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Surface-Specific Iodination of Membrane Proteins of Viruses and Eucaryotic Cells Using 1,3,4,6-Tetrachloro-3α,6α-diphenylglycoluril†

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ABSTRACT: The use of the iodinating reagent 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (chloroglycoluril) to selectively label membrane surface proteins was investigated with the following systems: enveloped viruses (Sendai and Newcastle disease viruses), human erythrocytes, and nucleated cells propagated both in suspension (EL-4) and in monolayer culture (BHK-21). Conditions are described for specifically iodinating surface proteins while maintaining full virus integrity or cell viability. Comparison of the chloroglycoluril method with the lactoperoxidase and chloramine-T methods for labeling surface membrane proteins shows that the chloroglycoluril method has a number of advantages: It routinely produces a 3- to 17-fold greater specific radioactivity without sacrificing viral or cellular integrity, it is technically simpler to use, it does not require the addition of extraneous protein to initiate the reaction nor a strong reducing reagent to terminate it. Chloroglycoluril also proved to be an effective substitute for chloramine-T in the nonvectorial labeling of viral and cellular proteins. Membrane protein samples were solubilized with the detergent sodium dodecyl sulfate before iodination or labeled in the presence of high iodide concentrations without prior solubilization. The resulting specific radioactivities generated by the use of chloroglycoluril were equal to or greater than those generated by the chloramine-T method. The effectiveness, simplicity of use, and versatility of chloroglycoluril recommend it as an iodinating reagent for both surface-specific and nonvectorial labeling of membrane systems.

The currently expanding interest in membrane architecture and surface phenomena such as hormonal action, cellular recognition, and chemotaxis demands methodology to label selectively externally disposed proteins, i.e., surface-specific (vectorial) methods. One of the most popular methods, lactoperoxidase-catalyzed iodination (LPO,[1] Philips and Morrison, 1971), using H_2O_2 alone or generated by the glucose-glucose oxidase system (GO, Hubbard and Cohn, 1972), requires the addition of extraneous protein to the system. This

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[1] Abbreviations used: LPO, lactoperoxidase; GO, glucose oxidase; chloramine-T, *N*-chloro-*p*-toluenesulfonamide; Tris, tris(hydroxymethyl)aminomethane; EDTA, (ethylenedinitrilo)tetraacetic acid; PBS, 150 mM NaCl in 5 mM sodium phosphate buffer at pH 8.0; DPBS, Dulbecco's phosphate-buffered saline (Dulbecco and Vogt, 1954); EL-4, C57 BL leukemia cells; BHK, baby hamster kidney cells; DPBI, Dulbecco's phosphate-buffered iodide (0.8% NaCl of DPBS replaced by 0.8% NaI); *Paramxyovirus* proteins; P, minor nucleocapsid protein; HN, major envelope protein associated with hemagglutinating and neuraminidase activities; NP, major nucleocapsid protein; F_1, envelope protein with role in hemolysis, cell fusion, and infectivity (possibly with F_2); M, matrix protein of the viral membrane; F_2, small envelope protein of unknown function; LETS, a large, external transformation-sensitive cellular glycoprotein.

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extraneous protein can itself become highly labeled during the iodination process. An alternative method employs chloramine-T. Under carefully defined conditions which include low iodide concentration, small amounts of this reagent, and brief time of exposure, preferential labeling of some surface proteins of viruses, bacteria, and eucaryotic cells has been achieved (Stanley and Haslam, 1971; Jone and Hager, 1976; Frost, 1977; Munford and Gotschlich, 1977). The possibility of protein denaturation by this powerful oxidant and the need for strong reducing conditions to terminate the reaction greatly restrict the utility of the chloramine-T method.

The introduction of 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril as a gentle, effective iodinating reagent for soluble and membrane proteins (Fraker and Speck, 1978) afforded the intriguing possibility that chloroglycoluril could be used as a nonenzymatic surface-specific reagent under proper conditions. This report describes the use of this reagent to vectorially label the externally exposed membrane proteins of erythrocytes and the envelope proteins of two paramyxoviruses, Sendai and Newcastle disease viruses. The paramyxoviruses possess a lipoprotein envelope which is derived from cell-surface membranes as the virus matures by exocytosis. These enveloped viruses constitute simple, well-defined membrane systems containing no more than a few major proteins. The relative positions of these proteins within the virion have been established using a variety of methods including accessibility to proteases (Calberg-Bacq et al., 1967), radiolabeling using lactoperoxidase and other surface probes (Moore et al., 1975; Li and Fox, 1975), and by isolation of envelope and nucleocapsid components following treatment with nonionic detergent (Scheid and Choppin, 1973; Shimizu et al., 1974; Lamb and Mahy, 1975; Li and Fox, 1975). In addition, retention of the biological integrity of the virion under iodinating conditions can be readily ascertained by measurements of its infectivity or ability to induce cell fusion. Extension of surface-specific labeling studies to human erythrocytes and nucleated cells demonstrates the versatility of chloroglycoluril as a surface-specific iodinating reagent.

Materials and Methods

Viruses. Sendai and Newcastle disease (strain HP-16) viruses were propagated in 10–11-day-old embryonated eggs. Allantoic fluid was harvested at 48-h postinfection. The virus was purified as described by Samson and Fox (1973) and stored frozen at −70 °C in 0.01 M Tris-HCl,[1] 0.10 M NaCl, 0.05 M Na_2EDTA at pH 7.4. The nomenclature for the paramxyovirus proteins described by Scheid and Choppin (1974) will be used throughout this paper.

Hemagglutination assays of Sendai virus were performed as described by Thacore and Youngner (1971) using washed chicken erythrocytes. The hemagglutination titer is expressed as the reciprocal of the greatest dilution of the virus suspension which caused agglutination of erythrocytes. The ability of Sendai virus to produce cell fusion was assessed using the procedure of Kohn (1965) with Madin-Darby bovine kidney cells grown in monolayer cultures in 16-mm diameter dishes (Linbro Chemical Co., Inc.). The fusion index is expressed as the average number of nuclei per cell.

Cells. Intact washed human erythrocytes were isolated from freshly drawn, heparinized blood by the method of Kant and Steck (1973). Ten milliliters of whole blood was mixed with 40 mL of ice-cold PBS [150 mM NaCl, 5 mM Na_2HPO_4 (pH 8.0)] and centrifuged for 10 min at 1100g and 4 °C. The supernatant fraction and buffy coat were removed by aspiration. The pellet was suspended in 50 mL of PBS and washed three additional times. The washed erythrocytes were finally suspended at a concentration of 8×10^8 cells/mL in PBS and were iodinated within 1 h of preparation. Unsealed ghosts (five-step ghosts) were prepared from the radiolabeled cells (Fairbanks et al., 1971) but with five wash cycles of 5 mM Na_2HPO_4 at pH 8.0 instead of three cycles. Erythrocytes were hemolyzed by suspension in 40 volumes of ice-cold 5 mM Na_2HPO_4 at pH 8.0 for 1 h. The membranes were pelleted by centrifugation for 20 min at 12 100g. After the supernatant fraction and tight pellet were removed by aspiration, the loose pellet was washed five additional times by suspension in 5 mM Na_2HPO_4 and centrifugation; the final five-step pellet was white. The supernatant fraction from the first lysate was centrifuged for 90 min at 75 000g to remove membrane vesicles.

Cells of a lymphosarcoma line, EL-4 (C57 BL, leukemia), were harvested from the peritoneal cavity of C57BL/6J mice and washed twice with Dulbecco's phosphate-buffered saline (DPBS) by centrifugation for 5 min at 270g. The cells were then incubated for 2 min at room temperature in 0.9% NH_4Cl in 10 mM Tris-HCl at pH 7.4 to lyse any contaminating erythrocytes and washed three additional times with DPBS before final suspension in DPBS at a concentration of 4×10^7 cells/mL.

Monolayer cultures of baby hamster kidney BHK-21(C-13) cells, American Type Culture Collection CCL10, were grown in a medium consisting of 10% tryptose phosphate broth, 10% fetal calf serum, and 80% minimum essential medium from Grand Island Biological Co. (Ham, 1965). The cells were used for iodination within 24 h of attaining confluency.

Viability of nucleated cells before and after iodination was assessed by exclusion of trypan blue dye. A 0.075% solution of trypan blue (Grand Island Biological Co.) in DPBS was applied to aliquots of cells in suspension or to monolayer cultures. The cells were incubated in the dye solution for 2 min and then counted. Viability is expressed as percentage of cells which excluded the dye. Samples of iodinated cells were subjected to mild trypsin digestion for 10 min with 10 μg/mL of trypsin (twice crystallized, Sigma Chemical Co.) as described by Hynes (1973).

Iodination Procedures for Viruses. Intact viruses (Sendai or Newcastle disease viruses) were iodinated using surface-specific LPO, chloroglycoluril, or chloramine-T methods. For each method, 100-μL aliquots of virus (100 μg of viral protein in 10 mM Na_2HPO_4 at pH 7.4) were reacted with 500 μCi of $Na^{125}I$ (carrier-free, Amersham/Searle Corp.) and the appropriate iodinating reagent at 0 °C. Samples containing chloramine-T (J.T. Baker Chemical Co.) were reacted for 1 min; all other samples were incubated for 10 min. Similar procedures were used to iodinate disrupted virions using nonvectorial chloroglycoluril or chloramine-T methods, but the viruses were solubilized in 2% sodium dodecyl sulfate prior to iodination and the iodination reactions proceeded at room temperature (21 °C). Radioiodination using chloroglycoluril was terminated by removal of the virus sample from the reaction vessel. Radioiodination using any of the other reagents was generally terminated by the addition of 5 μL of β-mercaptoethanol and 25 μmol of carrier NaI.

Samples iodinated using chloroglycoluril were used immediately for gel electrophoresis at this point and produced the same electrophoretic patterns as samples which had undergone a further centrifugation or dialysis purification step. Intact virus samples containing LPO, GO, or chloramine-T were centrifuged through a 30% (w/v) sucrose shelf for 90 min at 75 000g. Disrupted virus samples were dialyzed for 36 h at 4 °C against 10 mM $NaHPO_4$, 0.01 mM sodium azide at pH 7.4 with two changes of solution. The data for each table or figure were produced by iodination experiments performed on the

same day with the same batch of virus and same lot of $Na^{125}I$.

Four different LPO methods to iodinate Newcastle disease virus were used at 0 °C, but otherwise as cited in the literature. The first two employ repeated additions of H_2O_2 (Philips and Morrison, 1971; Li and Fox, 1975). The second two employ the H_2O_2-generating system of glucose–glucose oxidase (Hubbard and Cohn, 1972; Hynes, 1973). LPO and GO were obtained from Sigma Chemical Co. and Worthington Biochemical Corp., respectively.

Chloroglycoluril-mediated iodinations were done in previously prepared reaction vessels. A quantity of 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (available as IODO-GEN from Pierce Chemical Co.) was dissolved in chloroform and plated onto the surface of a glass reaction vessel (test tubes, scintillation vials, or coverslips) by drying under a stream of N_2 gas at room temperature. When the solvent was allowed to evaporate overnight by drying in open air instead of under a forced stream of gas, the reagent tended to flake off the surface of the vessel. Vessels plated with chloroglycoluril were stored in a desiccator at room temperature and used within 6 months. The vessels were rinsed with the iodinating buffer immediately before use to remove any loose flakes of chloroglycoluril.

Intact paramyxoviruses were routinely iodinated by adding virus to a 12 × 75 mm test tube (Van-Lab disposable Pyrex culture tubes) which had been plated previously with chloroglycoluril. The chloroglycoluril-driven reaction was stopped by removing the virus sample from the reaction vessel. The reaction vessel was rinsed twice with 100 μL of 10 mM phosphate at pH 7.4. The rinses were combined with the iodinated virus sample. A similar procedure was used to iodinate virus solubilized in 2% sodium dodecyl sulfate but the reaction proceeded at room temperature instead of 0 °C. Carrier iodide (25 μmol) was added to the samples before electrophoresis to hold isotope adsorption and consequent contamination of the apparatus to a minimum. Intact or disrupted virus samples were iodinated using 4 (Frost, 1977) or 100 μg of chloramine-T (Hunter and Greenwood, 1962) for 1 min as cited. Intact virus was iodinated at 0 °C; disrupted virus was iodinated at room temperature. An excess amount (10 or 250 μg) of sodium metabisulfite was added in addition to 25 μmol of carrier NaI to terminate the chloramine-T reaction.

Iodination Procedure for Cells. One milliliter of the washed erythrocyte suspension in PBS was added to scintillation vials (Packard low potassium glass vials for liquid scintillation counting). The vials had been previously plated with 10, 100, or 250 μg of chloroglycoluril. A 100-μL aliquot containing 12.4 units of LPO and 1 unit of GO (Hynes, 1973) or 3.6 milliunits each of LPO and GO (Hubbard and Cohn, 1975) in DPBS was added to other vials containing 5 mM glucose. The iodination reactions were initiated by the addition of 500 μCi of $Na^{125}I$ to each vial and allowed to proceed for 10 min with gentle agitation at 21 °C. The samples were then diluted with ice-cold PBS containing 5 mM carrier NaI and centrifuged for 10 min at 2500*g* to separate the cells from the enzyme catalysts and unreacted $Na^{125}I$ before preparing erythrocyte ghosts.

EL-4 cells (10^7 cells in 1 mL of DPBS) were radiolabeled in suspension using the chloroglycoluril and Hynes LPO-GO procedures described previously for erythrocytes but with 40% the amount of $Na^{125}I$, enzymes, and chloroglycoluril to compensate for the lower cell number. Cell viability was determined by trypan blue exclusion after the iodination reaction was terminated.

Confluent cultures of BHK-21 cells grown on 10-cm plastic dishes (Corning) were washed three times with 5 mL of DPBS before being overlaid with 3 mL of DPBS containing 5 mM glucose. Two glass coverslips (Gold Seal, 22 × 22 mm), previously plated with 100 μg of chloroglycoluril, were floated on one dish. As described by Hynes (1973) for the labeling of monolayer cultures, LPO (2.5 units) and GO (200 milliunits) were added to the second dish. Carrier-free $Na^{125}I$ (5 μL, 500 μCi) was added to each tissue culture dish, and the reaction mixtures were incubated and gently agitated by rotary shaking for 15 min at room temperature. After the dishes had been washed ten times with 5-mL aliquots of DPBI and then 5 mL of DPBS, viability of the cells was determined by trypan blue exclusion. To prepare the sample for polyacrylamide gel electrophoresis, BHK cells were solubilized by adding 1 mL of 3% sodium dodecyl sulfate to each dish, and the suspension was then drawn through a 25-gauge needle five times to reduce the viscosity of the preparation.

In order to label total cellular protein, 50-μL aliquots of erythrocyte ghosts and EL-4 and BHK-21 cells containing 100–200 μg of protein were solubilized in 10 mM Na_2HPO_4–2% sodium dodecyl sulfate at pH 7.4 by boiling for 2 min and labeled using 10 μg of chloroglycoluril and 500 μCi of $Na^{125}I$ for 10 min at room temperature. The preparations were decanted into test tubes containing 25 μmol of carrier NaI.

For each set of surface-specific iodination experiments, a control incubation was conducted concurrently by adding 500 μCi of $Na^{125}I$ to the virus or eucaryotic cell preparation and by allowing the control sample to incubate under the same conditions of time and temperature as the experimental samples but in the absence of external iodinating reagents. Results were used only if the control had a total incorporation of $\leq$1% of other samples, since oxidation products of $Na^{125}I$ can react to produce significant levels of nonspecific incorporation.

Gel Electrophoresis in Sodium Dodecyl Sulfate. Iodinated samples were prepared for gel electrophoresis by the addition of sodium dodecyl sulfate, glycerol, β-mercaptoethanol, and Bromophenol blue to achieve final concentrations of 2, 10, 5, and 0.008%, respectively. Samples not used on the day of preparation were immediately frozen and stored at −20 °C. All samples were used within 2 weeks of preparation. No differences were observed between fresh and frozen samples following electrophoresis. Molecular weight markers (*Escherichia coli* β-galactosidase, phosphorylase A, bovine serum albumin, ovalbumin, egg-white lysozyme, soybean trypsin inhibitor, and horse heart cytochrome *c*) were prepared, and 5 μg of each was electrophoresed on the same slab gel with iodinated samples. The known molecular weights of bands 1 and 2 of the erythrocyte ghost were also used in constructing calibration curves. Acrylamide, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine were purchased from Eastman, sodium dodecyl sulfate (Sequanal grade) was from Pierce, and ammonium persulfate was from J.T. Baker Chemical Co.

The procedure used for polyacrylamide slab gel electrophoresis was a modification of the discontinuous high-resolution system described by Laemmli (1970) for disc gels. The 1-mm thick slab gels generally consisted of 11.5 or 17.8 cm of a 5–12.5% acrylamide continuous gradient resolving gel of pH 8.8 and 1 cm of a 3% acrylamide stacking gel at pH 6.8. However, a 5–7.5% gradient gel was needed to resolve the NP and F proteins of Newcastle disease virus. To produce the two-dimensional gel of erythrocyte ghost proteins, a 11.5-cm slab was electrophoresed without the addition of mercaptan in the first dimension, sliced into its respective lanes, reduced with β-mercaptoethanol, and affixed with 1.5% agarose to the top of a 17.8-cm gel for the second dimension. After electro-

TABLE I: Comparison of Methods for Iodination of Newcastle Disease Virus.[a]

labeling method	quantity of iodinating reagent or reference	sp act.[b] (dpm/mg of protein)	HN/NP[c]	HN/NP intact[d] / HN/NP solubilized
intact virus				
control	none	0.00068×10^7	1.9	3.4
control with H_2O_2	none	0.29×10^7	2.6	4.5
LPO	Li & Fox (1975);	7.59×10^7	3.4	5.8
	Philips & Morrison (1971)	4.12×10^7	3.8	6.5
LPO-GO	Hubbard & Cohn (1972);	2.72×10^7	5.1	8.9
	Hynes (1973)	3.38×10^7	5.5	7.8
chloroglycoluril	4 μg	15.56×10^7	12.0	20.6
	10 μg	25.51×10^7	10.8	18.8
	100 μg	56.78×10^7	6.9	11.9
	10 μg + 1 mM NaI	18.02×10^7	2.4	4.1
chloramine-T	4 μg	2.55×10^7	1.6	2.7
	100 μg	10.17×10^7	0.7	1.2
solubilized virus[e]				
chloroglycoluril	10 μg	12.69×10^8	0.56	
	25 μg	24.15×10^8	0.55	
chloramine-T	4 μg	0.37×10^8	0.61	
	100 μg	10.56×10^8	0.59	

[a] The same preparation of Newcastle disease virus was iodinated by various methods (see Materials and Methods) using 100 μg of protein and 500 μCi of $Na^{125}I$ in a 100-μL reaction volume. Carrier NaI was added to one of the chloroglycoluril iodination mixtures to a final concentration of 1 mM. Iodinations of intact virus were performed at 0 °C; iodinations of solubilized virus were performed at 21 °C. [b] Specific activity was calculated from protein measurements (Markwell et al., 1978) and from radioactive measurements of protein samples precipitated with trichloroacetic acid (see Materials and Methods). [c] HN and NP proteins were resolved by electrophoresis on 5-7.5% slab gels. Their respective Coomassie blue stained bands were cut from the gel and counted in a gamma scintillation spectrometer. [d] The HN/NP ratio derived from iodination of the intact virus was divided by the average HN/NP ratio of the iodinated solubilized virus. [e] Virions were treated with 2% sodium dodecyl sulfate before iodination.

phoresis, the gels were stained with Coomassie brilliant blue R250, destained using the procedure of Fairbanks et al. (1971), and then dried under vacuum onto Whatman 3MM chromatography paper.

Radioactivity Measurements. Kodak no-screen X-ray film, double-coated NS-5T and single-coated SB-5, was used for autoradiography of the dried gels. In order to calculate the specific activity of the viral or eucaryotic cellular sample, protein was precipitated with cold 10% trichloroacetic acid containing 50 mM KI onto Whatman GF/C glass-fiber paper and washed using the disc-batch method outlined by Hubbard and Cohn (1975). A simulated ^{125}I standard was used to correct for counting efficiency. Protein was measured according to Lowry et al. (1951), as modified for use with membrane proteins (Markwell et al., 1978). To determine the percent distribution of radiolabel incorporated by individual viral proteins, Coomassie blue stained bands were cut from the dried gel and counted in a Packard gamma scintillation spectrometer. The ratio HN/NP was calculated from the amount of label incorporated into the major surface-exposed protein of the virus envelope (HN) divided by the amount incorporated into the major nucleocapsid protein (NP). This ratio was used as an indication of the surface specificity of the iodination method for viral samples.

Results

Iodination of Newcastle Disease Virus. The use of chloroglycoluril as a surface-specific reagent for membrane protein iodination was first explored with Newcastle disease virus, a membrane-enveloped paramyxovirus. The surface iodination selectivities of the chloroglycoluril and widely used LPO and chloramine-T methods were tested by determining the relative amounts of radioiodine incorporated into the major externally displayed (HN) and internally displayed (NP) viral proteins (Table I). All four LPO methods produced similar levels of radioiodine incorporation and surface specificity, as indicated by the HN/NP ratios. Slightly greater specific activities were obtained with LPO methods which rely on the addition of H_2O_2 (Philips and Morrison, 1971; Li and Fox, 1975) than with those which generate it using the glucose–glucose oxidase system (Hubbard and Cohn, 1972; Hynes, 1973), but the HN/NP ratios were slightly lower with procedures which employ added H_2O_2. In a control reaction containing only viral protein and ^{125}I, virtually no iodination of virus occurred. A small amount of nonenzymatic iodination, 3.8% of that obtained with the complete system, occurred when H_2O_2 was added to the control reaction (Table I).

The chloroglycoluril method produced substantially greater specific activities (5- to 17-fold) and higher HN/NP ratios than did any of other methods (Table I). The effect of chloroglycoluril concentration on the surface specificity of iodination of Newcastle disease virus was determined by varying the amount of reagent plated under standard conditions onto a 12 × 75 mm test tube (see Materials and Methods). Surface-specific labeling of NDV, as indicated by the HN/NP ratio, is sensitive to chloroglycoluril concentration. The 4–10-μg range of chloroglycoluril was optimal for the surface-specific iodination of 100 μg of viral protein. Higher concentrations of reagent produced an increase in specific activities but with corresponding decreases in the HN/NP ratio (Table I). Chloroglycoluril concentrations lower than those employed here (Table I) often led to a moderate loss of surface-specific labeling (data not shown, see also Table III). When the amount of viral protein was decreased tenfold to 10 μg, the amount of chloroglycoluril required for optimal surface-specific labeling decreased tenfold also. The effect of chloroglycoluril-mediated iodination of viral infectivity was judged by its effect on plaque formation using chick secondary monolayers (Samson and Fox, 1973). Samples of intact virus, labeled using 10 μg of chloroglycoluril, produced the same number of plaques (4.0×10^{10} pfu/mL) as did a noniodinated control sample (3.8×10^{10} pfu/mL).

TABLE II: Comparison of Proteins in Egg-Grown Newcastle Disease and Sendai Viruses.[a]

polypeptide	apparent mol wt in Newcastle disease virus	apparent mol wt in Sendai virus	position in virion	accessibility to surface-specific labeling by chloroglycoluril
P		78 000	nucleocapsid	−
HN	74 000	70 000	membr exterior	+
NP	55 000	60 000	nucleocapsid	−
F_1	53 000	47 000	membr exterior	+
M	34 000	34 000	membr interior	−

[a] Apparent molecular weights of viral proteins were calculated from molecular weight standards by coelectrophoreses with viral proteins on the 5–12.5% gradient gel shown in Figure 1.

TABLE III: Comparison of Methods for Iodination of Sendai Virus.[a]

labeling method	quantity of reagent or reference	sp act. (dpm/mg of protein)	HN/NP	HN/NP intact / HN/NP solubilized
intact virus:				
LPO	Li & Fox (1975);	5.00×10^7	2.1	3.6
	Phillips & Morrison (1971)	3.85×10^7	2.4	4.1
chloroglycoluril	1 μg	6.78×10^7	2.1	3.6
	10 μg	16.96×10^7	3.2	5.6
	100 μg	69.56×10^7	1.8	3.1
solubilized virus[b]				
chloroglycoluril	1 μg	4.78×10^8	0.58	
	10 μg	18.52×10^8	0.55	
chloramine-T	4 μg	0.23×10^8	0.58	
	100 μg	10.00×10^8	0.61	

[a] Aliquots of Sendai virus (100 μg of protein) were iodinated in a 100-μL reaction mixture containing 500 μCi of $Na^{125}I$ in 10 mM Na_2HPO_4 at pH 7.4. Iodinations of intact virus were performed at 0 °C; iodinations of solubilized virus were performed at 21 °C. Specific activity, HN/NP ratio, and (HN/NP intact)/(HN/NP solubilized) ratio were determined as in Table I. [b] Virions were treated with 2% sodium dodecyl sulfate before iodination.

Low concentrations of chloramine-T are reported to be suitable for iodination of intact polyoma virus and adenovirus (Frost, 1977). When used with Newcastle disease virus, chloramine-T, even at low concentrations, did not appear to discriminate adequately between labeling of surface membrane (HN) and nucleocapsid (NP) proteins and produced lower specific radioactivities than did chloroglycoluril-mediated iodination (Table I).

Generalized iodination of both membrane and nucleocapsid proteins (nonspecific or nonvectorial iodination) was achieved by two different approaches using chloroglycoluril as the iodinating reagent. In the first approach, carrier NaI was added to the reaction vessel while the intact virus sample was iodinated at 0 °C. The addition of carrier iodide at 1 mM (500 μCi of ^{125}I is 5 μM in this system) did not significantly reduce the specific activity obtained with 10 μg of chloroglycoluril (Table I) but dramatically increased the amount of label incorporated by the nucleocapsid protein, as evidenced by the 4.5-fold decrease in the HN/NP ratio. In a separate experiment, the addition of sodium iodide at 0.02, 0.10, and 1.0 mM produced specific activities of 75, 154, and 119% and HN/NP ratios of 93, 59, and 34% of those observed where no carrier iodide was added.

In the second approach to nonvectorial iodination, the virions were first solubilized in 2% sodium dodecyl sulfate solution, and the preparation was added to a vessel plated with 10 μg of chloroglycoluril for a 10-min reaction at 21 °C. This resulted in a lower HN/NP ratio than achieved with the first approach. An increase in chloroglycoluril concentration to 25 μg raised the specific activity twofold, but did not change the HN/NP ratio. Iodination of solubilized virus using chloramine-T produced the same HN/NP ratios as did chloroglycoluril-mediated iodination. In the presence of sodium dodecyl sulfate, 10 μg of chloroglycoluril and 100 μg of chloramine-T were equally effective in iodinating solubilized viral proteins (Table I).

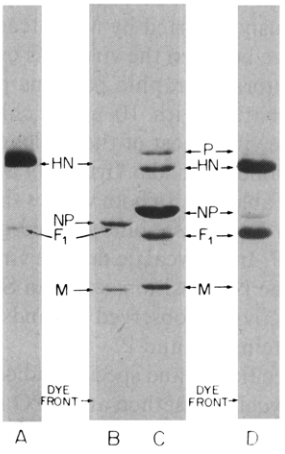

FIGURE 1: Comparison of Newcastle disease and Sendai viral proteins. The Coomassie blue stained patterns of Newcastle disease (B) and Sendai viral proteins (C) electrophoresed in adjacent lanes on the same 5–12.5% gradient slab gel are shown next to their respective autoradiographs (A and D) of proteins from intact viruses iodinated using 10 μg of chloroglycoluril.

Optimal Conditions for Surface-Specific Iodination of Sendai Virus. A second paramyxovirus, Sendai virus, is closely related to Newcastle disease virus in protein structure but it is more difficult to iodinate the surface specifically. This is consistent with reports that ordinary preparations of Sendai

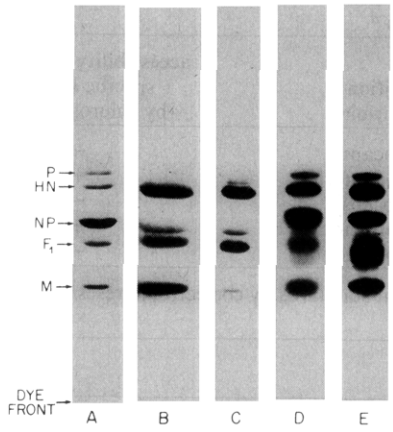

FIGURE 2: Comparison of methods for iodination of Sendai virus. Intact Sendai virus (Coomassie blue stained pattern of Sendai viral proteins resolved on a 5–12.5% gradient gel, A) was iodinated using LPO (B) or 10 μg of chloroglycoluril (C). Disrupted virus was iodinated in the presence of sodium dodecyl sulfate using 10 μg of chloroglycoluril (D) or 100 μg of chloramine-T (E).

virus consist of a mixture of virions with intact and damaged envelopes (Shimizu et al., 1976). This labile virus may further deteriorate during purification and handling (Lonberg-Holm and Philipson, 1974).

The Coomassie blue dye profiles of Sendai and Newcastle disease viral proteins run in adjacent lanes on a 5–12.5% slab gel are compared in Figure 1 and Table II. The envelope glycoproteins HN and F_1 of Newcastle disease virus have greater apparent molecular weights than do their counterparts in Sendai virus, the major nucleocapsid protein NP has a smaller molecular weight, and the M protein has approximately the same molecular weight. Newcastle disease virus lacks the minor nucleocapsid protein P. These findings are consistent with those previously reported by Mountcastle et al. (1971). Another difference between the viruses is evident from comparison of the autoradiographic patterns produced by surface-specific iodination with 10 μg of chloroglycoluril. In Newcastle disease virus most of the radioactivity is incorporated into HN, while in Sendai virus HN and F_1 are equally labeled (see also Table IV). In both viruses the most prominent protein species in Coomassie blue stained gels is the nucleocapsid protein NP. In Newcastle disease virus no radioactive band is seen at the NP position, while in Sendai virus some detectable radioactivity is observed in bands corresponding to nucleocapsid proteins NP and P.

The surface specificities and specific radioactivities achieved with the chloroglycoluril method and LPO methods were determined with the same preparation of Sendai virus (Table III). Similar levels of incorporation of radioiodine and surface specificities were observed with the LPO methods of Li and Fox (1975) and Philips and Morrison (1971). The Philips and Morrison method yielded a 23% lower specific radioactivity but a slightly higher HN/NP ratio (Table III).

Optimal surface-specific labeling of Sendai virus was achieved with a ratio of 1 μg of chloroglycoluril for 10 μg of viral protein (Table III), which is consistent with the data for Newcastle disease virus (Table I). The same optimal ratio of 1:10 was obtained using 10 or 100 μg of viral protein. At optimal concentration, chloroglycoluril produced a threefold greater specific activity and a HN/NP ratio equal to or greater than those achieved with either of the LPO methods (Table III). Surface-specific iodination of Sendai virus using 10 μg of chloroglycoluril did not affect the ability of the virus to induce hemagglutination of erythrocytes and cell–cell fusion. Aliquots of purified Sendai virus (100 μg) had the same hemagglutination titer and fusion index before and after iodination using standard reaction conditions [hemagglutination titer: before = 5120/mL, after = 5120/mL; fusion index: before = 1.96, after = 1.94].

A study of label incorporation and distribution vs. reaction time at 0 °C with 10 μg of chloroglycoluril revealed that incorporation of label by 100 μg of Sendai viral protein was maximal at 10 min and remained constant for at least 20 additional min. Approximately 75% of the label was incorporated during the initial 5 min. The HN/NP ratio was the same for 5- and 30-min reaction periods.

Welton and Aust (1972) had recommended the inclusion of an antioxidant such as butylated hydroxytoluene in iodination mixtures to prevent lipid peroxidation and to increase the incorporation of ^{125}I into protein. The inclusion of 0.005% (w/v) butylated hydroxytoluene had no significant effect on the total incorporation of label or on the HN/NP ratio achieved under standard reaction conditions, i.e., iodination of 100 μg of viral protein using 10 μg of chloroglycoluril for a 10-min reaction period at 0 °C.

Iodination of solubilized Sendai virus using either chloroglycoluril or chloramine-T produced a HN/NP ratio of 0.58 (Table III), the same ratio observed with Newcastle disease virus (Table I). Thus, the more selective surface-specific labeling of Newcastle disease virus (HN/NP of 11 vs. 3 at 10 μg of chloroglycoluril) was not due to a proportionally smaller content of NP. As previously noted with Newcastle disease virus, Sendai virions disrupted by sodium dodecyl sulfate were labeled to an equal or greater specific radioactivity with 10 μg of chloroglycoluril than with 100 μg of chloramine-T. Reduction in the amount of either iodinating reagent resulted in a substantial decrease in the amount of ^{125}I incorporated, but the HN/NP ratio remained constant.

Figure 2 shows a comparison of the results obtained with surface-specific and nonspecific iodinations of Sendai virus. The Coomassie blue profile of the noniodinated virus is shown in lane A. The two proteins HN and F_1, which are externally displayed on the surface of the virion, are intensely labeled by both LPO (B) and chloroglycoluril (C) methods, and the two nucleocapsid proteins NP and P incorporate much less label. The third membrane protein M, which appears to be associated with the inner surface of the bilayer only (Rott and Klenk, 1977), is iodinated substantially by the LPO but not by the chloroglycoluril method.

Some of the radioactivity seemingly incorporated by NP in Sendai virus is due to a minor component of approximately 57 000 daltons. This polypeptide cannot be totally separated from NP but appears as a heavily labeled leading edge of the NP band in preparations labeled by surface-specific methods (Figure 2B,C), and it partially accounts for the low HN/NP ratios attained with surface-specific labeling of Sendai virus. A similar polypeptide migrating between Sendai viral proteins NP and F_1 has been observed in preparations of Sendai virus grown in chick embryo lung cells and Madin-Darby bovine kidney cells (Lamb and Mahy, 1975; Mountcastle et al., 1971). The possible derivation of this protein from HN was indicated by its migration with HN in the nonreduced dimension of a two-dimensional slab-gel system and the appearance of a 57 000 and a 17 000 M_r protein component after reduction (Markwell and Fox, unpublished data).

The autoradiographs of gels of the detergent-treated virus preparation labeled by chloroglycoluril (Figure 2D) or chloramine-T (E) methods are similar in appearance to the Coomassie blue dye profile of the viral proteins (A). The F_1 protein of the virus preparation iodinated by chloramine-T formed a

TABLE IV: Distribution of Radioactivity in the Proteins of Sendai Virus.[a]

viral protein	labeling of intact virus: lacto-peroxidase[d]	chloroglycoluril 1 μg	chloroglycoluril 10 μg	labeling of solubilized virus[b]: chloroglycoluril, 10 μg	chloramine-T, 100 μg	^{14}C in vivo[c] labeling
P	45	26	17	41	33	57
HN	100	100	100	100	100	100
NP	48	48	31	181	164	168
F_1	120	77	91	97	108	86
M	88	37	29	111	121	109
F_2	78	18	21	19	43	26

[a] Aliquots of the iodinated Sendai virus samples used in Table II were electrophoresed on 5–12.5% slab gels to resolve the major viral proteins. Their corresponding Coomassie blue stained bands were cut from the gel and counted in a gamma scintillation spectrometer. Values were normalized by designating the amount of radioactivity incorporated into HN as 100. [b] The virus sample was solubilized in 10 mM Na_2HPO_4, 2% sodium dodecyl sulfate at pH 7.4 before iodination. [c] Values reported by Hosaka and Shimizu (1977) for egg-grown Sendai virus using a mixture of ^{14}C-labeled amino acids. [d] Li and Fox (1975) method.

more diffuse band than with any of the other iodinated preparations.

A portion of each virus sample used to determine the specific radioactivities expressed in Table III was electrophoresed on a 5–12.5% gradient gel. The distribution of ^{125}I among the major proteins of Sendai virus was determined for each method by cutting the Coomassie blue band corresponding to the respective protein from the dried gel and counting it in a gamma counter (Table IV). As previously mentioned, the exposed proteins of the viral envelope HN and F_1 incorporate the bulk of the radioactivity with surface-specific labeling methods. The matrix protein M and the small envelope glycoprotein F_2 are far more effectively labeled when intact virions are iodinated by the LPO method than by the chloroglycoluril method. The major viral proteins HN, F, and NP display a similar incorporation of label using either the chloroglycoluril or LPO methods. Radioiodination of isolated Sendai virions by the chloroglycoluril and chloramine-T methods in the presence of sodium dodecyl sulfate produces a distribution of label among the viral proteins which closely reflects that produced in ovo by incorporation of a mixture of ^{14}C-labeled amino acids (Hosaka and Shimizu, 1977). This indicates an essentially uniform distribution of iodinatable residues among the viral polypeptides.

The reproducibility of labeling patterns obtained by iodination of intact Sendai virus by the chloroglycoluril technique was tested with two different 100-μg aliquots of protein from the same preparation of Sendai virus at a 1-week interval and electrophoresis on separate gels. The major viral protein bands were cut out and the radioactivity was determined. Values are expressed normalized to HN as described in Table IV and shown as viral protein, sample 1:sample 2—HN, 100:100; NP, 31:31; F_1, 91:92; M, 29:29; F_2, 21:22.

Fraker and Speck (1978) had indicated that chloroglycoluril if properly plated should remain in the reaction vessel after the reaction mixture had been decanted by virtue of the reagent's insolubility in aqueous media. Therefore, the simple removal of the iodinated sample from the reaction vessel should effectively terminate the reaction without the addition of strong reducing reagents. To illustrate this, the following experiment was performed. Intact Sendai virus (100 μg of viral protein) was iodinated by the standard method (see Materials and Methods) with 10 μg of chloroglycoluril for 5 min instead of 10. Half of the iodinated sample was then transferred to a second test tube containing no chloroglycoluril. After a 1-min interval to allow reaction of small amounts of soluble iodinating species such as molecular iodine which may have been formed, 15 μg of *E. coli* β-galactosidase was added to both test tubes and the samples were incubated for an additional 5 min at 0 °C. The first virus sample was then removed from the original reaction vessel containing the chloroglycoluril, and both virus samples were prepared for resolution of viral proteins by gel electrophoresis. In the first sample, which was left in contact with chloroglycoluril for the entire 11 min, the β-galactosidase incorporated 11% of the total radioactivity. In the second sample, which was removed from the chloroglycoluril before the addition of β-galactosidase, the band contained less than 1% of the total radioactivity (approximately twice the amount incorporated by a control containing ^{125}I but no chloroglycoluril). When the same experiment was performed in the presence of 2% sodium dodecyl sulfate, more radioactivity (about 3% of the total) was found in the β-galactosidase band of the second sample. This indicates a tendency for chloroglycoluril to become suspended in the reaction mixture in the presence of sodium dodecyl sulfate.

Radioiodination of Human Erythrocytes. Fraker and Speck (1978) have used chloroglycoluril to label intact sheep erythrocytes and LPC-1 tumor cells, but their report gave no indication as to whether the labeling was surface specific or not. We have compared the iodination of various cell types by the chloroglycoluril and LPO methods.

Results of the iodination of intact washed human erythrocytes using surface-specific LPO–GO and chloroglycoluril procedures (see Materials and Methods) are compared in Table V. The sample containing only erythrocytes and $Na^{125}I$ (listed as control in Table V) incorporated <1% of the radioactivity of any of the complete systems. Deletion of any of the reagents (LPO, GO, or exogenous glucose) in the LPO–GO systems reduced the incorporation to <1% of that incorporated by a complete system (data not shown). All of the iodination procedures appeared to be surface specific to the extent that only 1–2% of the total incorporated radioactivity was found in the supernatant fraction of the first lysate (Table V). Both LPO–GO methods produced similar levels of radioiodine incorporation by the membrane fraction (first pellet): the chloroglycoluril method gave a substantially greater level of radioiodine incorporation than did either of the LPO–GO methods.

The distribution of radioactivity between purified membrane and cytosol fractions from iodinated intact erythrocytes was examined for two of the methods (Table VI). Five-step ghosts which contained no detectable hemoglobin (see also Figure 3) were prepared as detailed under Materials and Methods. The supernatant fraction of the first lysate was centrifuged at 75 000*g* for 90 min to remove membrane vesicles. The chloroglycoluril method produced a fourfold greater level of total

TABLE V: Comparison of Surface-Specific Methods for Iodination of Human Erythrocytes.[a]

method	quantity of iodinating reagent or reference	radioact. (dpm/8 × 10^8 cells) in 1st pellet	radioact. (dpm/8 × 10^8 cells) in 1st supernat fract	% of radioact. in supernat fract
control	none	0.09 × 10^6	0.12 × 10^6	55
LPO-GO	Hubbard & Cohn (1972);	26	0.56	2
	Hynes (1973)	39	0.81	2
chloroglycoluril	10 μg	141	1.38	1
	100 μg	495	3.53	1
	250 μg	734	8.75	2

[a] Washed human erythrocytes (8 × 10^8 cells/mL) were iodinated at 21 °C by LPO-GO and chloroglycoluril methods. The control contained erythrocytes and 500 μCi of $Na^{125}I$. Radioactivities found in acid-precipitable material from the first lysate pellet and supernatant fractions were measured in a gamma scintillation spectrometer.

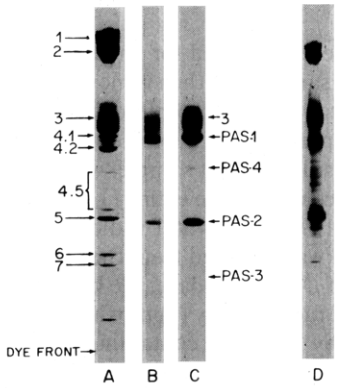

FIGURE 3: One-dimensional gradient gel electrophoresis of iodinated erythrocyte ghosts. Five-step ghosts (Coomassie blue stained pattern, A) which contained no detectable hemoglobin were isolated from intact red blood cells that had been iodinated using LPO (B) or chloroglycoluril (C). Isolated ghosts were iodinated using chloroglycoluril after solubilization with detergent (D) and electrophoresed on a 5–12.5% gradient gel.

TABLE VI: Distribution of Radioactivity after Iodination of Intact Erythrocytes.[a]

labeling method	fraction	sp act.[b] (dpm/mg of protein)	relative enrichment
chloroglycoluril	total cell	0.570 × 10^6	1
	5-step ghosts	48.4 × 10^6	85
	supernatant	0.018 × 10^6	0.03
LPO-GO	total cell	0.138 × 10^6	1
	5-step ghosts	11.4 × 10^6	83
	supernatant	0.003 × 10^6	0.02

[a] Washed human erythrocytes were iodinated at 21 °C by chloroglycoluril (100 μg) and LPO-GO (Hynes, 1973) methods using 500 μCi of $Na^{125}I$ for 8 × 10^8 cells. Unsealed ghosts were prepared from the iodinated cells and washed five times with 5 mM Na_2HPO_4 at pH 8.0. The supernatant fraction was prepared from the first lysis supernatant fraction by centrifugation for 90 min at 75 000*g* to remove membrane vesicles. [b] Specific activity was calculated as in Table I.

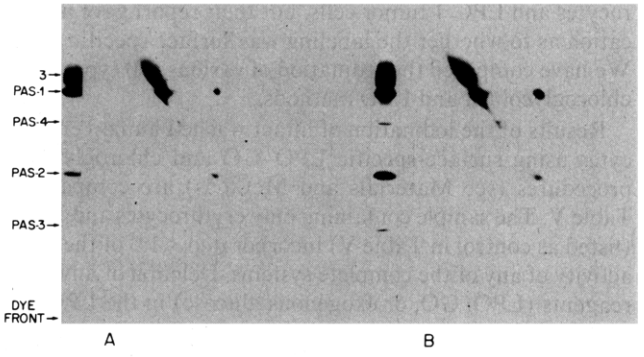

FIGURE 4: Two-dimensional gel electrophoresis of iodinated erythrocyte ghosts. Two-dimensional gel electrophoresis was performed on ghost proteins isolated from erythrocytes that had been iodinated using LPO (A) or chloroglycoluril (B) surface-specific methods. Autoradiographs of one-dimensional 5–12.5% gradient gels of each of the samples are shown to the left of the two-dimensional gels for comparison.

cell incorporation of radioiodine and fourfold greater specific activity in the purified ghost fraction than did the Hynes LPO-GO method. The specific activity values and 83-fold enrichment attained with the Hynes LPO-GO method are consistent with the data reported by Hubbard and Cohn (1972) for their LPO-GO method.

To determine if the same membrane proteins were iodinated by the chloroglycoluril and LPO methods, the autoradiographs of slab gels of ghost proteins isolated from cells labeled by the two methods were compared with each other and with their Coomassie blue dye patterns (Figure 3). The dye patterns were identical to each other and to that of the noniodinated sample (lane A). The autoradiographs produced by the two methods (lanes B and C) were also identical, except in intensity. The major membrane glycoproteins, band 3, PAS-1 and PAS-2 and the minor glycoproteins PAS-4 and PAS-3 were reactive in intact cells. No radioactivity was apparent in spectrin bands 1 and 2. Identification of the labeled proteins was made on the basis of migration rate, as reported by Steck (1974); the band nomenclature for erythrocyte ghost proteins was that of Fairbanks et al. (1971). Similar labeling patterns have been obtained with LPO or LPO-GO procedures (Philips and Morrison, 1971; Hubbard and Cohn, 1972; Reichstein and Blostein, 1973). The autoradiograph (Figure 3D) of gels of erythrocyte ghost proteins which had been solubilized in detergent before chloroglycoluril-mediated iodination resembles the Coomassie blue dye pattern; labeling of spectrin bands 1 and 2 is evident. A specific radioactivity of 19–24 × 10^8 dpm/mg of protein was routinely achieved during iodination of solubilized ghosts.

Erythrocyte ghost proteins were also electrophoresed in a two-dimensional slab-gel system (Figure 4) where the two dimensions are identical in composition (5–12.5% acrylamide), except for the inclusion of a reducing agent in the second dimension. In the two-dimensional gel of the control sample (cells exposed to ^{125}I without any external iodinating reagent), no major dye spots appeared below the diagonal in the Coomassie blue dye pattern, indicating the absence of native intermolecular disulfide linkages as shown previously by Wang and

TABLE VII: Comparison of Methods for Iodination of Cells in Suspension and Monolayer Culture.[a]

cell type	iodination method	% viability[b]	sp act. (dpm/mg of protein)[c]
EL-4			
intact cell:	control	97	0.01×10^7
	chloroglycoluril (10 μg)	88	1.2×10^7
	chloroglycoluril (100 μg)	92	5.4×10^7
	lactoperoxidase	95	1.7×10^7
solubilized cell:[d]	chloroglycoluril (10 μg)		75.0×10^7
BHK-21			
intact cell:	control	98	0.03×10^6
	chloroglycoluril (100 μg)	95	3.3×10^6
	lactoperoxidase	93	2.6×10^6
solubilized cell:[d]	chloroglycoluril (10 μg)		202.0×10^7

[a] EL-4 cells (10^7 cells in 1 mL of suspension medium) and confluent 10-cm dishes of BHK-21 cells were iodinated at 21 °C using the chloroglycoluril and lactoperoxidase–glucose oxidase methods. Control samples contained the same amount of $Na^{125}I$ as experimental samples (100 μCi for EL-4 samples, 500 μCi for BHK-21 samples) but contained no external iodinating reagent. [b] Viability was assessed by trypan blue dye exclusion after iodination. [c] Specific activity was calculated as in Table I. [d] Cells were solubilized in 10 mM Na_2HPO_4, 2% sodium dodecyl sulfate at pH 7.4 before iodination.

Richards (1974). There were no spots below the diagonal in Coomassie blue dye patterns and autoradiographs of ghosts isolated from LPO or chloroglycoluril-treated cells (Figure 4). Thus, exposure of these reagents did not result in oxidation of sulfhydryl groups to form new disulfide bonds. The spot above the diagonal evident in both autoradiographs was produced by the reassociation of some of PAS-2 to form the dimer PAS-1 (Marton and Garvin, 1973; Furthmayr and Marchesi, 1976) before electrophoresis in the second dimension.

Iodination of Nucleated Cells. Surface-specific iodination techniques employing chloroglycoluril and developed with viral and erythrocyte systems were also applied to nucleated cells propagated in suspension (EL-4) or in monolayer culture (BHK-21). Iodination of the latter was technically more difficult because coverslips plated with chloroglycoluril had to be carefully floated above the cells so as not to shear them. Three different concentrations of chloroglycoluril were used with EL-4 cells. The lowest two (10 and 100 μg) produced levels of radioiodine incorporation similar to or slightly greater than that obtained with lactoperoxidase-catalyzed iodination (Table VII). An increase in the amount of chloroglycoluril to 250 μg resulted in a dramatic loss of cell viability (<10% viable). With BHK-21 cells, the extent of incorporation of radioiodine and retention of viability were essentially identical when the LPO and chloroglycoluril-driven processes were applied. Solubilization of cellular protein before iodination results in specific radioactivities of 60- to 600-fold greater than those produced by surface-specific labeling methods. The labeling patterns of cells solubilized prior to iodination (Figure 5D) differed dramatically from those of intact cells (Figure 5B). The inclusion of 1 mM NaI during iodination of intact cells produced nonvectorial labeling patterns similar to those of cells disrupted by detergent, but the addition of NaI did not affect cell viability. Treatment of BHK cells labeled at high iodine concentration by mild tryptic digestion as described by Hynes (1973) had little effect on the radioactive band profile. Mild tryptic digestion of cells labeled at a low iodide concentration (surface-specific labeling) led to a dramatic loss of the label, especially from the 250 000 molecular weight protein band (data not shown).

The Coomassie blue dye patterns of proteins from nucleated cells (Figure 5A) are more complex than those of erythrocytes and paramyxoviruses, and the distribution of iodinatable residues among the proteins is less uniform. Some protein bands which stain intensely with Coomassie blue dye are noticeably absent in autoradiographs of cellular protein iodinated in the presence of sodium dodecyl sulfate (Figure 5D). The autoradiographs obtained after surface-specific labeling by chloroglycoluril and LPO methods (Figure 5B,C) are indistinguishable from each other for each cell type and distinctly different from the Coomassie blue dye pattern (Figure 5A) and the autoradiographs of cells solubilized in detergent before iodination (Figure 5D). A heavily labeled protein migrating at a molecular weight of 250 000 was observed in the autoradiographs of surface specifically labeled BHK-21 cells, a normal fibroblast line, but does not appear, as expected, in autoradiographs of the iodinated proteins of EL-4, a lymphosarcoma line. The autoradiographs of surface specifically labeled BHK cells (Figure 5B,C) resemble those previously published by Hynes and Pearlstein (1976) in which they identified the heavily labeled, high-molecular-weight protein as the LETS protein (fibronectin).

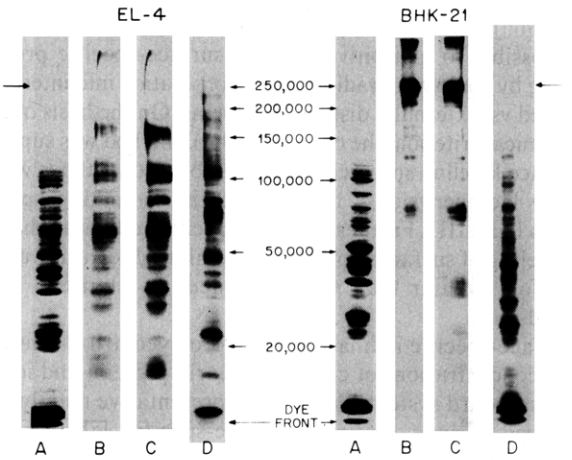

FIGURE 5: Comparison of methods for surface-specific iodination of nucleated cells. The Coomassie blue patterns (A) and autoradiographs of proteins from EL-4 cells iodinated in suspension and BHK-21 cells iodinated in monolayer culture using LPO (B) or chloroglycoluril (C) are shown next to autoradiographs of proteins from cells solubilized with detergent before chloroglycoluril-mediated iodination (D). An arrow marks the position of the LETS glycoprotein.

Discussion

Chloroglycoluril provides an effective, gentle, and technically simple means for labeling externally displayed proteins

on the surfaces of viruses and cells. This method avoids the introduction of extraneous proteins into the reaction system, obviating the problems in interpretation which may be encountered when the added protein catalysts have molecular weights similar to those of a cell or virus surface protein being studied. Since essentially all the chloroglycoluril remains in the reaction vessel after the iodinated sample has been removed, additional procedures are not required for separating the sample from the iodinating reagent. Iodinated samples can be used immediately for electrophoresis or for biological and chemical studies in which the presence of unreacted iodide does not interfere.

Optimal conditions for the use of chloroglycoluril as a surface-specific protein iodinating reagent for membrane-enveloped systems were developed with members of the paramyxovirus group and with human erythrocytes. Both systems are characterized by a single membrane containing a limited number of proteins. These proteins have been thoroughly characterized for membrane vs. soluble fraction localization. The extrinsic vs. intrinsic nature and the external vs. internal display characteristics have also been fastidiously established for the major membrane proteins of both systems. It is therefore possible to quantitively assess surface-specific protein labeling by comparing radioiodine incorporation into internally localized vs. externally displayed proteins. On the basis of this biochemical criterion, the chloroglycoluril method was superior in surface labeling specificity to four published methods which employ lactoperoxidase to generate the iodinating species (Tables I and III). Furthermore, the specific activities achieved for labeling of surface proteins using chloroglycoluril were 3- to 17-fold greater than those produced by standard LPO methods.

Surface-specific iodination by chloroglycoluril is sensitive to the concentrations of chloroglycoluril, protein, and iodide in the standard system. With two representative membrane-enveloped viruses, optimal surface-specific labeling was achieved at 4–10 μg of chloroglycouril/100 μg of protein. An increase in chloroglycouril concentration above 10 μg resulted in a loss of optimal surface-specific labeling. Similarly, when the protein concentration was decreased from 100 to 10 μg, the maximal concentration of chloroglycoluril for optimal surface-specific labeling also decreased by a factor of 10 (data not shown). This indicates that a ratio of 10 μg of chloroglycoluril/100 μg of protein should not be exceeded when optimal surface-specific labeling is desired. Similar studies with erythrocytes indicate that optimal surface-specific labeling was lost when the chloroglycoluril concentration exceeded 100 $\mu g/10^7$ cells. With this standard protein or cell/chloroglycoluril ratio, spectrin, which underlies the erythrocyte membrane (Steck, 1974), and the matrix (M) protein, which lies at the paramyxovirus envelope/capsid interface (Rott and Klenk, 1977), were not effectively labeled under conditions where surface-exposed proteins were highly labeled.

Iodination leading to surface-specific labeling of proteins by the chloroglycoluril method predominated at the low iodide concentrations (5 μM) used in this study. Significant loss of vectorial specificity was observed at iodide concentrations of 100 μM or greater. At a high iodide concentration (1 mM), iodination using chloroglycoluril became essentially nonvectorial (Table I), and the reaction mixture developed a yellow color. These data are consistent with chloroglycoluril-mediated generation of iodine, a membrane-permeable iodinating species. It is not clear, however, whether high iodide concentration favors: (1) excessive production of the same iodinating species produced at low iodide concentration, but in amounts sufficient to more than saturate rate-favored, reactive groups on membranes or (2) production of a new and more membrane-permeable protein-iodinating species. The former explanation is consistent with the fact that surface-specific labeling of enveloped viruses by the chloroglycoluril method is sensitive to decreased protein concentration at constant chloroglycoluril and iodide concentrations. The latter mechanism, generation of a new iodinating species, has been proposed to explain nonvectorial labeling achieved by use of LPO (Tsai et al., 1973) and chloramine-T (Montelaro and Rueckert, 1977) at high iodide concentrations. Iodination at high iodide concentrations in the absence of detergents has proved useful for nonvectorial labeling of viral membrane and nucleocapsid proteins without disrupting the membrane structure (Table I).

Nonvectorial labeling was also achieved by solubilization of viral or cellular proteins with detergent, e.g., sodium dodecyl sulfate, prior to iodination. This procedure produced greater specific activities and a more general distribution of label than did the use of high iodide concentrations, but necessarily disrupted membrane structure. With this approach, chloroglycoluril produced specific activities equal to or greater than those achieved with chloramine-T (Tables I and III).

Surface-specific iodination of nucleated cells was achieved using conditions optimized for chloroglycoluril-mediated, surface-specific labeling of erythrocytes and membrane-enveloped viruses. Chloroglycoluril-mediated labeling of intact BHK-21 cells grown in monolayer culture produced a pattern of radioiodination which was indistinguishable from that produced by cells treated with an LPO method (Figure 5, lanes B and C). It also closely resembled the surface-specific labeling pattern previously reported for BHK cells by Hynes and Pearlstein (1976). This indicates that the chloroglycoluril method may have general applicability for surface-specific labeling of membrane-enveloped systems. Iodination of nucleated eucaryotic cell systems using chloroglycoluril has not been biochemically characterized beyond the qualitative determination of feasibility described in Figure 5. These nucleated cellular systems are far too complex to allow analysis by the relatively simple biochemical techniques reported here for characterization of the viral and erythrocyte systems. The more reliable indicators of surface-specific labeling in complex cellular systems are relatively cumbersome and include autoradiographic analysis at the level of electron microscopy and characterization of membrane-enriched preparations (Hubbard and Cohn, 1975). A simple indication of surface-specific labeling in these complex cellular systems can be achieved, however, by testing for trypsin sensitivity of proteins labeled by surface-specific and general methods (Hynes, 1973). When BHK cells were labeled by the chloroglycoluril method in the presence of high (1 mM) or low (5 μM) concentrations of iodide and subsequently subjected to mild tryptic hydrolysis by the method of Hynes (1973), virtually all of the radioactive bands disappeared in the sample labeled with low iodide, but the majority of the bands resisted tryptic hydrolysis in the sample in which the cellular proteins were generally labeled with high iodide.

A final consideration is choice of the temperatures and buffer systems used in chloroglycoluril-mediated iodination. Our choices of temperature and buffer were dictated by the limits of stability of the biological system rather than some intrinsic property of the chloroglycoluril method. Erythrocytes were routinely iodinated in PBS at pH 8.0 and viruses in 10 mM Na_2HPO_4 at pH 7.4. However, no difference in specific activity or the HN/NP ratio was observed when Sendai or Newcastle disease viruses were iodinated in 10 mM Tris-HCl, 0.10 M NaCl at pH 8.0 (data not shown). With erythrocytes,

we routinely employed 21 °C as the reaction temperature, but obtained surface-specific labeling at 0 °C also (data not shown). Viral samples, especially Sendai virus, were routinely iodinated at 0 °C because of the lability of these viruses (Lonberg-Holm and Philipson, 1974).

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