Isozymes of Pyruvate Kinase in Liver and Hepatomas of the Rat

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Summary

Pyruvate kinase (PK) (EC 2.7.1.40) isozymes were assayed in normal rat liver and a series of transplantable rat hepatomas ranging widely in growth rate and degree of differentiation, with the use of gradient elution by chloride ion from columns of DEAE-cellulose. In agreement with other studies, three noninterconvertible forms were found in rat tissues: isozyme I, the major form in adult rat liver; isozyme II, the sole form in heart and skeletal muscle; and isozyme III, the sole form in poorly differentiated hepatomas, the major form in normal kidney and lung, and the minor form in adult liver. In normal adult rat liver, PK I makes up 90% of the total activity at 57 units/g tissue, and is decreased to 30 units/g after a 48-hr fast. Carbohydrate refeeding restores the normal PK I level in 24 hr and increases its activity to twice the normal level after 72 hr. Little or no change occurs in PK III, which remains at 3 to 6 units/g. In regenerating liver after partial hepatectomy, PK I was slightly below the normal fasted level, but PK III at 72 hr had about twice the normal activity. Fetal liver of 19 to 21 days had equal PK I and PK III levels, whereas early fetal liver or whole fetus contained almost entirely PK III.

Although one highly differentiated hepatoma, 9618A, has a PK isozyme pattern similar to that of liver, other well- and highly differentiated hepatomas had much lower total activities than liver, with a preponderance of PK III. In contrast, the rapidly growing, poorly differentiated hepatomas had extremely high total PK activity, virtually all consisting of PK III. These results provide further evidence of a profound alteration of gene expression in hepatomas resulting in the loss of a specific liver-marker isozyme with loss of differentiation and its replacement in poorly differentiated hepatomas by high activities of an isozyme normally very low in the normal liver.

Introduction

It is becoming increasingly evident that liver neoplasia is associated with profound alterations in isozyme pattern (10, 23, 32, 41, 57). These alterations involve the replacement of those isozymes that are under dietary and hormonal control by the host, and that have important metabolic functions in the adult differentiated liver by other isozymes which are normally either low in, or absent from, the adult tissue. As part of an ongoing study of this phenomenon, we have examined the alteration of PK (EC 2.7.1.40) isozymes in the Morris hepatomas (30, 31), a series of chemically induced, transplantable rat hepatomas ranging widely in growth rate and degree of differentiation. This enzyme occupies a key position in the metabolism of cells and, as we pointed out previously (27, 28, 57), high levels of this enzyme may profoundly affect the carbohydrate metabolism and may be a determining factor in the high aerobic glycolysis of tumors by way of competition with the respiratory system for the available ADP. In this study, we report data on the activity levels and the molecular forms of this enzyme in the "spectrum" of Morris hepatomas and compare these with similar data on fetal and adult normal liver of the rat (preliminary reports of this work have appeared (12, 13, 28, 57)).

Tanaka et al. (49) first demonstrated the existence of multiple forms of PK in rat tissues and established the presence of 2 forms in rat liver. One of these (PKL) was the form shown previously to be dependent on diet and insulin (24, 49, 55, 56), whereas the other form (PKM) had an electrophoretic mobility and kinetic as well as immunological properties similar to those of skeletal muscle PK. Since then, a host of studies has appeared that point to the presence, depending on the method of separation used, of as many as 6 isozymes (9, 10, 33, 48), but it is difficult to establish the exact number, owing to the ease with which they can undergo various modifications (6, 17, 22, 26, 47, 52). It appears from more recent work that there are only 3 distinct molecular forms (19, 20, 21), each of which probably undergoes conformational changes or intertype hybridization (8, 17, 19).

That liver neoplasia involves alterations of PK isozyme patterns was suggested by several previous studies. Imamura and Tanaka (20), using acrylamide gel electrophoresis, found that the major form in the AH 130 ascites hepatoma was identical with the minor (M) form in liver. Walker and Potter (53), also using starch gel electrophoresis, found an increased level of the minor liver form in the preneoplastic...
liver, in the hyperplastic liver nodules, and in the hepatomas that develop after feeding of the carcinogen 3'-methyl-4-dimethylaminoazobenzene to rats. Taylor et al. (51) reported that the poorly differentiated Morris hepatoma 3924A had a PK that differed kinetically from the rat liver isozyme. In our laboratory we found that the liver type was strongly held by DEAE-cellulose, whereas the isozymes of the Novikoff and Morris 3924A hepatomas, which were present in extremely high activity, were not bound by the resin (13). The use of DEAE-cellulose chromatography has now made it possible to verify the existence of 3 forms of the enzyme in rat tissues and to assay quantitatively the activities of each in a series of normal and neoplastic tissues.

MATERIALS AND METHODS

Tumors used in this study were induced originally in Buffalo and ACI rats by feeding of carcinogenic chemicals. Complete descriptions of their induction, properties, growth rates, and histological characteristics have been reported previously (30, 31). They were maintained by serial transplantation in female Sprague-Dawley rats purchased from the Holtzman Company, Madison, Wis. Transplantation was either i.m. or s.c., at either the National Cancer Institute or at Howard University, Washington, D. C., and were routinely shipped to Philadelphia for enzyme studies. The Novikoff hepatoma was maintained in Philadelphia by serial transplantation in female Sprague-Dawley rats purchased from the Holtzmann Company, Madison, Wis. Transplantation was either i.m. or s.c. routes or, where the tumor grows in the ascites form, i.p. Animals were routinely fed a commercial rat pellet diet, except in the dietary experiments. All chemicals were the purest available commercial products.

PK Assays. The routine assay procedure is based on those of Bücher and Pfleiderer (7) and Llorente et al. (26), with modifications as indicated throughout this paper. Animals were decapitated and bled; tissues were rapidly excised and homogenized in a motor-driven coxial homogenizer with a Teflon pestle, with 1 volume w/v of a pH 7.5 triethanolamine buffer solution until 5 ml was collected. The mixture contained, in a 0.4-ml volume, 50 mM glycylglycine, 4 mM acetylcysteine, and 2.5 mM MgCl2. After centrifugation for 1 hr at 105,000 x g in a Spinco Model L ultracentrifuge at 2º, the supernatant layer was carefully removed and stored at 0º. This preparation was diluted appropriately to give a ΔA of 0.03 to 0.1/min with a pH 6.0 buffer solution containing 1% bovine serum albumin, 0.5 M sucrose, 10 mM Tris, 10 mM EDTA, 4 mM acetylcysteine, and 2.5 mM MgCl2. After centrifugation for 1 hr at 105,000 x g in a Spinco Model L ultracentrifuge at 2º, the supernatant layer was carefully removed and stored at 0º. This preparation was diluted appropriately to give a ΔA of 0.03 to 0.1/min with a pH 6.0 buffer solution containing 1% bovine serum albumin, 0.5 M sucrose, 10 mM Tris, 10 mM maleate, 1 mM EDTA, 4 mM acetylcysteine, and 1 mM MgCl2. For liver and well-and poorly differentiated hepatomas, respectively, the usual dilutions were 100-, 25 to 50-, and 200-fold. The assay mixture contained, in a 0.4-ml volume, 50 mM glycylglycine, pH 7.5; 100 mM KCl; 10 mM MgCl2; 0.15 mM NADH; 5 or 13 mM phosphoenolpyruvate; and 0.75 unit lactate dehydrogenase. The reaction was started by the addition of ADP to a final concentration of 2 mM and, with a Gilford spectrophotometer, readings were made for about 10 min at a temperature of 25º. Under the conditions used, the reaction rates were linear with time and were proportional to the amount of enzyme, and blanks without substrate or enzyme were negligible.

Separation of Isozymes by Chromatography on DEAE-Cellulose. Tissue was homogenized in an equal volume, w/v, of a solution of 0.5 M sucrose 25 mM Tris HCl, 2.5 mM EDTA, 4 mM acetylcysteine, and 2.5 mM MgCl2 adjusted to pH 7.5 while cooling in the ice-salt bath, and the resultant suspension was centrifuged at 105,000 x g for 1 hr at 0º. The clear supernatant solution was applied directly to a column, 600 x 11 mm, containing a depth of 400 mm of DEAE-cellulose, prepared according to the method of Peterson and Sober (34) and equilibrated with a pH 7.5 solution containing 0.25 M sucrose, 10 mM Tris HCl, 1 mM EDTA, 1 mM MgCl2, and 4 mM acetylcysteine. The column was then washed with at least 50 ml of the same solution, and elution was conducted with a linear KCl gradient, with 500 ml of this solution in Chamber 1 and 500 ml of the same pH 7.5 buffer solution containing 0.5 M KCl in Chamber 2 of a Buchler Varigrad gradient maker. With the use of a Buchler polyostatic pump, the rate of flow was adjusted to 60 ml/hr, and 5-ml fractions were collected. Chloride was determined gravimetrically by precipitating AgCl and weighing. Fractions were assayed as described above except for the use of 5 instead of 13 mM phosphoenolpyruvate. At the peaks, where enzyme activity was high, the eluates were diluted with 1% bovine serum albumin containing 0.5 M sucrose, 10 mM Tris-HCl, 10 mM maleate, 1 mM EDTA, 4 mM acetylcysteine, and 1 mM MgCl2 at pH 6.0. When necessary, fractions were concentrated by vacuum dialysis against the homogenization buffer solution, using Sartorius collodion bags supplied by Science Essentials, Anaheim, Calif. These preparations are stable for at least 1 month when kept frozen.

Use of DEAE-Cellulose Chromatography for Assay of PK Isozymes. To expedite assays of nonadsorbing and adsorbing forms of PK, a micromodification of the DEAE-cellulose column procedure was used. The resin was thoroughly washed with 0.01 M EDTA solution, then treated by repeated suspension and decantation of a solution of 0.25 M mannitol in 50 mM triethanolamine at pH 7.5. To separate the nonadsorbing from the adsorbing forms, the fractionation was carried out in glass and plastic tubes approximately 0.5 x 8 cm, usually the barrel of a 2-ml disposable syringe. The bottom was plugged with glass wool and the tube was then packed with 2 ml of the washed DEAE-cellulose. Several successive 5-ml portions of the ice-cold mannitol/triethanolamine buffer solutions were washed through the column. The receiver was then replaced with a graduated centrifuge tube packed in ice, and a 0.2-ml portion of a 105,000 x g supernatant of a 33 or 50% w/v tissue homogenate was applied to the top of the column and washed through with small portions of the mannitol/triethanolamine buffer solution until 5 ml was collected. The receiver was then replaced and the elution was repeated with 5 ml of the same buffer solution containing 0.25 M KCl. The adsorbed and nonadsorbed fractions were then assayed, as already described, with 13 mM phosphoenolpyruvate. This procedure effectively separated the nonadsorbed from the adsorbed enzymes. Molecular weights of the isozymes isolated by DEAE-cellulose fractionation were determined by elution from Sephadex G-200 according to the procedure of Andrews (5). A column of 2.5 x 100 cm was filled to a depth of 88 cm with Sephadex G-200 equilibrated with a
buffer, pH 7.5, composed of 0.1 M KCl, 10 mM Tris HCl, 1 mM EDTA, and 1 mM MgCl₂. Purified standard proteins, i.e., hemoglobin, yeast hexokinase, liver alcohol dehydrogenase, and rabbit muscle aldolase, covering the range 32,000 to 160,000 were dissolved in 1 ml of the buffer, placed on the column, and eluted with the same buffer. The various PK specimens were applied in the same way. The molecular weight was estimated from the straight line obtained by plotting log molecular weight of the standard proteins against the ratio, elution volume/void volume.

RESULTS

Separation and Identification of PK Isozymes by Gradient Elution from DEAE-Cellulose. Although PK isozymes have been separated by electrophoresis (47, 49, 54) and by isoelectric focusing (9, 10, 18), our objective required a more convenient, reliable, and rapid procedure that would separate the forms of this enzyme from crude tissue preparations, and would give reasonably quantitative recoveries. This has been achieved by means of gradient elution with Cl⁻ from DEAE-cellulose. Using the methods described in “Materials and Methods,” recoveries of applied activity ranged between 75 and 100%, and only 3 distinct forms can be identified. As shown in Chart 1A, 2 forms are present in rat liver. One form is not held by the resin and is eluted in the void volume. It is a minor form, representing about 10% of the total activity in livers of adult, carbohydrate-fed rats, but in 19- to 20-day fetal rat liver this is a major form, representing about 60% of the total activity (Chart 1B). As shown in Chart 1A, the major form in adult liver is tightly bound to the resin and is eluted at Cl⁻ concentrations between 0.10 and 0.15 M. This form is also present in fetal liver and, as shown in Chart 1B, always appears as a double peak, one of which elutes slightly before the adult liver form.

Chart 1C shows that in rat skeletal muscle there is a single form which is also held by the resin, but which elutes before the liver form, at a Cl⁻ concentration of 0.01 to 0.05 M Cl⁻.

These 3 forms are the only ones seen in either normal or neoplastic tissues of the rat. All 3 forms, when rechromatographed separately or together, show absolutely no evidence of interconvertibility or alteration in their elution properties from DEAE-cellulose. These data are in agreement with those of Imamura et al. (21), who separated the PK isozymes by acrylamide gel electrophoresis, as well as with those of Middleton and Walker (29), who used cellulose acetate electrophoresis and DEAE-cellulose chromatography; and with those of Ibsen and Trippet (18), who used isoelectric focusing. Different investigators have used various terminologies for the designation of these PK isozymes. It is clear that our nonbound form corresponds to the M₁ form of Imamura et al. (20), the PK III of Walker and Potter (54), and the K form of Ibsen and Trippet (18); our tightly bound form corresponds to the major liver form of these authors, and the loosely bound form corresponds to their skeletal muscle form. Because these isozymes are not necessarily associated with a single tissue type, we feel it is more appropriate to avoid this connotation, and have therefore adopted the terminology used by Walker and Potter (54) of numbering these forms as I, II, and III, in the inverse order of their degree of binding to DEAE-cellulose. Thus, the major liver form is PK I, the skeletal muscle form is PK II, and the minor liver form is PK III.

Elution patterns of the hepatomas under study are shown in Chart 2. The highly differentiated, very slow-growing Morris hepatoma 9618A gave a pattern very similar to that of adult liver, with most of the activity in type I and minor activity in type III (Chart 2A). All of the well-differentiated hepatomas had low activities of both types, as seen for Hepatoma 9618B in Chart 2B. In contrast, all of the fast-growing, poorly differentiated hepatomas exhibited only type III, as a single, sharp peak in the void volume exemplified by the Novikoff ascites hepatoma pattern in Chart 2C.

Range of PK Isozyme Composition in Rat Liver. To
provide a baseline for quantitative comparative studies on PK isozymes in hepatomas, a study was made of the range of normal variation of activity in rat liver. To assay the 2 PK isozymes, a micromodification of the DEAE-cellulose elution procedure was used, as described in “Materials and Methods.” Tanaka et al. (49) previously showed by both electrophoretic and immunochemical criteria that dietary modification of liver PK is due solely to changes in the L form, and this is corroborated in Table 1. These data show that after a 48-hr fast, there was no significant change in type III activity, whereas type I activity dropped by about 50% on a per g liver basis, and by 75% on a total liver basis.

On repletion with a high carbohydrate diet, there was a remarkable restoration of type I activity, which by 48 and 72 hr was over twice that of the normally fed rats, while little if any change occurred in type III activity. As shown in the last column, the mean type I/type III activity ratio was 11 in the normally fed rats, dropped to 6 in the 48 hr-fasted rats, and reached extremely high values of over 30 in the carbohydrate-refed rats.

Regenerating livers, through the 24- to 72-hr period of rapid regeneration, displayed PK activities that were at the low, fasting normal levels, and activities in the whole liver were also quite low, reflecting the low liver weights, which ranged from about 3 to 6 g over the 72-hr regeneration period as compared with about 10 g for the normal rats. However, in contrast to the dietary effects, type III PK increased to more than double the normal level during the period of rapid proliferation at 48 to 72 hr posthepatectomy. During this period, the type I/type III ratio was below 3.

Although the total activity in 19- to 20-day fetal liver was fairly constant at about 30 units/g, there was a much higher proportion of type III and a lower proportion of type I than in adult liver. The mean ratio of the 2 forms was about unity; however, there was a wide spread of individual ratios, ranging from 0.7 to 1.8. This wide variance is not surprising since this is a period during which isozyme III is rapidly decreasing and isozyme I is increasing. Using an indirect method of calculation, based on differences in activities at 0.25 mM phosphoenolpyruvate and differences in inhibition by Cu²⁺, Middleton and Walker (29) obtained a type I/type III ratio of about 1.5 at this time of prenatal development, with total activities about the same as ours.

**PK Isozyme Composition in Hepatomas.** With this background of information on normal liver, the PK isozyme activities for hepatomas are presented in Table 2. Three
hepatomas classified histologically as highly differentiated were assayed. One of these, Hepatoma 9618A, is in a special category in growing extremely slowly, requiring up to 1 year between transfers, having a normal chromosome number and karyotype, and high levels of glucokinase (44) and liver-type phosphorylase activity (39). Its PK isozyme pattern was virtually identical with that of adult liver, with 94% of type I and only 6% of type III activity. The other 2 highly differentiated hepatomas, 9611B and 7787, had total activities characteristic of normal fasting liver but had only 26% of their total activity as type I. The 13 well-differentiated hepatomas had very low total activities, with a type I/type III ratio of about 0.5. It is evident that the major difference between normal liver and the well-differentiated hepatomas is a loss of most of the type I isozyme.

In contrast, there was a drastic reversal in the isozyme composition of the poorly differentiated hepatomas. In these tumors, which grow sufficiently rapidly to kill their hosts within 1 month or less, the total activities were very high, from 3 to 7 times that of normal rat liver, and they had nearly or completely lost the type I isozyme. The value of 8% for PK I given in Table 2 is certainly too high and may be an artifact. As shown in Chart 2C, by DEAE-cellulose chromatography, only type III was evident in the Novikoff ascites hepatoma, which exemplified other poorly differentiated hepatomas. In the micromethod used in these studies, elution of type III was not always complete when applied in high activity, and it was found that the enzyme that appeared to be held back appeared mainly in the void volume on rechromatography (12). Thus, we can conclude that with decreased differentiation and increased growth rate, there was a virtually complete disappearance of the PK isozyme which is the major form in adult liver, this form having been replaced by high activity of a form normally very low in adult liver.

Properties of the Novikoff Ascites Hepatoma Isozyme, PK III. A determination of the kinetic constants of isozyme III, obtained from the Novikoff ascites hepatoma partially purified by DEAE-cellulose chromatography, gave data identical with those of Imamura et al. (20) with respect to $K_m$ for phosphoenolpyruvate and ADP, allosteric activation by FDP, and inhibition by ATP. However, we observed a difference in molecular weight. According to Imamura et al. this isozyme, obtained in highly purified form from the AH-130 Yoshida sarcoma, had a molecular weight of 216,000. Although we also observed similar values ranging from M.W. 150,000 to 200,000 for different preparations of the Novikoff PK III, determined according to the method of Andrews et al. (5), when 4 mM FDP was present in the elution buffer, values of 105,000 to 125,000 were obtained when FDP was omitted. These preliminary data suggest that the conformation changes induced by FDP may involve aggregation of subunits. It is likely that these low-molecular-weight forms of PK III are the same as the 2 forms of kidney PK found by Ibsen and Trippet (18) to have molecular weights of 116,000 and 178,000.

**DISCUSSION**

Identity of PK III with PK Isozymes in Other Tissue. Despite the massive and confusing literature on the various forms of PK in animal tissues, owing to its pleotropic nature (18, 22, 47, 54), it appears that in the rat only 3 noninterconvertible forms exist (17, 20, 54). PK III, separated by chromatography on DEAE-cellulose, appears to be identical with the tumor form observed by Imamura and Tanaka (20) and Farron et al. (14) by means of gel electrophoresis; this form has been further identified with a form observed in tissues such as kidney, adipose tissues, fetal tissue, and cultured liver cells by means of electrophoresis (18, 20, 33, 54) or by isoelectric focusing and DEAE-cellulose chromatography (29, 33). The present study thus amplifies and

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**Table 2**

<table>
<thead>
<tr>
<th>Type of tumor</th>
<th>No. of tumors</th>
<th>No. of assays</th>
<th>Total activity</th>
<th>% of isozymes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Highly differentiated</td>
<td>2</td>
<td>5</td>
<td>33 (16-44)</td>
<td>26</td>
</tr>
<tr>
<td>9618A, Generation 7</td>
<td>1</td>
<td>7</td>
<td>96 (76-140)</td>
<td>94</td>
</tr>
<tr>
<td>Well differentiated</td>
<td>13</td>
<td>58</td>
<td>13 (3-25)</td>
<td>32</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>4</td>
<td>27</td>
<td>259 (150-460)</td>
<td>(8?)</td>
</tr>
</tbody>
</table>

*The tumor numbers and generations used for these experiments are listed as follows: highly differentiated: 9611B (Generations 2 to 6), 7787 (Generations 7 and 8), and 9618A (Generation 7); well differentiated: 5123A (Generations 60 to 71), 5123C (Generations 58 to 62), 5123TC (Generations 66), 7288C (Generations 53 to 59), 7793 (Generations 14 to 16), 7794A (Generations 18 to 21), 7795 (Generation 23), 7800 (Generations 27 to 34), 8999 (Generation 4), 9108 (Generation 11), 9121 (Generation 10), 9633 (Generations 4 and 5), and Reuber H-35 (Generations 43 to 48); poorly differentiated: 3683F (Generations 342 to 379), 3924A (Generations 219 to 248), 7288C (Generations 102 to 122); and Novikoff ascites (generation unknown, > 1000).
extends these previous studies in confirming, by means of DEAE-cellulose chromatography, the identity of the hepatoma PK III with those of other fetal and adult rat tissues, in confirming the existence of 3 isozymic forms, and in providing quantitative data on the activities of these forms in normal liver and in a series of hepatomas.

The pattern of PK isozyme alteration in hepatomas is similar to those patterns observed for the glucose ATP phosphotransferases (44), the aldolases (2), and the phosphorylases (39). Highly differentiated hepatomas retain an isozyme pattern similar to that of liver, with a preponderance of PK I. In less differentiated hepatomas, there is a variable loss of PK I without remarkable change in PK III; however, with further loss of differentiation and increased growth rate, PK I is nearly or completely lost, while PK III attains extremely high activity.

Role of PK III in Tumor Glycolysis. We have already pointed out (13, 28) that the high PK activity of poorly differentiated hepatomas may be an important determinant of the high aerobic glycolysis of these tumors. Recent information on PK III, together with the results of this study, lends further plausibility to this concept. It has been known for several years that the major liver isozyme, PK I, exists in 2 interconvertible forms. One of these is highly allosteric with a high K0.5 for phosphoenolpyruvate, a low K1 for ATP and alanine, and a powerful activating effect of FDP, which abolishes the alanine and ATP inhibition and drastically lowers the K0.5 for phosphoenolpyruvate. The other form is insensitive to FDP, but already has a very low K0.5 and is not appreciably inhibited by ATP or alanine (6, 26, 43). In a recent paper, Walker and Potter (54) demonstrated that the PK III in cultured liver cells also exists in 2 interconvertible forms. One of these forms (IIIa) has a relatively high K0.5 of 0.33 mM and was inhibited by ATP, alanine, and phenylalanine at low concentrations. The other form, IIIb, had a low K0.5 and was not inhibited by ATP, and was inhibited much less by alanine and phenylalanine. A crucial difference between the allosteric forms of PK III and PK I is that, whereas the allosteric PK I is strongly activated by FDP, the allosteric PK IIIa is actually converted to the nonallosteric PK IIIb, either by FDP or Mg2+ ions. Walker and Potter point out the similarity of this system to the interconversion of forms of the PK III of adipose tissue (36) and kidney (18), and suggest that this interconversion may be a factor in the regulation of glucose metabolism. According to the work of Walker and Potter (54),9 cells grown in glucose have all of the PK III in the b or active form, presumably owing to the resultant high intracellular level of FDP. It is conceivable, therefore, that the high aerobic glycolysis in the poorly differentiated hepatomas is enhanced not only by the high activity of PK III, but also by the glucose-induced rise in FDP concentra-

9 Recently, Walker and Potter (54) reported that cultured liver cells have only PK III. In preliminary unpublished studies (J. B. Shatton, G. Williams, E. Farber, and S. Weinhouse), we found that in several liver cell lines PK III was the main form but was also accompanied by variable, small amounts of PK I.

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REFERENCES


PK in Liver and Hepatomas


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