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*This paper is dedicated to Professor Włodzimierz Ostrowski*
***Review***

# Tumor-specific pyruvate kinase isoenzyme $M_2$ involved in biochemical strategy of energy generation in neoplastic cells

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**Key words**: tumor aerobic glycolysis, sensitivity to L-cysteine, Pasteur and Crabtree effects, tumor-specific variant $\gamma_3$ of pyruvate kinase isoenzyme $M_2$

**The differences in properties of pyruvate kinase (EC 2.1.7.40) from normal tissues and animal or human tumors are described and their significance for various metabolic abnormalities is reviewed. The tumor variant $\gamma_3$ from $M_2$ isoenzyme of pyruvate kinase sensitive to L-cysteine inhibition, when over-expressed, can be used as a marker of tumorigenic transformation. It seems that this variant represents a tumor-specific oncoprotein, involved in a novel metabolic strategy of energy generation during increased cell proliferation.**

Since the first epoch-making Warburg's [1, 2] discovery of high glycolytic activity in tumors, much work has been devoted to this metabolic abnormality in various *in vitro* comparative studies on tissue slices, cell suspensions or even reconstructed systems containing subcellular fractions from normal and tumor materials [3–6]. The high rate of aerobic glycolysis and diminution or lack of the Pasteur effect represent the major biochemical differences between normal and neoplastic materials. Moreover, the diminished Pasteur effect is accompanied by the Crabtree effect, i.e. inhibition by glucose of oxygen consumption [7–9]. This effect has been observed in all tumors, in primary cell cultures, as early as 24–48 h of *in vitro* cell adaptation to growth on glass [10], and during the replicative phase of virus DNA synthesis after cell infection with oncogenic polyoma virus [11]. However, it was also observed in a few proliferating normal cells or tissues characterized by a high rate of glycolysis, such as placenta, embryo, retina, platelets and epithelial cells [9].

Many explanations of the Crabtree effect have been suggested, mainly related to abnormal compartmentation of cell metabolites [9], especially of adenyl nucleotides [8,12]. However, understanding of its molecular mechanism was possible only after the discovery of key regulatory enzymes [13].

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**Abbreviations**: PEP, 2-phosphoenolpyruvate; FDP, fructose-1,6-bisphosphate.

## THIOL COMPOUNDS, GLYCOLYTIC ACTIVITY AND ATP LEVEL

Horn *et al.* [14,15] observed that cysteine and other compounds with a free thiol group (e.g. reduced glutathione) may favour, in the heart muscle homogenates, a shift of the glucose metabolism towards the anaerobic pathway, even in the presence of oxygen, and a diminution of the Pasteur effect. At the same time a defective cysteine metabolism, connected with a decrease in cysteine desulfhydrase, rhodanase [16,17] and 3-mercaptopyruvate sulfurtransferase activities [18] was described in neoplastic tissues. In this situation an accumulation of cysteine was expected, which could cause activation of aerobic glycolysis in tumors.

However, contrary to the initial assumption that compounds with thiol groups might increase aerobic glycolysis, it has been found that DL-cysteine inhibits this process in intact tumor cells and their cytosols [19] and restores the Pasteur effect (Table 1). Although DL-homocysteine as a homologue, and DL-serine as a structural analogue of DL-cysteine does not inhibit aerobic glycolysis when applied separately, they exerted a significant inhibitory effect when present in the medium together, due to endogenous formation of cysteine [19].

It appears that only L-cysteine inhibits aerobic glycolysis and restores the Pasteur effect in tumor cells, whereas D-cysteine has no influence on this process [19] (Fig. 1). This stereospecific inhibitory effect of L-cysteine results, at the most, in 50% inhibition. Since the effect of DL-penicillamine was similar to that of DL-cysteine [19] the number of carbon atoms in the effector molecule is probably of secondary importance as long as the thiol group is in the β-position.

L-Cysteine, parallelly with the inhibition of lactate formation, causes a decrease in cell ATP [19], and pyruvate contents, and an increase in 2-phosphoenolpyruvate (PEP) (Fig. 2). The observed cross-over in concentrations of these intermediates suggested that the cysteine-induced stereospecific inhibition of tumor glycolysis with restoration of the Pasteur effect *in vitro* occurs at the stage catalyzed by pyruvate kinase [19, 20].

Since glycolysis plays a crucial role in cytosolic energy generation, special attention should be paid to inhibitors of this fundamental process which may affect tumor growth.

**Table 1. Effects of cysteine (1 mM final concentration) on lactate production in Ehrlich ascites tumor cells, in their supernatants, and in supernatants from normal tissues [19]**

| Experimental material | Lactate production | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Cells (n=10) | | | | Supernatants (n=8) | | | |
| | $\bar{X}$ | ±S.D. | $\bar{X}$ | ±S.D. | $\bar{X}$ | ±S.D. | $\bar{X}$ | ±S.D. |
| | (μmol/h per mg protein) | | (% of control) | | (μmol/h per mg protein) | | (% of control) | |
| **Ehrlich ascites tumor** | 1.39 | 0.10 | 100.0 | 7.2 | 1.04 | 0.03 | 100.0 | 2.9 |
| DL-Cysteine | | | 67.4 | 4.8 | | | 63.6[b] | 17.3 |
| L-Cysteine | | | 60.7 | 10.0 | | | 62.5[a] | 23.4 |
| **Normal mouse liver** | – | | | | 0.33 | 0.01 | 100.0 | 3.0 |
| L-Cysteine | | | | | | | 90.9 | 2.7 |
| **Skeletal muscle** | – | | | | 0.72 | 0.02 | 100.0 | 2.8 |
| L-Cysteine | | | | | | | 94.4 | 2.6 |

[a] $0.005 < P < 0.01$; [b] $P < 0.001$

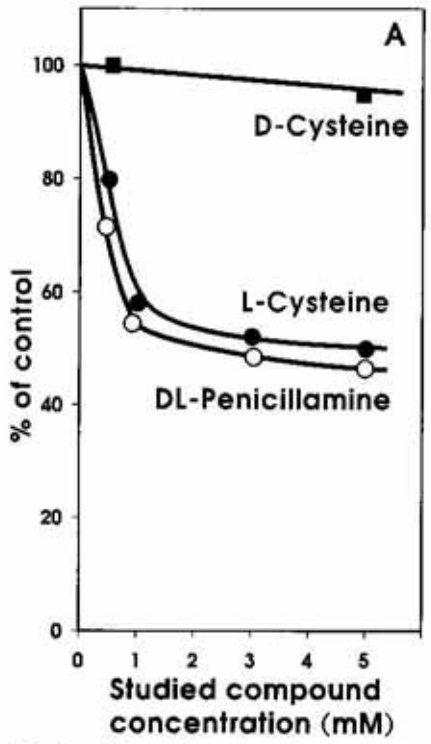


**Figure 1. The effect of L-cysteine, D-cysteine and DL-penicillamine on lactate formation in Ehrlich ascites tumor cells [19].**

The results are in percentage of control value.

## KEY GLYCOLYTIC ENZYMES

Among eleven glycolytic enzymes, three key enzymes: hexokinase, phospho-fructokinase and pyruvate kinase [21] catalyze reactions which are practically irreversible because of energy barriers or instability of products. Weber [13] has shown that the key cooperating enzymes are produced on the same functional genetic unit. Using a broad spectrum of hepatomas differing in growth rate he demonstrated that the activities of key enzymes of the glycolytic pathway correlate positively with the growth rate of these tumors. Glycolysis and activities of its three key enzymes in slowly growing hepatomas are similar as in liver, while in rapidly growing hepatomas with high glycolysis, a parallel increase in three key enzymes correlated with increased tumor growth rate. Weinhouse and coworkers [22], in line with the Warburg's hypothesis, discovered that respiration in slowly growing, well-differentiated hepatomas was as high as in liver slices, whereas in rapidly growing and poorly differentiated hepatomas, respiration was very low. Thus, the decreased Pasteur effect reflected the competition for ADP between respiration and a key glycolytic enzyme, pyruvate kinase. The increased hepatoma growth rate pointed to a parallel increase in ATP formation and protein, RNA and DNA biosynthesis [23].

In agreement with these findings we have shown that rapidly growing neoplastic cell lines, e.g. BHK-21 cells, had a high activity pattern of key glycolytic enzymes [24]. After infection with oncogenic polyoma virus, when viral genome became incorporated into cell genome as a transforming agent, a further increase in the activity of key glycolytic enzymes, especially of pyruvate kinase was observed. This correlated with a rapid increase in tumor antigen synthesis [24] and oncogene

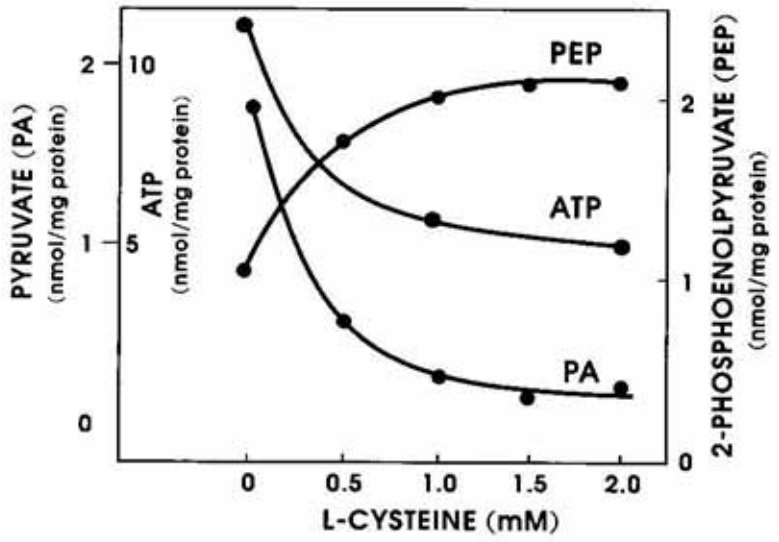


**Figure 2. The effect of L-cysteine on pyruvate (PA), 2-phosphoenolpyruvate (PEP) and ATP levels in Ehrlich ascites tumor cells [19].**

activation which, by changing the cell genotype, influenced cell metabolism and tumor phenotype [25, 26].

## PYRUVATE KINASE (EC 2.7.1.40) IN NORMAL AND TUMOR CELLS

In various normal tissues, pyruvate kinase has a rather low activity while it is over-expressed in many tumor cells or cultured cell lines [27, 28]. For example, in rat lung explants cultivated *in vitro*, pyruvate kinase activity started to increase as early as on the second day after explantation and preceded an increase in glycolysis [29]. On the 7th day after the explantation, pyruvate kinase activity was increased 5–7 fold and approached values found in Yoshida ascites tumor cells of rats. The enzyme activity correlated well with the doubling time of permanent cell lines established from lung explants by "spontaneous" malignant transformation [29] and tested by inoculation into newborn Wistar rats, in which progressive tumor growth was taken as a sign of malignancy.

Gosalvez *et al.* [30] have found that there is a competition for ADP between respiration and glycolysis (Fig. 3). Over-expressed pyruvate kinase utilizes more ADP and, in consequence, after addition of glucose respiration is inhibited (the Crabtree effect). High activity of pyruvate kinase in many cells is the main cause of higher ADP utilisation in glycolysis than in the respiratory pathway. In contrast, ATP, which is the end product of oxidative phosphorylation, normally inhibits pyruvate kinase by a feed-back mechanism [31]. Exogenous ATP diminishes aerobic glycolysis in tumor cell homogenates by inhibiting pyruvate kinase and in this way it even abolishes the Crabtree effect in cultured tumor cells [12]. However, when the activity of pyruvate kinase was very high after malignant transformation [29], a decrease in its inhibition by ATP has been considered to be the cause of lack or significant diminution of the Pasteur effect [28].

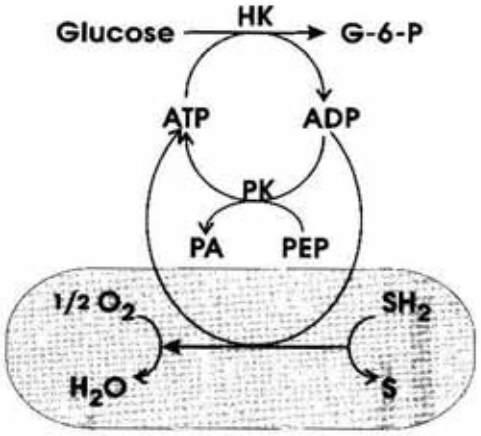


**Figure 3. Competition for ADP between cytosolic pyruvate kinase (PK) and mitochondrial oxidative phosphorylation as the cause of the Crabtree effect.**

HK, hexokinase; G-6-P, glucose-6-phosphate; PA, pyruvate; PEP, 2-phosphoenolpyruvate; S, $SH_2$, oxidised and reduced substrate.

## TUMOR PYRUVATE KINASE SENSITIVE TO L-CYSTEINE

The cross-over of the metabolite pattern [19] observed in metabolic studies with L-cysteine, indicates that this compound directly diminished the tumor pyruvate kinase activity, but had no effect on other key glycolytic enzymes or on pyruvate kinase activity of normal tissues [19]. Stabilisation of glycolytic activity at 50% of the control value indicates that in tumors, in addition to pyruvate kinase sensitive to L-cysteine, there is a cysteine-insensitive pyruvate kinase fraction. Thus the question arose, whether the cysteine-sensitive variant of the enzyme could be a marker of neoplastic transformation in various experimental tumors.

As it has been shown, pyruvate kinase activity becomes significantly increased on "spontaneous" or viral cell transformation *in vitro* [24, 29]. In Ehrlich ascites cells, in addition to increased activity, an unusual sensitivity of this enzyme to the inhibitory action of L-cysteine has been found [19, 32].

**Table 2. The effect of L-cysteine (0.05 mmol/l final concentration) on pyruvate kinase activity in cytosolic fractions of different tumors and normal tissues [33].**

| Material | Control (mIU/mg protein) = 100% (n = 6) | | + L-Cysteine (% of control) (n = 4) | |
|---|---|---|---|---|
| | $\bar{X}$ | ±S.D. | $\bar{X}$ | ±S.D. |
| **Mice solid tumors** | | | | |
| Mammary adenocarcinoma | 5652 | 868 | 75.4 | 19.7 |
| Madison lung carcinoma | 10418 | 1209 | 60.6[a] | 8.0 |
| Lewis lung carcinoma | 3543 | 133 | 74.4 | 18.8 |
| Colon carcinoma C-38 | 9561 | 2605 | 71.4 | 24.2 |
| Colon carcinoma C-26 | 3882 | 1100 | 76.3 | 19.7 |
| Melanoma B-16 | 7854 | 2438 | 94.2 | 20.2 |
| Polyoma | 2171 | 826 | 70.9 | 20.9 |
| Leukemia L-1210 (n = 2) | 8499 | 2977 | 76.2 | |
| **Mice ascites tumors** | | | | |
| Ehrlich ascites tumor | 3943 | 970 | 50.8[a] | 9.5 |
| Leukemia L-1210 | 3324 | 753 | 74.5[c] | 3.8 |
| Leukemia P-388 | 4337 | 1038 | 61.7[b] | 7.1 |
| Leukemia AKSL-4 | 6903 | 2473 | 83.1 | |
| **Normal mice tissues** | | | | |
| Skeletal muscle | 4552 | 1833 | 99.6 | 10.2 |
| Liver | 435 | 155 | 108.2 | 16.6 |
| Spleen | 1060 | 250 | 105.7 | 8.6 |
| Mouse embryo | 4793 | 149 | 100.0 | 0.0 |

[a] $0.001 < P < 0.005$; [b] $0.01 < P < 0.05$; [c] $0.05 < P < 0.1$

Pyruvate kinase sensitive to L-cysteine is present in a broad spectrum of mouse experimental tumors transplanted into syngenic recipients [33] and in rat hepatoma [34] but not in normal tissues [33] (Table 2). The activity of pyruvate kinase in cytosolic fraction of various mouse tumors as well as of mouse embryo and normal skeletal muscle was much higher (about 3000–10000 mIU/mg protein) than in corresponding fraction of mouse spleen or mouse liver (about 250–1000 mIU/mg protein). L-Cysteine (0.05 mmol/l) decreased pyruvate kinase activity, in the solid tumors studied to about 61–77% of the initial control values. The effects were slightly greater in ascitic tumors (51–75%). In contrast to tumor materials, L-cysteine had no effect on pyruvate kinase from cytosolic fractions of normal tissues (100–108%), irrespective of whether the initial enzyme activities were low (liver and spleen) or high (skeletal muscle and embryo).

## PYRUVATE KINASE ISOENZYMES. SEPARATION OF THE L-CYSTEINE SENSITIVE VARIANT

Three pyruvate kinase isoenzymes are known, namely isoenzyme L from liver,

**Table 3. Pyruvate kinase activity in cytosol and chromatin extracts of Ehrlich ascites tumor, Morris hepatoma 7777, normal mouse and rat livers [34], and selected human urothelial cell lines (TGr I–III) [43], and the effect of L-cysteine (results expressed as percentage of control activity without L-cysteine).**

| Material | Pyruvate kinase | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Cytosolic fractions | | | | | Chromatin extracts | | | | |
| | (mU/mg protein) | | | L-Cysteine concn. (mM) | | (mU/mg protein) | | | L-Cysteine concn. (mM) | |
| | $\bar{X}$ | ±S.D. | (n) | 0.05 | 0.1 | $\bar{X}$ | ±S.D. | (n) | 0.05 | 0.1 |
| | | | | (% of control activity) | | | | | (% of control activity) | |
| Ehrlich ascites tumour | 9083 | 2775 | (5) | 86 | 79 | 2541 | 879 | (5) | 85 | 80 |
| Mouse liver | 1457 | 372 | (5) | 100 | 100 | 969 | 259 | (5) | 100 | 100 |
| Morris hepatoma 7777 | 6750 | 2327 | (6) | 60 | 56 | 1225 | 190 | (4) | 50 | 42 |
| Rat liver | 908 | 161 | (6) | 100 | 100 | 621 | 112 | (4) | 100 | 100 |
| Selected human urothelial cell lines [55] | | | | | | | | | | |
| TGr: Cell line: | | | | | | | | | | |
| TGr I Hu 1125 | 980 | – | (2) | 100 | 100 | 829 | – | (2) | 100 | 100 |
| TGr II Hu 609 | 932 | 159 | (3) | 100 | 100 | 861 | 149 | (3) | 100 | 100 |
| HCV 29 | 1513 | 269 | (4) | 100 | 100 | 1124 | 143 | (4) | 100 | 100 |
| TGr III Hu 609 $T_{LLH}$ | 5729 | 1130 | (3) | 62 | 51 | 1070 | 182 | (3) | 90 | 66 |
| Hu 609 $T_{MV}$ | 4765 | 1045 | (3) | 78 | 60 | 718 | 187 | (3) | 81 | 55 |
| HCV 29 T | 3839 | 985 | (4) | 78 | 52 | 1428 | 478 | (4) | 85 | 62 |
| T 24 | 3238 | 135 | (4) | 60 | 41 | 1400 | 356 | (3) | 87 | 60 |

TGr, transformation grade.

isoenzyme M from skeletal muscles and brain, and isoenzyme $M_2$ (called also K, S, A or III) from kidney, lungs, adipose tissue, placenta, embryo and tumors [35–41]. These three isoenzymes are products of three distinct genes and differ in the amino-acid composition [37]. Normal livers contain two pyruvate kinase isoenzymes showing different pI values — the dominating isoenzyme L and isoenzyme $M_2$ [37]. The third isoenzyme M, more cathodic, with pI 7.5 is absent in liver [36, 37]. Isoenzyme L can be salted out at 0.21–0.30 ammonium sulfate saturation (fraction A), and isoenzyme $M_2$ at 0.51–0.70 saturation (fraction B) [33, 42].

It has been found that isoenzyme L is insensitive to L-cysteine. The sensitivity to L-cysteine was observed only in tumor fraction B, containing also isoenzyme $M_2$ [33, 42] but not in fractions B from normal tissues.

All events occurring in cell nuclei are highly energy consuming. It is accepted that this energy is supplied by glycolysis, since a positive correlation has been observed between increased tumor growth rate and glycolytic activity [13, 43, 44]. In addition to investigations on cytosolic pyruvate kinase, much work has been done on this enzyme from cell nuclei after their isolation, purification and lysis. For pyruvate kinase extraction from

chromatin pellets, changes in pH and ionic strength were used [45, 46]. This procedure has been applied to Ehrlich ascites tumor cells, Morris hepatoma 7777, normal mouse and rat livers [34], as well as to human urothelial cell lines in transformation grades (TGr) I, II and III cultivated *in vitro* [43]. Most of these cell lines (Table 3) were established at the Fibiger Institute (Copenhagen), as previously described [43]. Although cytosolic pyruvate kinase activities from animal tumors and human TGr III cell lines were much higher than from corresponding normal mouse or rat livers, and from cells in TGr I or TGr II, the enzyme activities in chromatin extracts from all materials studied were of a rather similar order of magnitude (Table 3). L-Cysteine inhibited tumor pyruvate kinase, both from cytosolic and nuclear compartments only to about 40–50%. This indicates that tumor chromatin extracts are inhomogeneous and contain also a cysteine-insensitive pyruvate kinase subfraction. Separation by polyacrylamide gel electrophoresis [47] of chromatin extracts at pH 8.3 [34, 43], has shown the presence of three pyruvate kinase variants: α, β, γ, but their mobilities were higher in chromatin extracts from normal liver and TGr I cells than in those from tumors (Fig. 4), both the experimental tumors [34] and urothelial cell lines in TGr II and TGr III [43].

Only the most cathodic, with the highest pI value, slowly migrating tumor variant γ, lo-

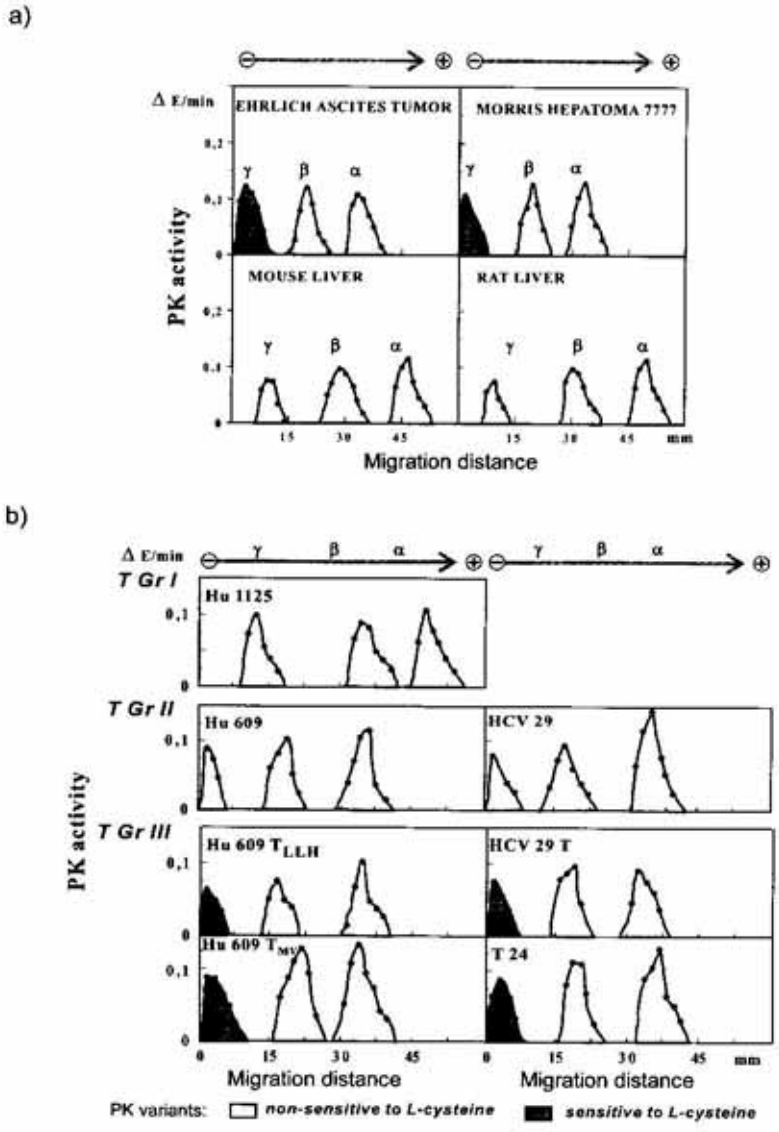


**Figure 4. Electrophoretic pattern of pyruvate kinase variants from chromatin extracts of: (a) Ehrlich ascites tumor, Morris hepatoma 7777, normal mouse and rat livers [34]; (b) Selected human urothelial cell lines of different transformation grades TGr I–III [43].**

calised at the gel origin, was sensitive to L-cysteine (hatched area in Fig. 4). The dose dependent inhibitory effect of L-cysteine, practically to zero value, points to homogeneity of the separated variant γ. The metabolic role of this new L-cysteine-sensitive variant in tumors remains unknown.

In urothelial cell lines [43] L-cysteine-sensitive variant can be used as a marker of multistage neoplastic transformation, as it reflects in TGr III cell lines their tumorigenic properties in nude mice. However, in premalignant immortal cell lines of TGr II, lower mobility of this variant than in TGr I cell line and the presence of a slowly migrating variant γ with a pI value of about 8.3, seem to be early characteristic changes of oncoming malignancy.

The variants from chromatin extracts purified by polyacrylamide gel electrophoresis [34, 43] were obtained, however, in amounts too small to allow their further characterization. Therefore affinity chromatography on Blue Sepharose CL-6B [42] was applied for obtaining the D-cysteine-sensitive variants. This method involving specific binding, separation and purification, followed by elution with competing ADP molecules of salted out cytosolic isoenzymes of pyruvate kinase, was successfully used for isolation of their variants from normal rat liver and Morris hepatoma 7777 [42].

## CHARACTERIZATION AND COMPARISON OF PYRUVATE KINASE VARIANTS FROM MORRIS HEPATOMA 7777 AND RAT LIVER

Pyruvate kinase fractions A and B, obtained from normal liver and Morris hepatoma cytosols by the salting out procedure correspond to isoenzymes L and $M_2$, respectively [42]. Characterization of the two isoenzymes and their variants is given in Table 4 and Fig. 5 [48].

The three variants of cytosolic isoenzymes L and $M_2$ differ in their properties [42, 48]. In fraction A (isoenzyme L) of normal liver, variants $\alpha_1$, $\beta_1$, $\gamma_1$, exhibit sigmoidal kinetics and greatest electrophoretic mobility [48]. They are sensitive to ATP and FDP. ATP, as a negative effector of pyruvate kinase in a feed-back mechanism, inhibits glycolytic energy generation [49] and FDP [50], in positive forward control, synchronizes the pyruvate kinase activity with that of phosphofructokinase. A similar electrophoretic pattern has been also observed in chromatin extracts from cell nuclei of normal rat and mouse livers [34] and of human urothelial cell cultures with limited life span, i.e. those of TGr I [43].

In fraction B (isoenzyme $M_2$) from normal rat liver pyruvate kinase, which is regulated by the same effectors as isoenzyme L [41], $\alpha_1$ and $\beta_1$ variants have similar mobility as variants in fraction A, but show linear kinetics and are insensitive to FDP. The third variant, $\gamma_2$ migrates slower towards the anode and is sensitive to both effectors [48].

Purified cytosolic fractions A and B from Morris hepatoma differ from corresponding fractions from normal liver. Purified cytosolic pyruvate kinase fraction B (isoenzyme $M_2$) from Morris hepatoma follows the tumor pattern. In addition to a severalfold increase in activity [34, 43, 48], qualitative changes have also been observed. Its kinetics is linear with respect to PEP [42, 48], it shows the greatest affinity to this substrate, and is completely insensitive to ATP and FDP. This confirms the suggestion that tumor isoenzyme $M_2$ is different from liver isoenzyme $M_2$. Purified cytosolic pyruvate kinase fraction B from Morris hepatoma 7777 shows a significantly decreased electrophoretic mobility of variants $\alpha_2$, $\beta_2$ $\gamma_3$, like the pyruvate kinase variants from chromatin extracts of Ehrlich ascites tumor [34] or urothelial cell lines in TGr III [43]. All three tumor pyruvate kinase variants from cytosol are practically insensitive to stimulation by FDP or inhibition by ATP [34, 48]. The slowest variant $\gamma_3$ is localised at pH 8.3 in the gel origin. Only this variant is sensitive to inhibition by L-cysteine (heached area) [48]. Unlike the purified fraction B from Morris hepatoma 7777, which is inhibited by high concentrations of L-cysteine to only 50% of the control activity, the purified variant $\gamma_3$ is inhibited by L-cysteine almost to zero value which points to its homo-

**Table. 4. Comparison of normal rat liver and Morris hepatoma 7777 cytosolic pyruvate kinase isoenzymes (purified by affinity chromatography [42]) and variants (separated by polyacrylamide gel electrophoresis, pH 8.3 [48])**

| | Rat liver | | | | | Morris hepatoma 7777 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Kinetics | $K_m/S_{0.5}$ (mM) | Spec.act. (IU/g protein) | Effector (0.1 mM) | % of control | Kinetics | $K_m/S_{0.5}$ (mM) | Spec.act. (IU/g protein) | Effector (0.1 mM) | % of control |
| Isoenzyme L | sigm. | 2.10 | 201000 | | | Isoenzyme L | sigm. | 0.75 | 295000 | | |
| (purif. grade: 250) | | | | | | (purif. grade: 118) | | | | | |
| ($M_m$ 200 kDa) | | | | | | ($M_m$ 208 kDa) | | | | | |
| **variant** | | | | | | **variant** | | | | | |
| $\alpha_1$ | sigm. | 2.00 | 42700 | – | 100 | $\alpha_1$ | lin. | 0.65 | 35300 | – | 100 |
| | | | | +FDP | 140 | | | | | +FDP | 100 |
| | | | | +ATP | 50 | | | | | +ATP | 90 |
| $\beta_1$ | sigm. | 1.70 | 47800 | – | 100 | $\beta_1$ | lin. | 0.85 | 54900 | – | 100 |
| | | | | +FDP | 175 | | | | | +FDP | 125 |
| | | | | +ATP | 65 | | | | | +ATP | 65 |
| $\gamma_1$ | sigm. | 2.10 | 42500 | – | 100 | $\gamma_2$ | lin. | 0.75 | 69000 | – | 100 |
| | | | | +FDP | 140 | | | | | +FDP | 100 |
| | | | | +ATP | 75 | | | | | +ATP | 80 |
| Isoenzyme $M_2$ | lin. | 1.20 | 46000 | | | Isoenzyme $M_2$ | lin. | 0.55 | 762000 | | |
| (purif. grade: 58) | | | | | | (purif. grade: 305) | | | | | |
| ($M_m$ 216 kDa) | | | | | | ($M_m$ 196 kDa) | | | | | |
| **variant** | | | | | | **variant** | | | | | |
| $\alpha_1$ | lin. | 1.20 | 19000 | – | 100 | $\alpha_2$ | lin. | 0.60 | 42700 | – | 100 |
| | | | | +FDP | 100 | | | | | +FDP | 100 |
| | | | | +ATP | 35 | | | | | +ATP | 100 |
| $\beta_1$ | lin. | 1.30 | 10300 | – | 100 | $\beta_2$ | lin. | 0.40 | 65300 | – | 100 |
| | | | | +FDP | 100 | | | | | +FDP | 100 |
| | | | | +ATP | 65 | | | | | +ATP | 100 |
| $\gamma_2$ | lin. | 1.10 | 12700 | – | 100 | $\gamma_3$ | lin. | 0.55 | 69000 | – | 100 |
| | | | | +FDP | 137 | | | | | +FDP | 100 |
| | | | | +ATP | 45 | | | | | +ATP | 100 |

kinetics with respect to PEP: $S_{0.5}$, sigm., sigmoidal; $K_m$, lin, linear; $M_m$, molecular mass

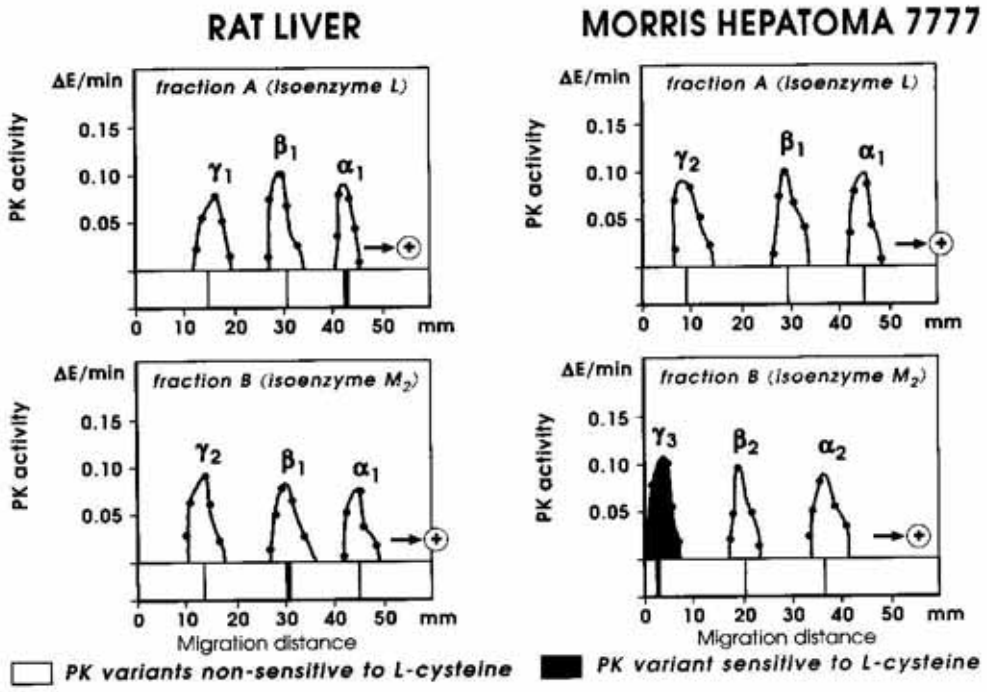


**Figure 5. Pyruvate kinase variants and protein discs separated by polyacrylamide gel electrophoresis from purified cytosolic pyruvate kinase isoenzymes of Morris hepatoma 7777 and normal rat liver [48].**

geneity. This L-cysteine sensitive variant seems to be one of protein products expressed on oncogene activation.

It has been suggested [39] that tumor isoenzyme $M_2$ is rather similar to isoenzyme M, which is more cathodic (pI 7.5) [36]. It was even suggested that it might be a hybrid $M_2M$ isoenzyme [37]. However, later on two forms of isoenzyme $M_2$ have been distinguished. One, predominating in tumors, placenta and kidney, is termed also type $III_b$ or alkaline type. Another form, predominating in the lung, is termed $III_a$ or acidic type [50]. Irving & Williams [51] identified in hepatoma two pyruvate kinase isoenzymes, a new L-type and a new $M_2$-type. Poorly differentiated tumors show little of the L-type and a severalfold increase in the new $M_2$-type.

The lack of sensitivity of tumor pyruvate kinase to ATP is consistent with the previous observation of Gosalvez *et al.* [52]. Since the enzyme remains very active even at low FDP and high ATP concentration, this points to its marked metabolic role in altered tumor metabolism, i.e., in diminution of the Pasteur effect and in appearance of the Crabtree effect.

Variant $\gamma_3$ of isoenzyme $M_2$ from Morris hepatoma may contribute to elevated overall aerobic glycolysis rate in tumors resulting from not only over-expression of tumor pyruvate kinase [42, 43, 48, 53], but also its enhanced affinity to PEP [42, 48], and lack of a fundamental regulatory feed-back mechanism, i.e., absence or diminution of the Pasteur effect [30, 52].

Since pyruvate kinase variants are also present in chromatin, the effect of DNA on their activities was also studied. All three tumor variants showed greater sensitivity to the inhibitory effect of DNA than the variants of normal livers [34]. Pyruvate kinase, similarly as other proteins with higher pI values, may exhibit an affinity to nucleic acids which was signalled by Baranowska & Baranowski [54]. However, it is not excluded that pyruvate kinase may also recognise particular base sequences of nucleic acid.

Different electrophoretic charge of the same isoenzyme variant could also be caused by FDP [50] or ATP [55] binding to the enzyme molecule, or reversible protein glycosylation or phosphorylation [56]. The appearance of sensitivity to L-cysteine of the slowly migrat-

ing tumor variant $\gamma_3$ points also to a change in the primary structure of tumor pyruvate kinase, and thus in the cell genotype, occurring during the multistage process of carcinogenesis.

## PHENOTYPIC AND GENOTYPIC CHANGES IN TUMOR PYRUVATE KINASE ISOENZYME $M_2$

Using highly purified pyruvate kinase isoenzymes L and $M_2$ from rat liver and Morris hepatoma, it was possible to answer the question what is the molecular basis of the changes observed [56].

The lowest content of *N*-acetylneuraminic acid (5 mol/mol protein) in tumor isoenzyme $M_2$, in comparison to liver isoenzyme $M_2$ (14 mol) and both normal liver (24 mol) and tumor isoenzymes L (15 mol) [56], points to a phenotypic modification of the protein molecule which could explain differences in negative charge of the variants. Another phenotypic difference is connected with the phosphate content. It is the highest (12 mol) in pyruvate kinase fraction B from hepatoma, twice as high as in fraction B of normal liver (6 mol) and four times as high as in fraction A, both from normal rat liver and Morris hepatoma (3 mol) [56].

Phosphorylation of pyruvate kinase may have a regulatory effect [50]. Phosphorylation at tyrosine residues was observed in pyruvate kinase of chicken liver cells transformed by Rous sarcoma virus containing the *src* oncogene coding for phosphoprotein $pp60^{v\,src}$ [57, 58]. Cell transformation caused a tenfold increase in total cell phosphotyrosine content, related to phosphorylation of several glycolytic enzymes and elevated rate of aerobic glycolysis [58].

Although tyrosine phosphorylation can be considered a post-translational regulatory change, it requires a genotypic change connected with an increase in tyrosine content. Recently, in variant $\gamma_3$ of hepatoma pyruvate kinase, twice as high tyrosine content has been found as in variant $\gamma_2$ of normal liver isoenzyme $M_2$ of pyruvate kinase. Differences in dicarboxylic amino acid content have been also found. Highly purified variant $\gamma_3$ with the slowest mobility contained by 26% less glutaminic acid than the normal variant $\gamma_2$ (Ignacak, Gumińska & Steczko, unpublished).

It has been revealed [56] that tumor pyruvate kinase fraction B has twice as many thiol groups as the corresponding isoenzyme $M_2$ from normal liver, which may explain greater affinity of the tumor enzyme to exogenous L-cysteine than that of normal liver pyruvate kinase. It should be, however, stressed that the inhibition by L-cysteine of the tumor isoenzyme is also stereospecific [18]. Neither D-cysteine nor other thiol group containing reactive compounds, are able to inhibit the enzyme. Thus in addition to thiol groups the whole structure enabling stereospecific binding of exogenous L-cysteine is necessary [19]. Although in addition to phenotypic modification of protein by glycosylation or phosphorylation of the tumor enzyme, which play an essential role in tumor metabolic strategy, genotypic changes in amino acid content have also been detected (Ignacak, Gumińska & Steczko, unpublished), but their participation in exogenous cysteine binding is doubtful.

Pyruvate kinase fraction B from hepatoma, especially its slowest tumor specific variant $\gamma_3$ sensitive to L-cysteine, should be regarded as one of the oncoproteins which modify the metabolic strategy of tumor cells in such a way as to ensure supply of substrates and energy for uncontrolled cell divisions.

## CLOSING REMARKS

Pyruvate kinase variant $\gamma_3$ from isoenzyme $M_2$ of hepatoma seems to be a tumor-specific oncoprotein. As an enzymatic mutant it loses the sensitivity to ATP [34, 48]. In consequence, glycolysis can proceed in aerobic conditions and the Pasteur effect disappears or is significantly reduced. The investigation of Gosalvez *et al.* [20] showed that in such conditions not ATP but L-cysteine can restore the Pasteur effect. However, the role of this signal reacting with pyruvate kinase tumor variant $\gamma_3$ has not been fully elucidated. It should be stressed that tumor pyruvate kinase variant $\gamma_3$ differing in its properties

from normal liver variant $\gamma_2$, resembles more closely the enzyme variant $\gamma_3$ from other types of neoplastic cells such as Ehrlich ascites tumor [34] or human bladder tumorigenic cell lines at the third stage of transformation [43] than the enzyme variant from comparable material of normal tissues, and therefore can be used as a marker of tumorigenic transformation.

## REFERENCES


1. Warburg, O. (1930) *Metabolism of Tumors*. Constable Co., London.
2. Warburg, O. (1956) On the origin of the cancer cells. *Science* **123**, 309–314.
3. Racker, E., Wu, R. & Alpers, J.B. (1960) Carbohydrate metabolism in ascites tumor and HeLa cells; in *Amino Acids, Proteins and Cancer Biology* (Edsall, J.T., ed.) pp. 175–189, Academic Press, New York, London.
4. Wu, R. & Racker, E. (1959) Regulatory mechanisms in carbohydrate metabolism. IV. Pasteur effect and Crabtree effect in ascites tumor cells. *J. Biol. Chem.* **234**, 1036–1041.
5. Chance, B. & Hess, B. (1959) Metabolic control mechanisms. IV. The effect of glucose upon the steady-state of the respiratory enzymes in the ascites cells. *J. Biol. Chem.* **234**, 2421–2427.
6. Gatt, S. & Racker, E. (1959) Regulatory mechanisms in carbohydrate metabolism. I. Crabtree effect in reconstructed systems. II. Pasteur effect in reconstructed systems. *J. Biol. Chem.* **234**, 1015–1024.
7. Crabtree, H.G. (1929) Observations on carbohydrate metabolism of tumors. *Biochem. J.* **23**, 536–545.
8. Ibsen, K.H., Coe, F.L. & McKee, R.W. (1958) Interrelationships of metabolic pathways in the Ehrlich ascites carcinoma cells. I. Glycolysis and respiration (Crabtree effect). *Biochim. Biophys. Acta* **30**, 384–400.
9. Ibsen, K.H. (1961) The Crabtree effect. A review. *Cancer Res.* **21**, 825–839.
10. Gumińska, M. & Borysiewicz, J. (1962) Studies on the Crabtree effect. I. Gaseous metabolism of tissue cultures. *Acta Biochim. Polon.* **9**, 71–81.
11. Gumińska, M., Skarżyński, B. & Porwit-Bóbr, Z. (1964) The metabolism of mouse embryo cells grown *in vitro* and infected with the oncogenic virus SE polyoma. *Acta Biochim. Polon.* **10**, 163–171.
12. Gumińska, M. (1962) Studies on the Crabtree effect. II. Factors inhibiting and enhancing the Crabtree effect in human neoplastic HeLa cells. *Acta Biochim. Polon.* **9**, 245–252.
13. Weber, G. (1968) Carbohydrate metabolism in cancer cells and the molecular correlation concept. *Naturwissenschaften* **55**, 418–429.
14. Horn, R.S., Haugaard, E.S. & Haugaard, N. (1965) The mechanism of the inhibition of glycolysis by oxygen in rat heart homogenate. *Biochim. Biophys. Acta* **99**, 549–552.
15. Horn, R.S. & Haugaard, N. (1966) Inhibition of carbohydrate metabolism by oxygen and *N*-ethylmaleimide in rat heart homogenates. *J. Biol. Chem.* **241**, 3078–3082.
16. Koj, A., Frendo, J. & Borysiewicz, J. (1964) Cysteine desulfhydrase and rhodanase activity in cells of tissue cultures and in transplantable tumors. *Acta Med. Polon.* **2**, 109–117.
17. Jackson, J.F. & Morse, F.A. (1970) Desulfurase activity and chromosome analysis of cultured rat hepatoma cells. *Rev. Eur. Études Clin. Biol.* **15**, 906–910.
18. Kun, E., Klausner, G. & Fanshier, D.W. (1960) The rate of the cleavage of β-mercaptopyruvate by rapidly dividing cells. *Experientia* **16**, 55–56.
19. Kędryna, T., Gumińska, M. & Marchut, E. (1983) The inhibitory effect of L-cysteine and its derivatives on glycolysis in Ehrlich ascites tumour cells. *Biochim Biophys. Acta* **763**, 64–71.
20. Gosalvez, M., Lopez-Alacron, L, Garcia-Suarez, S., Montalvo, A. & Weinhouse, S. (1975) Stimulation of tumor cell respiration by inhibitors of pyruvate kinase. *Eur. J. Biochem.* **55**, 315–321.

**21.** Gumińska, M. (1971) Regulation of glycolytic pathway of energy formation. *Monografie Biochemiczne*. **24**, 47–69 (in Polish).

**22.** Lo, C., Cristofalo, V.J., Morris, H.P. & Weinhouse, S. (1968) Studies on respiration and glycolysis in transplanted hepatic tumors of the rat. *Cancer Res.* **28**, 1–10.

**23.** Weber, G. (1997) Enzymology of cancer cells. *New Engl. J. Med.* **296**, 486–493; 511–541.

**24.** Gumińska, M. & Porwit-Bóbr, Z. (1972) Synthesis of tumor antigen and changes in activities of glycolytic enzymes induced by polyoma virus. *Acta Virol.* **16**, 183–190.

**25.** Bishop, J.M. (1983) Cellular oncogenes and retroviruses. *Annu. Rev. Biochem.* **52**, 301–354.

**26.** Anderson, M.W., Reynolds, S.H., You, M. & Maronpot, R.M. (1992) Role of proto-oncogene activation in carcinogenesis. *Environ. Health Perspect.* **98**, 13–24.

**27.** Gumińska, M. & Kieler, J. (1967) 2-Phosphoenolpyruvate (PEP) metabolism in non-malignant and malignant cells. *4th FEBS Meeting Abstract*. Norvegien Soc. for Biochem. Physiol. Universitetsforlaged. Oslo, p. 20.

**28.** Gumińska, M. (1969) 2-Phosphoenolpyruvate metabolism in experimental neoplastic cells. *Folia Med. Cracov.* **11**, 363–407 (in Polish).

**29.** Gumińska, M., Briand, P., Daehnfeldt, J.L. & Kieler, J. (1969) Changes in the pyruvate kinase and lactate dehydrogenase activity in rat lung during cultivation and "spontaneous" development of malignancy *in vitro*. *Eur. J. Cancer* **5**, 597–604.

**30.** Gosalvez, M., Garcia, J.P. & Weinhouse, S. (1974) Competition for ADP between pyruvate kinase and mitochondrial oxidative phosphorylation as a control mechanism in glycolysis. *Eur. J. Biochem.* **46**, 133–140.

**31.** Weber, G. (1969) Regulation of pyruvate kinase. *Adv. Enzyme Regul.* **7**, 15–40.

**32.** Gumińska, M., Kędryna, T. & Marchut, E. (1986) The effect of levamisole on energy metabolism in Ehrlich ascites tumor cells. *Biochem. Pharmacol.* **35**, 4369–4374.

**33.** Marchut, E., Gumińska, M., Kędryna, T., Radzikowski, Cz. & Kuśnierczyk, H. (1988) A pyruvate kinase variant in different mouse transplanted tumours. *Experientia* **44**, 25–27.

**34.** Gumińska, M., Stachurska, M.B. & Ignacak, J. (1988) Pyruvate kinase isoenzymes in chromatin extracts of Ehrlich ascites tumor, Morris hepatoma 7777 and normal mouse and rat livers. *Biochim. Biophys. Acta* **966**, 207–213.

**35.** Ibsen, K.H. & Tripped, P. (1972) Interconvertible and non-interconvertible forms of rat pyruvate kinase. *Biochemistry* **11**, 4420–4442.

**36.** Ibsen, K.H., Tripped, P. & Basabe, J. (1975) Properties of rat pyruvate kinase isozymes; in *Isozymes* (Markert, C.L., ed.) vol. 1, 543–559, Academic Press, New York.

**37.** Ibsen, K.H. (1977) Interrelationships and function of the pyruvate kinase isoenzymes and their variant forms. A review. *Cancer Res.* **37**, 341–353.

**38.** Feliu, J.E., Diaz, J.J., Garcia-Canero, R., Gosalvez, M. (1975) Interconvertible forms of class A pyruvate kinase from Ehrlich ascites tumor. *FEBS Lett.* **50**, 334–338.

**39.** Tanaka, T., Harano, Y., Sue, F., Morimura, H. & Mori, R. (1965) Evidence for the presence of two types of pyruvate kinase in rat liver. *Biochem. Biophys. Res. Commun.* **29**, 444–449.

**40.** Tanaka, T., Harano, Y., Sue, F. & Morimura, H. (1967) Crystalization, characterization and metabolic regulation of two types of pyruvate kinase isolated from rat tissues. *J. Biochem.* (Tokyo) **62**, 71–91.

**41.** Imamura, K., Taniuchi, K. & Tanaka, T. (1972) Multimolecular forms of pyruvate kinase. II. Purification of $M_2$-type pyruvate kinase from Yoshida ascites hepatoma 130 cells and comparative studies on the enzymological and immunological properties of the three types of pyruvate kinases L, $M_1$ and $M_2$. *J. Biochem.* (Tokyo) **72**, 1001–1015.

**42.** Ignacak, J. & Gumińska, M. (1993) Comparison of pyruvate kinase variants from rat liver and Morris hepatoma 7777, obtained by an affinity chromatography on Blue Sepharose CL-6B. *Acta Biochim. Polon.* **40**, 261–267.

**43.** Gumińska, M., Stachurska, M., Christensen, B., Tromhold, V., Kieler, J., Radzikowski, Cz. & Duś, D. (1989) Pyruvate kinase inhibited by L-cysteine as a marker of toumorigenic human urothelial cell lines. *Experientia* **45**, 571–574.

**44.** Criss, W.E. (1969) A new pyruvate kinase isozyme in hepatomas. *Biochem. Biophys. Res. Commun.* **35**, 901–905.

**45.** Rickwood, D. & Birnie, G.D. (1976) Preparation, characterization and fractionation of chromatin; in *Subnuclear Components Preparation and Fractionation* (Birnie, G.D., ed.) pp. 129–133, Butterworths, London.

**46.** Bloom, K.S. & Anderson, J.W. (1978) Fractionation and characterization of chromosomal proteins by the hydroxyapatite dissociation method. *J. Biol. Chem.* **253**, 4446–4450.

**47.** Davis, B.J. (1964) Disc electrophoresis. II. Method and application to human serum protein. *Ann. N.Y. Acad. Sci.* **121**, 404–427.

**48.** Gumińska, M. & Ignacak, J. (1996) Electrophoretic pattern of cytosolic pyruvate kinase fractions A and B (type L and $M_2$) from normal rat liver and Morris hepatoma 7777. *Biochim. Biophys. Acta* **1292**, 99–105.

**49.** Atkinson, D.E. (1968) The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. *Biochemistry* **7**, 4030–4034.

**50.** Eigenbrodt, E., Reinacher, M., Scheefers-Borchel, U., Scheefers, H. & Friis, R. (1992) Double role for pyruvate kinase type M in the expansion of phosphometabolite pools found in tumor cells. *Crit. Rev. Oncogenesis* **3**, 91–115.

**51.** Irving, M.G. & Williams, I.F. (1976) Kinetic properties of pyruvate kinase isolated from rat hepatic tumors. *Biochim. Biophys. Res. Commun.* **68**, 284–291.

**52.** Gosalvez, M., Garcia-Suares, S. & Lopez-Alarcon, L. (1978) Metabolic control of glycolysis in normal and tumour permeabilized cells. *Cancer Res.* **38**, 142–148.

**53.** Board, M., Humm, S. & Newsholme, E.A. (1990) Maximum activities of key enzymes of glycolysis, pentose phosphate pathway, and tricarboxylic acid cycle in normal, neoplastic and suppressed cells. *Biochem. J.* **265**, 503–509.

**54.** Baranowska, B. & Baranowski, T. (1976) Pyruvate kinase complexes with RNA in human skeletal muscles. *XIV Polish Biochem. Soc. Meeting,* Lublin Abstracts, pp. 167–168 (in Polish).

**55.** Gumińska, M. & Ważewska-Czyżewska, M. (1979) Congenital non-spherocytic haemolytic anaemia variants with primary and secondary pyruvate kinase deficiency. II. Enzymatic studies. *Brit. J. Haematol.* **41**, 125–132.

**56.** Ignacak, J. & Gumińska, M. (1997) *N*-Acetylneuraminic acid, phosphate and thiol groups of pyruvate kinase isoenzymes from Morris hepatoma 7777 and normal rat liver. *Acta Biochim. Polon.* **44**, 201–208.

**57.** Presek, P., Glossmann, H., Eigenbrodt, E., Schöner, W., Rubsamen, H., Friis, R.R. & Bauer, H. (1980) Similarities between a phosphoprotein pp60-associated protein kinase of Rous sarcoma virus and a cyclic adenosine 3′:5′-monophosphate-independent protein kinase that phosphorylates pyruvate kinase type M. *Cancer Res.* **40**, 1733–1741.

**58.** Presek, P., Reinacher, M. & Eigenbrodt, E. (1988) Pyruvate kinase type M is phosphorylated at tyrosine residues in cell transformed by Rous sarcoma virus. *FEBS Lett.* **242**, 194–198.