A critical appraisal of the effect of oxidized glutathione on hepatic glucose 6-phosphate dehydrogenase activity

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(Received 1 March 1983/Accepted 20 June 1983)

1. Experiments were undertaken to elucidate the mechanism of the reversal of NADPH inhibition of rat liver glucose 6-phosphate dehydrogenase by oxidized glutathione alone and in combination with a putative cofactor described by Eggleston & Krebs [(1974) *Biochem. J.* **138**, 425–435]. 2. Evidence is presented that this reversal is largely an artifact, caused by the incorrect application of a control assay procedure and a spurious effect of Zn^{2+} (added in order to inhibit glutathione reductase) in crude enzyme solutions. 3. When the proper assay procedure is used and glutathione reductase is inhibited with low concentrations of Hg^{2+} , glutathione addition has no effect on NADPH inhibition of glucose 6-phosphate dehydrogenase. 4. No evidence was found for the existence of a cofactor that mediates an effect of glutathione on glucose 6-phosphate dehydrogenase.

It is generally recognized that G6P dehydrogenase, which catalyses the first reaction in the HMP shunt, is a critical control site. A key difficulty in understanding the mechanism of regulation of this enzyme, stated clearly by Eggleston & Krebs (1974), is its potent inhibition *in vitro* under conditions known to occur generally *in vivo*, i.e., high concentration ratios of NADPH to NADP^+ . In rat liver cytoplasm, for example, the ratio of free NADPH to free NADP^+ is approx. 100 (Veech *et al.*, 1969), yet inhibition of G6P dehydrogenase *in vitro* is virtually complete when this ratio exceeds 9 (Eggleston & Krebs, 1974). Thus the question is: how can G6P dehydrogenase function *in vivo* under conditions that inhibit completely *in vitro*? The kinetic mechanism of rat liver G6P dehydrogenase was reported to be ordered sequential, with co-enzyme binding first (Thompson *et al.*, 1976). Increased hepatic G6P concentration cannot, therefore, directly relieve NADPH inhibition, unlike the situation in the lactating mammary gland, where G6P dehydrogenase catalyses a random-order mechanism that enables G6P concentrations to modulate NADPH inhibition (Shreve & Levy, 1980).

Eggleston & Krebs (1974) sought to determine what factors overcome NADPH inhibition of hepatic G6P dehydrogenase. Of the large number of

cellular components that they tested, GSSG was the only one effective at physiological concentrations (Eggleston & Krebs, 1974). The role of hepatic glutathione reductase (Mize & Langdon, 1962; Carlberg & Mannervik, 1975) in mediating this effect by re-oxidizing NADPH was ruled out by Eggleston & Krebs (1974), who claimed that this enzyme activity was not measurable at concentrations of GSSG that relieved NADPH inhibition of G6P dehydrogenase. They also showed that relief of NADPH inhibition still occurred in the presence of $33\text{ }\mu\text{M}$ - ZnCl_2 , which was reported to inhibit glutathione reductase activity completely (Mize & Langdon, 1962). Dialysis of liver extracts rendered G6P dehydrogenase insensitive to the GSSG effect, suggesting to Eggleston & Krebs (1974) that a cofactor might participate in its action. They were able to separate this putative cofactor from G6P dehydrogenase by filtration on Amicon Centriflo membrane cones. Once separated from the enzyme, the cofactor was very unstable and Eggleston & Krebs (1974) were unable to identify it. Rodriguez-Segade *et al.* (1978) reported that GSSG overcomes NADPH inhibition of both G6P dehydrogenase and 6-phosphogluconate dehydrogenase in mussel hepatopancreas. Subsequently Rodriguez-Segade *et al.* (1979, 1980) claimed that the cofactor, which they isolated from both mussel hepatopancreas and rat liver, is a protein of mol.wt. 10 000–15 000.

Our studies were initiated in order to extend the observations of Eggleston & Krebs (1974) and

Abbreviations used: G6P, glucose 6-phosphate; GSH, reduced glutathione; GSSG, oxidized glutathione; HMP, hexose monophosphate.

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Rodriguez-Segade *et al.* (1978, 1979, 1980). Instead, we found that the effects described by these authors are largely the results of several artifacts. Numerous other studies have described the effects of glutathione on the activities of the HMP shunt and G6P dehydrogenase (Jacob & Jandl, 1966; Watanabe *et al.*, 1972; Hilf *et al.*, 1978; Reitzer *et al.*, 1980; May, 1981; Puente *et al.*, 1981; Zimmer *et al.*, 1981). In view of the continuing interest in the regulation of the HMP shunt we considered it of value to describe our results and to offer our conclusions, namely that there is no evidence, yet, either for a direct effect of glutathione on hepatic G6P dehydrogenase or for the existence of a cofactor that mediates such an effect.

Experimental

Insofar as possible, we have used the same procedures as those described by Eggleston & Krebs (1974). Male rats (Wistar strain, except where noted) were obtained from the Charles River Breeding Laboratories, Wilmington, MA, U.S.A. They were maintained at 25°C on laboratory chow and water *ad libitum* on a 7½h-light/16½h-dark schedule, a regimen that was found to enhance the efficacy of the GSSG effect (Eggleston & Krebs, 1974). The livers were excised, rinsed with ice-cold 0.1 M-potassium phosphate, pH 7.4, containing 1.0 mM-EDTA (hereafter referred to as phosphate-EDTA), blotted to remove excess buffer, minced in an ice-cold beaker and homogenized in 4 vol. of ice-cold phosphate-EDTA in a Potter-Elvehjem homogenizer. Portions (5–10 ml) of this homogenate were stored frozen.

For some experiments Sprague-Dawley rats were used, rats were maintained on a 12h-light/12h-dark schedule or homogenates were prepared from livers perfused *in situ* with phosphate-buffered saline (0.138 M-NaCl / 2.68 mM-KCl / 6.46 mM-Na₂HPO₄ / 1.47 mM-KH₂PO₄, pH 7.2). These changes from the standard procedure did not materially affect the experimental results.

Partially (approx. 1500-fold) purified G6P dehydrogenase was prepared from rat liver homogenates by the procedure of Nevaldine *et al.* (1974) through the second (NH₄)₂SO₄ precipitation and dialysis. The enzyme was further purified by affinity chromatography on a adenosine 2'-phosphate 5'-phosphate ('2',5'-ADP') matrix as described by DeFlora *et al.* (1975), followed by precipitation at 60%-satd. (NH₄)₂SO₄. The precipitate was dissolved in 0.035 M-phosphate, pH 7.5, containing 7.0 mM-2-mercaptoethanol, 1.0 mM-EDTA and 20% (v/v) glycerol and stored at 4°C. The specific activities of several such enzyme preparations were 9–16 units per mg of protein (1 unit represents 1 µmol of substrate transformed per min at 25°C).

Assays were performed as described by Eggleston & Krebs (1974), with two pairs of cuvettes, one pair with and one pair without 70 µM-NADPH; one cuvette in each pair (the 'control') lacked substrate and the other contained 1.33 mM-G6P. Reactions were monitored at 340 nm and 25°C in a Gilford 240 spectrophotometer with an attached Gilford 6051 recorder, generally set for a full-scale absorbance of 0.2. Average values of duplicate determinations are reported. Like Eggleston & Krebs (1974), we report the absorbance change for 10 min, beginning 1 min after initiation of the assay. In order to facilitate interpretation of the results, we also report the absorbance changes in all four cuvettes, as well as the net changes (i.e. the change in the presence of G6P minus the change in the absence of G6P). Except where noted, all reaction mixtures contained, in 1.0 ml volumes, 0.05 M-Tris/HCl, pH 7.4, 3.3 mM-MgCl₂, 1.33 mM-G6P and 20 µM-NADP⁺ (Eggleston & Krebs, 1974); the concentrations of other components, added to some assays, are given in the Tables. When the enzyme was not pre-incubated, assays were always performed as follows. Tris/HCl, water, MgCl₂ and NADP⁺ were added in that order; GSSG and NADPH, when present, were added next; the enzyme was added, the reaction mixture stirred and allowed to stand for 30 s in the cuvette compartment; G6P was added to one pair of cuvettes, all four cuvettes were stirred and the reaction was monitored at 340 nm. In those experiments where the enzyme was incubated before assaying, details of the procedure are given in the Tables.

Glutathione reductase was assayed under the same conditions used for G6P dehydrogenase assays, but substituting 70 µM-NADPH for NADP⁺ and 0.1 mM-GSSG for G6P. NADP⁺, NADPH, G6P, GSSG, glutathione reductase and α-oxoglutarate were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Glutamate dehydrogenase was a product of Boehringer-Mannheim Biochemicals, Indianapolis, IN, U.S.A. *Leuconostoc mesenteroides* G6P dehydrogenase was prepared in our laboratory and was devoid of other detectable proteins (Haghighi *et al.*, 1982). All other chemicals were of reagent-grade. Minicon B15 or CS15 concentrators (15000-mol.wt. cut-off) and CF-50A Centriflo cones (50000-mol.wt. cut-off) were obtained from Amicon Corp., Danvers, MA, U.S.A.

Results and discussion

Effects of glutathione

When a high-speed supernatant solution from liver homogenate was used as the enzyme source, the addition of GSSG appeared to relieve NADPH inhibition (Table 1). The net activity in the presence of GSSG was virtually identical in the absence or

in the presence of NADPH. It can be seen, however, that there are two factors in this experiment that complicate the interpretation of the data: first, there is substantial activity in the absence of added substrate and NADPH; and secondly, there is a 'negative rate' (i.e., NADPH oxidation) in the control cuvette containing NADPH plus GSSG. These difficulties were recognized by Eggleston & Krebs (1974), who described means to overcome them.

Like Eggleston & Krebs (1974), we found that the activity in the absence of added substrate is virtually eliminated by concentrating the enzyme with a Minicon B-15 concentrator (Table 1). According to Eggleston & Krebs (1974), this removes endogenous G6P without removing cofactor. However, we could accomplish the same result by subjecting the enzyme to dialysis for 24 h against phosphate-EDTA or by centrifuging the enzyme with a Centriflo membrane cone (Table 1). According to Eggleston & Krebs (1974), these latter treatments remove cofactor from the enzyme preparation, rendering it insensitive to the effect of GSSG. In all of our experiments, however, the enzyme appeared to retain its sensitivity to GSSG as calculated from net rates. In the experiment with the Minicon-treated enzyme, for example, the net activity in the presence of 0.1 mM- (80 units) or 2.0 mM-GSSG (85 units) plus 70 μM-NADPH is nearly identical with that in the absence of both GSSG and NADPH (84 units) consistent with the conclusion that GSSG totally counteracts the inhibitory effect of NADPH. Like Eggleston & Krebs (1974), we found that GSSG

alone appeared to inhibit G6P dehydrogenase (however, see below).

Eggleston & Krebs (1974) showed that the effectiveness of GSSG in reversing NADPH inhibition was enhanced by pre-incubating the enzyme with NADP⁺, Mg²⁺ and GSSG. We were unable to confirm this finding; the data from experiment 5 (Table 2), in which enzyme was pre-incubated for 30 min before assaying, shows results comparable with those from experiment 4, in which assays were performed without pre-incubation.

The 'negative rates' that are seen in the control cuvettes in the presence of NADPH plus GSSG (Table 1) are due to endogenous glutathione reductase activity. Eggleston & Krebs (1974) claimed that in their preparations there was no measurable glutathione reductase activity when the concentration of GSSG was below 0.2 mM. This is surprising because under optimal conditions (stated to be 0.33 mM-GSSG and 0.1 mM-NADPH) they found an activity of 3.3 μmol/min per g for glutathione reductase (compared with 2.6 μmol/min per g for G6P dehydrogenase). We found that the 'negative rates' increased with increasing GSSG concentration (Table 1) and direct assays confirmed the presence of highly active glutathione reductase activity, even at suboptimal concentrations of GSSG well below 0.2 mM, in all liver extracts prepared in our laboratory.

Effect of Zn²⁺

Eggleston & Krebs (1974) found that the addition

Table 1. Effects of GSSG on G6P dehydrogenase preparations treated in various ways

Rat liver homogenate, prepared as described in the Materials and methods section, was centrifuged for 25 min at 39000 g at 4°C. The supernatant solution was designated 'high-speed' supernatant (E). E was concentrated over 25-fold for 17 h at 4°C in a Minicon B-15 concentrator and reconstituted to the original volume with phosphate-EDTA to generate EM. ED was prepared by dialysing E against 400 vol. of phosphate-EDTA for 24 h at 4°C with two changes of dialysis buffer, followed by centrifugation at 39000 g for 30 min at 4°C. EC was prepared by centrifuging 2.0 ml of E at 2500 rev./min in a pre-soaked Amicon CF-50A Centriflo cone for 60 min at 4°C. The material remaining in the cone (0.4 ml) was diluted to 2.0 ml with phosphate-EDTA and treated in the same way once more with a fresh, pre-soaked Centriflo cone. The material remaining in the cone (0.2 ml) was diluted to 2.0 ml again with the same buffer to generate EC. Absorbance changes were measured at 340 nm.

Enzyme treatment	[GSSG] (mM)	10 ³ × Absorbance change from 1 to 11 min						Activity with NADPH (%)
		-NADPH			+ 70 μM-NADPH			
		-G6P	+G6P	Net	-G6P	+G6P	Net	
High-speed supernatant (E)	0	33	65	32	5	29	24	75
	0.1	16	53	37	-9	27	36	97
E concentrated with Minicon (EM)	0	1	85	84	-5	48	53	63
	0.05	2	56	54	-44	31	75	139
	0.1	2	50	48	-66	14	80	167
	0.2	2	38	36	-87	-2	85	236
E dialysed for 24 h (ED)	0	2	84	82	-1	46	47	57
	0.1	2	51	49	-52	26	78	159
E centrifuged with Centriflo cone (EC)	0	7	95	88	-1	64	65	74
	0.1	1	60	59	-72	28	100	169

Table 2. *Effects of GSSG on G6P dehydrogenase in the presence of Zn²⁺ or Hg²⁺*

EM, prepared as described in Table 1, was used as enzyme. The same preparation was used for Expts. 1–3; a different preparation was used in Expts. 4 and 5. Metal ions were added as ZnCl₂ or mercuric acetate. In Expt. 5 enzyme was incubated for 30 min at 25°C with all the reaction components except G6P and NADPH. The reaction was initiated by the addition of G6P. NADPH, when included, was added just before substrate. Absorbance changes were measured at 340 nm.

Expt. no.	Metal ion added	GSSG (mM)	10 ³ × Absorbance change from 1 to 11 min						Activity with NADPH (%)
			-NADPH			+70 μM-NADPH			
			-G6P	+G6P	Net	-G6P	+G6P	Net	
1	None	0	4	76	72	-4	48	52	72
		0.1	-1	48	49	-35	20	55	112
2	33 μM-Zn ²⁺	0	24	76	52	11	47	36	69
		0.1	15	60	45	3	48	45	100
3	0.66 μM-Hg ²⁺	0	3	78	75	-5	45	50	67
		0.1	2	66	64	-9	44	53	83
4	2.0 μM-Hg ²⁺	0	1	38	37	0	21	21	57
		0.1	2	45	44	-2	23	25	57
5	2.0 μM-Hg ²⁺	0	0	37	37	-2	18	20	54
		0.1	1	42	41	-3	18	21	51

of 33 μM-ZnCl₂, known to inhibit rat liver glutathione reductase (Mize & Langdon, 1962), completely inhibited glutathione reductase activity in their preparations. Our results initially appeared to confirm this (experiment 2, Table 2). Thus, when a Minicon-concentrated enzyme preparation was used, the control cuvette showed no 'negative rate' in the presence of NADPH, GSSG and ZnCl₂, and the net rates in the absence and in the presence of NADPH suggested that, under these conditions, GSSG can overcome inhibition by NADPH. Further investigations revealed, however, that the extent to which 33 μM-ZnCl₂ appeared to inhibit glutathione reductase in different experiments was variable. In addition, we noted that there was substantial 'activity' in the presence of ZnCl₂ and in the absence of NADPH or substrate (see Table 2). This 'activity' is not due to G6P dehydrogenase. When the concentration of ZnCl₂ was increased from 33 μM to 67 μM and 167 μM in assays using 50 μl of a 'high-speed' supernatant solution of liver homogenate, the rate of absorbance increase at 340 nm rose from 0.018 per min to 0.136 per min to a rate too fast to measure accurately. The Zn²⁺-dependent increase in absorbance occurred in the absence of added NADP⁺ or G6P; it was not affected by the addition of α-oxoglutarate, NH₄Cl and glutamate dehydrogenase; and it was characterized by an absorbance maximum, not at 340 nm, but below 260 nm. The effect was observed in G6P dehydrogenase preparations concentrated with a Minicon concentrator (Table 2), dialysed against phosphate-EDTA (Table 3) or dialysed and gel-filtered on Sephadex G-100 in 50 mM-Tris/HCl, pH 7.0 (results not shown), but not in partially purified rat liver G6P dehydrogenase (Table 3). The absorbance increase

was more pronounced in 0.05 M-Tris/HCl, pH 7.4, than in 0.05 M-potassium phosphate, pH 7.4, and was markedly decreased in the presence of as little as 10 μM-EDTA (results not shown). (It was also found that the activity of glutathione reductase, assayed in either phosphate or Tris, is stimulated over 10-fold when 0.5 mM-EDTA is present in the assay mixture.) Like G6P dehydrogenase, this 'activity' is inhibited by both NADPH and GSSG (Table 2). Thus, this absorbance change, which appears to result from the interaction of Zn²⁺ with a macromolecular component in crude rat liver enzyme preparations, can easily be mistaken for G6P dehydrogenase activity unless appropriate controls are performed. It is not possible to simply subtract the rate observed in the presence of Zn²⁺ and in the absence of NADP⁺ or glucose 6-phosphate, as these reactants also modulate the rate of absorbance change.

Rodriguez-Segade *et al.* (1978, 1979, 1980) used 0.33 mM-ZnCl₂ [ten times the concentration employed by Eggleston & Krebs (1974)] in their assays. At this concentration of Zn²⁺ marked artifactual absorbance changes occur that invalidate the G6P dehydrogenase assays.

The virtual absence of this spurious Zn²⁺-induced absorbance change in phosphate-EDTA offered one means of overcoming this difficulty, but 0.1 M-phosphate was found to inhibit rat liver glucose 6-phosphate dehydrogenase substantially (40% inhibition under our assay conditions). We tested other metal ions, reported by Mize & Langdon (1962) to inhibit glutathione reductase, and found that low concentrations of Hg²⁺ (0.66–2.0 μM) inhibited effectively without introducing the spurious absorbance changes noted with Zn²⁺ (Table 2, experiments 3 and 4). Under these conditions, GSSG

Table 3. *Effects of 'cofactor' and GSSG on G6P dehydrogenase*

ED was prepared as described in Table 1. Purified enzyme was prepared as described in the Materials and methods section from Sprague-Dawley rats. 'Cofactor' was a Centrifo cone filtrate of the liver homogenate. Metal ions were added as ZnCl_2 or mercuric acetate. Absorbance changes were measured at 340 nm.

Expt. no.	Enzyme	Metal ion	[GSSG] (mM)	'Cofactor' (μl)	$10^3 \times$ Absorbance change per minute						Activity with NADPH (%)	
					-NADPH			+70 μM -NADPH				
					-G6P	+G6P	Net	-G6P	+G6P	Net	Net	+G6P*
1	ED	33 μM - Zn^{2+}	0	0	12	49	37	9	31	22	59	63
			0.1	0	8	35	27	-1	30	31	115	86
			0	10	29	43	14	10	28	18	129	65
			0.1	10	23	36	13	8	27	19	146	75
2	ED	2 μM - Hg^{2+}	0	0	2	62	60	0	33	33	55	53
			0.1	0	1	60	59	-2	37	39	66	62
			0	10	43	65	21	18	36	18	86	55
			0.1	10	43	65	22	18	37	19	86	57
3	Purified	50 μM - Zn^{2+}	0	0	-2	87	89	-2	48	50	56	55
			0.1	0	1	92	91	-2	52	54	59	57
			0	10	39	89	50	13	55	43	86	62
			0.1	10	38	89	51	13	54	41	80	61

* These activities were calculated from the rates uncorrected for controls lacking G6P (see the text).

produced little, if any, reversal of NADPH inhibition. The apparent, slight reversal in experiment 3 (Table 2) is the result of some residual glutathione reductase activity. For total inhibition of glutathione reductase, 2 μM - Hg^{2+} was required; at this concentration G6P dehydrogenase is inhibited approx. 15% (results not shown).

The data in Table 2 (experiments 4 and 5) also demonstrate that GSSG does not inhibit G6P dehydrogenase. The apparent inhibition observed by Eggleston & Krebs (1974) and seen in our experiments (Table 1; experiments 1 and 3, Table 2) is due to glutathione reductase activity.

The search for a cofactor

Eggleston & Krebs (1974) located a cofactor, required in their experiments for the GSSG-mediated reversal of NADPH inhibition, in Centrifo cone filtrates of rat liver homogenates, or of Minicon-concentrated homogenates. Rodriguez-Segade *et al.* (1979) obtained a cofactor with the same properties by gel filtration of tissue homogenates on Sephadex G-50 and claimed to have characterized it as a protein with a mol.wt. of approx. 10000. The implications of this finding, if substantiated, can be appreciated if one considers that thioredoxin (Engstrom *et al.*, 1974), glutaredoxin (Luthman & Holmgren, 1982a,b) and thioltransferase (Axelsson *et al.*, 1978; Axelsson & Mannervik, 1980) are all proteins with mol.wts. of 11000–12000 that can catalyse thiol/disulphide exchange reactions in which other proteins are substrates. Hepatic G6P dehydrogenase activity was found to correlate with the concentration of GSSG-protein (Isaacs &

Binkley, 1977). Francis & Ballard (1980) have presented evidence for the involvement of a thiol/disulphide exchange mechanism in the modulation of rat liver G6P dehydrogenase; in their studies the enzyme was inactivated by exposure to disulphides. Pea chloroplast G6P dehydrogenase is modulated by light via a mechanism that involves thiol/disulphide exchange (Anderson *et al.*, 1978; Ashton *et al.*, 1980).

The identification of the 'cofactor' as a protein with a mol.wt. of approx. 10000 appears questionable. Rodriguez-Segade *et al.* (1979) began their isolation of 'cofactor' by gel filtration of homogenate (from either mussel hepatopancreas or rat liver) on Sephadex G-50. Their data indicate, however, that the bulk proteins of their homogenate, including G6P dehydrogenase, are eluted at a position corresponding to a mol.wt. of approx. 25000–30000. This is not consistent with the expected molecular weight of hepatic bulk proteins or, specifically, of G6P dehydrogenase (Holten, 1972). We attempted to establish the need for a cofactor with the properties described by Eggleston & Krebs (1974), although, in contrast with their findings, in our hands (Table 1) enzyme centrifuged with a Centrifo cone appeared to be still as sensitive to the effect of GSSG (when glutathione reductase was active) as the Minicon-concentrated enzyme. Likewise, the enzyme retained its sensitivity to GSSG after gel-filtration on Sephadex G-75 (results not shown).

We attempted to prepare cofactor by the procedure of Eggleston & Krebs (1974). None of our preparations displayed properties resembling the

cofactor of Eggleston & Krebs (1974) when the data are analysed correctly. The results from three such experiments are shown in Table 3. In the first experiment, performed with crude, dialysed enzyme and assayed in the presence of $33\text{ }\mu\text{M-ZnCl}_2$, addition of GSSG, 'cofactor', or both appeared to cause relief of NADPH inhibition when calculated from net rates. This is due to the presence of G6P in the 'cofactor' (see below) and the spurious effect of Zn^{2+} . By ignoring the rates of the 'control' cuvettes lacking G6P (see below), most of the apparent relief disappears. In the second experiment, when Hg^{2+} was used instead of Zn^{2+} , there appears to be relief of NADPH inhibition when 'cofactor' is added, irrespective of the presence of GSSG. This is also due to the presence of G6P in the 'cofactor' preparation; again, calculations made by ignoring 'control' rates show no relief of NADPH inhibition. In the third experiment we used partially purified enzyme, which is insensitive to the spurious Zn^{2+} effect. Even $50\text{ }\mu\text{M-Zn}^{2+}$ causes no absorbance changes in the absence of G6P. The results of this experiment are the same as those of experiment 2.

On the assumption that the 'cofactor' might be tightly associated with G6P dehydrogenase, we also attempted to prepare it as follows. The pH of Minicon-concentrated enzyme was lowered to 5.7 with ice-cold acetic acid. The resulting precipitate was removed by centrifugation and the pH of the supernatant solution was adjusted to 6.5. One portion of this acid-treated solution was gel-filtered on a Sephadex G-75 column equilibrated with phosphate-EDTA, pH 6.5, and the fractions containing the highest activity of G6P dehydrogenase were combined and used as the enzyme. The other portion was treated with a Centriflo cone and the filtrate used as 'cofactor'. Acid treatment is used in the early stages of the preparation of thioltransferase (Carlberg & Mannervik, 1975; Axelsson *et al.*, 1978) and thioredoxin (Luthman & Holmgren, 1982b) from rat liver. This procedure also failed to yield material with 'cofactor' activity.

The presence of G6P in the 'cofactor' preparation used in experiments 1 and 2 of Table 3 was demonstrated using NADP^+ and homogeneous G6P dehydrogenase from *Leuconostoc mesenteroides* (Haghighi *et al.*, 1982). The G6P concentration in the 'cofactor' was 5.9 mM; thus the addition of $10\text{ }\mu\text{l}$ of this preparation to the 1.0 ml assay solutions produces a G6P concentration of 0.059 mM. From the reported value for the K_m of rat liver G6P dehydrogenase with G6P as substrate (Thompson *et al.*, 1976), this concentration should give a rate approx. 67% of that with saturating substrate. The 'control' rate in experiment 2 (Table 3) was 66% of the rate in the presence of 1.33 mM-G6P (46 times the K_m value). When 'cofactor' was prepared by the procedure of Eggleston & Krebs (1974) from

Minicon-concentrated enzyme, it contained no G6P and its addition to enzyme had no effect on NADPH inhibition (results not shown).

Table 7 of the paper by Eggleston & Krebs (1974) shows that they used Centriflo cone filtrates of rat liver homogenates as their source of cofactor. They state that if these filtrates were treated with a Minicon concentrator, the cofactor was completely removed (although they also claim that such treatment did not remove cofactor from the homogenates). We showed that Centriflo cone filtrates contain G6P and that it is completely removed by Minicon treatment. Treatment of enzyme with a Centriflo cone essentially eliminates the control rate observed in the absence of G6P and NADPH, as does treatment with a Minicon concentrator (Table 1). If the cofactor preparations of Eggleston & Krebs (1974) had contained G6P, they would have given a high control rate in the absence of G6P and, as the reported rates in Table 7 of their paper were corrected for such control rates, the effect would have been to cause an apparent alleviation of NADPH inhibition by GSSG, similar to that seen in our experiments (Table 3). As the 'cofactor' in these experiments contained G6P, it caused an absorbance change in the 'control' cuvettes. For reasons explained below, this 'control' rate should not be subtracted from the rate measured in the presence of G6P. We are not suggesting that all their 'cofactor' effects can be explained by the presence of G6P, but it seems probable that this may have been responsible for a major part of the effects.

The use of inappropriate controls

All the data reported by Eggleston & Krebs (1974) on rates of G6P dehydrogenase activity were corrected for controls without G6P. Although they do not show uncorrected rates in controls and experimental cuvettes, their net rates are sufficiently similar to those we have found (e.g., compare their Table 2 with our Table 1) that one may presume that the rates in control and experimental cuvettes were similar in the two laboratories. We have included these rates in our Tables as well as the net rates. It is clear that, unless the crude enzyme is treated with a Minicon concentrator, or dialysed, or treated with a Centriflo cone, there are large absorbance changes in the absence of G6P and NADPH. This was invariably true in all enzyme preparations obtained in our laboratory. Eggleston & Krebs (1974) state that some tissue preparations had a high 'blank' and that, in those cases, endogenous substrate was removed by Minicon treatment, but it is not clear from their data when this was done. In any case, it is incorrect to subtract this rate, measured in the absence of added G6P, from the experimental rate for the following reason. The concentration of G6P used in the assays (1.33 mM) is 46 times the K_m for

G6P (Thompson *et al.*, 1976). Thus, the enzyme is 98% saturated with substrate and, no matter how much G6P is present in the enzyme solution, its presence cannot increase the reaction rate in the assay solution significantly. The interpretation of the effect of GSSG on NADPH inhibition is very different, depending on whether or not the control rates are subtracted from the experimental rates in those experiments in which untreated enzyme is used (see Table 1). It is not possible to assess the effect of this erroneous correction in the paper of Eggleston & Krebs (1974) because the magnitude of the correction is not stated.

Conclusions

We conclude, on the basis of the above considerations and on the data, that no evidence exists so far for a direct effect of GSSG on rat liver G6P dehydrogenase that counteracts inhibition by NADPH, or for the existence of a cofactor that mediates such an effect. Instead, we believe that GSSG acts indirectly, probably via glutathione reductase, to oxidize NADPH to NADP⁺ in experiments carried out *in vitro*. The apparent relief of NADPH inhibition observed by Eggleston & Krebs (1974) can be accounted for in part by their use of ZnCl₂ and by the incorrect procedure of correcting their absorbance changes with rates measured in the absence of added G6P. What mechanisms, if any, operate *in vivo* to counteract NADPH inhibition remains unclear. In the light of the numerous reports of the involvement of thiol/disulphide ratios in the regulation of metabolism in general, and of the activities of G6P dehydrogenases from pea chloroplasts and rat liver in particular, a role for the operation of such a mechanism in counteracting NADPH inhibition of hepatic G6P dehydrogenase should not be lightly dismissed. Evidence for such a mechanism, however, has yet to be established.

This work was supported by grants AM07720 from the United States Public Health Service and PCM8102164 from the National Science Foundation.

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