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Glycogen phosphorylase isoenzymes from hepatoma 3924A and from a non-tumorigenic liver cell line

Comparison with the liver and brain enzymes

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Glycogen phosphorylase isoenzymes were isolated from normal rat liver, rat brain, the glycogen-poor Morris hepatoma (MH) 3924A, and the glycogen-rich non-tumorigenic liver cell line C_1I. Electrophoretic and immunological characterization of the enzymes showed that tumour and C_1I cells expressed a phosphorylase isoform similar to the brain type; the liver type was not detectable. All enzymes were obtained as dimers; the M_r of the subunits was 96000 (liver), 93000 (brain and MH 3924A) and 92000 (C_1I). Isoelectric focusing revealed a main band of pI 6.34 for liver phosphorylase *a*, pI 5.67 for the enzymes from MH 3924A and brain, and pI 5.68 for C_1I phosphorylase. Partial kinetic characterization of the AMP-independent forms of the isoenzymes yielded K_m values for glucose 1-phosphate of 3.5 ± 0.5 mM (liver), 3.9 mM (brain), 1.9 ± 0.3 mM (MH 3924A) and 2.5 ± 0.5 mM (C_1I); K_m values for glycogen were 0.4 mM (liver) and 0.3 mM (MH 3924A and C_1I), calculated as glucose equivalents. The AMP-independent phosphorylase was inhibited by glucose 6-phosphate (Glc6*P*) with K_i values of 0.32 ± 0.03 mM (C_1I), 0.50 ± 0.04 mM (MH 3924A) and ~ 5 mM (brain). The inhibition could be abolished by 1 mM-AMP, indicating that AMP and Glc6*P* may partially compete for the same site on the protein. Liver phosphorylase *a* was not inhibited by up to 25 mM-Glc6*P*. In contrast with liver and brain isoenzymes, phosphorylase from the cell lines was not affected by NaF and Na_2SO_4. The data show that both the hepatocellular carcinoma and the non-malignant immortalized liver cells express a phosphorylase isoform different from the liver type. Furthermore, there is some evidence that the enzyme from MH 3924A and C_1I cells is distinct from brain phosphorylase *a*, in spite of electrophoretic and immunological resemblance, and that this isoenzyme is subject to altered metabolic regulation.

INTRODUCTION

Tissue-specific, and particularly tumour-specific, gene expression has recently become a matter of intense investigation. Tumours often express isoforms of key enzymes of metabolism which are different from those expressed in the tissue of origin, and which often are not subject to metabolic regulation [1–3]. In the course of our studies on alterations in enzyme expression during transformation of hepatocytes, we observed that, in hepatocytes transformed either spontaneously *in vitro* or during chemically induced hepatocarcinogenesis *in vivo*, glycogen phosphorylase (1,4-α-D-glucan: orthophosphate α-D-glucosyltransferase, EC 2.4.1.1) showed a marked decrease in activity, and exhibited different biochemical and immunological properties from those observed in normal rat liver [4–7]. We supposed that in transformed hepatocytes a different phoshorylase isoenzyme is expressed as compared with normal hepatocytes [7].

Mammalian glycogen phosphorylases were found in at least three isoenzymic forms that could be distinguished by structural and functional properties [8–14]. In the rat, the brain form has been shown to be the predominant isoenzyme in fetal tissues and in many adult organs. Exceptions are adult liver and skeletal muscle, which express primarily the liver and muscle isoenzymes respectively (for references see [15]). Although all forms of phosphorylase catalyse the same reaction, the phosphorolysis of glycogen to yield glucose 1-phosphate (Glc1*P*), each phosphorylase isoenzyme fulfils different physiological requirements. The muscle form is best characterized; it provides energy for muscle contraction. Its physiological role is distinct from that of the liver isoenzyme, which plays a central role in the maintenance of blood glucose homoeostasis, and from the brain form, which is involved in the provision of an emergency glucose supply during periods of anoxia and hypoglycaemia [13–17]. Phosphorylase isoenzymes exhibit differences in regulatory properties that may be related to their distinct physiological roles, especially with regard to their response to the allosteric ligand AMP [11,18,19], which is high for the muscle but low for the liver isoenzyme.

Some years ago, Sato and co-workers [2,20–22] described the occurrence of a phosphorylase isoenzyme in transplantable rat hepatomas, which was different from the liver isoenzyme but exhibited similar electrophoretic mobility to the brain- or fetal-type enzyme, and cross-reaction with antibodies raised against the brain isoenzyme. So far, only the *b*-form of the phosphorylase isoenzyme from Novikoff hepatoma has been isolated and partly characterized biochemically and immunologically [21,22]; extracts from other hepatomes, e.g. Morris hepatoma (MH) 3924A, have been used for immunological studies [22]. The *a*-form, active *in vivo*, of a tumour phosphorylase has not been investigated so far. In this paper, we describe the isolation and partial characterization of glycogen phosphorylase from hepatoma MH 3924A, grown either as a solid transplantation tumour or kept as a cell line in culture, and from the non-tumorigenic epithelial liver cell line C_1I which was derived from adult rat hepatocytes [6]. Electrophoretic and immunological studies indicate that the phosphorylase isoform observed in both cell lines is similar to the brain type. Kinetic data, however, raise some evidence that the AMP-independent glycogen phosphorylase expressed in the cell lines may be distinct from brain phos-

Abbreviations used: Glc1*P*, glucose 1-phosphate; Glc6*P*, glucose 6-phosphate; IEF, isoelectric focusing; MH, Morris hepatoma; PBS, phosphate-buffered saline.

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phorylase *a*. These findings permit some insight into mechanisms of aberrant metabolic regulation in transformed cells.

EXPERIMENTAL

Materials

Ham's F12 medium and 100 × BME (Basal Medium Eagle) amino acids were purchased from Biochrom KG (Berlin, Germany). Fetal-calf serum, α-1,4-1,6-amyloglucosidase (EC 3.2.1.3), Glc1*P*, glucose 6-phosphate (Glc6*P*), AMP and ATP were from Boehringer Mannheim (Mannheim, Germany). Acrylamide and *NN'*-methylenebisacrylamide were from Bio-Rad (München, Germany). *NNN'N'*-Tetramethylethylenediamine (TEMED) was from Serva (Heidelberg, Germany). DEAE-cellulose DE52 was from Whatman Biosystems (Maidstone, Kent, U.K.) 5'-AMP–Sepharose 4B, Protein A–Sepharose 4B, Ultrodex, Ampholine 3.5–10, PhastGel IEF 5–8, homogeneous 7.5% and 12.5% PhastGel media, PhastGel gradient 10–15%, and native and SDS buffer strips for the PhastSystem were obtained from Pharmacia (Freiburg, Germany). Centricon 30 Microconcentrators were from Amicon G.m.b.H. (Witten, Germany). Rabbit muscle phosphorylase, bovine muscle phosphorylase kinase and peroxidase-conjugated anti-rabbit IgG were from Sigma (München, Germany). α-D-[U-^{14}C]glucose 1-phosphate (potassium salt; 150 mCi/mmol) was from Amersham Buchler (Braunschweig, Germany). Falcon 3003 tissue-culture dishes were from Becton/Dickinson (Heidelberg, Germany). All the other substances used were of the purest grade available.

Animals and cell lines

Male Sprague–Dawley rats (200–300 g body wt.) were obtained from the Zentralinstitut für Versuchstierzucht (Hannover, Germany), and ACI rats (~ 200 g body wt.) were from Harlan Industries (Indianapolis, IN, U.S.A.). Both were kept on Altromin standard diet (Altromin, Lage/Lippe, Germany) and water *ad libitum*. Chinchilla rabbits were from Ivanovas (Kisslegg, Germany).

Morris hepatoma (MH) 3924A (taken from the tumour bank of the German Cancer Research Center, Heidelberg, Germany) was obtained as a solid tumour after subcutaneous transplantation in ACI rats. MH 3924A cells were adapted to tissue culture from the ascitic form of the tumour as described in [23]. The non-tumorigenic epithelial liver cell line C_1I was established and grown as described [6,7] in Ham's F12 tissue-culture medium supplemented with amino acids, 10% (v/v) fetal-calf serum and 1 μM-dexamethasone. Culture conditions were 37 °C, 5% CO_2 in air atmosphere and 100% humidity. C_1I cells store glycogen for up to ~ 150 passages in culture, and thereafter reproducibly lose this ability without any other significant morphological alterations. Morphological transformation occurs only at > 500 passages. The results presented in this paper were obtained from cells of the glycogen-rich phase (around 50 passages). The same experiments were performed on glycogen-poor cells (~ 350 passages), but the data obtained from cells of the two phases did not show significant differences in the properties of glycogen phosphorylase.

Purification of glycogen phosphorylase from rat liver

Rat liver phosphorylase was isolated by modifying slightly the procedures of Appleman *et al.* [24], Wolf *et al.* [25] and Kobayashi & Graves [26]. Livers from well-fed rats were homogenized in 4 vol. of buffer A (10 mM-Tris/HCl, pH 7.4, 2 mM-EDTA and 5 mM-mercaptoethanol). Cellular debris, nuclei and mitochondria were removed by centrifugation at 6000 *g* (15 min), and glycogen particles were sedimented at 40000 *g* (2 h). The glycogen pellet containing most of the phosphorylase protein [24,27] was suspended in buffer A and incubated with 25 μg of α-1,4-1,6-amyloglucosidase/ml for 1 h at 30 °C, in order to digest the glycogen and to solubilize phosphorylase. During this step, phoshorylase was converted into the *b*-form. After centrifugation at 10^5 *g* for 1 h, the opaque glycogen pellet was no longer observed and phosphorylase was in the supernatant. This was dialysed against buffer A and loaded on a DEAE-cellulose column (3 cm × 15 cm). The column was eluted with a linear gradient of 0–0.4 M-NaCl in buffer A (50 ml each). Fractions containing phosphorylase activity were pooled, dialysed against buffer A and loaded on a 5'-AMP–Sepharose 4B column (2 cm × 8 cm) for affinity chromatography [28]. Phosphorylase was eluted with 10 mM-AMP in buffer A and dialysed thoroughly against 50 mM-Mes (pH 6.8)/20 mM-NaF.

Isolation of glycogen phosphorylase from rat brain and from solid MH 3924A

Rat brain or tumour tissue was homogenized in buffer A. Since no glycogen particles could be obtained from these tissues, phosphorylase was isolated from the 2×10^5 *g* supernatant (1 h centrifugation time) by DEAE-cellulose and 5'-AMP–Sepharose chromatography as described for the liver enzyme.

Isolation of glycogen phosphorylase from MH 3924A and C_1I cells

MH 3924A cells were harvested by trypsin treatment and pelleted. Then 10 g of packed cells (about 2×10^9 cells) were homogenized in 50 ml of buffer A. Since these cells do not contain glycogen, phosphorylase was found in the soluble fraction. The 10^5 *g* supernatant (1 h) was applied on a DEAE-cellulose column. The elution and further purification of MH 3924A phosphorylase were performed by the same methods as described for liver phosphorylase.

Phosphorylase from C_1I cells was isolated by the same procedure. However, the first centrifugation step at 6000 *g* was preceded by amyloglucosidase digestion. Glycogen particles as described for rat liver could not be obtained from C_1I cells, since the major part of phosphorylase activity was sedimented in the first centrifugation step at 6000 *g*.

Activation of glycogen phosphorylase from rat liver and brain

With the procedure described, liver and brain phosphorylases were isolated in AMP-dependent forms. The enzymes were converted into the AMP-independent form by incubation of 10 ml portions of the dialysed AMP–Sepharose eluates with 3.6 mM-ATP, 8 mM-magnesium acetate and 50 units of phosphorylase kinase for 30 min at 30 °C. Subsequently ATP and magnesium acetate were removed by dialysis against 50 mM-Mes (pH 6.8)/20 mM-NaF.

Trials to inactivate glycogen phosphorylase from C_1I and MH 3924A

Homogenates of C_1I and MH 3924A cells in buffer A were incubated at 30 °C in the presence of 5 mM-glucose with or without 0.5 mM-caffeine. Samples were taken at 10, 20 and 30 min and assayed for phosphorylase activity. In another experiment, liver homogenate in buffer A was preincubated at 30 °C for 1 h for complete inactivation of the enzyme. A 10^5 *g* supernatant was run and incubated with phosphorylase from MH 3924A cells.

Phosphorylase assay

Glycogen phosphorylase activity was determined in the direction of glycogen synthesis by measuring the incorporation of glucose from [^{14}C]glucose 1-phosphate into glycogen by the

procedure of Wang & Esmann [29], with modifications described previously [7]. The standard assay was run at 30 °C for 30 min and contained 50 mM-Mes, pH 6.8, 100 mM-NaF, 4 mM-EDTA, 0.1 mM-dithioerythritol, 100 mM-Glc1*P*, 0.4 μCi of [^{14}C]Glc1*P*/ml, 5 mg of glycogen/ml ($\approx$ 28 mM calculated as glucose equivalents) and, for measuring the *b*-form, 1 mM-AMP. In some assays phosphorylase *a* was assayed at 50 mM-Glc1*P*. If required, Na_2SO_4 and caffeine were added at 0.5 M and 0.5 mM respectively. The specific activity of the enzyme is given as nmol of substrate converted/min per mg of protein or m-units/mg of protein. All experiments described were run at least in triplicate.

Protein determination

Protein was determined by an Amidoschwarz protein assay (crude extracts) [30] or by the Lowry procedure (purified proteins) [31], with BSA as standard.

Preparation of antiserum

Antibodies against rat liver phosphorylase were raised in Chinchilla rabbits by injection of 1 mg of electrophoretically purified phosphorylase protein (see below) emulsified in complete Freund's adjuvant, followed by three booster injections each of 0.5 mg of protein in incomplete Freund's adjuvant. The presence of antibodies was demonstrated by the Ouchterlony double-immunodiffusion assay [32]. Immunoglobulins were isolated from the serum by chromatography on Protein A–Sepharose [33]. Antibodies against rat brain phosphorylase were generously given by Professor K. Sato, Hirosaki University, Japan.

Electrophoretic procedures

(1) Analytical isoelectric focusing (IEF). This was performed with the PhastSystem (Pharmacia), using PhastGel IEF 5–8 with a linear pH gradient from 5 to 8 and appropriate marker proteins with pI between 5.65 and 7.72. Focusing conditions were 510 V-h at 15 °C, according to the instructions in the User Manual [34]. Activity staining was performed after completion of the separation by incubating the gel for 30 min at 30 °C in a solution containing 100 mM-Glc1*P*, 5 mM-AMP, 10 mg of glycogen/ml, 50 mM-Mes, pH 6.8, 50 mM-NaF, 4 mM-EDTA and 0.1 mM-dithioerythritol. Elongated glycogen was stained by immersing the gels in Lugol's solution (1% $KI \cdot I_2$ in water) for 1–2 min, followed by washing with water to remove excess iodine. Gels focused in parallel (two gels can be run simultaneously in the PhastSystem) were stained with Coomassie Brilliant Blue R250 [34]. The gels were evaluated with a high-resolution gel scanner (Elscript 400; Hirschmann, Unterhaching, Germany).

(2) Preparative IEF. This was performed in a flat-bed gel-electrophoresis system (LKB–Pharmacia). The gel bed volume was 100 ml, containing 4% Ultrodex, 5% Ampholine pH 3.5–10, and phosphorylase protein (15 mg) after purification on AMP–Sepharose. The gel slurry was dried to a pre-determined limit according to the instructions of the producer. Focusing started at an initial voltage of 340 V; the final voltage was 1200 V. Separation of the proteins was complete after 20 h at 4 °C. In order to observe the focused protein bands within the gel bed, a filter paper was soaked with the assay mixture described for IEF. The dried filter paper was placed on the surface of the gel to allow adsorption of small amounts of focused proteins. The filter paper was then cut in two halves for demonstration of proteins (0.01% Coomassie Brilliant Blue R250) and enzyme reaction. At the position of the phosphorylase protein, a blue activity band was observed. The corresponding fraction was taken out of the gel bed and phosphorylase was eluted from the gel with buffer A.

(3) Immunoblotting. Proteins were separated in the PhastSystem using native PhastGel gradient gels with 10–15% acrylamide [34], and blotted on to nitrocellulose sheets by using the Phast-Transfer Semidry Transfer Kit. The transfer buffer was 25 mM-Tris, 192 mM-glycine, 20% (v/v) methanol, pH 8.3. Electroblotting conditions were 20 V, 25 mA, 1 W, 5 V-h at 15 °C. The proteins were detected on the membrane with 0.2% Ponceau S in 3% (w/v) trichloroacetic acid for evaluation of the completeness and quality of the blot. Thereafter, the membrane was destained in phosphate-buffered saline (PBS; 2.7 mM-KCl/1.5 mM-KH_2PO_4/6.5 mM-Na_2HPO_4/137 mM-NaCl), blocked overnight at 4 °C in 2% BSA and 0.05% Tween 20 in PBS, and washed twice in PBS. After incubation with the anti-phosphorylase IgG (diluted 1:10000 with PBS/Tween 20) for 2 h at room temperature, the membrane was washed three times with PBS and incubated with the second antibody (peroxidase-conjugated goat anti-rabbit IgG, diluted 1:1000 with PBS/Tween 20) for 2 h at room temperature. The bands were made visible with 0.05% diaminobenzidine/0.01% H_2O_2 in 0.2 M-Tris/HCl, pH 7.6.

(4) Native PAGE and activity staining. Proteins were separated on 7.5% or 12.5% homogeneous or 10–15% gradient native PhastGels [34]. After electrophoresis, the gels were incubated for activity staining as described for analytical IEF gels. The M_r of the native proteins was determined from the gradient gels by using the log-M_r-versus-D (migration distance) relationship with appropriate marker proteins [34].

(5) SDS/PAGE. Proteins were denatured at 100 °C for 3 min in the presence of 2% SDS and 5 mM-mercaptoethanol and separated on 12.5% homogeneous PhastGels [34]. M_r values of phosphorylase subunits were determined from R_F values and appropriate marker proteins.

RESULTS

Isolation of phosphorylase isoenzymes

Liver phosphorylase *b* was purified ~ 196-fold to a specific activity of 9.8 units/mg of protein (Table 1) assayed in the presence of 100 mM-Glc-1*P* and 1 mM-AMP. In accordance with findings by Tan & Nuttall [19], we measured considerable activity of phosphorylase *b* in the absence of Na_2SO_4. This might be due to the high NaF concentration included in the standard assay, which stimulates phosphorylase *b*. Conversion into phosphorylase *a* by phosphorylase kinase resulted in a 2.5-fold increase in activity measured in the absence of AMP. Addition of AMP did not significantly increase the activity of the activated enzyme. The specific activity of the isolated liver enzyme was in the same

Table 1. Purification of glycogen phosphorylase isoenzymes

The enzyme was isolated from rat liver, rat brain, C_1I cells (a non-tumorigenic liver cell line) and MH 3924A, grown either in tissue culture or as a transplantable solid tumour. The liver and brain enzymes were obtained in the *b*-form. Activation to the *a*-form was achieved by incubation with muscle phosphorylase kinase. Phosphorylase from C_1I and MH 3924A cells could not be activated by muscle phosphorylase kinase. One unit is given as 1 μmol/min.

Phosphorylase isoenzyme	Specific activity (m-units/mg of protein)		Purification (fold)
	Homogenate	Isolated enzyme	
Liver (*b*-form)	49.8	9800	196
(*a*-form)	–	24500	
Brain (*b*-form)	45.3	1450	32
(*a*-form)	–	7105	
C_1I cells	17.3	1630	94
MH 3924A cells	19.5	1900	97
solid	6.2	940	151

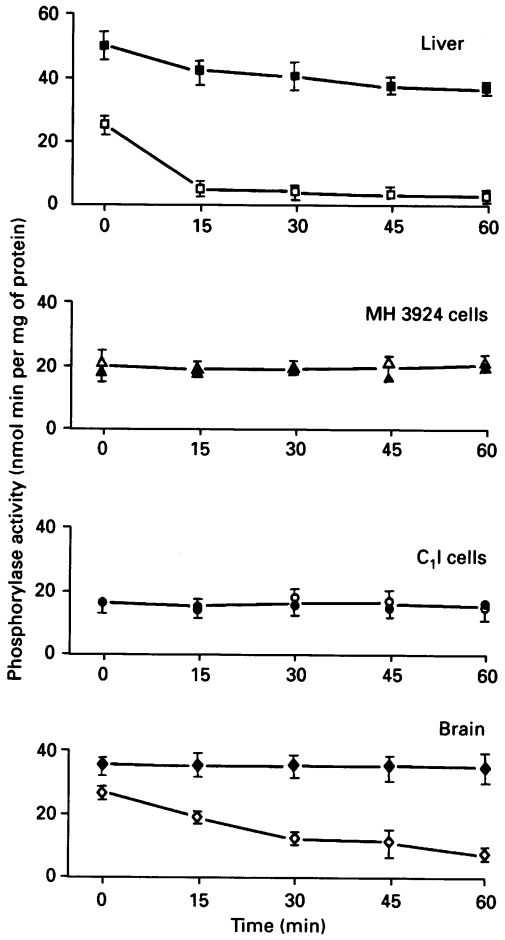

Fig. 1. Inactivation of phosphorylase in tissue homogenates

Homogenates of normal rat liver (□, ■), MH 3924A cells (△, ▲), C_1I cells (○, ●) and rat brain (◇, ◆) in 10 mM-Tris/HCl (pH 7.4)/2 mM-EDTA/5 mM-mercaptoethanol were incubated at 30 °C in order to inactivate glycogen phosphorylase by endogenous phosphorylase phosphatase. Samples were assayed at the time points indicated for AMP-independent phosphorylase (□, △, ○, ◇) and for total phosphorylase in the presence of 1 mM-AMP (■, ▲, ●, ◆). Data are means ± S.D. of three experiments.

range or somewhat lower compared with that described by others for the enzyme from rat [19,35], or rabbit [16,27] liver. Higher activities were reported for bovine and pig liver phosphorylase [24,27]. Phosphorylase from brain was also obtained in the AMP-dependent form. It was enriched only 32-fold, to a specific activity of 1.45 units/mg of protein. The brain enzyme could be activated 4.9-fold by muscle phosphorylase kinase (Table 1). Phosphorylase from C_1I cells was purified 94-fold to a specific activity of 1.63 units/mg of protein, and phosphorylase from MH 3924A cells 97-fold to 1.9 units/mg of protein. The enzymes from C_1I and MH 3924A cells and from the solid tumour were obtained in an AMP-independent form by the methods used. The enzyme from liver was almost homogeneous; the enzymes from C_1I and MH 3924A cells were only slightly contaminated by other proteins. The brain enzyme showed a more pronounced contamination by other proteins, which on the one hand may be due to the omission of a 'glycogen particle step', and on the other hand to the higher total protein concentration measured in brain supernatants as compared with those from the cell lines.

Characterization of phosphorylase isoenzymes

Surprisingly, the enzymes from C_1I and MH 3924A cells were not inactivated by endogenous phosphatase during a 1 h incubation of the cell homogenates at 30 °C in buffer A (Fig. 1), whereas the enzymes from liver and brain (Fig. 1) were easily inactivated. Furthermore, these enzymes were not significantly inactivated by incubation of the cell homogenates in the presence of glucose or glucose and caffeine. After incubation for 30 min, 90% of AMP-independent enzyme activity was retained in MH 3924A cells and 85% in C_1I cells. When the enzyme from MH 3924A cells was incubated for 40 min with preincubated liver supernatant, about 70% of AMP-independent phosphorylase activity was recovered. Hence, if there is an inactivation of the tumour enzyme by liver phosphatase, it is supposed to be very slow. The enzymes isolated from C_1I and MH 3924A cells could not be activated by muscle phosphorylase kinase.

Table 2 summarizes the effects of salts and caffeine on phosphorylase from brain (either inactivated by endogenous phosphatase or activated by muscle phosphorylase kinase) and from C_1I cells. Phosphorylase from C_1I cells was not significantly activated or inhibited by 0.5 M-Na_2SO_4, 0.1 M-NaF and 0.5 mM-caffeine. On the other hand, the AMP-dependent form of brain phosphorylase was clearly stimulated by NaF and Na_2SO_4 and inhibited by caffeine in the absence of AMP. Sulphate was a stronger activator than fluoride; the effects of the two salts were additive. In the presence of 1 mM-AMP, addition of the sulphate and caffeine led to a slight inhibition. The activated brain phosphorylase was only assayed in the presence of NaF, in order to avoid inactivation during the assay by endogenous phosphatase. It was markedly inhibited by Na_2SO_4; addition of caffeine resulted in a slight inhibition. These data clearly reveal differences between the brain enzyme and that from epithelial liver cells.

Electrophoretic characterization. In native homogeneous and gradient gels, phosphorylase from liver could easily be separated from those isolated from brain, C_1I and MH 3924A cells. Although the liver enzyme had migration properties similar to

Table 2. Effect of fluoride, sulphate and caffeine on phosphorylase activity

Assays were performed in dialysed 10^5 *g* supernatants of rat brain (inactivated by endogenous phosphorylase phosphatase, or activated by muscle phosphorylase kinase; means ± S.D. of two brains) and C_1I cells (untreated). Activity is expressed as m-units/mg of protein. Abbreviation: n.d., not determined.

Additions	AMP (1 mM)	Rat brain		C_1I
		Inactivated	Activated	
None	−	78.2 ± 13.6	n.d.	24.2
	+	206.2 ± 26.4		21.5
0.1 M-NaF	−	100.4 ± 8.9	630.1 ± 10.1	25.8
	+	211.9 ± 14.5	837.8 ± 15.1	19.5
0.5 M-Na_2SO_4	−	120.9 ± 19.8	n.d.	21.0
	+	166.3 ± 23.8		18.9
0.1 M-NaF	−	141.0 ± 10.7	410.1 ± 25.8	26.3
+0.5 M-Na_2SO_4	+	145.1 ± 18.5	497.6 ± 12.3	24.9
0.1 M-NaF	−	67.7 ± 12.5	428.9 ± 16.1	20.5
+0.5 mM-caffeine	+	188.8 ± 17.7	432.6 ± 69.3	21.5
0.5 M-Na_2SO_4	−	94.7 ± 18.9	n.d.	n.d.
+0.5 mM-caffeine	+	205.9 ± 35.4		
0.1 M-NaF	−	122.7 ± 10.8	355.9 ± 64.7	n.d.
+0.5 M-Na_2SO_4	+	186.5 ± 30.0	419.1 ± 46.0	
+0.5 mM-caffeine				

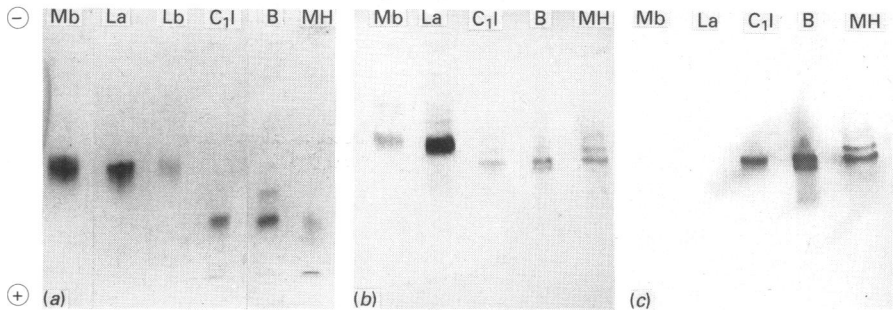

Fig. 2. Electrophoretic resolution, activity staining and immunoblotting of phosphorylase isoenzymes

Glycogen phosphorylase isoenzymes were separated in native 7.5%-acrylamide homogeneous (*a*) and 10–15% gradient gels (*b*, *c*) with subsequent activity staining (*a*) and immunoblotting (*b*, *c*). Antibodies were raised against rat liver phosphorylase (*b*) or rat brain phosphorylase (*c*). A 5 μg sample of protein from the purest fractions available was applied per lane. Abbreviations: Mb, rabbit muscle phosphorylase *b*; La, rat liver phosphorylase *a*; C_1I, phosphorylase from a rat liver cell line; B, rat brain phosphorylase; MH, phosphorylase from MH 3924A.

Table 3. Electrophoretic characterization of phosphorylase isoenzymes

M_r values of phosphorylase subunits were determined in homogeneous 12.5%-acrylamide/SDS PhastGels, and M_r values of the native enzymes in 10–15% gradient PhastGels, by using log M_r/distance plots [34]. pI values were determined from pH-gradient gels (pH 5–8). Data shown represent means ± S.D. from at least three gels.

Phosphorylase isoenzyme	M_r		pI	
	SDS/PAGE	Native PAGE	Main band	Minor band
Liver	96000 ± 1000	230000 ± 6000	6.34 ± 0.02	6.42 ± 0.03
Brain	93000 ± 1000	177000 ± 1500	5.67 ± 0.03	5.87 ± 0.06
MH 3924A	93000 ± 1000	180000 ± 5000	5.67 ± 0.02	5.65 ± 0
C_1I	92000 ± 800	177000 ± 2000	5.68 ± 0.08	–

Table 4. Enzyme kinetic parameters of glycogen phosphorylase isoenzymes

Assays were done with AMP–Sepharose eluates. Abbreviation: n.d., not determined.

	AMP-independent phosphorylase			AMP-dependent phosphorylase			
Tissue	K_m for Glc1*P* (mM)	K_m for glycogen (mM)	K_i for Glc6*P* (mM)	K_m for Glc1*P* (mM)	*h*	K_a for AMP (mM)	K_i for Glc6*P* (mM)
Rat liver	3.5 ± 0.5	~ 0.4	> 25	> 30	1.3	0.52	3.7 ± 0.1
Rat brain	~ 3.9	n.d.	~ 5	~ 3.9	–	0.0053	n.d.
MH 3924A	1.9 ± 0.3	~ 0.3	0.50 ± 0.04	No AMP-dependent form obtained			
C_1I	2.5 ± 0.5	~ 0.3	0.32 ± 0.03	No AMP-dependent form obtained			

that from muscle, the enzymes from C_1I and MH 3924A cells migrated to the same distance as the brain isoenzyme (Fig. 2*a*). In the homogeneous 7.5% gels the enzymes exhibited microheterogeneity. In 10–15%-acrylamide gradient gels the phosphorylase isoenzymes migrated as dimers of M_r 230000 (liver), 180000 (MH 3924A cells) and 177000 (brain, C_1I cells) (Table 3). The M_r of the subunits as determined from SDS gels was 96000 (liver), 93000 (brain, MH 3924A cells) and 92000 (C_1I cells) (Table 3).

Analytical IEF of phosphorylases with subsequent activity or protein staining yielded different pI values for the liver isoenzyme on the one hand and for those from brain and the cell lines on the other (Table 3). Phosphorylase from liver exhibited a major band with pI 6.34 and a minor band with pI 6.42. MH 3924A phosphorylase showed a main band with pI 5.67 and a very weak band with pI 5.65. The enzyme from C_1I cells focused on a single band at pI 5.68. For brain phosphorylase a main band with pI 5.67, and a minor band with pI 5.87 was obtained. The reaction product of the phosphorylases on the gels stained with Lugol's solution was dark blue, which indicates that the branching enzyme has been removed during the isolation procedure.

In the immunoblot, phosphorylase from liver showed a prominent reaction with the antibody raised against liver phosphorylase, whereas the enzymes from C_1I and MH 3924A cells reacted only very weakly with this antibody (Fig. 2*b*). An antibody raised against brain phosphorylase reacted strongly with the enzymes isolated from brain, MH 3924A and C_1I cells, but not with the enzyme from liver (Fig. 2*c*).

The data obtained from electrophoretic studies indicate that the enzymes isolated from brain, MH 3924A and C_1I cells are very similar, but different from the liver isoenzyme.

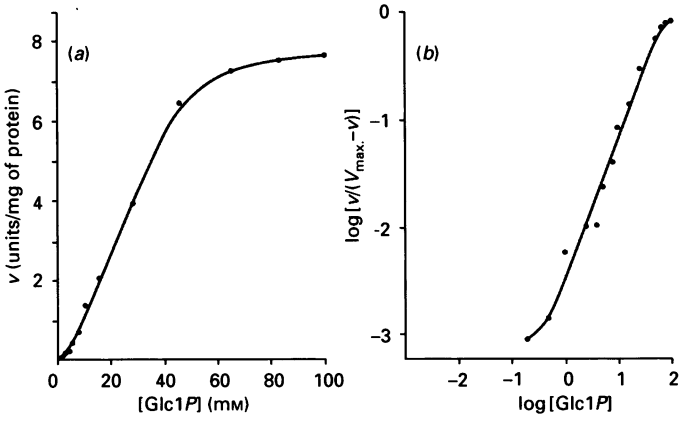

Fig. 3. Effect of Glc1*P* on the activity of glycogen phosphorylase *b* from rat liver

The *v* versus *s* plot (*a*) shows a sigmoid curve; the Hill plot (*b*) yields a positive co-operativity, $h = 1.3$.

Enzyme kinetics. Enzyme-kinetic studies were performed on the AMP-Sepharose eluates of the phosphorylase isoenzymes dialysed exhaustively against 50 mM-Mes (pH 6.8)/20 mM-NaF. Kinetics of phosphorylase *a* from rat liver was measured after conversion of phosphorylase *b* into the *a* form by phosphorylase kinase.

The kinetics of rat liver phosphorylase *a* with increasing concentrations of Glc1*P* as substrate in the absence of AMP was of the Michaelis–Menten type. The K_m value of phosphorylase *a* for Glc1*P* was 3.5 ± 0.5 mM (Table 4) in the presence of 5 mg of glycogen/ml. For glycogen, a K_m of about 0.4 mM, calculated as glucose equivalents, was obtained (Table 4) in the presence of 50 mM-Glc1*P*. It has been shown [18,19] that the K_m of phosphorylase for Glc1*P* is dependent on the concentration of glycogen, and vice versa. Maddaiah & Madsen [18] described a K_m for Glc1*P* of 0.98–0.32 mM at glycogen concentrations between 1.11 and 53.4 mM and a K_m for glycogen of 6.7 mM at 0.75 mM-Glc1*P*. These values were obtained with assays not containing fluoride. Tan & Nuttall [19] described a K_m for rat liver phosphorylase of 0.7 mM for Glc1*P* in the presence of 20 mM-glycogen, and a K_m of 1.8 mM for glycogen in the presence of 15 mM-Glc1*P* and 133 mM-fluoride. These data agree with observations made by us on a less purified fraction (10^5 *g* supernatant), where we found a K_m for glycogen of 1.4 mM in the presence of 15 mM-Glc1*P* (results not shown).

Incubation of liver phosphorylase *b* with increasing Glc1*P* concentrations in the presence of 1 mM-AMP yielded a sigmoid curve (Fig. 3*a*), which could not be linearized in the Lineweaver–Burk plot. From the Hill plot we obtained a positive co-operativity, with a Hill coefficient $h = 1.3$. K_m for Glc1*P* was > 30 mM (Table 4). In order to be sure that the co-operativity of phosphorylase *b* was not due to the experimental conditions, e.g. the sequence of enzyme and substrate addition, the *h* was determined under various conditions of enzyme incubation. These variations did not result in alterations in *h*. The data agree with findings by Tan & Nuttall [19] and Stalmans & Gevers [36], who also described a positive co-operativity of rat [19] and rabbit [36] liver phosphorylase *b* in the presence of AMP when measured in the direction of either glycogen synthesis [19] or degradation [36]. According to Tan & Nuttall [19], AMP decreased the apparent K_m of phosphorylase *b* for Glc1*P* from ~ 270 mM to 80 mM.

Activation of phosphorylase *b* by increasing AMP concentrations resulted in a hyperbolic curve. By linearization of the Lineweaver–Burk plot a K_a of 0.52 mM-AMP was obtained

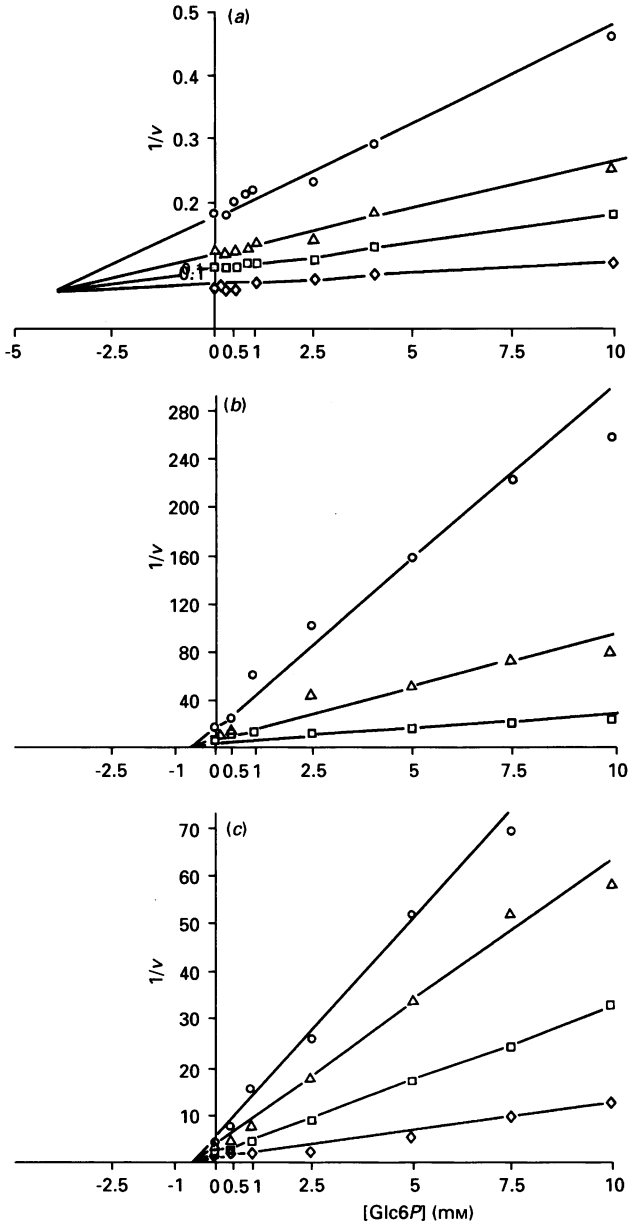

Fig. 4. Inhibition of phosphorylase by Glc6*P*

(*a*) Liver phosphorylase *b*. Glc1*P* concentrations were: ○, 25 mM; △, 37.5 mM; □, 50 mM; ◇, 75 mM. AMP was 2 mM. (*b*) AMP-independent phosphorylase from MH 3924A cells. Glc1*P* concentrations were: ○, 5 mM; △, 25 mM; □, 50 mM. AMP was absent. (*c*) AMP-independent phosphorylase from C_1I cells. Glc1*P* concentrations were: ○, 5 mM; △, 10 mM; □, 25 mM; ◇, 50 mM. AMP was absent. *v* is expressed in units/mg of protein.

(Table 4); in these assays [Glc1*P*] was 100 mM. The value described in the literature is about 0.5 mM [19,35,36], and is not significantly dependent on the Glc1*P* concentration present [19].

AMP-independent glycogen phosphorylase from both solid MH 3924A tumour and MH 3924A cells had a K_m of 1.9 ± 0.3 mM for Glc1*P*, and a K_m for glycogen of about 0.3 mM (Table 4). The K_m for Glc1*P* of AMP-independent phosphorylase from C_1I cells was 2.5 ± 0.5 mM and about 0.3 mM for glycogen (Table 4). Glycogen and Glc1*P* concentrations were 5 mg/ml and 50 mM respectively.

The phosphorylase isoenzymes from rat liver and MH 3924A and C_1I cells exhibited different behaviour towards Glc6*P*.

Phosphorylase *b* from rat liver was inhibited by Glc6*P* with K_i of 3.9 ± 0.1 mM in the presence of 100 mM-Glc1*P* and 1 mM-AMP (Fig. 4*a*). Phosphorylase *a* was not inhibited by Glc6*P* up to 25 mM when assayed in the presence of 50 mM-Glc1*P*. Similar observations were reported by Stalmans & Gevers [36]. Maddaiah & Madsen [18] obtained a K_i of 2.4 mM-Glc6*P* for rat liver phosphorylase *a*; however, [Glc1*P*] was only 2 mM in their assay. On the other hand, when assayed under the same conditions the AMP-independent enzymes from C_1I and MH 3924A cells were inhibited by Glc6*P* with a K_i of 0.32 and 0.50 mM respectively, as obtained from Dixon plots (Figs. 4*b* and 4*c*). The Dixon plots [37] suggest that the inhibition of rat liver phosphorylase *b* by Glc6*P* with respect to Glc1*P* may be of a competitive type, whereas the inhibition of the enzymes from C_1I and MH 3924A cells seems rather to be a non-competitive type. Re-plotting of the data of Figs. 4(*b*) and 4(*c*) according to Cornish-Bowden (s/v versus i [37]) or in $1/v$ versus $1/s$ diagrams yielded similar results. However, none of the phosphorylases from the cell lines were inhibited by Glc6*P* when 1 mM-AMP was added to the assay. This indicates a competition between AMP and Glc6*P* similar to that described for phosphorylase *b* by Wang *et al.* [38], who described very complex kinetics between glucose phosphates and nucleotides, which were interpreted as partially competitive because of the structural dissimilarity of AMP and Glc6*P*.

An AMP-dependent form of phosphorylase from MH 3924A and C_1I cells could not be isolated so far by the methods used; therefore the kinetic constants of the phosphorylase *b* forms of the cell lines could not be determined.

Brain phosphorylase was obtained in two interconvertible forms, one being AMP-independent (probably an *a* form) and the other AMP-dependent (*b* form). Both forms showed a hyperbolic saturation curve with increasing Glc1*P* concentrations. After linearization, K_m values of about 3.9 mM were calculated for both forms; they are in the same range as that obtained for liver phosphorylase *a*. Activation of brain phosphorylase *b* by increasing AMP concentrations yielded a hyperbolic curve with a K_a of 0.0053 mM. Hence the affinity of the brain enzyme for AMP was about 100-fold higher compared with the liver enzyme. A similarly high affinity for AMP of rabbit brain phosphorylase *b* was described previously [11,15,39], and, in contrast with the liver and muscle enzymes, the brain enzyme was activated by AMP in a non-cooperative way [11,15].

Brain phosphorylase *a* was inhibited by Glc6*P* with a K_i of about 5 mM. This agrees with findings summarized by Newgard *et al.* [15], who reported weak Glc6*P* inhibition of brain phosphorylase. Brain phosphorylase *b* did not show simple kinetics with Glc6*P*. This enzyme form was activated by low Glc6*P* concentrations (up to 0.5 mM) and was inhibited at [Glc6P] > 2 mM. This effect was consistently observed at different Glc1*P* concentrations (2, 4 and 8 mM). The curves resulting from these assays could not be linearized in double-reciprocal or Dixon plots.

DISCUSSION

Our investigations of the biochemical mechanism underlying the unusually high glycogen storage in pre-neoplastic hepatocytes, which represent pre-stages of hepatocellular carcinomas [4,5,7], suggested that altered expression of glycogen phosphorylase may be a central event leading to aberrant metabolic regulation [5,7,40]. The aim of the present study was first to identify the types of phosphorylase isoenzymes expressed in solid and cultured hepatoma cells and in the non-tumorigenic liver cell line C_1I, which exhibits glycogen storage similar to that in pre-neoplastic hepatocytes [6,7,23,40], and second, to compare the tumour isoenzyme with the liver- and brain-type enzymes.

Comparative electrophoretic and immunological studies of the enzymes isolated from MH 3924A and C_1I cells with those isolated from liver and brain showed that the enzyme expressed in the two cell lines exhibited characteristics similar to those of the brain isoenzyme, which, on the other hand, has been described to be closely related to the enzyme expressed in fetal liver or in 14-day whole embryo [15,20–22]. The enzymes from the cell lines and from brain could easily be separated electrophoretically from the liver enzyme in native homogeneous and gradient gels, owing to differences in mobility (Fig. 2) and M_r (Table 3) respectively. The differences in M_r were low, particularly those determined for the isoenzyme subunits, which may be more precise than those obtained for the native enzymes. However, these differences were consistently obtained with several enzyme preparations and varying protein loads, and hence point to true small differences in the M_r values of the liver and tumour isoenzymes. Reaction with the respective specific antibodies confirmed the expression of a phosphorylase isoenzyme similar to the brain type in the two cell lines.

Surprisingly, the tumour isoenzymes could not be inactivated in cell and tissue homogenates by endogenous phosphorylase phosphatase under conditions where the liver and brain enzymes were readily dephosphorylated to the *b* form. Therefore it cannot be decided at present if the cell lines express a phosphorylase isoform that is interconvertible in two forms. However, we showed previously [7] that phosphorylase of C_1I cells can be transiently inactivated by addition of 5 mM- or 50 mM-glucose to intact cells. Therefore it may be assumed that in the homogenates of the two cell lines and of the solid tumour phosphorylase phosphatase exists in an inactive or inhibited form, and that this might be a consequence of low glucose levels in these cells. The observation that phosphorylase is not inactivated when cell homogenates are incubated in the presence of glucose is not understood. It might be due to the destruction of a highly organized multi-enzyme system during homogenization and dilution. Since for our studies the properties of the AMP-independent forms of the enzyme, usually supposed to be the form active *in vivo*, were of greater interest than those of the inactive AMP-dependent form, we did not make further efforts to obtain a *b*-form of the phosphorylase from the cell lines. Phosphorylase *b* from rat liver, however, was included in the study to prove the comparability of our data with those published in the literature.

Differences were found in the activation/inactivation behaviour of the liver and brain isoenzymes. The isolated liver enzyme was activated 2.5-fold by muscle phosphorylase kinase; the brain enzyme was activated 4.9-fold (Table 1). During progressive conversion of phosphorylase *a* into *b*, a decrease of about 20–25% of total activity (+AMP) was observed in liver homogenate (Fig. 1), which agrees with the increase in activity of the purified enzyme after phosphorylase kinase treatment (Table 1). However, total phosphorylase activity did not decrease in brain homogenates during conversion of phosphorylase *a* into *b* (Fig. 1). Treatment of brain supernatants with muscle phosphorylase kinase resulted in a 2.9-fold activation (Table 2). Hence the measurable activation of brain phosphorylase by phosphorylase kinase seems to be dependent on the degree of purification, a finding which was not further investigated.

The relatively high K_m of liver phosphorylase *a* as compared with that (0.7 mM-Glc1*P*) published by Maddaiah & Madsen [18] may be due to the high fluoride concentration in our assay. As shown by Lederer & Stalmans [41] for human liver phosphorylase, the affinity of phosphorylase *a* for Glc1*P* is decreased about 4.5-fold by 0.15 M-NaF. Under our conditions, the liver, brain and tumour isoenzymes exhibited similar K_m values for Glc1*P* and glycogen (Table 4), and the kinetic constants de-

termined for the enzymes isolated from solid MH 3924A tumour and cultured cells were nearly identical (results not shown).

One important difference between phosphorylases from liver, brain and the cell lines was the inhibitory effect of Glc6*P* on the last. Although the liver enzyme was not inhibited by Glc6*P* concentrations up to 25 mM at 50 mM-Glc1*P* (a slight inhibition was observed at $\geqslant$ 50 mM-Glc6*P*), phosphorylase from C_1I and MH 3924A cells was inhibited by Glc6*P* under the same experimental conditions, with K_i values of 0.32 and 0.50 mM respectively, the brain enzyme was less sensitive (Table 4). It has been convincingly shown for the muscle enzyme [15,38] that Glc6*P* and AMP may bind to the same allosteric site. If this is also the case for the other isoenzymes, it might explain why Glc6*P* inhibition of the isoenzyme from C_1I and MH 3924A cells can be abolished by 1 mM-AMP. At low Glc1*P* concentration (2 mM) Maddaiah & Madsen [18] observed an inhibitory effect of Glc6*P* on liver phosphorylase *a*, which could also be prevented by AMP. This creates a very complex situation for the regulation of phosphorylase by several different ligands [38].

The preliminary kinetic data on brain phosphorylase (Table 4) suggest that the isoenzymes isolated from brain and from the cell lines were not entirely identical. This conclusion is supported by the different effects of lyotropic salts and caffeine (Table 2) on the enzymes from different sources. Although fluoride, sulphate and caffeine exerted similar effects on brain phosphorylase to those described for the liver enzyme [19,35], none of these substances was found to stimulate or inhibit the enzyme from the two cell lines. On the other hand, it is evident from electrophoretic and immunological studies that the enzymes from brain and the cell lines are much more closely related than the enzymes from liver and the cell lines.

In glycogen-storing C_1I cells the intracellular Glc6*P* concentration was found to be very high, namely 0.720 ± 0.095 μmol/g wet wt. [7]; in MH 3924A cells it was about 0.05 μmol/g wet wt. [40]. Regarding the K_i values of phosphorylase from C_1I and MH 3924A cells, it is conceivable that glycogen storage in C_1I cells is, besides stimulation of glycogen synthesis, a consequence of phosphorylase inhibition by intracellular Glc6*P*.

There is no agreement in the literature about the question whether any brain-type phosphorylase is expressed in adult liver. In our preparations of liver phosphorylase, the brain form was not detectable after electrophoretic separation and immunoblotting. This might be due to the procedure using glycogen particles as a source for the enzyme. Other authors used organ extracts that were obtained by low-speed centrifugation (10^4 up to 2.7×10^4 *g*). After electrophoretic separation or immunoblotting most of them could detect exclusively the liver isoenzyme [42–45] in livers from rat, pig and man. Only Kato *et al.* [46] found considerable concentrations of brain phosphorylase in human liver (< 10% of the activity found in brain), using an enzyme immunoassay, and Kässner *et al.* [47] concluded from precipitation studies with an antibody against liver phosphorylase that 2% of total glycogen phosphorylase activity in rat liver which could not be precipitated by the antibody should represent brain-type phosphorylase and should be confined to non-parenchymal liver cells.

It has been shown previously by immunohistochemical methods [5] that chemically induced primary hepatocarcinomas *in vivo* lose the liver-type enzyme. Our present results show that both malignant hepatoma cells and non-malignant immortalized liver cells do not express the liver isoenzyme of glycogen phosphorylase, but an enzyme which is related to the brain type. Whether this switch in enzyme expression is a consequence of prolonged culture or is related to transformation requires further investigation. The question whether the loss of liver-specific enzyme expression and occurrence of other non-hepatic isoenzymes is a concomitant or a consequence of transformation cannot yet be answered.

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