Tissue Glycoproteins in Rats Bearing Walker 256 Carcinoma*

Robert A. L. Macbeth and J. George Bekesi
(Department of Surgery and the Surgical-Medical Research Institute, University of Alberta, Edmonton, Canada)

Summary
In total, 137 rats have been sacrificed at intervals following the intramuscular implantation of Walker 256 carcinoma and the protein-bound hexosamines and N-acetylneuraminic acid determined in a variety of tissues. The tumor itself contained relatively large amounts of these carbohydrates, and the muscle adjacent to the tumor contained amounts significantly in excess of those found in normal muscle. The most interesting observation, however, was the early elevation observed in the protein-bound hexosamines of liver.

These observations, coupled with previous observations on plasma glycoproteins under similar circumstances, have been interpreted as favoring the liver as the source, at least of the initial plasma glycoprotein elevation observed in malignant disease.

Glycoprotein metabolism has rapidly become a field of intense interest to the biochemical research worker, and particularly the cancer investigator, if one can judge by the voluminous literature appearing on all facets of this complex problem in recent years. It is well known that plasma glycoproteins are elevated in malignant disease in humans (1, 6, 8, 20, 22, 26, 29, 30, 37, 38) and in experimental animals harboring experimental tumors (3, 10, 17, 21, 23, 28, 34, 36), and the references listed are by no means exhaustive.

However, in spite of this activity, very little is known about the mechanism by which these changes are achieved and even less about their significance. Some investigators (26) believe that the observed plasma elevation merely represents liberation into the plasma of breakdown products of tissue necrosis. Others (24, 28, 29) believe that they represent the liberation of a product of the metabolic activity of rapidly reproducing cells—i.e., they mirror hyperplastic processes. There are also those (10) who, on a basis of their observation that the tissue adjacent to a malignant focus has a greater than normal content of glycoprotein, have suggested that the elevated plasma glycoprotein reflects the depolymerization of the ground substance of this adjacent connective tissue, with subsequent release into the circulation. Additional support for this thesis has been reported by other observers on the basis of their studies on nonmalignant disease processes (13, 14). Still others (9, 24, 33) have suggested that the mechanism is far more complex and in some way involves increased hepatic synthesis and release of glycoproteins into the circulation in response to an appropriate stimulus such as tumor, inflammation, etc. Even more recently Patterson et al. (25) have presented results that would suggest that the protein-bound carbohydrate elevation observed in transplantable tumors is a function of bacterial contamination rather than of the growth of the neoplastic tissue per se. If confirmed this study would make it necessary to re-assess the literature relative to neoplasia and glycoproteins.

Since none of these hypotheses may be accepted as substantiated and since the mechanism of production of the elevated plasma glycoproteins observed in malignant disease is of importance even in the event that it is bacteria-initiated, it was decided to study the tissue glycoproteins of various organs of the rat in serial fashion following transplantation of Walker 256 carcinoma in the hope that these observations might contribute to resolving this controversy.

Materials and Methods
For this study male Sprague-Dawley rats weighing from 250 to 300 gm. were used. They were housed two per cage and maintained on a standard grain diet (19) with tap water ad libitum. Twenty-five rats served as untreated controls, and 137 animals received tumor implants. Transplantation was performed as previously described (21); no transplantation failures occurred.

Test animals were sacrificed in groups commencing 24 hours following implantation, whereas the control rats were killed in five separate groups at 7-day intervals. The rats were lightly anesthetized with ether, and at laparotomy as much blood as possible was removed from the abdominal aorta. With the needle in situ each animal was immediately perfused via the aorta with 20 ml. of normal saline and the procedure repeated. After the perfusion liver, kidney, spleen, lung, leg muscle from the tumor-free hind limb (left), leg muscle adjacent to the tumor (right), and Walker 256 carcinoma were removed and placed in dry ice. The tissue samples thus obtained were homogenized with

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The isolation and purification of tissue N-acetylnearaminic acid (sialic acid) were achieved by the method of Gottschalk (15). Part of the acedean-afected tissue was hydrolyzed in 0.1 n sulfuric acid at 80° C. for 1 hour. The hydrolysate was removed and the procedure was repeated 3 times. After isolation with Dowex 2-x8, 200-400 mesh resin, sialic acid was determined by Svennerholm's reisorbmate method (33). The effluents were also subjected to paper chromatography (31) with butanol:propanol:0.1 N HCl (1:2:1), and the compound moved as a single spot which corresponded with the movement of purified N-acetylnearaminic acid.

Tissue protein content was determined by the micro-Kjeldahl method of Archibald (2). To estimate possible interference from retained blood in the various organs, hemoglobin determinations were carried out on the tissue homogenates with the quantitative benzidine reaction (11). It is of interest to note that Burton et al. (9) observed that liver perfusion in the rat had no significant effect on the level of any of the tissue proteins or protein-bound carbohydrates studied by them. In addition, their calculations based on blood volume in the liver and the level of various blood proteins also suggested that blood contamination was unimportant. Our own findings with respect to the hemoglobin content of various tissues of the normal rat are recorded in Table 1, expressed on a basis of both acetone dry tissue and tissue protein. Subsequently, only the latter method of presentation will be utilized. A considerable range from tissue to tissue is apparent. In the case of kidney, lung, and spleen no significant variation from the normal values was observed with respect to either the hexosamines or sialic acid on serial determinations following Walker 256 carcinoma transplantation. For this reason they are not referred to in subsequent tables.

Observations on the effect of tumor growth on tissue protein-bound hexosamines are recorded in Table 3. It is noteworthy that the first significant change occurs in the liver. In this organ the hexosamines are significantly elevated above normal as early as the 2d day, reach a peak

### Table 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Without perfusion* (per cent)</th>
<th>Double saline perfusion* (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>5.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Kidney</td>
<td>4.3</td>
<td>1.2</td>
</tr>
<tr>
<td>Muscle</td>
<td>2.8</td>
<td>1.6</td>
</tr>
<tr>
<td>Tumor</td>
<td>2.0</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* Expressed as percentage of dry weight.

9 parts of ice-cold acetone and then centrifuged. The residue was then re-suspended in 30 ml. acetone, which was changed twice at 24-hour intervals. The acetone-defatted and air-dried samples were then subjected to analysis. Estimation of hexosamine content was carried out by hydrolyzing a part of the defatted tissue sample in 4 ml of 3.5 n hydrochloric acid for 15 hours at 100° C. in a sealed serum bottle, and after isolation with Dowex-5 ion exchange resin hexose was determined by the method of Elson and Morgan (12) as modified by Boas (7).

The natural history of the progress of implanted Walker 256 carcinoma as observed in our laboratory has already been recorded (21).

The protein-bound hexosamines and sialic acid content of various tissues of the normal rat are recorded in Table 2, expressed on a basis of both acetone dry tissue and tissue protein. Subsequently, only the latter method of presentation will be utilized. A considerable range from tissue to tissue is apparent. In the case of kidney, lung, and spleen no significant variation from the normal values was observed with respect to either the hexosamines or sialic acid on serial determinations following Walker 256 carcinoma transplantation. For this reason they are not referred to in subsequent tables.

### Table 2

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Protein-bound hexosamines</th>
<th>Protein-bound N-acetylnearaminic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tissue protein/ 100 gm acetone-dried tissue</td>
<td>Mg/100 gm tissue protein*</td>
</tr>
<tr>
<td>Liver</td>
<td>66.5</td>
<td>525</td>
</tr>
<tr>
<td>Kidney</td>
<td>85.3</td>
<td>685</td>
</tr>
<tr>
<td>Lung</td>
<td>93.7</td>
<td>760</td>
</tr>
<tr>
<td>Spleen</td>
<td>91.6</td>
<td>518</td>
</tr>
<tr>
<td>Leg muscle</td>
<td>87.4</td>
<td>195</td>
</tr>
</tbody>
</table>

* Including the standard deviation = \( \sqrt{\frac{\sum(x - \bar{x})^2}{n}} \).

### Table 3

<table>
<thead>
<tr>
<th>Tumor age (days)</th>
<th>No. animals</th>
<th>Liver*</th>
<th>Muscle adjacent to tumor*</th>
<th>Muscle contralateral extremity*</th>
<th>Walker 256 carcinoma*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>10</td>
<td>788 ± 17</td>
<td>255 ± 17</td>
<td>222 ± 19</td>
<td>242 ± 25</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>876 ± 49</td>
<td>211 ± 19</td>
<td>242 ± 25</td>
<td>242 ± 25</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>1293 ± 71</td>
<td>315 ± 21</td>
<td>300 ± 13</td>
<td>300 ± 13</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>1251 ± 75</td>
<td>270 ± 18</td>
<td>192 ± 20</td>
<td>192 ± 20</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>1310 ± 105</td>
<td>370 ± 36</td>
<td>262 ± 14</td>
<td>262 ± 14</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>1362 ± 92</td>
<td>261 ± 23</td>
<td>208 ± 6</td>
<td>208 ± 6</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>1398 ± 89</td>
<td>342 ± 13</td>
<td>245 ± 17</td>
<td>245 ± 17</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>1037 ± 98</td>
<td>442 ± 21</td>
<td>280 ± 24</td>
<td>280 ± 24</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>1639 ± 113</td>
<td>430 ± 9</td>
<td>291 ± 14</td>
<td>291 ± 14</td>
</tr>
<tr>
<td>12</td>
<td>5</td>
<td>1373 ± 91</td>
<td>359 ± 18</td>
<td>270 ± 10</td>
<td>270 ± 10</td>
</tr>
<tr>
<td>14</td>
<td>5</td>
<td>1080 ± 60</td>
<td>369 ± 15</td>
<td>224 ± 4</td>
<td>224 ± 4</td>
</tr>
<tr>
<td>16</td>
<td>5</td>
<td>930 ± 69</td>
<td>436 ± 32</td>
<td>310 ± 16</td>
<td>310 ± 16</td>
</tr>
<tr>
<td>18</td>
<td>4</td>
<td>740 ± 87</td>
<td>362 ± 26</td>
<td>292 ± 9</td>
<td>292 ± 9</td>
</tr>
<tr>
<td>20</td>
<td>4</td>
<td>925 ± 50</td>
<td>332 ± 24</td>
<td>270 ± 12</td>
<td>270 ± 12</td>
</tr>
<tr>
<td>22</td>
<td>4</td>
<td>668 ± 53</td>
<td>450 ± 10</td>
<td>290 ± 19</td>
<td>290 ± 19</td>
</tr>
<tr>
<td>24</td>
<td>3</td>
<td>892 ± 40</td>
<td>357 ± 19</td>
<td>225 ± 9</td>
<td>225 ± 9</td>
</tr>
</tbody>
</table>

* Tissue protein-bound hexosamines in mg/100 gm tissue protein ± the standard deviation.

† P <0.01.
level on the 10th day, and return to a normal value by the 16th day following tumor implantation.

The muscle adjacent to the tumor also manifests a significant change in the content of protein-bound hexosamine with tumor growth. A statistically significant elevation appears first on the 4th day, whereas from the 6th day on no significant variation from normal values occurred.

Table 4 records our observations with respect to tissue protein-bound N-acetylneuraminic acid or sialic acid.

Sequential data with respect to liver hexosamines have been included for comparison with liver hexosamines, although no significant variation from normal values occurred.

In the case of muscle adjacent to the tumor a significant elevation of the protein-bound sialic acid is apparent as early as the 3d day. The elevated concentration of this compound appears to manifest two plateaus—an early lower plateau extending from the 3d to the 10th day and a later higher plateau which is apparent on the 12th day and continues to the conclusion of the experiment. Again the muscle of the contralateral limb manifests no significant change in its sialic acid concentration with tumor growth.

The Walker 256 carcinoma itself, once large enough for assay, exhibits a rapid increase in sialic acid content up to the 6th day, a temporary reduction during the two subsequent observations, and then reaches a relatively steady state on the 12th day. On the last day of observation a particularly high concentration of this sugar is recorded.

Table 5 records the ratios of protein-bound N-acetylneuraminic acid to protein-bound hexosamines in various rat tissues. The significance of these observations will be referred to in the discussion. The average values for serum have been derived from material presented in a previous paper (20). Two values are given for muscle adjacent to the tumor in transplant experiments. This appeared desirable in view of the apparent double plateau of elevation of the protein-bound N-acetylneuraminic acid previously referred to.

**DISCUSSION AND CONCLUSIONS**

Shetlar (27) has recently drawn attention to the essential obstacle to our understanding of the relationship of plasma glycoproteins to various disease processes when he states: "The significance of the changes that occur in the serum glycoproteins is fundamentally related to their origin. If a clear concept of the origin of these compounds existed, the question of their significance could more readily be answered." The present investigation would suggest that the liver plays a prime role as the source of at least the initial glycoprotein elevation observed in animals harboring malignant neoplasms.

It would appear from this study that the first demonstrable deviation from normal following tumor transplantation is an increased concentration of protein-bound hexosamine in the liver. This finding precedes any demonstra-
ble increase in either protein-bound hexosamines or N-acetyleneuraminic acid in the muscle adjacent to the tumor and also precedes any measurable concentration of these monosaccharides in the developing tumor itself.

It is interesting, in retrospect, to look at our previous findings with respect to plasma protein-bound monosaccharides following tumor transplantation (21). Although significant elevation occurs relatively late, there is evidence of a change in the nature of the total plasma glycoprotein as early as the 1st day. The ratio of hexosamines to N-acetyleneuraminic acid may be seen to be 1:0.72 prior to transplant, 1:0.82 on the 1st, 1:0.92 on the 2d, and 1:1.06 on the 3d post-transplant day. Following this a relatively constant ratio persists throughout the remainder of the period of observation. We interpret this to indicate that, whatever the source of the glycoprotein liberated into the circulation in response to the presence of malignant disease, it occurs early after transplantation and is relatively N-acetyleneuraminic acid-rich relative to the average composition of the plasma glycoprotein present in a state of health.

If the findings reported at this time are interpreted to implicate the liver as the source of the initial rise in plasma glycoproteins, the failure to demonstrate an early increased concentration of liver protein-bound N-acetyleneuraminic acid following transplantation is difficult to reconcile with the plasma findings. It is possible, however, since the hexosamines have been shown to be the binding sugar of the oligosaccharide prosthetic group (16), that the initial steps in the synthesis only are mirrored in these data and that the N-acetyleneuraminic acid is subsequently added elsewhere or that once the complete N-acetyleneuraminic acid-containing oligosaccharide is synthesized in the liver it is almost instantaneously released into the circulation.

The liver has been previously suggested as the site of origin of the elevated plasma glycoproteins observed in various disease states, but direct evidence has been lacking. The types of observations that have led to this suggestion include first the demonstration of depressed plasma glycoprotein levels in acute parenchymous disease of the liver (24, 34) and second the demonstration, in normal animals, with radioactive tracer techniques, that glucose and glucosamine are the precursors of protein-bound glucosamine and that this binding is manifest first in the liver and only subsequently in the plasma (27). More recently Burston et al. (9) and Tombs et al. (33) have provided more direct evidence for this hypothesis in their study of various protein fractions and their associated carbohydrates in the liver tissue of patients with and without malignant disease. The present findings do confirm the previous observations (5, 10, 33) that there is a significant concentration of protein-bound carbohydrates in the developing tumor itself and a significant elevation in these compounds in the tissue adjacent to the tumor. However, the temporal relationship between these changes and those observed in the plasma would not support the thesis that the tumor (28, 29) or the tissue adjacent to it (10) is the source of the initial plasma elevation, although they may contribute to it at a later date. Wada et al. (34) have presented the only direct evidence which has come to our attention in support of this thesis. They interpret their results as indicating increased glycoprotein levels in the efferent venous drainage of a fowl sarcoma as compared with its afferent blood supply. However, on the other hand, Kent and Gey (18) have demonstrated that serum glycoproteins are preferentially utilized by tumor cells growing in tissue culture, and one might therefore expect the tumor to remove such compounds from the circulation rather than add them to it.

Although the present study does not prove that the liver is the origin of the increased plasma glycoproteins observed in malignant disease, it does add support to such a hypothesis and suggests further avenues of investigation that might be expected to clarify this question.

REFERENCES

1. Almqvist, P. O., and Lausing, E. A Study of Serum Glyco-
Seligson (ed.), Standard Methods of Clinical Chemistry,
3. Baldwin, R. W., and Harris, H. J. Serum Protein and Glyco-
protein Changes during Growth of Experimental Tumors in the
4. Barcelo, R.; Roipel, P.; and Legresley, L. P. Effects of
Oxymethabutazon (Tandearil) on Plasma Seromucoids. I. In
5. Barker, S. A.; Stacey, M.; Tupper, D. J.; and Kirkman,
J. H. Some Observations on Certain Mucopolysaccharides Contain-
6. Bernasconi, C.; Buscarni, L.; and Ezeheli, S. Phospho-
tase, Protein and Glycoprotein of Serum in Patients with
Prostatic Carcinoma. Effects Induced by Estrogen Treatment.
7. Boas, N. F. Method for the Determination of Hexosamines in
8. Bonomo, E., and Boncelli, M. I. Mucopolisaccaridi Serici
Comportamento Nei Pazienti Portatori di Neoplasia Tumori,
9. Burston, D.; Tombs, M. P.; Apsey, M. E.; and Maclagan,
N. F. The Perchloric Acid Soluble Basic and Acidic Proteins of
10. Catchpole, H. R. Serum and Tissue Glycoproteins in Mice
11. Crossby, H., and Damaskin, W. The Significance of Hemoglo-
binemia and Associated Hemosiderinuria with Particular
Reference to Various Types of Hemolytic Anemia. J. Lab.
for the Determination of Glucosamine and Chondrosamine.
13. Engel, M. B. Mobilization of Mucoprotein by Parathyroid
15. Gottschalk, A. The Chemistry and Biology of Sialic Acids
16. ———. The Relation between Structure and Function in Some
17. Hokkanen, P. K.; Pyorala, K.; and Tyskalin, E. The Mucop-
protein Level of Serum and Ascitic Fluid during Development of
ITB Aspects Tumor of the Rat. Acta Microbiol. Scandinav.,
20:15-20, 1956.
18. Kent, H. N., and Gey, G. O. Selective Uptake of Serum Gelo-
bulins and Glycoproteins by Cells Growing in Vitro. Science,
19. Kowalewski, K. Serum and Liver Lipids in Rats Treated


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