Protein-Bound Carbohydrates in Breast Cancer.
Liquid-Chromatographic Analysis for Mannose, Galactose, Fucose, and Sialic Acid in Serum

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We describe high-resolution chromatographic analysis for protein-bound sialic acid in serum, with use of a cerate oxidimetric detector. Values for sera from normal women averaged 680.5 mg/liter, with a coefficient of variation of 23%. Including data obtained by previously developed chromatographic procedures for protein-bound mannose, galactose, and fucose, we assessed sera from breast-cancer patients whose malignancy had been categorized as either stable, responsive, or progressive (based on clinical observations spaced from two to five months apart). All of 12 responsive patients had decreases of protein-bound fucose averaging 34.5% (SD, 18.1) and all of 10 patients with progressive disease had increases averaging 38.3% (SD 21.5). Changes in fucose averaged less than 6.7% (SD 4.9) for eight patients with clinically stable breast cancer. Changes in protein-bound mannose, galactose, and sialic acid did not correlate as well as did fucose with the clinical disease status of the patients.

Additional Keyphrases: cerate oxidimetric detector • monitoring cancer • serum glycoproteins • normal values

The existence of glycoproteins, and their importance in many forms of life, has been recognized for more than 15 years. However, much about the biological function of the sugar moieties in glycoproteins remains a mystery. In some cases their function is fairly obvious. For example, mucins have a lubricating function, which can be related to carbohydrate residues. In other cases a particular sugar residue may act as a signal for recognition, either by other proteins or by whole cells (1). Thus, the carbohydrate moieties may influence growth and cell-to-cell interactions and be of importance in the development of cancer. Increased concentrations of serum glycoproteins have been reported in human subjects with malignant diseases (2) and in serum from laboratory animals having induced tumors (3). However, increased concentrations of serum glycoproteins in serum are not a specific sign of malignant disease; they may appear in patients with renal, rheumatic, or infectious diseases, cardiovascular disorders, or biliary obstructions (4–7).

The real role that carbohydrates play in moderating the activity of protein is one of the least-understood phenomena in human biochemistry. Some interesting studies on the role of sialic acid in this context have been reported recently. Watkins et al. (8) reported in vitro studies showing that serum with abnormally high protein-bound sialic acid from a cancer-free human non-specifically blocked host immune response to tumor cells, and this blocking effect could be eliminated by partial enzymatic removal of sialic acid from serum glycoproteins with neuraminidase (EC 3.2.1.18). Other studies, performed by injecting animals with radioactively labeled glycoproteins, have demonstrated that glycoproteins whose terminal sialic acid residues were stripped off by neuraminidase to expose galactose residues were subject to rapid removal from the circulation by the liver. The same glycoproteins with terminal sialic residues intact displayed normal turnover in the circulation (see ref. 1). The implication of these studies seems to be that terminal sialic acid residues might protect a foreign protein from rejection caused by host immune response.

Rosato et al. (9) reported an apparent relationship between increased concentrations of protein-bound fucose in serum and the presence of malignant breast masses. Previously, we reported methodology for the chromatographic analysis of the three neutral protein-bound carbohydrates (mannose, galactose, and fucose) in serum and analytical data on samples from normal women and those with metastatic breast cancer (10). Concentrations of the three carbohydrates were generally increased in hydrolysates of serum from the patients with cancer, the increase in fucose being pro-

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portionately higher than that in mannose and galactose. The present study extends the analytical methodology to include sialic acid in addition to the three neutral carbohydrates and describes the variation of these four protein-bound carbohydrates with relation to the clinical status of breast cancer. The purpose of this work is to investigate the potential of these molecular entities as biochemical markers of breast cancer, not so much in a diagnostic sense, but more as a means of measuring the population of malignant cells to guide chemotherapeutic treatment in maximizing their destruction.

Methods and Materials

Chromatographic System

The chromatographic system used for the analysis of the neutral carbohydrates of glycoproteins was described in a previous publication (10). This system was modified for the chromatographic analysis of protein-bound sialic acid. A shorter column (100 × 0.22 cm i.d.) was used, and its jacket temperature was maintained at 60 °C by a constant-temperature circulating bath. The detection system used was the cerate oxidimetric detector as described previously (10). A linear relation of both the peak area and peak height to concentration was demonstrated for amounts of sialic acid ranging from 6.5 to 39.1 μg eluted from the anion-exchange column (Figure 1).

Initial separations of hydrolyzed serum samples were performed by using a concentration gradient of ammonium acetate/acetic acid (pH 4.3). The gradient caused sialic acid to be eluted after about 7 h. Few extraneous chromatographic peaks were observed (no other major peaks); consequently, conditions were sought that would enable a more rapid analysis of sialic acid without the necessity of column regeneration. A number of different concentrations of the acetate buffer were tested to find isocratic elution conditions that would enable separation of the peak corresponding to sialic acid from the large peak appearing at sample breakthrough. We found that the combination of an eluent consisting of 0.2 mol/liter ammonium acetate/acetic acid (pH 4.4) and a column temperature of 60 °C yielded a satisfactory separation of serum hydrolysates (Figure 2).

Analytical Method

The neutral protein-bound carbohydrates of serum glycoproteins were analyzed as described previously (10). Sialic acid, more labile than the carbohydrates, was analyzed separately as described below:

1. Precipitate the protein in 0.5 ml of serum by adding 3 ml of ethanol (95%) followed by centrifugation (15 min, 5000 rpm); discard the supernate.
2. Wash the precipitate by suspension in 3 ml of ethanol, again centrifuge and discard the supernate.
3. Dissolve the protein by gentle agitation in 1.0 ml of 0.1 mol/liter NaOH.
4. Add 2.0 ml of 0.1 mol/liter H2SO4; mix by swirling, deaerate with nitrogen, and seal tightly with a septum-sealed cap (Teflon-faced septum).
5. Place the sample in a heating block maintained at 80 °C for 1 h; then cool to room temperature and neutralize to an approximate pH of 4 with 0.25 ml of 1 mol/liter NaOH. Centrifuge the hydrolysate and remove the supernate for chromatographic analysis.

Evaluation of Experimental Variables

The neutral carbohydrates of glycoproteins were hydrolyzed in 1.0 mol/liter HCl for 4 h at 100 °C as described previously (10). Under similar hydrolytic conditions, sialic acid loses about 25% of its nitrogen content in the form of ammonia (11, 12). To avoid degradation of this labile compound, milder hydrolysis conditions are generally used for the release of protein-
To further test the method, we did multiple analyses of a pooled serum sample to establish a baseline value for the sample. Known amounts of sialic acid were added to the precipitated protein from this pooled serum and the samples were analyzed in the usual manner. The results (Table 1) showed the average recovery to be 92.4% of the added material, with a standard deviation of 3.8%.

Effect of Freeze-Thaw Cycles on Sialic Acid Analysis

A pooled serum was prepared from freshly thawed sera (stored at −70 °C). The pooled sample was thawed for about 1 h, sampled for analysis, and then refrozen for three days before thawing again. This thaw-freeze cycling was continued through three cycles. Analytical results indicated thaw-induced changes in sialic acid content with an increase of 7.8% after the second cycle and a decrease of 18.3% after the third cycle (Table 2). These results suggest that samples may undergo at least two thaw-freeze cycles without significant change in sialic acid content.

Clinical Classification of Breast Cancer Patients Included in Study

Patients included in this study of the variation of protein-bound carbohydrates (mannose, galactose, fucose, and sialic acid) with changing malignancy status had clinically demonstrable and biopsy-proved metastatic breast cancer. Local therapy used for these patients was either a radical or a modified radical mastectomy; in a few patients this was followed by radiotherapy. Further therapeutic regimes employed were: (a) bilateral oophorectomy, (b) diethylstilbestrol, or (c) 28-day cycles of either cyclophosphamide, methotrexate, and 5-fluorouracil; or cyclophosphamide, Adriamycin, and 5-fluorouracil; or Adriamycin and dibromodulcitol. Samples collected from patients receiving chemotherapy were obtained at least 10 to 21 days after the preceding dose of drug.

The patients were classified into three different clinically defined disease categories: responsive, pro-

### Table 1. Analytical Recovery of Sialic Acid Added to Three Pooled Samples of Serum

<table>
<thead>
<tr>
<th>Pool</th>
<th>Initial analysis</th>
<th>Added Sialic acid, mg/liter</th>
<th>Found</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>639.6</td>
<td>(1.4)&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>403.6</td>
<td>1019.7</td>
</tr>
<tr>
<td>II</td>
<td>628.0</td>
<td>(2.9)&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>403.6</td>
<td>987.6</td>
</tr>
<tr>
<td>III</td>
<td>493.2</td>
<td>(4.0)&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>558</td>
<td>994.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>558</td>
<td>1039.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>558</td>
<td>1007.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>558</td>
<td>993.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Coefficient of variation, %.
<sup>b</sup> Three analyses of the same hydrolysate.
<sup>c</sup> Four analyses of four separate hydrolysates.

mean 92.4 ± 3.8
gressive, and stable. A responsive classification was defined, for lesions measured in two dimensions, as at least a 50% reduction in the sum of the products of the longest perpendicular cross-sectional measurements of the lesions in the organ system. In situations where lesions were measurable in only one dimension (e.g., liver enlargement) a 30% reduction was required. An estimated 50% return to normality was required for tumors that could be evaluated but not measured (e.g., lymphangitic pulmonary metastases). This reduction in tumor burden had to persist for at least 28 days in at least half of the involved organ systems to qualify for a disease response classification. The second classification—progressive disease—was defined as the appearance of any new lesion or a greater than 25% increase in any existing lesion, measured as described previously, so long as the increase was at least 2 cm² in bidirectionally measured lesions and 2 cm in unidirectionally measured lesions and persisted for more than six days. A stable clinical status was designated in those situations where criteria did not yet exist for either responsive or progressive disease status.

**Results and Discussion**

**Protein-Bound Sialic Acid in Serum from Healthy Women**

We did chromatographic analyses for protein-bound sialic acid in sera from 19 healthy women subjects. The mean was 680.5 mg/liter (CV, 23.3%). The range was 294 to 919 mg/liter; the two SD range for the results was 383 to 996 mg/liter.

We have previously reported data on the protein-bound neutral carbohydrates in sera from normal women (10). Mannose averaged 444 mg/liter, galactose 436 mg/liter, and fucose 48 mg/liter for nonfasting healthy women.

**Effects of Changing Clinical Status of Breast Cancer on Protein-Bound Carbohydrates**

In an attempt to evaluate the efficacy of protein-bound carbohydrates as potential biochemical markers of breast cancer, we assayed a series of serum samples from patients with clinically defined breast cancer. Patients in each of the three disease categories—stable, responsive, and progressive—were selected, and at least two serum samples, obtained over periods of time ranging from two to five months were analyzed for protein-bound mannose, galactose, fucose, and sialic acid. All analyses were performed blind; i.e., patient

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**Table 2. Effect of Thawing and Refreezing Serum on Analysis for Sialic Acid**

<table>
<thead>
<tr>
<th>Thaw cycle</th>
<th>Sialic acid, mg/liter</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>657</td>
<td>3.6a</td>
</tr>
<tr>
<td>Second</td>
<td>708</td>
<td>2.5a</td>
</tr>
<tr>
<td>Third</td>
<td>537</td>
<td>3.4a</td>
</tr>
</tbody>
</table>

*Four analyses of four different hydrolysates.*

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![Fig. 5. Comparison of variations in protein-bound sialic acid for breast-cancer patients with clinical status of the malignancies](image-url)
classification was not revealed until after the results were collated.

Figure 4 shows a comparison of the changes in protein-bound fucose for breast-cancer patients whose disease has been classified into the three clinical categories. Figure 5 compares, for many of the same patients (same symbols in each category represent the same patient), protein-bound sialic acid for each of the three clinical categories. It is immediately apparent that changes in protein-bound fucose in every case correctly mirrored the clinical assessment of the patient's malignancy, whereas this was not true for sialic acid. Changes in protein-bound fucose averaged less than 6.7% (SD, 4.9%) for eight patients whose disease was classified as clinically stable. All 12 patients classified as having clinically responsive disease projected decreases in protein-bound fucose that averaged 34.5% (SD, 16.1%). All 10 patients with progressive malignancies displayed increases in fucose, averaging 38.3% (SD, 21.5%). Decreases in sialic acid were noted for the five patients whose clinical status was classified as stable, with a rather dramatic decrease noted for one patient (Figure 5). Six of eight patients whose disease was responding to treatment showed declines in sialic acid, while only three of eight patients showed increases where the malignancy was progressive. Note that most of the data for sialic acid are within ±2SD of the mean for the normal data, whereas 67% of the initial fucose measurements for responsive disease and 60% of the final measurements for progressive disease exceeded +2SD from the mean.

Figures 6, 7, and 8 compare fucose, sialic acid, mannose, and galactose for the three clinical conditions. A single patient has the same symbol for each of the four carbohydrates. Note that 10 of 12 patients showed declines in mannose and galactose for responsive disease (Figure 7), paralleling the fucose data. One of the two patients showing a reversal in this trend with increased galactose also showed corresponding increases in mannose and sialic acid. The decrease in fucose for this patient (solid reversed triangle, Figure 7) was not as significant as noted for the remainder of the responsive patients; thus it is possible that this particular patient was undergoing a change in the status of her malignancy.

In general, for progressive disease, decreases in one of the three carbohydrates (mannose, galactose, or sialic acid) also appeared to be reflected in the other two
Both fucose and sialic acid are terminal molecules in the carbohydrate chains of glycoproteins, and so it might be anticipated that an increase in the number of residues of fucose might be accompanied by a corresponding decrease in sialic acid. However, our initial results do not seem to show such an interrelationship between these two compounds. Our results do show good correlations of increasing protein-bound fucose with progressive breast cancer and decreasing fucose with responsive malignancy, and this is encouraging. Further studies are needed on other types of malignancies, with use of analytical methods of high specificity and with emphasis on protein-bound fucose and sialic acid.

Changes in protein-bound sialic acid, mannose, and galactose show only limited correlation with the clinical status of the malignancy. Of necessity, because of a delay in developing methodology, the samples which were analyzed for sialic acid had previously been thawed, analyzed for fucose, mannose, and galactose, then refrozen. Although the effect of one freeze-thaw cycle on sialic acid analysis seems relatively small (Table 2), it would be desirable to perform all analyses at the time the sample was first thawed.

References