New Positive, Tetrazolium-Linked, Staining Method for Use with Electrophoresis of Phosphoglycerate Kinase

Phosphoglycerate kinase (PGK; EC 2.7.2.3) has been the subject of considerable recent interest. It has been shown to be a sex-linked enzyme in the human population[1,2] and, as such, promises to be a useful tool in studies involving *X*-linked genes. This enzyme has an important functional role in metabolism as one of the adenosine triphosphate (ATP)-generating enzymes of glycolysis. In humans, a deficiency of this enzyme leads to a type of congenital nonspherocytic anemia[1,3]. Another factor adding to the importance of this enzyme is that, in red blood cells, it can be bypassed by a two-enzyme shunt which produces 2,3-diphosphoglycerate (2, 3-DPG). This intermediate has been extensively studied because of its role in modifying the oxygen affinity vurve of hemoglobin[4]. Thus, the relative activities of PGK and the 2,3-DPG shunt enzymes become important in determining red cell levels of 2,3 -DPG and ATP. Figure 1 illustrates the glycolytic role of PGK and its relation to the 2,3-DPG shunt.

Previous electrophoretic studies of PGK have been concerned with human red cell PGK and have used absence of fluorescence as an indicating system[2,5]. This communication describes a new staining technique for PGK activity utilizing a positive stain linked to a tetrazolium dye.

Methods. The basic strategy for our PGK strain is similar to that described by BREWER[6] for pyruvate kinase and creatine kinase, that is, the ATP formed by the PGK reaction is detected by a hexokinase (HK)/glucose-6-phosphate dehydrogenase (G6PD)/3-(4, 5 Dimethylthiazolyl-2)-2, 5 diphenyl tetrazolium (MTT) system. Phosphoglycerate kinase is detected in starch gels by means of an agar overlay containing 1, 3-diphosphoglycerate (1, 3-DPG) and adenosine diphosphate (ADP). The PGK produces ATP, which, in turn, acts on the HK/G6PD/MTT system and leads to colored bands of reduced MTT. The present commercial unavailability of 1, 3-DPG necessitates a generating system for this substrate. The 1, 3-DPG is generated in vitro by the glyceraldehyde-3-phosphate dehydrogenase (GAPD) enzymatic reaction

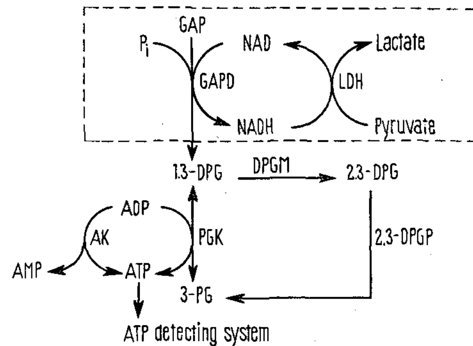

Fig. 1. Reactions relating phosphoglycerate kinase to its stain, the 2, 3-diphosphoglycerate shunt, and adenylate kinase. The 1, 3-DPG generating system is blocked off in the above figure. Abbreviations: GAP, glyceraldehyde-3-phosphate; Pi, inorganic phosphate; GAPD, glyceraldehyde-3-phosphate dehydrogenase; NAD and NADH, oxidized and reduced nictotinamide adenine dinucleotide, respectively; LDH, lactate dehydrogenase; 1,3-DPG, 1,3-diphosphoglycerate; DPGM, diphosphoglycerate mutase; 2, 3-DPG, 2, 3-diphoshoglycerate; DPGP, 2, 3-diphosphoglycerate phosphatase; ADP, adenosine diphosphate; PGK, phosphoglycerate kinase; 3-PG, 3-phosphoglycerate; ATP, adenosine triphosphate; AK, adenylate kinase; and AMP, adenosine monophosphate.

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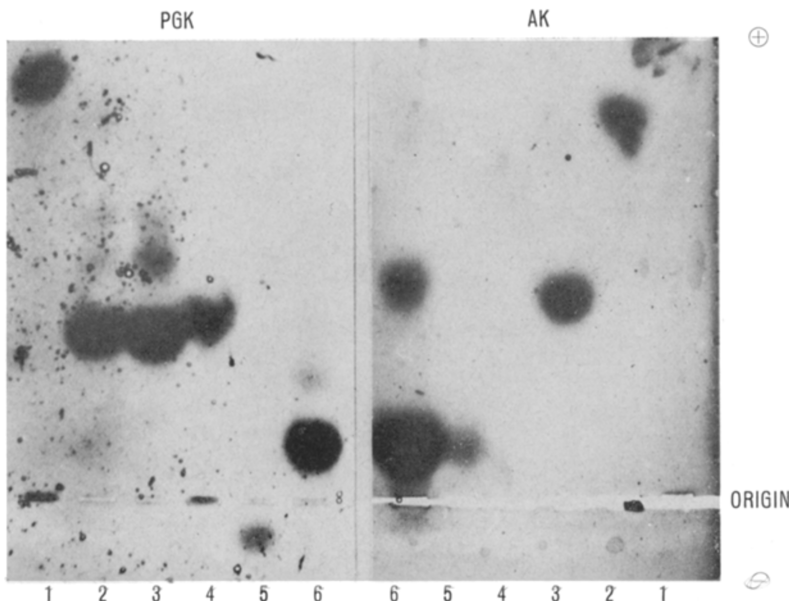

Fig. 2. Phosphoglycerate kinase (PGK) and adenylate kinase (AK) banding patterns. Samples are as follows: Slot 1, extract of a single fruit fly; Slot 2, rat blood hemolysate; Slot 3, Rhesus monkey blood hemolysate; Slot 4, mouse blood hemolysate; Slot 5, rabbit blood hemolysate; and Slot 6, human blood hemolysate. All the hemolysates are diluted with 2 parts water. Areas of true PGK activity are those which do not also show AK activity. The PGK face was photographed after about 15 min of development time while the AK was allowed to develop for about 45 min before photographing. Mouse AK migrates anodally and goes off the gel in the 8 h period.

using the appropriate enzyme substrates. Figure 1 should be consulted to clarify the overall steps in the staining procedure.

In the following description, all concentrations are expressed in terms of the final concentration in the overlay.

The 1, 3-DPG generating system consists of 0.00219 M glyceraldehyde-3-phosphate (GAP), 0.00033 M nicotinamide adenine dinucleotide (NAD), 0.01 M K_2HPO_4, 0.06 M pyruvate, 6.1 units lactate dehydrogenase (LDH) per ml, and 2.1 units GAPD per ml. These components are dissolved in a third of the *Tris*-HCl, 0.1 M pH 7 buffer to be used and this is incubated at 37 °C for 30 min. Pyruvate and LDH are used to keep the levels of reduced NAD (NADH) low since high levels can produce too dark of a background in the overlay.

To the 1, 3-DPG generating system, after 30 min incubation, are added the reagents of the ATP detection system, which are 0.0011 M ADP, 0.01 M glucose, 0.11 M $MgCl_2$, 0.00043 M nicotine adenine dinucleotide phosphate, 0.00039 M phenazine methosulfate, 0.00029 M MTT, 0.5 mg G6PD per ml, and 0.1 mg HK per ml. The resultant solution is mixed with a molten (40 °C) Ion-agar solution made from the remaining 2/3 buffer to give a final agar concentration of 0.75%. The final agar solution is then poured over the surface of the starch gel and incubated at 37 °C.

Bands of PGK activity will appear well within 30 min and should be photographed or scored. Because of the presence of components of the 1, 3-DPG generating system, the overlay will darken with more time and bands will be difficult to see.

Some of the bands seen may be due to adenylate kinase (AK) activity and not PGK. Since AK can convert ADP to ATP without the need for any other cosubstrates, staining the other face of the gel with an overlay similar to the above but minus the 1, 3-DPG generating system will indicate which are AK bands. AK bands usually take much longer to develop than PGK bands.

Starch gels were run for 8 h using the pH 7 histidine gel and citrate bridge buffer system and electrophoretic setup described by BREWER[6] for pyruvate kinase.

Results and discussion. In Figure 2 can be seen the starch gel PGK banding pattern of selected samples. The specificity of the stain is attested to by the fact that the PGK bands do not appear if either GAP or GAPD are omitted from the 1,3-DPG generating system. Further, a commercial PGK preparation (yeast) was found to produce a band only with the complete stain. Separate staining for LDH also removed the remote possibility that these bands could represent this activity.

The previous system for detecting PGK activity utilizes the reverse reaction of PGK. In this system 1, 3-DPG is formed at the site of enzyme activity on gels and is detected by a GAPD/NADH system. The areas of activity can be visualized under UV-light as less fluorescent areas (NAD) against a fluorescent background (NADH)[5].

The commercial availability of 1, 3-DPG would, of course, greatly simplify the positive staining technique described in this communication and add to its attractiveness. Nevertheless, our experience with the positive stain has been more satisfying than that with the fluorescent technique. In fact, our difficulties in visualizing banding patterns under UV-light (as required with the fluorescent technique) were the impetus for developing the positive stain.

In a study presently in progress involving over 15 species (both plant and animal) we have had no trouble obtaining PGK bands with the positive technique and distinguishing them from AK bands. Thus, the technique most likely has wide species applicability.

Zusammenfassung. Eine Methode zur positiven Anfärbung der Phosphoglycerat-Kinase (EC 2.7.2.3) nach Stärkegelelektrophorese wird beschrieben. Das in der Vorwärtsreaktion des Enzyms gebildete Adenosin-Triphosphat wird mit einem Indikatorsystem (Hexokinase, Glukose-6-phosphat-Dehydrogenase, Tetrazoliumsalz) erfasst.

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An Apparatus Suitable for Serial Determinations of Fibrinolytic Activity

Various methods have already been described for determining the influence of drugs on the fibrinolytic activity of plasma in vitro[1–9, 11, 13] or in vivo in pretreated animals[12]. Since the number of samples to be tested may be very large, the construction of a time-saving device to facilitate the estimation of fibrinolytic activity appears well worth the effort. Such an apparatus is particularly useful when, instead of a straight-forward measurement of lysis time, the clots have to be treated in a certain way or a sample of the incubation medium taken in order to define the mode of action of a preparation[1, 12].

We have therefore designed an apparatus permitting the lysis of 20 clots to be studied in various respects. One point to which we attached particular importance was that the formation and treatment (e.g. rinsing) of the clots, as well as the removal of samples of the incubation medium, could be effected simply and as nearly as possible simultaneously in all specimens.

The apparatus consists essentially of the following parts (Figure 1): rectangular cabinet box (A); a support plate (B); exchangeable clot rods (C) and racks for test tubes (D) of different sizes.

Cabinet. The cabinet is a rigid box, on the base (a) of which special test-tube racks are placed (D). A vertical

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