Biochemistry and Enzyme Induction in MC-29 Virus-induced Transplantable Avian Hepatoma

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SUMMARY
For the biochemical characterization of a new transplantable hepatoma derived from the MC-29 virus-induced liver tumor, the macromolecular content and the inducibility of glucose-6-phosphatase, glucose-6-phosphate dehydrogenase, and aryl hydrocarbon hydroxylase were compared in chicken liver and in this hepatoma. The alteration of the nucleocytoplasmic ratio was deduced from measurements of DNA, RNA, protein, and phospholipid contents of the whole cell homogenate and cell fractions. The increased nuclear and decreased cytoplasmic content of macromolecules suggests a dominancy of the nuclei in the tumor cells.

Glucose-6-phosphatase and aryl hydrocarbon hydroxylase activities were lower by 60 and 80%, respectively, in the highly proliferating hepatoma than in the liver. In contrast, glucose-6-phosphate dehydrogenase activity increased in the hepatoma. However, enzyme inducers, such as methylcholanthrene, hydrocortisone, and insulin, were able to enhance the activity of these enzymes in the liver but had no stimulating effect on the hepatoma.

INTRODUCTION
Experimental hepatomas have been comprehensively investigated in cancer research, since they are malignant forms of a highly differentiated tissue that could be reasonably well characterized by morphological and biochemical methods (16, 18, 19, 21, 29). They offer a promising tool for a better understanding of the essential features in the process of neoplastic transformation and also for a comparison of the structure and function between malignant and normal tissue. In this field the Morris hepatomas received special interest by providing an experimental system for studying the biological and biochemical parameters associated with the different tumor growth rates (18).

Studies in this field revealed that the morphological and biochemical properties of liver were still preserved in the slowly growing tumors, while these properties gradually disappeared as the tumor growth rates increased. Among the numerous contributions derived from the introduction of the chemically induced hepatomas, 2 conclusions are especially worthy of mention.

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2 The abbreviations used are: VTH, virus-induced transplantable hepatoma; PCA, perchloric acid; AHH, aryl hydrocarbon (benzpyrene) hydroxylase; BP, benzo(a)pyrene; 3-DH-BP, 3-hydroxybenzo(a)pyrene; G-6-Pase, glucose-6-phosphatase; G-6-P: glucose-6-phosphate; G-6-P-DH, glucose-6-phosphate dehydrogenase.

MATERIALS AND METHODS
Tissues. In our experiments we used the transplantable line of the MC-29 virus-induced hepatoma (VTH) and liver from healthy chickens. Tumors from Passages 4 to 15 and 30 to 46 were used and each experimental group was comprised of 4 to 10 animals. The hepatoma was maintained in both sexes of "Hunia hybrid" chicken as described by Lapis et al. (12). Ten days after transplantation the animals were sacrificed by decapitation. Liver and tumor were removed, weighed, and stored at −25 °C if not used immediately. Tissues were homogenized in 0.1 M potassium phosphate buffer, pH 7.5, containing 0.25 M sucrose, with a Teflon homogenizer. An aliquot of the homogenate was stained with orcein, and the number of nuclei was counted in a Buerker hemocytometer (33). Cell fractions were obtained by differential centrifugation of the homogenate (10). Further purification of the nuclei was carried out by sedimentation through 2.2 M sucrose at 40,000 × g for 90 min (5).

Nucleic Acid, Protein, and Phospholipid Measurements. For determination of the quantity of DNA, RNA, phospholipid-
ids, and protein, the cell homogenate and cell fractions were successively extracted with 0.5 N cold PCA-ethanol-ether (3:1). 0.5 N PCA at 70° for 20 min, and the final pellet was dissolved in 0.1 N NaOH. The phosphate content of the ethanol-ether extract measured by the Fiske-SubbaRow method was multiplied by 25, thus supplying the value for the quantity of phospholipids (7). DNA and RNA were measured in hot PCA fraction by applying the diphenylamine and orcin reagents, respectively (3, 4). Protein was measured in the final pellet according to the procedure of Lowry et al. (15).

Enzyme assays. AHH activity was determined according to the method of Nebert and Gelboin (20) as modified for whole tissue homogenate by Diamond et al. (6). Enzyme activity was expressed as pmoles of phenolic product of BP per mg protein per 60 min. The 0.1-ml homogenates corresponding to 0.01 g wet weight of tissue were incubated for 30 min at 37° in 1.1 ml of 0.25 M sucrose:3 mM MgCl:0.05 M Tris-HCl buffer, pH 7.5, containing 1 mg NADPH and 100 nmole BP. The reaction was stopped by the addition of 1 ml acetone and extracted with equal volumes of hexane and 1 N NaOH. The 3-OH-BP in the alkaline phase was assayed in a Farrand spectrofluorometer with excitation at 400 nm and emission at 520 nm. 3-OH-BP used as a standard was a gift of Dr. Gelboin (National Cancer Institute, Bethesda, Md.) through the courtesy of Dr. Tomatis (International Agency for Research on Cancer, Lyon, France). G-6-Pase activity was estimated by measuring P~ liberated from G-6-P (9). For measuring G-6-P-DH activity, the production of NADPH by the 15,000 x g supernatant of the cell homogenate was followed at wavelength 340 nm at 25° (14).

Stimulation of enzyme activities was carried out by in vivo treatment of the hepatoma-bearing and normal chicken with 1 of the following chemicals: hydrocortisone, 250 mg/kg i.p., insulin 100 IU/100 g, and methylcholanthrene, 25 mg/kg i.p.

Chemicals. 3,4-Benzpyrene was purchased from Sigma Chemical Co., St. Louis, Mo. Methylcholanthrene was purchased from Fluka AG Chemische Fabrik, Buchs, Switzerland. NADP and NADPH were products of Serva Feinbiochemica GmbH Co., Heidelberg, West Germany. Insulin (crystalline) and hydrocortisone were purchased from Gedeon Richter, Budapest, Hungary.

RESULTS

Macromolecular Content of VTH. The comparison of the macromolecular content in the liver and hepatoma showed definite differences, as seen in Table 1. In the hepatoma the quantity of DNA increased by a factor of 2, while the protein and phospholipid values substantially decreased. At the same time, the quantity of RNA decreased only to 73.9% in the hepatoma, compared to that of the liver.

The question has arisen as to which cellular fractions contribute to the difference in the macromolecular content between normal liver and hepatoma. Therefore, cell fractions were obtained by successive centrifugation of the whole cell homogenate at 600, 15,000, and 105,000 × g. Among these fractions only the cell nuclei, sedimented at

Table 1

<table>
<thead>
<tr>
<th>DNA, RNA, and phospholipid content of whole cell homogenate and cell fractions of chicken liver and hepatoma</th>
<th>Liver</th>
<th>Hepatoma</th>
<th>Hepatoma</th>
<th>Hepatoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(pg/10⁶ cells)</td>
<td>(pg/10⁶ cells)</td>
<td>(pg/10⁶ cells)</td>
</tr>
<tr>
<td>Cell fraction</td>
<td>10</td>
<td>150,000 × g supernatant</td>
<td>150,000 × g pellet</td>
<td>Nucleus</td>
</tr>
<tr>
<td>Whole cell homogenate</td>
<td>5</td>
<td>2.57 ± 0.28</td>
<td>0.012</td>
<td>0.012</td>
</tr>
<tr>
<td>Nucleus</td>
<td>5</td>
<td>2.30 ± 0.28</td>
<td>0.017</td>
<td>0.017</td>
</tr>
<tr>
<td>15,000 × g</td>
<td>5</td>
<td>4.65 ± 0.36</td>
<td>0.359</td>
<td>0.359</td>
</tr>
<tr>
<td>15,000 × g</td>
<td>5</td>
<td>4.68 ± 0.42</td>
<td>0.341</td>
<td>0.341</td>
</tr>
</tbody>
</table>

*Mean ± S.D. Numbers in parentheses, percentages.

P < 0.05, statistically significant.

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activity of this enzyme was observed in the Novikoff hepatoma (32) and also in the Morris hepatomas (26, 35).

Chart 1 shows the steps of cell metabolism that are connected to the activity of these investigated enzymes. It can be seen that G-6-P plays a central role in carbohydrate metabolism, because it represents the initial reactant for glycogen synthesis, glycolysis, pentose phosphate cycle, and also for the conversion to glucose. The latter 2 reactions are the subject of our present study. The 1st step in the pentose phosphate cycle is governed by G-6-P-DH while NADPH is formed, which is used by AHH. G-6-Pase hydrolyzes the G-6-P, yielding glucose, which is released into the blood.

The activities of G-6-Pase, G-6-P-DH, and AHH in the liver of normal chicken and VTH are summarized in Table 2. In the VTH there was a decrease in the activity of the 2 mitochondrial enzymes (G-6-Pase and AHH), whereas G-6-P-DH was increased as compared to the activities found in normal liver. For investigation of the differences in the behavior of these enzymes between VTH and liver cells, their response to endogenous or exogenous factors was studied.

Treatment in vivo with methylcholanthrene for 24 hr brought about an enhancement of AHH activity in normal liver but none in VTH (Table 3).

Hydrocortisone induced a 2-fold increase in the hepatic G-6-Pase activity after 7 hr of treatment. AHH was also stimulated by this hormone. However, no changes were found in the hepatoma in the activity of either enzyme (Chart 2).

Three hr after insulin administration, increased liver G-6-P-DH activity could be detected. At the same time the relatively high initial activity of this enzyme in the VTH became lower. AHH was also stimulated in the liver but not in the VTH (Chart 3).

**DISCUSSION**

The aim of this study was to outline the biochemical features of a transplantable hepatoma derived from a virus-induced liver tumor (VTH) that recently had been introduced among the experimental tumor models (12).

Measurements of the concentration of biopolymers in various cellular fractions revealed a marked difference between normal chicken liver and VTH. These differences involved alterations in the quantity or distribution of the macromolecules among the cell fractions pointed to the alteration of the nucleocytoplasmic ratio as an essential feature of the malignant cells. A substantially larger proportion of the whole cell protein and RNA was observed in the nuclei of the hepatoma than in the normal liver. Comparing the distribution of protein and RNA between the nucleus and the cytoplasmic fractions, it was observed that while chicken liver nuclei contained 5% of the total cellular protein and RNA, these figures both increased to 14% in the hepatoma.

**Studies on Enzyme Stimulation.** For establishing functional differences between the VTH and normal liver, those enzyme activities that could be related to liver function were examined. G-6-Pase and AHH appeared to be appropriate for this purpose, since their activities are much higher in liver than in other tissues. They are not essential for the life of the cells, but their activities take part in the differentiated function of the liver. As AHH plays an important role in the detoxication of foreign compounds and G-6-Pase is one of the key enzymes of gluconeogenesis, both contribute to the liver functions that maintain the homeostasis of the whole organism.

G-6-P-DH activity was also studied because an increased activity of this enzyme was observed in the Novikoff hepar-
Table 3
Effect of methylcholanthrene on AHH and G-6-Pase activity of chicken liver and hepatoma

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Treatment</th>
<th>AHH</th>
<th>G-6-Pase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Liver</td>
<td>MC</td>
<td>526</td>
<td>90</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>MC</td>
<td>1</td>
<td>112</td>
</tr>
</tbody>
</table>

* Percentage of untreated liver or hepatoma.
\(^{b}\) MC, methylcholanthrene.
\(^{c}\) Statistically significant, \(p < 0.05\).
\(^{d}\) Statistically not significant.

The decreased activity per cell of G-6-Pase (to 35%) and of AHH (to 9%) is in agreement with observations in the rapidly proliferating hepatomas (28, 34). The increased G-6-P-DH activity in the average cell of the virus-induced hepatomas (35%) is also in good agreement with reports on the increased activity of this enzyme in the Morris hepatomas (34) and in the Novikoff hepatoma (32). In investigating the adaptive changes of these enzyme activities by applying chemical or hormonal inducers, a striking difference was observed between liver and hepatoma. In the normal liver AHH could be stimulated by 20-methylcholanthrene, hydrocortisone, and insulin, G-6-Pase could be stimulated by hydrocortisone, and G-6-P-DH could be stimulated by insulin. However, the same treatment brought about no stimula-
The effect of hydrocortisone on the activity of AHH and G-6-Pase in chicken liver (I) and VTH (II).

Treatment with hydrocortisone, 250 mg/kg i.p., was carried out for 1, 7, and 24 hr. Enzymes were measured as described in "Materials and Methods." The values are percentages of the enzyme activity in the untreated corresponding tissues, given as means ± S.D. from 5 determinations. S.D. = \( \frac{\sum x^2 - (\sum x)^2/n}{n-1} \).

The effect of insulin on the activity of AHH and G-6-P-DH in chicken liver (III) and VTH (IV). Treatment with insulin, 100 IU/100 g s.c., was carried out for 1, 3, and 6 hr. Enzymes were measured as described in "Materials and Methods." The values are percentages of the enzyme activity in the untreated corresponding tissues, given as means ± S.D. from 5 determinations.

In evaluating our studies on enzyme activities, the following conclusions could be drawn. The presence of AHH and G-6-Pase in the VTH suggests that the genetic information, which is responsible for the differentiated function of the liver, could still exist and operate after malignant transformation, at least to a minor extent. Clinical experience indicates that various differentiated functions may be present, decreased, increased, or even derepressed in the different types of neoplasms. Our present study, which demonstrates the loss of response to enzyme induction in the hepatoma, indicates the importance of deficiency in cell regulation as a substantial element of cancer.

Pitot and Cho (25) surveyed the literature showing that tumors appear to be less responsive to enzyme inducers than do normal cells.

On the basis of the experimental evidence reported in this paper, we concluded that the reprogramming of gene expression that is manifested in chemically induced transplantable rodent hepatomas (8, 29, 30–37) also appears to apply to virally induced transplantable avian hepatoma.

ACKNOWLEDGMENTS

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REFERENCES


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