Protein Turnover in a Study of Host-Tumor Relationships

JANET GREENLEES and G. A. LEPAGE

(University of Wisconsin Medical School, McFarland Memorial Laboratory, Madison 6, Wis.)

A recent approach to the problem of protein turnover in neoplastic tissue made by LePage et al. (7) involved the determination of total radioactivity in tumor, as compared with liver, kidney, and spleen over a 6-day time period following the injection of glycine-2-C\(^{14}\). The experiment was carried out with the rapidly growing Flexner-Jobling rat carcinoma. To determine the effect of limiting the nitrogen turnover to a host-tumor relationship, half the animals were fasted after the injection of isotope. It was found that unlike normal tissues, such as liver, kidney, and spleen, tumor continued to grow and to increase in total radioactivity in spite of the stress of fasting. The Flexner-Jobling carcinoma was chosen as an example of a very anaplastic growth. The authors suggested that less malignant tumors might give a picture from which growth could be correlated inversely with the rate of exchange of amino acids with the body pool. Some of the following experiments were designed to test this premise.

A series of mammary adenocarcinomas originating in Strain A mice and varying in degree of autonomy (5) were used in experiments similar to the one on Flexner-Jobling carcinoma.

A study of the more quantitative aspects of nitrogen turnover was made with labeled tumor ascites cells. After the injection of radioactive tumor cells into the host, radioactivity entering the host’s metabolic pool was determined by measuring the C\(^{14}\) recovered in the tumor cells, in normal tissues, in respiratory CO\(_2\), and in urine.

EXPERIMENTAL

TUMOR GROWTH IN FED AND FASTED MICE

The tumors included 15091A,\(^1\) which is no longer strain-specific and represents the counterpart of the Flexner-Jobling carcinoma of the rat. The TA3,\(^2\) which has a shorter history and is strain-specific but sufficiently anaplastic to be converted to ascites cell form, was also included. To study more recently developed tumors, two primary mammary adenocarcinomas in Strain A females were used at successive stages in their earliest transfer generations. Since Strain A mice were not available in sufficient numbers, CAF\(_1\) male mice, obtained from the Bar Harbor Laboratories, were used when 8–16 weeks of age.

A preliminary experiment determined the time at which maximum incorporation of radioactive glycine into liver, tumor, and kidney protein occurs. Fifteen mice, each bearing two 10-day-old subcutaneous TA3 tumors, were divided into five groups, given injections of 1.5 \(\mu\)g. glycine-2-C\(^{14}\)/gm body weight, and sacrificed at 2-hour intervals starting 5 hours after injection. The proteins of liver, kidney, and tumor were isolated and counted according to the procedure described later. The results are recorded in Chart 1.

For each of the first group of experiments, 60 CAF\(_1\) male mice were divided into ten groups. Five groups were given trocar implants of solid tumors (subcutaneous injections in the case of TA3 ascites tumor) at two sites on the abdomen of each mouse. The mice were kept on a stock grain diet until the first two groups were sacrificed, at which time food was removed from the cages of the groups to be fasted. When the tumors had reached a size estimated to be 60–150 mg., the mice were given intraperitoneal injections of glycine-2-C\(^{14}\), 1.5 \(\mu\)g/gm body weight (about 30,000 counts/gm). Two control groups, one bearing tumors, were decapitated 10 hours later. Two groups each of tumor and nontumor, fed and fasted mice remained to be sacrificed 92 and 4 days later. Four days proved to be the maximum time the fasted mice could survive. Each group was sacrificed by decapitation. Liver, kidney, and tumors were removed. Livers from all members of the group were

\(^1\) Obtained from Dr. Theodore S. Hauschka, The Institute for Cancer Research, Philadelphia, Pa.

\(^2\) Obtained from the Roscoe B. Jackson Memorial Lab., Bar Harbor, Maine.
pooled and weighed, as were the kidneys and tumors in each case. The tissues were then homogenized in 4 or 9 parts of water and the suspensions made to a final concentration of 5 per cent with trichloroacetic acid (TCA). The precipitates were washed twice with 5 per cent TCA, 3 times with 95 per cent ethyl alcohol, extracted 3 times with a 3:1 alcohol-ether mixture at 40° C. to remove lipids, washed twice with acetone, and dried in vacuo. This material was then ground to a powder and weighed. Samples of each protein preparation were plated on aluminum plates and counted in internal flow counters. The radioactivity was corrected for self-absorption and expressed as counts/min/mg. All samples were counted for a sufficiently long period to obtain a 10 per cent statistical accuracy.

The results of two experiments on the 15091A mammary tumor were averaged and are shown in Chart 2. The results resemble those obtained in the Flexner-Jobling experiment (7). The top part of Chart 2 depicting gm wet weight of tissue/mouse over the 4-day period shows a weight loss in body, kidney, and liver in the fasted groups and a slight gain in the fed groups, due to growth. As is seen from the chart, tumor weight increased even under the stress of fasting. The second row indicates that there was a drop in the specific radioactivity in all tissues. The fed mice showed a rapid dilution of labeled material with exogenous metabolites. The fasted mice showed a slower dilution, since the metabolic pool was smaller and supplied only by breakdown of more susceptible tissues. Of greatest interest is the bottom row, showing the total activity of each tissue. Whereas liver and kidney were rapidly losing counts owing to dilution by unlabeled metabolites, or to tissue breakdown in the fasted animals, the tumor showed an over-all gain.

The results with the TAB tumor varied so slightly from those with 15091A that an average of two experiments gave a picture almost identical to that in Chart 2, and consequently these data have been omitted.

In contrast to these results were the data obtained from a spontaneous tumor arising in a Strain A mouse and transplanted twice before it was in condition to use in these experiments. The results for liver and kidney from this experiment resemble those of the previous experiments. This tumor presents quite a different picture, however, from that given by 15091A. The tumor wet weight of fasted mice declined rapidly after the 2nd day, and the total radioactivity behaved in the same manner as that in the normal tissues. At this early stage the tumor still retained some characteristics of the tissue of origin and was subject to the stresses of fasting.

The question next arose whether some transition would be made in successive generations. Experiments were done with fifth, seventh, and twelfth transplant generations of this tumor. Chart 3 shows that by the fifth generation a change had taken place, and the tumor increased in weight and in accumulated radioactivity by the 4th day. The seventh and twelfth transplant generations, starting with smaller tumors, confirm the fact that this tumor had become independent of fasting stress.

**Protein Studies in Labeled Ascites Cells**

Experiments were set up using the TAB ascites tumor in CAF1 male mice. In each experiment, the 6th day after a mouse had been given inoculations of 0.2 ml. of a 5 per cent suspension of ascites cells, it received an intraperitoneal injection of glycine-2-C14, 1.0 mg. in 0.3 ml saline solution equivalent to 25.9 × 10⁶ counts/min. After 24 hours' incubation, the ascites cells were drawn out and washed 4 times with isotonic saline solution to re-
duce the glycine content. In the final wash the cells were centrifuged for 7 minutes in the flat yoke of an International Clinical Centrifuge. A 40 per cent suspension of the packed cells in saline was made, and 0.5 ml. was injected intraperitoneally into each of two normal mice, which were then placed in separate respirometers for the next 24 and 48 hours, respectively. A third 0.5-ml. aliquot was precipitated by 5 per cent TCA, washed once with 5 per cent TCA and twice with acetone, dried, weighed, plated, and counted as before. The acid and acetone washes were collected, plated, and counted. When a mouse was removed from a respirometer, the ascites cells were drawn out, and the intraperitoneal cavity was washed thoroughly with isotonic saline. The mouse was killed, and liver, kidney, and spleen were removed. Each tissue was weighed and

![Chart 2](chart2.png)

**Chart 2.**—The changes in weight and radioactivity of body and tissues of fed and fasted mice bearing mammary adenocarcinoma 15091A.

- **O—O** = fed control mice; **O—O** = fasted control mice; **•—•** = fed tumor-bearing mice; **O—O** = fasted tumor-bearing mice.
homogenized and the protein isolated and counted as described earlier. An aliquot of the urine was plated and counted. The respiratory CO$_2$ was absorbed in NaOH solution, precipitated, and counted as BaCO$_3$. From a preliminary experiment in which a 2-hour incubation time was used (Table 1), it was discovered that the acid-soluble fraction contained a large proportion of the total radioactivity of the injected ascites cells. On analysis of this extract, adenine was found to contain almost a quarter of the activity. The balance was present as glycine. The adenine was separated on a Dowex-50 column and eluted with 6 N HCl. A water solution of the adenine was plated and counted. Glycine was determined as described by Barton (8). An approximation of the total glycine was obtained from the amount of dilution resulting on the addition of known amounts of nonradioactive glycine. To give the cells a chance to reduce the amount of this free glycine, a 24-hour incubation period was used.

Table 2 shows an example of the results obtained in three experiments. The per cent recovery of isotope is listed for each experiment in Table 3. The radioactivity in the acid-soluble fraction was reduced to 11 per cent of the total radioactivity of the ascites cells. A comparison of the specific activities of liver, kidney, and spleen, as well as total counts respired and excreted in the 2- and 24-hour experiments, shows the result of decreased radioactivity in the acid-soluble fraction.

![Chart 3](image-url)

**CHART 3.**—The changes in tumor weight and total radioactivity of fed and fasted mice bearing implants of early transplant generations of the first spontaneous mammary adenocarcinoma used.

- • = fed mice; • = fasted mice

**TABLE 1**

THE DISTRIBUTION OF RADIOACTIVITY 24 HOURS AFTER INJECTION OF A MOUSE WITH ASCITES CELLS LABELED FROM A 2-HOUR INCUBATION WITH GLYCINE-$\delta$-C$^4$

<table>
<thead>
<tr>
<th>Ascites cells:</th>
<th>0 hr.</th>
<th>24 hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein and nucleic acids</td>
<td>276,000 c.p.m.</td>
<td>375,000 c.p.m.</td>
</tr>
<tr>
<td>Acid-soluble extract</td>
<td>307,000</td>
<td>375,000</td>
</tr>
<tr>
<td>Acetone wash</td>
<td>70,000</td>
<td>70,000</td>
</tr>
<tr>
<td>Total</td>
<td>662,000</td>
<td>662,000</td>
</tr>
<tr>
<td>Liver</td>
<td>12,800</td>
<td>12,800</td>
</tr>
<tr>
<td>Kidney</td>
<td>1,500</td>
<td>1,500</td>
</tr>
<tr>
<td>Spleen</td>
<td>1,300</td>
<td>1,300</td>
</tr>
<tr>
<td>Urine</td>
<td>15,000</td>
<td>15,000</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>107,000</td>
<td>107,000</td>
</tr>
</tbody>
</table>
Some trial recoveries were made to determine the amount of loss involved in manipulation. Two normal mice were given injections of 0.5 ml. of a 40 per cent suspension of cells labeled from a 2-hour incubation with glycine C\textsuperscript{14}. After each injection the cells were immediately drawn out, a process which takes 10—15 minutes, and were treated in the usual manner, saving the acid wash. Recoveries of radioactivity were 62 and 67 per cent. This procedure was repeated with cells labeled from a 24-hour incubation with isotope. Both of two animals gave 92 per cent recoveries of the radioactivity. This seemed to indicate a very rapid turnover of the acid-soluble components to the metabolic pool.

The high activity found in the acetone extraction is not due primarily to dissolved lipids but probably to proteinaceous material which settles out on standing for 3 or 4 days in the cold. In one case this sediment was dried and counted. The radioactivity of this fraction constituted 95 per cent of the total activity in the acetone wash.

**DISCUSSION**

The increase in the liver weight of tumor-bearing animals over that of nontumor-bearing animals noted by several workers (1, 7, 10) involved relatively large tumors and was not observed in these experiments. According to Sherman (9), in rats with Walker Carcinoma 256, protein precursors are obtained from the diet until the tumor is 10 per cent of the total body weight. There then follows a period when dispensable stores of nitrogen in the tissues are broken down, at which time there is liver hypertrophy. It appears that nitrogen is accumulated in the liver owing to the functional demand on it to dispose of the split products from necrotic tumors. In the present studies, the tumors never constituted more than 7 per cent of total body weight, and toxic effects from necrotic tissue should be negligible.

The reason for a decrease in total radioactivity in tumor at the 92nd day is not known, although in experiments on the fifth transplant generation of the newly derived tumor this dip was also quite marked. It is possible that during the first 2 days of fasting the energy stores are depleted, and the cell then more rapidly converts protein to fuel, at which point dilution of the radioactive glycine is increased. This may occur earlier in experiments in which no loss in radioactivity is shown.

Photomicrographs were made of the third and 921st transplant generations of the newly derived tumor. Although the growth rate had not increased measurably by the 921st generation, some change in the histology was visible. The alveolar pattern was largely replaced by clusters of cells in irregular arrangement. The size and staining qualities of the nuclei had become more varied. However, no transition to a sarcomatous-like growth had occurred, even though the general appearance was one of greater malignancy. This is important, since Ehrlich and others (4, 6) have found sudden changes in mammary tumors occurring in ninth, sixth, or even second-generation transplants. The change reported involves the
spontaneous appearance of spindle cells and an abrupt acceleration in growth rate. If this had occurred one might expect some radical changes in the response of the tumor to stress. The mammary tumor transplants used in the present experiments grew for over a month without becoming outwardly necrotic.

The fact that in Chart 3 there is a decrease in total radioactivity in tumors of fed mice after the 92d day and that at no time is the incorporation of C\(^{14}\) comparable to that found in the tumor of Chart 2 indicates that in the case of this relatively recent tumor there is exchange of glycine with the body pool. In the third transplant generation the turnover is apparently about as rapid as that of normal tissue.

The experiments with labeled ascites cells indicated that, at least in the case of the TA3 ascites tumor, there continued to be some amino acid exchange, and nitrogen metabolism was not entirely a one-way passage. Although there was variation among the individual mice, two of the three cases showed a loss in tumor radioactivity during the 92d day, and in all three experiments there was an increase in activity outside the tumor, usually found in urine and CO\(_2\). The low recovery at 48 hours in experiment II and at 24 hours in experiment III may have been due to cells which lodged in the mesentery and formed solid tumors. Between 44 per cent and 47 per cent of the total radioactivity in the CO\(_2\) was respired during the second 24-hour period. The specific activity of the organs did not decrease, as one would expect if the body pool were not being supplied with labeled carbon. The percentage of the ascites cell radioactivity transferred to the organs, urine, and CO\(_2\) suggests that the loss of isotope from the tumor cells is continuous and regular, averaging 9 per cent every 24 hours. This radioactivity could not be due solely to the isotope in the acid-soluble fraction of the injected cells. The data from the trial recovery experiments implied that the turnover rate of the acid-soluble components was extremely rapid. Sixty to 70 per cent of the activity from this fraction was lost in the first 24 hours when equilibrium with the body pool was established, and there was only a slight decrease thereafter. The radioactivity in the acetone wash appeared to be stable, since there was an increase rather than a decrease in activity over the 2-day period.

Babson and Winnick (2), using Walker carcinoma labeled with tyrosine or leucine-C\(^{14}\), tried to determine the amount of nitrogen turnover in experiments similar to the ascites cell tests done here, analyzing only the tumor. After an initial 50 per cent loss of radioactivity on the 1st day, resulting from cell breakdown during fragmentation of the tissue and from initial autolysis, they found very little slope to the recovery curve. Although some of the decrease in radioactivity may be a result of necrosis, since the most highly labeled portion would soon become surrounded by newly synthesized material and be deprived of its nutrient supply, the authors concluded that the Walker carcinoma was not a “nitrogen trap.”

In the light of the data presented here it seems that nitrogen turnover decreases as the tumor becomes more anaplastic. Tumor per se is a “nitrogen trap” in the relative sense only. When the growth rate of a tumor has increased to such an extent that the tumor requires all the available building blocks for protein synthesis and the degradation products have little time to escape, it approaches this character.

**SUMMARY**

Control mice and mice bearing implants of mammary adenocarcinomas in varying stages of autonomy were given injections of glycine-2-C\(^{14}\).

Groups of fed and fasted mice were sacrificed at intervals up to 4 days. The total radioactivity in liver and kidney was found to decrease, but that of the more malignant tumors increased in both fed and fasted animals. The first few transplant generations after primary tumors responded to fasting in a manner similar to liver and kidney. By the fifth transplant generation, the total radioactivity of the tumor showed an increase, although some protein turnover was evident.

TA3 ascites cells were labeled in vivo with glycine-2-C\(^{14}\). The cells were drawn out, washed, and injected into normal mice. Injected mice were killed 24 and 48 hours later. The total radioactivity remaining in the ascites cells as well as that found in liver, kidney, spleen, urine, and respiratory CO\(_2\) was measured. Data from three experiments indicated that the TA3 tumor is a “nitrogen trap” in the relative sense only, that approximately 9 per cent of the radioactivity was lost from the tumor cells every 24 hours.

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