

Regulation of the Oxidative Phase of the Pentose Phosphate Cycle in Mussels

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1. The mechanisms that control the oxidative phase of the pentose phosphate cycle in mussel hepatopancreas were investigated. 2. The effects of GSSG (oxidized glutathione) on the inhibition of glucose 6-phosphate dehydrogenase by NADPH [Eggleston & Krebs (1974) *Biochem. J.* 138, 425-435] extend to 6-phosphogluconate dehydrogenase. 3. The effect of GSSG on both enzymes increases as the $[NADP^+]/[NADPH]$ ratio decreases; greater percentage deinhibition always was obtained for 6-phosphogluconate dehydrogenase. 4. Increasing concentration of GSSG increased the percentage deinhibition. This effect is more pronounced with 6-phosphogluconate dehydrogenase. 5. We confirmed the apparent imbalance between the activities of the two enzymes [Sapag-Hagar, Lagunas & Sols (1973) *Biochem. Biophys. Res. Commun.* 50, 179-185] in the presence of 10 mM-Mg^{2+} . 6. The imbalance practically disappears when the substrate concentrations are less than saturating and Mg^{2+} approaches physiological concentrations. 7. The addition of GSSG at physiological concentrations allows the activities of both enzymes to be measured at high $[NADPH]/[NADP^+]$ ratios and the co-operative action of GSSG and Mg^{2+} on the imbalance between the two enzymes to be verified. 8. The control of the activity of the two enzymes of the pentose cycle could be carried out by deinhibition of the two dehydrogenases and by the intracellular concentrations of substrates and inorganic ions.

The regulation of the oxidative phase of the pentose phosphate cycle offers at present various problems that impede our understanding of its operation *in vivo*. Sapag-Hagar *et al.* (1973) have reported that the ratio of activities of 6-phosphogluconate dehydrogenase/glucose 6-phosphate dehydrogenase under standard optimum conditions for lipogenesis in extracts of rat liver is only 0.2. Moreover, if assayed at nearly physiological concentrations of substrates and $[NADP^+]/[NADPH]$ ratios the above quotient can become as low as 0.01. This suggests an apparent physiological imbalance between the two dehydrogenases of the hexose monophosphate shunt. Sapag-Hagar *et al.* (1973) have suggested that under physiological conditions the imbalance between the two enzymes should be more marked, since it is recognized that the oxidative phase of the pentose cycle is controlled by the cytosolic $[NADP^+]/[NADPH]$ ratio (Kather *et al.*, 1972). NADPH is a competitive inhibitor of both dehydrogenases, although the K_i for 6-phosphogluconate dehydrogenase is lower (Beutler, 1957; Boivin & Galand, 1965; Veech *et al.*, 1969; Sapag-Hagar *et al.*, 1973; Benöhr & Waller, 1974; Silva Pando, 1976).

Abbreviations used: GSSG, oxidized glutathione; GSH, reduced glutathione.

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The inhibition of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase is total when the $[NADPH]/[NADP^+]$ ratio nears 9 *in vitro* (Eggleston & Krebs, 1974). In the cytoplasm of rat liver the free nucleotide ratio is of the order of 100 (Veech, 1968; Veech *et al.*, 1969). With these considerations in mind, Eggleston & Krebs (1974) commenced a systematic investigation to find some factor capable of counteracting the inhibition of glucose 6-phosphate dehydrogenase by NADPH. Of the many cell constituents tested, only GSSG has a marked effect on glucose 6-phosphate dehydrogenase at physiological concentrations. They likewise showed that for GSSG to act the presence of an unknown cofactor, which is highly unstable, is required. This has led us to investigate the factors that may modify the apparent imbalance between glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase and in particular the reversing effect of GSSG discovered by Eggleston & Krebs (1974) for glucose 6-phosphate dehydrogenase from rat liver. The experiments show that GSSG has a very marked effect depending on the $[NADPH]/[NADP^+]$ ratio. We also show that the imbalance between glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in mussel hepatopancreas is practically non-existent at near intracellular concentrations of substrates and inorganic ions.

Experimental

Special substrates and crystalline enzymes were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Solutions of NADPH and NADP⁺ were freshly made each day. To obtain crude enzyme preparations, weighed samples of tissues from mussels (*Mytilus edulis* L.; from N.W. Spain) were homogenized with 3 vol. of ice-cold 0.1M-potassium phosphate buffer, pH 7.4, containing 1mM-EDTA. After centrifugation at 27000g for 30min at 2°C the supernatants were used for assay of enzyme activities.

Assay of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) activity was carried out spectrophotometrically at 24–26°C in groups of two cuvettes containing 1 ml of reactants, including (final concn.) 60mM-Tris/HCl buffer, pH 7.4, and various concentrations of MgCl₂, glucose 6-phosphate (or 6-phosphogluconate), NADP⁺ and NADPH and samples of enzyme and tissue preparations freshly diluted with water. The amounts of tissue extracts added were the equivalent of 1–10mg fresh weight, depending on their activity.

Reactions were started by the addition of substrate, and spectrophotometer readings were taken at 340 nm while the reaction proceeded linearly with time (about 10min); later the reactions ceased to be linear with time, mainly because of the fall in the concentration of NADP⁺.

Conditions for comparing glutathione reductase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities

Substrate concentrations were optimal, i.e. 1mM-glucose 6-phosphate, 1mM-6-phosphogluconate and 0.4mM-NADP⁺ in the dehydrogenase assays, and 1mM-GSSG and 0.2mM-NADPH in the reductase assay. Tris/HCl buffer (60mM, pH 7.4) and 5mM-MgCl₂ were included in the reaction mixture. Blanks without glucose 6-phosphate, 6-phosphogluconate or GSSG were run simultaneously and the maximum linear rate of A_{340} changes was measured for up to 6min at 25°C after the addition of tissue homogenate. The A_{340} changes in the absence of substrates and added NADPH were small (0.005–0.015 absorbance unit in the first 5min). Rates are expressed as μ mol of substrate used/min per g fresh wt. at 25°C and pH 7.4.

Test system for effect of GSSG

The method of Eggleston & Krebs (1974) was modified to determine the effect of GSSG on the inhibition of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.

(a) *Determination of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.* The reaction mixture was composed of 60mM-Tris/HCl buffer, pH 7.4, 5mM-MgCl₂, 0.02mM-NADP⁺, 0.033mM-ZnSO₄, 1mM-glucose 6-phosphate or 1mM-6-phosphogluconate and tissue homogenate. Blanks without glucose 6-phosphate or 6-phosphogluconate were run simultaneously. Rates are expressed as μ mol of substrate used/min per g fresh wt. at 25°C and pH 7.4.

(b) *Action of NADPH on the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.* To reaction mixture (a) was added NADPH at a concentration 4 times that of NADP⁺ (0.08mM-NADPH).

(c) *Action of GSSG on the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.* To reaction mixture (a) was added 0.1mM-GSSG.

(d) *Addition of GSSG and NADPH on the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.* To reaction mixture (b) was added 0.1mM-GSSG.

Calculations and expression of results

To calculate the percentage activation exercised by GSSG against the inhibition by NADPH of glucose 6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase activity we used the following expression:

$$\% \text{ activation by GSSG} = 100 \left(1 - \frac{x}{y} \right)$$

where x = % inhibition exercised by NADPH, calculated from the results of experiments (a) and (b), and y = % inhibition exercised by NADPH in the presence of GSSG, calculated from the results of experiments (c) and (d).

The activating effect may also be expressed as the increase in activity that the two dehydrogenases experience when, inhibited by NADPH, GSSG is added to them. Then:

$$\% \text{ increase in activity} = 100 \left(\frac{x'}{y} - 1 \right)$$

where x' = value obtained for the enzyme activity in experiment (d) and y = value obtained for the enzyme activity in experiment (b).

The 6-phosphogluconate dehydrogenase/glucose 6-phosphate dehydrogenase ratio at different concentrations of substrates (6-phosphogluconate, glucose 6-phosphate, NADP⁺) with and without effectors (GSSG, NADPH, Mg²⁺) is calculated for determination of the imbalance between the 6-phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase activity.

The results are expressed as mean values \pm s.d. (numbers of determinations in parentheses). Student's *t* test was used to assess the significance of differences between means.

Results and Discussion

Effects of GSSG on the enzymic activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in mussel hepatopancreas

The inhibition of glucose 6-phosphate dehydrogenase by NADPH was first described by Negelein & Haas (1935) in yeast and confirmed for enzymes from other origins by various authors (Glock & McLean, 1954; Schachet & Squire, 1971; Bonsignore & De Flora, 1972; Silva Pando, 1976). It is recognized that the activity of 6-phosphogluconate dehydrogenase is controlled by the cytosolic ratio [free NADP⁺]/[free NADPH] and that changes in this ratio from 0.01 to 0.33 produce a 25-fold increase in the activity of the enzyme. It proved impossible to estimate the percentage inhibition of 6-phosphogluconate dehydrogenase *in vitro* at physiological concentrations of the [free NADP⁺]/[free NADPH] ratio. Veech *et al.* (1969), using indirect techniques, found a value of 0.01 for the [free NADP⁺]/[free NADPH] ratio. The K_i/K_m ratios for reduced and oxidized NADP are 1 for glucose 6-phosphate dehydrogenase and 0.56 for 6-phosphogluconate dehydrogenase (Silva Pando, 1976).

Eggleston & Krebs (1974) found that, among more than 100 cell constituents of rat liver tested, only GSSG reverses, at physiological concentrations, the inhibition that NADPH exercises on glucose 6-phosphate dehydrogenase. With this starting point, we have determined the percentage reversal that GSSG exercises on the two dehydrogenases of the pentose phosphate pathway at different [NADP⁺]/[NADPH] ratios (Table 1). For both enzymes the reversing effect of GSSG increases as the [NADP⁺]/[NADPH] decreases due to decreasing [NADP⁺]. In all cases greater percentage reversal is found for 6-phospho-

gluconate dehydrogenase than for glucose 6-phosphate dehydrogenase.

Uncertainty exists as to the absolute concentrations of free NADP⁺ and free NADPH in tissue, which limits precise prediction. The concentration of free plus bound NADPH in rat liver is approx. 0.38 mM (Williamson *et al.*, 1971). Since the [free NADPH]/[free NADP⁺] ratio in liver is about 100 (Veech, 1968; Veech *et al.*, 1969; Gumaa *et al.*, 1971) it is probable that the majority of the total NADPH is free; otherwise the concentration of bound NADP⁺ would have to be extremely small. With very low [NADP⁺]/[NADPH] ratios (0.05, 0.033 and 0.025), close to those described *in vivo*, no appreciable activity is observed in glucose 6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase in the absence of GSSG. Nonetheless, activity is manifested when 0.1 mM-GSSG is added to the reaction mixture.

Eggleston & Krebs (1974) used 33.3 μ M-ZnCl₂ to completely inhibit glutathione reductase from liver (Miza & Langdon, 1962). In mussel hepatopancreas we have used higher concentrations of Zn²⁺ to inhibit glutathione reductase (Table 2). Concentrations of Zn²⁺ of 0.33 mM completely inhibit glutathione reductase, although hardly affecting the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Under these conditions we studied the action of variable concentrations of GSSG on the inhibition by NADPH of the two dehydrogenases (Table 3). In the range of concentrations studied different percentage reversals are observed, increasing with the concentration of GSSG. The effect is more pronounced for 6-phosphogluconate dehydrogenase than for glucose 6-phosphate dehydrogenase. This fact could be related to the observation that NADPH inhibits 6-phosphogluconate dehydrogenase more strongly than glucose 6-phosphate dehydrogenase (Silva Pando, 1976), a more marked deinhibition therefore being necessary in the case of 6-phosphogluconate dehydrogenase for operation *in vivo* in the pentose phosphate pathway.

Table 1. *Effect of the [NADPH]/[NADP⁺] ratio on the percentage activation exercised by GSSG on glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in mussel hepatopancreas*

Details of the procedure are given in the Experimental section under 'Test system for effect of GSSG'. Corrections were made by using the controls without glucose 6-phosphate or 6-phosphogluconate. The results are expressed as mean values \pm s.d. (numbers of determinations in parentheses). Each determination was carried out for homogenates representative of five hepatopancreases.

Initial [NADP ⁺] (μ M)	Initial [NADPH] (μ M)	Ratio [NADPH]/[NADP ⁺]	Activation by GSSG (%)	
			Glucose 6-phosphate dehydrogenase	6-Phosphogluconate dehydrogenase
20	80	4	32 \pm 13 (11)	50 \pm 20 (12)
16	80	5	42 \pm 10 (4)	67 \pm 16 (4)
12	80	6.6	63 \pm 5 (4)	88 \pm 13 (4)

Table 2. Action of Zn^{2+} on the activities of glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and glutathione reductase from mussel hepatopancreas

Details of the assay methods are given in the Experimental section and in the text. Corrections were made by using controls without glucose 6-phosphate (for glucose 6-phosphate dehydrogenase), 6-phosphogluconate (for 6-phosphogluconate dehydrogenase) or GSSG (for glutathione reductase). The activities of dehydrogenases and glutathione reductase were assayed under optimum conditions in normal homogenates. The data given in the Table are representative of six experiments.

Enzyme activities in normal homogenates at 25°C and pH 7.4 ($\mu\text{mol}/\text{min per g}$)

[ZnSO ₄] (μM)	Glucose 6-phosphate dehydrogenase	6-Phospho- gluconate dehydrogenase	Glutathione reductase
0	1.87	0.94	0.35
33	1.79	0.90	0.04
66	1.70	0.86	—
330	1.65	0.80	—
660	0.76	0.39	—
990	0.68	0.28	—

Table 3. Effect of GSSG on the inhibition of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase by NADPH in mussel hepatopancreas

Cuvettes contained (final concn.) 60 mM-Tris/HCl buffer, pH 7.4, 5 mM-MgCl₂, 1 mM-glucose 6-phosphate or 1 mM-6-phosphogluconate, 0.2 mM-NADP⁺, 0.08 mM-NADPH, 0.33 mM-ZnSO₄, various amounts of GSSG, and mussel hepatopancreas supernatant (about 0.25 mg of hepatopancreas). The absorption changes have been corrected by using blanks from which glucose 6-phosphate or 6-phosphogluconate was omitted. The results are expressed as mean values \pm S.D. (numbers of determinations in parentheses).

[GSSG] (μM)	Increase in activity (%)	
	Glucose 6-phosphate dehydrogenase	6-Phosphogluconate dehydrogenase
25	27 \pm 12 (4)	45 \pm 22 (4)
50	42 \pm 21 (5)	67 \pm 11 (5)
75	62 \pm 36 (4)	153 \pm 44 (4)
100	81 \pm 46 (6)	178 \pm 67 (8)

The tissue concentration of GSSG found by Wendell (1970) in rat heart, with *N*-ethylmaleimide to prevent oxidation of GSH, is 0.03–0.05 mM. He likewise calculated the intracellular concentration of GSSG in rat liver from the data of Güntherberg & Rapoport (1968) to be about 0.06 mM. In mussel hepatopancreas, L. Sánchez Lopez (unpublished

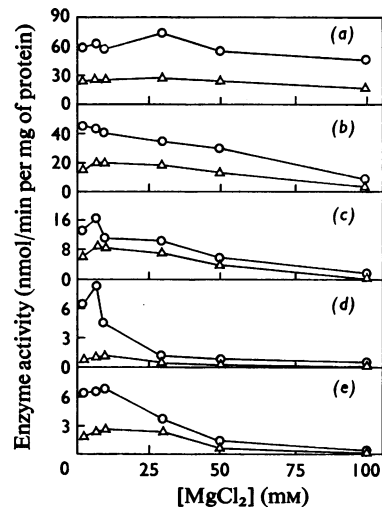


Fig. 1. Effect of Mg^{2+} concentration on the activities of the glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase

All the cuvettes contain (final concn.) 60 mM-Tris/HCl buffer, pH 7.4, different concentrations of MgCl₂ and mussel hepatopancreas supernatants (about 0.25 mg of hepatopancreas). Individual cuvettes contained: (a) 1 mM-glucose 6-phosphate and 0.4 mM-NADP⁺ for the glucose 6-phosphate dehydrogenase assay (○) and 1 mM-6-phosphogluconate and 0.4 mM-NADP⁺ for the 6-phosphogluconate dehydrogenase assay (Δ); (b) 1 mM-glucose 6-phosphate and 0.02 mM-NADP⁺ for the glucose 6-phosphate dehydrogenase assay (○) and 1 mM-6-phosphogluconate and 0.02 mM-NADP⁺ for the 6-phosphogluconate dehydrogenase assay (Δ); (c) 0.01 mM-glucose 6-phosphate and 0.04 mM-NADP⁺ for the glucose 6-phosphate dehydrogenase assay (○) and 0.01 mM-6-phosphogluconate and 0.04 mM-NADP⁺ for the 6-phosphogluconate dehydrogenase assay (Δ); (d) 0.01 mM-glucose 6-phosphate and 0.02 mM-NADP⁺ for the glucose 6-phosphate dehydrogenase assay (○) and 0.01 mM-6-phosphogluconate and 0.02 mM-NADP⁺ for the 6-phosphogluconate dehydrogenase assay (Δ); (e) 22 μM -glucose 6-phosphate, 0.01 mM-NADP⁺ and 0.01 mM-NADPH for the glucose 6-phosphate dehydrogenase assay (○) and 11 μM -6-phosphogluconate, 25 μM -NADP⁺ and 0.14 mM-NADPH for the 6-phosphogluconate dehydrogenase assay (Δ). The data have been corrected by using blanks from which glucose 6-phosphate or 6-phosphogluconate was omitted. For each determination a group of six mussels was used.

work) found values for GSSG of approx. 0.08 mM. Given that these values are within the limits of those used in the present experiments (0.1 mM), the results obtained should have a clear physiological significance.

Imbalance between the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase

The majority of bivalves, and in particular *Mytilus edulis*, are euryhalines. They are considered osmoconformist since the concentrations of the main ions of sea-water, such as Na^+ , Mg^{2+} , Cl^- and SO_4^{2-} , are the same as in their organic fluids (Robertson, 1949; Vernberg & Vernberg, 1972). Given that Mg^{2+} is an activator of the oxidative enzymes in the pentose pathway, and that its concentration in sea-water (and therefore in mussel tissue) is quite high, approx. 30–50mM (Robertson, 1941), we have studied its action, at different concentrations and in different states of substrate saturation, on the apparent imbalance between the two dehydrogenases of the pentose pathway (Fig. 1). The results of the present investigation confirm the apparent imbalance between the two dehydrogenases, described by Sapag-Hagar *et al.* (1973) in rat liver, in mussel hepatopancreas in the same conditions of substrate saturation and in the presence of 10mM- MgCl_2 . However, we have observed that, when the conditions of substrate concentration of Mg^{2+} near physiological concentrations, the imbalance between the two dehydrogenases is practically non-existent. To achieve experimental conditions approximating to those existing *in vivo*, both dehydrogenases have been tested at concentrations of substrates and of inhibitor (NADPH) in the order of their respective kinetic parameters (K_m and K_i). The K_i/K_m ratios for reduced and oxidized NADP⁺ are 1 for glucose 6-phosphate dehydrogenase and 0.56 for 6-phosphogluconate dehydrogenase. In this situation and at physiological concentrations of Mg^{2+} , the imbalance between the two dehydrogenases practically disappears.

We determined the effect exercised by GSSG at physiological concentrations (0.1 mM) on the catalytic imbalance between the two dehydrogenases (Table 4). The results show that, for an [NADPH]/[NADP⁺]

ratio of 4, the addition of GSSG provokes a rise in the 6-phosphogluconate dehydrogenase/glucose 6-phosphate dehydrogenase ratio, which under these conditions means a 24% levelling of the imbalance. With very high [NADPH]/[NADP⁺] ratios (20, 30 and 40) *in vitro*, it is not possible to detect an imbalance between the two enzymes, given that neither glucose 6-phosphate dehydrogenase nor 6-phosphogluconate dehydrogenase presents activity at [NADPH]/[NADP⁺] ratios above 9 (Eggleston & Krebs, 1974; Silva Pando, 1976). It may nevertheless be observed (Table 4) that the addition of 0.1mM-GSSG counteracts the inhibition and allows activities of both enzymes to be measured at high nucleotide ratios. Likewise, minimal imbalance values are

Table 5. *Effects of GSSG and Mg²⁺ on the 6-phosphogluconate dehydrogenase/glucose 6-phosphate dehydrogenase activity ratio in mussel hepatopancreas*

Cuvettes contained (final concn.) 60mM-Tris/HCl buffer, pH7.4, 1mM-glucose 6-phosphate or 1mM-6-phosphogluconate, 0.02mM-NADP⁺, 0.08mM-NADPH, 0.33mM-ZnSO₄, 2.5 or 30mM-MgCl₂, and various amounts of GSSG, and mussel hepatopancreas supernatant (about 0.25mg of hepatopancreas). Corrections were made by using controls without glucose 6-phosphate or 6-phosphogluconate. The data given in the Table are representative of six experiments.

[GSSG] (μM)	Ratio of 6-phosphogluconate dehydrogenase/ glucose 6-phosphate dehydrogenase	
	2.5mM-MgCl ₂	30mM-MgCl ₂
0	0.23	0.40
25	0.29	0.42
35	0.31	0.48
50	0.32	0.50
75	0.34	0.53
100	0.38	0.56

Table 4. *Effect of GSSG on the 6-phosphogluconate dehydrogenase/glucose 6-phosphate dehydrogenase activity ratio at different nucleotide ratios*

Cuvettes contained (final concn.) 60mM-Tris/HCl buffer, pH7.4, 5mM-MgCl₂, 1mM-glucose 6-phosphate or 1mM-6-phosphogluconate, 0.33mM-ZnSO₄, various amounts of NADP⁺ and NADPH, with and without 0.1mM-GSSG, and mussel hepatopancreas supernatant (about 0.25mg of hepatopancreas). The results are expressed as mean values \pm s.d. (numbers of determinations in parentheses). Each determination was carried out with homogenates representing five hepatopancreases. For further details see the Experimental section and Table 3.

Initial [NADP ⁺] (μM)	Initial [NADPH] (μM)	Ratio [NADPH]/ [NADP ⁺]	Ratio of 6-phosphogluconate dehydrogenase/ glucose 6-phosphate dehydrogenase	
			Without GSSG	With GSSG
20	80	4	0.33 \pm 0.08 (11)	0.41 \pm 0.11 (11)
16	80	5	0.31 \pm 0.04 (4)	0.34 \pm 0.04 (4)
10	200	20	—	0.87 \pm 0.10 (3)
10	300	30	—	0.62 \pm 0.08 (3)
10	400	40	—	0.77 \pm 0.12 (3)

observed (0.62, 0.77 and 0.87). We went on to test the joint action of Mg^{2+} and GSSG on the imbalance between the two dehydrogenases at a constant nucleotide ratio (Table 5). At all the concentrations of GSSG tested the imbalance is less in the presence of 30 mM- $MgCl_2$ (close to physiological concentrations) than with 2.5 mM- $MgCl_2$. The imbalance is less in the combined presence of Mg^{2+} and GSSG than in the presence of either alone.

In the light of the present experiments it is possible to suppose that the regulation *in vivo* of the activities of the two dehydrogenases of the oxidative phase of the pentose phosphate cycle constitutes a complex system in which are involved the deinhibition of the two dehydrogenases and the intracellular concentrations of substrates and inorganic ions.

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