The Metabolism of Plasma Glycoproteins

II. Studies on the Rate of Incorporation of Glucosamine-1-\(^{14}\)C into Protein-bound Hexosamine in the Rat Bearing Walker 256 Carcinoma

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Summary

The origin of the elevated serum glycoproteins observed in rats bearing 6- and 12-day-old Walker 256 carcinoma has been studied utilizing glucosamine-1-\(^{14}\)C as a precursor. The results indicate that the liver is quantitatively the major site of serum glycoprotein biosynthesis as is the case in the normal animal. While the rate of incorporation of glucosamine-1-\(^{14}\)C into hepatic protein-bound hexosamine was not altered from normal in the early stages of tumor development, the 12-day-old Walker carcinoma provoked a significant increase in the hepatic binding of glucosamine-1-\(^{14}\)C. In both tumor age groups the biologic half-life of the hepatic protein-bound hexosamine was longer than in normal animals.

In vitro studies following hepatectomy, with and without isolation of the tumor, and in vitro studies would strongly suggest that the tumor too is metabolically active with respect to glycoprotein biosynthesis and release. Furthermore, the rate of synthesis appears to depend on the volume of viable tumor rather than its age.

Evidence is presented which indicates that, in tumor bearing rats, the biosynthesis of the seromucoid fraction is significantly increased. In tumor bearing rats seromucoid synthesis represents 23-27% of the total serum glycoprotein synthesized as compared with a figure of 8.9% for normal animals. The biologic half-life of both the total serum glycoprotein and the seromucoid fraction is, however, significantly shorter in tumor bearing than it is in normal rats.

Analysis of the urine in both tumor age groups revealed that over 90% of the radioactivity that was recoverable in the urine was associated with free glucosamine and other as yet uncharacterized compounds.

The incorporation of administered glucosamine-1-\(^{14}\)C into body tissues, other than liver and tumor, was minimal in comparison.

Introduction

It is now well established that the growth of a malignant tumor is accompanied by an elevation of the serum glycoproteins in the human (11, 16, 35, 40, 45) and in experimental animals harboring transplantable tumors (4, 20, 37, 43). It has also been demonstrated that total surgical excision of a malignant tumor in the human is followed by a gradual reversion of the serum glycoprotein level to normal levels (16, 40).

Estimation of the carbohydrate constituents of the seromucoid fraction of serum proteins has shown that the composition, as well as the concentration, of seromucoid differs from normal in rats bearing Walker 256 carcinoma (18). Tissue protein-bound carbohydrate studies in the same experimental model have also demonstrated an increase in hepatic protein-bound hexosamine and an increase in both protein-bound hexosamine and N-acetylneuraminic acid in the leg muscle adjacent to the transplanted tumor (17). In this latter study the tumor too was found to be rich in bound carbohydrate with levels that reached a relatively steady state 6 days after implantation. A similar observation was made by Catchpole (4) who found increased serum and tissue protein-bound carbohydrate levels in mice implanted with fibrosarcoma. On the basis of these observations a number of hypotheses as to the origin of the elevation of serum glycoproteins in malignant disease have been proposed (4, 35, 37, 43). However none of the hypotheses have been adequately substantiated and the origin of the observed elevation remains in doubt.

Extensive studies on the site of biosynthesis of normal serum glycoproteins utilizing glucose-1-\(^{14}\)C or glucosamine-1-\(^{14}\)C in vitro have demonstrated the liver to be the major, if not the sole, organ involved (2, 24, 25, 30, 32, 36, 39). Studies utilizing the isolated perfused rat liver have also provided direct evidence for the hepatic synthesis of serum glycoproteins, and in addition, have confirmed that the rate of incorporation of labeled glucose into the seromucoid fraction occurs more rapidly than into the other serum glycoproteins (31, 33, 34).

While reports on human studies are infrequent it has been shown that the decay of administered \(^{131}\)I-labeled orosomucoid occurs more slowly in patients with parenchymatous liver disease and more rapidly in patients with inflammatory disease as compared with normal individuals (44).

In a previous paper the metabolism of the serum glycoproteins in the intact rat utilizing glucosamine-1-\(^{14}\)C as a precursor has been reported (19). Since all previous studies dealing with the biosynthesis of serum glycoproteins have been carried out in normal biologic systems it appeared timely to investigate the rate of incorporation of glucosamine-1-\(^{14}\)C into the protein-bound hexosamine of various tissues, total serum glycoproteins, and the seromucoid fraction in a pathologic setting. The rat bearing Walker 256 carcinoma has been selected for this study.
Materials and Methods

Adult male Sprague-Dawley rats weighing from 280 to 320 gm were selected. Animals were housed 4 to a cage, and maintained on a standard grain diet (15) with tap water ad libitum. Male Sprague-Dawley rats bearing 6-day-old Walker 256 carcinoma served as donors. Donor animals were anesthetized with Nembutal and the entire tumor removed intact and transferred to a beaker containing 0.85% sodium chloride solution. Tumors were then dissected free of connective tissue and blocks of apparently viable tumor tissue were transferred to a glass homogenizer. Three parts of sterile saline were added to the combined tumor tissue blocks and gently homogenized for 30–40 sec. Microscopic examination of the homogenate demonstrated that 85–90% of the cancer cells were intact. Implantation of 0.15 ml of this homogenate into the thigh musculature of the right hind limb of the recipient rat was carried out by means of a 1-ml tuberculin syringe fitted with a 25-gauge needle. A total of 121 animals received tumor implants and were divided into 2 groups: (a) 60 animals were studied on the 6th day following transplantation, and (b) 61 animals were studied on the 12th day following transplantation. The natural history of the progress of implanted Walker 256 carcinoma in our laboratory corresponds to that recorded by Weimer et al. (43).

Freshly prepared D-glucosamine-1-14C, with a specific activity 1.70 mc/m mole, was obtained from New England Nuclear. Each rat in the above groups received a single dose of 15.5 µc of glucosamine-1-14C (9.1 µmoles) in 0.5 ml of 0.9% NaCl i.p. at zero time. At the time of sacrifice the tumor bearing rats were lightly anesthetized with ether and as much blood as possible withdrawn from the abdominal aorta. With the needle in situ each animal was immediately perfused via the aorta with 20 ml of 0.85% sodium chloride solution and this procedure repeated. The rationale for double saline perfusion has previously been presented (17). After perfusion, liver, kidney, spleen, lung, muscle adjacent to tumor, and normal leg muscle from the non-tumor bearing hind limb as well as viable and necrotic Walker 256 carcinoma were removed and placed in Dry Ice. The tissue samples, 1.5–2.0 gm wet weight, were homogenized with 9 parts of ethanol and centrifuged. The residue was then resuspended in 30 ml of acetone at room temperature for 1–2 hr and centrifuged. This latter procedure was repeated. The acetone defatted, air dried, samples were then subjected to analysis.

Measurement of Expired CO2 (14). Nine rats were subjected to this assay; 3 animals representing each of the groups, normal, 6- and 12-day tumor bearing rats. Following administration of a single dose of 5.0 µc of glucosamine-1-14C i.p. at zero time animals were placed in metabolic bottles and room air continuously provided. Expired CO2 was trapped in carbon dioxide free 4 N NaOH. Samples were removed every hour following injection of the tracer up to 7 hr. In the case of each sample a 0.2-ml aliquot was made up to 1.0 ml with distilled water in a counting vial and 15 ml of polyethylene-611 counting solution was added. All analyses were performed in triplicate. Radioactivity was measured in a Nuclear-Chicago liquid scintillation counter.

Urine Analysis. Following many attempts, bladder catheterization in the rat was discarded as a satisfactory means of urine collection since mechanical trauma with bleeding was consistently produced. Metabolic cages, too, appeared inadequate for urine collection for this study. As a result the following technique was employed.

Under light ether anesthesia, two 3-0 silk ties were placed about the penis just proximal to the cartilaginous tip in a group of 12, 6-, and 12-day tumor bearing rats. Each animal then received 5.0 µc of glucosamine-1-14C i.p. at zero time and animals were permitted to recover. At hourly intervals, ranging from 1 to 10 hr, 2 rats in each group were lightly anesthetized and through a relatively bloodless midline suprapubic incision the bladder was readily visualized. Bladder aspiration, without significant trauma, was readily performed by means of a 27-gauge needle and tuberculin syringe. Repeat bladder aspiration was carried out on the same animals after an interval of 3 hr. Radioactivity of urine samples was measured in a Nuclear-Chicago liquid scintillation counter utilizing the method of Jeffay and Alvarez (12).

Hepatectomy Experiment. A total of 41 animals were utilized for this study. Nine 12-day tumor bearing rats were subjected to hepatectomy and 9 to sham operation as previously described (19). Nine 6-day tumor bearing rats were subjected to each of these procedures. An additional 5 animals bearing 6-day Walker tumors were studied after isolation of the tumor bearing limb combined with hepatectomy. Isolation was achieved by ligation of the femoral artery and vein and application of a tourniquet at the groin to occlude the remaining circulation.

Each rat then received a single dose of 5.0 µc of glucosamine-1-14C dissolved in 0.5 ml of 0.9% saline i.v. via the vena cava at the conclusion of the operative procedure. Animals were killed at appropriate intervals following isotope administration and serum and tissue subjected to the identical procedure to that carried out on intact tumor bearing rats.

In Vitro Experiment (6, 7, 14, 21, 38). Normal and tumor bearing rats were used to provide normal liver, viable tumor, and normal muscle for studies on the rate of in vitro incorporation of glucosamine-1-14C into protein-bound hexosamine. Animals were anesthetized with ether and the liver, tumor, and muscle tissue quickly removed and placed in beakers containing ice cold Krebs-Ringer phosphate buffer solution. Tissue samples were sliced 50–100 µ in thickness using a Stadie-Riggs tissue slicer and 2 gm of wet tissue immediately placed in an Erlenmeyer flask containing 8 ml of Krebs-Ringer phosphate buffer solution at pH 7.4. Flasks were then shaken at 37°C for 2 min in a Dubnoff metabolic shaker at 70 oscillations per min to achieve temperature equilibrium. Glucosamine-1-14C, 1.0 µc, was then added and incubation continued for selected time intervals. After incubation, 8 ml of ice cold distilled water was added to each flask and the reaction stopped by the addition of 4.0 ml of 3.5 N perchloric acid. The flask contents were transferred quantitatively into homogenizer flasks and homogenized for 2 min in a chilled VirTis homogenizer. The homogenates were then transferred to centrifuge tubes and centrifuged at 4000 rpm in a refrigerated Serval centrifuge.

The acid insoluble protein was resuspended in 25 ml of 95% ethanol and, following centrifugation, the residue was washed twice with acetone. The dry samples were then subjected to acid hydrolysis in order to permit estimation of the specific activity of the protein-bound hexosamine after column purification (1). Isolation and Purification of Serum and Tissue Protein-bound Hexosamine (1, 8). The isolation and purification of tissue, serum, and seromucoid protein-bound hexosamine was carried out as previously described (19).
ISOLATION OF SEROMUCOID FROM THE SERUM OF TUMOR-BEARING RATS (42). In order to estimate the rate of incorporation of labeled glucosamine into seromucoid hexosamine the seromucoid was first isolated by the method of Weimer and Moshin (42). The seromucoid fraction was then hydrolyzed and the hexosamine isolated and measured as described above.

DETERMINATION OF RADIOACTIVITY (3, 5, 29). Purified samples of hexosamine isolated from various tissue, from the total serum protein and the seromucoid fraction, from urine samples, and from sodium hydroxide containing respiratory carbon dioxide, were studied. The effluent (0.2–1.0 ml) was pipetted into counting vials and 10–15 ml of scintillation phosphor (35, 36) were added. The samples were counted twice for 10 min on a model 725 Nuclear-Chicago liquid scintillation counter by a channel ratio method (3).

The instrument was standardized daily with an unquenched ¹⁴C standard. A set of 6 quenched standards (Nuclear-Chicago) were also counted daily in order to establish the correction curve. The counts were corrected for quenching. The results are expressed as dpm/µmole of hexosamine or as percentage of the administered dose.

IDENTIFICATION OF HEXOSAMINE. In order to establish the purity of hexosamine after Dowex-50 column purification, samples from liver, kidney, serum, and Walker 256 carcinoma were directly chromatographed on Whatman No. 1 paper or on thin layer chromatographic plates with an n-butanol-ethanol-water (4:1:1) (41) or an ethyl acetate-pyridine-water-acetic acid (5:3:1) solvent system (9). For positive identification of the hexosamine from tissue and serum the following pure carbohydrate standards were also simultaneously chromatographed: d-glucosamine, d-galactosamine, d-mannosamine, d-galactose, d-mannose, and N-acetylglucosamine.

In order to further characterize the nature of the hexosamine isolated from tissues and serum the column purified samples were lyophilized and crystallized (10). The crystallized samples were dried over phosphorous pentoxide and subjected to chromatographic analysis as described above.

Confirmation of the identity of the standard amino sugars and the hexosamine isolated from tissue and serum was achieved with silver nitrate (27), anisidine phtholate (28), and the Morgan and Elson (27) color reactions. A 2nd set of paper chromatograms was carried out using a long wave length, ultraviolet lamp. The hexosamine isolated from tumor, liver, and kidney proved to have glucosamine as the major constituent although trace quantities of galactosamine was also detectable. The radioactivity was, however, limited to the area corresponding to the glucosamine. The hexosamine isolated from rat serum was composed of a single amino sugar, namely glucosamine.

Results

The data presented in the following tables and charts are expressed as dpm X 10⁻³/µmole of hexosamine, or as total activity in microcuries calculated from the mean pool of hexosamine of various tissues and of serum. In the tables the latter is expressed as a percentage of the administered dose.

RATE OF INCORPORATION OF GLUCOSAMINE-¹⁴C INTO THE LIVER, SERUM, AND SEROMUCOID PROTEIN-BOUND HEXOSAMINE

The specific activity of liver, serum, and seromucoid-bound hexosamine in rats bearing 6-day-old tumor is presented in Table 1. Rapid incorporation of labeled glucosamine into liver protein-bound hexosamine occurs, reaching a maximal specific activity 60 min after the introduction of the isotope. At that time 13.7% of the injected glucosamine-¹⁴C was found to be incorporated into the protein-bound hexosamine of the liver. The maximal specific activity of serum and seromucoid was observed 5 hr after the injection of isotope. Although the peak for both serum and seromucoid occurred at the same time, the magnitude of serum specific activity was consistently higher than that of serum suggesting that the seromucoid fraction may be released into the circulation at a faster rate.

In order to establish the total rate of incorporation of glucosamine-¹⁴C into the serum and seromucoid bound hexosamine, the mean pool of hexosamine was calculated from the hexosamine level assuming that the serum volume was 4% of the body weight (19, 39). Utilizing these figures the total incorporation of labeled glucosamine into serum and seromucoid hexosamine of both 6- and 12-day tumor bearing animals was calculated. It can be seen
in Table 1 that at the peak of specific activity 20.99% of the injected dose was present as serum protein-bound hexosamine and 4.94% as seromucoid-bound hexosamine. The seromucoid-bound hexosamine to total activity of serum protein-bound hexosamine was compared it was found to be 0.235 comparable ratios in healthy animals were found to be 0.09 for hexosamine and 0.89 for radioactivity (19).

Table 2 summarizes the data obtained relative to the liver, serum, and seromucoid protein-bound hexosamine of rats bearing 12-day-old Walker 256 carcinoma. Incorporation of glucosamine-1-14C into liver protein-bound hexosamine occurred at a faster rate than that observed in 6-day tumor bearing rats. The maximum specific activity was attained 30 min after injection of the labeled amino sugar. It can be seen that, at the point of maximal specific activity, 21.08% of the injected dose appeared in the form of liver protein-bound hexosamine compared to a maximal figure of 12.84% in the normal animals (19), and 13.7% in 6-day tumor bearing rats. It is apparent that the maximal specific activity of both serum and seromucoid-bound hexosamine occurs 2 hr after isotope administration. This is significantly earlier than the 5 hr required to achieve maximal specific activity in normal rats (19, 36, 39) or rats bearing 6-day-old tumor. It is also apparent that the activity curves of serum and seromucoid-bound hexosamine do not exhibit the same decay pattern as is observed in normal and 6-day tumor bearing rats. At the point of maximal specific activity 18.65% of the injected glucosamine-1-14C is present as protein-bound hexosamine, 78.44 ± 5.42 amoles for 12-day-old tumor protein-bound hexosamine, and 68.49 ± 6.02 amoles for necrotic tumor protein-bound hexosamine.

### Table 2

**Rate of Incorporation of Glucosamine-1-14C into Liver, Serum, and Seromucoid Protein-bound Hexosamine in Rats Bearing 12-Day-old Walker 256 Carcinoma**

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>No. of Rats</th>
<th>Liver</th>
<th>Serum*</th>
<th>Seromucoid*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Specific activity (dpm X 10^6)</td>
<td>Specific activity (dpm X 10^6)</td>
<td>Specific activity (dpm X 10^6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(μmol/ml)</td>
<td>(μmol/ml)</td>
<td>(μmol/ml)</td>
</tr>
<tr>
<td>6</td>
<td>0.25</td>
<td>96.05</td>
<td>17.48</td>
<td>3.27</td>
</tr>
<tr>
<td>6</td>
<td>0.50</td>
<td>115.82</td>
<td>21.08</td>
<td>8.96</td>
</tr>
<tr>
<td>6</td>
<td>1.00</td>
<td>111.68</td>
<td>20.33</td>
<td>27.38</td>
</tr>
<tr>
<td>6</td>
<td>1.50</td>
<td>104.44</td>
<td>19.01</td>
<td>48.24</td>
</tr>
<tr>
<td>6</td>
<td>2.00</td>
<td>79.05</td>
<td>14.39</td>
<td>69.63</td>
</tr>
<tr>
<td>6</td>
<td>3.00</td>
<td>49.98</td>
<td>9.03</td>
<td>55.04</td>
</tr>
<tr>
<td>4</td>
<td>4.00</td>
<td>36.87</td>
<td>6.71</td>
<td>52.47</td>
</tr>
<tr>
<td>4</td>
<td>5.00</td>
<td>30.79</td>
<td>5.01</td>
<td>50.85</td>
</tr>
<tr>
<td>4</td>
<td>7.00</td>
<td>28.11</td>
<td>5.12</td>
<td>45.86</td>
</tr>
<tr>
<td>4</td>
<td>10.00</td>
<td>14.34</td>
<td>2.61</td>
<td>35.58</td>
</tr>
<tr>
<td>4</td>
<td>15.00</td>
<td>14.22</td>
<td>2.59</td>
<td>22.83</td>
</tr>
<tr>
<td>4</td>
<td>25.00</td>
<td>14.51</td>
<td>2.64</td>
<td>17.67</td>
</tr>
<tr>
<td>4</td>
<td>48.00</td>
<td>10.5</td>
<td>1.91</td>
<td>6.50</td>
</tr>
</tbody>
</table>

**a** Mean serum hexosamine level 7.58 ± 0.71 μmoles/ml of serum.

**b** Mean seromucoid hexosamine level 2.16 ± 0.21 μmoles/ml of serum.

**c** T.A. = Total activity.
earlier than that observed in the serum. However, the maximal old tumor occurs 3 hr after the administration of isotope, 2 hr earlier than that observed in the serum. It is also apparent that the Walker tumor is the next tissue to exhibit significant binding of glucosamine-1-14C into the protein-bound hexosamine prior to its discharge into the circulation. It is of interest to note that the magnitude of the maximal specific activity of the protein-bound hexosamine of 6- and 12-day-old viable tumor is almost identical.

It is apparent that, at the time of maximal specific activity, 2.58% of the injected glucosamine-l-14C is incorporated into the protein-bound hexosamine of 6-day-old tumor while 10.08% is incorporated into that of 12-day-old viable tumor. This magnitude of incorporation exceeds that of all tissues except the liver.

In order to obtain further information on the metabolism of serum glycoproteins under pathologic conditions the biologic half-life of the protein-bound hexosamine of liver, tumor, serum, and seromucoid was determined. The logarithm of the specific activities of these organs in 6-day as compared with 12-day tumor bearing animals the average specific activity at 8 selected times was assumed to be linear up to 10 hr for liver and 15 hr for tumor, serum, and seromucoid. They were then plotted against time by means of the least squares method. The biologic half-life data thus obtained is recorded in Table 4. It can be seen that the biologic half-life of liver protein-bound hexosamine in both tumor age groups is longer than in the control group. It is apparent too that the decay of the serum glycoprotein occurs at a significantly faster rate in tumor bearing animals than in the control group. It is worthy of note that the degradation of seromucoid-bound hexosamine is identical in the non-tumor-bearing extremity, the total activity of the muscle adjacent to a tumor may be metabolically involved in the biosynthesis of the elevated serum glycoprotein. In order to test this hypothesis the rate of incorporation of glucosamine-1-14C into the protein-bound hexosamine of the muscle adjacent to the tumor of 6- and 12-day tumor bearing rats as well as into that of normal muscle was also studied.

For comparative purposes the data on the specific activity of the protein-bound hexosamine of 6- and 12-day-old Walker 256 carcinoma are also recorded in Chart 1 along with that of the muscle adjacent to the tumor and that of normal muscle.

Although the protein-bound hexosamine of the muscle adjacent to the tumor in both 6- and 12-day tumor bearing rats manifest a significant increase in activity as compared with the muscle of the non-tumor-bearing extremity, the total activity of the muscle adjacent to the tumor, in both tumor age groups, is less than 0.5% of the injected glucosamine-1-14C. It may be worthy of note that the incorporation of glucosamine-1-14C into muscle protein-bound hexosamine is identical in the non-tumor-bearing extremity of a tumor bearing rat and in comparable muscle of the extremity of a non-tumor-bearing rat.

Incorporation of glucosamine-1-14C into the protein-bound hexosamine of normal muscle and muscle adjacent to Walker 256 carcinoma. It is obviously essential, in a study such as this, to determine the effect of the presence of a malignant tumor on the ability of tissues other than liver to incorporate glucosamine-1-14C into protein-bound hexosamine. Since there was no significant difference in the specific total activities of these organs in 6-day as compared with 12-day tumor bearing animals the average specific activity at 8 selected times has been recorded to indicate the metabolic activity of these 3 organs.

The specific activity curves of the protein-bound hexosamine rapidly increased and reached a maximum specific activity at 3 hr in 6-day-old tumor and 4 hr in 12-day-old tumor.

Table 4: Biological Half-Life of Bound Hexosamine in Normal Rats and Rats Bearing Walker 256 Carcinoma

<table>
<thead>
<tr>
<th></th>
<th>Liver (hr)</th>
<th>Serum (hr)</th>
<th>Seromucoid (hr)</th>
<th>Tumor (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>2.55</td>
<td>19.4</td>
<td>10.9</td>
<td>12.44</td>
</tr>
<tr>
<td>Rats bearing 6-day-old Walker 256 carcinoma</td>
<td>3.75</td>
<td>6.9</td>
<td>6.8</td>
<td>12.44</td>
</tr>
<tr>
<td>Rats bearing 12-day-old Walker 256 carcinoma</td>
<td>3.05</td>
<td>8.85</td>
<td>8.6</td>
<td>18.8</td>
</tr>
</tbody>
</table>

* The data are taken from our previous paper (19) and presented here for the purpose of comparison only.
of kidney, spleen, and lung are depicted in Chart 2. It can be seen that each organ has limited ability to incorporate labeled glucosamine-1-14C into protein-bound hexosamine. Also, the presence of a transplantable tumor did not stimulate these organs to incorporate more glucosamine-1-14C than that previously observed in non-tumor-bearing rats (19), and this was equally true of hepatectomized and intact animals (vide infra).

**INCORPORATION OF GLUCOSAMINE-1-14C INTO PROTEIN-BOUND HEXOSAMINE IN RATS BEARING 6- AND 12-DAY-OLD WALKER 256 CARCINOMA AFTER TOTAL HEPATECTOMY.** It is tempting, on a basis of the data thus far presented, to suggest that viable Walker 256 carcinoma has the ability to incorporate free glucosamine-1-14C into protein-bound hexosamine and contribute to the elevation of serum glycoproteins observed in the tumor bearing rat. Since the liver has been demonstrated to be the source of the serum glycoproteins in the normal rat (19) the question naturally arises as to whether the tumor has the ability to synthesize and release serum glycoprotein into the circulation in the absence of the liver. In order to answer this question a group of 6- and 12-day tumor bearing rats were subjected to total hepatectomy, while comparable control groups were subjected to a sham operation. Each animal received 5 µCi of glucosamine-1-14C i.v. at 0 time. It is apparent from Table 5 that, in the absence of the liver, the Walker 256 tumor has the ability to incorporate glucosamine-1-14C into tumor protein-bound hexosamine and release it into the circulation. It is interesting to note that the rate of incorporation of the labeled glucosamine into tumor protein-bound hexosamine was not altered by the absence of hepatic tissue.

Utilizing the mean serum hexosamine levels from Tables 1 and 2 the total incorporation of glucosamine-1-14C into the serum protein-bound hexosamine of sham operated animals was 20.2% of the injected dose in 6-day, and 17.8% of injected dose in 12-day tumor bearing rats, 3 hr after administration of isotope. These data were compared to those obtained in tumor bearing animals which were subjected to total hepatectomy. It was estimated that the average blood loss due to the surgical procedure was 4.2 ± 1.2 ml. This would result in a decrease in plasma volume from an estimated 14 ml to 9.8 ml in a 350-gm rat as was utilized for the hepatectomy experiments. The corresponding mean serum hexosamine levels are 58.8 μmoles for 12-day tumor bearing hepatectomized rats respectively. The ratios of the total activity of serum protein-bound hexosamine obtained from 6- and 12-day tumor bearing hepatectomized rats to the total activity of serum protein-bound hexosamine obtained from the correspond-
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The data from the combined hepatectomy and tumor isolation experiment presented in Table 5 strongly suggest that the majority of the labeled protein-bound serum hexosamine observed in 6- and 12-day tumor bearing rats after hepatectomy is of tumor rather than of some other extrahepatic origin.

**Rate of in Vitro Incorporation of Glucosamine-1-14C into Protein-Bound Hexosamine by Liver, Tumor, and Muscle.**

Since even in the combined hepatectomy and tumor isolation experiment it was impossible to separate the action of the tumor from that of some other tissue in the tumor bearing limb an attempt was also made to study the rate of incorporation of glucosamine-1-14C into the protein-bound hexosamine of liver, viable Walker 256 carcinoma, and normal leg muscle in an in vitro system. The results are presented in Chart 4. These results would further substantiate the thesis that the tumor itself has the necessary requirements for the effective synthesis of protein-carbohydrate complexes and may therefore, under appropriate conditions, actively contribute to the elevated serum glycoprotein level which is observed in the presence of malignant disease.

The appearance of the tracer compound in the expired CO₂ and urine of tumor bearing rats. It is apparent from Chart 5 that only 1.5% of the injected tracer dose was converted to respiratory CO₂ in 7 hr in both tumor age groups. This is almost identical to the results obtained from normal rats (13). On the other hand, 30% of the injected radioactivity was present in the urine in 6-day tumor bearing rats, and 39.0% in 12-day tumor bearing rats, within 10 hr following the administration of labeled glucosamine. In order to establish the nature of the labeled compound excreted in the urine, pooled urine samples collected 4-6 hr after the injection of isotope were subjected to gel filtration on a long (1.5 × 90 cm) Sephadex G-50 column. In both tumor age groups 2 peaks were present. The 1st small peak was protein in nature and contained a small amount of labeled protein-bound hexosamine. The radioactivity of this bound hexosamine represented only 2.5% and 7.4% of the total activity of the original urine samples in 6- and 12-day tumor bearing rats respectively. The 2nd peak, which contained over 90% of the radioactivity found in the urine, was of small molecular size. It was eluted from the column just before the salt peak occurred and gave a positive color reaction with the Elson and Morgan reagent (8). Spectrophotometric analysis however, revealed the presence of 2 peaks, 1 peak corresponding with that of pure D-glucosamine, while the other had a maximal optical density at 460 μm.

Similar results were obtained when samples of the 2nd peak were hydrolyzed in 3 n HCl for 4 hr at 100°C and column purified on Dowex 50 H+ columns prior to the color reaction. Although the compound behaved like hexosamine, the preliminary information obtained from paper chromatography suggests the presence of another labeled compound in addition to glucosamine. It was also observed that neither free nor bound N-acetylneuraminic acid was present in either of the urine samples.

**Discussion**

It is apparent from this study that the metabolism of serum glycoproteins in the presence of malignant disease is significantly different from that observed in normal animals.

In 6-day tumor bearing rats the injected glucosamine-1-14C is rapidly incorporated into the protein-bound hexosamine of the liver reaching a peak at 60 min. Discharge of protein-bound hexosamine from the liver into the circulation also occurs at a rapid rate. However, the specific and total activity of hepatic protein-bound hexosamine and its calculated biologic half-life, do not manifest any significant alteration from similar data obtained from normal animals. It is also apparent from this portion of the study that the 6-day-old tumor is the next tissue to incorporate...
significant amounts of labeled glucosamine into protein-bound hexosamine. The peak of tumor activity is reached at 3 hr which is earlier than that observed either in the total serum or the sero-mucoid fraction. Although the specific activity curves of the total serum and the seromucoid fraction are almost identical, reaching a maximum at 5 hr, the total activity curve of the seromucoid fraction clearly indicates that increased biosynthesis of this fraction occurs in the tumor bearing rat, as compared with the normal animals (19). The half-life studies on both the total serum and the seromucoid fraction appear to be identical in the 6-day tumor bearing rat but are significantly shorter than those observed in normal animals (19).

The metabolism of serum glycoproteins in the more advanced 12-day Walker 256 tumor bearing rat exhibits further departures from normal. The administered glucosamine-1-14C is rapidly incorporated into hepatic protein-bound hexosamine, reaching a peak at 30 min. This peak occurs earlier than that observed in either normal (19, 32, 36, 39) or 6-day tumor bearing rats. Moreover, total activity studies indicate that binding of glucosamine-1-14C to hepatic protein-bound hexosamine is quantitatively increased in 12-day tumor bearing rats. The maximal specific and total activities of both the total serum glycoproteins and the seromucoid fraction occur at 2 hr which is significantly earlier than that observed in normal (19, 32, 36, 39) or 6-day tumor bearing rats. The data obtained from this study would strongly support the view that one of the characteristic effects of the presence of a malignant tumor on the metabolism of glycoproteins in the host is an increased synthesis of the seromucoid fraction. In the normal animal at the peak of activity the total activity of the seromucoid fraction represents 8.9% of that of the total serum proteins while in the 12-day tumor bearing rat it represents 27%. A quantitative increase in the seromucoid fraction of serum in patients harboring malignant neoplasms and animals bearing transplantable tumors has previously been reported (11, 17, 40, 45).

The viable portion of the 12-day tumor plays a prominent role in the protein binding of administered glucosamine-1-14C. Although its maximal specific activity occurs at 4 hr, later than that of either the total serum or seromucoid protein-bound hexosamine, its total activity at this point represents 10% of the injected dose. The hepatopometric experiments and hepatopathy plus tumor isolation experiments reported would appear to indicate clearly that the tumor is capable of protein binding of administered glucosamine-1-14C and of its subsequent release into the circulation as glycoprotein. Furthermore, the in vitro experiments demonstrate that tumor tissue is in fact capable of such biosynthesis independent of the presence of the liver while comparative studies on the activity of the tumor and the muscle tissue adjacent to the tumor in this regard would implicate the tumor itself as the extrapathetic site of glycoprotein synthesis in the tumor bearing rat. Similar observations have been made in our laboratory on the in vitro metabolism of human cancer and tissue adjacent to it utilizing glucosamine-1-14C. The rate of incorporation of labeled glucosamine by 1 rectal and 1 colonic carcinoma proved to be 4–5 times as great as that observed in either colon adjacent to the tumor or colon at a distance from the lesion. This would suggest that human cancer, too, may contribute to the elevated serum glycoprotein levels observed in patients with malignant disease. Recent studies on glycoprotein synthesis of ascites tumor cells, in vivo and in vitro, utilizing glucosamine-1-14C would further support the hypothesis that the tumor itself is capable of contributing to the elevated serum glycoprotein levels observed in the presence of malignant disease (22, 23, 26, 46).

It would appear from these results that, while the liver may be capable of increased synthesis and release of glycoproteins into the circulation in the presence of well established tumor, the tumor itself makes a contribution to the observed serum glycoprotein elevation. This is of particular interest in view of the recent demonstration of a qualitative, as well as a quantitative, difference in the composition of the seromucoid fraction of rats bearing established Walker 256 carcinoma (18) and raises the question as to whether the glycoprotein of tumor origin may not differ qualitatively from that of hepatic origin. This latter possibility suggests the need for studies directed towards the characterization of, and the establishment of the mechanism of synthesis of, the tumor glycoprotein as well as the demonstration of its biologic function.

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