Extraction of a Factor from Ehrlich Ascites Tumor Cells That Increases the Activity of the Fetal Isozyme of Pyruvate Kinase in Mouse Liver

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SUMMARY

Isoelectrofocusing studies of mouse tissue extracts show mice to have a pyruvate kinase isozyme pattern very similar to that of the rat. Moreover, electrophoresis or kinetic assays conducted on liver extracts from normal mice and from mice bearing Ehrlich ascites tumors show that the latter have a higher proportion of the fetal K-isozyme of pyruvate kinase. Serial injection of the supernatant remaining after centrifugation of homogenized tumor cells at 100,000 × g, or of the phenolic extracts from the latter, produced a similar shift in the liver isozyme pattern. This shift in the liver isozyme pattern involves both a decrease in L-isozyme activity and an increase in K-isozyme activity. However, only the increase in activity of the K-isozyme appears to be a specific response to injection of the extracts. The presence of a specific factor in these extracts was confirmed by the observation that similar extracts prepared from normal adult tissues did not increase activity of the K-isozyme. On the other hand, phenolic extracts from fetal mice did increase K-isozyme activity as did injections of serum from tumor-bearing mice or of the cell-free ascites fluid. Evidence is presented supporting the concept that the factor is proteinaceous in nature, and that it acts by derepressing synthesis of the K-isozyme.

INTRODUCTION

Recent data indicate that there are at least 3 distinct isozymes of mammalian pyruvate kinase (6, 11, 12, 14, 15, 18–20, 29, 32, 36, 37, 39, 44, 45). Near term, the dominant fetal isozyme (K type) of the rat is replaced by a 2nd isozyme (M type) in muscle and brain and by a 3rd isozyme (L type) in the parenchymal cells of the liver (26, 30, 36, 41). This normal developmental pattern is reversed in dedifferentiated neoplasms of either muscle or liver, i.e., the characteristic adult enzyme is replaced by the normal fetal enzyme (1, 2, 9, 11, 12, 31, 41–43).

Suda et al. (33, 35, 38, 45) have shown that noncancerous liver from tumor-bearing animals or livers perfused with the blood of tumor-bearing animals (34) also undergo a shift in the pyruvate kinase isozyme pattern; again L-isozyme activity decreases, and the fetal (K) isozyme activity increases. This shift in the pyruvate kinase isozyme pattern would appear to be part of a general shift toward fetal proteins which may be induced in liver by tumors (13, 33, 35). The increased activity of at least the K-isozyme of pyruvate kinase in the livers of tumor-bearing rats would appear to be due to de novo protein synthesis, as was shown by using antibiotics and by precipitating labeled enzyme with appropriate antibodies (35). A similar shift in pyruvate kinase isozymes was induced by injection of cell-free extracts of Ehrlich ascites cells into otherwise normal mice. However, the factor involved has not been characterized much beyond concluding it has a molecular weight of about 50,000 or less (35, 38).

Inspired by these studies, we have undertaken studies of the mouse isozyme pattern and the influence of ascites tumor growth and extracts on liver isozyme pattern. The data to be reported confirm the observations of the Japanese investigators (33, 35, 38, 45) and extend them by showing that the factor can be extracted with phenol and that it may be obtained from fetal tissues but not from normal adult tissues. Moreover, evidence is presented suggesting the factor is a polypeptide and that it acts as a derepressor.

MATERIALS AND METHODS

Materials. Mice used in these studies were young male (22 to 26 g) C57/6J × DBA/2J F, hybrids, usually obtained from The Jackson Laboratory, Bar Harbor, Maine. Free and agarose-bound pancreatic RNase A, RNase T1, from Aspergillus oryzae Grade III, Streptomyces griseus protease (type V), and l-tryptophan were obtained from Sigma Chemical Co., St. Louis, Mo. Chymotrypsin was a product of Worthington Biochemical Corp., Freehold, N. J. Other biochemical reagents were purchased from the California
Preparation of Phenolic Extracts. Ehrlich ascites tumor cells obtained from Dr. Ralph W. McKee were maintained in ascites form by serial passage, usually in C57BL/6J x DBA/2J F1, mice. Cells were harvested 9 to 12 days after inoculation with 0.2 ml cells (2 × 10^6 cells). Except for the data of Table 2, harvested cells were treated sonically 5 mm at about 7 amperes with a Bronson sonic oscillator and then centrifuged at 100,000 × g for 1 hr at 4°. One part of this high-speed supernatant was mixed with 5 parts of the homogenizing Medium A, described by Moldave (27) and 6 parts of water-saturated phenol. The general procedure described by Moldave (27) for the "isolation of C4-labeled S-RNA" was then followed, i.e., the mix was stirred in the cold for 1 hr, centrifuged, and the aqueous layer was drawn off. The phenolic and interface layers were reextracted with an equal volume of cold water and discarded; 0.1 volume of 20% potassium acetate, pH 5.0, and 2 volumes of 95% ethanol were added to the combined aqueous layers; this mixture was stored overnight at −20°; the resultant precipitate was taken up in either cold distilled water or in phosphate-buffered saline (10 mM sodium phosphate, pH 7.0, in 0.9% NaCl solution) and dialyzed against phosphate-buffered saline for 2 hr.

The same procedure was followed when phenolic extracts were made from solid tissues except the tissues were first homogenized in 3 parts (w/v) of homogenizing medium A or in 0.25 M sucrose and then mixed with 5 parts homogenizing Medium A prior to extraction with phenol.

Incubation with RNase and Proteases. Dialyzed phenolic extracts were incubated with 60 IU (1 mg/mL) of soluble pancreatic RNase A (Type I-A) at room temperature 15 min past cessation of the hyperchromatic effect or in 10 IU/mL of agarose-bound pancreatic RNase for 30 min. At this time the agarose-bound RNase was centrifuged out. Some RNase-incubated phenol extracts were further incubated with 46 IU (1 mg/mL) chymotrypsin for an additional 30 min at 37°, or with S. griseus protease for 18 hr at room temperature. Whenever such experiments were conducted, all samples to be injected (i.e., with or without the added hydrolyases) were incubated under the same conditions. The extended incubations were conducted to promote self-destruction of the proteases. That this was only partially successful was demonstrated by the fact the animals given injections of proteases alone died, although those given injections of extract plus protease demonstrated no adverse symptoms.

Injection Schedule. Unless otherwise noted, 0.3 mg of material (calculated from the 260-nm absorption, assuming A 260 = 0.04 μg/mL) was injected i.p. daily for 4 days and the animals were sacrificed on the 5th day.

Assay for Pyruvate Kinase Isozymes. Mice were decapitated, exsanguinated, and hepatectomized. The livers were freed from the gall bladder, vessels, and obvious connective tissues, homogenized in 0.25 M sucrose, and centrifuged at 100,000 × g for 1 hr (15). The total enzyme activity in the supernatant was determined by assay at pH 7.0 under conditions described (15) using, however, 0.5 mM ADP and 1.5 mM phosphoenolpyruvate. The relative proportion of K- and L-enzymes was determined by isoelectric focusing (15) or by measuring the rates in the same assay system but at pH 7.5 and with 0.36 mM phosphoenolpyruvate and 0.34 mM ADP in the presence and absence of 5 mM tryptophan, which was found to be a potent specific inhibitor of the mouse K-enzyme, as is the case for the rat enzyme (17, 21). The enzyme was preincubated for about 5 min with all components except ADP, which was used to start the reaction. Under these conditions (lowered phosphoenolpyruvate concentration and higher pH) both isozymes were near their V_max but the K-enzyme was inhibited almost completely while the L-enzyme retained 85 to 90% of its activity. Thus the percentage change induced by tryptophan × 1.17 was used to calculate the percentage L-enzyme in the assay mixture. This technique gave reliable data when tested on crude extracts of tissue with known isozyme compositions, with the various conformers of the 2 isozymes previously isolated by isoelectric focusing, and with appropriate mixtures. The following values for percentage L-enzyme were obtained when the same liver extracts were analyzed by isoelectric focusing and tryptophan inhibition, respectively: 2 livers from untreated mice, 79 and 77.4%, 84 and 87.1%; a liver from a tumor-bearing mouse, 66 and 66.5%; and a liver from a mouse that received injections of a phenolic extract, 68 and 68.2%.

Specific activity then was obtained by multiplying the total activity of enzyme by the fraction of that enzyme and dividing by the mg protein in the high-speed supernatant. Protein concentration was estimated by the Lowry technique (25).

Isoelectrofocusing. High-speed supernatants were subject to electrofocusing by techniques previously described (15). Extracts from the tissues were first electrofocused on pH 3.5 to 10 gradients in order to determine the range of pI values involved. The pI values reported were obtained using pH 5 to 8 ampholines. The proportion of the isozymes was obtained by estimating the areas under the respective activity peaks. Isoelectrofocusing of phenolic extracts was done on pH 3.5 to 10 gradients. Counts were obtained by adding 0.2 ml of each fraction to 10 ml Bray’s solution.

Gel Electrophoresis. Some phenolic extracts were subjected to analysis on polyacrylamide gels, using the basic techniques and precautions described by Loening (24) and Bishop et al. (3).

RESULTS

Mouse Isozyme Pattern. Table 1 summarizes isoelectric focusing patterns obtained when mouse tissue extracts were subjected to isoelectrofocusing. Approximately 70% of the pyruvate kinase activity from fetuses (that had their livers removed) was obtained as a twin band of activity at pH 6.6 and 6.9. Of the remaining activity, 15% had a pI value of 5.6, 7% had a pI value of 7.8, and 8% had a pI value of 7.3. The ascites tumor pyruvate kinase and the pyruvate kinase in an extract of spleens and lungs also yielded most of the activity at pH 6.6. Moreover, reincubation of pH 6.6 activity peaks converted them to pH 6.9 enzyme and then to a pH 5.5, 7.6, and 7.8 enzyme. Thus it may be concluded...
<table>
<thead>
<tr>
<th>Tissue extracted</th>
<th>K-isoenzyme variants</th>
<th>L-isoenzyme variants</th>
<th>M-isozyme</th>
<th>Undefined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Approximate pI values</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetal*</td>
<td>5.6 (15)</td>
<td>6.6 (50)</td>
<td>6.9 (20)</td>
<td>7.8 (7)</td>
</tr>
<tr>
<td>Ehrlich ascites tumor, fresh*</td>
<td>5.5 (0, 2)</td>
<td>6.6 (100, 80)</td>
<td></td>
<td>7.8 (0, 15)</td>
</tr>
<tr>
<td>Ehrlich ascites tumor, pH 6.6 peak†</td>
<td>5.5 (22)</td>
<td>6.9 (47)</td>
<td>7.6 (21)</td>
<td>7.8 (6)</td>
</tr>
<tr>
<td>Spleen plus lung†</td>
<td>6.5 (75)</td>
<td>6.9 (20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen plus lung pH 6.5 peak†</td>
<td>6.9 (100)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult skeletal muscle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal adult liver</td>
<td>6.6 (10)</td>
<td>6.9 (4)</td>
<td>7.8 (0-1)</td>
<td>5.2 (20)</td>
</tr>
<tr>
<td>(mean of 4 runs)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver from tumor-bearing mice*</td>
<td>6 days*</td>
<td>6.6 (27)</td>
<td>6.9 (4)</td>
<td>5.2 (17)</td>
</tr>
<tr>
<td></td>
<td>12 days</td>
<td>6.6 (30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16 days</td>
<td>6.6 (25)</td>
<td>6.9 (10)</td>
<td>5.2 (&lt;1)</td>
</tr>
</tbody>
</table>

* Approximately 13 days of gestation. Average weight, 0.68 g. Livers were removed.
† Numbers in parentheses, percentage of total activity.
‡ Results from 2 different runs.
§ First incubated at 37° for 30 min, then electrophoresed. Almost all activity was found at pH 6.6. The peak tubes were then reincubated and refocused twice, first with 10 mM EDTA, then at pH 7.8 in the presence of fructose 1,6-diphosphate-1-phosphatase (15). After EDTA treatment, little change was observed, except small amounts of pH 6.9 material were formed. The results reported were obtained after the 2nd refocusing experiment.
‖ These organs were obtained from normal adult mice and combined in order to obtain sufficient enzyme to electrophoresize. Rat spleen and rat lung were found to contain only the K-isozyme when studied by electrophoretic (14) or by isoelectrofocusing techniques (unpublished).
¶ Enzyme isolated between pH 6.35 and pH 6.64 was incubated 37° for 30 min and then reelectrophoresized.
** Ehrlich ascites tumor cells.
†† Days postinoculation.
that the pH 5.5, 6.6, 6.9, 7.6, and 7.8 pyruvate kinases represent various conformational and/or "pseudoisozymic" (21) variants of the basic fetal (K-type) isozyme. That is, of the pl variants found in fetal extracts, only the pH 7.3 form cannot be defined as an interconvertible form. The distribution, the number of variant forms, and the pattern of inhibition induced by amino acids (unpublished data) of the mouse K-type pyruvate kinase bear close analogy to that of the rat (11, 12, 14–20).

Table 1 also shows pyruvate kinase of skeletal and heart muscle to have a pl value of 7.8. As in rats (15, 18), this enzyme differs kinetically from the K-type enzyme with a similar pl value.

Liver extracts from normal mice yield about 80% of the total activity at pH 5.2 or 6.0. Kinetic studies indicate these enzymes to be an R, T conformational set showing a pattern of inhibition similar to the rat L-isozyme, R, T set (15, 18). The remaining 20% of activity had pl values of amino acid-inhibition pattern consistent with these forms being K-isozyme variants. Thus normal C57BL/2J × DBA/2J F1 mouse liver was found to yield 80% L-isozyme and 20% K-isozyme.

The electrofocusing patterns reported herein for liver and muscle extracts are similar to those reported by Yanagi et al. (45). However, these investigators reported the major ascites tumor band to have a pl value of about 7.8 and therefore characterized it as an M-isozyme rather than, as suggested by these data, a variant form of the basic K-isozyme.

Effect of Tumor Growth on the Liver Isozyme Pattern. Table 1 also shows the relative quantity of K-isozyme in liver extracts from tumor-bearing animals to be about twice that of controls. The proportion of K-isozyme was not greater in extracts from animals subjected to 16 days of tumor growth than from animals subjected to 6 days of tumor growth, indicating the change was not caused by metastases. Interestingly, tumor growth does increase the fraction of the pH 6.0 isozyme, i.e., the probable T conformer of the L-isozyme. This shift from the R to the T conformer probably reflects a change in the animals' carbohydrate metabolism toward gluconeogenesis. This assumption is supported by the observation of similar shifts for the L-isozyme in fasted rats (15) and for the K-isozyme in cultured cells deprived of glucose (40).

Liver extracts from animals bearing the DBA-mastocytoma ascites tumor showed a similar isozyme pattern shift and growth of Ehrlich ascites tumor in normal and diabetic C57 black mice also shifted the liver isozyme pattern toward the fetal pattern by increasing the specific activity of the K-isozyme.

Changes of Specific Activity Induced by Tumor Growth and Fractionated Tumor Cells. A more intensive investigation of these effects required a more convenient assay of the pyruvate kinase isozymes; thus, the kinetic technique described in "Materials and Methods" was developed. The data shown in all subsequent tables were obtained by means of this assay. Table 2 summarizes early data that differ from those of subsequent studies in that the tumor cells were broken under relatively mild conditions and the organelles separated by conventional differential centrifugation. However, the mean control values shown are those obtained in all studies performed. These data show that tumor growth, in the ascites or solid form, increased the specific activity of the K-isozyme more than 2-fold. Moreover, injection of the high-speed (100,000 × g) supernatant also increased K-isozyme activity when assayed in liver extracts. However, the material precipitated by ethanol from the aqueous phase of phenolic extracts of such supernatants appears to be a still more reliable source of the factor responsible for changing the liver isozyme pattern. On the other hand, the microsomal pellet appears to be inactive or, at best, to be marginally active.

Injection Frequency. The Japanese workers (35, 38) generally gave their animals one injection of tumor extracts and then assayed 4 days later. The data derived in the previous section were obtained using the philosophy that it

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals</th>
<th>Adult Mean specific activity (IU/mg protein)</th>
<th>Fetal Mean specific activity (IU/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control values*</td>
<td>17</td>
<td>0.721 (0.384–1.089)*</td>
<td>0.198 (0.090–0.270)</td>
</tr>
<tr>
<td>Growing Ehrlich’s ascites tumor (16 days)</td>
<td>1</td>
<td>0.838</td>
<td>0.404</td>
</tr>
<tr>
<td>Growing Ehrlich’s tumor, solid form (Sub Q) (60 days)</td>
<td>1</td>
<td>0.233</td>
<td>0.465</td>
</tr>
<tr>
<td>Injection of high-speed supernatant† (100,000 × g)</td>
<td>4</td>
<td>0.579 (0.322–1.197)</td>
<td>0.312 (0.197–0.510)</td>
</tr>
<tr>
<td>Injection of the &quot;microsomal&quot; pellet formed after centrifugation at 100,000 × g</td>
<td>2</td>
<td>0.294 (0.230 and 0.358)</td>
<td>0.227 (0.198 and 0.256)</td>
</tr>
<tr>
<td>Injection of phenol-extracted 100,000 × g supernatant</td>
<td>4</td>
<td>0.600 (0.379–0.646)</td>
<td>0.358 (0.298–0.430)</td>
</tr>
<tr>
<td>Injection of ascites fluid‡</td>
<td>2</td>
<td>0.757 (0.516 and 0.996)</td>
<td>0.258 (0.180 and 0.336)</td>
</tr>
</tbody>
</table>

* See Table 4.
* Numbers in parentheses, range.
† All injections were made once daily for 4 consecutive days, analyses being performed on the 5th day. In these experiments, the material injected was equivalent to the amount obtained from about 0.3 ml tumor (≈ 3 × 10⁷ cells). The cells were homogenized and fractionated under conditions designed to yield functional organelles. The basic procedure used was described by Koobs and McKee (23).
‡ Supernatant remaining after the tumor suspension was centrifuged at 485 × g for 5 min.
would be better to inject too much rather than too little material. Since extensive investigation, using this philosophy, would be wasteful of time and material, an attempt was made to determine an optimal injection sequence. These data (Table 3) suggest that the injection schedule fortuitously adopted (daily injections for 4 days) may be the minimal schedule required to increase the level of K-isozyyme. However, shorter periods of injection did lower the activity of the L-isozyme. Somewhat incongruously, the L-isozyme level returned to near normal levels in the animal given the greatest number of injections. As discussed below, the changes in L-isozyme activity may be due to nonspecific effects. It also may be that a minimal dose is required before any response of the K-isozyme occurs, e.g., 1 injection of a very active preparation conceivably may suffice. The observed positive relationship between amount of material injected and the response of the K-isozyme (38) could support this idea. However, it was considered to be more prudent to continue the protocol of 4 daily injections.

Statistical Evaluation of the Effect of Phenolic Extracts. The data of Table 4 indicate that the effects of phenolic extracts on the level of the fetal K-isozyme are statistically significant when compared to the values obtained with livers from normal untreated animals or from animals given daily injections of phosphate-buffered saline. The same is true when the parameter compared is the percentage of either isoyme, as shown for the L-isozyme. On the other hand, the effect of these extracts on the activity of the L-isozyme is statistically significant when compared to the untreated controls, but not when compared to the phosphate-buffered saline-injected animals.

The 15 experiments listed represent 15 different phenolic extracts. Thirteen of these extracts yielded specific activity values for the K-isozyme that were greater than that obtained for any control. One extract yielded a value in the upper range of normal controls (0.253 IU/mg) and 1 yielded a value of only 0.100 IU/mg. Presumably, this latter preparation did not contain active factor. Statistics calculated by excluding this 1 extract yielded p values of <0.0005 for the K-isozyme when compared to any of the control sets, but the p value for the L-isozyme compared to phosphate-buffered saline-injected controls still was not significant, <0.20. However, compared to nontreated controls, the value was significant (≈ 0.0025).

Distribution of the Factor in Mouse Tissues. Table 5 summarizes results obtained when various mouse tissues...
were extracted. It was not possible to extract the factor from normal adult tissues, even if the K-isozyme of pyruvate kinase predominated. On the other hand, fetal tissue is a source of the factor. Values obtained from newborn mice suggest they also synthesize the factor but in lesser quantities than do fetal mice.

As reported by Suda et al. (34), serum from tumor-bearing animals also contained the factor (Table 5).

**Nature of the Factor.** Gel electrophoretic analyses of phenolic extracts showed they contained variable amounts of 4 S, 5 S, about 18 S and higher-molecular-weight forms of 260 nm-absorbing material, and that the most active preparations were relatively rich in the lower-molecular-weight forms. The soluble material [lower-molecular-weight forms including tRNA (10)] remaining after extraction with 1 m NaCl retained activity, but the insoluble material [higher-molecular-weight forms, (7)] was inactive.

Treatment of such phenolic extracts with pancreatic RNase-A enhanced activity of various preparations (Table 6). Gel electrophoresis of such RNase-treated phenolic extracts shows a general degradation of the RNA present, resulting in a large unhomogenous mass of 260 nm-absorbing material extending beyond the 5 S material. The data of Table 6 also show that proteolytic enzymes tend to decrease the effectiveness of the factor. These data suggest the factor may be a polypeptide. In an attempt to confirm this concept, tumor-bearing mice were given injections of tritiated lysine, and the factor was extracted and electrofocused. Results of a typical experiment are summarized in Chart 1. These data show that labeled lysine is carried through the extraction. Moreover, prior to treatment with RNase, the label is found at very low pH values, as would be expected if bound to RNA. On the other hand, after treatment with RNase the radioactivity is found at higher pH values, as would be expected if the protein was freed from the RNA. In the experiment shown, the fraction obtained at pH 4.98 had biological activity. Despite the fact that RNase-treated and electrofocused preparations tend to lose their potency, biological activity has been found between pH 4.8 and 5.1 in 3 different experiments, 2 using [3H]lysine and 1 using [3H]tyrosine. Electrofocusing of the ethanolic precipitate from a tumor grown in the presence of [4C]uridine and [3H]lysine showed that, prior to RNase treatment, 4C and 3H counts both peaked at pH 2.7. On the other hand, after treatment with RNase's, the 3H counts moved to higher pH values, as in Chart 1, while the 4C counts, if anything, moved to a more acidic pH value. Unfortunately, this preparation had no biological activity. In a final experiment designed to show that the lysine was incorporated into a protein, one aliquot of an extract was treated with RNase while another aliquot was treated with RNase and *S. griseus* protease. Focusing of these preparations showed that the protease treatment reduced the amount of radioactivity obtained at the lower pH values and led to the production of a major radioactive peak between pH 10 and 12.7.

**DISCUSSION**

**Existence of a Factor.** Assay by 2 different techniques shows that tumor growth and serial injections of subcellular fractions of ascites tumor cells change the pyruvate kinase isozyme pattern of normal liver by increasing the proportion of the fetal type isozyme. These results confirm the data of Suda et al. (33, 35, 38, 45) who used an immunological technique to measure the relative proportion of the 2 isozymes. Moreover, it was shown that injection of phenolic extracts of Ehrlich ascites tumor cells produced a statisti-
Specific activity (IU/mg protein) of the K-isozyme in liver extracts from experimental animals given injections of RNase and proteases.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Type of extract used</th>
<th>Control animals</th>
<th>Extract</th>
<th>RNase-treated extract</th>
<th>RNase plus protease-treated extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ascites fluid</td>
<td>0.125±0.010</td>
<td>0.180</td>
<td>0.295</td>
<td>0.287</td>
</tr>
<tr>
<td>2</td>
<td>Ascites fluid</td>
<td>0.205±0.010</td>
<td>0.336</td>
<td>0.364</td>
<td>0.287</td>
</tr>
<tr>
<td>3</td>
<td>A high-speed supernatant solution</td>
<td>0.125±0.010</td>
<td>0.228</td>
<td>0.364</td>
<td>0.287</td>
</tr>
<tr>
<td>4A</td>
<td>Phenol extract of a high-speed supernatant</td>
<td>0.195±0.005</td>
<td>0.300</td>
<td>0.501</td>
<td></td>
</tr>
<tr>
<td>4B</td>
<td>Phenol extract of a high-speed supernatant</td>
<td>0.169±0.005</td>
<td>0.253</td>
<td>0.385</td>
<td>0.161</td>
</tr>
<tr>
<td>4C</td>
<td>Phenol extract of a high-speed supernatant</td>
<td>0.236±0.005</td>
<td>0.484</td>
<td>0.584</td>
<td>0.444</td>
</tr>
<tr>
<td>4D</td>
<td>Phenol extract of a high-speed supernatant</td>
<td>0.230±0.005</td>
<td>0.311</td>
<td>0.354</td>
<td>0.307</td>
</tr>
<tr>
<td>4E</td>
<td>Phenol extract of a high-speed supernatant</td>
<td>0.222±0.005</td>
<td>0.283</td>
<td>0.344</td>
<td>0.270</td>
</tr>
</tbody>
</table>

Mean ± S.E.* (4A to E) 0.209 ± 0.010 0.326 ± 0.040 0.434 ± 0.036 0.296 ± 0.058

* In all experiments but 4E, soluble RNase and chymotrypsin were used. In Experiment 4E, agarose-bound RNase and S. griseus protease were used. See "Materials and Methods" for details.

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Table 6

Influence of RNase and proteases on the activity of factor obtained from Ehrlich ascites tumor extracts or ascites fluid

The Factor as a Derepressor. Actinomycin S and p-fluorophenylalanine inhibit the increase of liver K-isozyme activity caused by tumor growth (35). Moreover, injection of labeled amino acids into normal and tumor-bearing animals and precipitation of the K-isozyme with appropriate antibodies yielded mean values of 7,410 and 36,950 cpm/mg precipitated enzyme, respectively. These data strongly sug-
suggest that the factor acts by stimulating the rate of de novo synthesis of the fetal K-isozyme (35).

Tumor growth and extracts both tend to increase the K-isozyme and to decrease the L-isozyme; however, the data reported suggest that the latter effect is not due to the effect of the factor. As indicated in Tables 2 to 4, a decrease in L-isozyme activity did not necessarily accompany an increase in the K-type enzyme. Besides being another indication that the effect observed is not due to an epigenetic transformation of the L-isozyme into the K type, these data suggest that 2 distinct mechanisms are operating. Conceivably, tumors elaborate both a repressor- and derepressor-like substance. This could account for the step-wise change in the isozymes observed in the hepatomas of graded degrees of dedifferentiation (11, 42, 43). However, since the level of the liver L-isozyme is dependent upon dietary and hormonal factors (11, 15, 37) and since the data of Table 4 suggest that the injection of phosphate-buffered saline alone has some effect on the level of the L-isozyme, it is suggested that the effects on the L-isozyme are due to some nonspecific effect. In either case, the factor under investigation may be thought of as a specific stimulator of K-isozyme.

Since residual K-type activity in the adult mouse or rat liver is a function of the nonparenchymal cell population (4, 8, 41) and since the factor probably does enhance de novo K-isozyme synthesis, the factor must act by 1 of 3 general mechanisms, namely, it could increase the population of nonparenchymal cells, it could increase K-isozyme synthesis in the nonparenchymal cells, or it could derepress K-isozyme synthesis in the parenchymal cells. The 1st mechanism appears unlikely since doubling enzyme activity implies doubling the nonparenchymal cell population, a possibility which is not logical, nor does it agree with the data of Suda et al. (35) who showed that, while tumor growth stimulated uptake of amino acids into pyruvate kinase, it did not stimulate uptake into other proteins. Increasing the synthesis of the K-isozyme in existing nonparenchymal cells suggests that, normally, the biosynthetic mechanisms are operating at no more than one-half the maximal rate and that there exists an actinomycin-sensitive factor that increases rather than starts the biosynthetic activity. While such an action is possible, it would seem illogical. The possibility is made even less probable by the observation that the K-isozyme level has been raised almost 4-fold above the mean control value (Table 4). These considerations leave as the most probable mechanism the possibility that the factor acts as a derepressor. Tanaka et al. (38) have previously hypothesized that the factor is a derepressor, on the basis of the evidence of de novo synthesis and the observation that strains of mice that normally had high levels of K-enzyme in the liver did not show as much response as did mice with a greater proportion of the L-isozyme.

Additional evidence supporting the contention that the factor acts as a derepressor is provided by the observation that the only tissues from which the factor could be obtained are rapidly growing ones (Table 5). A positive relationship between derepression of K-isozyme expression and replicative rate has been noted (4, 11, 40, 41). Thus it seems possible that the factor can be isolated only from those tissues that may have derepressed their own expression of the K-isozyme.

Chemical Nature of the Factor. The observation that the factor was present in phenolic extracts of the high-speed supernatant led to the initial hypothesis that the factor was a tRNA. This concept was compatible with the rough estimate of the molecular weight reported (35, 38), the fact tumor and fetal tissues manufacture unique tRNA molecules (5), and the initial gel electrophoretic studies described.

An indication that this tRNA hypothesis was wrong was provided by the observation that the addition of 14 g cresol (to further remove protein (22)) and 0.1 g 8-hydroxyquino-line per 100 g of phenol resulted in extracts having little or no activity. Furthermore, 1 inactive preparation that had no discernible 4 or 5 S material but that did have higher-molecular-weight material became active after treatment with RNase. As shown in Table 6, enhancement of activity by RNase generally does occur. Moreover, the addition of proteases appears to inhibit the activity of extracts. These observations led to the present working hypothesis, namely, that the factor is a polypeptide extracted in a form bound to tRNA. This hypothesis appears to be confirmed by the following observations: (a) extracts from tumors grown in the presence of tritiated tyrosine or lysine and then electrofocused yielded radioactivity mainly at pH values below 3.0, but similar extracts treated with RNase's yield radioactivity at pH values greater than 4.0; (b) H peaks with pl values of about 5.0 also have biological activity; (c) treatment with proteases after RNase treatment reduced the amount of radioactivity found; (d) extracts labeled with [14C]uridine as well as [3H]lysine show counts mainly at pH values less than 3.0; and (e) treatment of double-labeled extracts with RNase before focusing causes the H counts but not the 14C counts to move toward higher pH values. These electrofocusing experiments also suggest that the factor has a pi value of about 5.0.

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Extraction of a Factor from Ehrlich Ascites Tumor Cells That Increases the Activity of the Fetal Isozyme of Pyruvate Kinase in Mouse Liver

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