Biochimica et Biophysica Acta, 389 (1975) 290-305

BBA 76939

STUDIES ON THE TURNOVER OF PLASMA MEMBRANES IN CULTURED MAMMALIAN CELLS

I. RATES OF SYNTHESIS AND DEGRADATION OF PLASMA MEMBRANE PROTEINS AND CARBOHYDRATES

JERRY KAPLAN* and MERWIN MOSKOWITZ

Department of Biological Sciences, Purdue University, West Lafayette, Ind. 47907 (U.S.A.)

(Received December 18th, 1974)

SUMMARY

The rate of turnover of membrane proteins and membrane-bound carbohydrates in exponentially growing and in confluent contact-inhibited cultures of strain MK-2 cells was investigated. Cells were labelled with [^{14}C]leucine and [^{3}H]glucosamine, incubated in isotope-free medium and, at various times thereafter, the cells were harvested and membranes isolated from them. The rate of decay of the protein and carbohydrate components was determined from specific activity dilution of the labeled components in the isolated membranes.

Although the rate of membrane synthesis is different in exponential and contact-inhibited cells, the rate of degradation (turnover) of membrane proteins and carbohydrates was found to be the same (25 % per generation (42 h) or 0.6 %/h).

INTRODUCTION

Although most studies on the plasma membrane have concerned its structure, composition and topology, interest has recently focused on the synthesis and degradation of plasma membrane components. Most of the work in this field has used the plasma membrane of the adult rat hepatocyte. Two major findings can be extracted from these studies. (1) While the hepatocyte divides on the average of once every three months to a year, the membranous systems of the hepatocyte have half lives of an average of 24–48 h [1]. (2) The constituent components (lipids, proteins) of adult hepatocyte endoplasmic reticulum and plasma membrane are synthesized and degraded (turned over) at heterogeneous rates [2–5].

Warren and Glick [6] have reported that the rate of turnover of the plasma membrane in cultured exponentially growing strain L mouse cells is 10 % per genera-

* Present address: Department of Physiology, University of Connecticut Medical School, Farmington, Conn., U.S.A.

tion (approx. 24 h). When cell division was inhibited by inoculation of a culture at high densities, the rate of membrane synthesis was 80 % that of an exponentially growing cell; however, all newly synthesized membrane was degraded and thus the rate of turnover was higher than in growing cells. Pasternak and Bergeron [7] demonstrated that in a line of cultured rat tumor cells phospholipid turnover was heterogeneous, with phosphatidylcholine being degraded more rapidly that phosphatidylethanolamine, and that the rate of lipid turnover was constant whether or not the cells were exponentially growing.

In this paper we report that in a contact-inhibited line of cultured mammalian cells the rate of membrane degradation is similar in both growing and non-growing cells even though there is a 4-fold difference in the rate of membrane synthesis.

MATERIALS AND METHODS

Cell line. The cell line used in this study was LLC-MK_2 (obtained from the Eli Lilly Research Laboratories, Indianapolis, Indiana). This strain is a normal monkey epithelial cell derived from a pool of kidneys removed from six adult Rhesus monkeys. Eagle's minimal essential medium (Grand Island Biological) containing 50 units/ml penicillin (E. R. Squibb and Sons) and 50 μg/ml dihydrostreptomycin (Mann Research Laboratories) was used as the routine culture medium and in all the experiments. Stock cultures were maintained in six oz. prescription bottles (Brockway Glass Company) and were subcultured by use of 0.25 % trypsin (Difco) every 5 days. Experiments were carried out on 100-, 60-, or 20-mm plastic culture petri dishes (Falcon Plastics).

Labelling of cells. For kinetic experiments (incorporation and specific activity dilution) cells were labelled with 0.5 μCi/ml of [^{3}H]leucine, or 0.2 μCi/ml of [^{14}C]-leucine, 0.5 μCi/ml of [^{3}H]glucosamine or 0.5 μCi/ml of [^{14}C]glucosamine. For determination of glucosamine pool sizes 1–2 μCi of [^{3}H]glucosamine/ml was used.

D-[6-^{3}H]Glucosamine (1.3 Ci/mmol), D-[6-^{14}C]glucosamine (58 μCi/mmol), and D-[G-^{3}H]fucose (4.6 Ci/mmol) were purchased from New England Nuclear. L-[4, 5-$^3H_2$]Leucine (58.1 Ci/mmol), and L-[U-^{14}C]leucine (316–388 mCi/mmol) were purchased from Schwartz/Mann.

Membrane purification. The procedure used to isolate membranes was a modification of Warren's fluorescein mercuric acetate procedure [7]. Cells scraped off a monolayer with a rubber policeman were washed three times with 0.9 % NaCl, and centrifuged for 4 min at 500 $\times g$ in an International PR2. The supernatant was decanted and saturated solution of fluorescein mercuric acetate in 0.02 M Tris/acetate (pH 8.2) was added to give a cell density of 2–3 $\cdot$ 10^6 cells/ml. The cells were allowed to sit at room temperature for 7–10 min, then on ice for 7–10 min, homogenized by 10–15 strokes in a tight-fitting Dounce homogenizer, and then sucrose was added.

In order to separate membranes from the rest of the cellular material, 4–5 ml of homogenate were layered over a discontinuous sucrose gradient that contained 5 ml each of 60, 50, 45, 30, 20 % sucrose. The gradient was spun at 4 °C in a refrigerated International centrifuge PR2 for 70 min (750 rev./min, approx. 400 $\times g$). The membranes, which were found predominantly in the 30–45 % layer, were removed with a syringe and bent needle. The suspension was diluted by addition of an equal volume of 5 mM Tris/acetate and centrifuged for 1 h at 1500 rev./min. The super-

natant was decanted, and the pellet resuspended in 1–2 ml of 5 mM Tris/acetate and layered over a discontinuous gradient containing 2 ml each of 60, 50, 45, 40, 20 % sucrose. The gradient was spun for 70 min at 750 rev./min. The membranes which were found exclusively in the 40–45 % layers were removed with a capillary pipette, diluted with Tris/acetate and pelleted by centrifugation.

Preparation of samples for counting. At specified times after the addition of label, samples were washed three times with cold Hanks balanced salt solution. After the final washing, the sample was solubilized by addition of either 1 M NaOH or a solution containing a 1 % mixture of sodium dodecylsulfate and deoxycholate. Aliquots of the solubilized sample were removed for protein determinations. The remainder was combined with an equal volume of cold 10 % trichloroacetic acid. After chilling the sample was filtered through a fiberglass filter (Reeve Angel) and the filter and filter holder washed with 10 ml of cold 5 % trichloroacetic acid. The filters were dried and then 10 ml scintillation fluid (5 g 2,5-diphenyloxazole (PPO), 100 mg *p*-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (POPOP) dissolved in 1 l of spectral grade toluene) was added. The samples were counted in a Packard Tri-Carb scintillation counter with an efficiency of approx. 40 % for ^{3}H and 75 % for ^{14}C when the isotopes were counted independently. The ^{3}H, ^{14}C efficiencies for double label counting were 34 and 65 %, respectively.

Extraction and analysis of pools. The cellular pools of leucine and glucosamine were extracted in two ways.

(1) For kinetic experiments. Cells grown on either 60- or 35-mm plastic petri dishes were labelled for various periods of time, the labelled medium was removed by aspiration and the monolayers washed three times with cold 0.9 % NaCl. After the last wash, 2 ml of cold 5 % trichloroacetic acid were added to the 35-mm dishes and 4 ml to the 60-mm dishes. The dishes were then placed in a refrigerator at 4 °C for a minimum of 12 h. At the end of this time the trichloroacetic acid solution was transferred to a conical centrifuge tube and centrifuged at 1000 rev./min for 10 min at 4 °C to remove any free cells. The supernatant was removed and extracted three times with equal volumes of ether at room temperature, and the ether/trichloroacetic acid layer was removed by aspiration. After extraction 0.5-ml aliquots of the remaining aqueous solution were added to 10 ml of modified Bray's solution and counted.

(2) Glucosamine pool size determination. Cells grown in 100-mm plastic petri dishes were exposed to [^{3}H]glucosamine for 4 h, the medium was removed and the monolayers were washed with 4 ml of cold 0.9 % NaCl. After the final wash, 2 ml of 0.9 % saline were added to each plate and the cells were removed by scraping with a rubber policeman. The dislodged cells were transferred to centrifuge tubes and an aliquot of the cell suspension was removed for protein determination. The cell suspension was centrifuged at 1000 rev./min for 10 min, after which the supernatant was removed and discarded, and 3.0 ml of 80 % ethanol were added to the cell pellet [9]. The resulting suspension was placed in a boiling water bath for 2 min and then centrifuged at 1000 rev./min to remove the cells. The supernatant was removed, placed on ice for 30 min, and centrifuged again. The supernatant was evaporated to dryness in a rotary evaporator and then resuspended in 2.0 ml of distilled water and aliquots were taken for carbohydrate determinations, radioactivity measurements, and paper chromatography.

Paper chromatography. Paper chromatography of amino sugars was carried

out according to the method of Strause et al. [10], and sugar standards were visualized by spraying with alkaline $AgNO_3$ [11]. This system readily separates glucosamine ($R_f = 0.44$) from *N*-acetylglucosamine ($R_f = 0.72$). Sugar standards: D-glucosamine, D-galactosamine, D-mannosamine, D-galactose, D-mannose, *N*-acetylglucosamine, *N*-acetylgalactosamine, *N*-acetylneuraminic acid, uridine diphospho-*N*-acetylglucosamine were purchased from Sigma. Hexosamines were quantitated according to the procedure of Gatt and Berman [12] using crystalline glucosamine as a standard.

Membranes were hydrolyzed in order to examine the constituent amino acids and carbohydrates. To examine amino acids, pelleted isolated membranes were made up into a suspension containing 6 M HCl in double-distilled water to final volume of 1 ml. The samples were placed in hydrolysis tubes and hydrolyzed for 24 h in vacuo at 100 °C. A similar procedure was utilized for carbohydrates with the exception that 2 M HCl was used for 4 h.

Electron microscopy. Samples of the plasma membrane preparation were examined by electron microscopy. The samples were fixed in 3.0 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) followed by fixation in 2.0 % OsO_4 in the same buffer. The sample was dehydrated in a graded series of ethanol followed by propylene oxide. The sample was embedded in epon and ultra thin sections were made. The sections were stained with uranyl acetate and lead citrate and examined in a Hitachi Hu-11E electron microscope.

Protein determination. The amount of protein per sample was determined by Oyama and Eagle's modification [13] of the Lowry method, using bovine albumin as the standard.

Ribonucleic acid determination. RNA was measured chemically by the orcinol reaction [28] with yeast tRNA used as a standard.

RESULTS

Properties of MK_2 cell line

The cell line (MK_2) used in this study exhibits the characteristic of contact inhibition of growth (or density-dependent inhibition). These cells, regardless of initial inoculum, grow exponentially until they reach their saturation density of $5.5 \cdot 10^5$ cells/cm^2, at which point cell division and protein accumulation cease (Fig. 1). Changing the medium causes a 30 % increase in cell number with no further growth after the initial enhancement. If fresh medium is incubated with either a confluent or exponential monolayer for 12 h and then added to a confluent monolayer, there is no growth stimulation or increase in net protein. This phenomenon allows us to manipulate the external environment of the cells without grossly changing the metabolic characteristics of the cell.

Characteristics of isolated plasma membranes

Plasma membrane preparations prepared from both exponentially growing and contact-inhibited cells, by phase contrast microscopy consists almost entirely of whole cell ghosts and large membrane fragments. The preparation exhibits a 5–6-fold increase in the specific activity of incorporated [^{3}H]fucose compared to the intact cell (Table I). By direct chemical analysis the preparation shows a 75 % decrease in RNA relative to the intact cell (Table I). Examination of thin sections by electron

TABLE I

CHARACTERIZATION OF ISOLATED MEMBRANE PREPARATION

(1) Exponentially growing cells were exposed to [^{3}H]Fucose (20 μCi/ml) for 48 h. Cells were then harvested and plasma membranes were isolated, and the specific activity of the isolated membranes determined. An aliquot of homogenized cells, were taken just prior to centrifugation for a specific activity determination. (2) Aliquots of isolated plasma membrane preparations, and whole cell homogenates were obtained from exponentially growing cells. RNA was determined by the orcinol procedure with yeast tRNA used as a standard.

Sample	A Specific activity of incorporated [^{3}H]Fucose (cpm/100 μg protein)	B Specific activity of RNA (μg RNA/100 μg protein)
Plasma membrane	1103.3	4.2
Homogenate	198.6	16.23

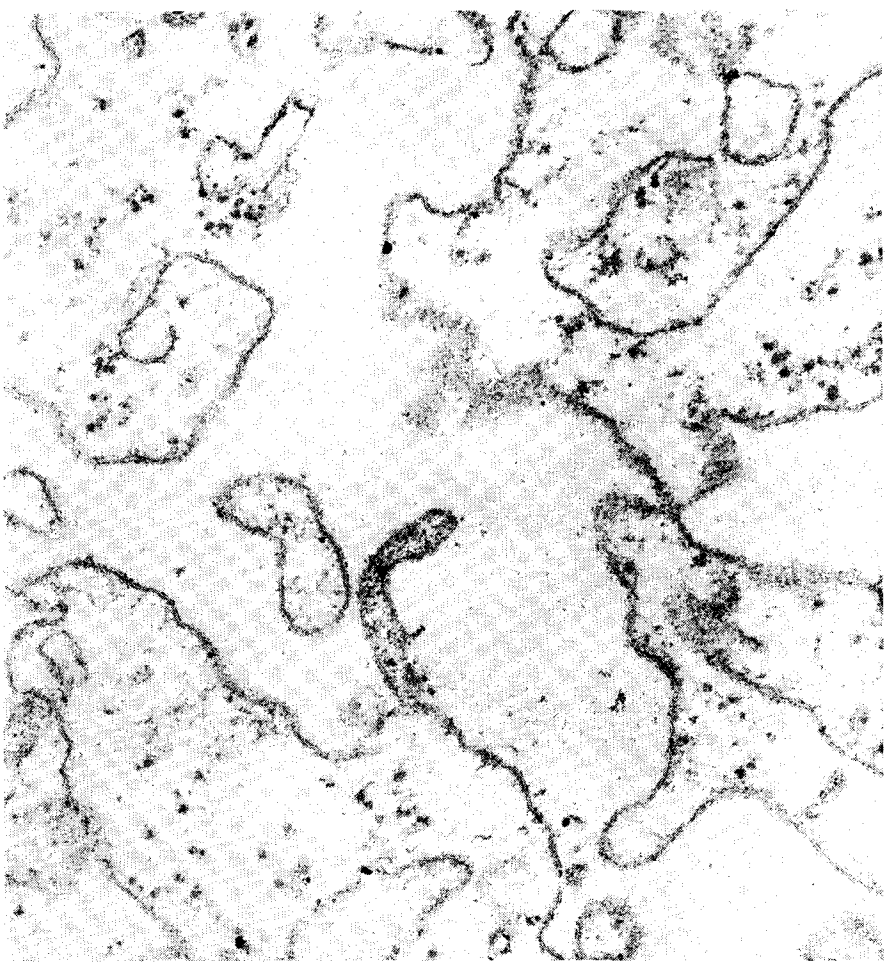

Plate 1. Electron microscopy of thin sections of plasma membranes isolated from MK_2 cells. Electron microscopy of plasma membranes from exponentially growing MK_2 cells. Membranes were isolated as described in Materials and Methods. The final magnification was 64 500.

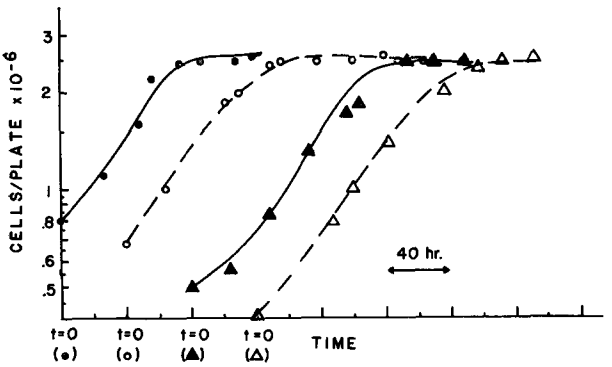

Fig. 1. Growth rate of MK_2 culture. Different numbers of cells were inoculated into 60-mm plastic petri dishes. At various times cells were removed and counted in a hemocytometer. Each curve represents a separate experiment.

microscopy (Plate 1) reveals large membranous sheets, devoid of mitochondria. There is, however, evidence for some small dense particulate matter adherent to these membranes suggesting the presence of ribosomes.

Contamination of the plasma membrane preparation by other cellular fractions during isolation was assessed by a reconstruction experiment. Purified [^{3}H]-leucine-labelled plasma membranes was added to a [^{14}C]leucine-labelled homogenate in which the plasma membranes had been previously removed. Upon reisolation less than 0.5 % of the [^{14}C]leucine label was found in the ^{3}H-labelled plasma membrane preparation.

The above data, in conjunction with published results [6, 7], suggest that while membranes isolated by the fluorescein mercuric acetate procedure may not be pure, they are certainly highly enriched in plasma membranes.

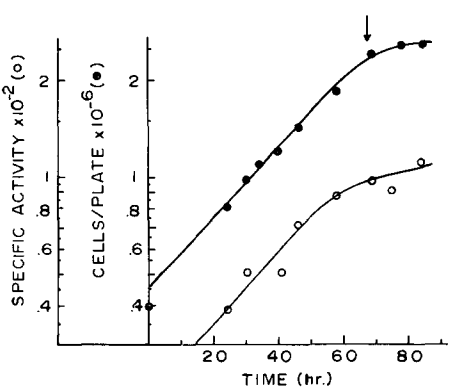

Fig. 2. Incorporation of leucine into membrane protein as a function of the growth curve. [^{14}C]-Leucine was added to a freshly inoculated culture on 60-mm plastic petri dishes. At the times specified cells were harvested, cell number determined, membranes isolated and the specific activity (cpm/μg protein) was determined. The arrow denotes the point at which the culture becomes confluent. ●, cells/plate; ○, cpm/μg protein (specific activity).

Rate of incorporation of membrane proteins

Fig. 2 shows the rate of leucine incorporation into membranes isolated from labelled cells as a function of the growth curve. It can be seen that the rate is constant throughout the exponential phase until the cells reach their saturation density, at which point the rate falls to 20–25 % that of the exponentially growing cells. This difference in the rate of synthesis was verified by pulse label studies, using 30-min labelling periods. These studies demonstrated an 80 % drop in the rate of incorporation of leucine into membranes isolated from contact-inhibited cultures relative to those isolated from exponentially growing cells.

Rate of "degradation" of membrane proteins

To determine the rate of membrane degradation, cells were labelled for three generations with [^{14}C]leucine and then incubated in cold medium. We determined that there was no difference in the rate of amino acid incorporation into membranes for scraped or trypsinized cells. Trypsinization affects the apparent loss of label from membranes for about 6 h, but does not have any effect on experiments that extend over many days. Fig. 3 shows that the rate of specific activity dilution follows the

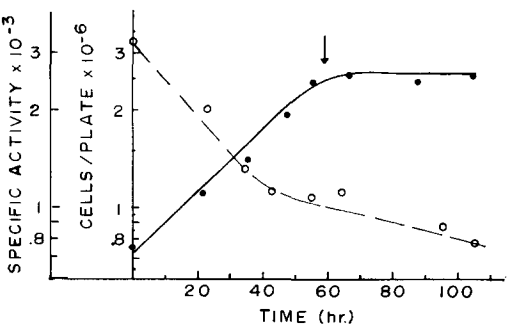

Fig. 3. Specific activity dilution of leucine-labelled membrane proteins as a function of the growth curve. Cells were labelled with [^{14}C]leucine for 72 h, harvested and inoculated onto 60-mm plastic petri dishes in the presence of media containing non-radioactive leucine. At the times specified cells were harvested, counted in a hemocytometer, and the specific activity (cpm/μg protein) of isolated membranes was determined. The arrow denotes when the culture becomes confluent. ○, specific activity (cpm/μg protein); ●, cells/plate.

kinetics of first-order decay until the cells become confluent, at which point the rate falls to a value 25 % that of the exponentially growing cells. The specific activity of the membranes from exponentially growing cells should decrease 50 % in each generation. However, the observed decrease in specific activity is greater than this theoretical value. The greater rate of isotope dilution is considered to represent the rate of membrane degradation, which is thus, 0.6 %/h. In confluent cells the rate of isotope dilution is 25 % per generation (42 h) but there is no net increase in protein. Therefore, all newly synthesized protein is replacing degraded protein, or protein released from the cells into the culture medium, and the rate of turnover is 0.6 %/h. Thus, although the rate of membrane synthesis is different in exponential and contact-inhibited cells, the rate of degradation (i.e. percent membrane replaced) is the same.

Greater than 90% of the incorporated label was found in the form of leucine. This was determined by hydrolyzing membranes that had been isolated from cells grown for 24 h in the presence of [^{14}C]leucine, and chromatographing the hydrolysate on Dowex 50W-X8 [13]. [^{14}C]Leucine was identified by co-chromatographing purified [^{3}H]leucine as a standard.

Since our turnover data is based on specific activity dilution which is highly dependent on the rate of re-utilization, experiments were carried out in an attempt to assess the extent of re-utilization. After a chase with unlabelled leucine there is no further incorporation of label into a membrane-associated molecule that is produced and exported (unpublished). In addition, the half-life of labelled leucine in the trichloroacetic acid-soluble pool was found to be much shorter than the half-life of labelled proteins (Figs 4a and 4b). These results suggest that there was little re-utilization of leucine in our system.

Fig. 4. Loss of label from the trichloroacetic acid pools of leucine-labelled exponential and confluent cells after incubation in unlabelled medium. Exponential and confluent monolayers grown on 60-mm plastic petri dishes were labelled for 4 h with [^{3}H]leucine. At this time the labelled medium was removed, the monolayers were washed once, and then incubated in unlabelled medium (confluent cells were incubated in "conditioned" medium). At the times specified the amount of trichloroactic acid-soluble cpm-plate was determined. (A) Exponential cells. (B) Confluent cells.

Although there is a difference in the ability of intracellular leucine to exchange with extracellular leucine, the fact that labelled leucine is observed in trichloroacetic acid-soluble pools hours after the chase is indicative of a continued breakdown of labelled proteins. While these data do not allow us to obtain absolute half-lives for membrane proteins, it suggests that the data we have obtained are at least an approximate estimate of membrane protein turnover.

Rate of synthesis of membrane-bound carbohydrates

To determine the rate of synthesis of membrane-bound carbohydrates, confluent and exponential monolayers were labelled with glucosamine since it has been shown to label membrane components almost exclusively [15]. It was found (Fig. 5) that there was no difference in the rate of incorporation of glucosamine into membranes from exponential and contact-inhibited cells.

Hydrolysis and chromatography of glucosamine-labelled membranes indicated that 90% of the incorporated label is in the form of glucosamine and galactosamine in a ratio of 3 : 1 in both contact-inhibited (Fig. 6) and exponential cells. Any sialic

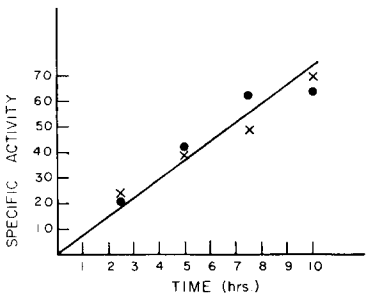

Fig. 5. Incorporation of glucosamine into membranes isolated from exponential and confluent cells. [^{3}H]Glucosamine was added to exponential and confluent cultures grown on 60-mm plastic petri dishes. At specified times cells were harvested and the specific activity (cpm/μg protein) of isolated membranes was determined. ●, exponential cells; ×, confluent cells.

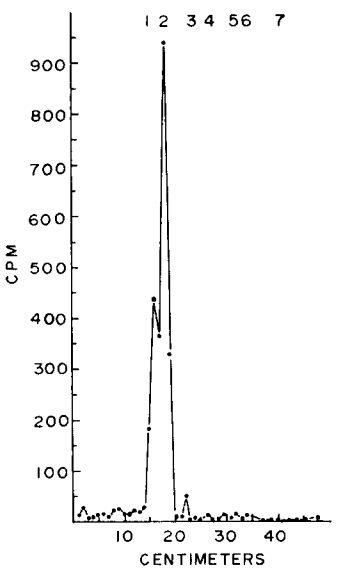

Fig. 6. Determination of the chemical form of the incorporated radioactivity in glucosamine-labelled cells. Isolated membranes from [^{3}H]glucosamine-labelled cells were hydrolyzed. The hydrolysate was run on paper chromatography. The paper was cut into strips and the amount of counts was determined. Duplicate chromatograms containing standard sugars as references were also run. This chromatogram was then stained with alkaline $AgNO_3$ to fix the position of the standards. Standards chromatographed are denoted at their location. 1, galactosamine; 2, glucosamine; 3, galactose; 4, glucose; 5, *N*-acetyl galactosamine; 6, *N*-acetyl glucosamine; 7, fucose.

acid present would be destroyed by the hydrolysis procedure. Since contact-inhibited cells synthesized membrane proteins at a rate 25 % that of exponential cells and the synthesis of glycoproteins and glycolipids involves synthesis of the protein or lipid moiety first with subsequent addition of the carbohydrate moiety [16, 17] this result presented a discrepancy. However, in as much as mammalian cells are absolutely dependent on exogenous leucine, but are capable of synthesizing glucosamine, a difference in the specific activity of the internal pool of glucosamine in exponential

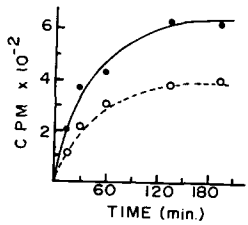

Fig. 7. Uptake of glucosamine into the trichloroacetic acid-soluble pools of exponential and confluent cells. Petri dishes were labelled with [^{3}H]glucosamine. At the times specified the amount of trichloroacetic acid-soluble cpm/cell was determined. ○, confluent cells: trichloroacetic acid-soluble cpm/cell; ●, exponential cells: trichloroacetic acid-soluble cpm/cell.

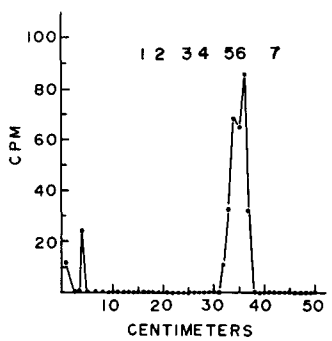

Fig. 8. Chromatography of material extracted from glucosamine-labelled cells. Exponential cells were labelled with [^{3}H]glucosamine for 4 h; the cells were extracted with cold trichloroacetic acid and the extracts were analyzed by paper chromatography. The paper was cut into strips and the amount of ^{3}H cpm/strips was determined. Duplicate strips were run with sugars as references and their locations were fixed by staining with alkaline $AgNO_3$. The reference standards are denoted in Fig. 6.

and contact-inhibited cells could account for the apparent discrepancy in incorporation. We therefore examined the rate of uptake of glucosamine into trichloroacetic acid-soluble material. Fig. 7 shows that exponential cells take up almost twice the amount of glucosamine that confluent cells do. Chromatography of the pools extracted by trichloroacetic acid (Fig. 8) (similar results were obtained with ethanol extraction) showed that 95 % of the label was in the form of either *N*-acetylglucosamine or *N*-acetylgalactosamine in a ratio of about 3 : 1 and that there was no difference between confluent and exponential cells in the relative ratio of the two. Table I shows the pool size and specific activities of glucosamine, extracted by the ethanol procedure from exponential or confluent cells labelled for 4 h with [^{3}H]glucosamine. It can be seen that the absolute pool size of exponential cells is greater than that of confluent cells but that the specific activity (cpm/μg sugar) was greater in confluent cells. Knowing the rate of incorporation (cpm/μg protein per time) and the specific activity of the pool (cpm/μg sugar) we calculate that exponential cells are synthesizing at least twice the amount of membrane-bound glucosamine as confluent cells. Further verification of this conclusion was obtained by labelling confluent and exponential monolayers with [^{3}H]fucose. This sugar is metabolized by a pathway that does not utilize UDP-*N*-acetylglucosamine [17], and is incorporated primarily into plasma

TABLE II

AMOUNT AND SPECIFIC ACTIVITY OF GLUCOSAMINE IN THE POOLS OF EXPONENTIAL AND CONFLUENT CELLS

Data are the averages of two separate experiments which were within 10 % of each other.

	Exponential	Confluent
Glucosamine*/sample (μg)	4.5	4.0
Specific activity (cpm/g sugar)*	4340	6500
Protein/sample	6.2	9.4
μg/sugar* per mg protein	1.55	.914

* Glucosamine was used as a standard.

membranes [17]. It can be seen in Table II that the trichloroacetic acid-soluble cpm/cell is the same, whereas exponential cells incorporate twice as much fucose as confluent cells. Thus, while differences in the pool specific activity of carbohydrates accounts for some of the apparent discrepancy between the reduction in membrane protein synthesis and membrane carbohydrate synthesis in contact-inhibited cells, there is still a significant disparity between the two. A possible explanation for this phenomenon will be presented in the Discussion.

Rate of "degradation" of membrane-bound carbohydrates

The rate of degradation of membrane-bound glucosamine was analyzed in the following manner. Exponential cells labelled for one generation were washed with unlabelled media and then incubated in unlabelled media. Fig. 9 demonstrates that the rate of turnover of membrane carbohydrate follows that of membrane protein giving a value of 20–25 % generation. Problems, however, were encountered in attempts to measure the rate of specific activity dilution in confluent cultures. For example, cultures were grown in the presence of label for 2 and 8 h, at which point they were washed and incubated in unlabelled conditioned medium. Fig. 10 depicts the rate of specific activity dilution of trichloroacetic acid precipitates of whole cells. It can be

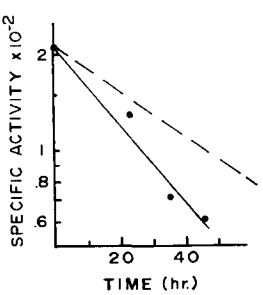

Fig. 9. Specific activity dilution of glucosamine in membranes of exponential cells. Exponential cultures labelled for 48 h with [^{3}H]glucosamine were harvested and inoculated into medium without added glucosamine at low densities on 60-mm plastic petri dishes. At the specified times cells were harvested and the specific activity (cpm/μg protein) of isolated membranes was determined. The theoretical specific activity is based on a 50 % reduction in labelled glucosamine content per cell. —, specific activity (experimental); - - -, theoretical specific activity.

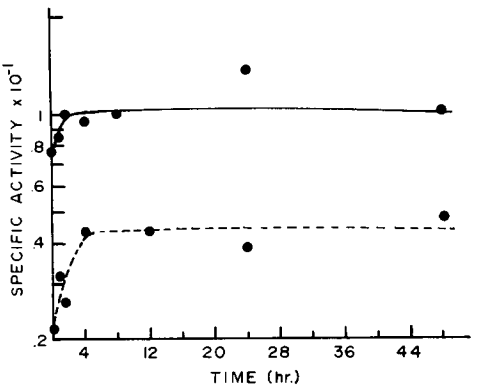

Fig. 10. Specific activity dilution of glucosamine-labelled confluent cells. Confluent cells were labelled for 2 and 8 h with [^{3}H]glucosamine and at the end of each time the media was removed and the cells were washed and incubated in unlabelled "conditioned" media. At the time specified cells were precipitated with cold 10% trichloroacetic acid and the specific activity (cpm/μg protein) of the precipitates was determined. - - -, 2 h prelabel; —, 8 h prelabel.

seen, that up until 4 h, after the end of the chase there is continued incorporation of label and that subsequently up to 40 h later there is no loss of label. These data suggest that there may be a significant amount of re-utilization of incorporated glucosamine in confluent cells. Schimke [19] has shown that the effect of re-utilization of amino acids is to increase the apparent half-life of proteins and organelles. To examine this hypothesis, prelabelled confluent cells were placed in unlabelled medium containing large amounts of unlabelled glucosamine. Table III shows that the specific activity of bound glucosamine is lowered when the amount of excess glucosamine is increased. Fig. 11 demonstrates specific activity dilution in confluent membranes prelabelled for 2 h, and subsequently incubated in medium containing 100 μg/ml of unlabelled glucosamine. The specific activity curve shows a bimodal distribution with a fast component exhibiting a half-life of 2 h and a slow component exhibiting a half-life of 180 h. The slow component(s) has a half-life identical to membrane protein from

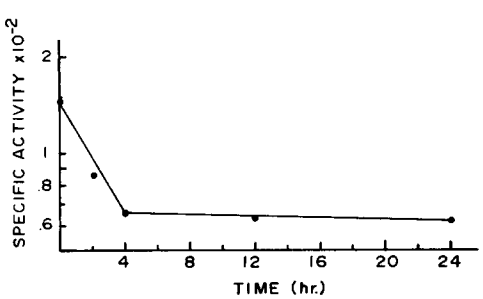

Fig. 11. Specific activity dilution of glucosamine in membranes of labelled confluent cells grown in the presence of unlabelled glucosamine. Confluent cultures grown on 60-mm plastic petri dishes were labelled for 2 h with [^{3}H]glucosamine. At the end of this time unlabelled conditioned medium was added containing 100 μg/ml of glucosamine. At the specified times cells were harvested and the specific activity (cpm/μg protein) of isolated membranes was determined.

TABLE III

UPTAKE AND INCORPORATION OF FUCOSE INTO TRICHLOROACETIC ACID-PRECIPITABLE FRACTIONS OF EXPONENTIAL AND CONFLUENT CELLS

Data are the averages of two separate experiments with differences of less than 10 %.

	Exponential	Confluent
Trichloroacetic acid-soluble material (cpm/μg protein)	1175	1157
Trichloroacetic acid-precipitable material (cpm/μg protein)	5.02	2.67

TABLE IV

EFFECT OF ADDED GLUCOSAMINE ON THE SPECIFIC ACTIVITY DILUTION OF LABELLED GLUCOSAMINE IN CONFLUENT CELLS

Confluent monolayers were labelled for 2 h with [^{3}H]glucosamine. At the end of that time the labelled medium was removed and unlabelled medium containing different amounts of unlabelled glucosamine was added. After 5 h the cells were harvested, precipitated with cold 10 % trichloroacetic acid and the specific activity (cpm/μg protein) of the precipitates was determined. The results are expressed relative to the activity at zero glucosamine concentration. Data represent the average of the two experiments. Results are within standard error of 10 %.

Unlabelled glucosamine/ml (μg)	0	50	100	150
Percent reduction of specific activity	–	13	40	40

confluent cells (Fig. 3) and membrane-bound carbohydrate as determined from long-term chase studies (unpublished). The fast component(s) represents a glycoprotein that is associated with the cell surface and released into the media (unpublished).

DISCUSSION

The results indicate that the rate of membrane degradation in exponentially growing and contact-inhibited cells is the same. This conclusion is based on (1) the deviation of the experimentally derived isotope dilution curve from a theoretical curve of isotope dilution for exponentially growing cells and (2) the rate of isotope dilution in confluent cells in which there is no increase in protein per cell. Warren and Glick [6] observed that the rate of membrane degradation equalled the rate of membrane synthesis in non-growing L cells. They also determined that the rate of membrane synthesis in non-growing cells was 80 % that of the growing cell. We have determined that the rate of membrane degradation in contact-inhibited cells equalled the rate of degradation in exponentially growing cells, and that contact-inhibited cells synthesized membrane at a rate that was 20 % of the exponentially growing cells. Thus, in both instances where non-growing cells are involved, the rate of degradation equals the rate of synthesis leading to no net gain or loss of membrane protein. Our results are

similar to those reported (25–30 %) for the rate of protein synthesis in other lines of contact-inhibited cells [21].

The rate of membrane-bound carbohydrate synthesis was estimated by determining the rate of incorporation of glucosamine, the specific activity of the glucosamine pool, and the rate of incorporation of fucose. Though we have not definitely characterized fucose as a plasma membrane component, Atkinson and Summers [18] have demonstrated that, in Hela cells, the plasma membrane is the location of more than 80 % of the fucose in the cell. It would appear that there is a greater inhibition of membrane protein synthesis than membrane carbohydrate synthesis when exponential cells become contact inhibited. However, we have not specifically separated membrane carbohydrate synthesis into glycoprotein, glycolipid, or mucopolysaccharides and perhaps there is a differential inhibition of synthesis of some of these components between exponential and confluent stages.

Evidence has been presented for a differential synthesis of at least one glycolipid in exponential and contact-inhibited cells. Hakomori [22] had demonstrated that lactoside cerebroside synthesis increases when BHK cells become contact inhibited. There is evidence, however, that the contribution of glycolipids and mucopolysaccharides to membrane carbohydrates is quantitatively small. Estimations of the percentage of membrane carbohydrates bound to glycolipids and mucopolysaccharides by gel electrophoresis indicate that only approx. 10–25 % of membrane-bound glucosamine is involved [23]. Furthermore, hyaluronic acid and chrondroitin sulfate, which are rich in glucosamine and glucosamine derivatives, are devoid of fucose and hence do not participate in the decline of fucose incorporation into membranes of contact-inhibited cells.

A further possibility is that there is a differential synthesis of glucosylated as opposed to non-glycosylated proteins in the membranes of contact-inhibited cells which would give rise to the differential inhibition of protein and carbohydrate synthesis and thus account for the apparent discrepancy. Results to be presented elsewhere do not support this possibility.

A hypothesis that we plan to explore is the possibility of a difference in the specific activity of the intracellular leucine pool in exponential or contact-inhibited cells. Our estimates of protein synthesis are based on the incorporation of an essential amino acid: if, in fact, there is a differential compartmentalization of leucine in either exponential or contact-inhibited cells, our present measurements would be a mere estimate of the rate of membrane protein synthesis. Experiments are under way in an attempt to measure not only the intracellular leucine pool, but also its relationship to membrane protein synthesis in exponential and contact-inhibited cells.

The errors inherent in determining rates by the method of specific activity dilution have already been mentioned and recent results have emphasized this point [26]. The possible error introduced into our system by the reutilization of leucine in the two different cell states was estimated in the following manner. Due to the facts; that at least 90 % of the incorporated label is found as leucine, the loss of leucine from the trichloroactic acid-soluble pool is rapid and; that after a chase no further label is incorporated into a molecule that has an extracellular fate and thus, cannot be reutilized, we feel, that our determinations of turnover rate, while certainly not absolute, are reasonable estimated. Similar reasoning applies to turnover rates based on the specific activity decline of incorporated glucosamine with the qualification that the

ability to chase glucosamine from the intracellular pool is dependent on both the amount of exogenous glucosamine and the growth phase of the culture.

Studies on the rate of turnover in bacteria have demonstrated that the rate of turnover during exponential growth is small compared to the rate of turnover during stationary or starvation conditions [24]. However, Eagle et al. [25] estimated that the rate of turnover for mammalian cells growing with different growth rates is similar. The rate of turnover in contact-inhibited cells appears to be similar to the rate of synthesis in this state leading to a condition of no net increase in cell mass (which is inferred from the fact that there is no increase in either cell number of protein/culture). That this condition is not overly detrimental to the cells is suggested by the fact that, with periodic feedings, they can be maintained in this condition for extensive periods of time and that in vivo, for many tissues and organs, lack of growth is the rule rather the exception.

ACKNOWLEDGEMENTS

The authors would like to gratefully acknowledge the many valuable suggestions and the advice of Dr D. A. Casciano and the superb typing of Ms J. Jannace. This work was supported in part by an N.I.H. traineeship (J.K.), Training Grant 5 T01 GM01392, and by the Jennie E. Hedges Memorial Grant for Cancer Research from the American Cancer Society (No. BC-44).

REFERENCES

1 Arias, I. M., Doyle, D. and Schimke, R. T. (1969) J. Biol. Chem. 244, 3303–3311
2 Omura, T., Siekevitz, P. and Palade, G. E. (1967) J. Biol. Chem. 242, 2389–2396
3 Dehlinger, P. J. and Schimke, R. T. (1971) J. Biol. Chem. 246, 2574–2583
4 Simon, F. R., Blumenfeld, O. D. and Arias, I. M. (1970) Biochim. Biophys. Acta 219, 349–360
5 Bock, K. W., Siekevitz, P. and Palade, G. E. (1971) J. Biol. Chem. 246, 188–195
6 Warren, L. and Glick, M. C. (1968) J. Cell Biol. 37, 729–746
7 Pasternak, C. A. and Bergeron, J. J. (1970) Biochem. J. 119, 473–480
8 Warren, L., Glick, M. C. and Knass, M. (1966) J. Cell Physiol. 29, 269–285
9 O'Brian, P. J. (1966) Methods in Enzymology, Vol. VIII, pp. 147–149, Academic Press, New York
10 Strause, Jr, J. H., Burge, G. W. and Darnell, J. E. (1970) J. Mol. Biol. 47, 437–448
11 Block, R. J., Durrum, E. L. and Zweig, G. (1958) in Paper Electrophoresis and Paper Chromatography, 2nd ed., p. 178, Academic Press, New York
12 Gatt, R. and Berman, E. R. (1966) Anal. Biochem. 15, 167–171
13 Oyama, L. I. and Eagle, H. (1956) Proc. Soc. Exp. Biol. Med. 91, 305–307
14 Schroeder, W. A., Jones, R. T., Cormick, J. and McCalla, K. (1962) Anal. Chem. 34, 1570–1575
15 Kornfeld, S. and Ginsburg, V. (1966) Exp. Cell Res. 41, 592
16 Bosmann, H. B., Hagopian, A. and Eylar, E. H. (1969) Arch. Biochem. Biophys. 130, 573–583
17 Roseman, S. (1970) Chem. Phys. Lipids 5, 270–297
18 Atkinson, P. and Summers, D. (1971) J. Biol. Chem. 246, 5162–5175
19 Schimke, R. T. (1971) in Mammalian Protein Metabolism (Munro, G. H., ed.), pp. 178–223, Academic Press, New York
20 Bekesi, J. G. and Winzler, R. J. (1970) J. Biol. Chem. 244, 5163–5175
21 Levine, E. M., Becker, Y., Boone, C. N. and Eagle, H. (1965) Proc. Natl. Acad. Sci. U.S. 56, 350–355
22 Hakomori, S. (1970) Proc. Natl. Acad. Sci. U.S. 67, 1741–1747
23 Sakiyama, H. and Burge, B. (1972) Biochemistry 11, 1366–1376
24 Goldberg, A. (1972) Proc. Natl. Acad. Sci. U.S. 69, 2640–2644
25 Eagle, H., Piez, K. A., Fleischmann, R. and Oyama, V. I. (1959) J. Biol. Chem. 234, 592–597

26 Righetti, P., Little, E. P. and Wolfe, G. (1971) J. Biol. Chem. 246, 5724–5732
27 Harris, E. D. and Johnson, C. A. (1969) Biochemistry 8, 512–518
28 Shatkin, A. (1969) in Fundamental Techniques in Virology (Habel, K. and Salzman, N., eds), pp. 231–237, Academic Press, New York