Anion Effects on the Kinetics of Yeast Phosphoglycerate Kinase

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Khamis, M. M. and Larsson-Raźnikiewicz, M., 1987. Anion Effects on the Kinetics of Yeast Phosphoglycerate Kinase. – Acta Chem. Scand., Ser. B 41: 348–355.

(A) The effects of phosphate, chloride, nitrate, pyruvate, malate, succinate and glutamate ions on the kinetics of yeast phosphoglycerate kinase (ATP: 3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) were studied with $MgATP^{2-}$ and 3-P-glycerate as variable substrates. Three types of patterns were obtained: (1) Nitrate, succinate, malate and glutamate ions, strictly noncompetitive versus both the substrates. (2) Phosphate and chloride ions, noncompetitive versus $MgATP^{2-}$ and mixed versus 3-P-glycerate. (3) Pyruvate ions, being very weak inhibitors, competitive with $MgATP^{2-}$ and noncompetitive with 3-P-glycerate. (B) Based on experiments with simultaneous inhibition by various combinations of two anions the following suggestions were made: The type 1 anions presumably bind to a site outside the active centre. These ions appear to bind to the enzyme independently of type 2. The latter also appears to include sulfate ions, which are competitive versus both the substrates as well as versus the phosphate and chloride ions. Sulfate and phosphate ions are electronically similar, but show different inhibition patterns, presumably due to various effects on the protein conformation. Type 3 inhibition exerted by pyruvate ions was shown earlier for 1-anilino-8-naphthalenesulfonate and salicylate ions, but as these two anions are supposed to bind to the adenine binding pocket of the catalytic centre, the results indicate that pyruvate ions might preferably compete with the nucleotide substrate for the polyphosphate binding site.

Anion effects on the phosphoglycerate kinase reaction have been of special interest. At low concentrations, many of these ions activate the catalytic reaction, whereas at higher concentrations they often act as inhibitors.[1,2] The optimal rate of the reaction is obtained at different concentrations for the various anions. Both activation and inhibition by sulfate ions are much more pronounced than for univalent anions. Scopes[2] suggests that these effects increase with the charge of the anion. Detailed studies have been made only on the effects of sulfate ions.[3] When acting as activators, the catalytic rate constants rather than the binding of the substrates is affected.[3] As inhibitors, sulfate ions exert their effects by competing with each of the two substrated $MgATP^{2-}$ and 3-P-glycerate. The enzyme appears also to possess regulatory sites available for the two substrates[4] and it shows strong interaction with polyanionic agents.[5] The single polypeptide chain of the enzyme is organized into two separate domains composed of the N-terminal and C-terminal halves of the chain.[6] The nucleotide substrate binds to the C-terminal domain of the crystalline enzyme.[6] There is some indirect evidence showing that 3-P-glycerate binds to the N-terminal lobe of the enzyme.[6] Anions are able to protect the single SH group in the enzyme from being modified.[7] The latter studies and X-ray crystallographic work[6] indicate that an anion binding site is located in the N-terminal lobe. There appear to exist two sulfate binding sites, one binding the activator and the other binding the inhibitor.[3] The question then arises: does the inhibition pattern obtained with the sulfate ions really imply that they bind to the catalytic centre? Strong evidence for conformational changes asso-

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ciated with the activating and inhibiting ranges of the sulfate ion concentrations have been reported.[1,8–10] Cleft-closing was suggested to be part of the catalytic mechanism.[6,11–12] To better understand the intimate details of these catalytic events, detailed studies of various anions have been performed.

Experimental

Phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) was prepared from baker's yeast[13] and the main electrophoretic component B was used.[14] Both gel electrophoresis and chromatofocusing showed that the enzyme was homogeneous. An absorbance coefficient of 0.50 ml mg^{-1} cm^{-1} at 280 nm and a molecular weight of 45 000 was used in calculating the enzyme concentration.[14] Glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12) from rabbit muscle was purchased from Boehringer Mannheim GMbH. Before use, the crystalline enzyme was centrifuged (27 000 × *g* for 20 min) and dissolved in 50 mM Tris-HCl buffer (pH 7.8, 25 °C) containing 100 mM NaCl. The enzyme was then dialysed against the same buffer overnight.

The barium salt of 3-phosphoglycerate, and the disodium salts of equine muscle ATP, yeast NADH, pyruvate, malate, glutamate and succinate were all purchased from Sigma Chemical Co. The ammonium salt of 1-anilino-8-naphthalenesulfonate (ANS) was obtained from Eastman Organic Chemicals. Before use, it was recrystallized several times from hot water after treatment with Norit.[15] An absorbance coefficient of 4.95 mM^{-1} cm^{-1} at 350 nm and a dissociation constant of 0.08 mM were used in the calculations of ANS[15] and $MgATP^{2-}$ [16] concentrations, respectively. 3-P-Glycerate was liberated from its barium salt with Na_2SO_4 and neutralized with NaOH. The concentration was determined as described earlier.[17]

All solutions were prepared from analytical grade reagents and Milli Q-filtered, distilled water. To remove contaminating metal ions, the solutions were shaken with dithizone in carbon tetrachloride[16] and then stored in acid-washed Duran glassware. Before use all standard solutions were adjusted to pH 7.8, 25 °C.

The activity of phosphoglycerate kinase was determined by the method of Bücher,[18] using a Zeiss PMQ3 spectrophotometer equipped with a SERVOGOR 120 recorder and a Paratherm U2 electronic thermostat under the conditions described earlier.[16] The initial velocity was expressed as $v = (dA_{366}/dt)_{t=0}$, in min^{-1}. About 0.2 μg of phosphoglycerate kinase and 0.2 mg of glyceraldehyde phosphate dehydrogenase were used per ml of the substrate solution. Unless stated otherwise, the assay mixture containing 1 mM $MgATP^{2-}$ / 2 mM 3-P-glycerate / 5 mM free Mg^{2+} / 0.5 mM NADH / and 50 mM Tris-HCl buffer (pH 7.8, 25) containing 100 mM NaCl. To compensate for the incident light absorbed by ANS a correction was made: for every reaction rate measured at a given ANS concentration, a relative velocity was measured with an identical substrate solution not containing ANS, but registered with a "filter cuvette" in the beam. The filter cuvette contained ANS at a concentration such that the total absorbance of the reference plus the filter cuvette was the same as that of the sample at 366 nm.[19]

Data obtained from simultaneous inhibition by two anions were treated in two different ways. First, according to Webb,[20] the concentration of the inhibitor was plotted against the concentration of a second inhibitor, counterbalancing any change in reaction velocity arising from the first inhibitor (cf. Fig. 3). Linearity and non-linearity were taken as evidence for exclusive and non-excluding binding, respectively, of the two inhibitors. Second, as suggested by Yonetani-Theorell,[21] graphs representing the variation in reciprocal velocity with the concentration of one inhibitor at various concentrations of a second inhibitor (λ) were constructed (cf. Fig. 4). Parallel straight lines were used to represent exclusive inhibition while intersecting lines indicate non-exclusive inhibition. In the most favourable cases both Webb and Yonetani-Theorell types of graphs were constructed for the same experiment.

Results and discussion

Kinetic effects of anions. Fig. 1 shows the effects of various anions, viz. phosphate, nitrate, succinate and pyruvate, on the catalytic reaction of phosphoglycerate kinase. In agreement with earlier reports[1–3] we found that the reaction velocity increases with an increase in anion concentration until it reaches an optimal point. From this point the reaction rate gradually decreases as the anion

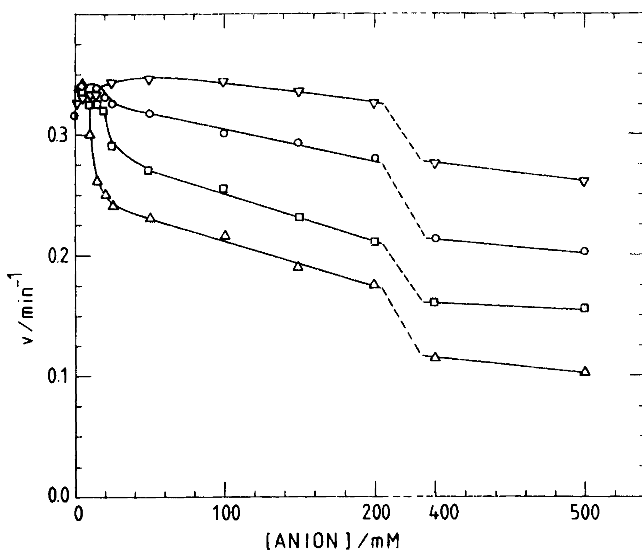


Fig. 1. The effects of anions on the activity of phosphoglycerate kinase. (▽) pyruvate, (○) succinate, (□) nitrate and (△) phosphate.

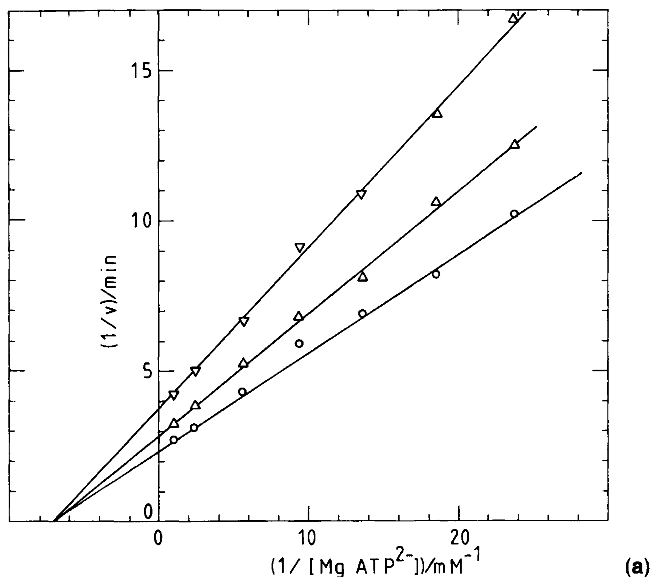
concentration is increased. The steepness of the rate profile in both the activation and inhibition regions seems to be less than that observed by Scopes.[2] This might be due to the higher substrate concentrations used in the present work.[2,3] Furthermore, it was observed earlier[1] that sulfate ion activates and inhibits more strongly than do monovalent anions. Scopes[2] suggested this phenomenon to be general for multivalent anions. In the present study (Fig. 1) it was noticed that, in most cases, inorganic anions are better modulators than are organic ions.

Work on sulfate ions[3] showed them to be competitive inhibitors versus both $MgATP^{2-}$ and 3-P-

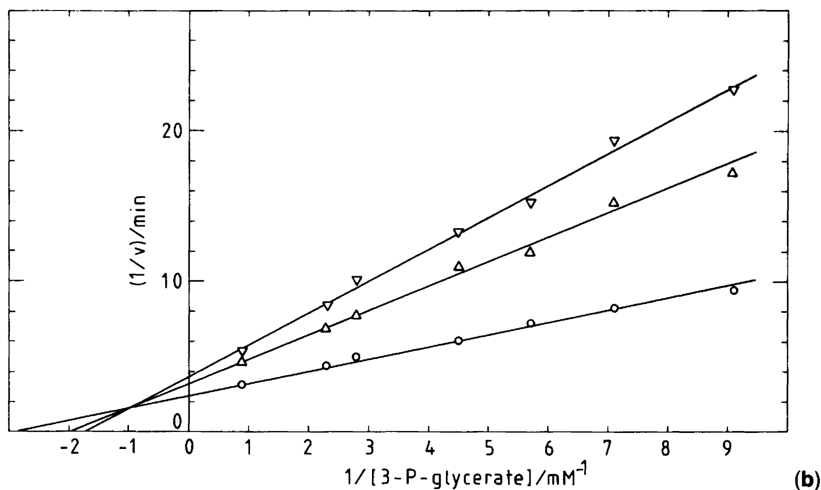
Table 1. Anion inhibition patterns of phosphoglycerate kinase.

Anion	Anion/mM	Variable substrate, $MgATP^{2-}$		Variable substrate, 3-P-glycerate		Ref.
		Non-competitive	Competitive	Non-competitive	Competitive	
Sulfate	40–100		+		+	
Phosphate	15–30	+		+[a]		
Nitrate	50–150	+		+		
Chloride	200–400	+		+[a]		
Pyruvate	400–800		+	+		
Malate	200–300	+		+		
Glutamate	400–800	+		+		
Succinate	150–500	+		+		
ADP^{3-}	0.1–1	+			+	23
$MgADP^{1-}$	0.1–1		+		+	23
AMP^{2-}	1–3		+	+		23
Salicylate	10–25		+	+		22
ANS	0.5–1		+	+		19

[a]A partially competitive behaviour exists. In all experiments the concentration of the variable substrate was in a range up to about 1 mM at fixed concentrations of the second substrate 3-P-glycerate or $MgATP^{2-}$ of 2 and 1 mM, respectively.



(a)



(b)

Fig. 2. The inhibition effects of phosphate ions on the phosphoglycerate kinase kinetics with varying (a) $MgATP^{2-}$ and (b) 3-P-glycerate concentrations, with (○) 0 mm, (△) 15 mM and (▽) 25 mM phosphate.

glycerate. The present kinetic studies on the influence of various anions on phosphoglycerate kinase activity showed that almost all the tested anions (see below, however) are non-competitive inhibitors versus the two substrates (Table 1). However, phosphate (Fig. 2) and chloride ions appear to exhibit partially competitive behaviour versus 3-P-glycerate, and pyruvate ions exhibit competitive behaviour versus $MgATP^{2-}$. Fairly high pyruvate concentrations were used in the experiment, and pyruvate inhibition kinetics at various Mg^{2+} concentrations were therefore measured. The results (not shown) appeared to exclude the possibility that this competitive behaviour arises as a result of the dissociation of the nucleotide-metal ion complex, however.

Double inhibition by sulfate and phosphate ions. It was suggested earlier[2] that sulfate and phosphate ions are mutually competitive because they are very similar electronically; like sulfate ions, phosphate ions were supposed to be competitive versus both $MgATP^{2-}$ and 3-P-glycerate (however, see Table 1 and Fig. 2). To check for differ-

ences in the inhibition patterns obtained, simultaneous inhibition by sulfate and phosphate ions was studied. The Webb plots in Fig. 3 show straight lines when the increase in phosphate concentration was compensated for by a decrease in sulfate concentration so as to keep the reaction rate constant at various fixed levels. These results indicate that the sulfate and phosphate ions are, indeed, mutually competitive. The non-competitive patterns exhibited by phosphate ions (Fig. 2, Table 1) compared to the earlier observed[3] competitive inhibition patterns exhibited by sulfate ions show that if these two inhibitors bind to the same site, this could be close to the active centre without sharing essential ligands with the substrates. Long range effects cannot be excluded, however. Different kinetic behaviour could be due to differences in effects on the protein conformation. Ultracentrifugation studies indicate[5] that the cleft between the two structural lobes of the enzyme closes and opens when sulfate ions acting as activators and inhibitors, respectively, bind to the enzyme. Closing of the cleft can also be induced by the substrates[10,11] (cf. also Ref. 19). Thus, the sulfate inhibition pattern might be due to competitive conformational changes exerted by the substrate and sulfate ions. Phosphate ions might bind to the enzyme without inducing such drastic changes in protein conformation.

Pyruvate and other anions competing with $MgATP^{2-}$ but not with 3-P-glycerate. Pyruvate ions exhibit strictly non-competitive inhibition versus 3-P-glycerate but competitive versus $MgATP^{2-}$ (Table 1). The same behaviour was noticed by Larsson-Raźnikiewicz and Wiksell in their studies of the effects of salicylate[22] and ANS[19] on the phosphoglycerate kinase reaction. These two ions were supposed to bind to the $MgATP^{2-}$ binding site in the active centre, primarily to the adenine binding area of the enzyme. Simultaneous inhibition by pyruvate and ANS or salicylate, indicates non-exclusive binding in both cases, however. It might be that pyruvate ions bind via their carboxyl and keto groups to the lysines that Watson *et al.*[6] suggested to interact with the β- and γ-phosphates of $MgATP^{2-}$. From other results[23] it appears evident that the adenine part of the nucleotide substrate as well as its polyphosphate chain are important for the interaction with the catalytic centre.

Of the anions listed in Table 1, pyruvate ions appear to behave as non-linear, parabolic inhibitors (for definition, see Ref. 24). This could be evidence for the multiple inhibitory effects of pyruvate ions. Studies of the simultaneous inhibition by pyruvate and nitrate ions show that they are competing (Table 2), and thus binding of pyruvate ions to the site for which most anions com-

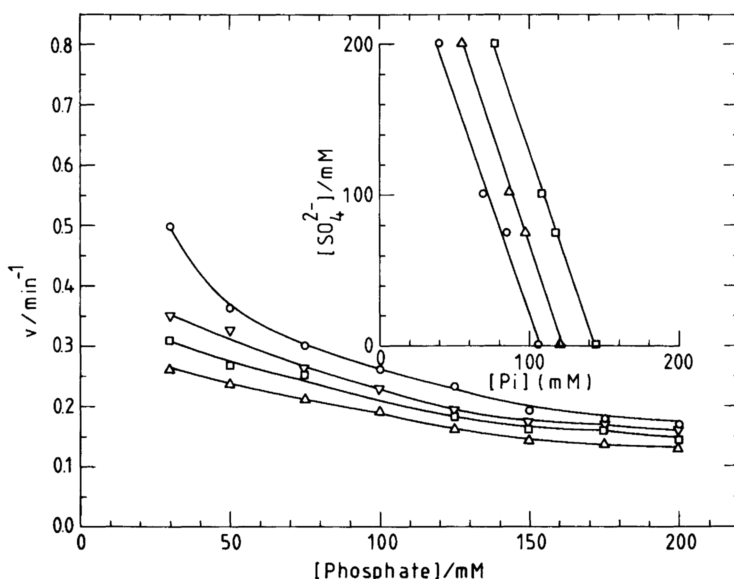


Fig. 3. Simultaneous inhibition by phosphate (P_i) and sulfate ions of phosphoglycerate kinase. The phosphate concentration was varied at sulfate concentrations of (○) 0 mM, (▽) 75 mM, (□) 100 mM and (△) 200 mM. The inserted figure was constructed from data obtained from the main figure when the increase in phosphate concentration was compensated for by a decreasing sulfate concentration to keep v constant at (○) 0.250, (△) 0.230 and (□) 0.205 min^{-1}.

Table 2. Multiple inhibition of phosphoglycerate kinase. Patterns for two simultaneous inhibitors.

Inhibitors		Non-exclusive	Exclusive
SO_4^{2-}	Phosphate		+
"	ANS	+	
"	NO_3^-	+	
"	Pyruvate	+	
"	$MgADP^-$	+	
"	ADP^{3-}		+
Pyruvate	ANS	+	
"	Phosphate	+	
"	Salicylate	+	
"	NO_3^-		+
NO_3^-	Salicylate	+	
Phosphate	Salicylate	+	

monly show non-competitive inhibition patterns (Table 1) appears likely also.

Multiple inhibition with sulfate ions and ANS or pyruvate ions in both cases indicated non-exclusive binding (Table 2, Fig. 4). Similar results were obtained in studies of simultaneous inhibition by phosphate ions and salicylate ions (Table 2). Sulfate and phosphate ions, when acting as inhibitors, appear to bind elsewhere at a site specific for them (see above), which does not share ligands with ANS, salicylate or pyruvate ions. Sulfate ions are competing with $MgATP^{2-}$ but not with $MgADP^-$ (Table 2). Binding studies by Scopes[25] showed similar results. Ultracentrifugation studies by Roustan *et al.*[5] showed that $MgADP^-$ does not affect the protein conformation in the same way as $MgATP^{2-}$. This supports the above made suggestion that the sulfate ion inhibiton involves conformational changes in the protein. ADP^{3-} appears to compete for the same form of the enzyme as do sulfate ions (Table 2). The inhibition patterns for ADP^{3-} (Ref. 23) and phosphate ions are similar (Table 1), exhibiting non-competitive behaviour versus $MgATP^{2-}$ and competitive versus 3-P-glycerate. It was suggested earlier[23] that ADP^{3-} as inhibitor does not bind to the site binding the nucleotide substrate.

General comments regarding anion inhibition and activation. Inhibitors competing with $MgATP^{2-}$, in the present case ANS,[19] salicylate,[22] pyruvate and sulfate[3] ions, are the most interesting ones in studies on structural and functional relationships of enzymes involved in energy metabolism; do they compete with the substrate for the same binding site or do they act via conformational changes?

It is reasonable to hypothesize that as inhibitors, salicylate, ANS and possibly also pyruvate bind to the active site region, and that the other anions bind to sites outside. Stinson,[7] Watson[6] and their coworkers suggested that the anions might bind in the N-terminal lobe. The large

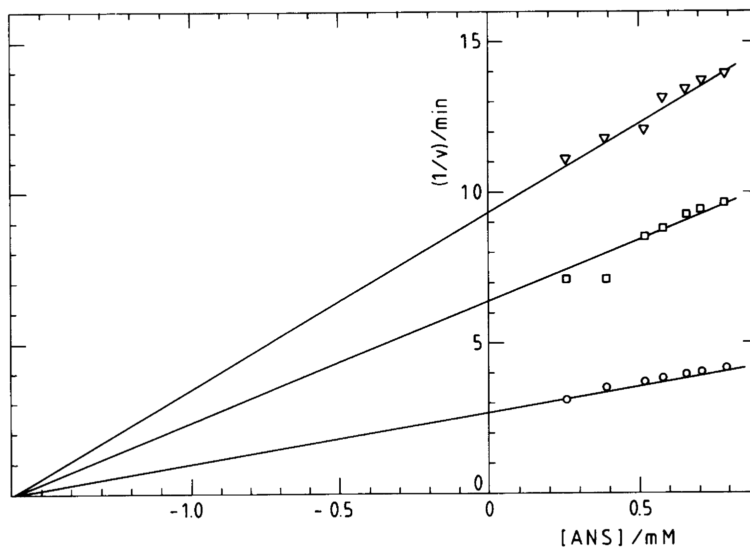


Fig. 4. Simultaneous inhibition by ANS and sulfate ions of phosphoglycerate kinase. (○) 0 mM, (□) 100 mM and (▽) 200 mM SO_4^{2-}. The assay mixture contained 0.5 mM ATP/1.0 mM Mg^{2+}. No NaCl was included in the buffer.

positively charged region certainly is able to coordinate anions in various ways.

One additional anion binding site, the activator site, is of special interest. Earlier results[3] showed that sulfate ions as activators do not affect substrate binding to the catalytic centre; instead, they affect the rate of the catalytic reaction. For most anions, the activating effect observed is small and difficult to study in detail, depending very much on the counteracting power exerted by the anion as an inhibitor. In any case, the optimal reaction rate was usually obtained at lower concentrations when the concentration of a second anion was increased to various fixed levels, thereby saturating the activator binding site. Such would be expected if the anions as activators bind to the same site. Additionally, in special cases apparent inhibition might be obtained by indirect effects, as shown by the pair of ions sulfate and pyruvate (Fig. 5). Unlike the latter, sulfate ions as inhibitors are competitive with 3-P-glycerate (Table 1); thus, their binding to the inhibitor binding site can be suppressed by a high 3-P-glycerate concentration. Under such conditions, the activation-inhibition profile of pyruvate in the presence of sulfate ions shows (Fig. 5) that inhibition with pyruvate is more pronounced after sulfate ions have been included in the assay mixture. These results also agree with the suggestion regarding identical activator sites for the two anions; sulfate ions, being better activators, are removed by pyruvate ions which do not have as good activating properties. Earlier results[3] indicated that sulfate ions and $MgATP^{2-}$, acting as activators, are also competitive.

Activation of the yeast phosphoglycerate kinase reaction occurred with all the anions tested except for the salicylate.[22] From earlier results[3] it is evident, however, that sulfate ions as activators affect the binding of neither $MgATP^{2-}$ nor 3-P-glycerate to the catalytic site. This might even be true for the other anions. It has been shown that sulfate ions in the activating concentration range modify the shape of the protein molecule, making it more compact.[10] The single SH group of yeast phosphoglycerate kinase is situated in the N-terminal lobe of the enzyme, where a large positively charged crevice exists.[6] Sulfate ions (at activating concentrations), ATP and 3-P-glycerate are each able to separately protect the SH group from reacting with 5,5-dithiobis-2-nitrobenzoate,[10] and the chromophoric properties of the labelled thiol group are affected by anions binding to the enzyme.[7] Besides their activating and inhibiting properties, anions may play very important roles in living organisms, for example by protecting phosphoglycerate kinase from proteolytic cleavage.[26]

The present work has been performed under conditions such that it was impossible to keep the ionic strengths under control (however, see Fig. 1 in Ref. 27 and p. 510 in Ref. 2), and this is probably the reason why inaccurate K_i values were obtained by estimation (not shown). Thus, the importance of this work is more qualitative than quantitative, even though ionic strength effects were shown earlier[27, 2] to have only minor importance. To obtain good crystals for crystallographic work, a high sulfate ion concentration has been a prerequisite, and an inactive form of the enzyme was obtained. As also pointed out by Watson *et al.*,[6] it is important to determine the

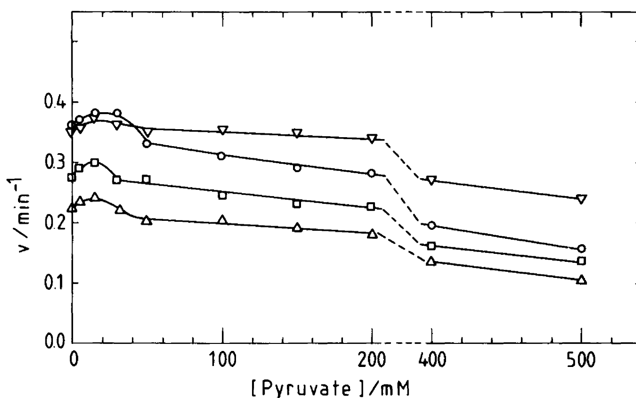


Fig. 5. The effects of pyruvate and sulfate ions on the activity of phosphoglycerate kinase at a high 3-P-glycerate concentration (10 mM) and various fixed sulfate ion concentrations: (▽) 0 mM, (○) 100 mM, (□) 150 mM and (△) 200 mM SO_4^{2-}. The assay mixture contained 0.5 mM ATP/2.0 mM Mg^{2+}. No NaCl was included in the buffer.

conditions necessary for crystallization of the active form of the enzyme. The present findings, possibly in combination with some solution studies on the structure–function relationships of the enzyme, should be helpful in this respect.

Acknowledgements. We wish to thank Mrs. E. Wiksell for helpful discussions and for writing computer programs, and Mrs. B.-M. Karlsson for preparing the enzyme and counter-checking some experiments. This work has been supported in part by the Swedish Council for Forestry and Agricultural Research, and by Sven and Ebba-Christina Hagberg's Foundation.

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Received December 15, 1986.