Constant Protein Catabolism of Walker Carcinoma 256 and Human Skin Epithelium in Tissue Culture*

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The capacity of a malignant tumor to obtain nitrogen at the expense of other tissues led Mider et al. (6) to propose, in 1948, that such a tumor is a nitrogen trap. LePage et al. (4, 9), working with Flexner-Jobling carcinoma in glycine-C\(^14\)-labeled rats, observed that the tumor was able to increase its protein N and total C\(^14\) radioactivity even under conditions of starvation. They concluded that the Flexner-Jobling tumor, in contrast to other tissues studied, exhibits essentially no protein catabolism.

Because the above observations can be explained equally well by enhanced uptake by tumor of amino acids released from other tissues, other workers have employed systems in which the tumor alone is labeled, avoiding uptake of label from extra-tumor sources. Babson and Winnick (1) injected a mince of tyrosine-C\(^14\)- and leucine-C\(^14\)-labeled Walker carcinoma into the hind legs of rats and noted a fall in tumor C\(^14\) radioactivity which, by their estimate, corresponds to a biological half-life of about 5 days. Moldave (8) demonstrated a loss of protein-bound radioactivity by Ehrlich mouse ascites carcinoma cells incubated both in vitro and in dialysis bags placed in the peritoneal cavity for periods up to 48 hours. Both concluded that tumor protein is in dynamic equilibrium with amino acids. Both admitted, however, that cell death incident to the experimental procedure is considerable in the systems employed.

The present study was undertaken with Walker tumor cells labeled with lysine-6-C\(^14\) in tissue culture, a system in which cell death is negligible, uptake of nonprotein lysine-C\(^14\) can almost completely be avoided by frequent change of medium, and small transfers of activity from the cell can be detected with precision. Lysine was employed as the labeling amino acid because it is not oxidized by nonhepatic tissues (7). The protein catabolic rate has been determined by measuring the transfer of radioactivity from C\(^14\)-labeled cell proteins to the nonprotein fraction of the nutrient medium under conditions in which the tumor cells are growing and multiplying actively.

MATERIALS AND METHODS

The culture medium used in all procedures was Eagle's basal medium (2), modified by the use of 10 per cent human placental cord serum, Simm's X-7 balanced salt solution as buffer, and the fresh addition of L-glutamine to each batch of medium prepared. Antibiotics were not added.

The culture of Walker 256 rat carcinoma cells has been maintained in continuous subculture for over a year since isolation by the authors. It has maintained its original appearance and growth characteristics. Continuing malignancy has been confirmed at intervals by injection into rats, with production of typical tumors and 100 per cent lethality. Cultures are maintained on glass in T-15 flasks without plasma clots. Stock cultures routinely are fed at 3-day intervals and subcultured into new flasks at 10-day intervals. The volume of nutrient per flask is approximately 3 ml.

Cell proteins are labeled by subculturing into standard medium containing added Dl-lysine-6-C\(^14\)-HCl (.012 mg/ml; sp. act., 15 ~c/mg). Cultures are allowed to grow for 3 days in labeled medium. The labeled medium is then removed, and the cultures are rinsed 3 times with Simm's X-7 containing 5 mg per cent unlabeled Dl-lysine-HCl. The cultures are reseeded with standard unlabeled medium and incubated for 1 day to rinse out residual Dl-lysine radioactivity and to establish equilibrium among the various compartments of the cell and the medium. Finally, the cultures are rinsed with Simm's X-7 containing Dl-lysine as above. Cultures so labeled have a total radioactivity of approximately 800 times the background of
For Experiment I, eight flasks containing labeled Walker cells were prepared as above. Three cultures (nos. 1, 2, and 3), designated "0 time" samples, were killed by the addition of 5 ml. of 6 per cent trichloroacetic acid (TCA). One ml. of 5 per cent DL-lysine was added immediately to minimize adsorption effects, and the killed cultures were stored in the cold.

The remaining five cultures were fed the standard unlabeled medium and were incubated at 37°C. At 24-hour intervals the medium was removed, the cells were washed 3 times with Stimm's solution containing DL-lysine, and fresh nutrient medium was added. On each day the washings from each culture flask were added to the respective nutrients just removed. These were designated as "nutrient samples." Each nutrient sample was centrifuged to remove cell debris, which was retained. Nutrient and debris samples were frozen at the end of the experiment.

After 2 days one culture (no. 4) was killed as above. At 4 days the remaining four cultures were killed. (The above procedure allows active growth with doubling of cell proteins in approximately 2 days, as estimated by the Folin-Lowry [5] analysis of aliquots.)

To each daily nutrient sample was added 1 ml. of 2 per cent DL-lysine•HCl. Each sample was made to 5 per cent with 100 per cent trichloroacetic acid, allowed to stand in the cold for 2 hours, centrifuged, and washed twice with 6 per cent TCA. The precipitate was assayed for C14 and expressed as nutrient protein C14 radioactivity; the combined supernatant and washings were assayed and expressed as nutrient nonprotein radioactivity. This nutrient nonprotein radioactivity is taken as the measure of protein catabolism in a 24-hour period.

To each cell debris sample was added 50 mg. of DL-lysine•HCl to provide sufficient organic material for efficient combustion of the total sample prior to radioactivity assay. Each culture of cells killed with TCA as above noted (whether killed at 0 time, 2 days, or 4 days) was scraped from its flask; to it was added 0.5 ml. serum to afford a mechanical carrier for tumor proteins. The resulting precipitate was centrifuged and washed twice with 6 per cent TCA. The precipitate was assayed as culture cell protein carbon-14 radioactivity; the supernatant and washings were assayed and expressed as nutrient nonprotein radioactivity. This nutrient nonprotein radioactivity is taken as the measure of protein catabolism in a 24-hour period.

Experiment II was designed to reveal possible variation in catabolic rate with drastic alterations of medium amino acid concentration. For Experiment II 40 flasks containing labeled Walker cells were prepared as in Experiment I. These were divided into eight groups of five each, selecting groups as follows: one group with standard nutrient; the other groups with nutrient at 50 per cent, 75 per cent, 125 per cent, and 150 per cent of standard amino acid concentration, respectively. Glutamine was not varied. At 2 days one culture from each group was scraped from its flask and divided for C14 radioactivity and protein determinations. At 4 days two cultures were scraped out and divided as above; the remaining two cultures were killed by precipitation in the flask with TCA.

In Experiment III, the amino acid concentration of the nutrient medium was varied in small increments about the optimum standard nutrient concentration to detect any major discontinuities in the catabolic rate as a function of amino acid concentration. Twenty labeled cultures were divided into six groups. The 0 time group of five flasks was terminated by precipitating three cultures in the flask with TCA, while two cultures were divided for radioactivity and protein determinations as in Experiment II. The remaining five groups of three flasks each were fed daily as follows: one group with standard nutrient; the other groups with nutrient at 50 per cent, 75 per cent, 125 per cent, and 150 per cent of standard amino acid concentration, respectively. Glutamine was not varied. At 2 days one culture from each group was scraped from its flask and divided for C14 radioactivity and protein determinations. At 4 days one of the two remaining cultures was removed and divided as above; the other was precipitated in the flask with TCA.

Experiment IV was carried out exactly as Experiment III, except that here the placental cord serum concentration was varied in small increments about the standard nutrient concentration. One culture group was fed standard nutrient, the other groups were fed nutrient at 50 per cent, 75 per cent, 125 per cent, and 150 per cent of standard serum concentration, respectively. Amino acids were not varied.

Experiment V was essentially the same as Experiment I, except that human skin epithelial cells were substituted for Walker cells. The cells containing no amino acids (except for the small amount contributed by the placental cord) were killed immediately.

Three of the five flasks were killed by precipitation with TCA and assayed for C14 radioactivity as in Experiment I. The cultures were scraped and rinsed from two flasks, and each was divided into two equal aliquots. One aliquot was precipitated with TCA and assayed for carbon-14 as in Experiment I; the other was analyzed for total protein by the Folin-Lowry method.

The remaining seven groups were fed daily with the following nutrient media: (a) standard nutrient as described at the beginning of this section; (b) nutrient at 50 per cent of the standard amino acid concentration; (c) nutrient at twice the standard amino acid concentration; (d) nutrient with lysine excluded; (e) nutrient at twice the standard lysine concentration; and (f) standard nutrient with glutamine excluded. The standard nutrient contains 50 mg. per cent amino acids (3.5 millimoles per cent). One culture from each group was scraped from its flask at 2 days and divided for C14 radioactivity and protein determinations. At 4 days two cultures were scraped out and divided as above; the remaining two cultures were killed by precipitation in the flask with TCA.

1 All organic samples were quantitatively oxidized to carbon dioxide with the digestion mixture of Van Slyke et al. (J. Biol. Chem., 191:299, 1951). The resulting carbon dioxide was transferred to a 1-liter ionization chamber with the aid of inert carrier carbon dioxide. The discharge rate of the chamber was measured in v/min by means of an Applied Physics Corporation vibrating reed electrometer. Electrometer readings have been converted to absolute units of radioactivity by calibration against U.S. Bureau of Standards Na2SO4O3. Reproducibility is approximately 1 per cent.
employed were taken from epithelioid outgrowths from bits of normal human epidermis cultured in T-15 flasks. They had been in culture approximately 1 month and had been serially subcultured twice at the time of the experiment. Eight culture flasks of labeled cells were prepared in a manner identical to that used for the Walker cells, of which three were terminated at 0 time. Of these three, one was precipitated by trichloroacetic acid in the flask, and two were scraped out and divided for protein determination and counting. The remaining five flasks were fed daily with standard nutrient as in Experiment I. One flask was terminated at 2 days by precipitation with trichloroacetic acid. Two cultures were killed similarly at 4 days, and two cultures were removed at 4 days and divided for protein determination and for C\(^{14}\) assay.

**RESULTS AND DISCUSSION**

Complete tables of data are given only for Experiment I because of space limitations. The data of the other experiments are summarized in the text and charts and will be published elsewhere (3).

In Table 1 are shown cell protein and nonprotein

**TABLE 1**

<table>
<thead>
<tr>
<th>EXPERIMENT I</th>
<th>0 TIME CULTURE RADIOACTIVITIES*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture 1</td>
<td>Culture 2</td>
</tr>
<tr>
<td>Nonprotein cell radioactivity</td>
<td>0.142</td>
</tr>
<tr>
<td>Protein cell radioactivity</td>
<td>2.690</td>
</tr>
<tr>
<td>Per cent radioactivity in cell proteins</td>
<td>95.0</td>
</tr>
</tbody>
</table>

* Radioactivities are in v/min; 100 v/min = approximately 1 μc.

C\(^{14}\) radioactivities for the three Walker cell cultures killed at the end of the labeling period. As noted above, virtually all the radioactivity was in the protein fraction.

In Table 2 are shown the C\(^{14}\) radioactivities of the protein, nonprotein, and debris transferred to the nutrient by each culture on each day, as well as the protein and nonprotein radioactivities remaining in the cells of each culture at the end of Experiment I (as in the newly labeled cultures, nearly all culture cell radioactivity was in the protein fraction). Zero time C\(^{14}\) radioactivity of each culture was obtained by adding all radioactivities.

The radioactivity of the culture cells on each succeeding day was obtained by subtracting the radioactivities transferred to the nutrient (see Table 3). The variation in total radioactivity...
did not affect catabolic rate determination, since
the rate was determined separately for each cul-
ture.

From the data of Tables 2 and 3 the per cent
radioactivity transferred on each day from the
culture cell protein to the nonprotein fraction
of the nutrient may be calculated. The biological
half-life for protein catabolism is readily obtained
by plotting the cell protein C\textsuperscript{14} decay curve for
each culture. Chart 1 shows the curve for culture
no. 5, as well as the range of values obtained for
the other cultures. Half-lives of 5.00, 4.89, 5.21,
and 5.02 days were obtained for cultures no.
5 through no. 8, respectively. The mean half-life
was 5.08 days ± 0.07 days standard error.

In pilot experiments with less refined technics
and not described here, a range of half-life of
4.76–5.50 days for six determinations was ob-
served.

The protein C\textsuperscript{14} catabolic decay curve may be
seen by inspection to conform very closely to
the curve $A = A_0 e^{-\lambda t}$, where $A =$ radioactivity
at time $t$, $A_0 =$ radioactivity at time 0, and $\lambda =$
catabolic rate. By substitution of the half-life,
the mean protein catabolic rate was found to be
0.57 per cent/hour, or 12.9 per cent/day, ± stand-
ard error of 0.2 per cent/day.

The constancy of catabolic rate from day to
day indicates that most of the labeled cell proteins
are being broken down at approximately the same
rate. If any significant amount of C\textsuperscript{14}-labeled
protein were being catabolized with a greatly
different half-life, the curve of Chart 1 would
not be a straight line but would be a curve, the
slope of which increases with time.

The response of catabolic rate to halving and
doubling of the nutrient amino acid concentration,
determined in Experiment II, is shown in Chart
2. Each point represents the mean of two deter-
minations on the Walker cell cultures killed at
the end of the experiment by precipitation in the

- **Chart 1.**—Experiment I. Decay curve for protein catab-
olism of Walker 256 cells.

- **Chart 2.**—Experiment II. Protein catabolic rate of Walker
256 cells at three nutrient amino acids concentration levels.

and with trichloroacetic acid. (Determinations in
which cultures were terminated by being scraped
from the flask before treatment with TCA are
not shown. They exhibit an erroneously higher
rate due to less efficient recovery of radioactivity,
as predicted by pilot experiments.) Approximate
95 per cent confidence intervals representing twice
the standard error are shown. It may be seen
that doubling the amino acid concentration pro-
duces no significant change in catabolic rate, while
the nonoverlap of confidence intervals for normal
and 50 per cent amino acids indicates a significant
depression of the observed catabolic rate at this
lower amino acid level. Growth rates are essentially
unchanged at the three levels, as estimated by
Folin-Lowry protein determinations of the total
culture cell protein at 0 time, 2 days, and 4 days.

When lysine was omitted from the medium,
the apparent catabolic rate fell to approximately

- **Table 3.**

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Culture 4</th>
<th>Culture 5</th>
<th>Culture 6</th>
<th>Culture 7</th>
<th>Culture 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.259</td>
<td>2.893</td>
<td>3.568</td>
<td>3.848</td>
<td>2.974</td>
</tr>
<tr>
<td>1</td>
<td>2.527</td>
<td>2.329</td>
<td>2.760</td>
<td>3.022</td>
<td>2.337</td>
</tr>
<tr>
<td>2</td>
<td>1.974</td>
<td>1.734</td>
<td>2.151</td>
<td>2.402</td>
<td>1.800</td>
</tr>
<tr>
<td>3</td>
<td>1.558</td>
<td>1.696</td>
<td>1.890</td>
<td>1.398</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.079</td>
<td>1.506</td>
<td>1.547</td>
<td>1.108</td>
<td></td>
</tr>
</tbody>
</table>

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day indicates that most of the labeled cell proteins
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lower amino acid level. Growth rates are essentially
unchanged at the three levels, as estimated by
Folin-Lowry protein determinations of the total
culture cell protein at 0 time, 2 days, and 4 days.

When lysine was omitted from the medium,
the apparent catabolic rate fell to approximately
10 per cent of normal, while the growth rate remained normal over the 4-day period. This, however, was an artifact due to the lack of a pool of unlabeled lysine, with consequent uptake of virtually all C14-labeled lysine released by the cells. This is confirmed by the observation that if growth and thereby lysine uptake are inhibited by the removal of all amino acids from the medium a normal catabolic rate is observed. The above observations also indicate that the activity released into the medium was lysine-C14, since the removal of the unlabeled lysine pool would not alter the net release of compounds other than lysine.

Doubling the lysine concentration of the medium introduced no significant change in catabolic rate while growth remained normal. This is indicative of the adequacy of the unlabeled lysine pool at the standard amino acid concentration.

Removal of the glutamine from the medium introduced no change in catabolic or growth rate. This is in contrast to the observation in our laboratory that freshly added glutamine is an essential nutrient component for the long-term maintenance of Walker cells. This glutamine requirement was not observed in the 4-day period of the present experiment.

From the data of Experiment III it is possible to plot a smooth function of the protein catabolic rate of Walker cells versus amino acid concentration (see Chart 3). If the standard deviation observed in Experiment II is assumed, no significant differences are observed between adjacent points, but the rate at 50 per cent amino acid concentration is significantly lower than that at standard amino acid concentration. The growth rate was unaffected at all amino acid levels used in this experiment.

In Chart 4 catabolic rate is plotted as a function of nutrient placental cord serum concentration, as determined by Experiment IV. No point is shown at the 50 per cent serum concentration, owing to loss from a laboratory accident. If, again, the standard deviation of Experiment II is assumed, the catabolic rate is shown not to be a function of nutrient serum concentration. Partial data obtained for the 50 per cent level support this conclusion. Growth, on the other hand, as measured by total cell proteins, was enhanced by increasing nutrient placental cord serum concentration. Protein catabolic rate was not, therefore, a function of growth rate.

The cell protein C14 decay curve for protein catabolism in the human skin epithelial cells of normal origin (Experiment V) is shown in Chart 5. The mean half-life was 7.2 days ± 0.2 days standard error. This corresponds to a decay rate of 9.2 per cent/day ± 0.3 per cent standard error. As in the Walker cell (see Chart 1), the catabolic rate was remarkably uniform from day to day and reproducible.

In all experiments with the Walker tumor cells there was observed a net transfer of soluble protein radioactivity from the intracellular compartments into the medium. The amount transferred was quite constant from day to day and was reproducible between culture flasks in the same experiment.

Chart 3.—Experiment III. Protein catabolic rate of Walker 256 cells as a function of nutrient amino acid concentration. (Glutamine not varied.)

Chart 4.—Experiment IV. Protein catabolic rate of Walker 256 cells as a function of nutrient serum concentration.
It was variable between experiments, however, varying from 3 per cent/day in Experiment II to approximately 10 per cent/day in Experiment IV. As may be verified from the data of Tables 2 and 3, the mean rate of protein transfer observed in Experiment I was 5.8 per cent/day ± 0.2 per cent standard error. Inasmuch as this variation was unaccompanied by any significant change in catabolic rate, the two very probably are not functionally related. Net protein transfer also was observed in the human skin epithelial cells studied, the rate being 3.2 per cent/day in the one experiment performed.

It may be argued that the observed catabolic rate is actually the result of cell death and autolysis. This view is hardly tenable, however, in the light of the constancy of the observed catabolic rate and the variability of protein transfer. It would require that cell death vary between the experiments in such a manner as to account for the variable total loss as nonprotein, protein, and cell debris, while the autolytic rate varied in such a compensating manner as to produce a constant percentage of nonprotein activity. Moreover, such an explanation would require the daily death of more than 20 per cent of the cells in some experiments. This is inconsistent with our daily observations of the cultures and the small losses as cell debris.

SUMMARY

The protein catabolic rates of the Walker rat carcinoma 256 and of a single line of human skin epithelial cells have been determined by labeling the cell proteins in vitro with lysine-6-C14 and measuring the subsequent transfer of radioactivity to the trichloroacetic acid-soluble fraction of the culture medium over a period of 4 days. Evidence presented supports the following conclusions:

1. The Walker carcinoma 256 was capable of catabolizing its protein.
2. In the tissue culture system described, the protein catabolic rate of Walker cells was remarkably constant at 12.9 ± 0.2 per cent per day in standard nutrient.
3. The protein catabolic rate was not a function of nutrient placental cord serum concentration or of the growth rate and was only a slowly varying function of nutrient amino acid concentration.
4. When lysine-C14 was the labeling compound, radioactivity was released as lysine-C14.
5. There was a net transfer of soluble C14-labeled cell protein into the medium at a rate that was constant within each experiment, but which was not reproducible between experiments.
6. At least one other cell type, i.e., human skin epithelium, exhibited a similar catabolic rate and net protein transfer in tissue culture.

ACKNOWLEDGMENTS

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