Chemical Modification of the Plasma Membrane Polypeptides of Cultured Mammalian Cells as an Aid to Studying Protein Turnover†

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ABSTRACT: The turnover of polypeptides in the plasma membranes of exponentially growing Chinese hamster ovary cells and fibroblasts derived from human skin has been examined. A number of techniques were tested in order to introduce radiolabel into proteins in such a manner as to avoid any reutilization of the labeled amino acids after protein hydrolysis by the cell. Of the methods employed, those most useful for this purpose were acetylation using radioactive acetic anhydride, in which label is recovered in the majority of the proteins in the plasma membrane, and iodination by the lactoperoxidase technique, which is selective, in that only a few components show incorporated radioactivity. Neither treatment appeared to affect cell viability. Generally cells were provided with either [^{14}C]- or [^{3}H]-L-leucine before chemical labeling, and, after treatment, returned to nonradioactive medium. Plasma membranes were harvested at various times and component proteins analyzed by polyacrylamide gel electrophoresis in presence of sodium dodecyl sulfate. The appearance of radioactivity in the medium was also followed. In neither cell type was there evidence for differential loss of any of the major protein species present. All seemed equally long-lived. A few minor proteins, in terms of their leucine content, which were detected by iodination did appear to be lost relatively quickly from the membrane. However, our results indicate that in rapidly dividing cell populations, plasma membrane proteins are largely conserved and there is little turnover.

There is now general agreement that cellular proteins experience turnover. That is, they are continuously being degraded and resynthesized. Turnover rates of proteins in cell membranes have generally been demonstrated in two ways. One method has been to label the tissue or cells with an appropriate radioactive amino acid and then, after withdrawal of the labeled precursor, follow the subsequent loss of radioactivity in the proteins under investigation (Arias *et al.*, 1969; Dehlinger and Schimke, 1971; Kiehn and Holland, 1970; Warren and Glick, 1968). An alternative method used for cells in tissue culture has been to follow the appearance and disappearance of certain marker molecules on the cell surface or to observe their transfer to the growth medium (Cone *et al.*, 1971; Gordon and Cohn, 1970; Harris *et al.*, 1969; Kapeller *et al.*, 1973; Kraemer, 1966,1967; Marcus, 1962). Using the former method Dehlinger and Schimke (1971) indicated that the plasma membrane of liver cells not only turns over rapidly, but that individual proteins show widely divergent rates of degradation. Their results suggested that proteins can associate with and dissociate from the protein–lipid complex independently of each other. This implies that membranes are neither synthesized nor degraded as units. By contrast, Warren and Glick (1968) found that in tissue culture cells, there was no differential turnover of protein, lipid, or carbohydrate of the plasma membrane. Indeed, in rapidly growing cells, the major components were to a great extent conserved. Only in stationary phase cells was there extensive loss of surface materials.

However, one particular problem with tissue culture studies is that they represent closed systems, in which the reutilization of a radioactive precursor, such as glycerol or an amino acid, may extend the apparent half-life of a given macromolecule. One way in which this problem might be circumvented is to incorporate a label into selected amino acids on existing proteins such that the derivative formed is unlikely to be reutilized following breakdown of those proteins. Clearly, however, if the technique is to have any value for studying turnover, the means for introducing label has to be mild enough in order to maintain cell viability and to preclude the selective degradation of the modified proteins. In this paper we have used a number of methods to follow turnover of plasma membranes in exponentially growing cultures of Chinese hamster ovary (CHO) cells and human skin fibroblasts. In doing so we have also been able to compare techniques that are believed to be selective, in the sense that they label only a limited number of accessible proteins on the cell surface, with others that appear to be more catholic in their action.

Materials and Methods

Radiochemicals. [4,5-^{3}H]-L-Leucine (38 Ci/mmol), [U-^{14}C]-L-leucine (348 Ci/mol), [1-^{14}C]acetic anhydride (24 Ci/mol), [^{3}H]-NaBH_4 (570 Ci/mol), potassium [^{14}C]cyanate (55 Ci/mol), and sodium [^{125}I]iodide (17 Ci/mg) were obtained from Amersham-Searle Corp.

Cell Growth. Human skin fibroblasts (line 37 of our stocks) were derived from a foreskin of a newborn child. After establishing monolayer culture, the fibroblasts were trypsinized (0.25% w/v in Hank's balanced salt solution) and subcultured into 75 cm^2 Falcon flasks (Bioquest, Cockeysville, Md.). When confluent (approximately 1×10^7 cells/dish) they were subcultured into two flasks. All experiments were performed on cells which had undergone between 10 and 25 such one-to-two passages. No major

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changes were noted either in population doubling time or plasma membrane composition during this period of "aging."

Cells were grown at 37° in an atmosphere of 95% air and 5% CO_2. They were maintained on 18 ml of Eagle's minimum essential medium in Earle's salts reinforced with 16.7% (v/v) heat inactivated fetal calf serum (Bioquest, Cockeysville, Md.) and containing antibiotic–antimycotic mixture (Grand Island Biological Co., New York, N. Y.). The population doubling time of this cell line was around 35 hr. In some experiments cells were grown in Blake bottles (200 cm^2) with 80 ml of medium.

Chinese hamster ovary (CHO)[1] cells derived from line K1 of Kao and Puck (1968) were grown on McCoy's 5A medium (Grand Island Biological) containing 10% fetal calf serum and antibiotic–antimycotic mixture. Under these conditions, the cell generation time was 16 hr and the line would reach a final cell density of 15×10^6 cells/75-cm^2 flask. These cells were also grown in Blake bottles. To assess all numbers, samples were counted in particle-free saline using a Coulter counter.

Cell Labeling by Iodination. Cells were grown on medium containing [^{14}C]-L-leucine (5 μCi/Falcon flask) for at least 24 hr prior to labeling with iodine. Then monolayer was washed several times with phosphate-balanced saline (PBS) and iodinated by the lactoperoxidase technique of Phillips and Morrison (1970). Approximately 300 μg of the enzyme (Calbiochem) and 200 μCi of $Na^{125}I$ dissolved in 3.0 ml of PBS were added to each flask. The reaction was initiated by addition of 20 μl of 0.06% w/v H_2O_2. Further amounts were added every minute for 10 min. Finally the cells were shaken gently for a further 15 min at 37° and the solution was then poured off. After washing with PBS new, unlabeled growth medium was added.

Labeling of Formaldehyde-Treated Cells by NaB^3H_4. The cell monolayer was washed thoroughly, and 10 ml of 10^{-3} or 10^{-5} M formaldehyde in Hank's balanced salt solution (HBSS) was added. After incubation at 37° for 15 min, this solution was poured off and replaced with 50 μCi of NaB^3H_4 (in 5 ml of HBSS) for 1 min in order to reduce Schiff's bases. The cells were then washed and new medium was added.

Labeling of Pyridoxal Phosphate Treated Cells by NaB^3H_4. A modification (Hunt and Brown, 1974) of the method described by Rifkin *et al.* (1972) was used to label free amino groups on the cell surface. Monolayer cultures (5×10^6 cells/plate) were allowed to react with 5 ml of 0.01 M pyridoxal phosphate in PBS for 30 min at 37°. The cells were washed twice with PBS and the reduction was carried out with NaB^3H_4 (100 μCi) in 5 ml of PBS for 15 min at 4°. The radioactive solution was then poured off, the cells were washed several times, and new medium was added.

Labeling with Potassium Cyanate. In order to modify cellular proteins with cyanate conditions closely to those described by Stark (1967) were employed. Cells were pregrown on [^{3}H]-L-leucine, washed free of radioactive medium, and allowed to react with 10 μCi of potassium [^{14}C]cyanate in 2 ml of PBS solution (pH 7.0). After shaking for 5 min at room temperature, the monolayer was washed and fresh medium added to each flask.

Galactose Oxidase Treatment and NaB^3H_4 Reduction. Galactosyl and *N*-acetylgalactosaminyl residues of cell surface glycoproteins and glycolipids may be oxidized using galactose oxidase. The exposed aldehyde groups on carbon atom 6 are then reduced using NaB^3H_4 (Morell *et al.*, 1966; Gahmberg and Hakomori, 1973). Cells were not preincubated with neuraminidase, however (Hunt and Brown, 1974).

Monolayer cultures were treated with 0.3 mg of galactose oxidase from *Dactylium dendroides* (Sigma Chemical Co.) in PBS for 90 min at 37° with occasional shaking and washed with PBS to remove excess enzyme. Reduction of oxidized groups was carried out by addition of 500 μCi of NaB^3H_4 and incubation for 10 min at room temperature.

Acetylation of Cells by [1-^{14}C]Acetic Anhydride. Cells were grown in medium containing [^{3}H]-L-leucine (10 μCi) at least 24 hr prior to acetylation. At this stage the monolayers were washed twice with PBS, the excess solution was poured off, and 0.2 ml of [^{14}C]acetic anhydride (10 μCi) in dimethyl sulfoxide was added to each flask. After shaking at room temperature for 5 min the labeling agent was poured off, the cells were washed several times, and new medium was added.

Isolation of Plasma Membranes. Plasma membranes were isolated by a slight modification of the method of Barland and Schroeder (1970). Since this method does not involve trypsinization of cells, membranes isolated by this method represent a complete structure with the trypsin susceptible molecules intact. Medium was decanted, and the almost confluent cells were washed twice with 0.16 M NaCl containing 0.01 M $CaCl_2$. Cells were then covered for 10 min at room temperature with 10 ml of solution containing four volumes of 10^{-3} M $ZnCl_2$ to one volume of dimethyl sulfoxide. This solution was then decanted and 30 ml of fluoroscein–mercuric acetate solution (2.2×10^{-3} M in 0.02 M Tris buffer (pH 8.1) added to each dish, which was then placed on an ice bath and shaken (120 rpm) for 30–45 min using a New Brunswick gyrotary shaker.

As a result of shaking, sheets of plasma membrane from the upper surfaces of the cells became suspended in the fluoroscein–mercuric acetate solution. As observed by Barland and Schroeder (1970), the cells and their contents remained firmly attached to the surface of the flask. The solution above the cells was decanted and centrifuged at 600*g* to pellet the membranes which were then suspended in 10^{-3} M $NaHCO_3$ and recentrifuged. A few nuclei and an occasional whole cell were noted in this membrane fraction. These were removed by sucrose density gradient centrifugation. The membrane preparation was suspended in 4 ml of 30% (w/w) sucrose layered above a 45% (w/w) sucrose bed (23 ml), and centrifuged for 1 hr at 150*g* using a refrigerated International centrifuge with a swinging bucket head. The interphase region, which contained the membrane sheets and which was devoid of nuclei or other contaminating particles, was collected, diluted with 10^{-3} M $NaHCO_3$, and centrifuged (1 hr; 10,000*g*). The membrane pellet was dissolved in a minimum amount of 0.04 M Tris-glycine buffer (pH 8.3) containing 2% (w/v) sodium dodecyl sulfate and boiled for 3 min. The solutions were then dialyzed for 16 hr against 1 l. of 0.1% SDS and 0.1% mercaptoethanol. Sucrose crystals were added to 10% by weight to increase the density of the samples, and up to 0.3 ml of sample (containing no more than 200 μg of protein) was used for electrophoresis.

For comparative purposes, an alternative method of

[1] Abbreviations used are: HBSS, Hank's balanced salt solution; PBS, phosphate-balanced saline; CHO, Chinese hamster ovary cells; SDS, sodium dodecyl sulfate.

membrane preparation has also been employed. Cells were detached from glass Blake bottles using 0.25% (w/v) trypsin and suspended in 20 ml of 0.15 M NaCl. They were sedimented by gentle centrifugation (250*g*; 5 min), washed once, swollen in hypotonic borate–EDTA (Warley and Cook, 1973), and finally lysed by a few strokes with a loose-fitting Dounce homogenizer.

Plasma membranes were then purified by the two-phase centrifugation system described by Brunette and Till (1971). These membranes were prepared for analytical electrophoresis in SDS as described earlier.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Polyacrylamide gels (7 × 0.6 cm) were prepared according to the method of Laemmli (1970) using the buffer systems of Davis (1964). The running gels whether 5, 7.5, 10, or 12% were prepared from a stock solution of 30% (w/v) acrylamide and either 0.75% (w/v) or 1.5% *N,N'*-methylenebisacrylamide, and polymerized chemically using tetramethylenediamine and ammonium persulfate. The buffer solution was 0.375 M Tris-HCl (pH 8.9) and 0.1% (w/v) SDS. The stacking gel (3.75% polyacrylamide and a length of 1 cm) contained 0.125 M Tris-HCl (pH 6.7), 0.1% SDS, and riboflavine (0.004%). They were photopolymerized under a fluorescent light.

After application of the sample, a current of 0.5 mA/tube was applied until the sample had concentrated at the top of the running gel. The current was then increased to 3 mA/tube and electrophoresis continued until the Bromophenol Blue marker had reached the bottom of the gel (*ca.* 3 hr). Prior to staining, the SDS was removed by shaking in ethanol–acetic acid–water (40:10:50 v/v) overnight. Gels were stained for protein using 0.125% Coomassie Blue, and diffusion destained in ethanol–acetic acid–water (10:7:83 v/v). Polyacrylamide gels containing 0.1% (w/v) SDS and mercaptoethanol were also run without stacking gels according to the procedure described by Weber and Osborn (1969). For molecular weight determinations the following proteins have been employed as standards: bovine serum albumin, trypsin, the light and heavy chains of human IgG, cytochrome *c*, and myoglobin.

Determination of Radioactivity. Unstained gels were fractionated using an autogeldivider (Savant Instruments, Hicksville, N.Y.) into approximately 60 fractions. The crushed gel fractions were stood overnight in 0.5 ml of water and their radioactive content was determined, after addition of 5 ml of toluene–Triton X-100 scintillant (Turner, 1968), by using a Beckman LS-300 scintillation spectrometer. Channels were so adjusted that no counts from ^{3}H were detected in the ^{14}C channel, while the extent of overlap of ^{14}C into ^{3}H channel was held at a fixed value (usually about 30%). There was no appreciable quenching of either ^{14}C of ^{3}H in these macerated gel samples as evidenced by single label experiments in which we checked the samples' channels ratio along such gels. Recovery of both ^{3}H and ^{14}C was also always greater than 85% of that applied. Aqueous samples were also checked for quench using the external channels ratio.

^{125}I was estimated using a Beckman autogamma counter. Alternatively, we have used liquid scintillation counting. The ^{14}C channel was so adjusted that the extent of overlap by ^{125}I was restricted to less than 0.25%, while ^{14}C was counted at about 20% efficiency.

Final values were computed using a Wang Model 700 programmable calculator and data are presented as percentages of the total ^{14}C or ^{3}H recovered from a gel.

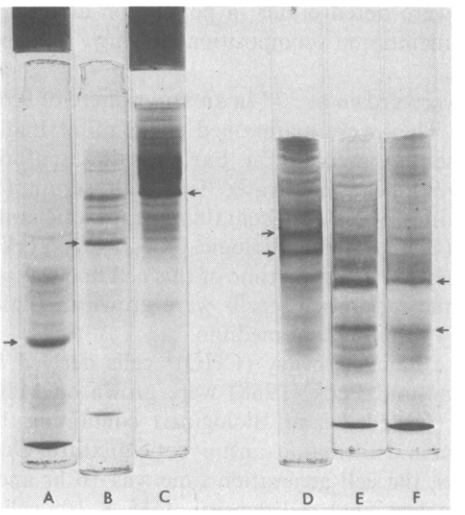

FIGURE 1: Polyacrylamide gel electrophoresis of the plasma membrane proteins of human skin fibroblasts (A–C) and CHO cells (D–F) in presence of sodium dodecyl sulfate. (A) separation using 7.5% w/v polyacrylamide with 0.185% *N,N'*-methylenebisacrylamide cross-linker; (B) using 10% w/v polyacrylamide with 0.25% cross-linker; (C) 12.5% w/v polyacrylamide with 0.31% cross-linker. A, B, and C were all run with a stacking gel according to the method of Laemmli (1970). D, E, and F all employed 7.5% w/v polyacrylamide and 0.375% cross-linker. However, D was run without a stacking gel by the procedure of Weber and Osborn (1969), while E and F were run according to the method of Laemmli (1970). Plasma membranes were all prepared according to Barland and Schroeder (1970) except F when the method of Brunette and Till (1971) was used. The major protein of skin fibroblast and two major proteins of CHO cells are shown by arrows.

Fractionation of Medium. At each time membranes were harvested, the amount of radioactivity in the medium was also analyzed. Aliquots were removed and protein was precipitated by addition of an equal volume of 15% w/v Cl_3CCOOH. The precipitate was washed twice with 7.5% Cl_3CCOOH dissolved in boiling 2% SDS and dialyzed overnight against water. The specific radioactivity of Cl_3CCOOH insoluble protein was then determined. The supernatant fraction after Cl_3CCOOH treatment was extracted three times with ether to remove excess Cl_3CCOOH, dialyzed, and its radioactive content measured.

Determination of Protein. The amount of protein in aqueous samples was measured by standard procedures (Campbell and Sargent, 1967). When β-mercaptoethanol was present a modification of this method was employed (Ross and Schatz, 1973) to avoid interference by thiols.

Results

Polyacrylamide Gel Electrophoresis of Membrane Polypeptides. Figure 1 shows the patterns of stained polypeptides from the plasma membranes of CHO cells and human skin fibroblasts separated under a number of different electrophoretic conditions. The plasma membrane of human skin fibroblasts contains at least 25 polypeptides that stain with Coomassie Blue, ranging from molecular weights greater than 130,000 to less than 10,000. One (mol wt 45,000) is clearly present in much greater amounts than the others.

In the case of the CHO cells, a large number of polypeptides can again be detected by Coomassie Blue staining. Two bands (mol wt 55,000 and 40,000) stain particularly deeply.

The gel patterns that are in Figure 1 are extremely repro-

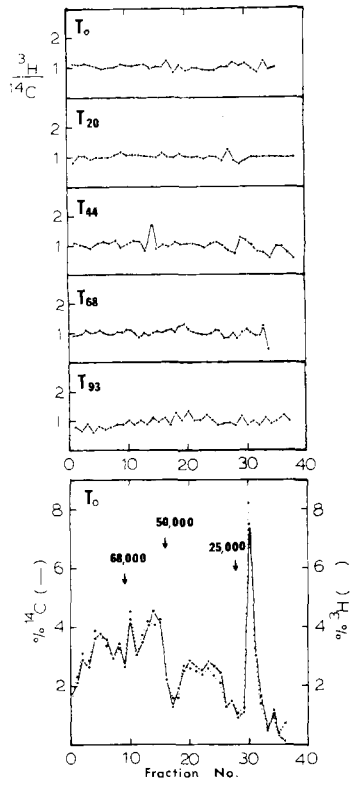

FIGURE 2: Changes in the radioactivity distribution with time in plasma membrane proteins of CHO cells maintained on radioactive L-leucine during log phase growth. The lower graph shows the radioactive profiles from zero time controls in which plasma membranes had been isolated from cells grown on either [^{14}C]- or [^{3}H]-L-leucine for 24 hr prior to harvest. The membrane preparations were mixed and proteins separated on the same gel. The upper figures show the ratios of per cent ^{3}H to per cent ^{14}C along such gels after ^{14}C-labeled cells had been transferred to ^{3}H medium for periods of 20, 44, 68, and 93 hr. Positions of three markers are shown. Electrophoresis was carried out as in Figure 1E.

ducible from plasma membrane preparation to preparation. Further, we have noticed no appreciable change after storage of the SDS-protein solutions at −20° for more than 6 months.

Pulse-Chase Experiments with Contrastingly Labeled L-*Leucine.* (a) CHO CELLS. CHO cells were introduced into Falcon dishes at a range of inoculum sizes, such that different groups of cells would reach approximately 90% confluency 24, 48, 72, or 96 hr later. Nine of these flasks received 5 μCi of [^{14}C]-L-leucine; one received 25 μCi of [^{3}H]-L-leucine. After 24 hr, the plasma membranes were harvested from one flask of ^{14}C-labeled cells and the single flask of ^{3}H cells was to act as zero time controls. These membrane preparations were mixed and dissolved in SDS prior to electrophoresis. Figure 2 (upper curves) indicates that both sets of membranes appeared to contain the same polypeptide components in similar proportions. It also emphasizes the reproducibility of the method employed for membrane isolation.

The solution in the remaining flasks was replaced by medium containing [^{3}H]-L-leucine. The cells were then grown on for periods up to 4 days. Surface membranes from duplicate flasks were harvested at regular intervals during this time and their component protein was analyzed by electrophoresis on polyacrylamide gels.

As expected there was a progressive fall in the specific ^{14}C radioactivity of the membranes and a rapid increase in their ^{3}H content (Table I). By 96 hr, after which the cells had undergone at least four population doubling periods (they had become confluent about 80 hr after transfer to ^{3}H medium), the specific ^{14}C radioactivity had declined about ninefold. This decline precluded any statistically valid experiments using electrophoresis extending beyond this time because of the dilution of ^{14}C in protein.

TABLE I: Specific Radioactivity of the Plasma Membrane Proteins of [^{14}C]-L-Leucine Labeled CHO Cells after Transfer to Medium Containing [^{3}H]-L-Leucine.

	Time after Transfer (hr)				
	0	20	44	68	93
Specific radioactivity[a] of membrane protein (dpm/μg of protein)					
^{14}C	96	43	30	27	11
^{3}H	0	361	405	422	350

[a] Results are obtained from separate flasks.

Clearly, the electrophoretic gel patterns during the "chase" on ^{3}H medium do not show any marked changes that might be indicative of differential loss of any polypeptide components from the membrane. We have also repeated these results using a number of other polyacrylamide gel concentrations. Proteins that turned over quickly might have been expected to have shown an enhanced incorporation of ^{3}H and an accelerated decay of ^{14}C that would have been detectable as a divergence of the radioactive profiles and the ratio of ^{14}C to ^{3}H along the gel. On the other hand, the experiments do not rule out the possibility that any ^{14}C-labeled leucine released might have been rapidly and preferentially reutilized for the biosynthesis of new protein. Indeed, the decline in ^{14}C radioactivity was somewhat less than that anticipated from the increase in cellular protein over the period of the "chase."

(b) HUMAN SKIN FIBROBLASTS. A similar experimental design was used in order to follow the replacement of ^{14}C-labeled membrane proteins by ^{3}H in human skin fibroblasts, although a shorter "chase" period was employed. Over a 24-hr period after transfer to ^{3}H medium, there was again no marked divergence of the ^{3}H or ^{14}C profiles (Figure 3). During this time the specific ^{14}C radioactivity of the membrane protein had fallen by more than 25% (Table II).

There was a gradual increase in Cl_3CCOOH soluble and insoluble materials in the medium, but only about 10% of the [^{3}H]-L-leucine provided was utilized. Clearly, provided that there is rapid equilibration between internal amino acid pools and the medium, any [^{14}C]leucine released by protein turnover would be rapidly diluted by a very large excess of ^{3}H-labeled amino acid. The ^{14}C specific radioactivity of the membrane at 48 hr was approximately 45% of what it was at T_0, a value close to that expected in a period 1.3 times that of the population doubling time of the cells.

Iodination of Skin Fibroblasts. Fibroblasts (3×10^6 cells) were transferred to MEM medium containing [^{14}C]-L-leucine (5 μCi/flask) for 48 hr. At this stage, when they were approximately 60% confluent, the medium was poured off and the cells were iodinated by the lactoperoxidase technique. After careful washing, new unlabeled medium was added and the cells were allowed to continue growth. Plasma membranes were harvested immediately following iodination and subsequently after a further 23, 31, and 45 hr.

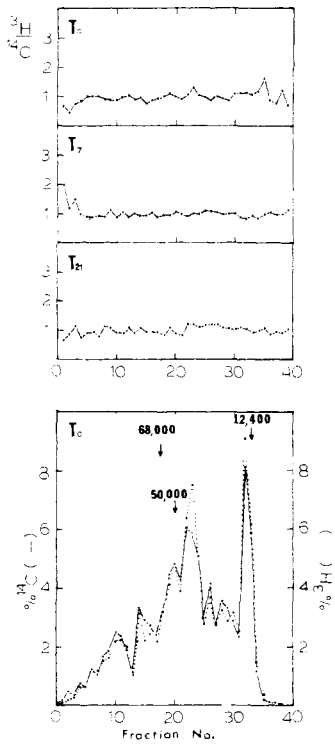

FIGURE 3: Changes in the radioactivity distribution with time in plasma membrane proteins of human skin fibroblasts maintained on radioactive L-leucine during log phase growth. The lower plot is the zero time control, and the upper figures show the ratios of per cent ^{3}H to per cent ^{14}C along gels after transfer of cells from ^{14}C- to ^{3}H-labeled L-leucine medium. Electrophoresis was carried out as in Figure 1A.

Membranes were not isolated between 0 and 23 hr, because within 1 hr of iodination, cells started to lift away from the plastic surface of the Falcon dish and to form large clumps. By 6 hr these clumps began to reattach, but it was almost 20 hr before the cells had spread sufficiently in monolayer to allow the plasma membranes to be isolated by the shake-off method. By 45 hr the cultures were confluent and showed no visible signs of gross cellular damage or death.

The specific ^{14}C and ^{125}I radioactivities of the plasma membranes declined steadily during the growth period on unlabeled medium (Table III). However, the ratio of ^{125}I to ^{14}C remained relatively constant suggesting that there was no large-scale, selective loss of iodinated macromolecules. We cannot discount the fact that some of the variation in the ratios may be accounted for by pipetting errors while dispensing small volumes of reagents for the iodination, under sterile conditions.

Figure 4 compares the gel profiles for ^{14}C and ^{125}I after polyacrylamide gel electrophoresis of the solubilized membrane preparations. As expected from our earlier results, there were no significant changes in the ^{14}C profile with increasing time. The same major polypeptides seemed to be present in roughly similar proportions. However, whereas the ^{125}I patterns at 23, 31, and 45 hr show a close similarity, that at T_0 is quite distinct. A major iodinated hand (mol wt 65,000) can be detected approximately 3 cm down the gel which is either lost or present in much smaller amounts by 23 hr. It does not correspond with a major peak of ^{14}C radioactivity and is probably not therefore a major component of the plasma membrane.

A second major peak of radioactivity migrated only a small distance into the gel (apparent mol wt 100,000). Whereas at T_0 this band contained only 28% of the ^{125}I recovered from the gel, at subsequent times it amounted for about 50% of the iodine label on a typical gel loaded with 50 μg of protein. The radioactivity in this protein fell approximately threefold from 7650 dpm at T_0 to 2210 at 45 hr. The ^{14}C specific radioactivity of the total membrane showed a proportionately similar decline, indicating that this high molecular weight material was not lost from the membrane at a rate significantly different than that of the majority of the other membrane proteins. Also significant is the fact that the iodine pattern is reproducible between 23 and 45 hr, suggesting that no differential turnover of iodinated proteins occurred during that time.

Iodination of CHO Cells. CHO cells were grown from a range of inoculum sizes such that they would become close to confluency 24, 36, and 48 hr later. All of the flasks received 2.0 μCi of [^{14}C]-L-leucine. After 24 hr, the cells were iodinated, new unlabeled medium was added, and growth, which appeared normal, was allowed to continue. Plasma membranes were isolated immediately after iodination and at various times subsequently. Figure 5 shows the

TABLE II: Radioactivity in the Medium and in the Plasma Membrane Fraction of [^{14}C]-L-Leucine Labeled Human Skin Fibroblasts after Transfer to Medium Containing [^{3}H]-L-Leucine.[a]

	Time (hr)				
	0	4	9	24	48
Cl_3CCOOH soluble fraction (dpm/flask)					
^{14}C	1506	3048	4695	5010	8934
^{3}H	50.2×10^6	45.9×10^6	46.0×10^6	44.9×10^6	40.2×10^6
Cl_3CCOOH insoluble fraction (dpm/flask)					
^{14}C	*b*	2980	3435	4091	3561
^{3}H	*b*	16,221	20,775	33,067	58,537
Specific radioactivity of membrane protein (dpm/μg of protein)					
^{14}C	118	106	88	84	49
^{3}H	*b*	52	114	387	504

[a] Cells were grown on Falcon dishes on MEM medium (15 ml) containing [^{14}C]-L-leucine (5 μCi) for 24 hr and washed three times with HBSS. New medium containing [^{3}H]-L-leucine (25 μCi) was then added. Results at each time point were obtained from single flasks. [b] Not determined.

TABLE III: Specific ^{125}I and ^{14}C Radioactivity of the Plasma Membrane Fraction of Human Skin Fibroblasts after Iodination with Sodium [^{125}I]Iodide and Transfer from Medium Containing [^{14}C]-L-Leucine to Unlabeled Medium.[a]

	Time (hr)			
	0	23	31	45
Specific radioactivity (dpm/μg of protein)				
^{125}I	412	340	227	141
^{14}C	76	69	61	27
Ratio $^{125}I/^{14}C$	5.4	4.9	3.7	5.2

[a] Cells had been preincubated on 15 ml of medium containing [^{14}C]-L-leucine (5 μCi) for 24 hr prior to iodination.

^{125}I and ^{14}C profiles along the polyacrylamide gels after electrophoresis of the plasma membrane proteins. As anticipated from previous experiments, there was little alteration in the pattern of ^{14}C. With the iodine, however, some slight changes were evident. One major iodinated protein of high molecular weight which accounted for about 15% of the label at T_0, had steadily increased its contribution to the total radioactivity to about 25% at 21 hr. This seemed to be due to the relatively greater rate at which ^{125}I was lost from some of the other proteins which migrated into the mid region of the gel.

Acetylation of Skin Fibroblasts. Skin fibroblasts were grown on MEM medium containing [^{3}H]-L-leucine (10 μCi/flask) for 24 hr. They were then acetylated with [^{14}C]acetic anhydride before new nonradioactive medium was added to each flask. Table IV shows the radioactivity recovered in Cl_3CCOOH soluble and insoluble products in the medium immediately following the pulse and at subsequent time intervals up to 21 hr. Plasma membrane was also isolated at each of these times. The acid-soluble radioactivity remained relatively constant after the first sample (T_0) was collected (approximately 5 min after addition of nonradioactive medium), again suggesting that there was a very rapid equilibration of the internal pools of the cells with their external environment. By contrast radioactivity in precipitated material gradually increased over the period of the experiment. Nevertheless, the ratio of ^{3}H to ^{14}C remained approximately constant, indicating that acetylated and leucine-labeled macromolecules were being released into the medium at similar rates.

Similarly, there was a progressive and parallel decrease in both the ^{3}H and ^{14}C specific radioactivities of membrane protein. These data suggest that acetylated proteins were not being selectively eliminated from the plasma membranes of the treated cells. However, the ratio of ^{3}H to ^{14}C in the membrane was higher than that observed for secreted proteins. The rate of decline in leucine radioactivity also seemed higher than observed in previous experiments with skin fibroblasts (Tables II and III). The reasons for these observations are unclear.

The patterns of ^{14}C and ^{3}H radioactivity on the gels after electrophoresis of the SDS solubilized plasma membranes were very similar. Clearly the majority of the membrane proteins had become acetylated by our procedure. There was no obvious selectivity as was observed after iodination (see also Carraway *et al.*, 1972). However, while the major

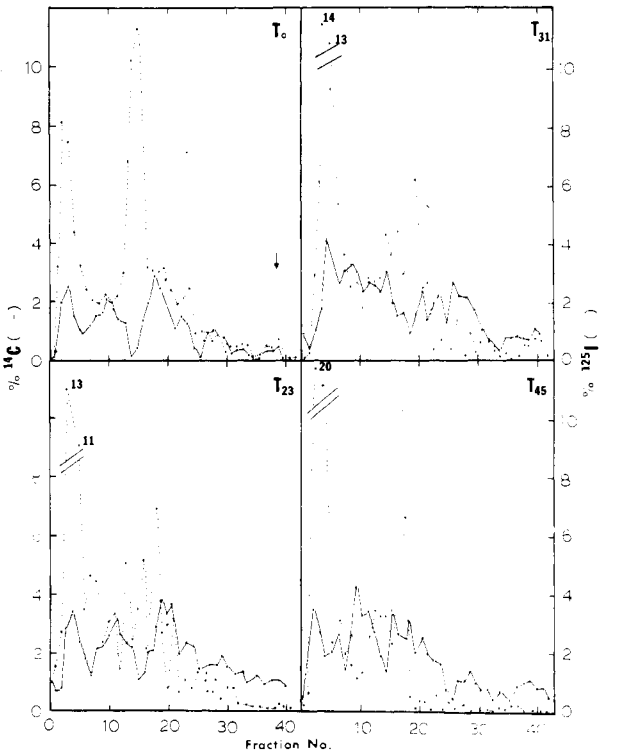

FIGURE 4: Changes in the distribution of ^{125}I with time in the plasma membrane proteins of human skin fibroblasts after lactoperoxidase-catalyzed iodination. For comparison the ^{14}C profile (●—●) is included. Membranes were harvested immediately after iodination and subsequently at 23, 31, and 45 hr. The dye front is marked with an arrow. Gels were 2.5% w/v polyacrylamide with 2.5% w/v cross-linker.

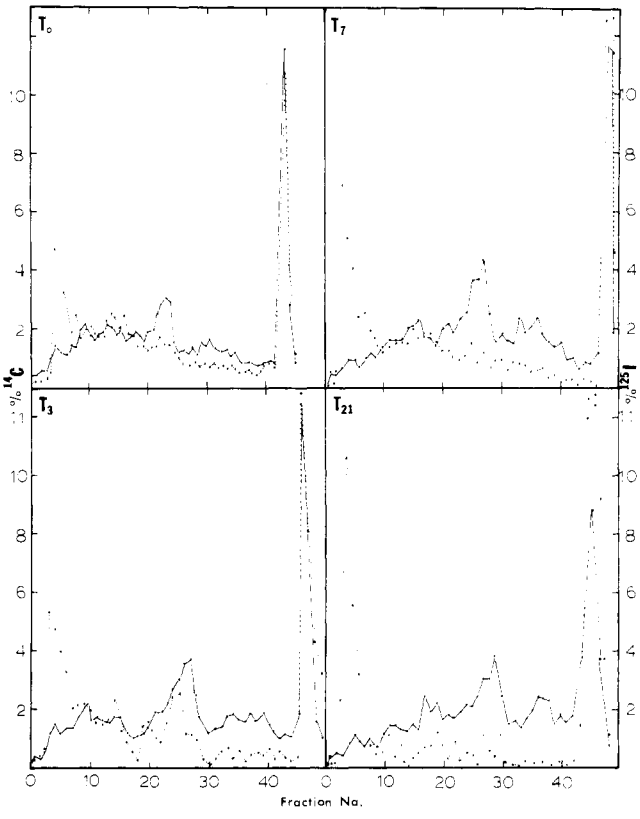

FIGURE 5: Changes in the distribution of ^{125}I (···●···●···) with time in the plasma membrane proteins of CHO cells after lactoperoxidase-catalyzed iodination. The ^{14}C profile (-●—●-) after incorporation of [^{14}C]-L-leucine is also included. Electrophoresis was carried out as in Figure 1E.

TABLE IV: Radioactivity in the Medium and in the Plasma Membrane Fraction of Human Skin Fibroblasts after Acetylation with [^{14}C]Acetic Anhydride and Transfer from Medium Containing [^{3}H]-L-Leucine to Unlabeled Medium.

	Time (hr)			
	0	4	8	21
Cl_3CCOOH soluble fraction (dpm/flask)				
^{14}C	16,368	20,016	19,020	20,766
^{3}H	83,982	85,940	89,315	80,850
Ratio ^{3}H/^{14}C	5.4	4.2	4.7	4.0
Cl_2CCOOH insoluble fraction (dpm/flask)				
^{14}C	20,011	39,996	44,286	70,485
^{3}H	90,910	149,328	162,530	332,370
Ratio ^{3}H/^{14}C	4.6	3.8	3.7	4.7
Specific radioactivity of plasma membrane (dpm/μg of protein)				
^{14}C	86	75	76	45
^{3}H	871	842	836	472
Ratio ^{3}H/^{14}C	10.1	11.2	11.0	10.5

protein species midway down the gel seemed to have incorporated proportionately more leucine than acetyl groups, the material of lower molecular weight running near to the dye front was enriched with ^{14}C. This pattern is not unexpected if it is assumed that while leucine is only likely to be present in proteins, both polypeptides and lipids would have become labeled after acetylation (Carraway *et al.*, 1972). It is well established that lipids as well as small peptides run close to the dye front during electrophoresis in SDS–polyacrylamide gels and this probably accounts for the higher amount of ^{14}C label in that region.

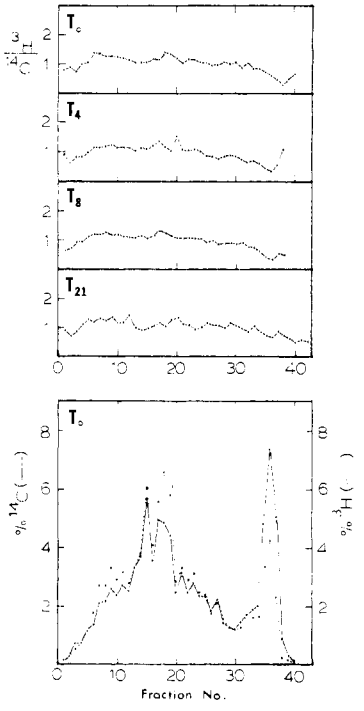

FIGURE 6: Changes in the distribution of ^{14}C (-●—●-) with time in the plasma membrane proteins of human skin fibroblasts after acetylation with [^{14}C]acetic anhydride. The lower plot shows the radioactive profile from zero-time controls in which membranes from cells grown in presence of [^{3}H]-L-leucine (···●···●···) were harvested immediately after acetylation. The ratios of per cent ^{3}H to per cent ^{14}C along such gels at 0, 4, 8, and 21 hr after acetylation are shown in the upper figures. Electrophoresis was carried out according to Figure 1B.

It is clear from Figure 6 that the distribution of ^{14}C and ^{3}H along the gel remains relatively unchanged over a 21-hr period after labeling. Total cellular protein per flask increased approximately 50% during this time. There was clearly no evidence for differential turnover of any of the protein components, unless it is assumed that acetylated amino acids are as readily reutilized for protein biosynthesis as leucine itself.

Radioactivity was also incorporated into the proteins of the plasma membranes of CHO cells after treatment with [^{14}C]acetic anhydride (Figure 7). The cells continued to divide at the predicted rate after acetylation and appeared normal in their morphology. The patterns of ^{3}H and ^{14}C along polyacrylamide gels were similar, but not identical. Again, the material running close to the dye front incorporated proportionately more ^{14}C than ^{3}H. Nevertheless, the ratio of ^{3}H to ^{14}C, though distinct in different regions of the gel, did not change significantly over a 47-hr growth period.

Alternative Method for Membrane Isolation. For comparative purposes, we have isolated plasma membranes from both CHO and skin fibroblasts by the method of Brunette and Till (1971) which involves homogenization of whole cells. The membranes were solubilized in SDS solution and the component polypeptides analyzed by polyacrylamide gel electrophoresis. Figure 1F compares the gel staining patterns for plasma membranes of CHO cells that are obtained by this method and by the shake-off technique employed earlier. Although there are some minor quantitative differences in relative staining intensities, it is clear that the same major protein bands are present on both gels.

In a further experiment, we coelectrophoresed ^{14}C-labeled membrane material from skin fibroblasts prepared by the Brunette and Till method with ^{3}H-labeled material prepared *via* the shake-off technique. Figure 8 shows the radioactive profiles that were obtained along a typical gel. Although it is clear that there was some quantitative variation in the relative amounts of polypeptides present, the patterns were not dissimilar. It should also be emphasized that membranes prepared by the Brunette and Till technique were isolated from cells that had been freed from the substratum using dilute trypsin. Consequently, some modification of exposed proteins is to be expected. Nevertheless, we believe that although the shake-off method gives rise largely to the upper plasma membrane surface, these fragments do not

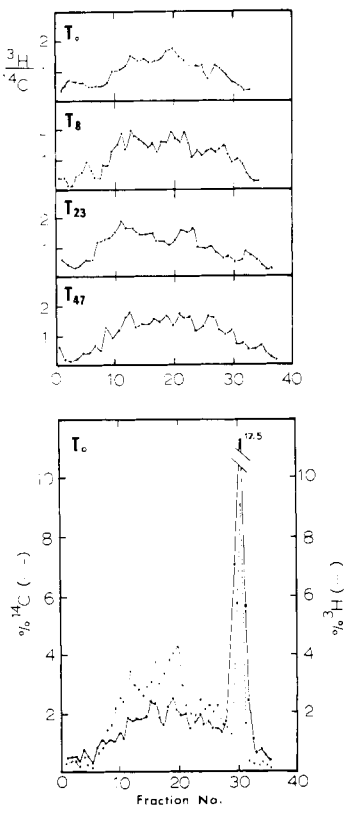

FIGURE 7: Changes in the distribution of ^{14}C (-●—●-) with time in the plasma membrane proteins of CHO cells after acetylation with [^{14}C]acetic anhydride. The lower plot shows the radioactive profiles from zero-time controls in which membranes from cells grown in presence of [^3H]-L-leucine (···●···●···) were harvested immediately after acetylation. The ratios of per cent ^3H to per cent ^{14}C along such gels at various times after acetylation are shown in the upper figures. Electrophoresis was carried out according to Figure 1E.

contain major polypeptide components significantly different from those of whole cell plasma membranes.

Other Labeling Methods. A number of other methods have been tried in order to incorporate radioactive label into proteins of the plasma membrane without killing the cells. When cells were treated successively with formaldehyde and NaB^3H_4, they continued to grow normally. The label introduced, however, did not appear to be associated with the major proteins of the membrane (Figure 9). Moreover, the pattern was indistinguishable from that noted when the pretreatment with formaldehyde was omitted. Because the nature of the radioactive product was unclear this method was abandoned.

Others have employed pyridoxal phosphate as a membrane impermeable reagent to react with exposed proteins at the surface. Again, however, we noted that most of the radioactivity introduced by the NaB^3H_4 in our attempts to reduce the Schiff's bases was nonspecific, and occurred whether or not the pyridoxal phosphate had been added. Moreover, the cells became inviable.

Carbamylation using sodium [^{14}C]cyanate did not appear to harm the cells as they continued to divide at an unchanged rate, but the amount of label introduced was very small and unsuitable for our experiments. Possibly the reagent could not penetrate the cells.

Labeling *via* the presumed membrane-impermeable enzyme, D-glactose oxidase, and the subsequent reduction of exposed aldehyde groups with NaB^3H_4, has recently been used to introduce ^3H into membrane glycoproteins. From

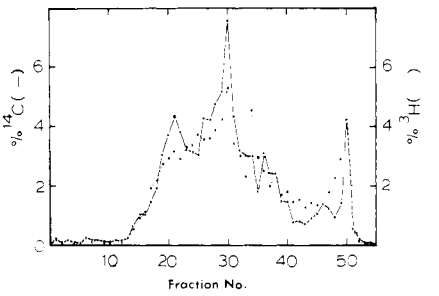

FIGURE 8: Comparison of plasma membrane polypeptides prepared by two different methods for isolating plasma membrane. Human skin fibroblasts were grown either on [^{14}C]- (-●—●-) or [^3H]- (···●···●···) L-leucine and membranes isolated by the method of Barland and Schroeder (1970) or Brunette and Till (1971), respectively. The preparations were mixed and component polypeptides coelectrophoresed as in Figure 1B.

our point of view, however, this method suffered from two major drawbacks: (1) a considerable amount of nonspecific label was introduced; (2) the cells detached and lost their viability.

Discussion

There is a considerable amount of evidence that cellular proteins are continuously being broken down and resynthesized. The process has been widely studied, and it has become clear that there is great diversity in the rates at which different enzymes turnover, and that both degradation and synthesis determine the level of an enzyme in a tissue (see Schimke and Doyle, 1970). Moreover, degradation of most soluble enzymes follows first-order kinetics (Schimke and Doyle, 1970; Schimke, 1964; Fritz *et al.*, 1969), implying that "aging" of proteins is not a signal for their destruction, but that once a particular molecule has been synthesized, its chance of being destroyed is no different from that of other members of its species.

What controls the rate of breakdown of a particular protein is unclear. In part, it may be determined by the nature of its polypeptide bonds and by its conformational stability in the milieu of the cytoplasm. Clearly, its interactions with ligands such as substrates and cofactors and with other macromolecules (for example, in membranes) may confer such stability. Schimke and coworkers (Arias *et al.*, 1973) have indicated that, in rat liver, larger proteins are generally (but not invariably) degraded faster than smaller ones, possibly because they have a greater chance of random collision with proteases. They have also indicated that these

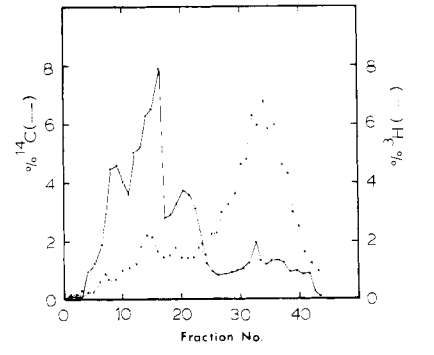

FIGURE 9: The distribution of ^3H (···●···●···) into plasma membrane polypeptides of human skin fibroblasts after successive treatment of cells with 10^{-3} M formaldehyde and NaB^3H_4. The distribution of polypeptides labeled with [^{14}C]-L-leucine is also indicated (-●—●-). Electrophoresis was carried out according to Figure 1C.

general rules in relation to molecular size and first-order decay seem to apply to the proteins of the endoplasmic reticulum and plasma membrane as well as the soluble phase of the cell. Thus, although both membrane systems had mean half-lives of 2–2.5 days, individual proteins turned over at widely divergent rates, with those of highest molecular weight being degraded and replaced most rapidly. Their results implied that proteins can dissociate from and be inserted into membranes independently of each other. Meldolesi (1974) came to similar conclusions when investigating guinea pig, pancreatic-acinar cells.

There is considerable evidence that the proteins of the plasma membrane of mammalian cells in culture also turnover. Labeled macromolecules similar to those of the membrane have been detected in the growth medium by a wide variety of different analytical techniques (Cone *et al.*, 1971; Kapeller *et al.*, 1973; Kraemer, 1967; Onodera and Sheinin, 1970). Materials are evidently being continuously released or shed into the immediate surroundings of the cell in undegraded form. Such results are not surprising if newly synthesized precursor arrives at the surface in greater amounts than that required to compensate for cell growth. Warren and Glick (1968) concluded that during this process of plasma membrane maintenance in stationary phase cells, proteins, glycoproteins, and lipids were turned over at equivalent and not asynchronous rates. They suggested that the plasma membrane is degraded and synthesized as a unit. However, in cultures growing in log phase, there was a greatly reduced turnover, and most of the plasma membrane substance was retained, although individual polypeptides were not investigated.

Our results using contrastingly labeled L-leucine are consistent with such a hypothesis. Thus, in both the CHO and fibroblast lines, there was a reduction in specific radioactivity of the membrane label following the "pulse" fairly close to that expected from the population doubling times of the cell in absence of protein destruction. There was no indication that individual proteins, even of widely different size, were turned over at different rates. Subbiah and Thompson (1974) have recently reported similar results with the protozoan *Tetrahymena pyriformis*. However, as emphasized earlier, the problem of reutilization of amino acids is an extremely important consideration. Proteins that turnover most rapidly might well be those that are most likely to reincorporate recycled amino acids most efficiently, although it is usually assumed that there is rapid equilibration of internal pools with the large excess of contrastingly labeled amino acids in the medium (Poole and Wibo, 1973). However, in liver a very high percentage of amino acids produced during degradation are reincorporated into new protein and label cannot be easily flooded out *via* exchange with the serum (Lotfield and Harris, 1956; Gan and Jeffay, 1967). Koch (1962) has also emphasized that the true turnover rate of protein cannot be calculated from such experiments. It might also be argued that reutilization is likely to be even more efficient in growing than in nongrowing cells such as those encountered in liver. However, there is strong suggestive evidence that during active growth most cell components are conserved significantly longer in both bacteria (Mandelstam, 1960; Koch and Levy, 1955) and mammalian cells (Goldberg *et al.*, 1974; Hershko and Tomkins, 1971).

The experiments in which proteins were chemically modified have provided much more clear-cut evidence that the majority of the plasma-membrane proteins turnover at slow and probably equivalent rates in dividing cell populations. Treatment of proteins with acetic anhydride is believed to introduce acetyl groups largely into exposed amino and hydroxyl groups (Riordan and Vallee, 1967) while iodination largely labels accessible tyrosine residues. It is highly unlikely that such modified amino acids would be reutilized for protein biosynthesis. The methods have also provided an interesting contrast, for while acetic anhydride appeared to react with all of the membrane proteins, presumably because of its ability to diffuse into the cells and membranes (Carraway *et al.*, 1972) the iodination reaction is thought to be surface specific, tagging only these proteins which project out from the bilayer (Phillips and Morrison, 1970). In our hands iodination was clearly selective, as only a few components became radioactive. By contrast, following withdrawal from ^{3}H-medium and acetylation with [^{14}C]-acetic anhydride, all of the major membrane proteins showed equivalent and parallel declines in their specific ^{14}C and ^{3}H radioactivities. There was, therefore, no evidence for large-scale selective reutilization of [^{3}H]leucine or for asynchronous turnover of any components.

There did, however, appear to be some differential loss of iodinated proteins from the surface of both the CHO cells and the fibroblasts. In the latter, one major component was very rapidly lost, although this may have been related to the cells becoming detached. The CHO cells, however, behaved normally following iodination. Nevertheless, it has to be emphasized that with iodination label may well have been introduced preferentially into a number of minor, and superficially attached membrane proteins, into materials in the process of being secreted, into a few dead cells (Juliano, 1974), or even into serum proteins absorbed from the medium. Finally, of course, this selective loss might be due to the cell's ability to recognize faulty proteins, quickly and efficiently. On the other hand, a large part of the iodinated protein fraction did appear stable, indicating that they too were relatively long lived.

In conclusion, therefore, it appears that in rapidly growing fibroblast cultures the total complement of plasma membrane proteins turn over at a relatively slow rate. It also appears that the major protein components do not readily exchange from such membranes. All of the proteins appeared to be equally conserved. It is clearly important to emphasize that the situation in nongrowing cells might be quite different from that described here. However, it should be possible, using these techniques, to investigate stationary phase cultures where turnover is expected to be much faster. Such experiments are in progress.

Acknowledgments

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Puromycin Analogs. Ribosomal Binding and Peptidyl Transferase Substrate Activity of a Carbocyclic Analog of 8-Azapuromycin†

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ABSTRACT: The synthesis of an 8-aza carbocyclic puromycin analog, 7-dimethylamino-3-{(*R*)-[(2*R*)-hydroxy-(3*R*)-(*p*-methoxyphenyl-L-alanylamino)]cyclopentyl}-*v*-triazolo[4,5-*d*]pyrimidine (**1**), is described. The previously described carbocyclic pyrimidine, 2α-acetamido-5β-(5-amino-6-chloro-4-pyrimidinylamino)cyclopentan-1α-ol (**2**), was diazotized and converted to the corresponding 8-azapurine (**4**). Treatment of **4** with aqueous dimethylamine followed by acid hydrolysis of the acetamido blocking group gave the amino nucleoside analog (**6**). Coupling of **6** with *N*-benzyloxycarbonyl-*p*-methoxyphenyl-L-alanine, followed by hydrogenolysis of the Cbz blocking group, gave a mixture of **1** and its diastereoisomer, 7-dimethylamino-3-{(*S*)-[(2*S*)-hydroxy-(3*S*)-(*p*-methoxyphenyl-L-alanylamino)]cyclopentyl}-*v*-triazolo[4,5-*d*]pyrimidine (**2**). The diastereomers were separated by chromatography. Biological testing with *Escherichia coli* ribosomes provides a comparison of the relative ribosomal binding affinities and peptidyl transferase substrate activities of puromycin and 8-aza carbocyclic puromycin. Kinetic data indicate that such compounds are capable of binding to the ribosomal acceptor site and act as peptidyl transferase substrates in a manner identical with the mechanism of action of puromycin.

A variety of analogs and isomers of the antibiotic puromycin have been prepared to define the structural requirements for protein biosynthesis inhibition. It has been well documented that puromycin binds to the ribosome and terminates protein synthesis by accepting the growing peptide

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