MEMBRANES OF ANIMAL CELLS

II. The Metabolism and Turnover of the Surface Membrane

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ABSTRACT

Turnover studies of the surface membrane and of cell particulate matter of L cells in tissue culture in logarithmic and plateau phase of growth have been made. The rate of incorporation of isotope into these fractions and the rate of fall of specific activities of labeled L-cell fractions have been observed. The following interpretation of the data appears most likely although other interpretations are possible. Growing and nongrowing cells synthesize approximately similar amounts of surface membrane and particulate material. In the growing cell the material is incorporated with net increases in substance. There is relatively little turnover. In the nongrowing cell newly synthesized material is incorporated, but a corresponding amount of material is eliminated so that there is turnover without net increase of substance. Our results suggest that there is no gross differential turnover between the protein, lipid, and carbohydrate of the surface membrane under the conditions of our experiments. Metabolic inhibitors or omission of amino acids in the culture medium lead to a decrease in synthesis of surface membrane and cell particulates and cause an equivalent decrease in the rate of degradation of surface membrane and of particulates; therefore the synthetic and degradative aspects of turnover appear to be coupled. As cultures of nongrowing cells in suspension or on a glass surface age, their synthetic and turnover capacities diminish. Our results suggest that the cell may exist in a nongrowing state with a level of synthesis similar to that of a growing cell. It can exist in this state with a high level of turnover.

INTRODUCTION

The literature on the turnover of components of cells and tissues constitutes a rather bewildering array of data. The earlier history runs from Folin's (10) conception of a stable endogenous body compartment to Schoenheimer and Rittenberg's dynamic state of body constituents (45) and then back to a view of the living cell as being metabolically stable (18).

One of the difficulties is that many of the data are not comparable because different systems and experimental approaches have been used. Metabolic turnover has been studied in bacteria (2, 18, 21, 23, 33, 41, 44), yeast (14, 15), and molds (29). Turnover has been studied in the whole animal (49, 50), in tumors (11, 12, 35, 36), and in various tissues and organelles (17, 29). Turnover of proteins of cells in tissue culture has also been investigated (6, 7, 19, 20, 22, 34).

There have been problems in interpreting turnover data from tissues because cell growth and cell death are difficult to estimate. Tissues may be made of more than one kind of cell, and there may

be cell secretions which complicate matters. Further, pool sizes and the reutilization or loss of degradation products especially in vivo can be very difficult to assess. However, some generalizations have emerged from the data. First, there may be little metabolic turnover in growing cells or in some of their components whereas there are significantly greater amounts of turnover when the cells do not grow (14–16, 30–32, 51). This statement does not stand without apparent contradiction, for Eagle and his coworkers (6, 7) have found a relatively constant and equal turnover in growing and nongrowing (amino acid–depleted) tissue culture culture cells. Second, different components of the cell turn over at different rates (3, 4, 38, 47).

Virtually nothing is known of the metabolic turnover of the surface membrane. With the development of new methods to isolate this structure (52, 53) we are now able to investigate the problem.

In this paper we present data on the metabolic turnover of the surface membrane and of cell particulate matter of the L cell, a mouse fibroblast growing in culture in logarithmic phase of growth and in the nongrowing state. Preliminary reports of this work have been presented (54, 55).

MATERIALS AND METHODS

Chemicals

Most chemicals employed were of reagent grade. Vinblastine sulfate (Velban) was purchased from Eli Lilly & Co. (Indianapolis, Ind.; puromycin-2-HCl, from Lederle Laboratories (Pearl River, N.Y.); trypsin 1:250, from Difco Laboratories (Detroit, Mich.); D-glucose-^{14}C (uniformly labeled) and D-glucosamine-HCl-*1*-^{14}C from New England Nuclear Corp. (Boston Mass.); L-valine-^{14}C and L-leucine-^{14}C (uniformly labeled) from Schwarz Bio Research Inc. (Orangeburg, N. Y.). Isotope preparations were lyophilized in sterile tubes and were transferred to culture bottles by washing with medium.

Fluorescein mercuric acetate (FMA) was purchased from Nutritional Biochemicals Corporation (Cleveland, O.). A saturated solution of FMA was made at a concentration of approximately 2.2×10^{-3} M with 0.02 M tris Cl buffer, pH 8.1. The FMA solution, after being stirred for 1 hr, was adjusted to pH 8.1 with 2 M tris base. Some FMA did not go into solution and was filtered off. This solution was used within 3 wk.

Sucrose solutions were made according to the table in the *Handbook of Chemistry and Physics*, 1943, 27th edition, page 1556.

Cell Culture

Mouse fibroblasts (L cells) were derived from stocks of Dr. John Littlefield of the Massachusetts General Hospital (Boston, Mass.) and were grown in suspension unless otherwise indicated. Eagle's medium was generally employed and contained 10% fetal calf serum. Twice the usual amount of essential amino acid mixture and vitamin mixture was employed. 1 ml of medium contained 14.3 μmoles of glucose, 2.0 μmoles of sodium pyruvate, 2.6 μmoles of glutamine, 0.24 μmole of NaH_2PO_4, 100 μg of streptomycin, and 100 U of penicillin G. The pH of the medium was not controlled although it never fell below 6.8 as indicated by phenol red indicator.

Early cultures of our cells were found by Dr. H. Morton (Department of Microbiology, University of Pennsylvania) to be negative for PPLO infection. Later cultures, upon periodical examination, were also negative in this respect as found by Dr. L. Hayflick (The Wistar Institute of Anatomy and Biology, Philadelphia, Pa.).

Cells were counted, and their size and size distribution were determined with a Coulter counter, model B (Coulter Electronics Hialeah, Fla.).

In the trypan blue test, 2 volumes of a filtered solution of 0.5% trypan blue were added to 1 volume of cell suspension, and the cells were counted after 5 min at room temperature.

Isolation and Analysis of Membranes

Membranes were isolated by a scaled down version[1] of the FMA method previously described (52, 53). All operations were carried out at 2°–4°C unless otherwise noted. The contents of a vessel containing 2×10^7–10^8 cells in tissue culture medium were centrifuged in a glass-stoppered, 50 ml, conical centrifuge tube at 400 *g* for 5 min. The supernatant was removed, and the cells were washed twice with 50 ml of 0.16 M NaCl warmed to 37°C. Saline was added to the pellet to a volume of 1.0 ml. The suspension was transferred to a small Dounce homogenizing tube (7 ml tube with a B pestle, Kontes Glass Co., Vineland, N. J.), and 3.0 ml of FMA solution were added while the suspension was being stirred. After 5 min at room temperature the tube was placed in ice until the suspension had reached 3°C. The cells were then broken with a tight pestle; about 25 strokes were required. The homogenizing process was monitored, as were successive steps in the isolation, by phase-contrast microscopy. 4 ml of 60% sucrose solution were added to the homogenate. After being stirred, the homogenate-sucrose mixture was placed on 10 ml of 45% sucrose solution in a 40 ml centrifuge tube with a blunt tip. The tube was centrifuged at 150 *g* (800 rpm) in a

[1] We wish to thank Mrs. Elsa Weinstein for adapting the FMA procedure to a small scale.

refrigerated International centrifuge (PR 2) for 1 hr. The upper phase was removed to within 1 mm of the meniscus by means of a syringe fitted with a No. 15 needle whose terminal 6 mm were bent at a right angle to the shaft, the bevel faced up. The fluid in the meniscus region was harvested separately. This interface fraction contained cells and nuclei as well as membranes. About 0.1 volume of water was added to this fraction, and the fraction was recentrifuged on a solution of 45% sucrose for 1 hr at 125 $\times$ *g* (750 rpm). The top layer was removed and combined with the top layer of the first centrifugation. The bottom layer containing cells, nuclei, and coarse debris was pooled with the bottom layer of the first centrifugation. The membrane fraction was virtually devoid of cells but contained fine debris. This fraction was then diluted with 0.1 volume of water and placed on 10 ml of 35% sucrose solution in a 40 ml glass centrifuge tube and was centrifuged at 1800 *g* (2900 rpm) for 1 hr. Fine debris stayed in the upper phase while the membrane formed a pellet that could be resuspended in a small volume of diluent. The supernatant fluid was removed and combined with the bottom phases of the first two centrifugations. Further treatment of this particulate fraction will be described below. A few drops of 35% sucrose solution were added to the pellet of surface membranes. After being stirred, the suspension was transferred to a tube containing sucrose solution in a linear gradient running from 45 to 65%. The tube was centrifuged in the SW-39 rotor of the Spinco Model L-2 ultracentrifuge for 1 hr at 33,000 *g*. An orange band containing membranes formed in the middle of the tube. The band of purified membrane was harvested with a 2 ml syringe fitted with a No. 18 needle with a bent tip. The suspension of membranes in sucrose solution was diluted with distilled water to 12 ml, stirred, and then centrifuged at 4000 *g* for 30 min. The supernatant solution was then removed, and 2 ml of water were added to the pellet. The final preparation contained virutally no nuclei, cells, or mitochondria. A minimum of debris was present. The yield of whole ghosts was 20–55%. Membranes were counted with the hemocytometer under the phase-contrast microscope. Proteins dissolved in 0.5 N NaOH were determined at four concentrations by using the method of Lowry et al. (28). Bovine serum albumin served as the standard. Fractions were plated in triplicate, and the radioactivity was counted in a Nuclear-Chicago Corporation (Des Plaines, Ill.) windowless counter (Model 8703). Glucose was determined by using the method of Park and Johnson (39).

For extraction of lipids, 1 ml of the membrane suspension was transferred to a 12 ml conical centrifuge tube and centrifuged at 4000 *g* at 4°C for 25 min. The supernatant fluid was carefully removed, and an aliquot was plated for radioactive counting. It contained a negligible number of counts (< 2% of counts in membranes). The pellet of surface membrane was extracted three times for 1 hr at room tempeature with 1.5 ml of chloroform and methanol (1:1). The organic phases were combined, and 1.2 ml of water were added. After being shaken vigorously, the tube was centrifuged for 10 min at 1800 *g*. The bottom phase was plated in triplicate for radioactive counting.

Particulate Fraction

The lower phase of the first two centrifugations and the supernatant fraction of the third centrifugation were combined. These phases contained virtually all of the nuclei, mitochondria, and endoplasmic reticulum of the cells and consisted largely of the internal membrane systems of the cells. FMA combined with these structures rendered them dense so that they formed a pellet upon centrifugation. The mixture was diluted with water and centrifuged at 8000 *g* for 30 min in a Sorvall RC-2 centrifuge. The pellet was then washed three times, each time with 30 ml of water. The radioactivity found in the wash after three centrifugations was negligible. The pellet was diluted to a volume of 3 ml with 0.1 N NaOH. The protein content was determined at four concentrations, and ^{14}C activity was determined in duplicate. The membrane fraction and the particulate fraction isolated contained about 15–20% of the total protein of the cell.

RESULTS AND DISCUSSION

Dilution or Loss of Isotope from Surface Membranes and Cell Particulates

GROWING CELLS: Fig. 1 illustrates the results of three experiments of similar design. Briefly, cells were grown in the presence of a labeled substrate, then washed, transferred to nonradioactive medium, and allowed to grow. Cells just out of the isotopic medium and while growing for 3 days thereafter in nonradioactive medium were processed. The results indicate that the reduction of specific activity of the surface membranes of rapidly growing cells closely follows what would be predicted on the basis of simple dilution due to growth in nonradioactive medium, i.e. if the cell number doubles, the specific activity should fall by 50%. The results, averaged over 20 generations of growth by using the isotopes listed in Fig. 1, showed that the specific activity (counts per minute per microgram protein) of the surface membrane is 7.1 $\pm$ 2.0% less per generation than what would be expected from simple growth dilution. The specific activity of the membrane, determined as counts per minute per cell ghost, was 7.5 $\pm$ 2.2% per generation less than calculated, while the cell particulate

specific activity (count per minute per microgram protein) was 9.1 $\pm$ 3.7% less per generation than calculated. The results were essentially the same with glucose-^{14}C, amino acids, or glucosamine as precursor.

Since we know how much ^{14}C there is in each cell membrane and the number of cells at the time of each sampling, we can calculate the absolute amount of ^{14}C in the membrane initially (day 0) and each day thereafter. This amount drops per generation by an amount corresponding to the deviation from calculations mentioned above. Thus, it would seem that most of the ^{14}C incorporated into the membrane was retained by the membrane during the course of the experiment. Deviation from the predicted specific activities and slow loss of ^{14}C can be explained by a relatively small turnover of the substance of membrane ($T\frac{1}{2}$ approximately 8–9 days) and particulate fraction ($T\frac{1}{2}$ approximately 7 days). However, we have not ruled out the possibility that, between the membrane and an internal pool, there is a more rapid turnover which is masked by efficient reutilization of isotope. This possibility will be discussed later.

From the values it can be seen that the fraction of the total ^{14}C of the membrane extracted into lipid solvents was relatively constant within each experiment, regardless of the source of ^{14}C. This indicates that there was no detectable gross turnover of the lipid portion of the membrane relative to the rest of the membrane. This result would appear to conflict with the results of other experiments on the exchange of membrane lipids with lipids of the nutrient medium (5, 13, 27, 37). However, the differences may be explained by the following possibilities: a small but rapidly exchanging fraction might have been missed in our experiments; exchange work has been done mainly with the red blood cell which may behave differently from a dividing tissue culture cell; exchange of lipids may be going on at adsorption sites on the membrane which are not integral parts of the membrane and are lost during isolation of the surface membrane.

The particulate fraction (nucleus, mitochondria, endoplasmic reticulum, and other particulates) that sedimented at 8000 *g* for 30 min after treatment with FMA behaved like the surface membrane. The ratio of counts per minute per microgram of membrane protein over that of particulate protein was frequently about 1.2 but could vary slightly. With amino acids-^{14}C the ratio varied from 1.1 to 1.3 and with D-glucosamine-^{14}C the ratio was 6.2–6.9 which indicates that the hexosamine went more selectively into *N*-acetyl hexosamines and sialic acids of the cell surface. The constancy of the ratios suggests that the turnover and behavior of other particulates of the cell, which are largely membranes of nucleus, mitochondria, and endoplasmic reticulum, is essentially the same as that of the surface membrane.

NONGROWING CELLS: The design of the experiment illustrated in Fig. 2 is similar to that of Fig. 1 except that the concentration of ^{14}C-labeled cells was raised to a level where no growth occurred during the experiment, and fresh medium was not added as aliquots were removed. It can be seen that in the first day (equivalent to one generation time) the ^{14}C specific activity of the surface membrane fell by about 50% (curve *A*). In other experiments the decrease on the first day ranged from 38 to 50%. The specific activity of cell particulates (curve *B*) and of whole, ethanol-extracted cells (curve *C*) also decreased sharply. This indicates that under the conditions of our experiments the nongrowing L cell turns over rapidly since cell number and cell size did not change significantly whereas the ^{14}C specific activity decreased. This result cannot be explained on the basis of cell death and replacement for, when nongrowing cells were cultured for 1 hr in the presence of tritiated thymidine, the specific activity (counts per minute per milligram protein) of these cells was only 8% that of L cells in log phase of growth.

The fraction of radioactivity in the membrane extracted by lipid solvent was quite constant throughout the experimental period (Fig. 2).

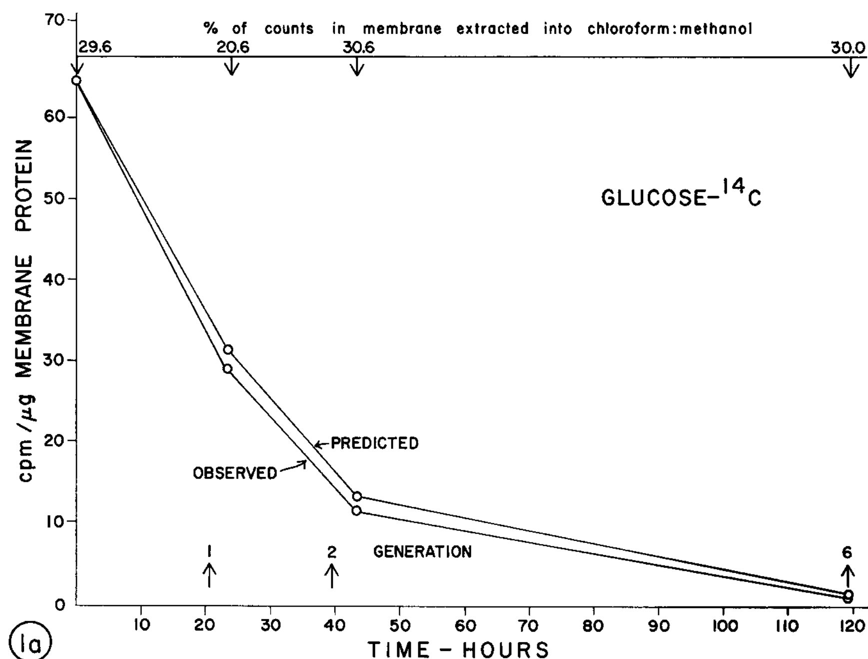
Experiments, similar to those described in Fig. 2, with L cells adherent to glass were also carried out. In an experiment in which a nongrowing confluent culture of L cells was labeled with glucose-^{14}C then washed, and incubated with nonradioactive medium, the specific activity of the membrane fell in 1 day 43.2%, calculated as counts per minute per microgram protein, or 50.1%, calculated as counts per minute per ghost. Thus, the surface membranes of cells on glass appear to behave like the surface membranes of cells in suspension culture.

Table I shows the results of an experiment in which L cells labeled with L-valine-^{14}C were cultured in nonradioactive medium. In 24 hr

the ^{14}C specific activity of surface membrane and cell particulates of nongrowing cells decreased 46 and 44%, respectively. In another vessel growing cells had increased 116% in number and the ^{14}C specific activity of the surface membrane and cell particulates had fallen by 58 and 59%, respectively. The calculated declines in specific activity, obtained by dividing the zero time value of specific activity of the growing culture by 2.16, were 22.4 for surface membrane and 23.8 for cell particulates. As seen in Table I the observed specific activities after 24 hr of growth were 20.6 cpm/μg of membrane protein (>91% of the value calculated on the basis of increase in cell number) and 21.0 cpm/μg particulate protein which is >88% of the calculated value. These results agree with those seen in Figs. 1 and 2 which show that the decrease in specific activity of isotope in growing cells closely follows the values predicted on the basis of increase in cell number. The results in Table I also agree with those of Fig. 2 which show that, when cells are not dividing and changing in size, there is a large decrease of specific activity which can be explained on the basis of turnover. A similar result has been obtained with cells labeled with glucosamine-^{14}C. The specific activity of sialic acid was found to drop 45% in 18 hr when the cells were in a nongrowing state in isotope-free medium.

Incorporation of Isotope into Surface Membranes and Cell Particulates

Fig. 3 illustrates the results of experiments in which glucose-^{14}C is incorporated into the



Figs. 1 *a–c* show the decay in ^{14}C specific activity of surface membrane of L cells labeled with (*a*) glucose-^{14}C, (*b*) L-valine-^{14}C and L-leucine-^{14}C, (*c*) D-glucosamine-^{14}C and subsequently grown in nonradioactive medium.

FIGURE 1 *a* L cells were grown in glucose-^{14}C (specific activity = 56,400 cpm/μmole). Cells were centrifuged and washed twice with saline (0.16 M Na Cl); a portion was taken for processing to obtain surface membranes and cell particulates; the remainder was placed in 100 ml of fresh nonradioactive medium and allowed to grow with a T_D of approximately 18–20 hr. At times indicated on the graph, one-half the culture was removed and processed. Equal volume of fresh nonradioactive medium was added back, and cells continued to grow. Cells appeared healthy, as observed with phase-contrast microscopy, and remained constant in size. Predicted curve was calculated on the basis of increase in cell number: if cells doubled in number in nonradioactive medium, predicted specific acitivity would be 50% of initial specific activity.

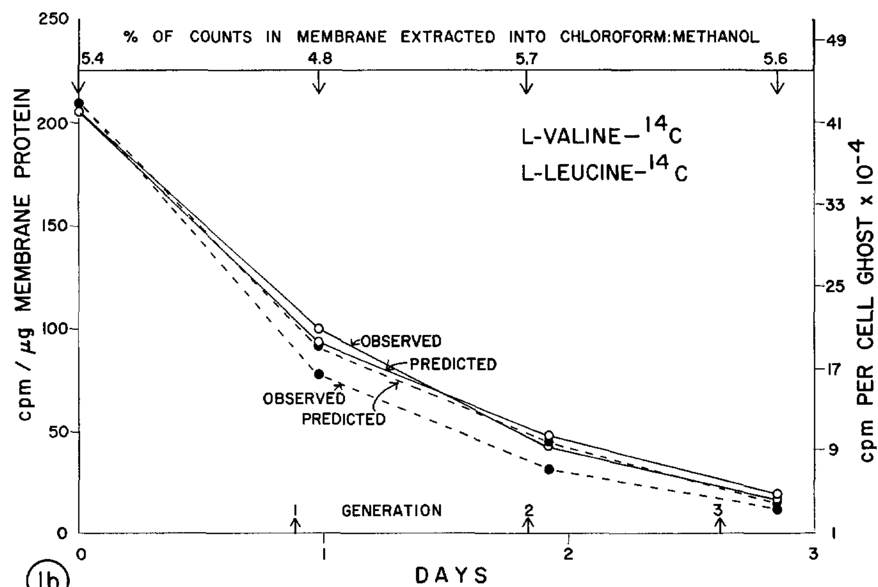


FIGURE 1 *b* This experiment was designed like that described in *a*. Cells were labeled with L-valine-^{14}C and L-leucine-^{14}C (specific activity of each amino acid was 546,000 cpm/μmole). L cells grew in nonradioactive medium as shown by doubling time on abscissa. Cells appeared healthy, as observed in the phase-contrast microscope, and remained constant in size. Open circles, surface membrane, cpm/μg protein, predicted and observed; closed circles, surface membrane, cpm per cell ghost $\times$ 10^{-4}, predicted and observed.

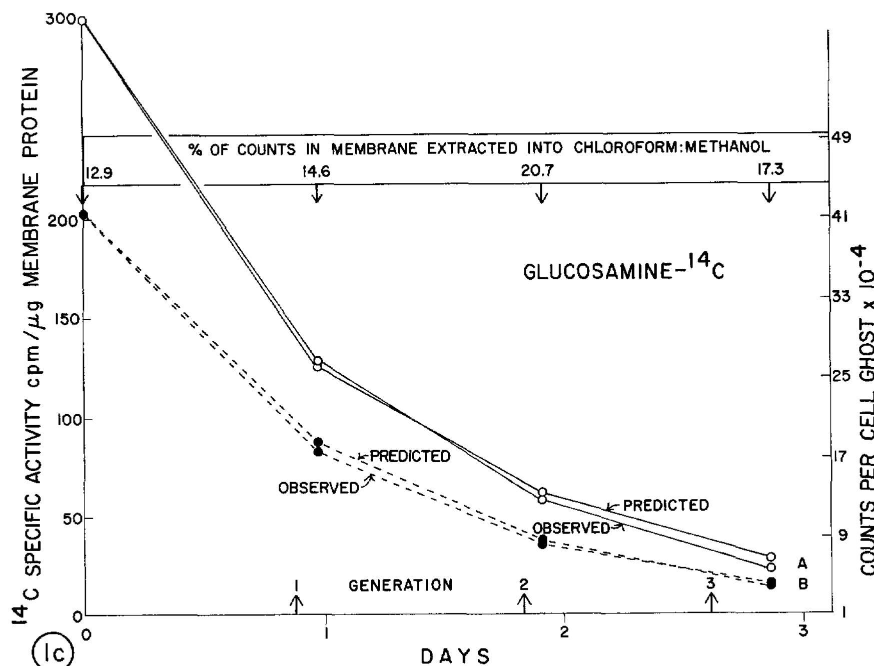


FIGURE 1 *c* The design of this experiment is the same as that described in *a*. Cells were labeled with D-glucosamine-HCl-^{14}C (1.2×10^6 cpm/ml). The L cells grew rapidly in nonradioactive medium as shown by the doubling time on the abscissa. The cells appeared healthy, as observed in the phase-contrast microscope, and remained constant in size. Open circles, surface membrane, cpm/μg protein, predicted and observed; closed circles, surface membrane, cpm per cell host $\times$ 10^{-4}, predicted and observed.

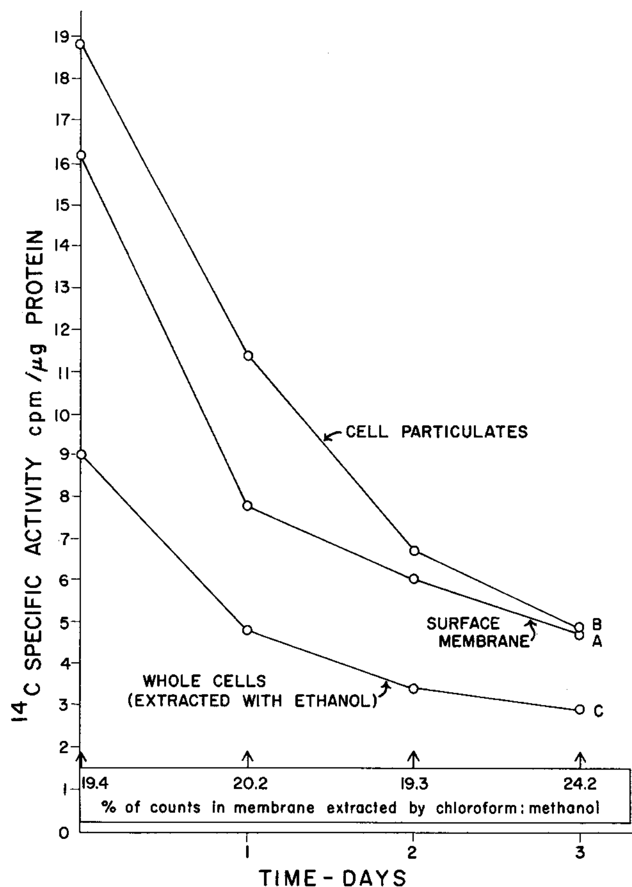


FIGURE 2 Decay of specific activity of nongrowing, ^{14}C-labled L cells. L cells in logarithmic phase were grown in presence of glucose-^{14}C (specific activity = 15,800 cpm/μmole glucose) for 3 days. Cells were harvested, washed twice with saline, and resuspended in 300 ml nonradioactive medium. Cell count was 2.05×10^6/ml. Sample was removed before resuspension and each day thereafter for 3 days. Cell number increased to only 2.34×10^6/ml on third day. There was no significant change in cell size during the experiment. At times indicated, one sample was removed to obtain surface membranes (curve *A*) and cell particulates (curve *B*) as described in Methods. Second sample was centrifuged; medium was removed; pellet was washed and extracted with 4 ml of 30% ethanol. Ethanol extraction was repeated twice. Cells, after treatment, appeared remarkably intact. Protein and ^{14}C contents of cell pellets were determined (curve *C*). Glucose content of medium on day 3 was 45 % that of fresh medium. Values for per cent of counts extracted from the membrane by chloroform:methanol were obtained as described in text.

surface membrane and cell particulate of nongrowing (Fig. 3 *a*) and growing (Fig. 3 *b*) cells. In this experiment growing and nongrowing cells were incubated in fresh medium containing glucose-^{14}C. Aliquots were removed every few hours and the surface membrane, and cell particulates were isolated. It can be seen that the rate of incorporation, as measured by the rise in ^{14}C specific activity of surface membrane and cell particulates, does not depend on cell growth. It should be pointed out that, since the specific activity of the substrate in the culture of nongrowing cells was 32% less than that of the growing cells, the sets of curves of Fig. 3 are directly comparable. Comparison of rates of incorporation of ^{14}C into growing and nongrowing cells over 1 day can also be made of the controls of Fig. 6 and Tables II and III. In general, the rate of rise of ^{14}C specific activity of surface membranes of nongrowing cells was close to that of growing cells.

In Fig. 3, the curve of incorporation of isotope into the membranes of growing cells is virtually straight and the comparable curve for the nongrowing cells is bowed. This difference may possibly be explained by the presence of a pool of precursors in nongrowing cells through which isotopic material would have to pass. Thus, a lag in incorporation of isotope would be seen in nongrowing cells.

The percentage of membrane counts extractable by lipid solvents shown by the numbers under the curve of specific activity of the membranes (Fig. 3) appears to be fairly constant throughout the period of the experiment.

The rate of incorporation of isotope into growing and nongrowing cells does not depend on the cell concentration. In experiments similar to those of Fig. 3, comparable rates of incorporation were obtained in a culture with 4.5×10^5 cells per milliliter that did not grow and in another culture with 2.2×10^6 cells which grew rapidly for 6 hr. The reasons for the unexpected growth or nongrowth in these experiments are unknown.

TABLE I

Decay in ^{14}C Specific Activity of Surface Membranes and Particulates of L Cells Labeled with L-valine-^{14}C and Subsequently Cultured in Nonradioactive Medium

Vessel	Time	Increase of cells	Specific activity	
			Surface membrane	Cell particulates
	hr	%	*cpm/µg protein*	
1	0	—	48.5	51.5
2	24	2	26.4	28.9
3	24	116	20.6	21.0

L cells were grown in log phase for 48 hr in the presence of L-valine-^{14}C (67,000 cpm/ml). The cells were centrifuged and washed twice with physiological saline. One-third of the cells was processed for surface membranes and cell particulates (time, 0 hr.). The remainder of the cells was suspended in two vessels in nonradioactive medium at concentrations of 2.6×10^6 and 2.1×10^5 cells per milliliter. After 24 hr the cells were harvested and processed. Cells at a concentration of 2.6×10^6/ml increased 2% in number while those at a concentration of 2.1×10^5/ml increased 116% in number. Cell size did not change significantly.

Continuous Incorporation of Isotope into L cells

In Fig. 4 *a* can be seen the course of incorporation of isotope into membranes and particulates of cells continuously growing in the presence of glucose-^{14}C. Incorporation reaches a maximum after about 4–5 days or generations. Without turnover the incorporation should be half-maximal after one generation, but in this experiment the specific activity of membrane was a little less than half-maximal On the other hand, the specific activity of the particulate fraction was approximately one-half the ultimate maximum after the first doubling in isotopic medium.

Fig. 4 *b* shows the rate of incorporation of isotope into surface membranes and particulates of nongrowing cells. The ultimate level reached after about 5 days is higher than that in the growing cells. The reason for this finding is not known. It can be explained in part by a higher specific activity of glucose-^{14}C in the nongrowing culture (see legend of Fig. 4 *b*). Lipid extraction of the membranes within each experiment yielded a fairly constant fraction of the total membrane counts; this indicates no apparent gross differential turnover of lipid relative to the rest of the membrane over 7 days. In growing cells, 38.1 ± 2.9% and in nongrowing cells, 26.1 ± 5.4%, of the membrane radioactivity were extracted with lipid solvents. At the present time no explanation for the differences in the precentage of counts in the lipid extracts can be given.

In another experiment, L cells confluent on a glass surface were cultured in the presence of glucose-^{14}C. The ^{14}C specific activity in the surface membrane and particulates of these nongrowing cells reached a maximum after approximately 4 days.

Effects of Puromycin and Actinomycin D on Labeling of Cells with Glucose-^{14}C

Puromycin and actinomycin D inhibit the incorporation of glucose-^{14}C and L-valine-^{14}C into the surface membrane and particulates of nongrowing L cells (Fig. 5, Table II). In the experiment described in Fig. 5, nongrowing cells were used as a control rather than growing cells since the inhibitors stop growth rapidly. Inhibition of isotope incorporation by actinomycin D can be seen after 4 hr in the cell particulates whereas incorporation into surface membrane was not affected for at least 6 hr. Inhibition of incorporation of isotope into membrane seems to be less severe than inhibition of incorporation into cell particulates. Puromycin inhibition was evident by the time the first aliquots were taken.

Effects of Vinblastine and Thymidine on Incorporation of Glucose-^{14}C and L-Valine-^{14}C

When L cells are cultured in the presence of vinblastine sulfate and thymidine, cell division ceases but there is incorporation of glucose-^{14}C (Fig. 6) and L-valine-^{14}C (Table III) into the surface membrane. Incorporation of glucose-^{14}C is less, relative to the controls, than is the incorporation of L-valine. The discrepancy might be explained by a partial inhibition of conversion of glucose-^{14}C to membrane precursors in cells treated with vinblastine sulfate and thymidine.

There is a considerable increase in the size of cells grown in the presence of vinblastine sulfate and thymidine (see legend of Fig. 6). In these experiments isotope was added after the cells had enlarged, and 60–70% of the cells showed mitotic figures.[2] Incorporation of isotope as measured

[2] We wish to thank Dr. M. K. Nass for the estimate of mitotic figures.

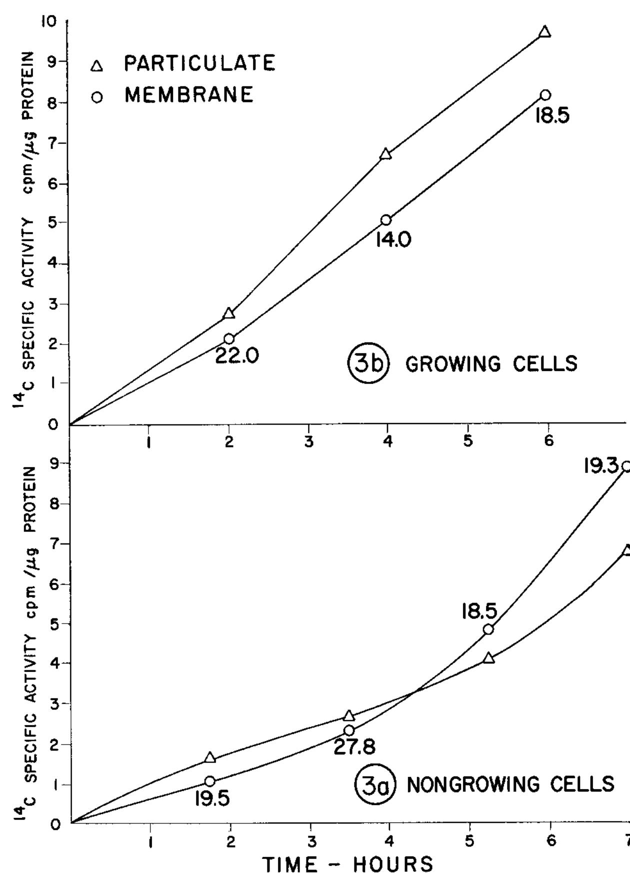


FIGURE 3 Incorporation of glucose-^{14}C into surface membranes and cell particulate fractions of growing and nongrowing L cells. *a*, nongrowing cells. L cells in plateau phase were resuspended in 100 ml of fresh medium which contained glucose-^{14}C (21,700 cpm/μmole). Cell number was adjusted to 2.2×10^6/ml. Spinner flask was gassed with 5% CO_2 in air and incubated at 37°C. Samples of 25 ml were removed every 1.75 hr, at which time a cell count and size estimation were made. No significant change in either was observed during the experiment. Cell samples (25 ml) were washed twice with saline and processed as described in Methods. *b*, growing cells: L cells which were growing logarithmically were centrifuged and resuspended in 300 ml of fresh medium containing glucose-^{14}C (28,700 cpm/μmole) in a spinner flask. The cell number was 5.13×10^5 ml at the beginning of the experiment and increased 20% without change in cell size during a 6 hr period at 37°C. Samples (100 ml) were removed every 2 hrs, washed twice with saline, and processed as described in Methods.

The numbers beside the points on the membrane curve refer to the percentage of the total counts of the membrane that were extractable into chloroform : methanol. Circles, surface membrane; triangles, cell particulates. It should be noted that the specific activity of the glucose in the medium used in *b* (28,700 cpm/μmole glucose) was 32% higher than in *a* (21,700 cpm/μmole glucose). Correction should be made in comparing the curves.

by specific activity (counts per minute per microgram protein) is somewhat lower than it is in controls when glucose-^{14}C is used, and is approximately the same as it is in controls when valine-^{14}C is the precursor. When isotope levels are calculated as counts per minute per cell ghost, it is found that incorporation per cell membrane is considerably higher than in the controls because the enlarged cell has a much larger surface membrane and apparently a greater biosynthetic capability.

It has been shown that cells in mitosis (40) or vinblastine-treated cells (42) are capable of only a small fraction of the protein and nucleic acid

TABLE II

The Effect of Actinomycin D and Puromycin on Incorporation of L-valine-^{14}C into Surface Membranes of L Cells

Vessel	Specific activity	
	Surface membrane	Cell particulates
	cpm/μg protein	
Control-growing cells	26.8	27.1
Control-nongrowing cells	22.7	21.6
Cells + actinomycin D	11.7	17.5
Cells + puromycin	5.2	4.9

The design of this experiment is the same as that of the experiment in Fig. 5, except that actinomycin D and puromycin were added with the isotope rather than 2.5 hr before. L-valine-^{14}C (67,000 cpm/ml of medium) was used. In 26 hr of culture in radioactive medium the cells in the first vessel had doubled in number whereas the cells in the last three vessels showed no significant increase in cell number. Cells were processed after 26 hr of culture in radioactive medium.

TABLE III

The Effect of Vinblastine Sulfate and Thymidine on Incorporation of L-valine-^{14}C into Surface Membranes of L Cells.

Vessel	Specific activity of surface membrane
	cpm/μg protein
Control, growing cells	25.6
Control, nongrowing cells	20.4
Cells + vinblastine sulfate	20.8
Cells + thymidine	24.4

The design of this experiment is the same as that of the experiment in Fig. 6. L-valine-^{14}C (67,000 cpm/ml of medium) was used. In 24 hr of culture in radioactive medium the cells of the first vessel had doubled in number whereas those in the second vessel had increased in number only 6%. There was no increase in cell number in the third and fourth vessels; the size of cells in them had increased markedly as described in the legend of Fig. 6. The cells were processed after 24 hr incubation in radioactive medium.

synthesis of the normal cell. It may very well be that the residual synthesis is that of surface membrane and, to some extent, of cell particulates. It is reasonable to expect that membrane synthesis goes on during mitosis and cytokinesis since this is a period when surface membrane is needed.

Capability of Nongrowing Cells to Incorporate L-Valine-^{14}C as the Cultures Age

In the experiment illustrated by Fig. 7, nongrowing cells were incubated in suspension for 15 days. At the times indicated, an aliquot was removed and centrifuged. Fresh medium containing L-valine-^{14}C was added to the cells and, after incubation for 6 hr, surface membrane and a cell particulate fraction were isolated.

As seen in Fig. 7, when cells remain in suspension in plateau phase their capability for incorporation of L-valine-^{14}C into membrane and cell particulates during a 6 hr period drops off. The cells on day 9 appeared to be healthy (see legend of Fig. 7) but it is difficult to evaluate the sample taken at 15 days when the cell number had declined precipitously. The FMA method for preparing membranes appears to select for living cells. Our impression is that unhealthy or damaged cells become small, dense masses when the FMA reagent is added. Thus, on day 15 we may be measuring incorporation into the surviving, intact cells of the culture.

These results can be interpreted as a nonspecific decline in vigor and synthetic capability of aging cells in a far from perfect environment. On the other hand, the possibility exists that the decline of synthetic capability may be a reflection of a physiological shutdown with decreased turnover that may occur when cellular interactions are increased as with contact-inhibited cells growing on a surface (26). Eidam and Merchant (8, 9) have shown that supplements of glucose, equivalent to the levels of glucose used in our experiments, prolong the period in which L cells can exist in an apparently healthy state in plateau phase at high cell concentrations.

The same type of experiment was carried out with confluent cells on a glass surface (Fig. 8). Here it can be seen that the synthetic capability of nongrowing cells on glass also falls as the cultures age. The decline in isotope incorporation (5 hr pulse) can be seen in the whole, ethanol-extracted cell, the cell surface membrane, and the particulates. The rate of incorporation into membrane appears to fall linearly and, if this decrease were to continue, would be zero in 16 days. The explanations for the decline are the same as those for the decline seen in the experiments with suspension

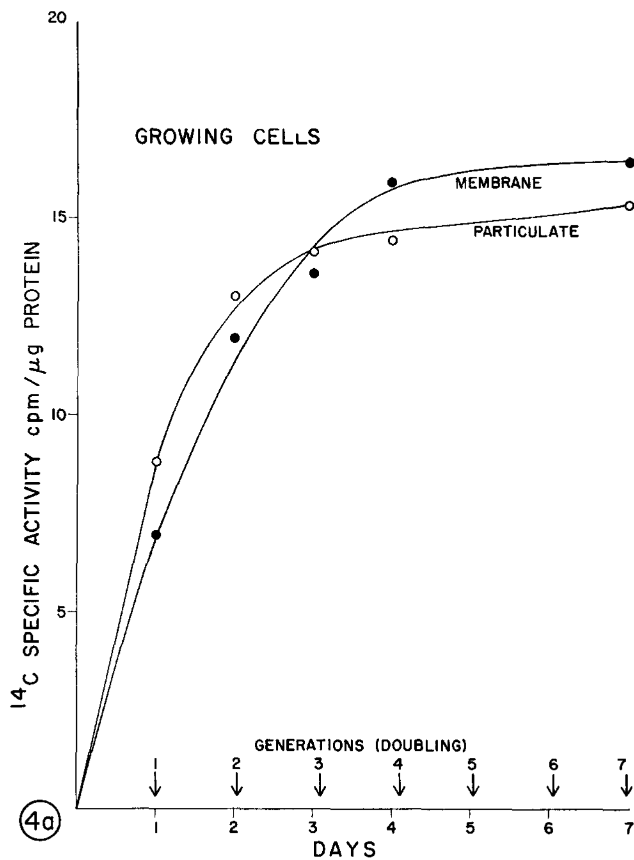


FIGURE 4 *a* Incorporation of isotope into L cells growing in the presence of glucose-^{14}C. L cells were added to 250 ml of medium containing glucose-^{14}C (18,400 cpm/μmole). These grew throughout the course of the experiment with a T_D of approximately 25 hr. At the times indicated on the graph, approximately half the culture was removed and the cells were processed as described in Methods. An equal volume of radioactive medium was added back to replace the medium removed and the culture was allowed to continue in this manner for 7 days. Cell volume remained constant. Samples were tested for dead cells by the trypan blue assay; the percentage of dead cells was never greater than 0.5. Surface membranes were extracted with chloroform:methanol. The percentage of the total counts of the membranes extractable was quite constant over the period of the experiment (38.1 ± 2.9%).

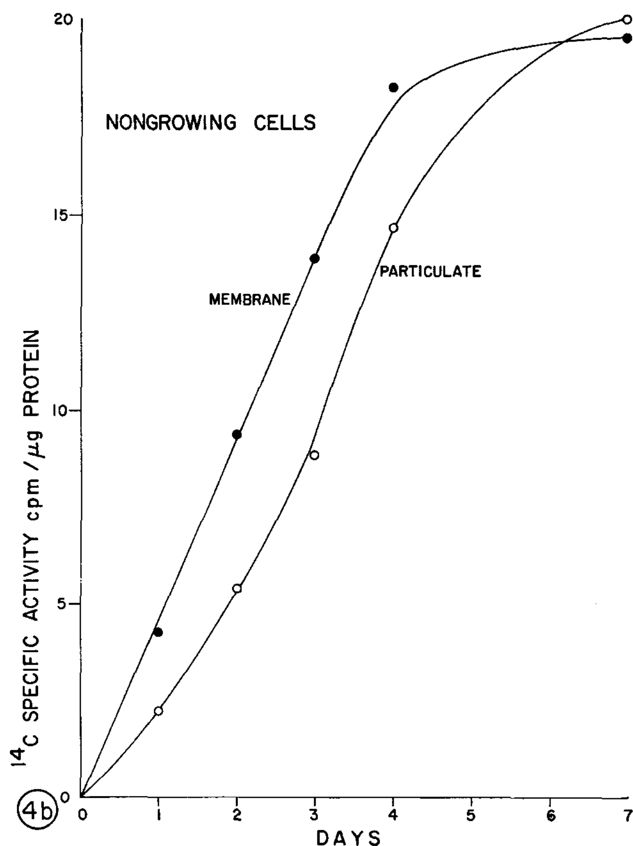


FIGURE 4 *b* Incorporation of isotope into L cells nongrowing in the presence of glucose-^{14}C. L cells were added to 250 ml of fresh medium containing glucose-^{14}C (specific activity, 20,200 cpm/μmole). The cell count initially was 870,000 cells per milliliter, and on the last day of the experiment it was 1.14×10^6 cells per milliliter which represents an increase of 31% in 7 days, essentially stationary culture. Cell size did not vary significantly. At the times indicated on the graph, samples of 40 ml were removed and the cells were processed as described in Methods. Fresh medium was not added back to the cultures. The glucose content of the medium on day 7 was 29.3% of the initial glucose content of the medium. The fraction of cells that took up trypan blue on days 3 and 4 was 1%. Surface membranes were extracted with chloroform:methanol. The percentage of the total counts of the membranes extractable was 26.1 ± 5.4%; the variability did not follow a regular pattern.

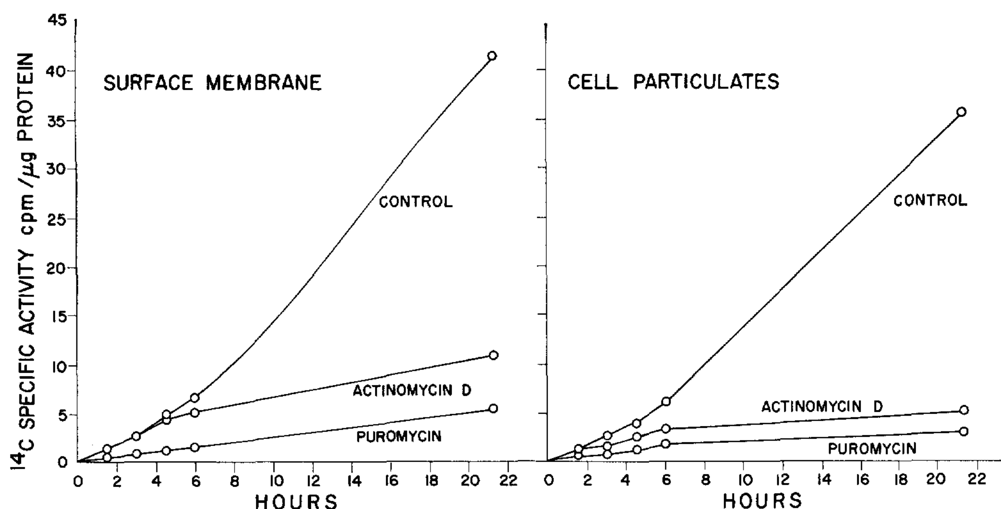


FIGURE 5 The effect of puromycin and actinomycin D on labeling of surface membrane and cell particulates of nongrowing L cells by glucose-^{14}C. Three spinner flasks, each containing approximately 2.8×10^8 cells in 250 ml of fresh medium (1.12×10^6 cells per milliliter), were set up. The first flask served as a control; to the second was added 35 μg puromycin per milliliter; to the third was added 5 μg actinomycin D per milliliter. The flasks were incubated for 2.5 hr at 37°. Glucose-^{14}C was then added to each flask (specific activity, 32,200 cpm/μmole) and the incubation was continued. Samples of 50 ml were removed every 1.5 hr for 6 hr, and a final sample was obtained 22.75 hr after the addition of isotope. Samples were washed twice with saline and processed as described in Methods. Cell number and size in each sample were determined. Cell growth either in number or in size did not occur during the experiment.

culture. However, the possibility is greater with adherent cells that a physiological explanation such as contact inhibition is more valid (26).

Effect of Inhibitors and Deficient Medium on Incorporation and Loss of Isotope from L Cells in Suspension Culture

Table IV *a* and *b* shows the results of experiments in which incorporation of glucose-^{14}C into surface membranes and cell particulates was inhibited to various extents by several inhibitors. These tables also shows that the inhibitors had a corresponding inhibitory effect on the loss of the isotope from the cell fraction. The direct correlation between the extent of inhibition on incorporation and release of isotope is fairly close in the case of surface membranes and less close in the case of the cell particulates.

It was previously shown by several workers that the degradative part of turnover (14, 30, 43), as shown by the release of isotope from liver slices, is inhibited by metabolic inhibitors and amino acid analogues (46, 48). Our experiment confirms the previous work and provides further evidence for a coupling of synthesis and degradation of cell components. In other words, there must be synthesis for degradation or turnover. It is unlikely that degradation depends on synthesis of degradative enzymes. The mechanism of the coupling is unknown.

The Differential Turnover of Surface Membrane Components

In several of the experiments discussed (Figs. 1–4) the membranes were extracted with chloroform-methanol by a method that has been shown to leave virtually no residual lipids.[3] Within each experiment the fraction of the total counts of the membrane extractable into lipid solvents was quite constant over several generations, regardless of the culture conditions or the isotope precursor used. This suggests that, grossly, there does not seem to be a differential turnover between the lipid component and the remainder of the membrane. If the rate of turnover of the lipid component were different from that of the rest of the membrane one would expect that the fraction of the total

[3] Experiments of Mr. D. B. Weinstein and Dr. J. B. Marsh.

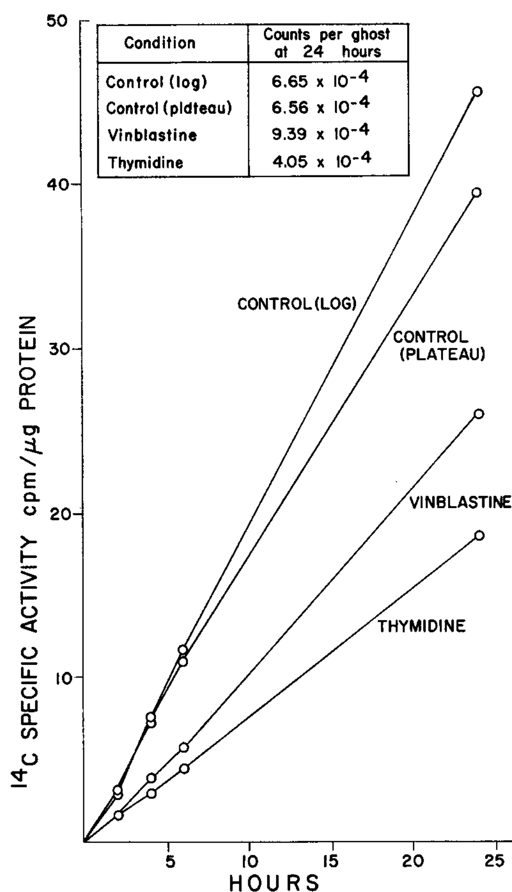


FIGURE 6 Effect of vinblastine sulfate and thymidine on incorporation of glucose-^{14}C into surface membrane of L cells. Two spinner flasks were set up, each with 500 ml of fresh medium containing 400,000 cells per milliliter. To one flask was added vinblastine sulfate, 0.1 μg/ml; to second flask was added thymidine to concentration of 10^{-2} M. Flasks were incubated at 37°C for 21 hr. During this time, cells treated with vinblastine sulfate had increased 18% in number and had increased in volume from 1880 to 3969 μ^3. Cells treated with thymidine had increased 17% in number and from 1880 to 2507 μ^3 in volume. At this point isotope was added to the above flasks and two other control vessels (third and fourth flasks). First flask, vinblastine sulfate (see above) (specific activity, 23,700 cpm/μmole glucose). Second flask, thymidine (see above) (specific activity, 21,500 cpm/μmole glucose). Third flask, log control. L cells from the same original culture that was used in the first and second flasks; 400 ml with 378,400 cells per milliliter (specific activity, 21,500 cpm/μmole glucose). The cells of this culture doubled in number in 19 hr without significant change in size. Fourth flask, plateau control. L cells of same origin as those used in the third flask were centrifuged and resuspended in 100 ml fresh medium at 2.25×10^6 cells per milliliter (specific activity, 16,650 cpm/μmole glucose). Cells of this culture did not change significantly in number or size during the experiment. The flasks were incubated at 37°C and samples were removed at 2, 4, 6, and 24 hr after the addition of isotope. Cells were washed twice with saline and were processed as described in Methods. Since there is some difference in the specific activity of the glucose in the four flasks, these have been made comparable by calculating to common specific activity of 41,000 cpm/μmole glucose. Inset table shows specific activities of the surface membranes of cells which had been in isotopic medium for 24 hr. Specific activities are calculated as counts per minute per cell ghost. (See Discussion).

isotope of the membrane residing in the lipid would change from one generation to the next.

Further support for this notion is provided by the observations that the pattern of change of the specific activities of membranes was the same when a general isotope precursor (glucose-^{14}C), protein precursors (L-leucine-^{14}C and L-valine-^{14}C), and a carbohydrate precursor (D-glucosamine-^{14}C) were used. In nongrowing cells the specific activity of membranes fell 40–50% per day with all ^{14}C precursors used. Growing cells also showed a similar pattern of change, regardless of the isotopic precursor employed.

The apparent absence of a differential turnover would seem to disagree with the work of Omura et al. (38) who reported that the turnover of the lipid of smooth and rough microsomal membranes is 10–30% faster than that of the protein component. However, our conclusion is only tentative since we have not measured systematically changes with time of specific activities of individual lipid, protein, and carbohydrate components of the membrane. It is very possible that there are substances in small quantity in the membrane that are turning over rapidly and that we would not ordinarily detect since the specific activities that we are measuring are gross.

GENERAL DISCUSSION

On the basis of the results presented, we propose the following scheme for the behavior of the surface membrane and particulates of the L cell.

The L cell synthesizes similar amounts of surface membrane and particulate material whether the cell is growing and dividing or not. If there is a demand for new membrane material, as there must be in the dividing cell, new membrane is

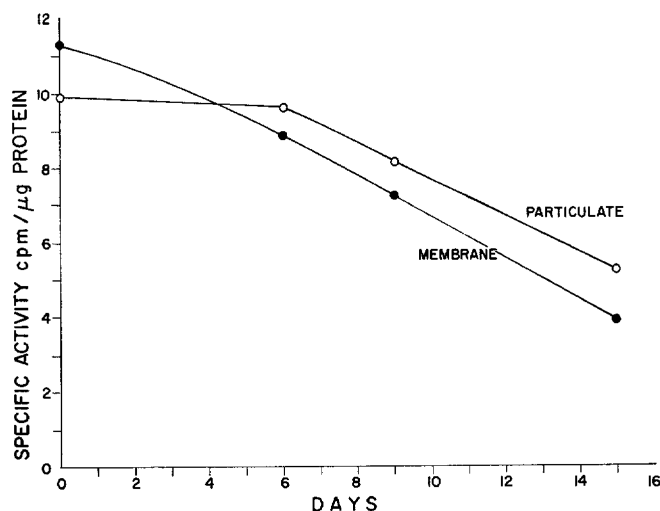


FIGURE 7 Capability of nongrowing L cells in suspension culture to incorporate L-valine-^{14}C as the culture ages. A suspension of L cells was allowed to grow to a stationary phase (1.2×10^6 cells per milliliter). On the days indicated on the graph, 40 ml were transferred to a small spinner flask containing fresh medium with L-valine-^{14}C (specific activity, 205,000 cpm/μmole). After incubation for 6 hr at 37°C the cells were harvested and processed for surface membranes and particulate fraction as described in Methods. From day 0 to day 9 the cells had increased only 25% in number, with an increase of diameter from 14.4 to 16.9 μ. After day 10 the cell number fell precipitously to 3×10^5 cells per milliliter and remained at this level until day 15 when the final sample was tested. Tests with trypan blue showed the following percentages of cells taking up dye: day 0, 1.1%; day 6, 1.3%; day 9, 1.1%; day 15, 13.4%.

incorporated to form new surface. If the cell is not growing or dividing, the newly synthesized membrane precursor enters the surface membrane while an equivalent amount is eliminated with no net change. This constitutes turnover, and it is considerably greater in the nongrowing than in the growing cell. There may be a slight excess of synthesis over demand in the growing cell because there is a small turnover here as well. It has recently been shown that the level of protein synthesis in the Jensen sarcoma cell is similar at different rates of proliferation: at the slower growth rates the products of protein synthesis were secreted into the medium (25). This material could be the product of cellular turnover.

Whether the general picture presented above can be applied to the whole cell remains an unanswered question. It should be emphasized that this surface membrane comprises only 3–5% of the total cell protein and that the isolated particulate fraction constitutes 15–20% of the cell protein.

Both the synthetic and degradative aspects of turnover are dependent on the metabolic integrity of the cell. If biosynthesis is inhibitied, this inhibition is soon reflected in the degradative half of turnover.

The above interpretations of the data seem to be the most likely. The greater turnover of components of the nongrowing cell compared to that of the growing cell is consistent with the results of other workers. It should be stressed that the system studied is very complex and that the data have not excluded other important possibilities. It is possible that in the growing cell there is an internal pool of surface membrane precursors which is in rapid equilibrium with the surface membrane itself so that there could be, in fact, a localized turnover of the surface membrane in the growing cell.

We tend to support the idea that surface material is ejected into the medium of nongrowing cells because we have found relatively large quantities of sialic acid-^{14}C–containing macromolecular material in the medium of these cell cultures. This material may be rejected surface membrane. We have been unable, until now, to correlate the formation of this material with turnover data because there is present in the medium too much other radioactive nondialyzable material (hyaluronic acid or collagen?). The nature of this

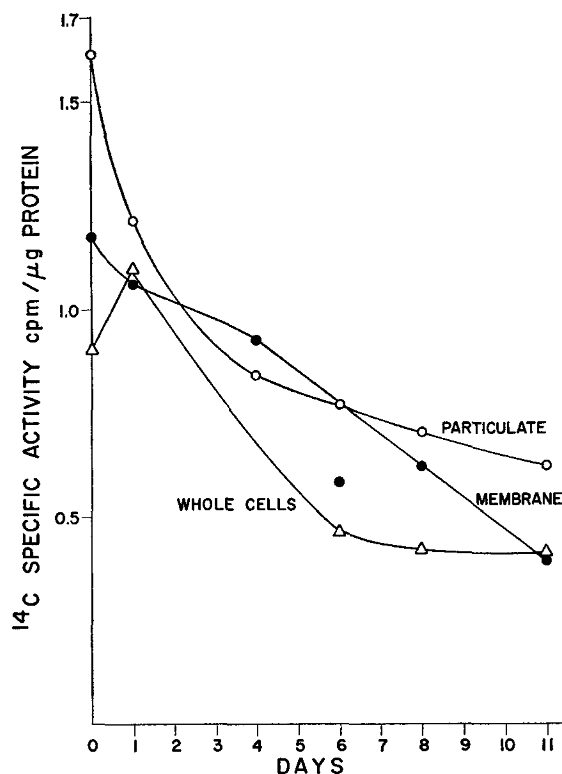


FIGURE 8 Capability of L cells, confluent on a glass surface, to incorporate glucose-^{14}C as the culture ages. 50 ml of a suspension of L cells (862,400 cells per milliliter) were added to each of six culture flasks with an area of 180 cm^2. 50 ml of fresh medium were added and the cells were allowed to grow at 37°C. The cells quickly adhered to the glass, and in 24 hr they had doubled in number. The next day (day 0–1) the cells increased only 27% in number and thereafter they were constant in number. On the days indicated in the graph, the medium from one bottle was poured off, and 100 ml of fresh medium with glucose-^{14}C (9,400 cpm/μmole glucose) were added. Incubation in radioactive medium was carried out for 5 hr. The medium was poured off and the cells on glass were washed twice with phosphate-buffered saline (PBS). 2.5 ml of 0.5% trypsin solution (Difco Laboratories 1:250) in 7.5 ml PBS were added and, after 10 min at 37°C, 20 ml of fresh medium containing trypsin inhibitor were added. The cells were removed from glass by jets of fluid. The suspension was washed into a test tube and the cells of this preparation were counted. The cells were washed twice in saline. Most cells were processed for surface membranes and cell particulates as described in Methods. About 20% of the cells was treated with 10% trichloroacetic acid, centrifuged, resuspended in saline, and treated twice more with 10% trichloroacetic acid. Analyses of protein and radioactivity were made on these cells. The medium was changed every 3 days in those cultures that were not harvested. The cells after removal from glass appeared healthy as observed in the phase-contrast microscope. Free cells in the overlying medium in each bottle were taken into consideration in calculating cell growth. Free cells usually constituted about 5% of the total number of cells. These were not processed.

material is now being studied. Other workers have also found that macromolecular material is secreted into the medium (9, 24). It is not known whether this material reenters the cell to a significant extent. In two imperfect experiments, which permit us to draw only tentative qualitative conclusions, there seemed to be only a small incorporation of isotope into unlabeled nongrowing cells cultured in used isotope-containing, dialyzed medium. This finding agrees with the work of Eagle et al. (7) who found little transfer of material from labeled to unlabeled cells, but disagrees with the results of Eidam and Merchant (9) who found significant incorporation into L cells of labeled

TABLE IV *a*

Effect of Metabolic Inhibitors and Deficient Medium on Incorporation of Isotope into and Loss of Isotope from Surface Membrane of L Cells

Method of inhibition*	Incorporation of isotope		Loss of isotope		
	Specific activity of membrane	Inhibition	Specific activity of membrane	Decrease in specific activity‡	Inhibition
	cpm/μg protein	%	*cpm/μg protein*		%
Control	6.0	—	2.7	1.8	—
Puromycin (35 μg/ml)	0.8	86.7	4.4	0.1	95.6
KCN (10^{-3} M)	4.8	20.0	3.0	1.5	16.7
DNP (10^{-4} M)	3.1	48.3	3.6	0.9	50.0
Omit glutamine	2.6	56.7	3.7	0.8	55.6
Omit valine, leucine, isoleucine	2.9	51.7	4.0	0.5	72.2

* Mediums shown in this column were contained in flasks 1–6, respectively.

‡ Initial specific activity of surface membrane before incubation of cells in nonradioactive medium with inhibitors was 4.5. 100% activity is a fall of specific activity from 4.5 to 2.7 = 1.8.

Incorporation of isotope. Six flasks were set up. The first contained 2.5×10^8 cells per milliliter (25 ml) and served as a control with nongrowing cells. The remaining five flasks each contained 100 ml of medium with 5×10^6 cells per milliliter.

Flasks were incubated for 2 hr at 37°C. Glucose-^{14}C was added (specific activity was 10,500 cpm/μmole glucose) and the flasks were then incubated for 23.5 hr. Cells were harvested and processed for membranes and particulates as described in Methods. Cell growth did not occur in any of the vessels, nor were there significant changes in cell sizes.

Loss of isotope. See explanatory material under Table IV *b* on facing page.

macromolecular material which had been produced by L cells.

It is unlikely that the observed turnover in nongrowing cells is an artifact due to cell death and cell replacement. In most of the experiments, the magnitude of the changes in specific activity, both in the synthetic and in the degradative directions, and the fact that the same characteristic changes took place at the beginning of the experiments as well as later on, argue against this possibility. The cell counts, made at frequent intervals, were constant; evidence of cell debris in the size-distribution curves made by the Coulter counter was lacking. By this method we could detect debris from 2–3% of the cells. Cell cultures were frequently examined under the phase-contrast microscope to see whether the cells appeared healthy and cell debris was present. Assays by the trypan blue test were also made. As mentioned previously, the rate of incorporation of tritiated thymidine into nongrowing cells was 8% that of cells in log phase of growth.

The fact that the surface membrane of nongrowing cells turns over whereas that of growing cells is apparently barely turning over may provide a basis for a qualitative difference between the membranes in these states: differences in permeability, receptivity to virus, antigenicity, and other properties.

It is surprising that in the tissue culture cell, the synthesis of cell components goes on at a fairly constant rate whether the cell is dividing or is in the G_1 phase (plateau), S phase (thymidine block), or the M phase (vinblastine block). Experiments are planned in which synchronized cells in various stages of the mitotic cycle are exposed to short pulses of isotope to clearly establish the relative rates of synthesis of surface membrane in these stages. The magnitude and constancy of synthesis of the nongrowing tissue culture cell studied here prompts us to suggest that, in addition to states of the cell, such as (*a*) dividing or (*b*) nondividing and relatively quiescent, in tissue cells in G_0 phase (1), or in contact-inhibited cells, there can be (*c*) nondividing cells with a high rate of turnover which exist in this state for significant periods of time. It is possible that this is characteristic of established cell lines in tissue culture. We

TABLE IV *b*

Effect of Metabolic Inhibitors and Deficient Medium on Incorporation of Isotope into and Loss of Isotope from Particulate Fraction of L Cells

Method of inhibition	Incorporation of isotope		Loss of isotope		
	Specific activity of particulates	Inhibition	Specific activity of particulates	Decrease in specific activity*	Inhibition
	cpm/μg protein	%	*cpm/μg protein*		%
Control	7.1	—	3.3	2.2	—
Puromycin (35 μg/ml)	1.1	84.5	5.3	0.2	90.9
KCN (10^{-3} M)	2.8	60.6	4.2	1.3	40.9
DNP (10^{-4} M)	4.4	38.0	4.2	1.3	40.9
Omit glutamine	4.4	38.0	4.8	0.7	68.2
Omit valine, leucine, isoleucine	3.5	50.7	3.7	1.8	18.2

* Initial specific activity of cell particulates before incubation of cells in nonradioactive medium with inhibitors was 5.5. 100% activity is a fall of specific activity from 5.5 to 3.3 = 2.2.

Incorporation of isotope. See explanatory material under Table IV *a* on facing page.

Loss of isotope. L cells were labeled by growing them in the presence of glucose-^{14}C overnight. The cells were harvested and washed twice with saline. One portion of the cells (3.4×10^7) was processed for surface membranes and cell particulates as described under Methods. The remainder was divided among six flasks as described for "incorporation of isotope." The medium in this experiment contained no isotope. Volumes of the medium, cell counts, and inhibitor concentrations were the same as for incorporation of isotope. The cells were incubated for 23.5 hr and then processed for surface membranes and cell particulates. The only difference between this experiment and that described for incorporation of isotope was that there was no preincubation with inhibitors for 2 hr. Cell growth and change of cell size were not significant.

assume that in normal tissues and in contact-inhibited cells synthesis and turnover have been depressed (26).

In contrast to ours, previous studies comparing the metabolic turnover rates of growing and non-growing tissue culture cells found no real differences (7, 8). In studies on bacteria (30, 32) some difference in turnover rates was evident. In these studies growth was prevented by the omission of an essential amino acid. As we have shown in Table IV, the absence of essential amino acids inhibits turnover, and this may explain why little or no difference in turnover rates between growing and nongrowing (amino acid-depleted) cells was observed. In our studies growth was prevented at high cell concentration in fresh medium which permitted extensive synthesis and turnover to take place.

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