Influence of the Walker 256 Carcinosarcoma on Muscle, Tumor, and Whole-Body Protein Synthesis and Growth Rate in the Cancer-bearing Rat

John A. Tayek, Nawfal W. Istfan, Catherine T. Jones, Karim J. Hamawy, Bruce R. Bistrian, and George L. Blackburn

ABSTRACT

The in vivo rates of protein synthesis were assessed in tumor tissue, skeletal muscle, and whole body of rats bearing the Walker 256 carcinosarcoma. Estimates of protein synthesis in the nontumorous tissues were compared to tumor-free controls. Changes in size of the whole animal and tumor (i.e., growth) were measured, and fractional rates of growth, synthesis, and breakdown were estimated. Muscle protein synthesis and whole-body growth were significantly reduced in rats bearing larger tumors, and both were negatively correlated with tumor size ($r = -0.723$ and $-0.825$, respectively; $P < 0.01$). Furthermore, whole-body and muscle protein synthesis were positively correlated with body growth ($r = 0.380$ and 0.563, respectively; $P < 0.05$). Tumor growth followed first-order kinetics between days 7 and 13 following implantation, with a mean rate constant of 34.3%/day for the larger tumors and 27.7%/day for the small tumors. The difference in tumor growth became statistically significant over the final 3 days of tumor volume measurements. Fractional protein synthesis was significantly lower in the larger compared to the smaller tumors (48.6 versus 84.8%/day; $P < 0.05$) as measured on day 14. This finding indicates a lower protein breakdown rate for the larger tumors (14.3 versus 59.0%/day; $P < 0.01$) and suggests that the process of protein breakdown could play a significant role in determining tumor size, leading support to the theory of tumors acting as nitrogen traps.

INTRODUCTION

The cachexia syndrome is associated with high rates of mortality in cancer patients. (1) Several mechanisms have been offered to explain the weight loss associated with the presence of cancer. These include insufficient dietary intake and absorption, (2) enhanced tumor nutrient requirements, (3) alterations in host metabolism, (4) or a combination of these processes. (5)

Weight loss in cancer cachexia has been explained mechanistically by increases in total body energy expenditure and reductions in muscle-protein synthesis that lead to muscle wasting. (6) Studies by Lundholm and others have shown that skeletal muscle protein synthesis is reduced with the onset of cancer cachexia (7) and may be reduced further with increasing tumor size. (8) Previous studies have utilized the technique of constant infusion of labeled amino acids and its specific activity in the plasma amino acid pool to estimate plasma protein flux, oxidation, and whole-body protein synthesis. This method may underestimate intracellular amino acid oxidation and whole-body protein synthesis. In comparison, using the specific activity of the intracellular amino acid pool to determine whole-body protein flux and synthesis assumes the intracellular pool to be a precursor pool for overall protein synthesis. The use of intracellular measurements makes possible comparison of tumor and tumor-free protein synthesis rates and also allows quantification of tissue-specific amino acid concentrations which may well be altered by disease. Expecting a rapid tumor turnover, similar to that reported earlier by Kawamura et al., (9) we selected the flooding dose technique which uses the free intracellular amino acid concentration as the precursor pool to investigate whole-body and tissue protein synthesis parameters.

This technique provides a large dose of amino acid so that the specific radioactivity in all possible precursor pools is nearly equal, therefore minimizing the error in the determination of the specific radioactivity of the free amino acid at the site of protein synthesis, both in the individual tissues and for the whole body. (10)

The purpose of this study was to explore the relationship between tumor protein synthesis and the rate of tumor growth and to evaluate the influence of the tumor on the whole-body and muscle protein synthesis. Fractional synthetic rates, $K_s$, were studied in tumor, muscle, and the whole body of young Walker 256 carcinosarcoma-bearing rats. The fractional growth and breakdown rates of the Walker 256 carcinosarcoma were determined, and the effects of tumor size on whole-body and muscle protein synthesis were examined. Our hypothesis was that the metabolic influence of cancer on the host's protein synthesis would be dependent upon the size of the tumor, and that the rate of tumor growth would correlate with tumor protein-synthetic rates.

MATERIALS AND METHODS

Rats and Experimental Design. Twenty-four male Sprague-Dawley rats (130–150 g) were obtained from Taconic Farms (Germantown, NY). The rats were maintained on a 12-h light, 12-h dark photoperiod at 27°C. The animals were fed a complete reconstituted powdered diet with 2% casein and given water ad libitum for the entire study period. (11) Eighteen of the rats were inoculated s.c. in the right flank with 0.1 ml of a Walker 256 carcinosarcoma (Arthur D. Little, Inc., Cambridge, MA) cell suspension of approximately $1 \times 10^7$ cells. Six rats received a saline injection and served as controls. Food intake and weights were measured daily. Each day the site of tumor inoculation was inspected, and the length, width, and depth of the tumor were measured with calipers. Estimates of tumor volume were made with the formula for a prolate spheroid

$$V = L \cdot W \cdot D \cdot \pi/6$$

where $L$ is the length, $W$ is the width, and $D$ is the depth of the solid tumor. The measured tumor volume was used to estimate tumor weight based on regression analysis for tumor volume and weight at sacrifice. Tumor regression was defined as a loss of greater than 40% of the maximum tumor volume during the study period, and this was noted in 3 of the 18 rats. In this study, the 18 tumor-bearing rats were divided into 2 groups of equal number dependent upon final tumor size (i.e., large and small).

After an overnight fast on the 14th day of tumor growth, the rats were given injections of L-[1-13C]leucine (50 mCi/mmol; ICN Radiochemicals, Los Angeles, CA).
were placed in duplicate in 5 ml of 10% SSA, and a 1-3 g portion was crystalline powder. Portions of the tumor (1-2 g) were placed in 5 ml of saline for total nitrogen measurements. Finally, the entire carcass, including the unused liver portion, was frozen in liquid nitrogen and all of the tissues were stored at -25°C until the time of analysis. The entire process required 3 min.

Analytical Methods. The heparinized blood was centrifuged at 2500 rpm for 15 min, following which time the plasma was removed and stored at -25°C. The plasma was thawed at time of analysis, and 1 ml was deproteinized with 0.2 ml of 30% SSA for measurement of plasma leucine concentration and specific activity. The deproteinized serum was vortexed, incubated at 25°C for 30 min, and centrifuged for 15 min at 4000 rpm. Fifty µl were analyzed for total leucine content using an Beckman LS-8000 liquid scintillation spectrometer.

The frozen carcass and tumor were brought down to -180°C with liquid nitrogen and each was wrapped in cloth and crushed with a mallet. The small pieces were then placed in a Waring blender with solid (O° C) to keep the tissue from thawing and were ground to a fine, crystalline power. Portions of the tumor (1-2 g) were placed in 5 ml of 10% SSA and 5 ml of saline. Portions (1-3 g) of the whole-body crystals were placed in duplicate in 5 ml of 10% SSA, and a 1-3 g portion was placed in 5 ml of saline.

All tissue samples in 10% SSA (muscle, tumor, and whole body) were homogenized (Polytron homogenizer; Brinkmann Instruments, Westbury, NY) and centrifuged at 4000 rpm in order to separate the protein (precipitate) and the free intracellular (acid-soluble) amino acids for determination of leucine-specific activities in these two fractions. The supernatant was further spun down at 15,000 rpm to remove any contamination from the protein-bound fraction, and 50 µl were analyzed by HPLC for leucine concentration. An additional 1 ml was further treated with 0.2 ml of 30% (v/v) H2O2 to eliminate radioactivity found in a-ketosiacaproate (13) and incubated at 35°C for 60 min and then at 70°C for 30 min. This was then centrifuged, and 0.5 ml of the supernatant was added to 10 ml of commercial scintillant (Instagel); United Technologies Packard, Downers Grove, IL) and analyzed for total 14C radioactivity with a Beckman LS-8000 liquid scintillation spectrometer.

The injection of 150 µmol of leucine/100 g body weight was used to ensure adequate distribution of leucine and specific radioactivity to the acid-soluble portion of tumor tissue, since the distribution of leucine throughout the Walker 256 tumor tissue has not been previously reported. Measurement of [14C]leucine incorporation into protein after a large bolus dose has two theoretical advantages: (a) because of the reduction in the differences between the specific radioactivity of the free amino acid in the plasma and tissue, the error from the inability to identify the specific radioactivity at the site of protein synthesis is minimized; (b) the isotopic steady state would be maintained for a short period (10 min) of study so that label recycling, which underestimates protein-synthetic rate in rapidly turning over tissue, such as liver and tumor, would also be minimized. Recent evidence supports that recycling after prolonged infusions makes an important contribution to protein flux and can lead to underestimates of protein flux of about 25%. (16) Large doses of leucine over a short period of time have failed to stimulate nonnontumorous protein synthesis, (17) and it is assumed that tumor tissue would respond in a similar way.

Calculations. In order to estimate the rates of protein synthesis and breakdown in the tumor, we used the model of Waterlow et al. (18) which accounts for changes in protein mass during non-steady states. In this model, synthesis, breakdown, and growth are represented by fractional constants and first-order kinetics. Fig. 1 shows the growth curve of the tumor, represented as mean values for the 15 tumor-bearing rats with nonregressing tumors. The weight of the tumor, as a function of time, could be approximated by

\[ W_2 = W_1 e^{K_p} \]  

(A)

In this equation, \( W_1 \) and \( W_2 \) are the tumor weights at times \( t_1 \) and \( t_2 \), and \( K_p \) is the fractional growth rate constant. This rate constant (\( K_p \)) is the algebraic sum of two other first-order rate constants, \( K_s \) and \( K_d \), which represent the fractional rates of protein synthesis and degradation, respectively.

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The abbreviations used are: SSA, sulfosalicylic acid; HPLC, high performance liquid chromatography.
W256 INFLUENCE ON PROTEIN METABOLISM AND GROWTH RATES IN RATS

There was a significant reduction ($P < 0.05$) in the fractional growth rate in rats bearing larger tumors to 1.8%/day. Total-body protein synthesis over the 6-day period of observation in the rats bearing larger tumors ($40.5 \pm 6.9$ g) was reduced, compared to rats with smaller tumors and controls ($56.2 \pm 6.9$ and $63.9 \pm 11.5$ g ($P = 0.14$), respectively).

In addition to differences in tumor size and body weight, there were also differences in the weight of liver, which was significantly larger in the tumor-bearing rats, ($P < 0.01$) and the size of the liver as percentage of nontumorous body weight correlated with tumor weight ($r = 0.918; P < 0.001$). The percentage of nitrogen composition of the liver was similar in both control ($3.04 \pm 0.22\%$) and tumor-bearing ($3.15 \pm 0.15\%$) rats; consequently, total nitrogen content of the liver was significantly increased in the tumor-bearing rats.

Fractional synthetic rates for whole body and muscle are represented in Table 2. The whole-body protein-synthetic rate was slightly but not significantly reduced in rats with larger tumors from 34.7 to 21.7%/day ($P < 0.07$). This estimate of whole-body $K_s$ of 34.7%/day for the non-tumor-bearing 220-g animals is similar to that measured by McNurlan and Garlick (20) using $[^{14}\text{C}]$leucine by the flooding dose method. The rate for whole-animal protein synthesis in g per day can be normalized to 5.0 g per 100 g nontumorous body weight/day, is comparable with previous estimates using the methodology of constant infusion of labeled amino acid $[4.7 \text{g/day} (21) 4.0 \text{g/day} (18)]$, and is slightly higher than the amount determined using a flooding dose in younger animals ($3.1 \text{g/day}$). (20) The mean daily muscle protein synthesized, assuming that skeletal muscle represents 40% of nontumorous body weight, (18) was significantly reduced in the animals bearing larger tumors (Table 2).

In the tumor-bearing rats, whole-body protein synthesis and muscle protein synthesis showed an inverse correlation with the weight of tumors at sacrifice ($r = -0.523$ and $-0.723; P < 0.05$ and $< 0.01$, respectively). In addition, body growth ($K_r$) was also negatively correlated with tumor weight ($r = -0.848, P < 0.01$). In comparison, whole-body and muscle protein synthesis directly correlated with body growth: $[(K_r); r = 0.380$ and 0.563; $P < 0.05$ and $< 0.01$, respectively]. Our data suggest then that the Walker 256 carcinosarcoma influences whole-animal protein synthesis by reducing both skeletal muscle and whole-body protein synthesis (Table 2). Tumor growth and protein kinetic data are listed in Table 3. Tumor $K_s$ was significantly lower in the large compared to the small nonregressing tumors ($48.6\%$ versus $84.7\%$; $P < 0.05$). The daily mean and final day tumor protein synthesis were similar in both tumor groups. Nitrogen content of the tumor averaged $2.05 \pm 0.11\%$ and did not vary with tumor size. The tumor leucine concentration was $7.66 \pm 0.50\%$ of the total nitrogen content.

Changes in tumor volume were used to calculate tumor growth which averaged $31.7\%$/day for the 15 nonregressing tumors. The regressing tumors ($n = 3$) followed complex growth curves and were excluded from this calculation. A curve showing tumor growth, derived from the means of 15 data points, is presented in Fig. 1 to illustrate the first-order kinetics pattern followed by these tumors in the period between 7 and 13 days following implantation. Growth rate constants were obtained for individual tumors and showed a faster growth rate (34.3 versus 27.7%/day) for the larger tumors as indicated in Table 3.

Tumor $K_s$ was determined by subtracting $K_r$ from tumor $K_s$ (Table 3). This estimate is determined from $K_s$ measurements in the fasting state since it has been shown that tumor tissue is...
W256 INFLUENCE ON PROTEIN METABOLISM AND GROWTH RATES IN RATS

Table 1 Clinical characteristics of Walker 256 carcinosarcoma-bearing and non-tumor-bearing rats

<table>
<thead>
<tr>
<th>Tumor size</th>
<th>Initial body wt (g)</th>
<th>Final body wt (g)</th>
<th>Tumor wt (g)</th>
<th>Food intake (g/day)</th>
<th>Nontumorous wt gain (g/day)</th>
<th>Liver wt (g)</th>
<th>Liver wt (% of nontumorous body wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy control (6)</td>
<td>143 ± 2*</td>
<td>223 ± 6</td>
<td>4.2 ± 0.8</td>
<td>7.4 ± 0.5</td>
<td>6.9 ± 0.4</td>
<td>3.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Small tumor-bearing (9)</td>
<td>148 ± 3</td>
<td>224 ± 5</td>
<td>10.7 ± 1.7</td>
<td>7.4 ± 0.5</td>
<td>8.5 ± 0.3</td>
<td>3.8 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Large tumor-bearing (9)</td>
<td>150 ± 3</td>
<td>202 ± 10</td>
<td>29.0 ± 3.2</td>
<td>2.3 ± 1.8</td>
<td>9.6 ± 0.3</td>
<td>4.8 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± SE.  
* P < 0.01.  
* P < 0.05.  
* P < 0.001.

Table 2 Fractional synthesis rates and protein synthesis

Fractional synthetic rates (Ks) of individual tissues and whole body of controls and tumor-bearing rats on day 14. Fractional rates of protein synthesis were determined in rectus muscle and total carcass at 1 min after a bolus of 150 µmol of L-leucine (containing 30 µCi of [14C]leucine)/100 g body weight. The total protein synthesis was determined by multiplying the fractional synthesis rate by the change in protein mass divided by the first-order growth rate constant determined for the period of observation (see equation D). Muscle mass was assumed to represent 40% of nontumorous body weight. The mean daily protein synthesis was then determined by dividing total synthesis by the time of the observation (6 days). The fractional synthetic rates for muscle were significantly reduced (P < 0.05, analysis of variance) and that of whole body was marginally reduced (P < 0.07) in the larger tumor-bearing rats. The daily muscle protein synthesis was significantly reduced (P < 0.05, analysis of variance) in this same group.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Muscle</th>
<th>Whole body</th>
</tr>
</thead>
<tbody>
<tr>
<td>%/day</td>
<td>g/day</td>
<td>%/day</td>
</tr>
<tr>
<td>Control (6)</td>
<td>22.4 ± 3.8*</td>
<td>2.8 ± 0.6</td>
</tr>
<tr>
<td>Small tumor-bearing (9)</td>
<td>20.3 ± 1.2</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>Large tumor-bearing (9)</td>
<td>15.0 ± 1.1*</td>
<td>1.7 ± 0.2*</td>
</tr>
</tbody>
</table>

* Mean ± SE.  
* P < 0.05.  
* P < 0.07.

Table 3 Growth and fractional synthesis and breakdown rates of nonregressing Walker 256 carcinosarcoma studied in vivo

Individual growth rates (Kg) were determined for tumor tissue by plotting tumor volume over the final 7-day period of observation with tumor volume following first-order growth rates for small and large tumors (r² = 0.85; 0.92). Fractional synthetic rates (Ks) of tumor tissue were determined by measurements of [14C]leucine incorporation into tumor protein. Smaller tumors had a significantly greater Kg than larger tumors after 14 days of growth (P < 0.01). Fractional breakdown rates (Kb) were determined by the difference of two other first-order rate constants, fractional synthetic rates (Ks) and Kg. Tumor Kg was significantly (P < 0.01; t test) reduced in the larger compared to the smaller tumor group. There was a slight but nonsignificant increase in tumor protein synthesis at day 13 for the large tumor group. There was no difference in mean protein synthesis between both tumor sizes.

<table>
<thead>
<tr>
<th>Tumor size</th>
<th>Kg (%/day)</th>
<th>Ks (%/day)</th>
<th>Kb (%/day)</th>
<th>Protein synthesis on day 14 (g/day)</th>
<th>Mean protein synthesis, days 7–13 (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small nonregressing (6)</td>
<td>27.7 ± 7.2*</td>
<td>84.8 ± 5.1</td>
<td>57.0 ± 11.2</td>
<td>1.2 ± 0.2</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Large nonregressing (9)</td>
<td>34.3 ± 5.6</td>
<td>48.6 ± 9.1*</td>
<td>14.3 ± 9.9*</td>
<td>1.6 ± 0.3</td>
<td>0.7 ± 0.1</td>
</tr>
</tbody>
</table>

* Mean ± SE.  
* P < 0.05.  
* P < 0.01.

DISCUSSION

The contribution of the tumor to the total percentage of protein synthesized (whole body plus tumor) was 8 to 10% when the tumor weight was 5 to 13% of total body weight (small and large tumors, respectively). These estimates, which are based on the mean protein synthesis rates between days 7 and 13 (Equation D and accompanying text), are shown in Fig. 2. In spite of similar dietary intakes, rats bearing larger tumors demonstrated a 36.4% reduction in muscle protein synthesis when compared to the rats bearing smaller tumors. A similar study, (24) using the Walker 256 carcinosarcoma in rats, also demonstrated reduced protein synthesis in muscle tissue by 26.1% when studied in vitro. The decreased incorporation of 14C into tissue protein was explained by a 29.4% increase in muscle leucine oxidation. Increases in whole-body protein oxidation in in vivo studies have been demonstrated, (25) although uncommon, (6, 9, 21) so that reductions in muscle and whole-body protein synthesis seen in the larger Walker 256 carcinosarcoma may be due to elevations in oxidation rates seen uniquely with this tumor. The flooding dose methodology, however, did not permit an evaluation of oxidation rates.

Many factors may contribute to the reduction in body growth rate in the rats bearing larger tumors including fuel availability and/or hormonally induced alterations in whole-body protein synthesis. In the present experiment, suppression of muscle protein synthesis contributed in part to reductions in whole-body protein synthesis observed in the rats bearing larger tumors and to the reduced overall growth rate. Tumor protein synthesis partially compensated for the reduction in muscle and whole-body protein synthesis in the rats bearing smaller tumors (Fig. 2). In the larger tumor group, despite an increase in the amount of tumor protein synthesis, there was a greater reduction in muscle and whole-body protein synthesis. It therefore

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Fig. 2. Estimate of protein synthesis. Mean whole-body protein synthesis in healthy rats was 10.7 g/day and was reduced to 6.7 g/day in rats with large tumors. Muscle protein synthesis was reduced significantly ($p < 0.05$) from 2.8 to 1.7 g protein per day in the same group. Muscle protein synthesis in healthy control animals represented 26% of whole-body protein synthesis, and this was reduced to 23% of whole animals (whole body plus tumor) protein synthesis for animals with larger tumors. Tumor protein synthesis represented 8 and 10% of total protein synthesis, respectively, of the animals with nonregressing small and large tumors.

It appears that the reduction in whole-body protein synthesis is not completely explained by changes in muscle and tumor compartments. Other sources of active protein synthesis, such as the gastrointestinal tract, must also contribute to the overall reduction in whole-body protein synthesis. Evidence to support this comes from Radcliffe and Morrison, (26) who documented over a 30% reduction in intestinal weight and presumably protein mass in rats of similar age and type after 20 days of Walker 256 carcinosarcoma growth.

Nevertheless, changes in whole-body and muscle-synthetic rates appear to be the major factors responsible for alterations in growth rates in the Walker 256 cancer-bearing rats. This observation is consistent with the earlier work of Millward et al., (27) which demonstrated that muscle mass is regulated primarily through alterations in protein synthesis. Whole-body growth, with or without tumor, had a parallel change with reductions in the synthetic rates, which suggests that alterations in growth may be dependent upon these changes.

The finding of reduced $K_p$ in the larger tumors is of interest since it suggests that a possible factor affecting tumor growth is regulated by the process of protein degradation. As indicated in Table 3, the average growth rate of the group of large tumors was faster than that of the small tumors. The fact that this difference did not achieve statistical significance is probably related to the variability inherent in tumor volume measurements early in tumor growth. Indeed, using the last 3 days of tumor measurements, the growth rates are significantly different for the two groups of tumors (data not shown). It should be noted, however, that a conclusion about reduction of $K_p$ in larger tumors is possible only if assumptions of first-order kinetics for both protein synthesis and breakdown hold throughout the period of observation. These assumptions have been discussed by Waterlow (18) with regard to the use of isotope tracer methods for measurement of protein synthesis. The growth curve in Fig. 1 suggests that tumor growth of the Walker 256 carcinosarcoma could be approximated reasonably well with first-order kinetics between days 7 and 13, thus making estimation of breakdown rates feasible as described ($K_d = K_e - K_p$).

It is also understood that for the calculated rates of protein breakdown to apply, the growth characteristics of the tumors at the time synthesis was measured should be similar to those represented by the growth curve from day 7 to 13. Theoretically, sudden and severe reductions in growth rates could have occurred on day 14 in a manner which would account in part for the reduced synthesis rates in the animals with larger tumors. However, such changes were not obvious in our study and would need to be further addressed by a different experimental design. In fact, using the last 3 days of tumor growth for regression analysis (data not shown) results in $K_e/K_p$ ratios of 0.46 and 0.85 ($p < 0.01$) in the large and small tumors, respectively. The reduction in protein synthesis in the larger tumors is not inconsistent with previous literature, (21, 28) and protein synthesis rates in tumor tissue have been negatively correlated with tumor volume. (28) Furthermore, low rates of protein breakdown have been reported in transformed cell lines (a bronchial carcinoma, MCF7 and T47D human breast tumors, and SV40-transformed 3T3 fibroblasts), which contribute to the rapid growth rate of cancer cells. (22) We conclude, therefore, that while this study is not conclusive, it does support the possibility that protein breakdown regulates tumor growth in the Walker 256 carcinosarcoma in rats.

Finally, these findings do not supersede the host of immunological, hormonal, and metabolic factors that play vital roles in the mathematical relationships defined in this study that determined the onset and rapidity of tumor growth. Rather, the combination of biochemical measurement of tumor $K_e$ and anatomical measurements of tumor growth ($K_p$) as done in this study provides a method of determining the theoretical protein breakdown and its relationship to tumor growth. The reduced protein breakdown observed in this study of cancerous tissue lends support to the theory of tumors acting as nitrogen traps (19) as well as providing insight into potential novel approaches to alter tumor growth. In particular, directing chemotherapeutic agents towards increasing tumor protein breakdown in solid tumors, in addition to the conventional ones of altering protein synthesis by antimitabolites, may offer a new avenue of therapeutic approach.

REFERENCES


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