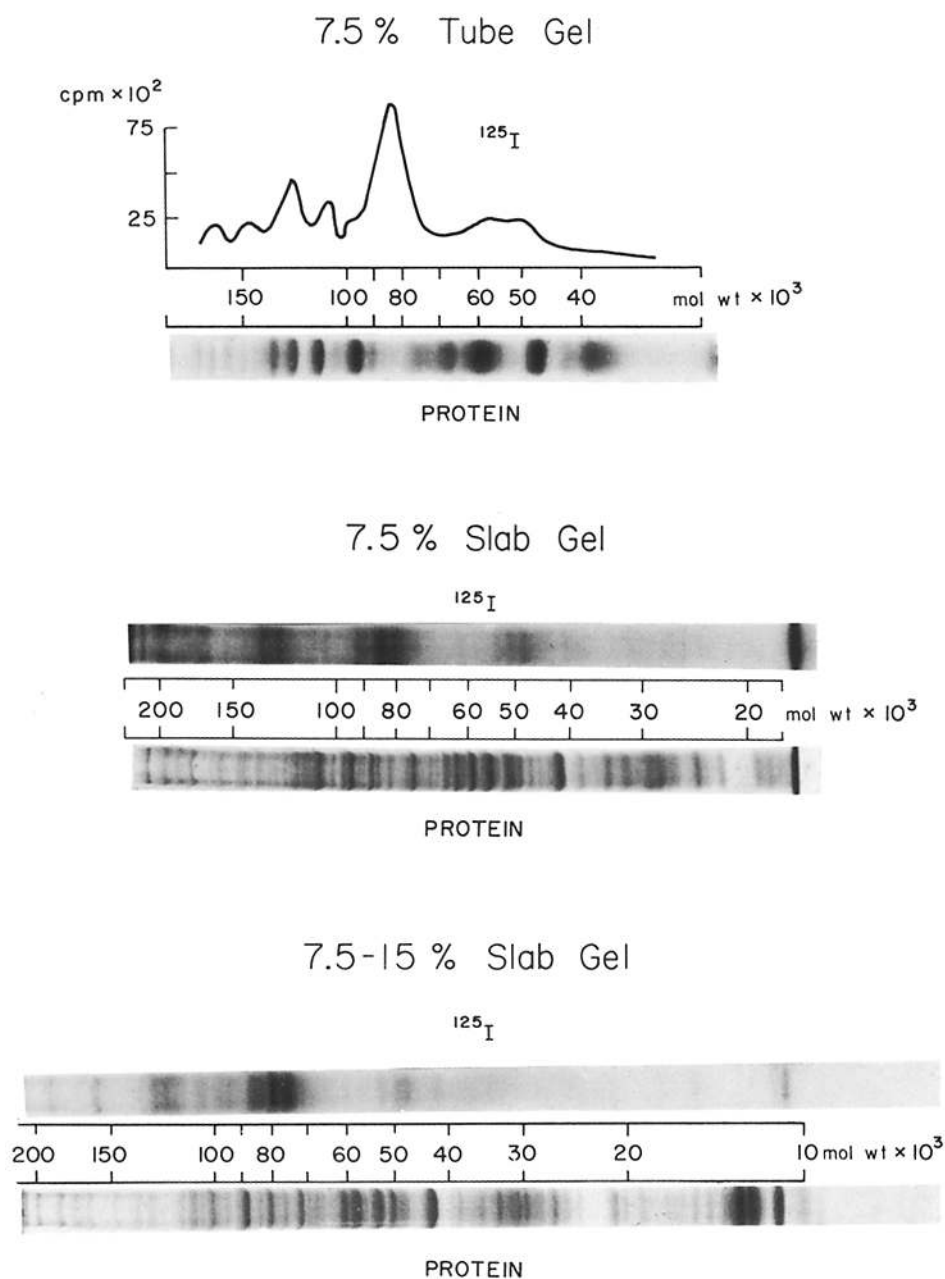


FIGURE 7 SDS polyacrylamide tube and slab gel electrophoresis patterns of iodinated L cells. Top gels (*I*): 7.5% acrylamide, 35:1 A/MBA, 6 mm diameter, 10-cm long tubes; electrophoresis 15 h at 6 mA/gel; gels stained with Coomassie blue, cut, and 1.8 mm sections counted. Middle gels (*II*): 7.5% acrylamide, 35:1 A/MBA (resolving gel), 3.0% acrylamide, 35:1 A/MBA, (spacer gel), 1-mm thick, 13-cm long slab, electrophoresis 7 h at 10 mA; gels stained with Coomassie blue (protein), dehydrated, and exposed 15 days to X-ray film (^{125}I). Bottom gels (*III*): 7.5% to 15.0% continuous gradient acrylamide, 35:1 A/MBA (resolving gel), 3.0% acrylamide, 35:1 A/MBA (spacer gel) 1 mm thick, 20-cm long slab, electrophoresis 20 h at 5 mA; gels stained with Coomassie blue (protein), dehydrated, and exposed to X-ray film for 3 days (^{125}I).

TABLE VI
Distribution of Radioactivity in SDS Polyacrylamide Gels

mol wt range	Region*	Radioactivity†	Number of peaks (tube)§	Number of bands (slab)
		%		
10,000	—	0	0	0
11,000–40,000	F	0¶ (5)	0	3
41,000–60,000	E	15–20	2	4
61,000–70,000	—	5	0	1
71,000–105,000	D	35	1–2	4
106,000–150,000	C	15	2	3–4
151,000–200,000	B	15	1	2
200,000	A	5–10	0–1	2
Total		90–100	6–8	19–20

* See Fig. 6 for description of regions.

† Obtained from counting tube and slab gels.

§ See Fig. 6.

|| See Fig. 7.

¶ Lost from tube gel.

L cells were grown in a variety of different media for up to 1 mo then iodinated, and cell samples electrophoresed (Fig. 9). The labeling pattern remained unchanged whether cells were grown in BSA, horse serum, bacto-peptone, or fetal calf serum. The level of incorporation per cell also remained comparable, indicating that proteins were *not* being removed without replacement. In addition, L929 cells and a new LM stock (LM26, American Type Culture Collection, less than 1 mo in the laboratory) were compared to the old LM line and found to be similar. A negative control consisted of the iodination pattern of HeLa cells (20) which was strikingly different from that of the LM cell although both were grown in FCS. It can be concluded that the L-cell labeling pattern on SDS gels reflected iodination of endogenous membrane proteins.

ADDITIONAL CONTROLS: LPO and GO were enzymatically iodinated with insolubilized enzymes (provided by Dr. J. P. Kraehenbuhl) incorporating 300,000 and 50,000 cpm ^{125}I /mg into LPO and GO, respectively. These labeled reagents were added for 10 min at 4°C to unlabeled L cells in the same concentrations used for routine iodination but in the absence of glucose and ^{125}I . 96% of the radioactivity was recovered in the first supernate and none remained by the fourth rinse. An additional control consisted of preincubating unlabeled LPO with L cells, rinsing the cells twice, then adding the remaining reagents.

Incorporation was less than 1% of the level obtained in the presence of LPO. These two controls indicated that LPO, iodinated or uniodinated, did not adsorb to L cells and contribute to the membrane labeling pattern seen on SDS gels (LPO, mol wt = 78,000) or cause localized, nonrepresentative iodination.

Other experiments were performed to confirm the gel labeling pattern. Cells were rinsed one to four times before iodination with no effect on the ^{125}I distribution. From 2,000 to 100,000 ^{125}I atoms were incorporated per cell without changing the pattern. Iodinated cells were solubilized in varying amounts and ratios of SDS and 2ME or dithiothreitol without changing the resulting protein or ^{125}I distribution patterns.

GEL ELECTROPHORESIS OF MEMBRANE PREPARATIONS—TUBE GELS: Coomassie blue staining patterns of gels from an L-cell homogenate and membrane fraction of one FMA II experiment were compared (Fig. 10 C). Two types of gels were prepared: a homogeneous 7.5% acrylamide gel (I), and a discontinuous 5.0% over 7.5% gel (II) which was designed to allow penetration of ~150,000 mol wt polypeptides well into the 5.0% gel but to retard smaller polypeptides in the 7.5% section. In gels I, at least 26 bands in the homogenate could be resolved and 15 in the membrane. The patterns were quite different, with three bands 200,000 daltons and larger predominating in the mem-

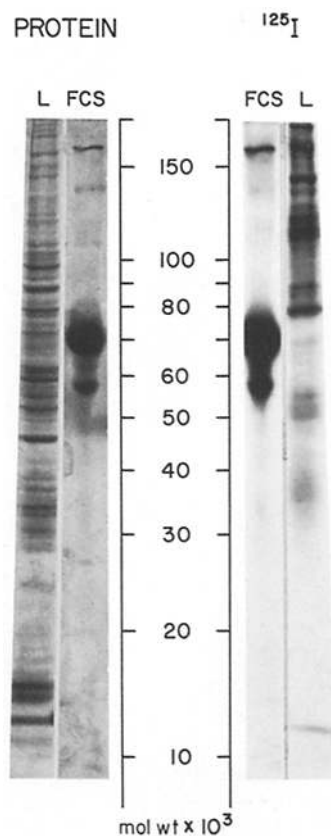


FIGURE 8 SDS polyacrylamide gradient slab gel electrophoresis patterns of iodinated FCS and iodinated intact L cells. Gels were stained for protein with Coomassie blue (left), dehydrated, and exposed to X-ray film for 17 days (FCS) or 30 days (L cells) (right). Resolving gel composition 7.0%–13% acrylamide, 35:1 A/MBA, 1 mm thick, 20 cm long. Electrophoresis 15 h at 7.5 mA.

brane but undetectable in the homogenate. The top band may represent aggregated proteins or protein associated with some mucopolysaccharides (49), as it migrated less than 1 cm into gel II and β -galactosidase, at 130,000 daltons, migrated to the 5%/7.5% interface. As can be seen in the radioactivity distribution pattern (Fig. 10 B), this band was not radioactive. The density of the two bands at $\sim 200,000$ mol wt was found to be quite variable among membrane preparations. Other major bands occurred at approximate molecular weights of 110,000, 55,000, and 50,000. These were quite reproducible among different preparations. Polypeptides smaller than 35,000 daltons were eluted from the gels

and not recovered. The intensely staining (non-radioactive) bands at $\sim 200,000$ daltons were less evident in Tris-HCl membrane preparations, but otherwise the protein staining pattern was similar. Attempts to demonstrate PAS-positive bands in these membrane preparations were unsuccessful although up to 150 μ g protein was applied.

Homogenate and FMA II membrane fractions from iodinated L cells were electrophoresed in 7.5% tube gels and the distribution of radioactivity was plotted (Fig. 10 A). All the peaks present in the homogenate were present in the isolated membrane. The relative specific activity in each peak was increased 14-fold (Table IV). Greater than 75% of the total label in the gels was present in three to four broad peaks of 70,000–200,000 daltons. No intense Coomassie blue bands corresponded to these major labeled peaks, suggesting that the accessible membrane proteins were a rather small percentage of the total proteins in the membrane-enriched preparation. Examination of the radioactivity distribution in gels from the nuclear pellet or any other fraction gave a pattern identical to that seen in Fig. 10 A. Recovery of applied radioactivity was always 92–95%. When total cell homogenates were solubilized and electrophoresed some label ($< 10\%$) was always found at the top of the gel. To ascertain whether very large polypeptides ($> 150,000$ mol wt) were labeled, 5%/7.5% discontinuous tube gels were prepared and the homogenate and membrane fractions electrophoresed. As seen in Fig. 10 B, there were no labeled peaks found in the 5% region although three prominent Coomassie blue bands were present (gels II). Again, the labeling pattern of homogenate and membrane fraction were identical, except that the latter contained 14 times more radioactivity than the homogenate on a protein basis. Tris-HCl membranes yielded similar results. If nonmembrane proteins (intra- or extracellular) were being iodinated, the homogenate labeling pattern would be more complex than that of the membrane. This was never found in 12 membrane preparations, substantiating the membrane localization of all the iodinated proteins. In addition, these results indicate that externally disposed membrane proteins that were iodinated were quantitatively retained in the membrane during the entire isolation procedure.

SLAB GELS: The electrophoresis pattern of a Tris-HCl membrane preparation is presented in

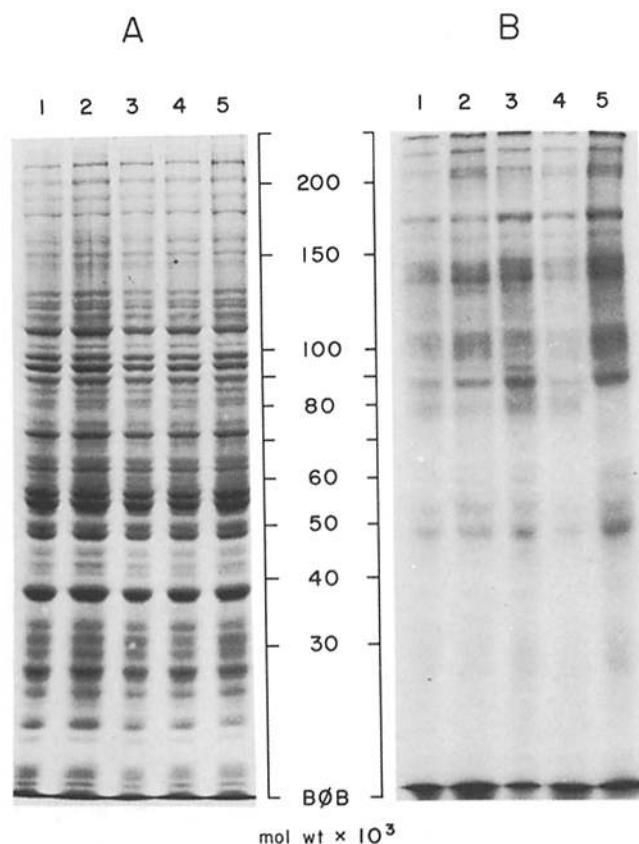


FIGURE 9 SDS polyacrylamide slab gel electrophoresis patterns of iodinated L cells grown in different media. A, gel stained for protein with Coomassie blue. B, autoradiogram of gel dehydrated and exposed to X-ray film for 5 days. Lane 1, LM cells grown in FCS; lane 2, L 929 cells in FCS; lane 3, LM cells in horse serum; lane 4, LM 26 cells in FCS; lane 5, LM cells in bactopectone. Resolving gel composition 7.5% acrylamide, 35:1 A/MBA, 1 mm thick, 13 cm long. Electrophoresis 7 h at 10 mA.

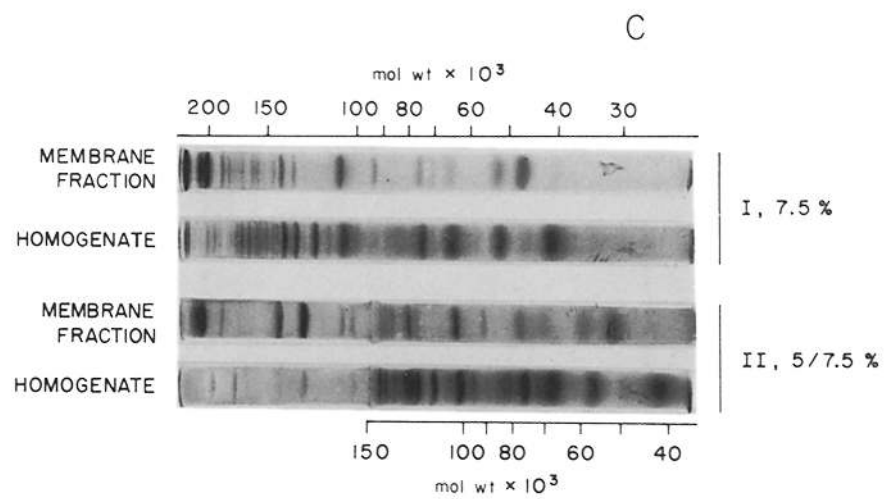
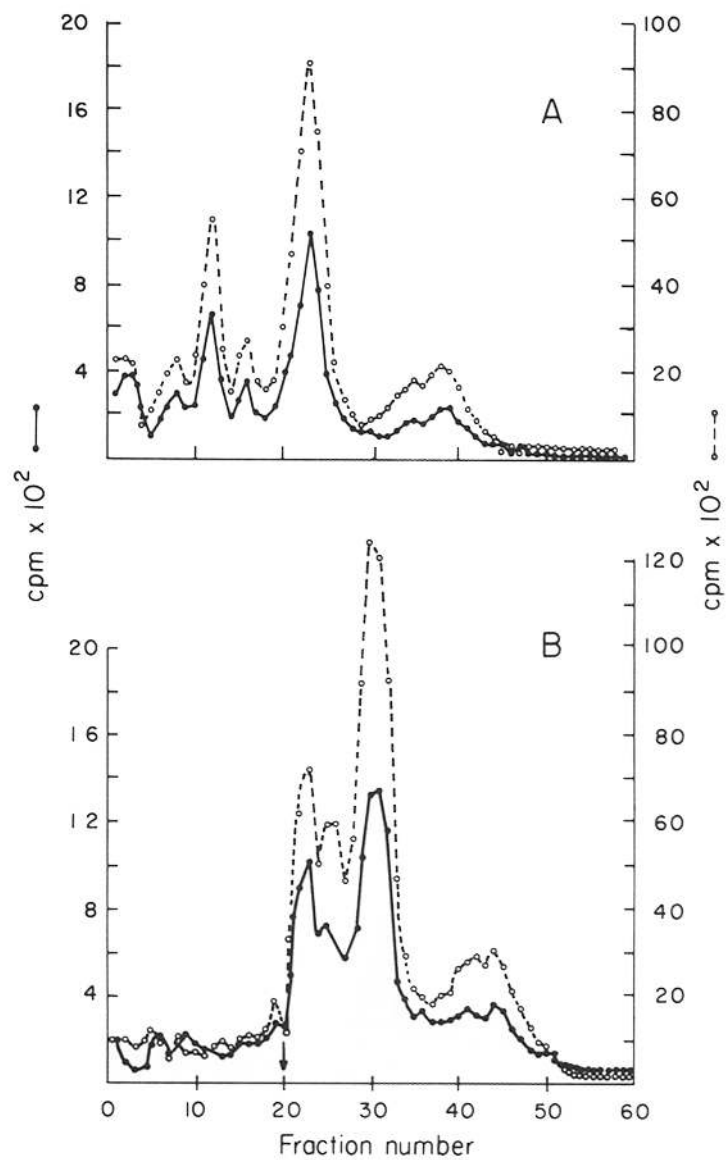
Fig. 11. More than 40 polypeptides were present as compared to the 15–17 found with tube gels. The range extended from below 10,000 to >200,000 daltons. As stated above, polypeptides below 35,000 daltons were eluted from tube gels during electrophoresis, yet 1 major and 12 minor bands were seen in the molecular weight range 10,000–35,000 on gradient slab gels. There were six major bands, the most prominent being 36,000, 50,000, and 62,000 daltons, all smaller than the bulk of the iodinated membrane proteins. Two labeled bands of 15,000 and 8,000 daltons represented 5–10% of the total radioactivity and were routinely eluted from tube gels. No label was present in regions below 8,000 daltons in gels immediately dried and autoradiographed or in those processed as in Fig. 11. Lipids migrate in this

region (32) and the lack of labeling provided additional evidence that these membrane constituents were not normally labeled.

DISCUSSION

General Technique

The enzymatic iodination technique used in a study of the red cell membrane (21) has been successfully extended to a nucleated cell, the L cell. The iodination conditions were very similar to those for the red cell except that iodination was performed at 4°C to minimize membrane activity. The lower temperature did not affect the incorporation efficiency which was 0.1–0.5% at 37°C or 4°C with carrier-free ^{125}I . If more iodide was introduced a Q_{10} of ~ 3 was observed, typical for



reactions catalyzed by enzymes and demonstrating that iodide is most probably the limiting reagent in our system. L-cell viability was not affected by the iodination as the membrane was "tagged" with 0.1–0.5% of the maximum number of iodide atoms capable of being incorporated (discussed in detail in the accompanying paper). Usually 10,000–50,000 atoms per cell were incorporated, or approximately 30 atoms of 3 Å diam per μm^2 of L-cell surface area.³ The effect of iodination on the individual labeled membrane proteins (inactivation or denaturation) must await their isolation and functional characterization; however, soluble proteins have been enzymatically iodinated with no loss in activity (12, 13, 44, 54). Controls similar to those performed in the red cell study demonstrated that nonenzymatic iodination was not occurring. Monoiodotyrosine was the predominant species iodinated. Lipid labeling accounted for less than 3% of the incorporated label and this amount may represent labeled proteins soluble in the organic solvents used.

There have been numerous reports on the use of the lactoperoxidase iodination system to probe the plasma membranes of eukaryotic cells (11, 39, 45, 42, 47, 26, 25, 19, 24, 43, 50, 51, 52, 55, 58, 61, 66), membranes of isolated subcellular organelles (3, 23, 29), and nonmembranous cell organelles (33). The conditions chosen by many investigators were similar to those originally employed by Phillips and Morrison (46). Our iodination conditions differ in the use of glucose-glucose oxidase to generate continuously micromolar quantities of H_2O_2 , of a very low LPO activity, and of carrier-free radiolabeled iodine. Due to these different conditions the kinetics of incorporation in the two iodination systems differ (26, 39, 58; but see reference 55). The extent of nonenzymatic iodination in either system was found to be negligible where carefully studied (26, 39). Poduslo et al. (47) reported that iodination of L cells in the absence of

³ Cell surface area $\sim 1,600 \mu\text{m}^2$.

LPO was 72% of that found in the presence of LPO. In addition, these investigators found that approximately 30% of the isotope was extractable with chloroform-methanol, but evidently a TCA precipitation was not performed before extraction. These results may represent the continued presence of unincorporated isotope in their system. We and others who have examined the possibility of lipid

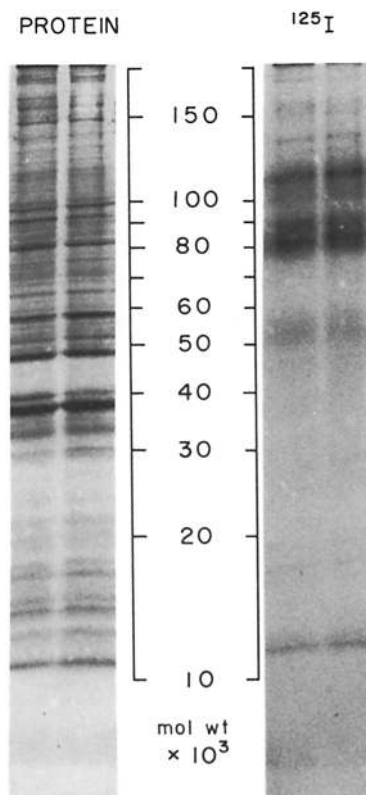


FIGURE 11 SDS polyacrylamide slab gel electrophoresis pattern of a Tris-HCl membrane preparation. Ca, 50 μg protein applied in each case, electrophoresed 8 h at 10 mA, the gels stained for protein with Coomassie blue (left lanes), dehydrated, and exposed to X-ray film for 24 h (right lanes). Resolving gel composition 7.5% to 15% acrylamide, 35:1 A/MBA.

FIGURE 10 SDS polyacrylamide tube gel electrophoresis patterns of iodinated L cells and membrane (FMA II) isolated from iodinated L cells. A, 7.5% acrylamide, 35:1 A/MBA, 10 cm gels cut and 1.8 mm sections counted. Electrophoresis for 15 h at 6 mA/gel. Graph represents distribution of ^{125}I in 80 μg total cell protein (●—●), 50 μg membrane protein (O—O). B, 4 cm of 5% acrylamide, 25:1 A/MBA, 6 cm of 7.5% acrylamide, 35:1 A/MBA. Electrophoresis conditions identical to A. Gels cut and counted and the results plotted as in A. C, Gels stained for protein with Coomassie blue. Composition of gels I identical to 10 A, gels II to 10 B, and electrophoresis conditions comparable but fractions from a different FMA II preparation. 40 μg homogenate protein and 30 μg membrane protein applied.

iodination have found only about 3% of the acid-insoluble label (nondialyzable by Vitetta and Uhr, [62]) to be soluble in organic solvents. Characterization of the iodinated species (MIT) is a very important prerequisite to subsequent electrophoretic or metabolic studies and has not generally been made.

EM Autoradiography

By EM autoradiography, 90% of the grains were associated with plasma membrane still present at the cell surface and proved that LPO was restricted to reacting at the external surface of the L cell. A variable 5–10% of the grains were found in the centrosphere region and appeared to be associated with vesicles. These grains could represent interiorization of labeled plasma membrane in the form of pinocytic vesicles during the iodination and rinse procedures. Measurements of uptake of a soluble protein added during the rinses would establish whether nonspecific pinocytosis was continuing or whether labeled membrane was being preferentially interiorized. Marchalonis et al. (39), and Kennel and Lerner (26) have also used EM autoradiography to localize incorporated ^{125}I to the plasma membrane of SIAT-4 lymphoma and WIL $_2$ -A3 human lymphoid cells, respectively. Other investigators (24, 25, 19, 43) have iodinated cells on a monolayer but have not established the membrane localization of the label incorporated. In initial experiments we found that a proteinaceous layer adherent to the culture dish was iodinated much more heavily than the plasma membrane of the cells spread on this layer. The extensive labeling of nonmembrane elements in a system obscures analysis of those membrane proteins which are labeled and should be avoided or recognized and carefully controlled.

Membrane-Enriched Preparations

In addition to autoradiographic localization of the incorporated label, membrane-enriched fractions from iodinated L cells were prepared. The enrichment of acid-insoluble label paralleled that of cholesterol, alkaline phosphodiesterase I, and whole membrane ghosts, further establishing the membrane localization of the incorporated ^{125}I . These results suggest that enzymatic iodination would be very useful as a specific plasma membrane marker in membrane isolation procedures and would give a better indication of membrane yield than enzymatic markers. The covalent nature

of the incorporated label makes it a more stable membrane component than an enzyme activity which might be inactivated (or stimulated) during isolation of the membrane. In addition, contamination of other subcellular fractions with plasma membrane can be assessed easily, since in the L cell and under our iodination conditions, all the labeled proteins are retained in the membrane through the isolation (see below). This is more difficult to prove with a single enzyme activity.

The relative enrichment of label in the membrane fraction over the homogenate ranged from 11- to 21-fold in 12 experiments with three different isolation procedures. Use of fluorescein mercuric acetate before homogenization and isolation resulted in 13–20% yields of L-cell membranes as measured by recovery of label, cholesterol, and/or membrane ghosts of six preparations. This was lower than the 25–40% yields reported by Warren and Glick (64). Better yields and membranes of comparable or higher purity (~14-fold) were obtained by simplifying the original Warren procedure (FMA II). FMA membranes prepared by either procedure displayed fibrillar material and many ribosome-like particles adsorbed to the cytoplasmic face, as reported by others using different tissue culture cells (28, 63). These particles did not appear to be attached to the plasma membrane in the intact cell (20) and exposure of the isolated membranes to a high salt concentration (0.5 M KCl) released most of them (1). We concluded that the interaction was an artifact of the isolation procedure. It was difficult to assess further contamination in FMA membranes, except morphologically, because enzyme activities were not retained after exposure to the sulfhydryl blocking reagent. Efforts to reverse the FMA binding with 2-ME early in the isolation procedure resulted in disintegration of the membrane ghosts.

The advantage of the Tris isolation procedure was in the retention of enzyme activities. However, this was offset by the low yields of L-cell membranes, from 8 to 13%. Tris membrane ghosts were not as stable as FMA membranes and continually vesiculated during isolation. The relative enrichment of ^{125}I in the membrane over the homogenate was slightly higher than in the FMA membranes, from 11- to 21-fold, but purer surface membranes could not be obtained, even after initial attempts of sonication and isopycnic centrifugation of the vesicles, a method which, as others have reported, significantly enriches the final fraction with plasma membrane fragments (6). One

Tris-HCl membrane preparation contained 8% of the label and 0.37% of the total protein, giving a 21.7-fold purification of label over the homogenate. Assuming that 90% of the acid-insoluble radioactivity was on plasma membrane-derived proteins and that 80% of the proteins in the fraction were also derived from the plasma membrane (10–20% contamination with lysosomal, microsomal, and ribosomal proteins), L-cell membrane proteins represent ~3.2% of the total cellular protein. Similar calculations led Kennel and Lerner (26) to conclude that as much as 16% of the total protein in WIL₂-A3 cells was membranous. Red blood cell membrane proteins represent only ~1.5% of the total (8, 21). Adequate controls to assess possible adsorption of cytoplasmic and extracellular proteins to the isolated membrane have not yet been performed but the extent of adsorption is likely to be high (40).⁴

We achieved an eightfold purification of alkaline phosphodiesterase I activity in one Tris membrane preparation where ¹²⁵I was enriched 11-fold. It is possible that this enzyme is not localized solely to the plasma membrane in L cells; however, the activity was quite low and accurate recoveries were difficult to obtain. It was not possible to assay 5' nucleotidase activity in membrane preparations because L cells exhibited no measurable hydrolytic activity toward adenosine 5'-monophosphate at pH 8.5.

The difficulties of obtaining pure plasma membrane preparations from tissue culture cells in sufficient quantities for analysis should be apparent. The use of enzymatic iodination as a specific membrane marker is useful in this regard, provided localization of the incorporated label to the periphery has been demonstrated.

Gel Electrophoresis

Intact iodinated L cells were electrophoresed in SDS-polyacrylamide tube gels so that 100% of the incorporated iodide could be examined. Approximately six labeled peaks ranging from 40,000 to 200,000 daltons were found. The predominant label (70%) occurred in four peaks between 70,000 and 150,000 daltons. Identification of the iodinated species as moniodotyrosine and the incorporation of [¹⁴C]tyrosine into the labeled bands⁵ confirmed the presence of this amino acid in the polypeptides labeled. However, the amount

of label in a given band in SDS gels depends on the concentration of the polypeptide at the cell surface, the number of tyrosine residues in the polypeptide, and the accessibility of these tyrosines to LPO iodination. These parameters have not yet been established.

SDS gels of solubilized membranes isolated from intact, iodinated L cells exhibited the same labeling pattern as that of intact cells but with 11–21 times more radioactivity recovered in each peak on a protein basis, reflecting the relative enrichment of these proteins in the isolated membrane preparation. These results are further evidence that only the externally disposed plasma membrane proteins were being iodinated in our system and that these proteins were firmly anchored in the membrane since none were lost during the isolation procedure. LPO-iodination is useful as a plasma membrane marker in subcellular fractionation only if the gel labeling pattern of cells iodinated while still intact is identical to that of plasma membranes derived from those cells. Others have examined the labeling pattern of their membrane-enriched preparations which contained only 10–20% of the total label (26, 58, 52). The presumption was that membrane was lost to other fractions during the isolation procedure; however, iodinated proteins could also have been lost from the membrane, thus reducing the recovery of label in the final membrane fraction.

Use of gradient slab gels has resolved the six broad labeled peaks into 15–20 bands ranging from 11,000 to 200,000 daltons. The four peaks comprising 70% of the label in tube gels were found to separate into at least nine bands. Recalling the relative simplicity of the red cell membrane labeling pattern (21), it is clear that a rapidly dividing mammalian cell such as the L cell has many more externally disposed membrane proteins that can be iodinated than are found in the red cell membrane. This finding has been substantiated by others using enzymatic iodination to label cell types different from the mouse fibroblast studied here (11, 52, 58, 42, 26). Generally one or two broad peaks of label predominated in the membrane preparations when analyzed by SDS tube gel electrophoresis; however, many minor peaks were also found. Slab gel electrophoresis of these preparations would probably reveal more labeled components.

The increased resolution of SDS slab gel electrophoresis over the tube gel system used was due to a number of factors. The gels were two to three times longer, a low-percentage acrylamide

⁴ J. D. Castle, personal communication.

⁵ A. L. Hubbard, unpublished data.

spacer gel was used to concentrate the sample, and the discontinuous pH of the system gave further band sharpening. The use of autoradiography also increased resolution because minor bands, which would not be discriminated by cutting and counting gel slices, were resolved visually.

Numerous controls established that the proteins iodinated were cell derived and not adsorbed serum components. L cells grown over 1 mo in horse serum or 2 wk in BSA or bacto-peptone and then iodinated incorporated amounts of ^{125}I per cell comparable to that of FCS-grown L cells and exhibited very similar SDS gel labeling patterns. If serum components had contributed to the gel pattern, their desorption, degradation, or dilution over a 2-wk period (at least 21 cell divisions) should have resulted in very low incorporations as well as different radioactivity distribution patterns but this was not found. The nonidentity of the labeling patterns obtained when intact HeLa cells and L cells, both grown in FCS, were iodinated is further evidence that serum components did not contribute significantly to the gel patterns.

Preliminary attempts to demonstrate the presence of glycoproteins in the L-cell membrane preparations were unsuccessful. ^{14}C glucosamine and ^3H fucose could be incorporated into acid-insoluble constituents by L cells, and when total cells were electrophoresed a few discrete bands in the range of 50,000 to 150,000 daltons could be resolved.⁵ Isolated membranes from these labeled cells did not contain enough radioactivity to electrophorese. Double-labeling experiments using ^3H fucose and ^{125}I to establish coincidence between glycoproteins and iodinated membrane proteins were unsuccessful due to the very low levels of fucose incorporation.

The gel electrophoresis results reported in our L-cell study contradict those of Poduslo et al. (47). These investigators found that one peak at ~230,000 daltons constituted over 25% of the label recovered in SDS tube gels, with the remainder distributed equally among twelve peaks from 50,000 to 150,000 daltons. The major peak corresponded to a Coomassie blue band which was also PAS-positive (16) and located at the top of their gels. Hynes (25) reported a somewhat similar finding using LX cells; however, monolayer cells were used (see Discussion above) and the subsequent analysis was only qualitative. We have found less than 10% of the label in this region. It is possible that different L-cell strains have different surface proteins, depending on the growth phase, age, or extent of transformation of the culture. It is

interesting that the virus-transformed cells in the studies of Hynes (25) and Hogg (19) exhibited labeling patterns similar to those found with the LM and L929 strains (transformed cells) used in this study. Another explanation for the differences found is that mucopolysaccharides which were synthesized intracellularly were secreted and adsorbed to the L cell membrane. The intact, iodinated L cell was not solubilized and electrophoresed in the study of Poduslo et al., and only 2% of the "incorporated" label per cell (not TCA precipitated) was reported to be localized to the membrane, suggesting possible cell damage and intracellular labeling. We found that 90% of the radioactivity was localized to the cell periphery by EM autoradiography.

CONCLUSION

In this study we have established the conditions necessary for the lactoperoxidase-specific iodination of tyrosine residues accessible in the plasma membrane polypeptides of living mouse L cells. In the accompanying paper we have used this specific labeling technique to study the metabolic fate of membrane proteins in the L cell and the flow and fate of surface membrane as it is interiorized.

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