QUANTITATIVE EVALUATION OF ELECTROPHORETIC ALLO- AND ISOZYME PATTERNS

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1. Introduction

The combination of zonal electrophoresis and activity staining is a powerful tool in mammalian enzyme genetics [1]. In the past, qualitative evaluations of allozyme and isozyme patterns were adequate for the detection of common and rare alleles, chromosomal assignments and studies on population genetics. Problems in developmental genetics now demand more accurate quantitative determinations of the proportions of the different enzyme forms [2,3]. We report a new fluorometric method for quantitation of enzyme activity in different bands following electrophoretic separation, and its application to two relevant examples:

(i) Allozyme patterns of X chromosomal phosphoglycerate kinase 1 (PGK-1, EC 2.7.2.3) caused by the pattern of mosaicism following X-chromosome inactivation in different tissues of the mammalian female embryo [4,5].

(ii) Isozyme patterns of phosphoglycerate mutase (PGAM, 2.7.5.3) extracted from hearts of newborn mice are demonstrated in relation to trisomy of chromosome 19 of the house mouse.

2. Methods

2.1. *Recording of fluorescence*

An earlier arrangement for the recording of NADH–NADPH-specific surface fluorescence of perfused rat liver has been modified [7]. A block diagram is shown in fig.1. Light of 365 nm is isolated by an interference filter (H) from the beam of Osram HG 15/1 Mercury arc lamp (G) and directed against the electrophoretic strip (D). The fluorescence excited is projected by an optical lens to a limiting slit (1 × 9 mm) and directed to a RCA 1 P 21 photomultiplier (F), guarded against excitation energy by a KV 418 'cut off' filter (E) (Schott and Gen. Mainz). An Eppendorf Photometer 1200 M (Eppendorf Gerätebau Netheler and Hinz GmbH, Hamburg) operates both lamp and photomultiplier, converts the fluorescence signal and feeds the pen of the chart recorder.

A chamber (A) of black polyvinylchloride (PVC), covered by a glass plate (B) sealed by a film of water, is horizontally moved under the optical beam as indicated. The chamber contains the electrophoretic strip (D) upside down in bubble-free contact with an acetylcellulose membrane (Sartorius Membranfilter

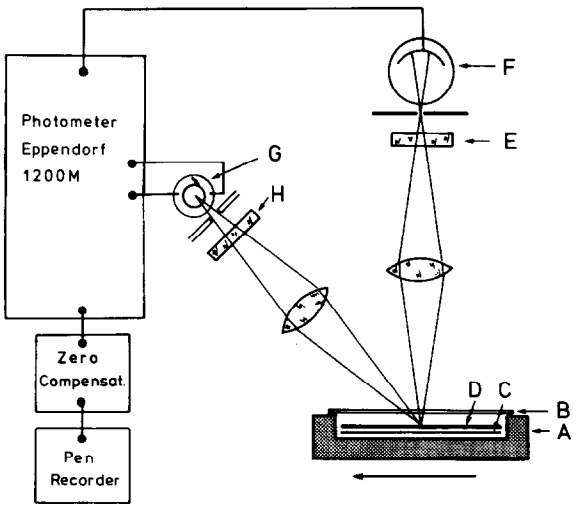

Fig.1. Block diagram of arrangement used for fluorometric testing of enzyme activity on electrophoretic slides. Further explanation is given in the text.

GmbH, Göttingen) of similar size impregnated with the substrates and enzymes of the test system. The development of fluorescence is followed by repeated recording.

2.1. *Indicator system*

In these measurements the indicator reaction is catalyzed by a surplus activity of glucose-6-phosphate dehydrogenase (G-6-PDH, EC 1.1.1.49). It is coupled to the test systems by ATP via the hexokinase reaction (HK, EC 2.7.1.1) [8,9].

$$\text{Glucose} + \text{ATP} + \text{NADP} \xrightarrow[H_2O]{\text{HK, G-6-PDH}}$$

$$\text{6-phosphogluconate} + \text{ADP} + \text{NADPH}_2$$

The system generates bright fluorescence of NADPH. It regenerates ADP at the same time. Our 'indicator stock solution' (13 × conc.) of metabolites contains in 0.1 M triethanolamine–HCl buffer (pH 7.6) the following (mM): glucose (150), ADP (25), NADP (40), $MgSO_4$ (130), adjusted with ~50 μl 2 N NaOH/ml to pH ~7.6. It is aliquoted and stored frozen.

2.2. *Electrophoresis*

Cellogel strips (5.7 × 14 cm) ~0.3 mm thick are used together with the commercial S 60 A chamber and 8.5 cm bridges (Celtec Diagnostica GmbH, Oberursel, semimicro equipment). Satisfactory separations require cooling. This is achieved by bubble-free contact of the impermeable underside of Cellogel strips to the slightly convex surface of a PVC-chamber, inserted into the commercial bridge and thermostated at 20°C (~5°C below the temperature of the laboratory) by water flow.

The Cellogel strip is washed for 5 min in electrophoresis buffer (see below). Dithioerythritol (25 mg) is dissolved in 250 ml electrophoresis buffer. The presoaked strips are soaked in this solution for 10 min before it is filled into the electrode compartments (each 125 ml). AMP (free acid) is added (0.36 mg/ml) to the cathodal buffer in order to promote migration of possibly disturbing adenylate kinase (EC 2.7.4.3) [11]. A prerun of 10 min at 200 V is performed before the samples (1 μl containing 10–200 μU, see also table 1) are applied by means of a Celtec semimicro (4 trace) 'stamp'.

2.3. *Samples*

Erythrocytes and homogenized tissues are extracted with 50 mM triethanolamine–HCl (pH 7.6) saturated with digitonin (~2 mg/ml) and containing 0.3 mg/ml dithioerythritol (DTE) and 0.5 mg/ml bovine serum albumin (BSA, grade I). The same DTE–BSA buffer mixture, without digitonin, is also used for dilution of auxiliary enzymes for the assays described below. To the supernatants of centrifuged tissue, homogenates are finally added 2 vol. mixture of 10 ml glycerol and 5 ml containing 6 mg DTE and 10 mg BSA. This measure stabilizes enzyme activity during deep freeze (–25°C) and avoids desiccation of samples applied to the applicator.

2.4. *PGK assay*

The resolution of PGK-1 allozymes is achieved by electrophoresis for 90 min at 200 V at pH 8.8 in the following electrophoresis buffer: 20 mM sodium barbital, 10 mM sodium citrate, 5 mM $MgSO_4$, 2 mM EDTA.

Due to its instability glycerate-1,3-diphosphate, substrate of PGK [12], must be prepared in situ. This is achieved by linking the reactions which are catalysed by aldolase (ALD, EC 4.1.2.13), glycerol-1-phosphate dehydrogenase (GDH, EC 1.1.1.8) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12). In presence of catalytic amounts of NAD–NADH this system catalyses an overall reaction which might be described as 'phosphorylating dismutation' of fructose-1,6-diphosphate, without disturbing stoichiometric production of fluorescent NADH:

$$\text{Fructose-1,6-diphosphate} + \text{phosphate} \longrightarrow$$

$$\text{glycerate-1,3-diphosphate} + \text{glycerol-1-phosphate}$$

PGK, when linked to this system, catalyses the transphosphorylation to ADP, thus providing ATP as key metabolite of the indicator system.

The 'PGK-assay stock solution', aliquoted and stored in the deep freezer contains in 0.1 M triethanolamine–HCl buffer (pH 7.6): 1.2 mM NAD, 40 mM K_2HPO_4 and 40 mM trisodium fructose-1,6-diphosphate.

The 'PGK-assay enzyme mixture' is prepared by dissolving commercial enzyme suspensions (in 3.2 M ammonium sulfate) in 70 μl DTE–BSA buffer:

20 μl G-6-PDH (100 μg) and 10 μl HK (100 μg), both from yeast, 20 μl ALD (200 μg), 10 μl GDH (100 μg) and 10 μl GAPDH (100 μg), all from rabbit muscle.

Five minutes before the end of the electrophoresis run the 'stain' is made by combining 0.4 ml 20 mM $MgSO_4$ in 0.1 M triethanolamine–HCl buffer (pH 7.6), 0.2 ml 'PGK-assay stock solution', 0.02 ml 'PGK-assay enzyme mixture' and 0.05 ml 'indicator stock solution' at room temperature. An acetyl cellulose membrane is impregnated with 'stain' and placed in the gel support. The staining reaction is initiated by placing the electrophoresis strip face down on this membrane.

2.5. *PGAM assay*

The resolution of the three PGAM isozymes is achieved during 50 min at 300 V in 20 mM sodium barbital (pH 8.0) containing 10 mM $MgSO_4$ and 2 mM EDTA. Linked reactions in the assay which lead to the conversion of ADP to ATP and join the system therefore to the above-mentioned indicator reactions are:

Glycerate-3-phosphate ⟷ glycerate-2-phosphate

Glycerate-2-phosphate ⟷ phosphoenolpyruvate + H_2O

Phosphoenolpyruvate + ADP ⟶ pyruvate + ATP

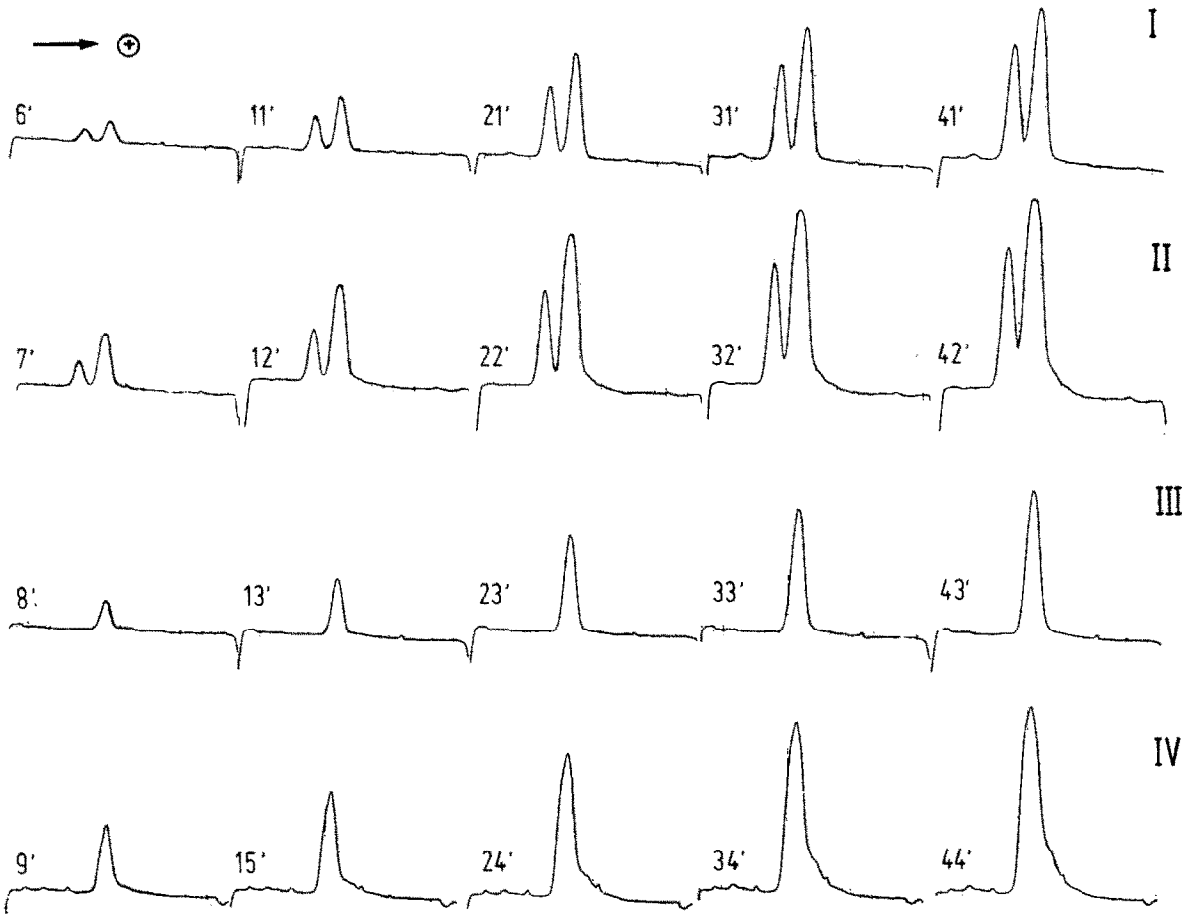

Fig.2. Development of fluorescent bands specific for PGK-1B (left) and PGK-1A (right) on an electrophoretic strip. Times of recording from 6–44 min are indicated in the figure. In the experiment [10] a femal mouse homozygous for X-linked PGK-1A (variant) has been mated with a male hemizygous for PGK-1B (wildtype). I-III extracts of tissues of a femal conceptus (13th day): Trace I embryo proper; trace II yolk sac mesoderm; trace III yolk sac endoderm. Trace IV is extract of the mothers liver (PGK-1A).

The 'PGAM-assay stock solution' contains 2 mM glycerate-3-phosphate and 5 μM glycerate-2,3-diphosphate in electrophoresis buffer.

The 'PGAM-assay enzyme mixture' is prepared by dissolving commercial enzyme suspensions in 3.2 M ammonium sulfate in 60 μl DTE–BSA buffer as follows: 20 μl G-6-PDH (100 μg) and 10 μl HK (100 μg), both from yeast, 10 μl enolase (EC 4.2.1.11, 100 μg) and 10 μl pyruvate kinase (EC 2.7.1.40, 100 μg) both from rabbit muscle.

About 2 min before the end of the electrophoresis run an acetylcellulose membrane is impregnated with the following 'stain': 0.4 ml electrophoresis buffer, 0.2 ml 'PGAM-assay stock solution', 0.05 ml 'indicator stock solution' and 0.02 ml 'PGAM-assay enzyme mixture'.

3. Results and discussion

The quantitative assay was originally developed in connection with isozyme studies in trisomy 19 of the mouse. However, with the detection of the PGK-1A variant in feral mice [13], and its use as an X-chromosomal enzyme marker in various experimental designs the evaluation of PGK-allozyme patterns has become particularly interesting.

3.1. *PGK-1 allozyme patterns*

Traces of 4 electrophoretic PGK-1 patterns, which have been repeatedly scanned at 10 min intervals are mounted in fig.2 in order to demonstrate the time course of the fluorometric assay. At lower levels the amount of NADPH which is generated in the assay, is directly proportional to the fluorescence output represented by the areas under the peaks in fig.3.

At higher levels of NADPH weakening of the exciting beam by absorption must be considered. However, the time course shown in fig.3 is similar to the one observed spectrophotometric when the same system is used as 'forward' test of PGK (not shown), except the pronounced retardation in the beginning. Establishment of a steady state of diffusion of enzymes and substrates in the sandwich can be taken as the major cause of this retardation. It is almost independent of the PGK activity in the electrophoretic bands. The non-linearity which will be observed later on in the time course is mostly due to product inhibition. It causes distortions of the recorded patterns in cases of pronounced

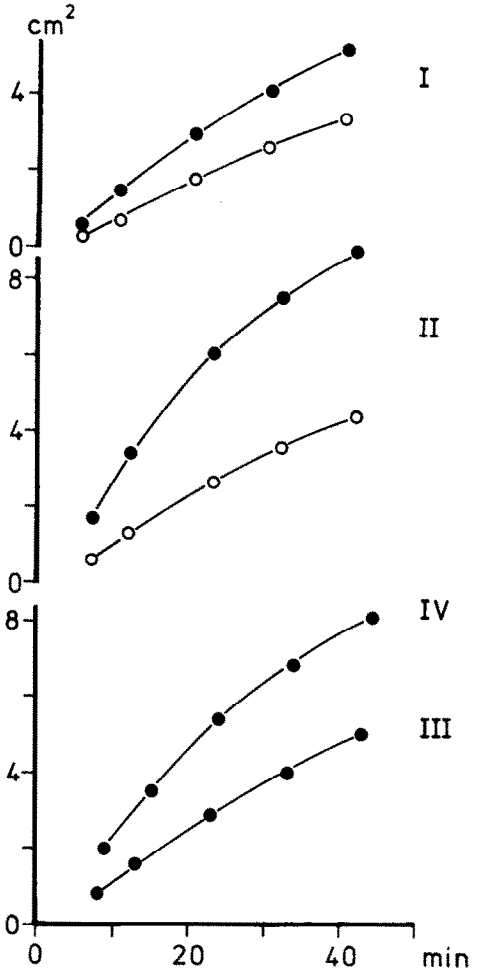

Fig.3. Areas of the peaks shown in fig.2 plotted against time of registration. (○) PGK-1B; (●) PGK-1A.

inequality of allozyme activities. The weaker band will show up as a higher proportion than in reality because, at the time necessary for evaluation of the lower activity the reaction rate of the stronger bands has slowed down. This is shown in fig.4 and table 1 where erythrocyte extracts of hemizygous PGK-1A and PGK-1B males have been mixed in different proportions.

Fig.4 allows also an estimate of the sensitivity of the methods which in fact discriminate between allozyme activities down to a proportion of 1/100 (data not shown) and, with prolonged times of scanning, assay activities of 1 μU.

The samples shown in fig.2 were kindly contributed from current studies by Dr M. Monk, MRC

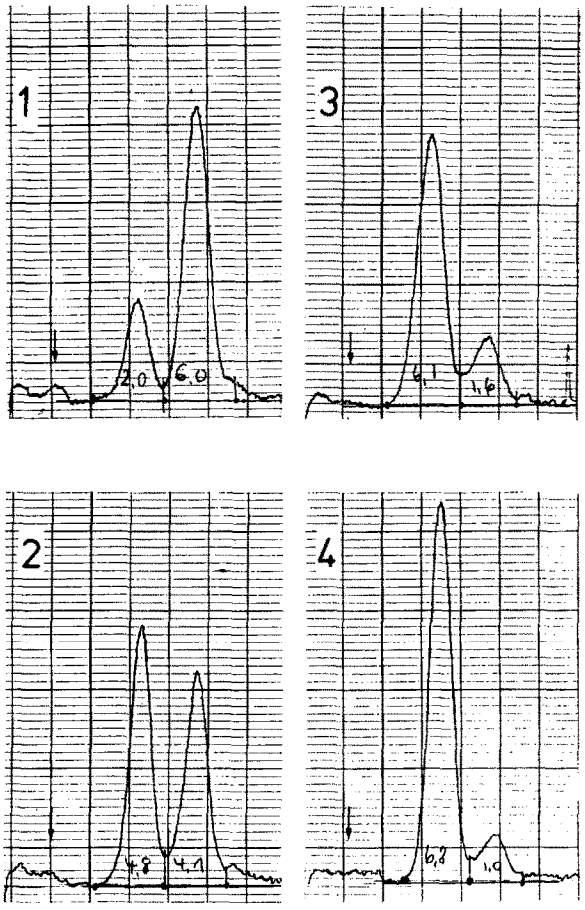

Fig.4. Electrophoretic traces (at 35 min) of artificial mixtures of PGK1-A and PGK1-B allozymes. See also table 1.

Table 1
Extracts of erythrocytes[a] from PGK-1A and PGK-1B males were photometrically tested, mixed in different proportions and resolved electrophoretically (fig.4)

Photometrically[b]				Fig.4[c]
Trace	PGK-1A	PGK-1B	%PGK-1B	%PGK-1B
1	127	38	23	25
2	79	95	55	54
3	32	151	83	79
4	16	170	91	87

[a] Dilution factor 250
[b] μU/μl; for the optical test the PGK assay described in the methods section was used: 500 μl buffer, 10 μl indicator substrate, 20 μl PGK substrate, 10 μl enzyme mix
[c] Percentage of area under left hand peaks of area under both peaks

Mammalian Development Unit, London. They demonstrate the very interesting phenomenon of inactivation of the paternal X-chromosome in the yolk sac endoderm of female mice [14,15]. The yolk sac is one of the outer extra-embryonic membranes that envelopes the developing fetus. Although the yolk sac endoderm expresses only the maternal X-chromosome, the yolk sac mesoderm and the fetus are functional mosaics by virtue of random X-chromosome inactivation in females [3–5].

3.2. *PGAM isozyme pattern*

In *Mus musculus* the A and B subunits of PGAM are expressed together in the heart, whereas liver and blood contain only the AA- and skeletal muscle only the BB-isozyme. In a first approximation the specific activity of the isozymes may be taken to be equal. This is assumed in the evaluation (table 2) of the scans presented in fig.5, which have been recorded 60 min after the establishment of the sandwich contact.

Animals trisomic with respect to chromosome 19 might express a higher dosage of subunit A and consequently affect the pattern of isozymes. The results seen qualitatively by comparison of the traces and quantitatively from the numbers in table 2 show that subunit A appears in a higher proportion in a randomized pattern with the AA and AB isozymes elevated at the expense of BB.

Trisomy 19 has been produced by the mating scheme developed by A. Gropp [16] using the Robertsonian translocations Rb (9.19) 163 H and Rb (8.19) 1 Ct. In a male heterozygous for these translocations one chromosome 19 is fused with chromosome 9 and the other with chromosome 8. These fusions lead to meiotic malsegregations (non-disjunctions) in the course of formation of male germ cells, some of which become disomic for chromosome 19 and consequently cause trisomy 19 when the male is mated to normal diploid females.

The trisomy 19 individual analysed in fig.5 was born live. Live births are rare for this syndrome

Table 2
Evaluation of areas under peaks in fig.5 (% total)

	AA	AB	BB	ξA/2	ξB/2	ξA/ξB
Control	17.7	31.6	50.6	33.5	66.4	0.5
Trisomy 19	27.5	41.8	30.6	48.5	51.5	0.94

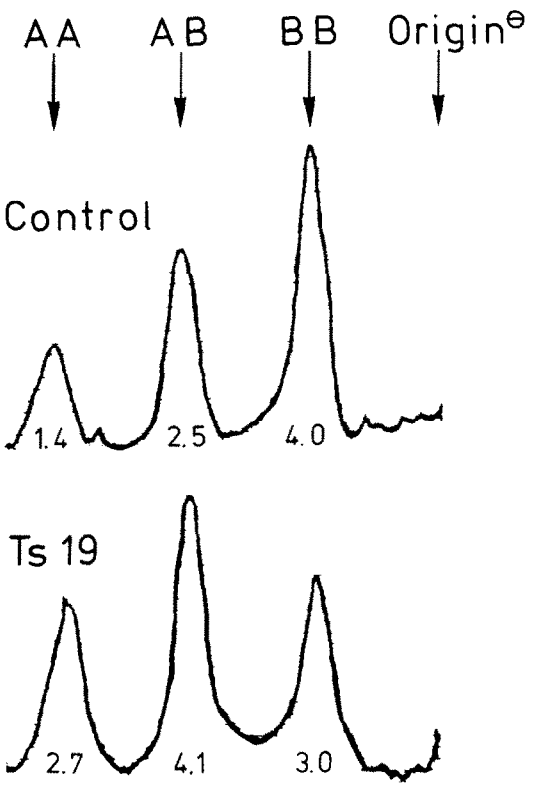

Fig.5. Isozyme patterns of phosphoglycerate mutase in cytosol extracts of the hearts of mouse siblings. Ts 19: 72 h old male trisomic for chromosome 19; control female litter mate. Sum of enzyme units extracted/g fresh weight: 13.1 in Ts 19 and 12.7 in control. Numbers under the peaks give their planimetric area as cm^2 (see also table 2).

[17,18] and when they do occur the pups are significantly retarded. One may therefore ask, whether the normal litter mate is a proper control. In the example of table 2 the increase of the proportion ξA/ξB exceeds the theoretical 150%. Significantly increased activity levels of subunit A-dependent isozymes have been measured in all developmental stages in trisomy 19 [19] and the interesting question of the exact increments can be answered by an extended study of the developmental course of the isozyme patterns of the heart.

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