Characterization of Glycogen Synthetases and Phosphorylases in Transplantable Rat Hepatomas

Kiyomi Sato, Harold P. Morris, and Sidney Weinhouse

The Fels Research Institute and Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania, 19140 [K. S., S. W.], and the Department of Biochemistry, Howard University, Washington, D. C. 20001 [H. P. M.]

SUMMARY

A study of the chemical and kinetic properties of a series of transplantable, chemically induced rat hepatomas has revealed profound alterations in isozyme patterns of glycogen synthetase and phosphorylase. Highly differentiated, slow-growing hepatomas retain the liver-type isozymes, but with decreased differentiation and increased growth rate these are largely or entirely replaced by nonhepatic isozymes. The phosphorylase of poorly differentiated hepatomas was a third form, shown by isoelectric focusing and by immunochemical criteria to be different from either the liver or muscle isozymes. This form was also present in 21-day fetal liver, together with the adult liver isozyme. All of the hepatomas contained the phosphatases and kinases that interconvert the a and b forms, and preliminary data indicate that the synthetase phosphatase of the poorly differentiated hepatomas also is different from the liver phosphatase.

Total synthetase activity was high in liver and muscle, lower in the well- to highly differentiated hepatomas, and very low in poorly differentiated tumors, except for the Novikoff ascites hepatoma, the only one of the poorly differentiated tumors that stored appreciable glycogen. In all tissues, b form predominated. Total phosphorylase activity was highest in muscle, predominantly in the b form; lower in liver; and still lower in tumors. There was no correlation between the activity of tumor phosphorylase and the degree of differentiation or the storage of glycogen, and most of the liver and hepatoma phosphorylase was in the a form.

INTRODUCTION

Recent work from our own and other laboratories (6, 14, 23, 32, 45) has shown that tumors arising from a single cell of origin, the parenchymal cell of the liver, may exhibit a wide diversity of metabolic behavior, paralleled by major alterations of enzyme activities. Among the results of these studies is the recognition that, with increased deviation from their morphological resemblance to the hepatocyte and with increased growth rate, there is a pattern of loss of those isozymes that play a functional role in liver metabolism and their replacement by isozymes that are low or absent in the adult, differentiated liver cell. In some instances the isozymes that are lost or greatly lowered in activity, e.g., glucokinase and liver-type pyruvate kinase, are adaptive and highly responsive to dietary and hormonal changes, whereas their replacements seem geared essentially to efficient utilization of substrates. Thus, these isozyme alterations provide a molecular basis for the lack of host control that characterizes neoplastic cells.

This study represents an extension of these studies to the glycogen synthetases (EC 2.4.2.11) and phosphorylases (EC 2.4.1.1). These enzymes are of special interest for studies on the loss or retention of liver "marker" enzymes in hepatic tumors. Each of these enzymes exists in active and inactive forms which are interconvertible by synergistic phosphorylations and dephosphorylations, mediated by cyclic AMP, the activity of which is in turn modulated in part by insulin, glucagon, and epinephrine (15, 25). Moreover, the active and inactive forms of both of these enzymes in liver differ chemically and kinetically from their counterparts in muscle (2, 5, 10, 28, 46). During the course of this work, we found (a) that poorly differentiated, rapidly growing hepatomas contain forms of both of these enzymes that differ from those of adult liver; (b) that the rat hepatomas have a 3rd set of phosphorylase isozymes that can be distinguished from both liver and muscle isozymes; and (c) that these nonhepatic isozymes are the sole or major forms in fast-growing, poorly differentiated hepatomas. The tumor phosphorylase, although not detectable in adult liver, is present in fetal liver. A comparison of these new forms of phosphorylase with the liver and muscle forms represents the major part of this report.

MATERIALS AND METHODS

Normal male rats of a pathogen-free (CFN) strain were obtained from Carworth Farms, New City, N. Y., and were used when they were about 6 months old and weighed 200 to 300 g. All animals were fed commercial rat food and were maintained in air-conditioned quarters on a 12-hr light-dark cycle. All experiments were conducted at uniform times to...
enzymes levels.

Morris hepatomas (18, 19) were transplanted in male or female Buffalo rats in Washington, D. C.; then rats were shipped to Philadelphia and maintained as above until tumors reached a diameter of 1 to 2 cm, when they were used in the experiments. Novikoff hepatomas (22) were transplanted i.m. for solid and i.p. for ascites tumor cells in female Sprague-Dawley rats obtained from the Holtzman Company, Madison, Wis. These fast-growing tumors were used within 7 to 10 days after inoculation.

Tissue Preparations. Rats were decapitated and exsanguinated, and tissue was quickly removed, chilled in ice-cold 0.9% NaCl solution, blotted, dissected away from connective tissue, minced, and homogenized in a glass coaxial homogenizer with a Teflon pestle. While the tissue was cooling in ice, 5 or 6 strokes of the pestle sufficed to disintegrate the tissue in 4 volumes (w/v) of a solution containing 0.5 M sucrose, 63 mM glycylglycine (pH 7.4), 6.3 mM EDTA (pH 7.4), 5 mM dithiothreitol, and 125 mM NaF when indicated. Skeletal muscle was homogenized in a Waring Blender, Model 1120 (Dynamics Corp.), for 1 min in the same solution. Novikoff ascites cells were drained off, centrifuged at 600 X g for 1 min, and washed and centrifuged 3 times with ice-cold 0.9% NaCl solution. The cells were then packed by centrifugation at 2000 X g for 5 min, suspended in 4 volumes of the above-described medium, and submitted to sonic vibration at 2000 X g for 5 min, suspended in 4 volumes of the above-described medium, and submitted to sonic vibration at 10 kc at 30-sec intervals, repeated 2 to 4 times with 30 sec in between, with a Bronson Model 124 Sonicator while the suspension was cooled with ice. This preparation was centrifuged at 5000 X g for 10 min, and the supernatant was decanted through glass wool for use in experiments. Through-out these studies, dilutions and washings were made with a solution containing 0.4 M sucrose, 50 mM glycylglycine (pH 7.4), 5 mM sodium EDTA (pH 7.4), 4 mM dithiothreitol, and 100 mM NaF when used.

Assay of Glycogen Synthetase. Glycogen synthetase was assayed by measuring the incorporation into glycogen of glucose from UDP-glucose uniformly labeled with 14C, essentially according to the method of Saheki et al. Assay mixture contained 50 mM glycylglycine (pH 8.2), 5 mM uniformly labeled UDP-glucose-14C (specific activity, 30 to 40 X 10⁸ cpm/mmoles), 10 mM glucose-6-P when indicated, 100 mM NaF, 2.0 mg of rabbit liver glycogen, and 0.05 ml of enzyme preparation in a total volume of 0.20 ml.

However, when the interconversion of the a and b forms was measured, 50 mM Tris-maleate at pH 7.4 was used as the buffer instead of glycylglycine at pH 8.2. After 5 min incubation at 30°, the reaction was stopped by addition of 2.5 ml of 30% KOH solution, and glycogen was recovered according to the method of Steiner et al. (36). The washed glycogen was dissolved in 1.2 ml of water, 1.0 ml of the solution was transferred into a vial, 10 ml of Aquasol (New England Nuclear, Boston, Mass) were added, and radioactivity was measured in a Packard Model 3375 liquid scintillation counter.

Vessels were run in duplicate, with and without glucose-6-P, and the total activity (without glucose-6-P) minus synthetase a activity (without glucose-6-P) was taken as the synthetase b activity. The conditions chosen were optimal with respect to substrates and cofactors, with assurance of linearity of reaction rate with time and enzyme concentration.

Assay of Glycogen Phosphorylase. Glycogen phosphorylase was measured by the incorporation into glycogen of glucose from glucose-1-P labeled uniformly with 14C, by a modification of the method of Sutherland. The standard assay mixture contained 50 mM Tris-maleate (pH 6.1), 45 mM uniformly labeled glucose-1-P-14C (specific activity, 3 to 5 X 10⁸ cpm/mmoles), 1.0 mM AMP (where indicated), 100 mM NaF, 2.0 mg of rabbit liver glycogen, and 0.05 ml enzyme preparation in a total volume of 0.20 ml. Skeletal muscle preparations were further diluted 10- to 20-fold before assay. After 5 min incubation at 30°, the glycogen was isolated and assayed for radioactivity as described for glycogen synthetase. Vessels were run in duplicate with and without AMP; the total activity (with AMP) minus the phosphorylase a activity (without AMP) was taken tentatively as phosphorylase b activity. (However, total liver phosphorylase b activity cannot be assayed, even with AMP present.) Conditions chosen were optimal for maximal activity, with assurance of linearity of reaction rate with time and enzyme concentration.

Interconversions of a and b Forms. Three ml of the crude extracts were incubated with or without 10 mM MgCl₂ at 30°, and at the indicated times 0.05-ml portions were assayed for both forms of glycogen synthetase and phosphorylase. After 60 min, 1.0-ml portions were transferred to another vessel containing 0.10 ml of a solution made up to give final concentrations of 10 mM MgCl₂, 10 mM ATP, and 10⁻⁵ M cyclic AMP when indicated. At the designated times, 0.05-ml portions were assayed for both a and b forms of each enzyme.

Glycogen and Protein Determination. One-half-ml portions of the 5000 X g supernatants were deproteinized by addition of 2.0 ml of 6% trichloroacetic acid, and the precipitate was centrifuged off. Two ml of the supernatant solution were treated with an equal volume of ethanol, and the glycogen obtained by repeated precipitation with ethanol was assayed by the atherone method. Protein was determined colorimetrically with a phenol reagent.

Chemicals. Labeled sugars and Aquasol were obtained from New England Nuclear, and other reagents were obtained from Sigma Chemical Company, St. Louis, Mo. Glycogen was washed with Amberlite MB-3 as described by Thomas et al. Freund's complete adjuvant was obtained from Difco Laboratories, Detroit, Mich.

Preparation of Enzymes for Kinetic and Immunological Studies. Rat skeletal muscle phosphorylase b was purified and crystallized twice according to the procedure of Sevilla and Fischer, and rat liver phosphorylase b was purified according to the procedure of Appleman et al. The phosphorylase b of the Novikoff hepatoma, which contains considerable glycogen, was purified partially as follows. The glycogen-microsome pellet, obtained by centrifugation at 105,000 X g, was suspended in one-third of the original volume of a solution containing 50 mM Tris-HCl, 1 mM EDTA, and 1 mM dithiothreitol and was digested overnight at 2° with human salivary amylase purified by the method of Shainkin and Birk. The enzyme thus solubilized was precipitated by ammonium sulfate between 30 and 60% saturation and then submitted to fractionation on a Sephadex...
Kiyomi Sato, Harold P. Morris, and Sidney Weinhouse

G-200 column according to the procedure of Kamogawa and Fukui (13). This procedure gave a 25% yield of enzyme purified 200-fold. Phosphorylases of other hepatomas, which contained little or no glycogen, were fractionated directly from the 105,000 g supernatant by ammonium sulfate precipitation between 30 and 60% saturation, followed by either dialysis against the dilution medium described above or "desalting" by passage through Sephadex G-25.

 Isoelectric Focusing. The procedure was essentially as described in the instructions provided by the manufacturer, LKB Instruments, Inc., Rockville, Md. One % solutions of the ampholytes containing 1 mM dithiothreitol were used to give the indicated pH gradient with a 0–45 (w/v) sucrose density gradient. For the broad pH range of 3 to 10, an LKB 8102 column with a volume of 440 ml was used. A 2-ml sample was positioned one-third of the way from the bottom, and the initial voltage was set between 100 and 150 to control the current at about 3 ma. Within 12 hr the voltage was increased gradually to 350, and focusing was conducted at this voltage for 48 hr, while keeping the temperature at 3°. Fractions of 3 ml were then collected and pH (at 2.5°) and enzymes were assayed in each fraction.

For the narrow pH gradient, 5 to 7, an 8101 column with a volume of 110 ml was used and a 1-ml sample was applied. The initial voltage setting was 150 to 200, and within 12 hr it was increased to 500 and maintained for 48 hr. One-ml fractions were then collected and pH and enzyme activities were determined.

Preparation of the Antiserum to Skeletal Muscle Phosphorylase b. Twice-crystallized rat skeletal muscle phosphorylase b was prepared as described by Sevilla and Fischer (33). An adult albino rabbit was immunized by 3 i.m. injections, at 1-week intervals, of 2 ml of an emulsion of 4 mg of crystallized rat muscle phosphorylase b and 2 ml of Freund's complete adjuvant. Three weeks after the last injection, 4 mg of the enzyme in 0.7% NaCl solution were injected s.c. as a booster, and after 1 week the blood was collected from the jugular vein. A γ-globulin fraction was obtained from the antiserum by collecting the fraction precipitated by ammonium sulfate at 20 to 33% saturation. The precipitate was dissolved in a solution consisting of 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 2 mM dithiothreitol, and 0.9% NaCl solution and dialyzed well against the same solution. The final concentrations of protein of the γ-globulin fraction was 100 to 110 mg/ml.

Inhibition of Phosphorylase by Antibody. The effect of the antibody to the skeletal muscle phosphorylase b on the phosphorylase activities of the various tissues was assayed as follows. Each phosphorylase preparation was diluted to approximately the same activity range. A fixed amount of phosphorylase (0.1 to 0.3 unit) and increasing concentrations of γ-globulin fraction were incubated at 30° in 0.1 ml of a mixture consisting of 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 2 mM dithiothreitol, 0.9% NaCl solution, and 1% normal rabbit serum added to prevent the loss of activity of the highly purified phosphorylase during the incubation. Controls were run with a similar globulin fraction obtained from a normal rabbit. After 20 min, 0.1 ml of the reaction mixture was measured for the assay of total phosphorylase, with AMP and with or without 0.4 M or 0.5 M Na2SO4, at 30° for 10 min.

RESULTS

Enzyme Levels in Liver and Hepatomas. General properties of the tumors under study are displayed in Table 1. These are arranged in order of increasing growth rate. As noted earlier (18, 19), the slow-growing tumors are well- to highly differentiated and contain little or no glycogen.

Table 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Generation</th>
<th>Age of tumor (mo.)c</th>
<th>Histological evaluation</th>
<th>Glycogen (mg/g)</th>
<th>Synthetase a</th>
<th>Phosphorylase a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.23</td>
<td>1.58</td>
</tr>
<tr>
<td>Host liver (4)b</td>
<td>60</td>
<td>16</td>
<td>W-HDc</td>
<td>15</td>
<td>0.29</td>
<td>2.08</td>
</tr>
<tr>
<td>Skeletal muscle (5)</td>
<td>2.6</td>
<td>1.68</td>
<td>1.20</td>
<td>3.6</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>Hepatomas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.23</td>
<td>1.58</td>
</tr>
<tr>
<td>9618B (2)</td>
<td>6</td>
<td>16</td>
<td>W-HDc</td>
<td>15</td>
<td>0.29</td>
<td>2.08</td>
</tr>
<tr>
<td>20 (2)</td>
<td>3</td>
<td>13</td>
<td>W-HD</td>
<td>&lt;1</td>
<td>0.15</td>
<td>0.60</td>
</tr>
<tr>
<td>66 (3)</td>
<td>3,4</td>
<td>8</td>
<td>HD</td>
<td>&lt;1</td>
<td>0.09</td>
<td>0.61</td>
</tr>
<tr>
<td>47C</td>
<td>7</td>
<td>4</td>
<td>HD</td>
<td>&lt;1</td>
<td>0.18</td>
<td>0.88</td>
</tr>
<tr>
<td>7794B</td>
<td>25</td>
<td>4</td>
<td>HD</td>
<td>&lt;1</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>7787</td>
<td>17</td>
<td>1.5</td>
<td>H-WD</td>
<td>0.12</td>
<td>0.19</td>
<td>3.77</td>
</tr>
<tr>
<td>7288C</td>
<td>121</td>
<td>1.0</td>
<td>PD</td>
<td>&lt;1</td>
<td>0.13</td>
<td>0.09</td>
</tr>
<tr>
<td>7777</td>
<td>73</td>
<td>1.0</td>
<td>PD</td>
<td>&lt;1</td>
<td>0.18</td>
<td>0.09</td>
</tr>
<tr>
<td>3924A (1)</td>
<td>291</td>
<td>1.0</td>
<td>PD</td>
<td>&lt;1</td>
<td>0.07</td>
<td>0.22</td>
</tr>
<tr>
<td>Novikoff hepatomas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.10</td>
<td>0.25</td>
</tr>
<tr>
<td>Solid (8)</td>
<td>0.3</td>
<td>PD</td>
<td>&lt;1</td>
<td>5.2</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td>Ascites (9)</td>
<td>0.25</td>
<td>PD</td>
<td>15</td>
<td>3.9</td>
<td>0.8</td>
<td></td>
</tr>
</tbody>
</table>

a Months between inoculation and use in experiments, when tumors were 1 to 2 cm in diameter.
b Number in parentheses, number of assays.
c WD, well-differentiated; HD, highly differentiated; PD, poorly differentiated.

726 CANCER RESEARCH VOL. 33
Glycogen Synthetases and Phosphorylases in Hepatoma
differentiated as revealed by histological examination. Glycogen content was high in only 2 tumors, and these represent the extremes of differentiation, namely, the slow-growing 9618B and the rapidly growing Novikoff ascites tumor. However, neither tumor, with 15 mg glycogen per g, contained anywhere near the level of 70 mg/g in liver. Other tumors had little or no glycogen. The total synthetase level, although lower in tumors than in liver, was considerably higher in the well-differentiated than it was in the poorly differentiated hepatomas, the difference being due essentially to synthetase b activity. Again, however, a conspicuous exception was the Novikoff ascites hepatoma, in which there were high levels of both the a and b forms, comparable with the levels in liver. In contrast with liver and the well-differentiated hepatomas, the activity of the synthetase in skeletal muscle was mainly in the a form. In contrast with synthetase activity, phosphorylase activity in the well-differentiated hepatomas was primarily in the a form, and there were no marked differences in this respect from the poorly differentiated hepatomas, including the Novikoff hepatomas. There is a roughly inverse correlation between the glycogen content of the tissues and the ratio of total phosphorylase to synthetase, e.g., with liver, muscle, and the Novikoff hepatomas, suggesting that this ratio may be a factor in glycogen storage; however, this relationship breaks down when we consider the ratio of activities of the active (a) forms of the respective enzymes. Synthetase a is far higher in muscle than in liver, whereas phosphorylase a is far higher in liver than in muscle.

Effects of pH on Synthetase Activity of Rat Liver and Muscle. Previous studies of Sato et al. (27, 28) revealed that, when assays are carried out in Tris-maleate buffer, rat liver synthetase b has a sharp pH optimum at pH 8.5 to 8.8, with essentially no activity at pH 7. When liver extracts were prepared as described in the text in the presence of fluoride to inhibit conversion of the b to the a form, the pH curves shown in Chart 1A were obtained. The effect was most marked with Tris-maleate buffer, but it was also evident with glycylglycine. There was only negligible activity in the absence of glucose-6-P, thus indicating that synthetase a was virtually absent. The effect of increasing concentration of maleate at pH 7.4 as well as that of Pi and sulfate may be seen in Chart 1B. Pi and sulfate inhibited to approximately the same extent, namely, about 90% at 10 mM, and maleate exhibited a similar curve, but at 10-fold higher concentration. However, these anion effects were very slight at pH 8.5. In contrast, muscle synthetase b exhibited a broad pH optimum, was nearly as active at pH 7 as at pH 8.5, and was not affected by the nature of the buffer (Chart 1C). As shown in Chart 1D, the muscle enzyme at pH 7.4 was unaffected by maleate up to 100 mM and was inhibited only to the extent of about 15% by 10 mM Pi and SO4²⁻.

Effect of pH on Synthetase Activity of Hepatomas. These differences in the kinetic properties of the respective liver and muscle synthetases were exploited to determine the nature of the hepatoma synthetases. Data obtained on the pH dependence and inhibitory effects of anions for the slow-growing, highly differentiated Morris Hepatoma 66, shown in Chart 2, A and B, are strikingly similar to those for liver, as shown in Chart 1, A and B. The synthetase was entirely in the b form, and the activity at pH 7 was only about one-tenth that at pH 8.

Chart 1. Effects of pH and anions on glycogen synthetase activities. A and C, o, 50 mM Tris-maleate buffer; o, 80 mM glycylglycine buffer; •, no glucose-6-P, 80 mM glycylglycine buffer. B and D, ---, pH 7.4; - - - - , pH 8.5; o, maleate; o, Pi; o, sulfate.

Chart 2. Effects of pH and anions on glycogen synthetase activities of Hepatomas 66 and 2924A. Designations are the same as in Chart 1. •, •, without glucose-6-P.
8.5. On the other hand, a rapidly growing, poorly differentiated hepatoma such as the 3924A exhibited a pattern closer to that of muscle than of liver (Chart 2, C and D). Inhibition was about 38% with both buffers at pH 7.0 and all of the activity was in the b form. Likewise, effects of the anions on this enzyme resembled those on the muscle enzyme; a moderate activation with maleate and a moderate inhibition, to about 35 to 38% at 10 mM P_i and SO_4^{2-}.

The synthetase assays with Tris-maleate buffer at pH 7.4 and 8.5 were applied to the series of tumors under study, and the data are shown in Table 2. The activity patterns ranged widely but fell roughly into 2 classes, those with a low ratio of activity at pH 7.4 to that at pH 8.5 and those with a ratio of 95 to 100%. In the former group were all of the well- and highly differentiated hepatomas; their synthetase b thus resembles the liver enzyme, and in the latter group were all of the poorly differentiated hepatomas, in which synthetase b differed from the liver enzyme and resembled the muscle enzyme.

Studies of the Interconversion of Synthetases a and b. As a further means of characterizing the tumor synthetases, their mode of conversion of the b to the a form was examined in crude extracts. In assaying the rate of conversion, we again took advantage of the fact that activity of liver synthetase b is very low in Tris-maleate buffer at pH 7.4, whereas that of synthetase a is as high at pH 7.4 as it is at pH 8.5. The typical pattern for conversion of the liver enzyme is shown in Chart 3A. Over the course of 1 hr incubation at 30°, the action of the synthetase b phosphatase resulted in substantial conversion to the a form as shown by the increase of activity without glucose-6-P, to the point where at least 80% is in the a form. That this conversion was due to phosphatase activity is indicated by its complete inhibition in the presence of 100 mM fluoride. [As reported by Sato et al. (27), when the conversion was assayed at pH 8.5 (data not shown) at which the synthetase b activity was optimal, a activity increased without change in total activity.] Addition of ATP, Mg^{2+}, and cyclic AMP caused a rapid and essentially complete reconversion of the a to the b form, presumably catalyzed by the cyclic AMP-activated protein kinase (15, 35). Incubation of the crude muscle extract under similar conditions resulted, as shown in Chart 3B, in a substantial conversion of the synthetase b to synthetase a; but since the muscle b form activity is unimpaired at pH 7.4, the total activity, assayed with glucose-6-P present, remained unchanged. Again, addition of ATP, Mg^{2+}, and cyclic AMP rapidly reconverted the a to the b form, while total activity remained unchanged.

As shown in Chart 3C, the pattern of conversion of synthetase b to a and its reconversion to b for the highly differentiated Morris Hepatoma 66 was essentially identical with that of liver. These findings make it clear that the synthetase b in this tumor is like the hepatic type and that this tumor, like liver, contains not only the synthetase b phosphatase but also the cyclic AMP-stimulated protein kinase. Chart 3C shows that little if any conversion of the Hepatoma 66 synthetase b to a occurred without Mg^{2+}. In this respect, it is also similar to the liver synthetase phosphatase, which also requires Mg^{2+} (11, 27).

The pattern for poorly differentiated hepatomas, however, differed markedly from the hepatic pattern, as exemplified by

![Chart 3. Interconversions of a and b forms of glycogen synthetases.](chart3.png)
Glycogen Synthetases and Phosphorylases in Hepatoma

Chart 3D for the Novikoff ascites hepatoma. Total activity changed minimally, while synthetase a increased markedly and dropped extremely rapidly after addition of ATP and cyclic AMP. A spontaneous rise in synthetase activity after 70 min denotes a subsequent further action of the phosphatase. Thus, it is clear that this hepatoma contains a synthetase of the nonhepatic type and also contains a highly active phosphorylase and a cyclic AMP-stimulated protein kinase. The Novikoff hepatoma phosphatase did not require Mg\(^{2+}\) but was stimulated somewhat by this ion. This behavior is in accord with that of the muscle phosphatase (42).

Interconversion of Phosphorylase a and b. A similar set of patterns for the interconversion of a and b forms of glycogen phosphorylase was obtained with the same crude preparations by taking advantage of the fact that liver phosphorylase b is inactive even in the presence of AMP (2, 46), whereas the muscle phosphorylase b in the presence of AMP is fully as active as muscle phosphorylase a in the absence of AMP (5). The pattern obtained for liver is shown in Chart 4A. With 1 hr incubation at 30°, there was a rapid and nearly complete conversion of the a to the b form, which was reversed partially by subsequent treatment with ATP, Mg\(^{2+}\), and cyclic AMP. In Chart 4B, for muscle we found that, since essentially all of the phosphorylase is in the b form, there was no further change due to phosphatase activity. However, conversion to the a form was very rapid and virtually complete after activation of the kinase system with ATP and cyclic AMP. Chart 4C reveals for Hepatoma 66 an interconversion pattern somewhat similar to that of liver but sufficiently different to suggest that this tumor may contain more than 1 form (see later). Chart 4D shows for the Novikoff ascites hepatoma a pattern close to that of muscle.

Effect of Sulfate on Phosphorylase Activity. According to the work of Appleman et al. (2), rabbit and pig liver phosphorylase b do not have an absolute requirement for AMP but are strongly activated by SO\(_4^{2-}\), whereas the muscle enzymes have an absolute requirement for AMP and are unaffected by SO\(_4^{2-}\). As shown in Chart 5, A and B, the same respective properties are exhibited by b phosphorylases of rat liver and muscle. As shown in Chart 5C, the highly differentiated Hepatoma 9618B phosphorylase b exhibited a sulfate activation pattern similar to that of liver; but the extent of activation was less and was maximal at 0.2 M as compared with >0.4 M for liver. Similar patterns were obtained for phosphorylase of 2 other well-differentiated Hepatomas 20 and 66. These differences may be due either to the presence of some liver phosphorylase a in the preparations, since the liver a form is strongly inhibited by SO\(_4^{2-}\) or the presence of another form of phosphorylase. As seen in Chart 5D, there was only very slight sulfate activation of the Novikoff hepatoma phosphorylase b, and there was a strong

Some of the well-differentiated hepatomas such as 7787, 20, and 7794B exhibited patterns that were intermediate between that for Hepatoma 66 and that for the poorly differentiated hepatomas such as 3924A and Novikoff, thus suggesting that tumors of intermediate states of differentiation may possess more than 1 type of synthetase as was found previously for other enzymes in these tumors (1). These same tumors also displayed phosphorylase b-to-a conversion patterns in experiments like those of Chart 5, thus pointing also to multiple forms of phosphorylase in these tumors.

---

Chart 4. Interconversion of a and b forms of glycogen phosphorylases. Conditions and designations are as described in the text, and the data on phosphorylases were obtained from the same experiments described in Chart 3 for the synthetases. Assays were conducted at the designated intervals as described in the text, with 1 mM AMP (○, ●) and without AMP (○, ●). HEP, hepatoma.

Chart 5. Activation of b phosphorylases by sulfate. Assays were conducted as described in the text, with 1 mM AMP (○, ●) and without AMP (○, ●) and with sodium sulfate concentrations as designated. HEP, hepatoma.
AMP requirement; this is in contrast to the properties of liver phosphorylase b.

Isoelectric Focusing of Synthetase and Phosphorylase Isozymes. The kinetic data thus far described disclose that, whereas the slow-growing, highly differentiated hepatomas contain phosphorylase and synthetase isozymes of the liver type, the rapidly growing, poorly differentiated hepatomas have isozymes that differ from the liver type and closely resemble the muscle type. The presence of nonhepatic synthetase and phosphorylase isozyme types in the poorly differentiated hepatomas prompted attempts at their further characterization by means of isoelectric focusing; patterns obtained for muscle, liver, and Novikoff hepatoma are given in Chart 6. This procedure provides an excellent means of separating the synthetases from the phosphorylases. The former as shown in Chart 6, A and C, exhibited a rather complex pattern, focusing over a broad pH range, with multiple peaks occurring between pH's of 3.9 and 4.7. However, the liver synthetase could not be recovered, owing presumably to heat lability and heavy precipitation of protein at this pH range. Further investigation of the isoelectric focusing of synthetase isozymes will be undertaken later. This procedure also effectively separates the phosphorylases from glycogen, which focuses at a low pH (near pH 4).

The 3 phosphorylase isozymes focused at discrete peaks, and it is evident that the Novikoff hepatoma phosphorylase (Chart 6C), despite its kinetic resemblance to the muscle phosphorylase, has an isoelectric point at 5.6 and is thus distinctly different from the muscle form (Chart 6A), with a pI of 6.1. The major peak differs also from the liver form, with a pI of 5.9, and is not affected by SO$_4^{2-}$. The Novikoff hepatoma pattern has a minor peak at 5.9, which is stimulated by SO$_4^{2-}$, suggesting the presence of some of the liver form; the liver pattern has a shoulder at a higher pH, also indicating some further heterogeneity. All 3 isozymes were primarily in the $b$ form, as indicated by the differences in activity with and without AMP.

Data on the phosphorylases of Morris Hepatoma 20 obtained by isoelectric focusing are given in Chart 7A. This tumor, as expected from the data of Chart 5, apparently possesses the liver as well as the tumor type isozyme, as revealed by the sulfate-requiring peak at pH 5.9. A similar pattern for 21-day rat fetal liver (Chart 7B) clearly points to the presence in this tissue of a form similar to the tumor isozyme, as indicated by its pi of 5.6 and lack of SO$_4^{2-}$ requirement. However, at this stage of fetal development the adult liver form was evidently predominant. Further work on the embryonic development of phosphorylase isozymes is in progress.

Immunological Studies. The preceding results clearly demonstrated that the tumor isozyme differs kinetically from that of liver and, although it appears similar kinetically to the muscle phosphorylase, it differs in isoelectric point as determined by isoelectric focusing. Further evidence that the tumor isozyme differs from that of the muscle has been obtained by an immunological study (Chart 8). An antibody to the rat skeletal muscle phosphorylase, prepared by injection of the purified isozyme into rabbits, completely inactivated the muscle isozyme, with close proportionality between the degree of inactivation and the quantity of antibody globulin added. However, there was no effect on the liver isozyme and little or no effect on either the Novikoff ascites hepatoma or the Morris Hepatoma 20 isozymes. The enzyme of the poorly differentiated Morris Hepatoma 3924A was inactivated to the extent of approximately 35%, giving a pattern suggesting that it may be a mixture of muscle and tumor isozymes.$^5$

For further characterization of the nature of these isozymes, the antibody "titrations" were conducted in the presence or absence of sulfate. As expected, the liver isozyme required sulfate, but neither the Novikoff nor the 3924A isozyme was affected. The well-differentiated Morris hepatoma 20 was not inactivated by the antibody but was inhibited about 50% in the absence of sulfate. This confirms the data in Chart 7A pointing to the presence in this tumor of both liver and tumor isozymes.

$^5$Since this tumor has been transplanted i.m., we considered the possibility that it may have carried with it some muscle tissue, which is extremely high in phosphorylase. However, an i.p. transplant of this tumor gave the identical pattern of immunological inactivation, suggesting that muscle-type phosphorylase may be an intrinsic part of the makeup of this tumor. This possibility is being investigated further.
**Glycogen Synthetases and Phosphorylases in Hepatoma**

**DISCUSSION**

Total Enzyme Activities and Glycogen Storage. It is evident that glycogen levels are generally low in tumors (21); this is particularly true with rat hepatomas in comparison with liver. However, Nigam (20) found that certain strains of the Novikoff hepatoma store considerable glycogen, and Saheki et al. (26) found the same for the AH-66F Yoshida hepatoma. Table 1 shows that the 9618B and the Novikoff hepatomas, which represent the extremes of differentiation and growth rate, are the only hepatomas that stored appreciable glycogen. Lea et al. (16) also found high levels of glycogen in slow-growing Morris Hepatomas 9618A and 7787. However, generally low glycogen levels in Morris hepatomas were reported earlier by Weber and Morris (43), Sweeney et al. (39), and Weinhouse (44).

Total glycogen synthetase activity in the highly differentiated, slow-growing 9618B hepatoma was comparable with that of liver and, although activity was lower in other slow-growing, well-differentiated hepatomas, it was much higher than in the rapid-growing, poorly differentiated hepatomas. However, a notable exception was the very-fast-growing Novikoff ascites hepatoma, the total synthetase activity of which was fully as high as that of liver. It is evident from Table 1 that only those tumors with high synthetase activity stored glycogen appreciably, although the enzyme was predominantly in the b or inactive form. Lea et al. (16) also found high and predominant b glycogen synthetase activity in 4 well-differentiated Morris hepatomas, and Nigam (20) found synthetase in predominant b form in the Novikoff hepatoma, with activities lower than in normal or host liver.

Total phosphorylase activities were much lower in the hepatomas than in normal or host liver and in general were predominantly in the a form. There was no discernible correlation between the degree of differentiation and the phosphorylase activity or between the latter and the storage of glycogen. These data are also in accord with those of Lea et al. (16), who found activities of predominantly phosphorylase a to be somewhat lower in 4 well-differentiated Morris hepatomas than in liver, and with Nigam (20), who found phosphorylase activity in Novikoff hepatomas to be lower than in liver and predominantly of the a form. The loss of phosphorylase activity in early stages of carcinogenesis, decreasing progressively in "precancerous" hyperplastic nodules of liver, was reported by Epstein et al. (8). The present results add little to our knowledge of those factors that control glycogen storage in tumors and only reinforce previous results, which point to little if any effect of total synthetase or phosphorylase activity on glycogen storage.

Isozyme Alterations. In previous studies we as well as others (6, 14, 23, 32, 45) found that, with decreased differentiation and increased growth rate of hepatomas, there was a loss of such typical hepatic isozymes as glucokinase, aldolase, and pyruvate kinase; with dedifferentiation and increased growth rate, these were replaced by high activities of corresponding
isozymes that are normally low in the adult liver. The present results for synthetase and phosphorylase are strikingly similar. The pH-activity curves and the patterns of interconversion of a and b forms reveal that hepatoma dedifferentiation is accompanied by a replacement of the liver form of each enzyme by a nonhepatic form. These data confirm and extend to the Morris hepatomas previous observations by Sato et al. (27, 28), who found from similar evidence that 2 Yoshida ascites hepatomas, AH-66F and AH-130, resembled the muscle rather than the liver synthetase. The tumor synthetase has not been further characterized, and its molecular relationship to the muscle form remains undisclosed.

The same pattern of retention and loss was observed for liver phosphorylase. The results of isoelectric focusing and the immunological data leave little doubt, however, that the phosphorylase that replaces the liver form is a 3rd form, probably differing in its primary structure from both the liver and muscle types. Forms of phosphorylase differing from either that of liver and muscle have been reported for rat and bovine kidney by Villar-Palasi and Gazquez-Martinez (41) and for bovine spleen by Kamogawa and Fukui (13). Further evidence of heterogeneity of phosphorylase has been reported by Yunis et al. (48) in rabbit heart muscle and in phosphorylase and synthetase of a rat choroma (3, 47). The relationships of these multiple forms to those described here remain for further investigation.

The apparent identity of the tumor phosphorylase with a fetal liver isozyme provides further support for the growing realization that many proteins which have been synthesized in fetal tissues, but in which synthesis has been suppressed during normal embryonic development, are reactivated in tumors (1, 37). In studies reported in preliminary form (31), we found that 15-day whole rat embryo, which contains no appreciable liver tissue, also has a form of phosphorylase that is inhibited by antibody to Novikoff hepatoma phosphorylase but not by antibody to adult rat muscle phosphorylase. The same is true for 17-day rat fetal skeletal muscle, thus indicating that the tumor phosphorylase is present in fetal tissues other than liver and that normal embryonic development of muscle also involves replacement of phosphorylase isozymes.

Interconversion of a and b Forms. The data shown in Charts 3 and 4 demonstrate that the tumors also contain enzymes for the interconversion of the a and b synthetases and phosphorylases. Generally, the phosphatases were weakly active in the crude tumor extracts, whereas the kinases were strongly active. These observations are in good accord with the predominance in the tumors of synthetase b and phosphorylase a. Whether these results reflect inherent activities of the enzymes themselves or allosteric effects remains uncertain, since synthetase and phosphorylase phosphatase activities are affected by glycogen, glucose, glucose 6-phosphate, adenine nucleotides, and some ions (7, 15). The presence in the hepatomas of kinases and phosphatases that interconvert the a and b forms also raises questions concerning their relationship to the liver kinases and phosphatases. The liver phosphorylase differs from the muscle form in requiring Mg++ for activation (11, 27). Bishop (4) reported that liver possesses both a Mg++-dependent and a Mg++-independent form of synthetase phosphatase. However, the muscle form is Mg++-independent (42). Sato and Tsuiki (30), in examining the differing glycogen storage capacities of the AH-66F and AH-130 hepatomas, found that the synthetase phosphatase of the latter was strongly inhibited by glycogen, whereas that of the former was relatively unaffected, thus probably accounting for the high glycogen level of the AH-66F hepatoma. The well-differentiated Hepatoma 66 phosphatase acting on the synthetase (Chart 3C) required Mg++, whereas the Novikoff phosphatase acting on its synthetase exhibited a much lesser Mg++ requirement. These findings taken together with those of Sato and Tsuiki (30) suggest that, along with a replacement of the liver synthetase, there may also be a replacement of the liver synthetase phosphatase in poorly differentiated hepatomas. Whether the protein kinases also differ in their kinetic properties is now under study. These enzymes, depending as they do for their activity on cyclic AMP, influence not only glycogen metabolism but also many other fundamental biological phenomena such as protein synthesis, gene expression, hormonal control, and autonomous growth (12), and their characterization in tumors holds promise of an explanation of some of the unique features of tumor growth and metabolism.

REFERENCES

11. Hizukuri, S., and Larner, J. Studies of UDPG: 1,4-Glucan


Characterization of Glycogen Synthetases and Phosphorylases in Transplantable Rat Hepatomas

Kiyomi Sato, Harold P. Morris and Sidney Weinhouse


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/33/4/724

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.