Enzymatic and Immunological Studies on Pyruvate Carboxylase in Livers and Liver Tumors

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SUMMARY

It was found that one Reuber hepatoma and seven Morris hepatomas with different growth rates and various degrees of differentiation contained pyruvate carboxylase (PC) activity ranging from 3 to 113% of control values. The levels of PC activity in the regenerating liver, spontaneous mouse hepatoma, and host livers of tumor-bearing rats were estimated in the range of normal rat liver. In the group of tumors studied, two hepatomas of intermediate growth rate and four highly and well-differentiated, slow-growing tumors, like rat fetal liver, had negligible or little PC activity. However, one hepatoma, 9618A, had a normal level of PC activity. Antibodies prepared in rabbits against the purified mitochondrial enzyme showed identical titration curves against the nuclear and cytosol PC of liver. A single continuous precipitation line was obtained on Ouchterlony double-diffusion precipitation analysis when purified mitochondrial enzyme, crude mitochondrial preparations of rat tissues and hepatomas, were compared. It was also found that the immunological, enzymatic, and electrophoretic properties of the tumor PC were the same as those of normal adult and newborn rat liver. The amount of enzyme protein was quantitatively reduced in all but one of the liver tumors studied.

INTRODUCTION

PC⁴ [pyruvate: carbon dioxide ligase (ADP), EC 6.4.1.1] catalyzes the carboxylation of pyruvate to form oxalacetate which is an important intermediate in the reactions involved in the processes of gluconeogenesis, lipogenesis, energy production, and other tricarboxylic acid cycle activities (17, 28). Its role in these functions is supported by the finding that when rat liver mitochondria are incubated with an excess of pyruvate, NaH¹⁴CO₃, activators, and cofactors necessary for carboxylation, the principal products containing incorporated ¹⁴C are citrate, malate, fumarate, and aspartate (9, 25), which are precursors of glucose, fatty acids, proteins, and nucleic acids.

It is probable that the function of this enzyme is important for the normal growth and metabolism of liver.

It was shown (23, 29, 33, 34) that the activities of enzymes important in gluconeogenesis are negatively related to the growth rate of liver tumors on the basis of studies of glucose 6-phosphatase, fructose-1,6-diphosphatase, and phosphoenolpyruvate carboxykinase. However, the scant data on PC were based on assays of total enzyme activity, and no data were available on mitochondrial PC in the highly differentiated, slow-growing hepatomas.

The elegant demonstration of isoenzyme alterations as anomalies of gene expression in the experimental hepatomas and the characteristic resemblance of the rapidly growing, poorly differentiated hepatoma to the rat fetal liver provide important knowledge for our understanding of the molecular basis of cancer (7, 35—37). Preliminary observations that rat fetal liver and slow-growing minimal-deviation hepatoma 9633 have negligible PC activity prompted this comparative study of PC in the liver-liver tumor model system. Comparisons were made of: rapidly growing tissues versus quiescent and slow-growing tissues, host or normal liver versus hepatoma, well-differentiated hepatoma versus poorly differentiated hepatoma, transplantable rat hepatoma versus spontaneous mouse hepatoma and fetal versus adult liver.

Some of the specific questions we wish to ask are: Are isoenzymes of PC involved in liver-liver tumor systems? Is there a correlation between PC activity and the growth rate of livers and liver tumors, including the highly differentiated hepatomas? Is the observed reduction in PC activity in tumor mitochondria due to a difference in the content of enzyme protein or to a difference in quality of the enzyme protein? This paper is concerned only with the problems related to these 3 questions.

MATERIALS AND METHODS

Animals and Hepatomas. Tumor-bearing and normal male rats were housed in individual cages under standard conditions (12-hr-cycle lighting schedule, 72°F). Purina laboratory chow and water were available ad libitum. The tumors were transplanted bilaterally s.c. or i.m. The growth rate, histological classification, and generation of the 8 hepatoma lines used in this study are summarized in Table 1. Host livers and livers from 1 strain of normal male rats (Buffalo) and 2 stocks of male rats (Sprague-Dawley and...
### Table 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Generation*</th>
<th>Growth rate*</th>
<th>Enzyme activity (μmoles CO₂ fixed/g/min)</th>
<th>Specific activity (μmoles/mg mitochondrial protein)</th>
<th>Mitochondrial protein (mg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rat liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffalo</td>
<td></td>
<td>Fast</td>
<td>4.71 ± 0.35</td>
<td>205 ± 12.7</td>
<td>21.0 ± 0.32</td>
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<tr>
<td>Sprague-Dawley</td>
<td></td>
<td></td>
<td>4.68 ± 0.42</td>
<td>225 ± 10.6</td>
<td>20.8 ± 0.43</td>
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<tr>
<td>Wistar</td>
<td></td>
<td></td>
<td>4.92 ± 0.21</td>
<td>250 ± 10.7</td>
<td>19.4 ± 0.39</td>
</tr>
<tr>
<td>Regenerating rat liver (24 hr)</td>
<td>14</td>
<td>Fast</td>
<td>3.55 ± 0.24</td>
<td>186 ± 12.3</td>
<td>17.6 ± 0.46</td>
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<tr>
<td>Host liver</td>
<td>14</td>
<td>Fast</td>
<td>2.81 ± 0.22</td>
<td>219 ± 16.8</td>
<td>12.8 ± 0.51</td>
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<tr>
<td>Host liver</td>
<td>72</td>
<td>Intermediate</td>
<td>0.41</td>
<td>52</td>
<td>7.5</td>
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<tr>
<td>Host liver</td>
<td>63</td>
<td>Intermediate</td>
<td>0.78</td>
<td>80</td>
<td>9.7</td>
</tr>
<tr>
<td>Host liver</td>
<td>9</td>
<td>Slow</td>
<td>2.85</td>
<td>158</td>
<td>18.0</td>
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<tr>
<td>Host liver</td>
<td>7787</td>
<td>Slow</td>
<td>2.79 ± 0.20</td>
<td>159 ± 34</td>
<td>18.1 ± 0.7</td>
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<tr>
<td>Host liver</td>
<td>21</td>
<td>Slow</td>
<td>3.25</td>
<td>141</td>
<td>23.0</td>
</tr>
<tr>
<td>Host liver</td>
<td>9633</td>
<td>Slow</td>
<td>4.11 ± 0.17</td>
<td>266 ± 16</td>
<td>16.2 ± 0.76</td>
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<tr>
<td>Host liver</td>
<td>13, 14, 16</td>
<td>Slow</td>
<td>0.31 ± 0.04</td>
<td>32 ± 9</td>
<td>10.4 ± 0.63</td>
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<tr>
<td>Host liver</td>
<td>9618A</td>
<td>Slow</td>
<td>4.30 ± 0.31</td>
<td>221 ± 29</td>
<td>19.5 ± 1.3</td>
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<tr>
<td>C3H-A♂ mice</td>
<td>7, 6</td>
<td>Slow</td>
<td>4.77 ± 0.57</td>
<td>202 ± 18</td>
<td>16.6 ± 1.0</td>
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<tr>
<td>Liver</td>
<td></td>
<td></td>
<td>2.80</td>
<td>191</td>
<td>14.5</td>
</tr>
<tr>
<td>Tumor</td>
<td></td>
<td>Slow</td>
<td>2.86</td>
<td>153</td>
<td>18.7</td>
</tr>
<tr>
<td>Rat fetal liver</td>
<td>(17-19 day gestation)</td>
<td>Fast</td>
<td>0.25 ± 0.03</td>
<td>41 ± 5.5</td>
<td>8.0 ± 0.9</td>
</tr>
</tbody>
</table>

*An inferior number after a generation number refers to number of the transfer in that generation.

*See Refs. 11, 19-22, 24, and 26.

*Mean ± S.E. (3 to 8 animals for hepatomas and livers).

Wistar) were used as controls. Rat regenerating livers were obtained by partial hepatectomy as described by Higginson and Anderson (10). C3H-A♂ mice were given by Dr. W. E. Heston of the National Cancer Institute. Pregnant rats were purchased from the Flow Research Laboratory, Dublin, Va.

**Chemicals and Enzymes.** All chemicals used in counting media, buffers, and standard solutions were of scintillation or analytical grade. Lithium coenzyme A, citrate synthase, and phosphotransacetylase were purchased from Boehringer Mannheim Corporation, New York, N. Y. Radioisotopes (14C-labeled sodium bicarbonate, 60 mCi/m mole) were obtained from Amersham/Searle, Arlington Heights, Ill. Lubrol WX was purchased from General Biochemicals, Chagrin Falls, Ohio.

**Preparation of Mitochondria.** Control and tumor-bearing rats were decapitated with a guillotine and allowed to bleed 20 to 30 sec. Liver and tumor were removed and cleaned of fat, connective tissue, and necrotic areas and were placed in cold STE medium, pH 7.6. In some cases, 1% bovine serum albumin was added to the STE medium for the preparation of tumor mitochondria. For routine crude preparation of mitochondria, the tissue was minced and homogenized in an ice bath with 5 passes of a motor-driven glass homogenizer with a Teflon pestle at approximately 2000 rpm in a volume of isolation medium 2 to 8 times the weight of the tissue (based on the mitochondrial protein content of the tissue). Additional homogenization was sometimes required for tumors. The homogenate was centrifuged at 550 × g for 10 min, at 0 to 2°C, to remove the nuclei. The procedure of isolation and purification of mitochondria and mitochondrial membranes was similar to methods described in a previous paper (5).

**PC Assay.** PC was routinely assayed in crude mitochondrial preparation by a modified method of Henning et al. as described by Adam and Haynes (1). The enzyme was activated by Lubrol WX at a concentration of 0.5 to 1.0 mg/mg mitochondrial protein. The maximal activity was assayed in a final volume of 0.5 ml in STE medium that contained the following substances: potassium pyruvate, 5.0 mM; MgCl₂, 12.5 mM; KHCO₃, 12.0 mM; potassium phosphate, 8.0 mM (pH 7.6); ATP, 5.0 mM; lithium coenzyme A, 0.5 mM; acetyl phosphate, 1.1 mM; and 0.25 μCi of NaH¹⁴CO₃. The assay was coupled with phosphotransacetylase (6 μg/ml) to maintain a constant level of acetyl-CoA and with citrate synthase (12 μg/ml) to form a relatively stable product, citrate. The reaction was initiated by addition of the mitochondrial preparation, con-
taining approximately 0.2 mg mitochondrial protein, to
the reaction mixture and incubation at 37°C with slow shak-
ing for 5 min. The reaction was stopped by addition of 1 ml of 5% TCA. Following centrifugation at 550 × g for 5 min, 0.5 ml of the supernatant fluid was cleared of acid-labile
14CO2 by 100% CO2, which was bubbled through it for 2 min. The 14C was counted in a liquid scintillation spectrometer. Enzyme activity is defined as μmoles CO2 fixed per g of tissue per min or nmols CO2 fixed per mg mitochondrial protein per min at 37°C. Incubations in the absence of pyruvate or enzyme were carried out as blanks. A blank incorporation of about 80 to 200 cpm was found; this value was subtracted from all assay measurements. The mitochondrial suspension without Lubrol treatment was also included and gave an incorporation of 14CO2 that was usually very low, indicating that the mitochondria were intact.

Measurement of Radioactivity. 14C radioactivity was measured by scintillation counting in a Beckman liquid scintillation spectrometer. The scintillator contained PPO, naphthalene, Cellosolve, and toluene (1:20:100:150) (w/w, v/v). Sufficient counts were recorded to give an accuracy of 1.0 to 1.5%; the efficiency of counting was about 96% for 14C.

Identification of Reaction Products. Separation of radioactive carboxylic acids on Dowex 1-formate resin was performed according to the method of Busch et al. (4) after removal of TCA with ether and neutralization of the extracts. After the nonacid components had been washed off the column with water, the acids were eluted with 4 N formic acid. The fractions containing the peak activity were pooled, air dried, and taken up in a small quantity of water. Aliquots of the sample from each step were counted, and the recovery of the activity was calculated. The labeled compounds were identified by means of silica gel thin-layer chromatography (31) and high-voltage paper electrophoresis (13).

Thin-Layer Chromatography. The labeled acids and standard solutions were applied on an Eastman Type K 301R silica gel sheet and were developed in a well-saturated tank with methanol:5 N ammonia solution (80:20) for 2 hr. The acids were located with a Brom cresol green aerosol (yellow spots on blue background). They were scraped off the plate with the silica gel and transferred directly to counting vials containing 15 ml of counting solution (5 g PPO, 0.3 g POPOP, and 4 g Cab-O-Sil in 1 liter toluene).

High-Voltage Paper Electrophoresis (Horizontal Plates). The labeled acids and standard solutions were applied to a strip of Whatman No. 3MM paper (6 × 28.5 inches). Electrophoresis was carried out in 2 N acetic acid solution (pH 2.2) at 7 kV, 29 to 33 ma, for 2 hr. The paper was dried and treated with steam to evaporate traces of acetic acid. Citric acid, malate, and other organic acids were detected by spraying with 0.05% methyl red in 0.01 M phosphate buffer, pH 7.7. The acid appeared as red spots on a yellow background. The distribution of radioactivity on the electrophoretogram was located by a Packard scanner. The areas in the electrophoretogram corresponding to the radioactive peaks were cut and placed in counting vials containing the scintillant.

Purification of PC. The procedure for the preparation of rat liver mitochondrial PC described by McClure et al. (18) was followed, with slight modifications. The initial 10 to 40% homogenate (depending on the mitochondrial protein content of the tissue) was centrifuged at 50 × g for 10 min, and the pellet was resuspended in STE medium and centrifuged again at 50 × g for 10 min. The combined supernatant fractions were centrifuged at 9750 × g for 10 min. The resulting pellets were washed twice with the same isolating medium. The remainder of the procedure of PC extraction and purification was the same as that described by McClure et al. (18). The elution pattern of PC from the sucrose gradient contained 2 protein peaks corresponding to the refractive indices 1.3600 and 1.3650. The PC activity peak coincided with the 1.3650 protein peak. The purity of this peak was examined immunologically and electrophoretically. The average specific activity of PC peaks was 25 units/mg protein. Peaks were pooled and used for preparation of antisera.

Immunization. Rabbit antisera against pure PC was prepared according to the method described by Williams and Chase (38) and Work and Work (39). Approximately 2 mg PC protein in 2 ml solution were well suspended in an equal volume of complete Freund's adjuvant. The emulsion was injected into 8 sites of a 2 to 3-lb rabbit (3 i.m. injections in each side of the rabbit and 2 s.c. injections in back of the neck). Six rabbits were used in this experiment. Blood samples taken before immunization served as control sera.

Three additional injections of enzyme were made during a 1-month period. The potency of the antisera was measured weekly by Ouchterlony double-diffusion-precipitation analysis.

Double Immunodiffusion in Agar Gel. The circular-well method of double immunodiffusion was used which involves the use of agar plates with wells for both antigens and antibodies. One % Ionagar No. 2S was made in barbitone:acetate buffer, pH 8.6 (final concentration of 0.05 ionic strength). For the double immunodiffusion, initial trials to fix a balanced proportion between antigen and anti-
sera were performed in 2 series, e.g., with a fixed amount of the antigen diffusing against a series of antiserum dilu-
tions and vice versa. The application of the reactants was standardized as much as possible in terms of protein concentration. After 48 hr of diffusion, the slides were washed for 48 hr in 0.15 M NaCl and for 24 hr in multi-
ple changes of deionized distilled water. Following these washings, the slides were dried and then stained 1 min with 0.5% Amido schwarz dye in methanol:acetic acid:water (5:1:5) and were washed with the solvent until well de-
colorized.

Single Radial Immunodiffusion in Agar Gel. The pro-
dure of Mancini et al. (16) was used for the quantitative immunological determination of PC protein. One part of 3%(w/v) agar in barbitone:acetate buffer containing 0.01% Merthiolate was mixed with 2 parts of anti-PC antiserum at 48°C. The agar:antisera mixture was poured onto prewarmed slides to give a layer 2.0 mm thick. After solidi-
fication of the gel in a humid atmosphere, wells 2.5 mm in diameter were punched out. These wells were filled with
antigen solutions by means of a calibrated microsyringe, with care being taken to deliver accurately measured volumes containing known amounts of protein. At the end of 5 days of incubation at 22°, the agar slides were washed for 2 to 3 days in 0.15 M NaCl and then in distilled water. They were dried and stained with Amido schwarz. A standard curve was plotted relating the area of the precipitate to the amount of purified PC preparation added. The relative concentration of PC protein in the crude mitochondrial samples developed under standard conditions was estimated by the standard curve.

**Protein Determination.** Protein concentrations were determined by the method of Lowry et al. (15) with bovine serum albumin as a standard. A spectrophotometric method (14) was used for the determination of protein content in the fractions from the sucrose gradient that was used in the purification of PC.

**RESULTS**

**Enzyme Activation.** The enzyme can be activated either by sonic disruption or by the non-ionic detergent Lubrol WX. The effectiveness of these 2 methods in dismembration of mitochondrial preparations was compared by following PC activity and the changes in turbidity at A₂₅₀. The PC activity rose during the 1st min of sonic disruption and remained at a similar level for up to 8 min, although the rate of clearance of the mitochondrial suspension was found to be proportional to the time of sonic disruption.

The results of the Lubrol effect on the dismembration and PC activity in the crude mitochondrial preparations of liver showed that PC activity increased with increasing Lubrol concentration. Four min after Lubrol treatment, 8 to 10-fold increases in activity were observed in most cases. Concentrations of 0.5 to 1.0 mg Lubrol per mg mitochondrial protein were chosen for routine assay, but concentrations as high as 1.8 mg Lubrol per mg protein caused no inhibition of the enzyme. The effect of Lubrol on tumor PC activity was similar.

**Maximal PC Activity in Liver and Tumor Mitochondria.** In order to ensure that the observed differences between liver and tumor were not the results of preparative artifacts, the following experiments were performed. (a) Tumor mitochondria prepared with the isolation media [used by Sordahl and Schwartz (30) and Schnaitman and Greenwald (27)] and 0.25 M sucrose, with and without bovine serum albumin, appeared to be metabolically functional. In the presence of the substrate succinate, the addition of ADP to the mitochondrial preparation caused an increase in respiratory activity. The respiratory control values obtained for hepatoma H35 were 2.7 to 5.6. (b) STE medium was routinely used for the crude preparation of liver mitochondria. The addition of 1% bovine serum albumin to STE medium for the preparation of tumor mitochondria did not change the measured amount of PC activity in the mitochondria; therefore, STE medium was used for both liver and tumor mitochondrial preparations. (c) The response of tumor mitochondria to Lubrol treatment in terms of turbidity change and activation of PC was another indication that the mitochondria were intact. (d) The PC activity in tumor mitochondria was studied in relation to the concentrations of the substrates, activator, and cofactors (either reduced to one-fifth or increased to 2-fold the concentrations described in "PC assay"). The PC activity of hepatoma 9633, at barely detectable levels, was not noticeably altered by the changes. On the other hand, hepatoma 9618A and host liver responded to the variations in the concentrations of these reactants in a similar fashion (Chart 1).

**Intracellular Location of PC.** The PC activity in the subcellular fractions of hepatoma 9633 and host liver was examined. About 70% of the enzyme activity in liver is localized in the crude mitochondrial preparation. About 10% was observed in the postmitochondrial fraction and 20% was observed in the nuclear fraction.

Qualitative characteristics of the PC present in these subcellular fractions of liver were examined immunologically. A single continuous precipitation line was obtained by Ouchterlony double-diffusion analysis when the mitochondrial, nuclear, and cytosol PC were compared. Furthermore, the results of immunochromatography tests showed that antibodies prepared in rabbits against the purified mitochondrial enzyme gave identical titration curves against the mitochondrial, nuclear, and cytosol PC of liver (Chart 2). Therefore, there is a high probability that all of the PC activity observed in all the subcellular compartments is derived from mitochondria and represents preparative artifacts and mitochondrial leakage.

Furthermore, the low enzyme activity in tumor 9633 does not appear to be caused by the presence of a dissociating endogenous inhibitor since the mixing experiment of host liver and tumor gave the mean activity of the 2 tissues.

**Identification of Reaction Products.** TCA extracts of the labeled reaction products in a 5-min incubation were prepared and analyzed by silica gel thin-layer chromatography and high-voltage paper electrophoreses. A single distinct peak was observed on the radioactive scanning tracing of the electrophoretogram. This peak, which
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![Graph](chart2.png)

Chart 2. Titration of PC in mitochondria (O), nuclei (Δ), and cytosol (D) against control serum (●) and antiserum (O, Δ, □) prepared from rabbits immunized with rat liver mitochondrial PC. The subcellular fractions were prepared by differential centrifugation, as described in "Materials and Methods." The supernatant of the Lubrol-treated subcellular fraction and antiserum were incubated at room temperature for 10 min and then in an ice bath for 1 hr. The solution was centrifuged and the activity remaining in the supernatant was assayed. For the enzyme assay, see "Materials and Methods."

coincided with the location of the citric acid standard, contained 91.6% of the total radioactivity.

Under the given conditions, the separation of the radioactive acids on the silica gel thin-layer chromatography was not as clear-cut as that obtained by high-voltage paper electrophoresis. About 25% of the total radioactivity remained at the origin of the spot. Nevertheless, it still showed that among 5 or 6 standard acids, only 1 radioactive peak was observed, and this had the same Rf value as citric acid. From these results, it can be said that a net synthesis of citrate was demonstrated in mitochondrial extracts under the assay conditions.

Enzymatic and Immunological Measurement of PC in Livers and Liver Tumors. Tables 1 and 3 summarize the data on PC activity and PC protein content in rat livers and hepatomas.

Eight lines of hepatomas, ranging from fast to slow growing, from poorly to highly differentiated, and from an abnormal to a completely normal karyotype, contain various amounts of PC activity ranging from 2 to 113% of control values. Rapidly dividing regenerating liver and nondividing normal and host livers contained a high level of PC activity. On the other hand, the rapidly growing fetal liver, hepatoma 9A, 2 intermediate types of hepatoma, and 4 slow-growing hepatomas (including 2 minimal-deviation types) contained little PC activity. It is interesting to note that the levels of the enzyme activity in the tissues of C3H-45° liver and tumor were within the normal range. Also interesting are the contrasting characteristics between the 2 minimal-deviation, very slow-growing hepatomas 9633 and 9618A. Hepatoma 9618A had a normal level of PC activity and a relatively high glycogen content (Ref 24, and our unpublished observations) compared with other hepatomas, whereas hepatoma 9633, with only about 8% of host liver PC activity, contained a negligible amount of glycogen.

The mitochondrial protein content in the hepatomas varied from 30 to 80% of their host liver values. These data are also listed in Table 1.

Table 2 summarizes the results of the Ouchterlony double-diffusion precipitation analysis of the anti-PC antiserum of rat liver mitochondria against PC from various rat tissues and hepatomas. A single continuous precipitation line was observed on Ouchterlony double-diffusion precipitation pattern when purified mitochondrial PC enzyme, crude mitochondrial preparations of rat livers, and hepatomas were compared. There was a good correlation between the immunoprecipitation data and the amount of PC activity measured enzymatically in these tissues. It can be said, from these data, that the mitochondrial PC of rat liver and rat hepatomas appear to have a common antigen.

Validity of the Immunochemical Evidence for PC Protein Deficiency in Hepatomas. The homogeneity of the PC preparations used for preparation of antibodies was examined by 4 and 7.2% polyacrylamide gel electrophoresis in 0.04 M Tris:glycine buffer at pH 8.8 (6, 8). The original liver homogenate separated 16 protein fractions on 7.2% gel. The number of protein bands gradually decreased in the following purification steps. The purified enzyme obtained from the sucrose gradient purification step separated on 7.2% gel as a single band with a mobility similar to the bovine serum albumin dimer. The enzyme preparation had an average specific activity of 25 units/mg protein. A single immunoprecipitation line produced by the anti-PC antiserum is additional evidence of the purity of the enzyme preparation.

Attempts were made to purify PC from hepatoma H35. It was found that the purified PC from both liver and hepatoma had the same electrophoretic mobility in 4.0 and 7.2% polyacrylamide gel in Tris:glycine buffer. Both liver and tumor PC activity were inhibited by avidin, and the preincubation of the avidin and biotin alleviated the inhibition action of avidin. Both liver and tumor PC had the same immunoprecipitation reaction with the antiserum made against liver mitochondrial PC. However, the yield and specific activity of PC were low in the tumor, about 13% of the normal rat liver value.

Approximately 40 milliunits of PC activity were inactivated by 20 μl of antiserum (Chart 2). In the in vitro inhibition test, a linear relation between the concentration of antiserum and percentage inhibition of PC activity was observed. No significant inhibition was found with the control serum.

A standard curve for the quantitation of PC protein was constructed according to the method of Mancini et al. (16). The amounts of PC present in livers and hepatomas determined by the single radial-diffusion method are pre-
PC in Livers and Liver Tumors

Table 2
Ouchterlony double-diffusion precipitation analysis

Summary of the results of Ouchterlony double-diffusion precipitation analysis of the rabbit antiserum against PC of the crude mitochondrial preparations from various rat tissues and hepatomas. The rabbit antiserum was made against rat liver mitochondrial PC.

<table>
<thead>
<tr>
<th>PC sources</th>
<th>Serum</th>
<th>Immunological reaction</th>
<th>Enzymatic PC activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified rat liver mitochondrial PC</td>
<td>Control serum</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Purified rat liver mitochondrial PC</td>
<td>Antiserum</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Crude mitochondrial preparations</td>
<td>Antiserum</td>
<td>-</td>
<td>Negligible</td>
</tr>
<tr>
<td>Normal rat liver without Lubrol</td>
<td>Antiserum</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Normal rat liver with Lubrol (or freeze-thaw)</td>
<td>Antiserum</td>
<td>-</td>
<td>Negligible</td>
</tr>
<tr>
<td>Host livers</td>
<td>Antiserum</td>
<td>-</td>
<td>(2-113% of control)</td>
</tr>
<tr>
<td>Eight lines of hepatoma</td>
<td>Antiserum</td>
<td>-</td>
<td>(2-113% of control)</td>
</tr>
<tr>
<td>Rat fetal liver</td>
<td>Antiserum</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rat regenerating liver, kidney, and adipose tissue</td>
<td>Antiserum</td>
<td>-</td>
<td>Negligible</td>
</tr>
</tbody>
</table>

Table 3
Immunooquantitation of PC in normal rat livers, hepatomas, and their respective host livers

<table>
<thead>
<tr>
<th>Tissue</th>
<th>PC protein (mg/g of tissue)</th>
<th>No. of determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal liver (Buffalo strain)</td>
<td>0.139 ± 0.006*</td>
<td>4</td>
</tr>
<tr>
<td>Hepatoma 9633</td>
<td>0.003</td>
<td>4</td>
</tr>
<tr>
<td>Host liver</td>
<td>0.119 ± 0.007</td>
<td>4</td>
</tr>
<tr>
<td>Hepatoma H35</td>
<td>0.004</td>
<td>2</td>
</tr>
<tr>
<td>Host liver</td>
<td>0.110</td>
<td>2</td>
</tr>
<tr>
<td>Hepatoma 9618A</td>
<td>0.146 ± 0.007</td>
<td>4</td>
</tr>
<tr>
<td>Host liver</td>
<td>0.156 ± 0.006</td>
<td>4</td>
</tr>
</tbody>
</table>

* Mean ± S.E.

DISCUSSION

The PC observed in the various subcellular fractions of liver in this study gave a reaction of identity supporting the concept of an exclusive localization of mitochondrial PC (2, 3, 12, 18, 32). The immunological and electrophoretic data on liver and tumor indicated that no isoenzymes of PC were present in the liver-liver tumor system. Since rat liver mitochondria contain 70 to 80% of the total PC activity and the immunological properties of the PC present in the nuclear and cytoplasmic fractions are the same as that in the mitochondrial, we may consider that the PC's of the subcellular fractions are similar proteins.

Among the 8 lines of hepatomas used in this study, 7 contained a reduced amount of PC (2 to 20% of their host liver values). Hepatoma 9618A, on the other hand, contained PC activity about 13% higher than that of its host liver. According to Nowell et al. (21, 22), most of the hepatomas had 42 chromosomes, and only hepatomas 9633 and 9618A were completely normal in karyotype. However, their most recent studies on hepatoma 9618A (Generations 6 and 7, which we used in this study) indicate that the karyotype is no longer "normal diploid" (P. C. Nowell, personal communication). The data on PC activity and mitochondrial membrane protein profiles of these 2 minimal-deviation hepatomas were most striking. Hepatoma 9618A had a normal level of PC, a normal mitochondrial membrane protein pattern, and a relatively high content of glycogen compared with other hepatomas. Hepatoma 9633 had a much reduced amount of PC, a negligible amount of glycogen, and an altered mitochondrial membrane protein pattern (our unpublished observations).

It was observed that the number of mitochondria in tumors was reduced in terms of mitochondrial protein content per g fresh weight of tissue (Table 1). Therefore, the decrease in the mitochondrial PC in the hepatomas may well partially reflect the decrease in the number of mitochondria in the tumors. However, the reduction in specific activity (nmoles CO₂ fixed per mg mitochondrial protein) as well signifies a true decrease in the amount of PC in tumor mitochondria.

Weber (33) and Weber and Morris (34) reported that the total tissue PC activity of fast-growing hepatomas 3924A and 3683 had 14 and 9%, respectively, of normal liver PC values, and slow-growing hepatoma 5123-D had 99% of control value. Together with the data on 3 other gluconeogenic enzymes, they postulated the positive correlation of a progressive decrease of key gluconeogenic enzymes with increasing growth rates of the tumors. However, minimal-deviation hepatomas 9633 and 7787 and 4 other hepatomas with intermediate and slow growth rate (H35, 7800, 21, and 6) used in this study also had an 80 to 90% reduction in PC activity. Furthermore, the slow-growing mouse hepatoma and the fast-growing regenerating liver had normal levels of PC. Therefore, these observations do not show a good correlation between the progressive loss
of PC and growth rate and degree of differentiation of the tumor cells. Except for hepatoma 9618A, the PC in the other 7 hepatomas was reduced to 2 to 20% of the control values.

Furthermore, immunological and enzymatic studies indicate that the PC proteins of rat liver and liver tumors appear to be the same. As far as we are aware, this is the first report showing that the changes in the amount of PC activity in the hepatomas result from variations in the amount of PC protein present.

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Enzymatic and Immunological Studies on Pyruvate Carboxylase in Livers and Liver Tumors

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