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# STUDIES ON THE TURNOVER OF PLASMA MEMBRANES IN CULTURED MAMMALIAN CELLS

## II. DEMONSTRATION OF HETEROGENEOUS RATES OF TURNOVER FOR PLASMA MEMBRANE PROTEINS AND GLYCOPROTEINS

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SUMMARY

The relative rate of turnover of individual membrane proteins and glycoproteins in exponentially growing and contact-inhibited $MK_2$ cells was investigated. Plasma membranes were isolated from cells that had been sequentially labelled with $^{14}C$ and $^{3}H$ isotopes of leucine and glucosamine. The membranes were then solubilized in sodium dodecylsulfate and their polypeptides separated by acrylamide gel electrophoresis. The $^{3}H/^{14}C$ ratios of the individual polypeptides reflected their relative rates of turnover. The proteins and glycoproteins of the exponentially growing cells exhibited markedly heterogeneous rates of turnover. In contrast, polypeptides in membranes of contact-inhibited cells exhibited a lesser degree of heterogeneity of turnover. In both exponential and contacted cell membranes a glycoprotein with a high apparent molecular weight exhibited the fastest rate of turnover.

## INTRODUCTION

Studies on the synthesis and degradation of membrane components suggest that they are turned over at different rates [1–5]. A number of workers have shown that the constituent lipids and proteins of endoplasmic reticulum of the adult rat hepatocyte have different half-lives [1, 2, 4]. Dehlinger and Schimke [3] and Simon et al. [5] have demonstrated by a sequential double-label technique that protein components of the rat hepatocyte plasma membrane have heterogeneous rates of turnover.

At present, only a limited number of studies on membrane turnover using cultured mammalian cells have been undertaken. Warren and Glick [7] found no difference in the half-life of incorporated leucine, glucosamine and glucose in the membranes of cultured L cells. Pasternak and Bergeron [8], however, found that plasma membrane phospholipids of cultured rat tumor cells were turning over heteroge-

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neously. Hakomori [9] has shown that the synthesis of lactosyl cerebroside is apparently preferentially suppressed when exponentially growing mammalian cells become contact inhibited.

In this report we present evidence that both membrane proteins and glycoproteins exhibit heterogeneous rates of turnover and that when exponentially growing cells become contact inhibited the relative rates of turnover of membrane components change.

## MATERIALS AND METHODS

The cell line used in this study was a permanent line of epithelial Rhesus monkey kidney cells LL-$MK_2$ ($MK_2$). These cells grew with a generation time of 42 h and division ceased when the cells reached a density of $5.5 \cdot 10^4$ cell/$cm^2$.

The cells were grown in Eagles minimal essential medium supplemented with 10 % horse serum (Grand Island Biochemicals). In working with cells that were contact inhibited, medium that was conditioned by prior exposure to exponential or confluent monolayers for 12 h was used. This conditioned medium did not induce division in confluent cultures and affected neither the rate of amino acid incorporation in both contact-inhibited and exponential cells nor the generation time in exponential cells.

*Labelling conditions*

Cultures were labelled with either 0.5 $\mu$Ci/ml [4,5-$^3H_2$]leucine (specific activity, 58.1 Ci/mmol) or [U-$^{14}$C]leucine (specific activity, 316–388 Ci/mmol) (Schwartz/Mann) in minimal essential medium containing 1/100 the normal concentration of leucine. Cultures were labelled in complete medium with either 0.5 $\mu$Ci/ml D-[G-$^3$H]glucosamine (specific activity, 1.3 Ci/mmol) or 0.2 $\mu$Ci/ml D-[G-$^{14}$C] glucosamine (specific activity, 58 $\mu$Ci/mmol) (New England Nuclear).

Plasma membranes were isolated by a modification of the fluorescein mercuric acetate procedure [10].

Isolated membranes were dialyzed extensively against a buffer containing 0.1 M Tris/acetate, pH 9.0, 1.0 % sodium dodecylsulfate, 1.0 % mercaptoethanol, 0.001 % ethylenediaminotetraacetic acid, a procedure which removes the fluorescein mercuric acetate. The solubilized membranes were heated at 100 °C for 1 min and then applied to a 5 % acrylamide gel according to the procedure of Kiehn and Holland [11]. The gel dimensions were 0.6 cm by 12 cm and were run at 5 mA/gel. At the end of the run, the gels were frozen on solid $CO_2$ and sliced into 1-mm segments with a Mickle gel slicer (Gomshall, Surrey). Each slice was placed in a scintillation vial, to which was added 0.4 ml of Nuclear Chicago Solubilizer (NCS) and 0.05 ml of water. The vials were capped, and incubated at 50 °C overnight, and then 10 ml of toluene POPOP were added to each vial. The vials were counted in a Packard three-channel scintillation counter with an efficiency of 32 % for $^3$H and 74 % for $^{14}$C on a double-label setting.

Protein was determined using the procedure of Oyama and Eagle [13] and bovine crystalline albumin as a standard.

## RESULTS

The initial procedure chosen to examine relative rates of membrane turnover

was that of Arias et al. [1]. Cells were labelled with one isotopic form of a precursor molecule (i.e. $^{14}C$), incubated in isotope-free medium, and then pulsed with the other isotopic form ($^{3}H$). At this point membranes were isolated, solubilized in sodium dodecylsulfate/mercaptoethanol/urea, and applied to acrylamide gels. The $^{3}H/^{14}C$ ratio of a component is a measure of its relative rate of degradation since a rapidly turning over component would have a higher ratio than one that has a lower turnover rate. Fig. 1 Adepicts the ratios obtained when exponentially growing cells were labelled for 6 h with [$^{14}C$]leucine. The label was then removed, and cells were grown in the presence of medium without labelled leucine for 1.5 generations (60 h), and then

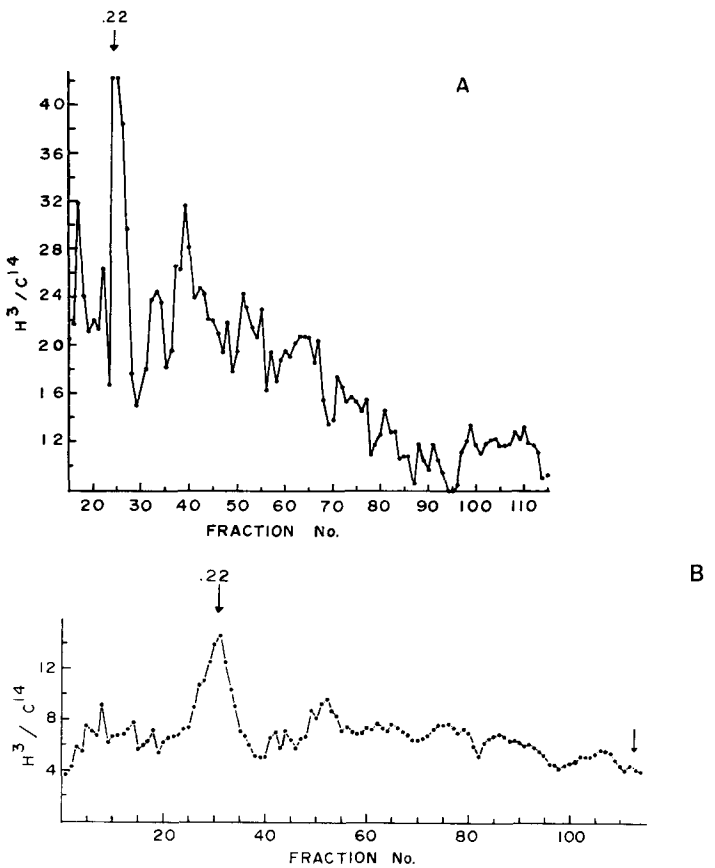


Fig. 1. Relative rates of turnover of membrane proteins as determined with sequential double-labe studies. Cells growing on 100-mm plastic petri dishes were labelled with [$^{14}C$]leucine in Eagles minimal essential medium containing 1 % of the normal amount of leucine for 6 h. The medium-containing label was then removed and the cells were then washed twice and incubated in regular medium for 60 h. The cells were then labelled with [$^{3}H$]leucine for 6 h in Eagles minimal essential medium containing 1 % of the normal amount of leucine. After this time, membranes were isolated, solubilized with sodium dodecylsulfate and analyzed with sodium dodecylsulfate/acrylamide gel electrophoresis. In this, as in all other electrophoretograms, the arrows denote the locations of a component with a migration rate ($R_F$) of 0.22 and the position of bromphenol blue. (A) $^{3}H/^{14}C$ ratios of membranes from exponential cells. (B) $^{3}H/^{14}C$ ratios of membranes from confluent cells.

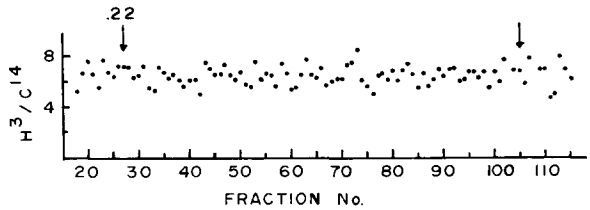


Fig. 2. Acrylamide gel electrophoresis of membranes from cells labelled with [$^3$H]leucine and [$^{14}$C]-leucine. One exponential culture was labelled for 6 h with [$^3$H]leucine and another exponential culture was labelled for 6 h with [$^{14}$C]leucine. At the end of this time, the cells from both cultures were pooled, membranes isolated, solubilized with sodium dodecylsulfate, analyzed by gel electrophoresis, and the $^3$H/$^{14}$C ratio determined.

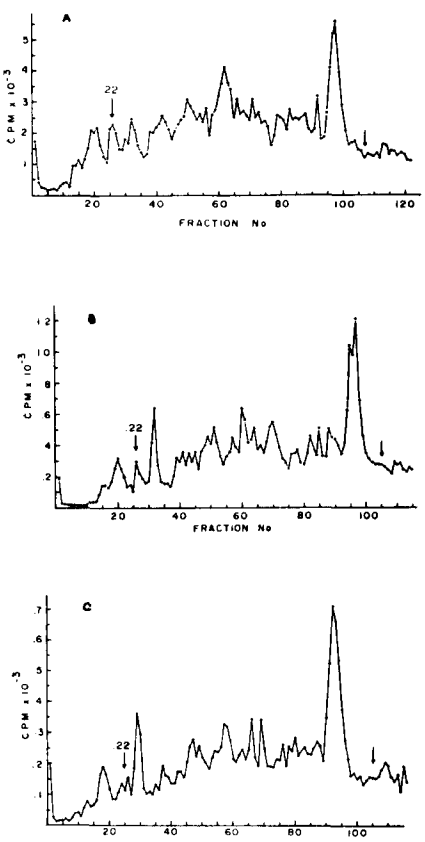


Fig. 3. Distribution of labelled proteins in membranes of exponential cells as a function of growth. Exponential cells grown on 100-mm plastic petri dishes were labelled with [$^3$H]leucine for 6 h, at which point the medium was removed and the dishes washed twice and incubated in unlabelled medium. At the time when labelled medium was removed, and at 24 and 48 h after the addition of unlabelled medium, membranes were analyzed as in Fig. 1. (A) t = 0 electrophoretogram. (B) 5 = 24 h electrophoretogram. (C) 5 = 48 h electrophoretogram.

labelled for 6 h with [$^3$H]leucine. Membranes were isolated from these labelled cells and the isolated membranes solubilized and electrophoresed. There is a great amount of heterogeneity in the $^3H/^{14}C$ ratios in these membranes which is not seen when two different batches of cells are labelled simultaneously, one with [$^3$H]leucine and the other with [$^{14}$C]leucine (Fig. 2). It is also noted that there is a correlation between components having higher apparent molecular weights and higher ratios, which is in agreement with the results of Dehlinger and Schimke [3]. Fig. 1B depicts the ratios obtained when confluent cells are treated in a manner similar to exponential cells, the only difference being that "conditioned" medium [10] was used to maintain the cells in the contact-inhibited state. The ratios obtained were non-uniform but the degree of heterogeneity was less marked than the ratios obtained from membranes of exponential cells. It is noted that one component, with an $R_F$ of 0.22, exhibits a much greater rate of turnover than that of the rest of the membrane components, in both exponential and confluent cells.

To test the assumption that the sequential double-label procedure measures relative rates of turnover, the following experiment was carried out. Exponentially grown cells were labelled for 6 h and the cultures were then divided into three aliquots. Membranes were isolated from the first aliquot immediately after the labelling period, and from the other two after 24 and 48 h growth in unlabelled medium. The isolated membranes were solubilized and run on acrylamide gels. It can be seen (Fig. 3) that there is a differential loss of counts in the various peaks as a function of time. There is also a good correlation with the sequential label experiment in that those peaks which lose label the fastest have the same $R_F$ as those membrane components which exhibit the highest ratios in the sequential double-label experiment.

The results of the double-label experiment with confluent cells present another method of gauging the accuracy of the double-label technique. The data in Fig. 1B suggest that there is one component(s) that has a much faster rate of turnover than the rest of the membrane. If this interpretation were correct, we should be able to detect the loss of this component in a specific activity dilution experiment having a period of labelling sufficiently short that a greater percentage of the label entered this component. Fig. 4 depicts the results obtained when confluent cells are labelled for 2 h with [$^{14}$C]-leucine and then chased in the presence of unlabelled leucine. It can be seen that there

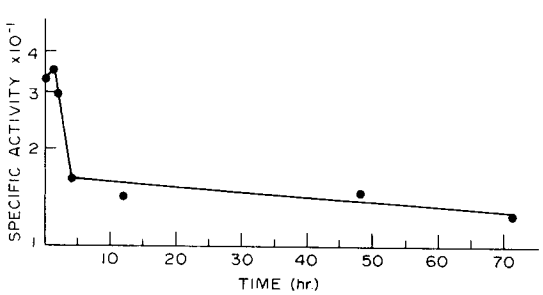


Fig. 4. Specific activity dilution of membrane proteins in confluent cells. Confluent cultures grown on 60-mm plastic petri dishes were labelled for 2 h with [$^3$H]leucine. At the end of this time, the dishes were washed twice and incubated in unlabelled conditioned medium. At the specified times, cells were harvested and the specific activity (cpm/$\mu$g protein) of isolated membranes was determined.

is a biphasic pattern of membrane specific activity dilution with a fast component(s) exhibiting a half-life of 2 h and a slow component with a half-life on the order of 160 h. The latter half-life is identical to that obtained from confluent membranes which has been prelabelled for extensive periods of time [10].

The sequential label experiments were also done with glucosamine to label the carbohydrate moiety of glycoproteins. In these experiments [$^3$H] glucosamine was given first and then followed by [$^{14}$C]glucosamine; during the chase, confluent cells were incubated in the presence of 150$\mu$g/ml of unlabelled glucosamine in order to reduce reutilization [10]. The results demonstrate that there are large differences in the relative rate of turnover of membrane glycoproteins in exponentially growing cells (Fig. 5A) and contact-inhibited cells (Fig. 5B).

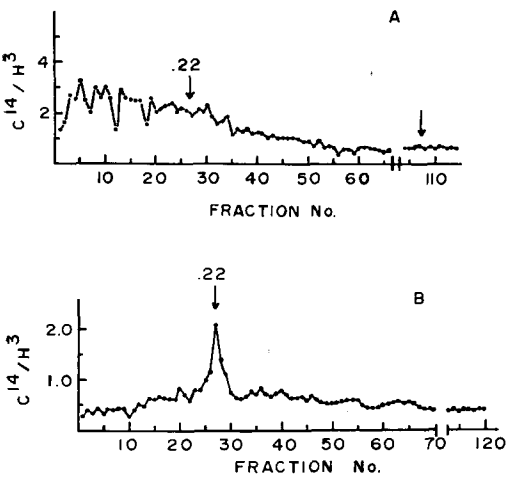


Fig. 5. Relative rates of turnover of membrane glycoprotein as determined by sequential double-label studies. The experimental procedure in Fig. 1 was followed, except that cells were labelled with glucosamine. (A) Exponential cells were labelled initially with [$^3$H]glucosamine and then labelled with [$^{14}$C]glucosamine 53 h later. (B) Confluent cells were labelled with [$^3$H]glucosamine and then labelled with [$^{14}$C]glucosamine 53 h later. Between the two labelling periods cells were incubated in "conditioned" medium containing 100 $\mu$g/ml of unlabelled glucosamine.

The ratios obtained from membranes of exponential cells show relatively broad regions of higher ratios, as opposed to discrete peaks. The lack of discrete peaks is not due to our inability to separate glycoproteins: many distinctly separated glycoproteins are observed in electropherograms of glycoproteins from membranes of exponentially growing cells (Fig. 6). Confluent membranes, however, show only a restricted area of increased ratio, the peak of which has an $R_F$ of 0.22, which is the same as the rapidly turning over leucine-labelled component in confluent cells. Specific activity dilution experiments using short labelling periods with glucosamine as a label in confluent cells also indicate a biphasic pattern of turnover with a fast component exhibiting a half-life of 2 h (not shown). Thus it appears, both from its $R_F$ value on acrylamide gel electrophoresis and from the similarities in half-lives obtained in specific activity dilution experiments, that this rapidly turned over component is a glycoprotein.

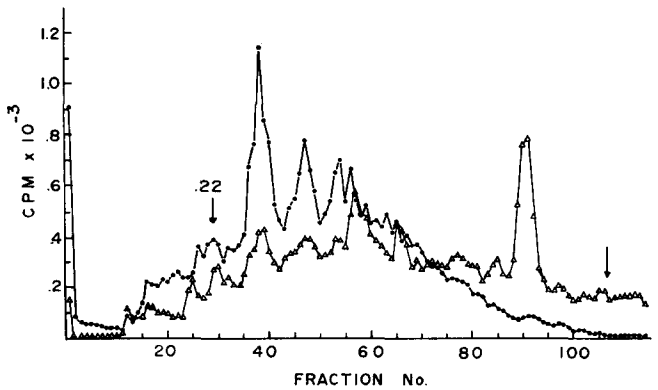


Fig. 6. Distribution of labelled leucine and glucosamine in membranes of exponentially growing cells. Exponentially growing cultures were labelled for 6 h with [$^3$H]glucosamine and [$^{14}$C]leucine. Membranes were isolated and analyzed as in Fig. 1. ●, [$^{14}$C]glucosamine; △, [$^3$H]leucine.

## DISCUSSION

The data presented suggest that membrane components are being actively synthesized and degraded at heterogeneous rates. These results are in agreement with previous studies on membrane turnover in rat liver [1–5] and with phospholipids in cultured rat cells [8]. The data are also in agreement with that of Dehlinger and Schimke [3] in that there is a correlation between components with "apparently" higher molecular weights having higher rates of turnover.

The results in Figs 1 and 3 demonstrate that there is a difference in the relative turnover ratio of membrane protein components in exponential and confluent cells. It should be noted that if the rate at which leucine is reutilized is different in exponential and contact-inhibited cells, it would affect the magnitude of the ratio difference. While we have not, at this point, obtained an absolute estimate for the rate of reutilization there is still a qualitative difference in the relative rate of turnover of membrane proteins in growing and non-growing cells. The results using glucosamine (Fig. 5) to label membrane glycoproteins also indicate that there is a difference in the relative rate of membrane turnover in exponential and confluent cells. The reasons for the lack of definition in the plot of ratios derived from exponential cells labelled with glucosamine is not due to our inability to separate membrane components, since we can delineate glucosamine-labelled components (Fig. 6), leucine-labelled components (Fig. 1A), and at least 20 components in stained preparations (Kaplan, J., unpublished data). A possibility that we are presently examining is that the carbohydrate moiety can be turned over independently of its conjugate polypeptide chain. Two independent pieces of evidence support this possibility. Kramer [12] demonstrated that neuraminidase-treated CHO cells can preferentially resynthesize the cleaved neuraminic acid. Roth and White [6] have demonstrated that mammalian cells have extra-cellular glycosyltransferases which they suggest have a physiological role in cell adhesion.

Although there is a general reduction of relative turnover rates in confluent membranes, there is one component which continues "turning over" at a relatively rapid rate. In view of the fact that leucine and glucosamine are turning over in this

component with similar half-lives, it is apparently a glycoprotein. Recent studies in this laboratory (Kaplan, J., unpublished data), indicate that this component is present in the cell membrane with the carbohydrate moiety on the exterior, contains a binding site for concanavalin A and is continuously being released into the medium (unpublished). The fact that it is released into the medium and is the only identifiable membrane component there suggests that the rest of the membrane is degraded by internal proteolysis. The data also point out that the presence of secreted molecules can complicate estimations of tissue or membrane turnover, since the secreted molecule is not degraded by proteolysis as is the rest of the organelle.

The results presented in Figs 1 and 6 suggest that those components which exhibit the greatest rate of turnover in exponential cell membranes are glycoproteins. With the low percentage acrylamide gels used here, these glycoproteins would migrate with anomalously high molecular weights [2]. For example, the $R_F$ 0.22 component in a 5 % gel has an apparent molecular weight of 300 000 and in a 7.5 % gel exhibits a molecular weight of 90 000 (Kaplan, J., unpublished data). Thus, the correlations between components of higher molecular weight having higher rates of turnover in our system may only be apparent based on sodium dodecylsulfate gels. This would suggest that glycosylated polypeptides have higher rates of turnover than non-glycosylated polypeptides.

Another theory that must be entertained is that these glycoproteins are located on the external surface of the cell. The possibility then exists that it is some aspect of this location that gives rise to higher rates of turnover and not glycosylation per se. We are, at present, attempting to discriminate between these possibilities.

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