The Synthesis of Hexosamine in Tumor Homogenates

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Leloir and Cardini (17) described the essential reactions leading to the enzymatic production of glucosamine. Using extracts from Neurospora crassa, they reported that glucosamine-6-phosphate was synthesized from hexose-6-phosphate and glutamine. Subsequent research by these and other investigators demonstrated glucosamine synthesis in pig kidney extracts (18), rat liver extracts (23, 94), and a spectrum of tissues from the growing rabbit (6). Of the rabbit tissues studied, the synthesis observed in epiphyseal cartilage homogenates was markedly high (6). Kulonen (16) described the Flexner-Jobling carcinoma, the Jensen hepatoma, were investigated with respect to amino sugar synthesis.

In view of the consistent observation of amino sugar synthesis in mammalian connective tissue, it was of interest to determine whether tumors of both epithelial and connective tissue origin retained enzymatic potential for the synthesis of amino sugars. Dunham and Stewart (10), in a review of transplantable animal tumors, described the Flexner-Jobling carcinoma, the Jensen sarcoma, and the Walker carcinosarcoma as being derived from epithelial tissue, connective tissue, and both epithelial and connective tissue. Therefore, these neoplasms, as well as azo dye-induced hepatomas, were investigated with respect to amino sugar synthesis.

MATERIALS AND METHODS

The Jensen sarcoma,1 the Walker carcinosarcoma 256, and a hepatocarcinoma (3'-Me-DAB tumor), induced by feeding 3'-methyl-4-dimethylaminoazobenzene, were carried in the Holtzman rat by the transplantation method described by Talalay et al. (31). The Flexner-Jobling carcinoma was carried subcutaneously, and the Novikoff hepatoma was carried intraperitoneally. The tumors were harvested at appropriate intervals after transplantation, freed of gross necrotic and hemorrhagic areas, and were homogenized in a Potter-Elvehjem homogenizer. The tumors were homogenized in a citrate buffer (6), pH 7.0, containing sufficient magnesium chloride to give a final concentration of 0.055 M in the reaction mixture.

For the studies of amino sugar synthesis in liver, normal animals were sacrificed by cervical fracture, and the livers were quickly excised and perfused with ice-cold physiological saline. The livers from three or more animals were pooled and homogenized in a 0.1 M phosphate buffer (14), pH 6.6.

All the homogenates were incubated in Warburg reaction vessels at 38°C in air, and the tissue concentration in these vessels was approximately 300 mg (wet wt.)/ml. Aliquots were removed from each reaction vessel for nitrogen determinations by the Kjeldahl procedure of Ma and Zuazaga (20). Cell suspensions of the solid Walker tumor were prepared by suspending tumor particles in 0.089 M phosphate buffer, pH 7.4 (1), containing 0.25 M sucrose, and by fragmenting these particles with a loose-fitting glass tissue suspender. The cells were separated from the debris by four successive passages through glass-wool filters. The filtrate was then centrifuged at 100 × g for 2 minutes and the supernatant removed by suction. Cells were resuspended in a citrate buffer (6) and again were centrifuged for 2 minutes at 100 × g. The supernatant was discarded and fresh citrate buffer added to give a final tissue concentration of ca. 600 mg (wet wt.)/ml. The concentration of glucosamine was determined upon 1-ml. aliquots removed from the reaction vessels at appropriate time intervals. The method described by Elson and Morgan (11), and subsequently modified by Blix (3), was used to determine the glucosamine content. Absorption measurements were carried out in a Beckman Model D.U. spectrophotometer at 512 mg.

RESULTS

When homogenates of the Walker tumor were incubated in the presence of fructose-6-phosphate and glutamine, a steady increase in substrate glucosamine was observed over a 3-hour period. During this time there was a three- to fourfold increase in the amount of the hexosamine. For example, glucosamine determinations after 0, 1, 2, and 3 hours of incubation were 14.3, 58.8, 70.9, and 81.2 μg/mg of homogenate nitrogen, respectively. When fructose-6-phosphate, glutamine and glutamine and L-asparagine, Mann Research Laboratories, New York, N.Y.

Sources were as follows: glucosamine hydrochloride, Eastman Organic Chemicals, Rochester, N.Y.; glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-diphosphate, Schwarz Laboratories, Inc., Mount Vernon, N.Y.; mannose-6-phosphate, Nutritional Biochemicals Corp., Cleveland, Ohio; L-glutamine and L-asparagine, Mann Research Laboratories, New York, N.Y.

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amine, or both were deleted from the reaction mixture, negligible amounts of hexosamine were synthesized. The omission of magnesium chloride from the system had no appreciable effect on glucosamine synthesis. These results were substantiated further by the quantitative methods of Tracey (32) and Boas (4), and the data agreed favorably with those obtained with the modified Elson-Morgan determinations.

The effects of pH on amino sugar synthesis in Walker tumor homogenates are shown in Chart 1. It can be seen that the optimum pH was between 6.8 and 7.0, while at pH 8.1 and 5.6 hexosamine synthesis was negligible. When the concentration of substrates was varied in the reaction mixture, it appeared that 0.04 M fructose-6-phosphate and 0.05 M glutamine would give the maximum yield of hexosamine. Consequently, these concentrations were used in all subsequent experiments with the Walker tumor.

Since fructose-6-phosphate was an essential reactant for glucosamine synthesis, other hexoses were tested for activity. The results are summarized in Table 1. It can be seen that neither glucose supplemented with adenosine triphosphate (ATP) nor fructose-1,6-diphosphate served as satisfactory hexose precursors. Walker tumor homogenates, however, were able to utilize glucose-6-phosphate and mannose-6-phosphate as well as fructose-6-phosphate. Attempts to replace glutamine with equimolar amounts of asparagine as a nitrogen donor yielded negligible glucosamine synthesis.

It was possible that the glucosamine synthesis observed in the crude homogenates could be due to the breakdown of some of the stroma components, or other tissue contaminants, and was not a product of neoplastic enzymatic activity. This possibility was tested by preparing tumor cell suspensions from freshly excised intramuscular tumor. These cell suspensions were then homogenized and used in the test system. At the end of 3 hours' incubation, 72 μg glucosamine/mg of homogenate nitrogen was present in the incubation mixture. Further, the omission of fructose-6-phosphate, glutamine, or both from the system resulted in no significant hexosamine synthesis. Since microscopic examination of the cell suspensions (prior to homogenization) showed them to be essentially free of debris and to be only contaminated with non-neoplastic cells to an extent of 4 per cent by cell count, the synthesis of glucosamine must have been due to the Walker tumor per se.

As a result of these experiments, the Flexner-Jobling carcinoma and the Jensen sarcoma were investigated for amino sugar synthesis, and the results are shown in Table 2. The glucosamine synthesis in the Jensen sarcoma was the same as that observed in the Walker tumor, while the synthesis in the Flexner-Jobling carcinoma was less (P < .01). Both tumors, however, showed appreciable amino sugar synthesis.

Since Pogell and Gryder (24) had reported that liver homogenates would synthesize limited amounts of glucosamine, experiments were initiated to determine optimal conditions for the synthesis of amino sugars in crude homogenates of perfused livers. The optimum pH for the synthesis of glucosamine had been determined.

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**TABLE 1**

<table>
<thead>
<tr>
<th>Hexose</th>
<th>Initial (μg/mg tissue N)</th>
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<tbody>
<tr>
<td>Glucose + ATP*</td>
<td>17.1 ± 3.7†</td>
<td>22.0 ± 3.5</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>7.5 ± 1.8</td>
<td>187.5 ± 19.1</td>
</tr>
<tr>
<td>Fructose-6-phosphate</td>
<td>9.7 ± 2.0</td>
<td>90.6 ± 8.3</td>
</tr>
<tr>
<td>Fructose-1,6-diphosphate</td>
<td>15.8 ± 0.3</td>
<td>21.0 ± 0.3</td>
</tr>
<tr>
<td>Mannose-6-phosphate</td>
<td>7.1 ± 1.9</td>
<td>108.0 ± 7.7</td>
</tr>
</tbody>
</table>

* ATP present at a concentration of 0.1 M.  
† Standard deviation about the mean.

Reaction mixture: hexoses, 0.04 M; glutamine, 0.05 M; MgCl₂, 0.025 M; citrate buffer, 0.1 M; pH 7.0; tissue, 300 mg (wet wt.)/ml; incubation, 3 hours at 38°C.
thesis of hexosamine in normal liver homogenates appeared to be 6.5–6.7, while the optimum concentration of fructose-6-phosphate and glutamine was 0.02 M and 0.03 M, respectively. When homogenates of two transplantable hepatomas (Novikoff and S'-Me-DAB tumor) were incubated under conditions established with the Walker tumor and liver homogenates were incubated under the conditions just described, data as shown in Table 3 were obtained. The amount of synthesis in the two hepatomas was significantly higher (P < 0.05) than that observed in the liver homogenates.

With respect to the synthesis of amino sugars, the authors have been unable to find any record of glucosamine synthesis by a tumor, although it may be suspected from other studies. For example, serum polysaccharide levels were elevated in cases of malignancy, and the protein-bound carbohydrates seemed to contain equivalent amounts of galactose, mannose, and glucosamine (15). Furthermore, in studies with the Rous sarcoma, Glaser

**TABLE 2**

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<thead>
<tr>
<th>COMPARISON OF THE AMINO SUGAR SYNTHESIS IN WHOLE HOMOGENATES OF THE FLEXNER-JOBLING CARCINOMA, THE WALKER CARCINOSARCOMA 556, AND THE JENSEN SARCOMA</th>
</tr>
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<tbody>
<tr>
<td><strong>TOTAL GLUCOSAMINE</strong></td>
</tr>
<tr>
<td><strong>REACTION MIXTURE</strong></td>
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<tr>
<td></td>
</tr>
<tr>
<td>Complete</td>
</tr>
<tr>
<td>Complete minus:</td>
</tr>
<tr>
<td>Fructose-6-phosphate</td>
</tr>
<tr>
<td>Glutamine</td>
</tr>
<tr>
<td>Fructose-6-phosphate plus glutamine</td>
</tr>
</tbody>
</table>

* Standard deviation about the mean.

**TABLE 3**

<table>
<thead>
<tr>
<th>COMPARISON OF THE SYNTHESIS OF AMINO SUGARS IN HOMOGENATES OF RAT LIVER, S'-ME-DAB HEPATOMA, AND THE NOVIKOFF HEPATOMA</th>
</tr>
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<tbody>
<tr>
<td><strong>TOTAL GLUCOSAMINE</strong></td>
</tr>
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**DISCUSSION**

Most of the studies concerning glucosamine and neoplasms have been in the area of anti-metabolite effects. Quastel and Cantero (25) reported that the intraperitoneal administration of glucosamine resulted in the degeneration of Sarcoma 37 in mice; however, no complete regressions were observed. Since that time a number of other papers have appeared regarding the anti-tumor effect of glucosamine, and they have been either inconclusive or negative (7, 9, 12, 19, 21, 22, 26, 27, 29, 30). Recently, Ball and co-workers (2) studied the effect of glucosamine on the growth of the Walker tumor in vivo. They found that administration of multiple daily doses resulted in some retardation of the growth of the tumor.

Brown (13) observed the in vitro synthesis of hyaluronic acid chains from uridine diphospho-N-acetylglucosamine and uridine diphosphoglucuronic acid.

The results obtained in the present investigation indicated that a spectrum of tumor homogenates of both epithelial and connective tissue origin can synthesize amino sugars. The results of the studies with livers and hepatomas showed that either carcinogenesis, subsequent transplantation, or both resulted in an increased production of amino sugars by the neoplastic tissue when compared with its tissue of origin. The resolution
of these possibilities may prove helpful in the
description of the biochemistry of carcino-
genesis.

The apparent specificity for certain hexose
moieties was interesting, particularly the observation that the Walker tumor could synthesize an amino
sugar from mannose-6-phosphate. This amino sugar,
however, may have been mannosamine, since Comb and Roseman (8) isolated an enzyme from
Clostridium perfringens which cleaved human plasma
N-acetylneuraminic acid to pyruvic acid and N-acetylmannosamine. Another explanation for
this phenomenon may have been that the Walk-
er tumor contained a phosphomannose isomerase
similar to that reported for rabbit muscle by
Slein (28). This facet merits further research.

SUMMARY

Homogenates of the Walker carcinosarcoma 256
produced hexosamines from hexose-6-phosphate
and glutamine. The reaction was shown to be en-
zymatic and had a pH optimum of 6.8-7.0. Amino
sugars were produced from glucose-6-phosphate,
fructose-6-phosphate, and mannose-6-phosphate.
The synthesis of glucosamine was shown to be
associated with neoplastic cells of the Walker
tumor and not associated with stroma or normal
tissue contaminants. Amino sugar synthesis was
observed in a spectrum of transplantable rat tu-
mors, and the synthesis in the hepatomas exceeded
that observed in homogenates of perfused normal
rat livers.

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