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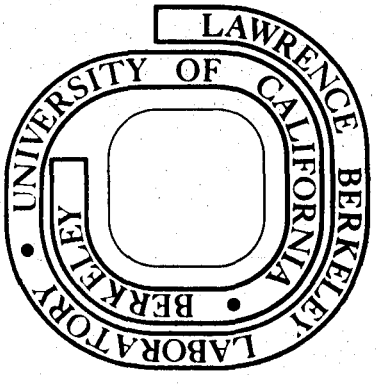

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REGULATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN SPINACH CHLOROPLASTS BY RIBULOSE-1,5-DIPHOSPHATE AND NADPH/$NADP^+$ RATIOS*

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SUMMARY

The activity of glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) from spinach chloroplasts is strongly regulated by the ratio of NADPH/$NADP^+$, with the extent of this regulation controlled by the concentration of ribulose-1,5-diphosphate. Other metabolites of the reductive pentose phosphate cycle are far less effective in mediating the regulation of the enzyme activity by NADPH/$NADP^+$ ratio. With a ratio of NADPH/$NADP^+$ of 2, and a concentration of ribulose-1,5-diphosphate of 0.6 mM, the activity of the enzyme is completely inhibited.

This level of ribulose-1,5-diphosphate is well within the concentration range which has been reported for unicellular green algae photosynthesizing in vivo. Ratios of NADPH/$NADP^+$ of 2.0 have been measured for isolated spinach chloroplasts in the light and under physiological conditions.

Since ribulose-1,5-diphosphate is a metabolite unique to the reductive pentose phosphate cycle and inhibits glucose-6-phosphate dehydrogenase in the presence of NADPH/$NADP^+$ ratios found in cloroplasts in the light, it is proposed that regulation of the oxidative pentose phosphate cycle is accomplished in vivo by the levels of ribulose-1,5-diphosphate, NADPH, and $NADP^+$.

It already has been shown that several key reactions of the reductive pentose phosphate cycle in chloroplasts are regulated by levels of NADPH/$NADP^+$ or other electron-carrying cofactors, and at least one key regulated step, the carboxylation reaction is strongly affected by 6-phosphogluconate, the metabolite unique to the oxidative pentose phosphate cycle. Thus there is an interesting inverse regulation system in chloroplasts, in which reduced/oxidized coenzymes provide a general regulatory mechanism. The reductive cycle is activated at high NADPH/$NADP^+$ ratios where the oxidative cycle is inhibited, and ribulose-1,5-diphosphate and 6-phosphogluconate provide further control of the cycles, each regulating the cycle in which it is not a metabolite.

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Abbreviations: RPP, reductive pentose phosphate; OPP, oxidative pentose phosphate; PMS, phenazine methosulfate; Ribul-1,5-P_2, ribulose-1,5-diphosphate; Ribul-5-P, ribulose-5-phosphate; Rib-5-P, ribose-5-phosphate; Fru-6-P, fructose-6-phosphate; Fru-1,6-P_2, fructose-1,6-diphosphate.

INTRODUCTION

Within chloroplasts of green plant cells, the reductive pentose phosphate cycle (RPP cycle, Calvin cycle) [1] operates during photosynthesis in the light, whereas the oxidative pentose phosphate cycle (OPP cycle, hexose monophosphate shunt) operates under conditions of cell respiration such as in the dark [2,3,4,5]. Although the RPP cycle is driven by ATP and NADPH formed by light reactions in the pigmented

chloroplast membranes, and the OPP cycle generates NADPH by the oxidative metabolism of carbohydrate reserves, mass action by the levels of these cofactors would be an insufficient regulatory mechanism for controlling these two cycles and preventing wasteful reactions. In particular, the free energy change accompanying the oxidation of glucose-6-phosphate to 6-phosphogluconate with the concurrent reduction of $NADP^+$ to NADPH is from -8 Kcal to -12 Kcal, depending on metabolite concentrations [6]. This reaction, mediated by glucose-6-phosphate dehydrogenase is clearly rate-limiting, even during operation of the OPP cycle. No conceivable physiological levels of the reactants and products could control it through mass action [6].

Kinetic tracer studies with unicellular algae demonstrated the immediate appearance of 6-phosphogluconate when the light was turned off [7] or in the light following addition of Vitamin K_5 [2,7]. The primary effect of Vitamin K_5 addition to the algae is thought to be the diversion of electrons coming from the light reactions, resulting in a decreased ratio of $NADPH/NADP^+$, similar to that which occurs when the light is turned off. The appearance of 6-phosphogluconate, which occurs also in isolated spinach chloroplasts upon addition of vitamin K_5, is considered to be an indication of operation of the OPP cycle [2,7].

Other results of kinetic tracer studies with algae and either light-dark changes or Vitamin K_5 addition suggested that activation of glucose-6-phosphate dehydrogenase and of the OPP cycle was accompanied by decreased activities of key regulated steps of the RPP cycle including the conversion of ribulose-5-phosphate to ribulose-1,5-diphosphate, mediated by phosphoribulokinase (E.C.2.7.1.19), the carboxylation reaction mediated by ribulose diphosphate carboxylase (E.C.4.1.1.39), and the conversions of

fructose-1,6-diphosphate and sedoheptulose-1,7-diphosphate to fructose-6-phosphate and sedoheptulose-7-phosphate respectively, mediated by hexose diphosphatase (E.C.3.1.3.11) [2]. Each of these reactions is unique to the RPP cycle, and each is characterized by a large negative free energy change under steady-state conditions in the light [6]. Thus there is an inverse relationship between the rate-limiting step of the OPP cycle (activated at low NADPH/$NADP^+$ ratios) and the rate-limiting steps of the RPP cycle (activated at high NADPH/$NADP^+$ ratios).

Besides these <u>in vivo</u> studies, some investigations of enzymatic activities suggested that NADPH/$NADP^+$ ratios could provide a general regulatory control in chloroplasts. Under certain conditions ribulose diphosphate carboxylase is activated by NADPH [8], while the activity of glucose-6-phosphate dehydrogenase is stimulated by $NADP^+$ [9]. In the case of the ribulose diphosphate carboxylase, however, enzyme activity was also strongly affected by physiological levels of 6-phosphogluconate. Thus, the metabolite unique to the OPP cycle was affecting a reaction unique to the RPP cycle. This led us to consider whether a metabolite unique to the RPP cycle might affect the activity of an enzyme unique to the OPP cycle.

Furthermore, with either darkness or Vitamin K_5 addition, the one metabolite of the RPP cycle which drops to a very low concentration compared with its concentration in the light is ribulose-1,5-diphosphate, the carboxylation substrate. This suggested the possibility that the high level of ribulose-1,5-diphosphate in the light might act to suppress the activity of glucose-6-phosphate dehydrogenase. Finally, Pelroy, et al. [10] found the glucose-6-phosphate dehydrogenase activity of several strains of blue-green algae to be inhibited by physiological levels of

ribulose-1,5-diphosphate. In this report we describe the effect of ribulose-1,5-diphosphate in amplifying the regulatory effects of NADPH/$NADP^+$ ratios on glucose-6-phosphate dehydrogenase activity, and as a function of pH.

MATERIALS AND METHODS

Young spinach leaves of approximately the same age were harvested from outdoor-grown plants after a 16-hour dark period. Chloroplasts were isolated according to the method of Jensen and Bassham [11]. The resulting chloroplast suspension in solution B contained about 2mg chlorophyll/ml. For comparative studies whole chloroplasts from the same batch of leaves were isolated and the soluble chloroplast protein solution was separated from the chloroplast lamellae using the method described by Bassham et al. [12], except that the chloroplasts from one batch were disrupted in 1 ml of solution Z [12]. The isolated chloroplasts and the soluble protein fraction were always kept in the dark at 2°C and were used immediately for the glucose-6-phosphate dehydrogenase assay.

Glucose-6-phosphate dehydrogenase standard assay mixtures contained 36 mM triethanolamine/HCl buffer (pH 7.6), 10 mM $MgCl_2$, 0.6 mM $NADP^+$, 1.0 mM glucose-6-phosphate and 0.1 ml of the chloroplast suspension or 0.2 ml of the solution of chloroplast soluble proteins in a final volume of 2.1 ml. The concentrations of different metabolites of the RPP cycle and of NADPH added to the assay mixture are indicated in the legends. The enzyme assays were carried out under a nitrogen atmosphere and all solutions were flushed with nitrogen before use. The chloroplasts were osmotically destroyed upon being pipetted into the hypotonic assay

solution, thus releasing the glucose-6-phosphate dehydrogenase and other soluble enzymes into the test medium. The reduction of $NADP^+$ was measured spectrophotometrically at 340 nm in quartz cuvettes of 1 cm light path using a Cary 11B spectrophotometer. Assays were performed at 20°C. The reactions were started by addition of the substrate. No detectable increase in absorbance was observed when substrate or $NADP^+$ was omitted. References always were run without substrate. Enzyme activity is expressed as amount of $NADP^+$ reduced per minute and per milligram chlorophyll and is calculated from the initial reaction velocity.

As both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (E.C.1.1.1.43) are present in spinach chloroplasts [13], three assays were performed to show that the presence of 6-phosphogluconate dehydrogenase does not interfere with assaying glucose-6-phosphate dehydrogenase. One assay contained 1.0 mM glucose-6-phosphate and 1.0 mM 6-phosphogluconate, the other two contained all ingredients plus glucose-6-phosphate or 6-phosphogluconate, respectively.

The sum of the initial reaction velocities of both enzymes determined in separate assays was equal to the total initial reaction velocity when both enzymes were assayed simultaneously in the same reaction mixture. Thus under the conditions described above, the presence of 6-phosphogluconate dehydrogenase did not interfere with the measurement of glucose-6-phosphate dehydrogenase activity.

Assay mixtures for the measurement of 6-phosphogluconate dehydrogenase were the same as for glucose-6-phosphate dehydrogenase except 1.0 mM 6-phosphogluconate was substituted for glucose-6-phosphate. Chlorophyll was determined according to Vernon [14].

For the determination of $NADP^+$ and NADPH levels in chloroplasts in the dark and during CO_2 fixation in the light whole choloroplasts were

isolated and were allowed to photosynthesize according to the procedure described by Jensen and Bassham [11]. A fluorometric method for measuring pyridine nucleotide levels in animal tissues described by Cartier [15] has been modified to detect very small amounts of pyridine nucleotides in chloroplasts while using a simple and rapid method for preparing samples from a kinetic experiment with photosynthesizing chloroplasts.

The following procedure was used: 0.02 ml samples were removed from a chloroplast suspension capable of fixing CO_2 in the light and were immediately injected into either 0.05 ml 0.1 N NaOH (to destroy the oxidized pyridine nucleotides and keep the reduced ones) or 0.05 ml 0.1 N HCl (to destroy the reduced pyridine nucleotides and keep the oxidized ones). The resulting samples were then immediately heated at 60°C for 20 min. Within that time the reduced pyridine nucleotides in the acid and the oxidized ones in the base are completely destroyed. After heating, the samples were kept at 0°C. Chloroplast debris were spun down at 12,000 x g for 10 min. The supernatant was used directly for the determination of $NADP^+$ and NADPH. Controls containing internal pyridine nucleotide standards were used for a standard calibration curve.

For the determination of $NADP^+$ and NADPH the following reaction sequence (continuous cycling) was used:

$$\text{Glucose-6-phosphate} + NADP^+ \xrightarrow[\text{dehydrogenase}]{\text{glucose-6-phosphate}} \text{6-phosphogluconate} + NADPH$$

$$NADPH + PMS_{oxyd.} \longrightarrow NADP^+ + PMS_{red.}$$

$$PMS_{red.} + \text{resazurine} \longrightarrow PMS_{oxyd.} + \text{resorufine}$$

The electrons from NADPH are transferred to resazurine, which is reduced to the highly fluorescent resorufine. In this redox chain the amount of resorufine is directly proportional to the amount of $NADP^+$ or NADPH if these coenzymes are present in concentrations lower than the K_m. The assay contained the following components in a final volume of 0.905 ml:

triethanolamine/HCl, pH 7.8, 77 mM (0.7 ml);

$MgCl_2$, 2.2 mM (0.02 ml);

glucose-6-P, 2.2 mM (0.05 ml);

dipyridyl, 14 µM (0.02 ml);

PMS, 0.55 mM (0.05 ml);

resazurine, 10 µM (0.05 ml);

glucose-6-phosphate dehydrogenase, 15 µg dissolved in triethanolamine buffer, 50 mM, pH 7.8 (0.015 ml);

0.005-0.02 ml samples for $NADP^+$ or NADPH determination. The concentrations indicate the final concentrations in the test system.

The optimal values for both the strength of the triethanolamine buffer and the concentration of the cofactor Mg^{2+} were determined by their respective stabilizing and enhancing effects on the enzymatic reaction. The buffer in the concentration used does not quench the fluorescent dye significantly in comparison to final buffer concentrations higher than 80 mM. Moreover, the capacity of the buffer is so high that the addition of the acidic or basic samples does not shift the pH of the test system significantly. In this way it is possible to avoid a procedure in which the samples with the pyridine nucleotides must be neutralized before measuring. Such neutralization procedures are difficult and time consuming because of the small sample sizes and the necessity of avoiding any localized pH "overshoot" which could destroy the pyridine nucleotides being measured.

The reaction was started by addition of the samples containing $NADP^+$ or NADPH after the base line of the recorder was adjusted and straight. The measurements were run in a 10 x 3 mm cuvette (3 mm side turned towards the excitation beam) at 20°C with a Hitachi MPF-2A spectrofluorometer coupled with a Hitachi recorder (sensitivity range 4, 5 or 6). The excitation wavelength was 540 nm with a band width of 8 nm; the increase of fluorescence was followed at 580 nm with a band width of 8 nm. The assay permits the determination of both $NADP^+$ and NADPH. NAD^+ and NADH do not interfere with the test system because NAD^+ cannot be reduced by glucose-6-phosphate dehydrogenase. NADH is of course oxidized by PMS to NAD^+, and PMS_{red} reduces resazurine; however, this reaction is very fast and the increase of resorufine only raises the base line, which is then adjusted before the enzyme reaction with the triphosphopyridine nucleotides is started.

The chemicals were obtained from Calbiochem, Sigma and MCB (resazurine). The acid forms of ribulose-1,5-diphosphate and 6-phosphogluconate were generated from solutions of the respective barium and tri-monocyclohexylammonium salts with Dowex-50.

RESULTS

Glucose-6-phosphate dehydrogenase from whole spinach chloroplasts incubated with 1 mM Ribul-1,5-P_2 was inhibited about 60% in the presence of just saturating amounts of its substrate glucose-6-phosphate and its coenzyme $NADP^+$ (Table I). Other intermediates of the reductive pentose phosphate cycle caused less than 10% inhibition except Ribul-5-P with an inhibitory effect of 18%.

The stability of the inhibitory effect of Ribul-1,5-P_2 over a certain period of time monitored in the test cuvette at 340 nm was directly dependent on the conditions under which the assay was conducted. Since there was a Mg^{2+} concentration of 10 mM in the assay, one had to expect possible CO_2 dark fixation in the presence of CO_2 and Ribul-1,5-P_2 [16] which would reduce the actual Ribul-1,5-P_2 concentration. Under aerobic conditions the inhibition of the enzyme activity by Ribul-1,5-P_2 was indeed slowly decreasing during the assay indicating the consumption of Ribul-1,5-P_2 by the carboxylation and/or oxygenase reaction. A further addition of Ribul-1,5-P_2 to the assay mixture during the assay brought the amount of inhibition back to the original value. Under anaerobic assay conditions, achieved under a nitrogen atmosphere, the inhibition remained stable during the time the enzyme activity was monitored. Therefore all assays were conducted under a nitrogen atmosphere and with solutions previously flushed with nitrogen. The anaerobic environment had no effect on glucose-6-phosphate dehydrogenase activity.

As the whole chloroplasts were lysed in the assay mixture, releasing glucose-6-phosphate dehydrogenase and the other soluble components from the compartment within the outer chloroplast membranes, the lamellae also remained in the test solution. This fact caused the problem of inhomogenity as the lamellae slowly sank to the bottom of the cuvette changing slightly the intensity of the 340 nm light beam going through the cuvette. This disadvantage could be eliminated by either using a reference cuvette without substrate or by using a soluble chloroplast protein solution without the lamellae as the enzyme source. The effects of different metabolites of the reductive pentose phosphate cycle on glucose-6-phosphate dehydrogenase in the soluble protein solution alone are not significantly different from those obtained by lysing whole chloroplasts in the assay mixture (Table I). As we added the same amount of enzyme to both kinds

of assay, these results are not unexpected. In preliminary experiments (data not shown) we already could show that the affinity of glucose-6-phosphate dehydrogenase for substrate and coenzyme did not change due to the different way of handling the enzyme before assaying. For all further experiments we used the soluble chloroplast protein solution as a source for glucose-6-phosphate dehydrogenase.

6-phosphogluconate dehydrogenase, the second key enzyme in the OPP cycle is unaffected by Ribul-1,5-P_2 and the other metabolites tested and was therefore not the subject of further investigations.

The inhibition of glucose-6-phosphate dehydrogenase by 1 mM RuDP could be demonstrated in the presence of just saturating substrate and coenzyme concentrations. However, under physiological conditions the concentration of the coenzyme $NADP^+$ is subject to drastic changes within the chloroplasts [17]. Fig. 1 illustrates the change of enzyme activity at decreasing $NADP^+$ concentrations and increasing Ribul-1,5-P_2 levels. Both decreasing the coenzyme concentration and increasing the Ribul-1,5-P_2 concentration results in a decrease in enzyme activity. In other words the lower the coenzyme concentration the more effective is the inhibition by Ribul-1,5-P_2. At a saturating $NADP^+$ concentration of 0.6 mM the enzyme activity can be inhibited to 50% by 0.7 mM Ribul-1,5-P_2, at 0.01 mM $NADP^+$ it can be inhibited to 50% by only 0.25 mM Ribul-1,5-P_2; that is an inhibition of about 90% of the activity found at saturating $NADP^+$ concentrations and without the addition of Ribul-1,5-P_2.

In a plot of 1/v versus 1/[$NADP^+$] at different levels of Ribul-1,5-P_2 using the data from Fig. 1 it can be shown that the inhibition of chloroplast glucose-6-phosphate dehydrogenase by Ribul-1,5-P_2 gives a noncompetitive inhibition pattern in respect to the coenzyme $NADP^+$ (Fig. 2). The K_m value for the coenzyme is calculated from the figure to be 55 µM. At that

$NADP^+$ concentration (half-saturating) there is about 50% of the enzyme activity at saturating concentrations. This can be decreased to 25% by addition of 0.5 mM Ribul-1,5-P_2.

The activity of glucose-6-phosphate dehydrogenase is, however, not only determined by the actual concentration of its oxidized coenzyme $NADP^+$ but also by the concentration of the reduced one [9]. NADPH is a competitive inhibitor of the enzyme activity in respect to the oxidized $NADP^+$ (Fig. 3).

To demonstrate how $NADP^+$ and NADPH levels are changing in intact spinach chloroplasts during dark-light-dark transitions, these two coenzymes were measured in chloroplasts which were allowed to photosynthesize. The levels of $NADP^+$ and NADPH levels are subject to very fast changes after a dark-light or a light-dark transition (Fig. 4). In the light $NADP^+$ is reduced very rapidly to NADPH. After about 1 min the levels of NADPH and $NADP^+$ reach steady-state values. Upon switching off the light a rapid oxidation of NADPH takes place. The changes of the NADPH level in the chloroplasts are always just opposite to those of the $NADP^+$ level. The NADPH/$NADP^+$ ratios are about 0.2-0.5 in the dark but increase after a dark-light transition very fast up to 1.5-2.5 depending on a previous dark phase or the pattern of dark-light-dark alternation (Fig. 4).

The antagonistic effect of $NADP^+$ and NADPH on chloroplast glucose-6-phosphate dehydrogenase is demonstrated in Fig. 5, where the enzyme activity is plotted against different ratios of NADPH/$NADP^+$. At pH 7.6 and a ratio of 1 the enzyme activity is inhibited up to about 50%. A complete inhibition can be reached at a ratio of 2.4 and higher. At pH 8.2, an increasing ratio decreases the enzyme activity much faster than at pH 7.6. The inhibition is already complete at a NADPH/$NADP^+$ ratio of 1.5. Full

enzyme activity is maintained at ratios up to about 0.25 at both pH values.

In a further experiment we were interested in the change of glucose-6-phosphate dehydrogenase activity in the presence of certain NADPH/$NADP^+$ ratios and by adding increasing amounts of Ribul-1,5-P_2. For this experiment we chose ratios of 0.5, 1.0, and 2.0 at a pH value of 7.6 (Fig. 6). Under these conditions Ribul-1,5-P_2 became an even more effective inhibitor in the presence of increasing NADPH/$NADP^+$ ratios. At a ratio of 1 the enzyme activity is decreased by about 50% (cf. Fig. 5) corresponding to the same enzyme activity at a half-maximal concentration of $NADP^+$ in the absence of NADPH (cf. Fig. 1 and 2). In the latter case a concentration of 0.5 mM Ribul-1,5-P_2 was necessary for the inhibition of the enzyme activity up to 75%, while at a ratio of 1, a concentration of 0.3 mM Ribul-1,5-P_2 has already the same inhibitory effect. At high NADPH/$NADP^+$ ratios such as exist in chloroplasts in the light _in vivo_ [17], the inhibitory effect of Ribul-1,5-P_2 becomes even stronger. Addition of 0.5 mM Ribul-1,5-P_2 at a ratio of 2 brings the inhibition to nearly 100%.

With an increase in pH of the assay mixture to a value of 8.2, the relative inhibition by Ribul-1,5-P_2 within a concentration range from 0.1-1.0 mM is not different from that at pH 7.6 (Fig. 7). Even with an NADPH/$NADP^+$ ratio of 0.5 there is no difference in the relative inhibition at the two pH values. However, the inhibitory effect of Ribul-1,5-P_2 increases at higher ratios. This effect is even more severe at the high pH (compare the two curves for the ratio of 1 in Fig. 6 and 7). Thus, Ribul-1,5-P_2 is a most effective inhibitor for glucose-6-phosphate dehydrogenase at high NADPH/$NADP^+$ ratios and in a slightly alkaline medium.

DISCUSSION

In an earlier study [6] standard physiological free energy changes of reactions of the reductive and oxidative pentose phosphate cycle were measured during steady-state photosynthesis in Chlorella pyrenoidosa. Using the criterion of large negative free energy changes as an indicator for reactions catalized by regulated enzymes, the oxidation of glucose-6-phosphate, mediated by glucose-6-phosphate dehydrogenase, with a free energy change of -8.4 kcal was already considered a regulated step in the oxidative pentose phosphate cycle in chloroplasts. Direct measurements of the enzyme activity in spinach chloroplasts have shown that the enzyme is activated in the dark and inhibited up to 70-80% in the light [9]. This inhibition in the light occurs within the first minute after a dark-light transition and is completed after 1-2 min illumination.

Another indication of the different activities of glucose-6-phosphate dehydrogenase in light and darkness is the immediate appearance of 6-phospho-gluconate acid in the dark or with vitamin K_5 addition in a substantial amount in tracer studies with spinach chloroplasts [2] or Chlorella pyrenoidosa [7]. This intermediate was not detectable in the light, without vitamin K_5 addition. These findings led to the concept of exclusive operation of the oxidative pentose phosphate cycle in the dark and of the reductive pentose phosphate cycle in the light [7].

From results first indicated in an earlier communication [9] and further elaborated above, it is clear that the general regulation of the activity of glucose-6-phosphate dehydrogenase is determined by the ratio of the reduced to the oxidized state of its coenzyme $NADP^+$. However, the reaction in vivo, with a ΔG^S of -8 to -12 Kcal, is essentially irreversible, and the concentrations of reactants and substrates have little

or no effect on rate via mass action, since all are in the 0.01 mM to 1.0 mM range. Thus a specific regulatory effect of NADPH and $NADP^+$ on enzyme activity is required.

As the outer chloroplast membranes are impermeable towards pyridine nucleotides, the total concentration of oxidized plus reduced pyridine nucleotides is quite constant under certain physiological conditions in chloroplasts of different sources [17]. However, as the present study shows, the ratio of NADPH/$NADP^+$ in spinach chloroplasts undergoes drastic changes after dark-light or light-dark transitons. Upon illumination of whole chloroplasts a rapid reduction of $NADP^+$ to NADPH by photoelectrons occurs, increasing the NADPH/$NADP^+$ ratio. This ratio changes from about 0.1-0.3 in the dark up to 2.0-2.5 in the light and drops again to its original value after a light-dark transition. In contrast to an earlier report by Heber and Santarius [17], the present study shows that the ratios in whole chloroplasts determined during a dark or light period are quite constant over many minutes. The ratio seems to fall off slightly in a longer light period always accompanied by a slight levelling off of the CO_2 fixation rate. It appears that the maintenance of a constant fixation rate is directly correlated with at least the ability of the photoelectron flux to keep a certain concentration of $NADP^+$ in its reduced form. It can be assumed that the NADPH/$NADP^+$ ratio is kept quite constant <u>in vivo</u> maintaining the highest level of reducing power for reducing 3-phosphoglycerate and for regulating enzyme activities. The total amount of NADPH plus $NADP^+$ in whole chloroplasts illuminated or kept in the dark remains almost unchanged, indicating that in our chloroplast preparations neither a light-induced conversion of NAD^+ to $NADP^+$ reported by Ch-Hama and Miyachi [19] occurs

nor is an active phosphatase present which cleaves either $NADP^+$ or NADPH to NAD^+ or NADH [20]. As the sum of the triphosphopyridine nucleotides concentration in our measurements is constant, the occurence of both kinase and phosphatase at the same time would at least have no effect on the overall value of the NADPH/$NADP^+$ ratio and the total amount of triphosphopyridine nucleotides.

The NADPH/$NADP^+$ ratios determined in whole chloroplasts during a light or dark period can be simulated in reconstituted chloroplast preparations capable of photosynthesis [12]. In such preparations, all enzymes of the reductive and oxidative pentose phosphate cycle are present as well as the chloroplast lamellae system. To this system is added known amounts of NADPH and $NADP^+$. In this system, glucose-6-phosphate dehydrogenase activity is a direct reflection of the NADPH/$NADP^+$ ratio present in the assay mixture. In the presence of high ratios (up to 2.5) corresponding to conditions during illumination in whole chloroplasts, the enzyme activity is strongly or totally inhibited, while with low ratios between 0.1-0.5 corresponding to the redox state in vivo during the dark, the enzyme is active. Ratios between these two ranges are passed quickly during a light-dark transition or dark-light transition.

In whole chloroplasts, the kinetics of the changes of the NADPH/$NADP^+$ ratio after turning off the light are similar to those of the appearance of 6-phosphogluconic acid in the dark in Chlorella [7], indicating the operation of glucose-6-phosphate dehydrogenase. A similar correlation can be seen between the kinetics of glucose-6-phosphate dehydrogenase inhibition (as indicated by 6-phosphogluconate disappearance) and increase in the NADPH/$NADP^+$ ratio after a dark-light transition.

It was reported earlier that the inhibition of glucose-6-phosphate dehydrogenase in spinach chloroplasts is complete after 20 min illumination [9]. However with the improved technique for assaying the enzyme reported here we now see that the inhibition of the enzyme activity is complete after 1-2 min light. This time period is the same as that in which the ratio NADPH/$NADP^+$ in chloroplasts increases rapidly and reaches the level in the light and 6-phosphogluconate disappears in Chlorella in light.

In simulating the NADPH/$NADP^+$ ratio measured in whole chloroplasts, the question of the proper concentrations of NADPH and $NADP^+$ arose. As we do not know the exact volume of the chloroplast compartment, we can only roughly estimate the total triphosphopyridine nucleotides concentration to be between 0.05-1.0 mM. Since we chose concentrations in the upper part of this range and since the reported inhibition by Ribul-1,5-P_2 becomes relatively stronger with decreasing $NADP^+$ concentrations, all the described inhibitions should be even stronger at lower $NADP^+$ concentrations. For glucose-6-phosphate and the Mg^{+2} those concentrations were used which gave the maximal velocity of enzyme activity under our assay conditions. A concentration of 0.4 mM Ribul-1,5-P_2 during steady-state photosynthesis inside the chloroplasts of photosynthesizing Chlorella pyrenoidosa has been estimated, based on steady-state levels of ^{14}C found during photosynthesis with $^{14}CO_2$ [6]. Thus, a concentration in the range of 0.1-1.0 mM RuDP in spinach chloroplasts photosynthesizing in air (0.03% CO_2) may be within the normal physiological range.

Another set of compounds undergoing change in concentration between light and dark are the adenylates. It is known that ATP is involved in the regulation of glucose-6-phosphate dehydrogenase in several

organisms [21-24]. However, ATP concentration has no effect on the enzyme activity in spinach chloroplasts (Table I). Experiments with vitamin K_5 added to spinach chloroplasts demonstrated that while the oxidative pentose phosphate cycle is activated upon addition of the vitamin in the light, the ATP level is almost unaffected [2]. Different ATP concentrations in the presence of NADPH also have no inhibitory effect. Thus there is no evidence for a regulatory participation of ATP in the operation of the oxidative pentose phosphate cycle in spinach chloroplasts.

Other key steps in the reductive cycle may also be affected by the redox level of cofactors in the chloroplasts. The addition of vitamin K_5 to Chlorella, diverting electrons and inducing the operation of the OPP as revealed by immediate 6-phosphogluconate appearance, also caused a sudden rise in fructose-1,6-diphosphate and sedoheptulose-1,7-diphosphate [2]. This is indicative of an inactivation of the respective diphosphatase activities. The hydrolyses of these two sugar diphosphates to their monophosphates have been identified as key regulated steps of the RPP cycle from a variety of kinetic and enzymatic evidence [2,5,7]. Moreover, now it appears that the conversion of ribulose-5-phosphate to ribulose-1, 5-diphosphate, mediated by phosphoribulokinase, also stops rapidly in the dark, or upon diversion of electrons with vitamin K_5 [2,5]. There thus appears to be a general regulatory mechanism in chloroplasts in which the ratio of reduced to oxidized cofactors controls the activities of the principal regulated steps of the OPP and RPP cycles. While $NADPH/NADP^+$ ratio is implicated in the glucose-6-phosphate dehydrogenase and ribulose diphosphate carboxylase mediated reactions, control of phosphoribulokinase and of hexose/heptose diphosphate mediated steps may be via the reduced to oxidized ratio of some other cofactor such as ferredoxin [25,26]. It is

possible that another general regulatory factor could be the ratio of adenylates, since ATP/ADP ratio, or "energy-charge" is important in other systems. However, there is little evidence for this in spinach chloroplasts to date.

Besides the general regulatory mechanism, it is now clear that there are some specific metabolite controls. Thus Ribul-1,5-P_2 effects the glucose-6-phosphate dehydrogenase only in the light and 6-phosphogluconate effects ribulose diphosphate carboxylase only under dark conditions, since these fine regulators are only present in measurable and effecting amounts under certain physiological conditions (light or dark). Both NADPH and $NADP^+$ have the same binding site on the glucose-6-phosphate dehydrogenase molecule, competing each other for this site. Ribul-1,5-P_2 is a noncompetitive inhibitor with respect to $NADP^+$, and thus shows the characteristics of allosteric binding, effecting the coenzyme binding site in favoring the binding of NADPH in the presence of Ribul-1,5-P_2. This explains the increasing inhibition of the enzyme activity by Ribul-1,5-P_2 at increasing NADPH/$NADP^+$ ratios. It further illustrates one kind of interaction between a general regulator and a specific regulator. The specific regulator, Ribul-1,5-P_2 in this case, amplifies the already present control by NADPH/$NADP^+$ ratio.

Among the intermediates of the RPP cycle, Ribul-1,5-P_2 is the only effective inhibitor of glucose-6-phosphate dehydrogenase in spinach chloroplasts. Its inhibitory effect must play an important physiological role in the chloroplasts metabolism in respect to the regulation of the oxidative pentose phosphate cycle. The slight inhibition by ribulose-5-phosphate found in the incubation experiments with the chloroplast enzymes (Table I) is perhaps due to some endogenous phosphorylation, mediated by

phosphoribulose kinase. During a dark period the NADPH/$NADP^+$ ratio is low, and thus the rate of the oxidative pentose phosphate cycle is uninhibited. Furthermore the level of Ribul-1,5-P_2 will be very low or zero [6]. Under such conditions, the oxidation of glucose-6-phosphate proceeds at a substantial rate. The reaction still is not reversible, and remains the rate limiting step for the OPP cycle. Given the large negative free energy change for the reaction [6], the concentration of glucose-6-phosphate would drop below the limits of detection if the oxidation of glucose-6-phosphate became very rapid. This does not occur although there is a drop to about 1/2 of the light steady state value [6]. Since the binding constant of 6-phosphogluconic acid dehydrogenase is about 0.09 mM [27], the concentration of 6-phosphogluconate rises to an appreciable level: about 0.07 mM in Chlorella pyrenoidosa [6]. This explains the appearance of labelled 6-phosphogluconic acid as an indicator of the operation of the oxidative cycle [2].

At the beginning of a light period $NADP^+$ is quickly reduced and ADP is phosphorylated resulting in a high NADPH/$NADP^+$ and ATP/ADP ratio. The high NADPH/$NADP^+$ ratio slows the activity of glucose-6-phosphate dehydrogenase immediately. Simultaneously the 6-phosphogluconate level decreases rapidly and can't further serve as an activator of ribulose diphosphate carboxylase but is replaced by NADPH as an activator [28]. The increased level of reduced cofactors apparently activates phosphoribulokinase, and with ATP produced by light reactions, ribulose-5-phosphate is converted to ribulose-1,5-diphosphate. This substrate for the carboxylation reaction also serves to inhibit further the oxidation of glucose-6-phosphate (Fig. 5-7). Moreover, the pH in the stroma increases in the light [29] due to proton pumping into the thylakoids [30]. This higher pH amplifies the inhibitory

effect of Ribul-1,5-P_2 and the high $NADPH/NADP^+$ ratio. In this way the OPP cycle is turned off and the RPP cycle is turned on without the occurence of wasteful reactions.

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TABLE I

THE EFFECT OF DIFFERENT METABOLITES OF THE RPP CYCLE AND ATP ON THE ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

The assay mixture contained 10 mM $MgCl_2$, 0.6 mM $NADP^+$, 1 mM glucose-6-phosphate and 0.1 ml or 0.2 ml chloroplast suspension or chloroplast soluble protein solution, respectively, in 36 mM triethanolamine/HCl buffer. The compounds were added with the enzyme. After mixing all ingredients the reaction was started after 1 minute. The control corresponds to an enzyme activity of 0.045 moles $NADP^+$ reduced x mg^{-1} chlorophyll x min^{-1} (= 100%) in both assays with whole chloroplasts or protein solution of the reconstituted system. The data results from at least four independent experiments.

Compound		% of Control (whole chloroplasts)	% of Control (Protein solution of reconstituted system)
control		100	100
Fru-1,6-P_2	1mM	91	92
Fru-6-P	1mM	103	99
PGA	1mM	94	95
Ru-5-P	1mM	97	95
Ribul-5-P	1mM	82	84
Ribul-1,5-P_2	1mM	38	40
ATP	1mM	96	99

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FIGURE CAPTIONS

Fig. 1. Inhibition of chloroplast glucose-6-phosphate dehydrogenase by Ribul-1,5-P_2 at different $NADP^+$ concentrations. Concentrations of $NADP^+$ are indicated in the figure. Coenzyme and inhibitor were added to the assay mixture before 0.2 ml of soluble protein solution was added. The controls without Ribul-1,5-P_2 corresponds to enzyme activities of 0.043, 0.026, 0.020 and 0.010 µmoles $NADP^+$ reduced x mg^{-1} chlorophyll x min^{-1} at $NADP^+$ concentrations of 0.600, 0.090, 0.045 and 0.010 mM, respectively.

Fig. 2. Inhibition of chloroplast glucose-6-phosphate dehydrogenase by Ribul-1,5-P_2 at different $NADP^+$ concentrations. Concentrations of Ribul-1,5-P_2 are indicated in the figure. The enzyme solution was added after all ingredients except the substrate were in the assay mixture. The reaction was started by adding the substrate.

Fig. 3. Inhibition of chloroplast glucose-6-phosphate dehydrogenase by NADPH at different concentrations of $NADP^+$. Concentrations of NADPH are indicated in the figure. All other ingredients of the assay mixture are those of the standard assay mixture.

Fig. 4. Changes in the levels of $NADP^+$ and NADPH in intact spinach chloroplasts in a dark-light-dark-light transition. The dark periods are indicated by black bars. $NaHCO_3$ in a final concentration of 6 mM was added during the first light period at 5 min (indicated by arrow). The NADPH/$NADP^+$ ratios are indicated in the lower part of the graph. The fixation rate of $^{14}CO_2$ into acid-stable products was 116 µmoles x mg^{-1} chlorophyll x h^{-1}.

Fig. 5. Inhibition of chloroplast glucose-6-phosphate dehydrogenase activity at different NADPH/$NADP^+$ ratios and at two different pH values (•—• pH 7.6; o—o pH 8.2). The concentration of $NADP^+$ was kept constant at 0.6 mM, while the concentration of NADPH was changed giving the ratios indicated in the figure. The two controls without NADPH were considered as 100% and correspond to enzyme activities of 0.045 (at pH 7.6) and 0.039 (at pH 8.0) µmoles $NADP^+$ reduced x mg^{-1} chlorophyll x min^{-1}.

Fig. 6. Inhibition of chloroplast glucose-6-phosphate dehydrogenase by Ribul-1,5-P_2 at different NADPH/$NADP^+$ ratios. The concentration of $NADP^+$ was kept constant at 0.6 mM while the concentration of NADPH was changed giving the following ratios: •—• without NADPH; x—x NADPH/$NADP^+$ = 0.5; ∘—∘ NADPH/$NADP^+$ = 1.0; □—□ NADPH/$NADP^+$ = 2.0. The assays were run at pH 7.6. The controls (= 100%) without Ribul-1,5-P_2 correspond to enzyme activities of 0.046, 0.034, 0.022, and 0.006 µmoles $NADP^+$ reduced x mg^{-1} chlorophyll x min^{-1} for the ratio of 0, 0.5, 1.0, and 2.0, respectively.

Fig. 7. Inhibition of chloroplast glucose-6-phosphate dehydrogenase by Ribul-1,5-P_2 at NADPH/$NADP^+$ ratios of 0.0 (•—•); 0.5 (x—x); 1.0 (o—o). The assays were run at pH 8.2. The controls (= 100%) without Ribul-1,5-P_2 correspond to enzyme activities of 0.039, 0.024, and 0.012 µmoles $NADP^+$ reduced x mg^{-1} chlorophyll x min^{-1} for the ratios of NADPH/$NADP^+$ of 0.0, 0.5, and 1.0, respectively. The other test conditions were the same as in Fig. 5.

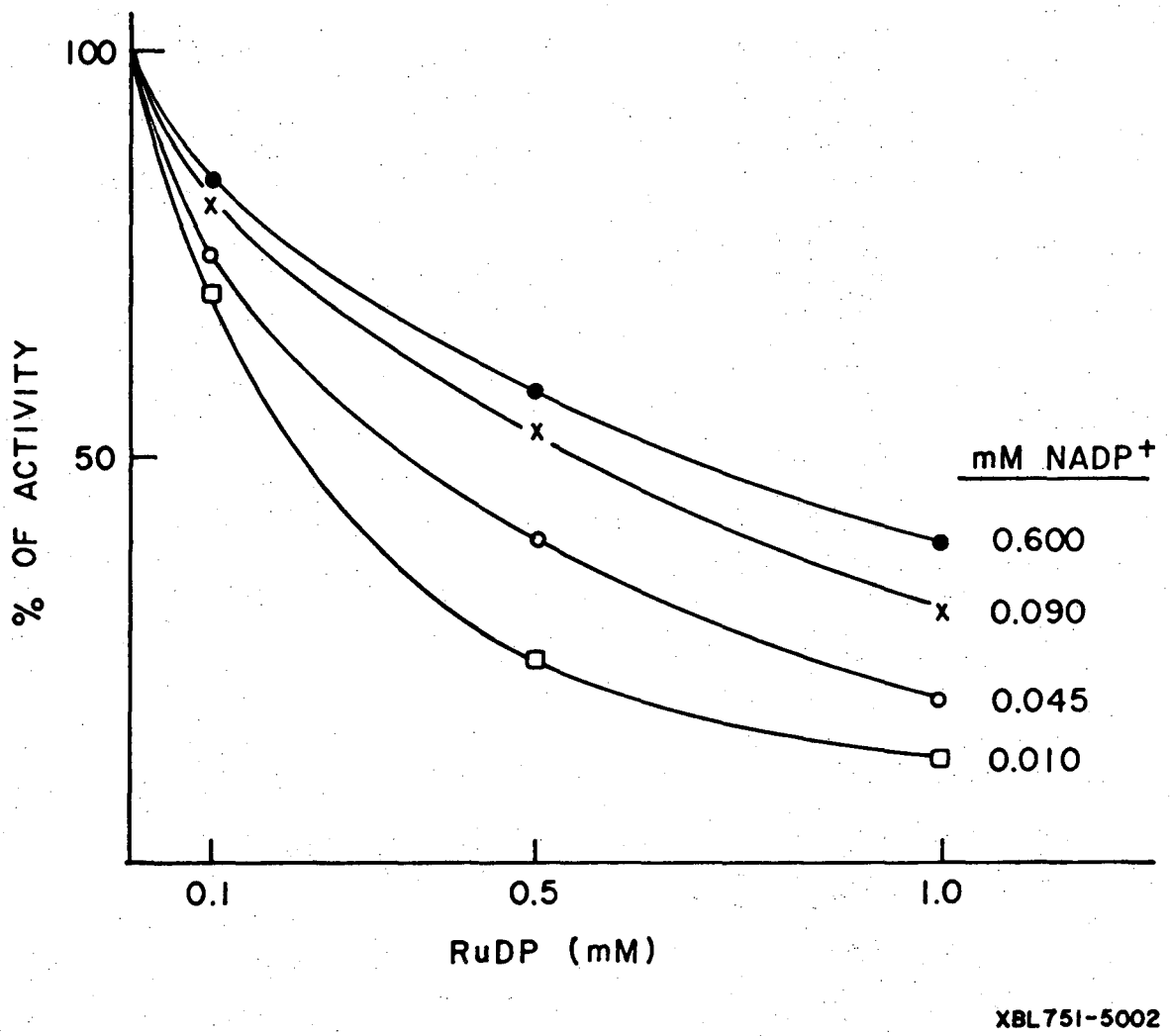
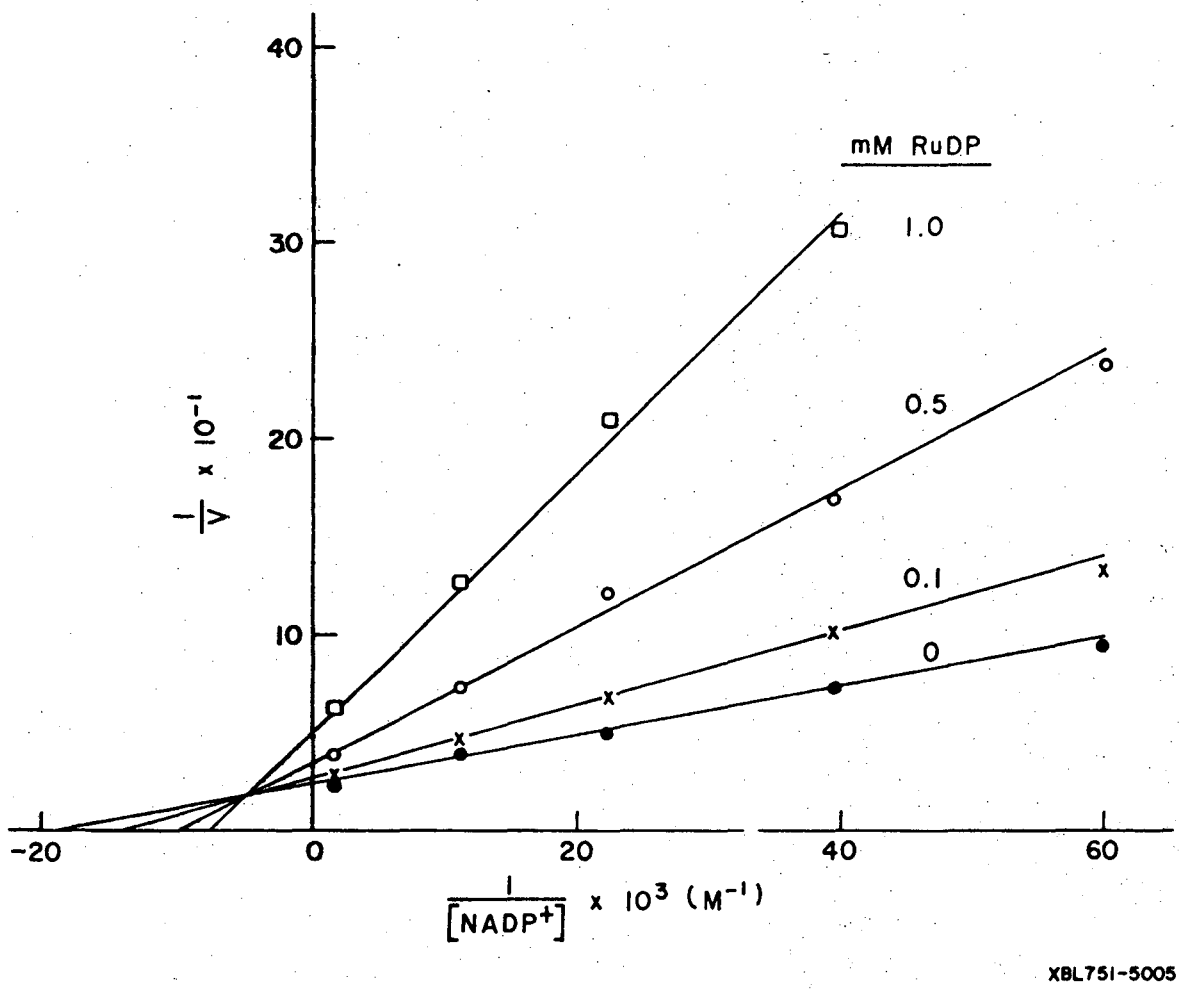


Fig. 1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30
20
10
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0.1
0
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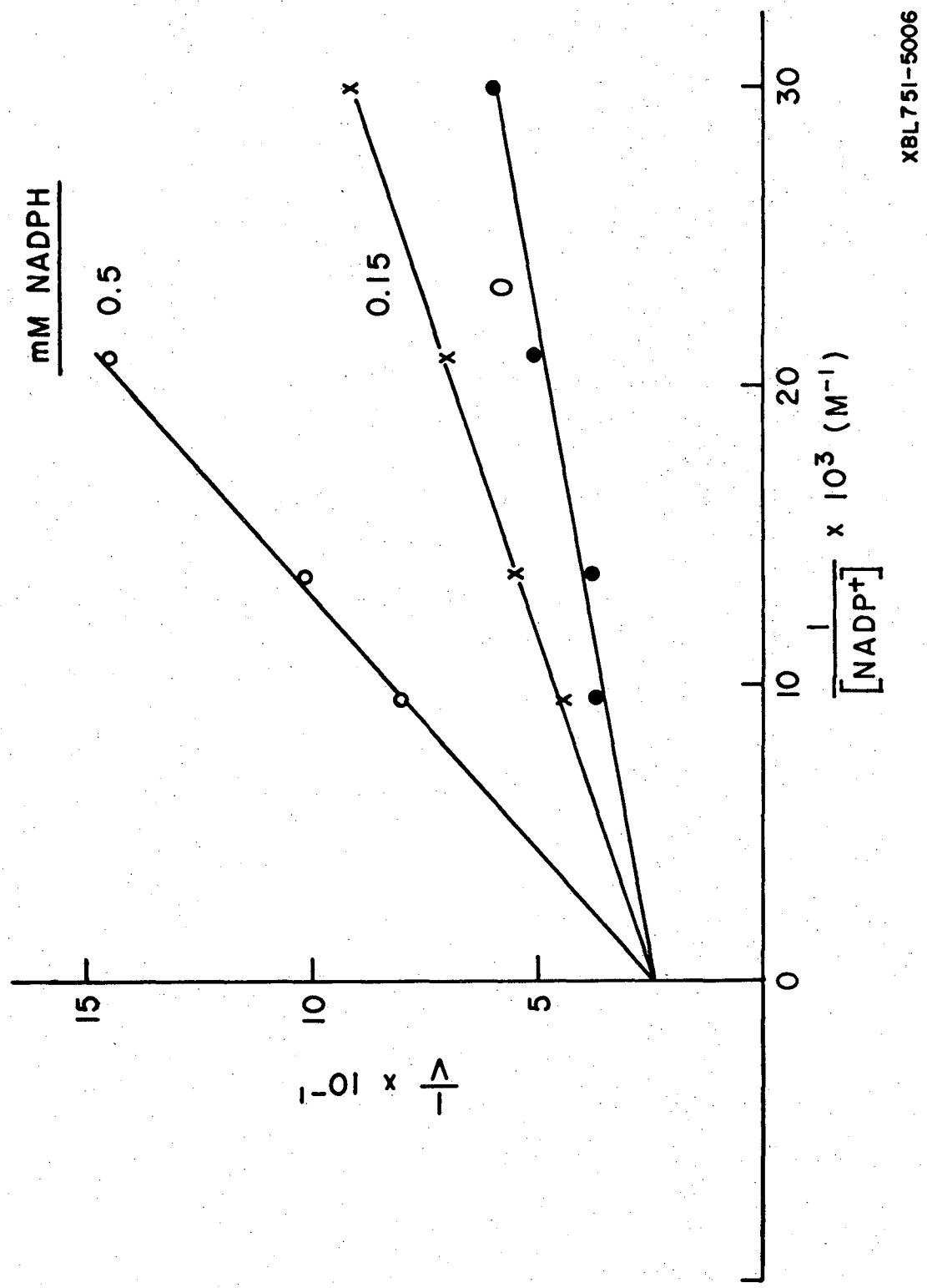
-20
0
20
40
60
$\frac{1}{[NADP^+]} \times 10^3 \ (M^{-1})$
XBL751-5005

Fig. 2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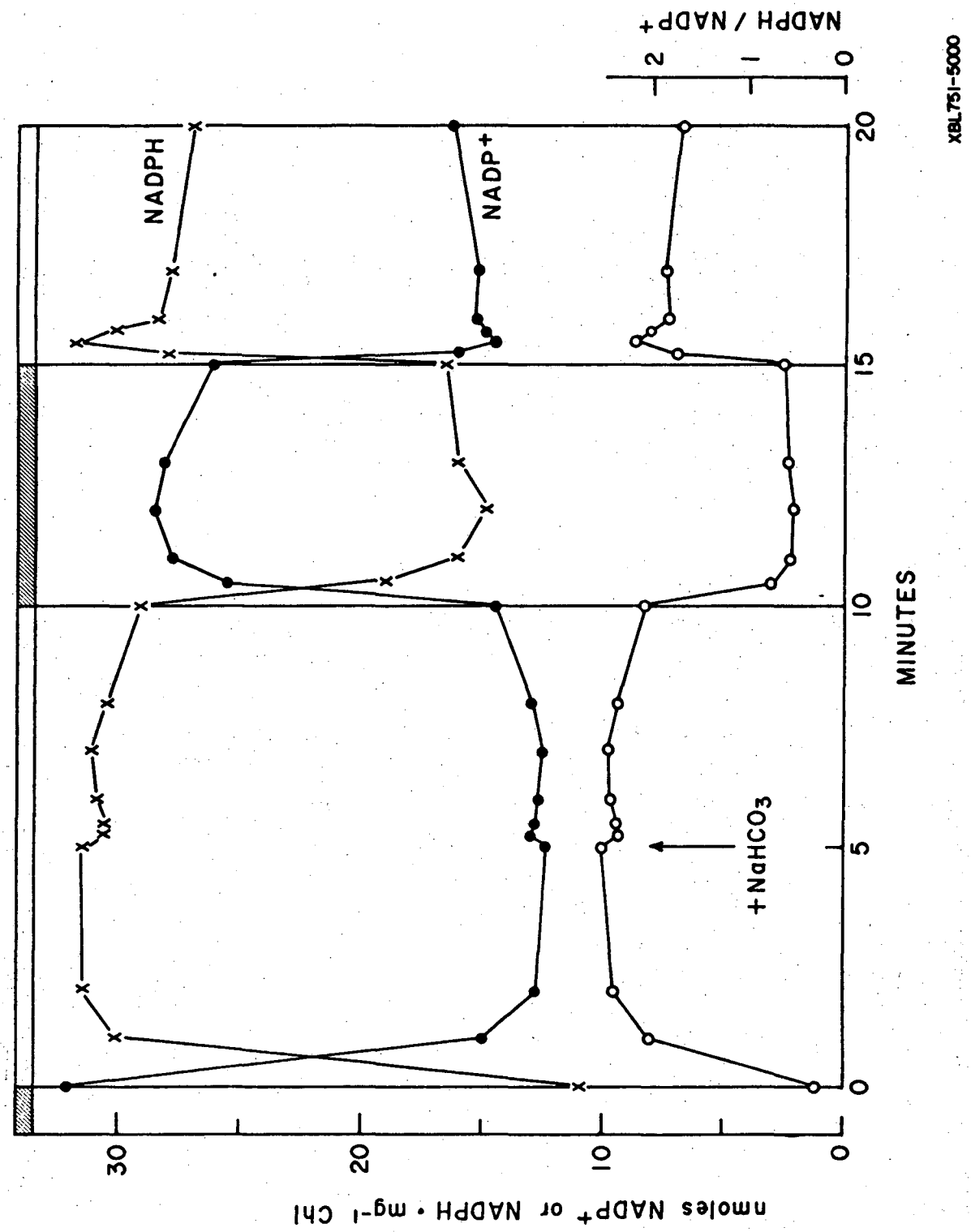
0.15
0
15
10
5
0
10
20
30
$\frac{1}{[NADP^+]} \times 10^3 \ (M^{-1})$
$\frac{1}{v} \times 10^{-1}$
XBL751-5006

Fig. 3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20
30
NADPH
NADP+
+NaHCO3
0
5
10
15
20
MINUTES
NADPH / NADP+
0
1
2
XBL751-5000

Fig. 4

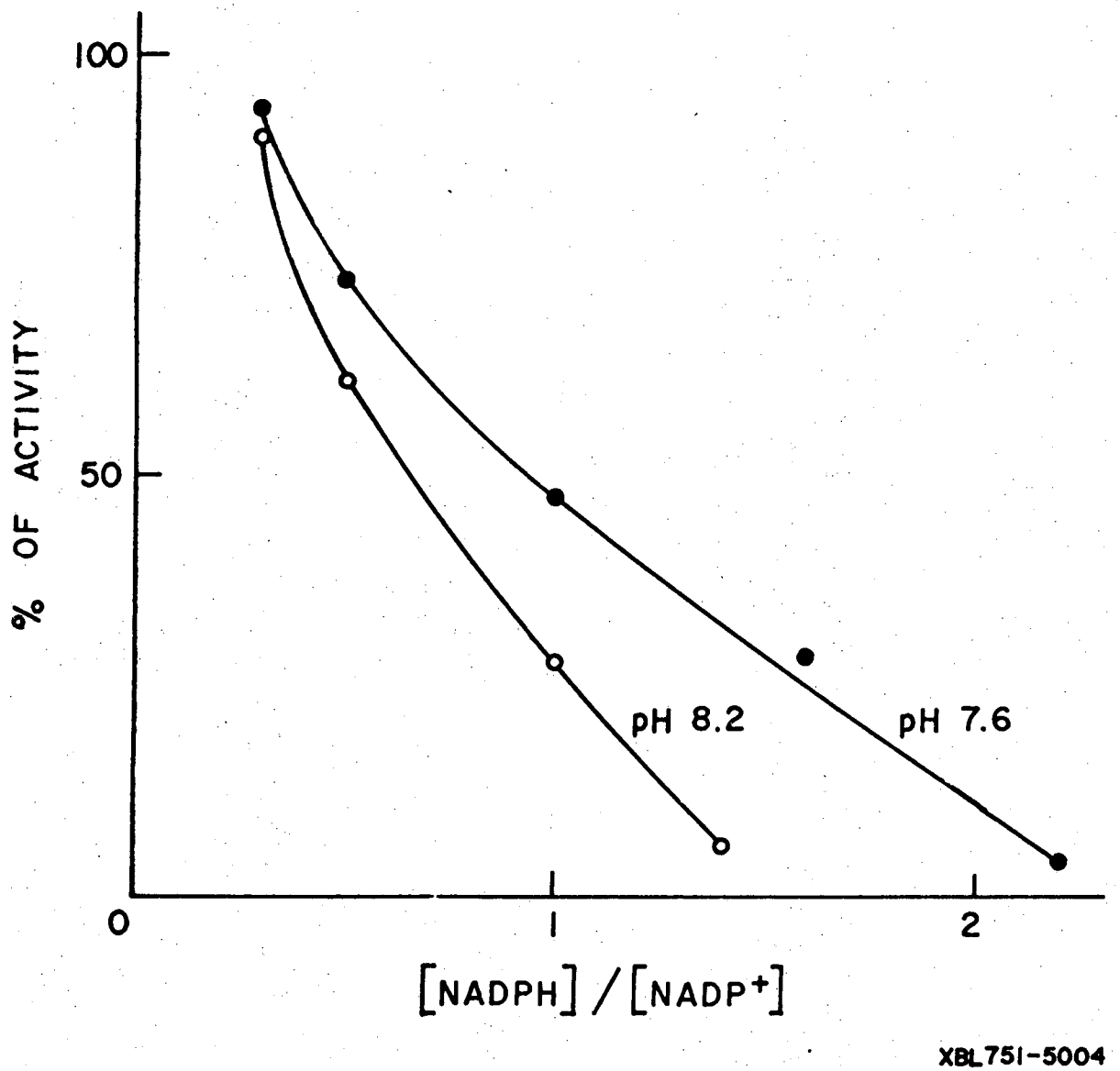

Fig. 5

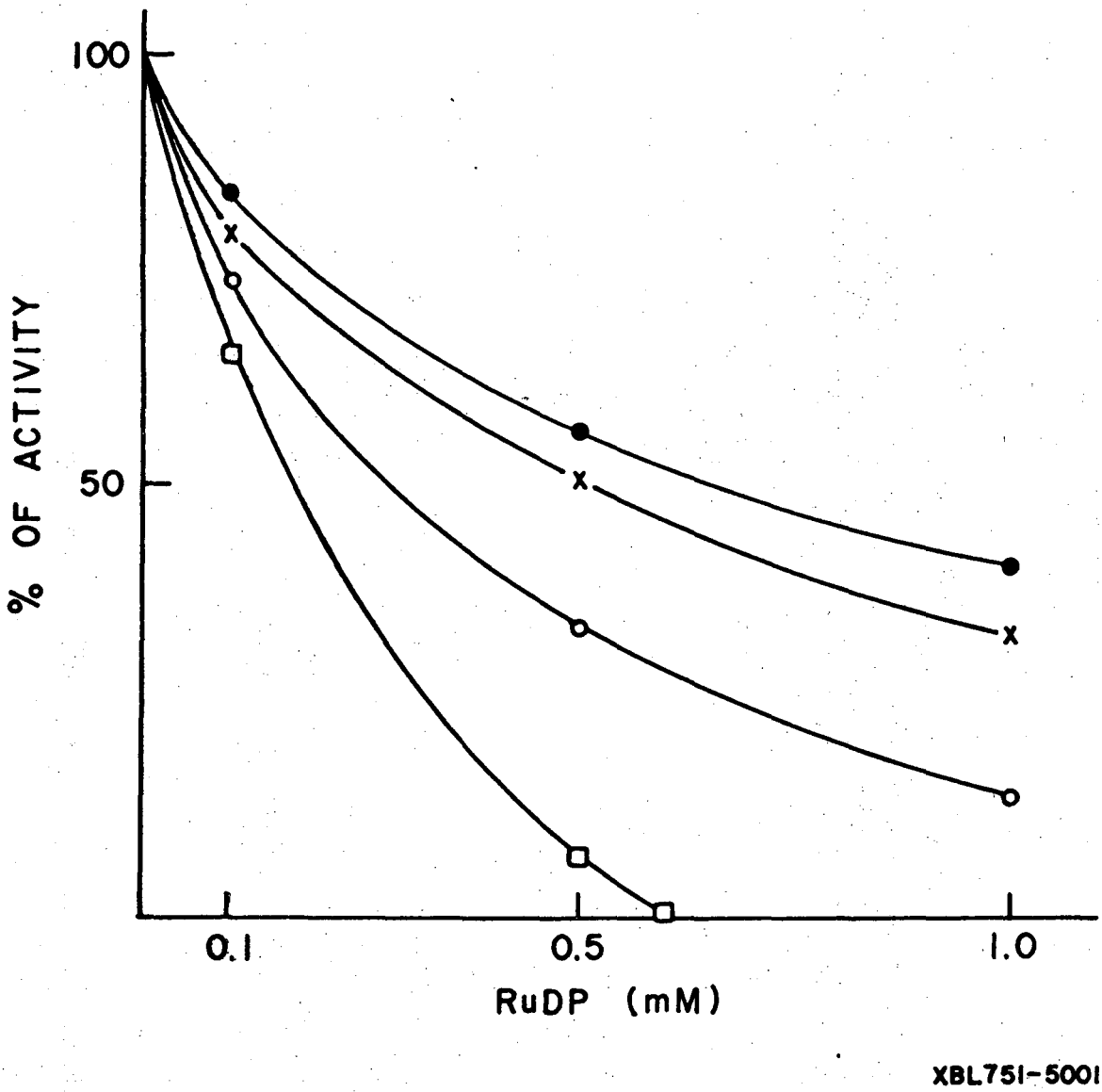

Fig. 6

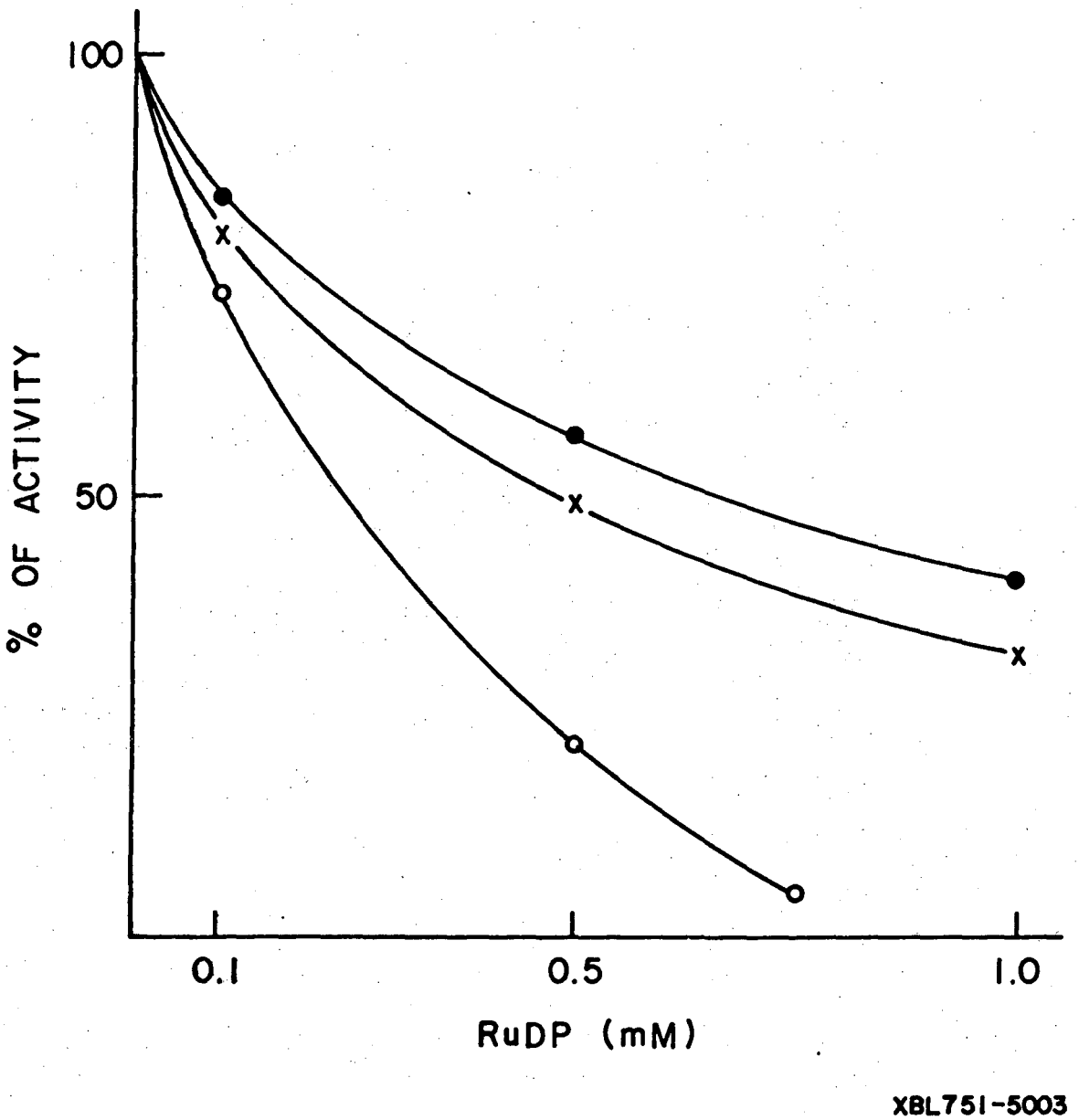

Fig. 7

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