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# Interrelationships and Functions of the Pyruvate Kinase Isozymes and Their Variant Forms: A Review[1]

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## Summary

The relationships among and the properties of the pyruvate kinase isozymes are reviewed, emphasizing their potential role in carcinogenesis. Particular consideration is given to evaluation of the concept that the three major nonreadily interconvertible forms are the products of distinct genes, the relationship of these forms to additional separable forms of pyruvate kinase, the types and possible functions of interconvertible forms of the major isozymes, and mechanisms affecting the genetic expression of the isozymes. Emphasis is placed upon the apparent derepression of the fetal isozyme in hepatomas and the influence of neoplasms and their extracts on the expression of pyruvate kinase in the liver of host animals.

## Introduction

Restriction of a hereditary deficiency of pyruvate kinase (EC 2.7.1.40) to erythrocytes suggested that this enzyme might exist in more than 1 molecular form (cf. Ref. 157). In subsequent studies, conducted on rat and human tissues, 2 distinct isozymes were found, the one associated with liver and RBC and the other with all remaining tissues but kidney. Kidney extracts seemed to contain an enzyme with intermediate properties and was postulated to be a hybrid (8, 17, 93, 94, 158, 159, 176). However, more recent investigations contradict these earlier ones, since most tissues are found to have an isozyme with properties in common with those of the major kidney isozyme, and this isozyme was found not to have properties compatible with the hybrid concept (5, 6, 18, 21, 28, 40–42, 64, 65, 67, 71–77, 82, 95, 119, 121, 125, 126, 139, 147, 153, 154, 160, 169, 171, 174, 179, 193). These studies, conducted on a wide variety of mammals, suggest there are 3 distinct isozymes, *i.e.*, the liver or L-type, found as the isozyme of hepatocytes, as a minor isozyme of kidney cortex and intenstine and perhaps as the RBC enzyme; the muscle or M-type, now thought only to be expressed in striated muscle, heart, and brain; and the kidney or K-type, found in all other adult and fetal tissues and in tumors. These 3 seemingly noninterconvertible forms of the enzyme may each exist in variant forms and may form hybrids with each other, leading to some diversity and confusion.

Since an isozyme switch occurs during neoplastic development, the pyruvate kinase isozyme system may play a role in carcinogenesis. Moreover, the expression of the pyruvate kinase isozymes is subject to experimental manipulation, suggesting that this system could be used fruitfully as a model system in studies of differentiation and dedifferentiation. There remains, however, confusion in the literature concerning the number, distribution, properties, and relationships among the isozymes. This review attempts to evaluate the available data relevant to the relationships among the various forms of pyruvate kinase and to examine their roles in neoplasms.

## Nomenclature

The initial confusion concerning the number and distribution of the isozymes, their pleomorphism, the presence of hybrid forms, and differences of opinion concerning the relationships among the isozymes has led to the development of a plethora of nomenclatures. Table 1 compares these various designations. Throughout this review the K,L,M nomenclature will be used. When relevant the subunit constitution of the isozymes will be designated by a subscript, *e.g*: $M_4$ for the tetrameric form of the M-isozyme and $K_2M_2$ to designate a hybrid with 2 subunits of the K-type protomer and 2 of the M-type protomer.

## Relationship among the K-, L-, and M-Isozymes

Although the K-, L-, and M-isozymes have distinct kinetic and physical properties and do not readily interconvert, proof that they are products of 3 distinct genes is still lacking. Therefore the possibilities that they are related as hybrids, as posttranscriptionally modified products, or as products of distinct genes must be evaluated.

**Hybrids.** The M-isozyme appears to be a homotetramer when examined by several criteria (20, 21, 29, 45, 62, 83, 86, 124, 130). The available evidence also suggests that the L-isozyme is a homotetramer (19, 97). These conclusions are fortified by *in vitro* hybridization studies which show that reassociation of dissociated mixtures of homogeneous preparations of the $L_4$ and $M_4$ isozymes yields only the 2 isozymes plus 3 hybrid forms (19, 21, 35). Moreover, since these hybrids do not have the electrophoretic properties of the K-isozyme, this isozyme cannot be a hybrid of the other 2. Further support for the homotetrameric nature of the isozymes is provided by the observation of KL (21, 138, 147) or KM (68, 147) hybrids in vertebrate tissue extracts. Thus it seems clear that none of the 3 basic isozymes is a hybrid of the other 2.

**Posttranscriptional Modification.** Marie *et al.* (101) propose the M-isozyme is a modified K-enzyme. The fact that

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Table 1
*Distribution and nomenclature used for the 3 major pyruvate kinase isozymes*

| Muscle and brain | Liver parenchymal cells kidney cortex cells, and perhaps RBC | Many adult cells, including kidney, also predominant in fetus and tumors | Nomenclature first used by |
|---|---|---|---|
| M | L | LM or M | Tanaka *et al.* (159), 1967 |
| A | B | C[a] | Susor and Rutter (153), 1968 |
| | 2 | 1 | Farina *et al.* (38), 1968 |
| III | I | II[b] | Bigley *et al.* (8), 1968 |
| Muscle | V | I | Schloen *et al.* (137), 1969[c] |
| | II | I | Weinhouse *et al.* (192), 1972 |
| II | I | III | Walker *et al.* (179), 1972 |
| $M_1$ | L | $M_2$ | Imamura and Tanaka (75), 1972 |
| M | L | K | Farron *et al.* (40), 1972 |
| M | L | S, K, and $M_2$[d] | Nakamura *et al.* (113), 1972 |
| 3 | 1 | 4 | Osterman *et al.* (121), 1973 |
| M | L | A | Carbonell *et al.* (18), 1973 |
| | 1 | 5 | Osterman and Fritz (120), 1974[e] |

[a] C- and D-isozyme added by Susor and Rutter (154). The D-isozyme is the erythrocyte enzyme and the C-isozyme is the K-isozyme. RBC pyruvate kinase has also been called the R-isozyme (75).
[b] Used specifically for kidney isozyme.
[c] *R. pipiens* tissues; isozymes II to IV are apparent KL hybrids.
[d] Variant forms of the K-isozyme.
[e] Isozymes 2 to 4 are either hybrids or K-isozyme variants found in intestine.

the K- and M-isozymes cross-react immunologically (77, 101, 153) and a reported progression during development of forms electrofocusing between the K- and M-isozyme are cited as evidence supporting this concept. That the L-isozyme may be phosphorylated by a cAMP[2]-activated protein kinase (60, 63, 100, 167) suggests phosphorylation-dephosphorylation as a possible mechanism of interconversion. However, this protein kinase will not phosphorylate the K- or M-isozymes (63); thus, it is unlikely that the interconversion between K- and M-forms is a consequence of phosphorylation. Furthermore, the L-isozyme seemingly could not be the unphosphorylated precursor of the other isozymes since phosphorylation should lower the pI value and the L-isozyme has the lowest pI values (*e.g.*, Ref. 67). Nontheless, some undefined epigenetic modification could produce one homotetramer from another. On the other hand, the observed progression from the K- or M-isozyme could be related to transient hybrid forms or be due to enzyme pleomorphism. These phenomena are discussed below.

**Products of 3 Distinct Genes.** The difference in amino acid composition between bovine L- and M-isozyme suggests that these isozymes are products of distinct genes (21). Moreover, the absence of consistent evidence supporting a reciprocal relationship between the K- and L-isozymes under conditions causing apparent induction or repression of synthesis of either of these isozymes (Table 2) suggests that they are not related by posttranscriptional modification. Thus, the available evidence strongly suggests that the L-isozyme is a product of a gene different from the one(s) coding for either the K- or M-isozyme, but there are little data concerning the relationship between the K- and M-isozymes. Certainly, these isozymes are closely related when judged by immunological criteria.

## Additional Forms of Pyruvate Kinase

Many more than 3 forms of pyruvate kinase have been reported. The extra forms may be products of additional genes, hybrid forms, or variant forms of 1 or more of the 3 basic isozymes and/or of their hybrids. These additional forms of pyruvate kinase may relate to unique forms occasionally reported to be isolated from tumors.

**Hybrid Forms.** Recombination and/or immunological data indicate that extra activity bands found in bovine kidney (147) and in *Rana pipiens* liver (138) extracts are KL hybrids. Similar data support the presence of KM hybrids in bovine (147) and chicken (68) tissue extracts. Data, generally obtained from electrophoretic studies but otherwise unsupported, suggest the presence of KL hybrids in kidney and intestinal extracts (75, 119, 120, 193) and of KM hybrids in several tissues (41, 74, 147, 193) obtained from various adult mammalian species.

As might be expected from the tissue distribution of the L- and M-isozymes, no evidence has been obtained for the existence of LM hybrid forms *in vivo*.

The L-isozyme of liver is found only in the parenchymal

[2] The abbreviations used are: cAMP, cyclic adenosine 3':5'-monophosphate; P-enolpyruvate, phosphoenolpyruvate; Fru-1,6-$P_2$, fructose 1,6-diphosphate.

Table 2
*Changes in isozyme levels reported for rat liver and hepatomas under various physiological conditions*

| Condition | Relative activity[a] for the K-isozyme | L-isozyme | Other |
|---|---|---|---|
| Adult | | | |
| High-carbohydrate diet[c] | 0.9–1.3 (56, 110, 133)[b] | 1.8–3.5 (56, 110, 133) | 1.0[d] (110) |
| Fasting[c] | 0.8–1.2 (39, 56, 159) | 0.4–0.5 (39, 56, 159) | |
| Diabetic | 1.0 (159) | 0.3 (159) | |
| Added insulin[c] | 1.2 (56) | 5.8 (56) | |
| Regenerating[c] | 1.3–5.0 (39, 159, 179) | 0.2–0.4 (39, 159, 179) | |
| Tumor-bearing | 5.4–10.2 (110, 150) | 0.3–1.0 (110, 150) | 1.4[d] (110) |
| Fetal (19–21 days) | 2.5 (39) | 0.3 (39) | |
| Preneoplastic nodular hyperplasia | 2.1 (195) | 0.7 (195) | |
| Hepatoma | | | |
| Highly differentiated | 1.1–4.8 (39, 110) | 0.2–1.6 (39, 110) | |
| Well-differentiated | 1.7–2.1 (39, 110) | 0.3–0.7 (39, 110) | |
| Poorly differentiated | 14.7–46.7 (39, 110, 161) | 0.0–0.4 (39, 110, 161) | |

[a] Specify activity obtained/specific activity of normal controls.
[b] Numbers in parentheses, references. Only references reporting tabular data in specific activity units and in which the isozymes were separated were used.
[c] Under conditions of maximal response.
[d] Reported as M-isozyme.

cells (hepatocytes) (13, 30, 49, 127, 172), and KL hybrids seem not to be found in extracts of normal adult liver (147). Thus the adult mammalian hepatocyte must have an effective mechanism for repression of K-isozyme synthesis. In marked contrast, *R. pipiens* liver extracts have large quantities of KL hybrid forms (138).

**Interconvertible Forms of the L-Isozyme.** Interconvertible forms of the L-isozyme may be obtained by electrofocusing (59, 71, 74, 102, 110). One form with a pI value of 5.3 in the pig and of 5.4 to 5.5 in the rat is a tetramer, yields hyperbolic rate plots for P-enolpyruvate and is not activated by but has a high affinity for Fru-1,6-$P_2$ (59, 71, 74, 110). Partial purification (59) relectrofocusing (71, 110), incubation with fructose 1,6-phosphatase (71, 74), or starvation (71) converts this variant to a pH 5.6 to 5.7 form. This form is also a tetramer, but it has sigmoidal kinetics for P-enolpyruvate and is activated by and driven to the lower pI form by Fru-1,6-$P_2$. These data led us to the conclusion that the pH 5.4 form was the R-conformer and the pH 5.7 form was the T-conformer of an R,T tetrameric set (71, 74). Further purification, additional isoelectrofocusing, or $(NH_4)_2SO_4$ treatment leads to the formation of at least 1 still higher pI form, which is also driven to lower pI forms by electrofocusing in the presence of Fru-1,6-$P_2$ (59, 74, 110). This form of the L-isozyme, with a pI value around 6.2, often shows evidence of heterogeneity (59, 74, 152). This high-pI form of the pig enzyme was apparently characterized by Kutzbach *et al.* (97), who report it to be a homotetramer susceptible to regulation by several metabolites.

Hess and Kutzbach (59) suggest that the lowest pI form of the pig L-isozyme has 2 moles of bound Fru-1,6-$P_2$, the middle pI form has 1, and the highest pI form has none. Although this concept is supported by the facts that there appears to be a high- and low-affinity binding site (79, 110) and that the lowest pI form certainly binds Fru-1,6-$P_2$ (59, 74), while the middle pI form might (74), this proposed relationship seems incongruous with the observations that incubation with Fru-1,6-Pase produces relatively little of the highest pI form of the isozyme, that the highest pI form seems never to be isolated from fresh tissue extracts, and that this latter form often is heterogeneous.

Marie *et al.* (102) find an L-isozyme variant with a pI value of 6.28 in human liver extracts. Upon purification it is converted to a form with a pI value of 5.85. These results are the converse of that reported for purification of the pig liver enzyme (59) and could not be caused by gaining Fru-1,6-$P_2$, since this effector was not added during purification. Marie *et al.* feel that a peptide in the extracts may be responsible for maintaining the enzyme in the higher pI form. These authors also find small amounts of pH ~ 5.0 L-isozyme in both extracts and purified preparations. Conceivably, this form is analogous to the lowest pI form of the 3 forms commonly found in rat or pig liver preparations or to a 4th, pH 4.9 form, found by Muroya *et al.* (110) in rat liver extracts. Gel isoelectrofocusing studies suggest there may be at least 5 different interconvertible forms of the human L-isozyme (102).

The 3 higher-pI more commonly obtained forms of the rat or pig L-isozyme cannot differ because of different degrees of phosphorylation, since they are interconvertible in both directions in the absence of added substrate or enzyme.

**Interconvertible Forms of the K-Isozyme.** Interconvertible forms of the rat K-isozyme with pH values of 6.2 to 6.4, 6.6 to 6.8, and 7.7 to 7.8 have been reported (71, 74, 110). Additional forms with pI values of 5.1, 5.4, and 7.4 were observed by Ibsen *et al.* (71, 74) but not by Muroya *et al.* (110). Both groups report the pH 6.2 to 6.4 and pH 6.6 to 6.8 forms to be tetramers. The pH 7.7 to 7.8 form of the K-

isozyme differed from the 2 predominant tetrameric forms by having a very high $K_{0.5\ S}$ value for P-enolpyruvate (71, 110, 113) and in being a dimer (71, 74, 110).

That a K-variant with a pI value of 7.4 truly does exist is supported by the following observations: small quantities of pH 7.4 enzyme appear to be formed from pH 7.7 and 6.4 K-enzyme variants (71); pH 7.4 enzyme thus formed has kinetic properties that differ markedly from that of the M-isozyme, which also has a pI value of 7.4 (71, 74); and pH 7.4 enzyme is found in electrofocused tissue extracts not generally found to have an M-isozyme, including liver, spleen, lung, and kidney (113). This pH 7.4 form also seems to be a dimer (71, 74). Evidence for a K-isozyme variant with a pI value similar to that of the M-isozyme has been found in several species (67).

The reported pH 5.1 and 5.4 forms of the rat K-isozyme were rarely found in electrofocused fresh extracts. However, upon incubation they were usually found, but in association with precipitated material (71). Although these observations could suggest that these low-pI forms are an artifact, perhaps arising from protein-protein interactions, low-pI forms of the K-isozyme were obtained from fresh extracts of chicken tissues. Moreover, it was shown that incubation with EDTA induced their formation from middle-pI forms (68). These observations led to the suggestion that 1 of these forms is analogous to the highly anionic rat isozyme variant isolated by Pogson *et al.* (125–127), which was also generated by EDTA treatment. Moreover, both Pogson's anionic variant and the low-pI form appear to be dimers. [Although Pogson felt that the difference in S value was too small for a tetramer-dimer interconversion, it is similar to the change obtained with the M-isozyme (29) when it undergoes dimerization.]

Because the tetrameric forms of the rat L- and K-isozymes predominate in fresh tissue extracts, it seemed probable that these were the true *in vivo* forms and that the others were derived forms (71, 74). However, as discussed below, it now seems probable that at least some of the additional pI forms have a physiological function.

**RBC Enzyme.** RBC lysates may yield a band of pyruvate kinase activity that migrates slightly more slowly than the L-isozyme does during electrophoresis (75, 76, 116, 121, 154, 193). This activity band has been suggested to be a distinct isozyme, possibly the product of a 4th gene (104, 154), and it has been called isozyme D or R (75, 154). However, it seems more likely to be a hybrid or an L-isozyme variant. Support for the hybrid concept is derived from: the aforementioned electrophoretic behavior; the observation that freezing converts the RBC form to the L-form (76); the ability to obtain 2 different protein bands by sodium dodecyl sulfate electrophoresis of homogeneous preparations of the human RBC enzyme (124); and by an almost 2-fold excess of methionine peptides after cyanogen bromide cleavage (124). However, the observations that the RBC and L-isozymes cross-react immunologically but that there is no cross-reactivity between RBC enzyme and antibodies to the K- or M-isozymes (93, 94, 99, 101, 115) do not seem to be consistent with the hybrid concept.

Like the L-isozyme, the RBC enzyme has been separated in multiple forms (2, 10, 11, 69, 70, 115). Indeed, the enzyme isolated from rat RBC lysates has a pI value and kinetic properties similar to the type of L-isozyme found in liver extracts prepared from fasted animals (74). The following observations also suggest that the L- and RBC isozymes are products of the same gene: incubation of the RBC enzyme with added liver extracts converts it to the L-form (115); the RBC enzyme isolated from patients with hepatitis migrates like L-isozyme (116); both the L- and RBC isozymes are probably indirectly induced by insulin (see below); liver L-isozyme is deficient in patients having classical pyruvate kinase deficiency hemolytic anemia (7, 76, 107, 116); and both the L- and RBC enzyme migrate more slowly than normal when obtained from a patient having a hemolytic anemia associated with an aberrant pyruvate kinase (115, 116).

## Physiological Rationale for the Isozymes

As predicted from studies on the intact cell, yeast pyruvate kinase was found to be regulated by metabolites (57). Subsequent studies showed that the mammalian L-isozyme (3, 24, 144, 159, 165) and RBC enzyme (25) were also inhibited by alanine and ATP and activated by Fru-1,6-$P_2$. These properties, the tissue distribution and the similarities between the enzymes from such divergent sources, suggest that these enzymes evolved to permit gluconeogenesis; *i.e.*, under conditions of rapid glycolysis the Fru-1,6-$P_2$ level would rise, thereby activating the enzyme and synchronizing it with phosphofructokinase activity, while under gluconeogenic conditions the expected high ATP or alanine levels and low Fru-1,6-$P_2$ levels would shut down the enzyme and prevent or control a possible futile energy-consuming cycle. Consonant with this concept is the observation that addition of alanine to the intact hepatocyte does stimulate gluconeogenesis and inhibit glycolysis (34). Apparent induction of the L-isozyme by insulin and/or carbohydrate is also consistent with this idea. On the other hand, these concepts may be an over simplification since the adipocyte, which reverses glycolysis at the level of pyruvate kinase, retains the K-isozyme (18, 74, 125, 126), and the liver of adult chickens (21, 68, 146) and *Xenopus laevis*[3] seems not to develop the L-isozyme. These data indicate that gluconeogenesis can occur in the absence of the L-isozyme. That $H^+$ is a positive effector and seems to override the effects of most known negative effectors, even at pH 7.0, suggests that $H^+$ may also play a role in regulation (72), as may divalent cations (78). That neither guinea pig (41) nor bovine (22) L-isozyme is inhibited by alanine also suggests that the role of this amino acid is not crucial for the physiological function of this enzyme in at least these animals. Similarly, the rabbit L-isozyme appears not to be inhibited by ATP (78), suggesting that this modulator also is nonessential.

The K-isozyme is subject to regulation by the same effectors as the L-isozyme, although the role of ATP inhibition is open to question (*cf.* Ref. 74). The ability of both these enzymes to exist in diverse forms, as well as their sensitivity to a great number of compounds, may explain reported differences in kinetic properties.

In addition to control by amino acids and MgATP, it has

[3] K. H. Ibsen, J. R. Basabe, T. P. Lopez and S. W. Marles, unpublished observations.

been suggested that the isozymes of pyruvate kinase are subject to feedback inhibition by NADH (187), fatty acids (8, 185), phosphocreatine (88), and magnesium 2,3-diphosphoglycerate (74, 128). Subsequent studies indicate that the NADH effect is an artifact caused by deviation from Beer's law at high absorbance values (72) and that the fatty acid effect is not a primary kinetic event (73) but is probably due to a secondary denaturation of the enzyme (123). Inhibition by phosphocreatine differs from that of other inhibitors in that it has a maximal effect at pH values below neutrality (74, 88). However, somewhat incongruously, the K-isozyme is most sensitive to phosphocreatine and the M-isozyme is least sensitive (74).

Although the M-isozyme is often assumed not to be a regulated enzyme, the rat isozyme is inhibited by phenylalanine in a manner that may be allosteric, and it is reactivated by alanine and Fru-1,6-$P_2$ (23, 66, 72, 73, 87, 140, 175, 180, 181, 183). It may also be isolated in different conformational forms (71, 87).

Gosalvez *et al.* (51) and Weinhouse *et al.* (190) adduced data that suggest that the K-isozyme from hepatomas, unlike the normal liver enzyme, can successfully compete with mitochondria for ADP. This latter observation may be related to the high anaerobic glycolytic rate of most tumor cells. In keeping with this concept, it has been reported that addition of inhibitory amino acids to intact Ehrlich ascites tumor cells increases the respiratory rate and decreases the glycolytic rate. Concomitant with these changes, there is an accumulation of intracellular P-enolypyruvate and a decrease in ATP and pyruvate, indicating that pyruvate kinase was inhibited and therefore consistent with the hypothesized role of the K-isozyme in the control of glycolysis and the Crabtree and Pasteur effects of tumor cells (50). These properties would also be of potential physiological significance in fetal tissues, which also use the K-isozyme.

## Possible Functions of Multiple Forms of the Isozymes

**Phosphorylation.** The phosphorylated L-isozyme loses activity, suggesting that this is a mechanism of control. That the enzyme can be reversibly dephosphorylated by a histone phosphatase indicates that potential mechanisms exist for the reversible transition between phosphorylated and unphosphorylated forms (167). Moreover, inhibition by phosphorylation makes sense in liver but would not in a tissue like muscle. That is, in the liver, glucagon would raise the cAMP level and promote gluconeogenesis both by activating phosphorylase and by inhibiting glycogen synthetase and pyruvate kinase. On the other hand, in muscle tissue, epinephrine would raise the cAMP level and promote glycolysis, thereby also activating phosphorylase and inhibiting glycogen synthetase, but could not achieve the desired end of increasing glycolysis if pyruvate kinase were also inhibited. The observation that cAMP added to hepatocytes does inhibit glycolysis at the pyruvate kinase level provides evidence for the *in vivo* role of phosphorylation in metabolism (9, 43, 131, 164). Glucagon has been reported to inhibit (9, 43, 163) and *not* to inhibit pyruvate kinase *in vivo* (26). Insulin counteracts the glucagon effect. However, there appears to be no evidence to suggest that any of the various forms of the L-isozyme isolated to date differ because of phosphorylation.

**Dimerization and High-$K_{0.5\ S}$ Forms.** Two different hypotheses propose that a slow dissociation reaction promotes the amino acid inactivation of the K-isozyme (61, 66). Both hypotheses are supported by the following observations: there is a reversible concentration-dependent tetramer-dimer interconversion (61); the dimer is stabilized by alanine (61, 143); the tetramer is favored by the presence of $Mg^{2+}$ and Fru-1,6-$P_2$ (74); the dimer-tetramer equilibrium is slowly adjusted toward the tetramer by P-enolpyruvate (61); protein concentration affects kinetic properties (61); and negative cooperativity may occur under varied conditions (61, 66, 74). The hypotheses differ in that Hofmann *et al.* (61) propose that alanine interacts only with the dimeric form, driving it to a still less reactive dimeric form, while Ibsen and Marles (66) propose that this amino acid reacts with the tetramer, stabilizing it in the T-conformation, which spontaneously but slowly dimerizes to a still less reactive form. The concept that the amino acid reacts with the tetramer is supported by the observation that both activating and inhibiting amino acids are weak competitive inhibitors of the R-conformer and that 2 tetrameric forms are found in electrofocusing studies (see above). Moreover, dimeric forms have very high $K_{0.5\ S}$ values for P-enolpyruvate (71, 74, 110).

Evidence that this tetramer-dimer equilibrium has physiological significance is provided by the presence of small quantities of dimers (71) or of very-high-$K_{0.5\ S}$ forms in fresh tissue extracts (12, 170). Alterations of pyruvate kinase associated with erythrocyte aging (122) could be related to dimerization. Certainly, the $K_{0.5\ S}$ value for P-enolpyruvate tends to increase when lysates are stored at pH values of 7.0 or greater, and low-$K_{0.5\ S}$, high-molecular-weight forms predominate in acid-citrate stored blood (69).

Van Berkel *et al.* (173) showed that sulfhydryl oxidation converted the L-isozyme to a very-high-$K_{0.5\ S}$ form. Badwey and Westhead (2) found a similar effect on the RBC enzyme. Both groups outline mechanisms of oxidation-reduction control relevant to the physiological function of the respective cell types. Seven distinct forms of the RBC enzyme are proposed by Badwey and Westhead (2). These include tetramers, dimers, and monomers in reduced and oxidized states. All 3 states of quaternary structure, as well as a possible pentamer, appear to have enzymatic activity (2, 69). Conceivably, some of the molecular weight variants are complexed with a peptide similar to the one reported to interact with and stabilize the K-isozyme of leukocytes (84). This factor could also account for some of the pI forms, since it makes the enzyme more anionic.

Modification of quaternary structure seems not to have been reported for the L-isozyme. However, by analogy with the K- and RBC isozymes, it seems probable that 1 or more variant forms of the L-isozyme is an active dimer. A molecular weight of 166,000 for the M-isozyme obtained by light scattering data (14) suggests that it too may exist as a dimer in solution in the absence of dissociating reagents. Evidence that the $M_4$ enzyme is an unsymmetrical dimer of dimers has also been adduced (33).

**High-pI Forms of the K-Isozyme and Anaerobic Glycolysis.** Although the suspected 6 forms of the K-isozyme seem to readily interconvert, our experience suggests that the

ratio of the different pI forms varies in a manner somewhat characteristic of the organ of origin. This tendency has also been noted by Nakamura *et al.* (113). Such variability would be consistent with the concept that the varied forms have physiological significance. More specifically, extracts from tumor tissues or other cells often exposed to hypoxic conditions seem to yield a greater fraction of high-pI forms of the K-isozyme, *e.g.*, extracts of hepatoma (31), Ehrlich ascites tumor cells (Ref. 194, but not Ref. 74), rhodamine sarcoma (113), fetuses (74), and dental pulp (112). Nakamura *et al.* (113) also report that the pH 7.8 form has both an unusually large $K_{0.5\ S}$ value for P-enolpyruvate and an unusually small $K_{0.5\ S}$ value for ADP. Such an enzyme could exert the previously discussed type of metabolic control observed by Gosalvez *et al.* (50, 51) for tumor pyruvate kinase. These possible relationships need to be investigated in a systematic fashion.

Although low-pI variants of the K-isozyme are rarely obtained from fresh extracts of rat or mouse tissues, they are the predominant form of K-isozyme obtained in those chicken or *X. laevis* tissue extracts that also have suspected KM hybrids (68).[3] This observation could suggest that the K-isozyme is not a homotetramer.

## Control of Isozyme Expression

**Normal Development.** The K-isozyme is the predominant fetal isozyme of the rat (38, 40, 75, 135, 154), guinea pig (41), bovine (147), and human (42, 101). It is also the isozyme of the early chicken embryo (21, 68, 146) and of *X. laevi* tissues from at least the gastrula through the early tadpole stage.[3] There has been, however, disagreement concerning the predominant isozyme of the fetal liver. While most investigators report that the isozyme of the early fetal rat liver is K-isozyme (39, 40, 75, 106, 135, 177, 179), Osterman *et al.* (121) find that at least one-half the total activity is due to the L-isozyme as early as 5 days before birth. A large fraction of L-isozyme has also been reported for fetal guinea pig (41) and fetal human liver (5, 6, 42, 101). A pyruvate kinase that differs from the usual K- or L-isozyme forms also exists in fetal rat liver (121, 129, 179). This enzyme is apparently associated with the hemopoietic cells (179) and is probably similar to the RBC enzyme, although in some systems it acts electrophoretically like the M-isozyme (129, 179). Conceivably, this enzyme might have been included as a part of the reported L-isozyme activity in some studies but apparently not in those reported for fetal guinea pig and human liver (41, 42). Despite the fact that significant quantities of other isozymes can be found in fetal liver, the K-isozyme seems, in general, to become more predominant in younger fetuses, suggesting that it is, indeed, the prototype isozyme as postulated by Imamura and Tanaka (75).

**Expression of the L-Isozyme.** The activity of the L-isozyme of rat liver continues to rise until the 4th week after birth even in the absence of solid food in the diet (177). However, L-isozyme activity in the adult rat falls during starvation, on a low-carbohydrate diet, or in diabetes and, conversely, rises upon refeeding the starved animal, particularly with a high-carbohydrate diet or upon treating the diabetic animal with insulin (Table 2) (1, 4, 27, 39, 56, 71, 96, 133, 141, 155, 156, 159, 184, 186–189, 192). The increase in activity due to insulin or diet is prevented by inhibitors of transcription and translation suggesting *de novo* protein synthesis (155, 156, 184, 186, 189). Mouse liver enzyme has been reported not to be inducible (4).

Weber *et al.* (184, 186–188) reported a parallel change in the "key" liver glycolytic enzymes, glucokinase, phosphofructokinase, and pyruvate kinase, and proposed that these enzymes are regulated as a "functional genome unit" analogous to an operon in prokaryotes. Takeda *et al.* (156) noted that glycerol feeding increases liver pyruvate kinase and glucokinase activity in normal rats but only pyruvate kinase activity in diabetic animals. Actinomycin D treatment prevented these increases. These observations suggested that the 2 enzymes are independently regulated and that, whereas insulin might directly induce glucokinase, a triose intermediate was the specific inducer of pyruvate kinase. A similar conclusion was arrived at by Bailey *et al.* (4) and Sillero *et al.* (141). Gunn and Taylor (56) correlated the change in activities of the key glycolytic enzymes with the concentration of various metabolites under various regimens and concluded that the enzymes are regulated independently and that dihydroxyacetone phosphate is likely to be the specific inducer of pyruvate kinase. However, these authors were troubled by the observation that there was no correlation between dihydroxyacetone levels and the increase in liver pyruvate kinase activity when starved animals were refed with protein. This apparent paradox in their data may be related to the observation that a protein-induced increase in pyruvate kinase activity was prevented by cycloheximide but not by actinomycin D, as is the carbohydrate- or insulin-induced increased in activity (155). Thus it seems possible that L-pyruvate kinase production might be regulated by both a transcriptional and posttranscriptional mechanism, the latter being related to a deficiency of amino acids.

The L-isozyme activities in rat intestinal mucosa (120) and kidney (133) are also responsive to diet.

Although experimental diabetes has been reported not to depress pyruvate kinase activity of erythrocytes or reticulocytes (48, 159), Kimura *et al.* (89) report a 10-fold increase in the pyruvate kinase level of erythrocytes from a patient with an insulinoma. Activity returned to normal within 47 days following extirpation of the tumor. These authors also noted a decrease in the pyruvate kinase level of erythrocytes from diabetic patients. Similarly, Sandoval and Carbonell (133) report that the activity of the reticulocyte enzyme is increased in animals consuming a high-fructose diet.

An inducible pyruvate kinase is a trait shared by some primitive eukaryotes (44, 47).

**Regulation of K- and M-Isozyme Expression in Normal Adult Tissues.** Generally, the K- or M-isozymes are considered to be constitutive in normal adult tissues. Exceptions to this generalization may exist for intestinal mucosa and uterus. This latter organ, from bovine sources, contains $K_4$ isozyme plus possible KM hybrids (147). Progesterone administration to the estradiol-treated rat (81) or estrogen administration to the gonadectomized rat (142) appears to induce pyruvate kinase activity. Thus K and/or M subunit synthesis may be controlled by steroids. Paradoxically, the estrogen effect is reported to be inhibited by progesterone (142). Perhaps the apparent contradictions in these studies

are related to different behavior of the isozymes. In the case of intestine, Osterman and Fritz (120) indicate that both K- and L-isozymes are similarly affected by fasting and refeeding.

**Expression of the K-Isozyme and Dedifferentiation.** Hepatoma proteins often differ from their normal liver counterparts by regressing to a fetal form (*cf.* Refs. 32, 40, 53, 90, 118, 134, 136, and 189 to 192). This regressive phenomenon has led to suggestions that neoplasia is a disease of cellular differentiation rather than a somatic gene mutation (103). Although it seems indisputable that a misprogramming of protein synthesis is associated with the neoplastic state, it is not clear whether the regressive phenomena are: chance events; events vital for transformation; and/or events vital for continued growth of the newly transformed neoplastic cell. Alternatively, it may be that primordial cells become neoplastic or are selected for during neoplasia. These cells are presumed to undergo an incomplete ontological development and thus never attain a full complement of adult proteins. Presently, the trend in thinking seems to be tending toward the belief that the misprogramming of protein synthesis associated with tumorigenesis is programmed and not random. For instance, the "Molecular Correlation" concept (182) suggests a vital relationship between differentiation and transformation, while the "Blocked Ontogeny" concept (129, 182) envisions a vital and incomplete differentiation of stem cells. Even the phenomenon of ectopic synthesis of polypeptide hormones by tumors, felt probably to be the consequence of a random disturbance of gene expression (39, 132), occurs in an orderly manner (8). The term "disdifferentiation" (151) suggests that the cancer phenotype may be a consequence of an abnormality of gene expression without implying a cause or effect. This problem is considered in detail by Knox (90).

Whether or not the expression of otherwise repressed genes in cancer is intimately related to the event of neoplastic transformation, it seems probable that some of these regressive phenomena help the developing tumor to survive in what must be an increasingly hostile environment. Thus, for a neoplasm to become a clinically significant mass, it may have undergone an initial transformation followed by 1 or more disdifferentiation events. Evidence for such a conclusion is provided by Farber's (37) observation that chemical hepatic carcinogenesis is a prolonged, multistep process that includes a reversible preneoplastic, hyperplastic nodule state. Thus, disdifferentiation may well be unprogrammed, but derepression or arrested expression of some genes will provide the nascent tumor mass a competitive advantage, which might eventually account for its ability to survive at the expense of the host.

Expression of the fetal isozyme of pyruvate kinase may be an example of a vital disdifferentiation event, since fetal K-isozyme replaces the L-isozyme in hepatomas (Table 2) (5, 6, 38–40, 76, 135, 160, 179, 189–192, 195) and the M-isozyme in a rhabdomyosarcoma (40). As discussed, the K-isozyme may be important in control of glycolysis under hypoxic conditions. Thus this isozyme switch may permit the tumor to grow rapidly by meeting the increased demand for energy in the face of a limited oxygen supply.

Despite these reports of K-isozyme in hepatomas, there are reports that liver tumors produce an M-isozyme or new isozymes, which if true could suggest that the disdifferentiation event is random and perhaps of little significance. However, early identification of tumor pyruvate kinase as new or as M-type was due to the earlier inability to distinguish between the K- and M-isozymes (*e.g.* Refs. 38, 159, and 166). Moreover, the suggested tendency of tumor extracts to contain forms of the K-isozyme with pI values similar to or greater than that of the M-isozyme may explain reports of "new" or of M-isozymes in electrofocused tumor extracts (31, 113, 194).

Tumor pyruvate kinases identified as a novel form or as the M-isozyme on the basis of criteria other than pI value may also prove to be K-isozyme variants. Possible examples include the following. On the basis of kinetic criteria, Irving and Williams (80) identified 2 hepatoma pyruvate kinases, one as a new L-type enzyme and the other as an M-type. However, the new L-type enzyme behaves much like one of the high $K_{0.5\ S}$ variants of the K-isozyme, while the so-called M-isozyme is inhibited by alanine. This latter response is uncharacteristic of the M-isozyme; on the other hand, the kinetic properties of this isozyme appear to be compatible with its being the tetrameric R-conformeric form of the K-isozyme. Similarly, Spellman and Fottrell (145) report that the tumor and placental pyruvate kinases differ from all the other human isozymes by being more cathodic. This observation is consistent with the concept that they are the highest pI form of the K-isozyme. They also report that the placental enzyme has one-half the molecular weight of the other isozymes, as do the high-pI forms of the K-variant (71, 74, 110). Seemingly, in these studies K-isozyme from other tissues acted electrophoretically like the M-isozyme rather than like the most cathodic form.

Thus it is conceivable that the reported unusual forms of tumor pyruvate kinase are K-type variants.

Farina *et al.* (39) and Weinhouse *et al.* (189, 191, 192), using the Morris transplantable hepatomas, and Tanaka *et al.* (161) and Yanagi *et al.* (195), using hepatomas newly induced by chemical carcinogens, studied the pyruvate kinase isozyme patterns in neoplasms with graded degrees of differentiation. The highly differentiated tumors had isozyme patterns not necessarily very different from those of normal liver [there consistently being, however, some increase in K-isozyme activity (Table 2)]. The well-differentiated tumors showed a consistent and marked decrease in the activity of the L-isozyme but still retained a relatively low but significantly greater than normal K-isozyme specific activity. However, the poorly differentiated tumors had little L-isozyme and showed a manyfold increase in K-isozyme specific activity (Table 2). Weinhouse *et al.* (189) point out that the sequential effects suggest that 2 different genes are functioning. Yanagi *et al.* (195) hypothesize that the decrease in L-isozyme is due to loss of the receptor sites responsible for the apparent inducibility of the L-isozyme by diet (see above) that and the increased activity of the K-isozyme is due to the production of a derepressor factor (see below) by the dedifferentiated tumor. Variations in L-isozyme levels could also be a consequence of a changed nutritional and/or hormonal state of the host animal.

There have also been several reports concerning the pyruvate kinase isozyme patterns of the preneoplastic, hyperplastic nodules induced by carcinogens. A minimal to a

marked increase in K-isozyme activity was observed (Table 2) (36, 179, 195). Yanagi *et al.* (195) suggest that the relative increase in K-isozyme activity is related to the type of carcinogen used. The increase in K-type pyruvate kinase occurs after relatively brief exposure to a carcinogen and is observed prior to a change in total pyruvate kinase activity, mitotic index, histological appearance, or protein and DNA content (36). Such a relationship is consistent with the concept that increased expression of the K-isozyme is an occurrence not far removed from the transformation event itself.

**Apparent Derepression of K-Isozyme Synthesis in Nonneoplastic Liver.** Greenstein and Andervont (54) noted that the activity of certain enzymes in the liver of tumor-bearing animals is altered. This led to the generalization that the host liver takes on immature and/or neoplastic features (53). This concept has been confirmed and quantified (58, 90, 92, 148) and, when coupled to the regressive tendencies of the tumors themselves, has led to the idea that tumors produce circulating factors that alter gene expression in liver.

An early attempt to exploit this concept by isolating the active factor causing suppression of liver catalase activity was undertaken by Nakahara and Fukuoka (111). They found an active fraction which they called "toxohormone." Attempts to purify and characterize this "toxohormone" have resulted in inconclusive and contradictory results (*cf.* Refs. 46, 52, 85, 117, and 196). Matuo *et al.* (105) report that purified "toxohormone" is a peptide with a molecular weight of 60,000 and a pI value of 5.0. It has been said to be isolated from the non-histone nuclear protein fraction (108); however, Nakamura *et al.* (114) suggest that there are 2 factors, one an acidic nuclear protein and the other a basic nuclear protein. On the other hand, Uenoyama and Ono (168) isolated a factor from tumor and liver cells that acts to inhibit catalase synthesis at the translational level; however, no attempt was made to relate this factor to toxohormone. Despite the apparent confusion in the literature, it seems that tumors do produce a factor or factors able to inhibit the expression of catalase or catalase activity in otherwise normal liver cells.

The pyruvate kinase isozyme system appears to be a good model system in which to study mechanisms controlling genetic expression; not only is there a shift away from the L-isozyme and toward the K-isozyme in hepatomas, but such a switch also tends to occur in regenerating liver (39, 159, 179), in hepatocytes cultured for more than 5 days (55, 178), in the liver of tumor-bearing animals (64, 113, 114, 148, 150, 161), in the liver of the parabiotic twin of a tumor-bearing animal (148, 150), in livers perfused with blood from a tumor-bearing animal (149), in the livers of animals given injections of the blood of tumor-bearing animals (64), in the livers of animals given injections of the cell-free fluid of ascites tumor cells (64), and in the liver of animals given injections of extracts prepared from tumor cells (64, 110, 113, 150, 161, 162). Whereas these data were obtained from rodents, an isozyme shift also was observed in liver samples obtained from humans with gastric cancer (149).

Although several mechanisms can be invoked to account for an increase in K-isozyme activity in the host liver in response to tumor growth or injection of subcellular fractions, it seems probable that the increase is due to increased protein content since it can be measured immunologically (148–150, 161, 162), tumor growth causes labeled amino acids to be incorporated into K-isozyme protein of host liver at a much greater rate than into liver protein in general (150), and the increase is prevented by compounds that inhibit protein synthesis at the transcriptional or translational levels (150). These latter observations make more remote but do not rule out possible control at the translational or degradational levels. As discussed, there is evidence for translational control of L-isozyme production. Also, shifts in variant forms, analogous to that observed for the L-isozyme in the fasted animal (71, 91), could represent a mechanism for controlling the rate of enzyme degradation. However, translational control and maintenance of a low K-isozyme level by a degradative mechanism would seem to be inefficient mechanisms for effectively eliminating the K-isozyme in the hepatocyte during the adult life of the normal animal, and the hepatocyte is stated, but not documented, to be the cellular site of increased K-isozyme production (161).

This latter observation would also rule out the possibility that the increase is due to proliferation of non-hepatocyte cells, a phenomenon observed in the liver of tumor-bearing animals (109). This possibility is made still more remote by the observation that a major shift to K-isozyme can be elicited within 2 hr by perfusion of the isolated liver with the blood from tumor-bearing animals (149).

Thus these observations suggest that the tumor-derived factor operates by inducing the hepatocyte to turn on the repressed fetal gene for the K-isozyme but does not indicate whether it acts directly or indirectly. That a similar isozyme shift occurs in hepatocytes cultured *in vitro* (55, 178) shows that the host animal is not necessarily required for apparent derepression of K-isozyme synthesis to occur. However, tumors may act by a different mechanism or a derepressor factor may be added via the growth medium. *De novo* production of K-isozyme also may occur as a response coupled to growth. Such a mechanism is consistent with the association of K-isozyme and dedifferentiation in tumors and of this isozyme and growth in the regenerating liver (179), and tumor growth does trigger DNA synthesis and cell proliferation in the host liver (109). Thus the substance obtained from tumor cells could be a growth factor.

However, the bulk of evidence suggests that the factor is itself a regulator of genetic expression; *i.e.*, there is an apparent parallelism between the effect of tumor or tumor extracts on the host animal liver and the isozyme shift that occurs in the hepatomas; the K-isozyme activity increase is prevented by inhibitors of transcription; factor with derepressor-like activity can be isolated from the chromatin of tumor cells (110, 113, 161) (Table 3) and from the chromatin of spleen, which also produces the K-isozyme (110); and the active substance appears to be a low-molecular-weight (160) polypeptide (64). Thus the factor is likely to be a nuclear protein (161). That nuclear proteins can be found in the circulatory system is substantiated by the observation that antibodies to chromatin protein, prepared from cultured fibroblasts, have been reported to show specificity to an $\alpha$-serum protein (197). It has been postulated that the K-isozyme-stimulating nuclear protein is released in greater

Table 3
*Ability of Ehrlich ascites cell nuclear fractions to increase K-isozyme activity*

| Fraction [Busch *et al.* (16)] | Enriched constituent [Busch *et al.* (16)] | Relative specific activity[a] | |
|---|---|---|---|
| | | Kuwata *et al.*[b] | Tanaka *et al.* (161) |
| Nuclei | | 2.1[c] | 1.8 |
| Nuclear supernatant | | 1.5 | 1.6 |
| 0.14 M NaCl wash | Nuclear sap proteins I | 2.2 | |
| 0.1 M Tris wash | Nuclear sap proteins II | 1.1 | 1.2[d] |
| ppt[e] from above | Washed chromatin | 4.8 | |
| 1st 2.0 M NaCl wash | Deoxyribonucleoprotein I | 2.4 | |
| ppt from above | Extracted chromatin | 3.6 | |
| 2nd 2.0 M NaCl wash | Deoxyribnucleoproteins II | 2.3 | 1.4[d] |
| ppt from above | Further extracted chromatin | | 1.6[d] |
| 0.05 N NaOH wash | Ribonucleoproteins | 1.3 | 1.5 |
| ppt from above | Residual proteins | | 1.6 |

[a] Relative specific activity is the specific activity of K-isozyme in liver extracts of experimental animals divided by the specific activity of the K-isozyme in liver extracts of control animals given injections of 0.9% NaCl solution.

[b] J. H. Kuwata, S. W. Marles, and K. H. Ibsen, unpublished information. Factor and isozyme activity was assayed as described previously (64). The 95% confidence limit is at a relative specific activity of 1.23. Most of the values for the experiments represent the mean obtained with 4 mice and 2 different preparations.

[c] Prepared by washing through 1.8 M sucrose [Busch *et al.* (15)]. Nuclei were contaminated by cytoplasm and unbroken cells.

[d] Steps apparently combined.

[e] ppt, precipitate.

than normal quantities from tumor cells because of their greater rate of proliferation and/or destruction (161).

That a K-isozyme-stimulating substance can be isolated from the high-speed supernatant fraction of sonically disrupted tumor cells as a peptide-RNA complex (64) could indicate that there is more than 1 factor, that the factor involved is produced in the nucleus but acts in the cytoplasm in association with RNA, that the factor is released from the nucleus and becomes associated by chance with RNA, or that the factor exists in the nucleus as an RNA-protein complex which is released by sonic disruption. That the active peptide survives phenol extraction and electrofocusing still complexed to RNA (64) suggests that it has a high affinity for RNA and that a specific interaction is involved.

In summary, data obtained concerning the K-isozyme-stimulating factor are sufficient to prove that a specific effect is occurring and suggest that the factor may be a nuclear protein that functions as a derepressor. However, other possibilities have not been excluded. Eventually, elucidation of the nature and role of this factor will depend upon its purification and the development of an *in vitro* assay system. Although formidable problems may hinder attainment of these goals, their realization should provide fundamental information concerning genetic regulatory mechanisms in higher eukaryotic cells.

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