Regulation of the Pentose Phosphate Cycle

By LEONARD V. EGGLESTON* and HANS A. KREBS

*Metabolic Research Laboratory, Nuffield Department of Clinical Medicine,
Radcliffe Infirmary, Oxford OX2 6HE, U.K.*

(Received 20 September 1973)

1. A search was made for mechanisms which may exert a 'fine' control of the glucose 6-phosphate dehydrogenase reaction in rat liver, the rate-limiting step of the oxidative pentose phosphate cycle. 2. The glucose 6-phosphate dehydrogenase reaction is expected to go virtually to completion because the primary product (6-phosphogluconate lactone) is rapidly hydrolysed and the equilibrium of the joint dehydrogenase and lactonase reactions is in favour of virtually complete formation of phosphogluconate. However, the reaction does not go to completion, because glucose 6-phosphate dehydrogenase is inhibited by NADPH (Negelein & Haas, 1935). 3. Measurements of the inhibition (which is competitive with NADP^+) show that at physiological concentrations of free NADP^+ and free NADPH the enzyme is almost completely inhibited. This indicates that the regulation of the enzyme activity is a matter of de-inhibition. 4. Among over 100 cell constituents tested only GSSG and AMP counteracted the inhibition by NADPH; only GSSG was highly effective at concentrations that may be taken to occur physiologically. 5. The effect of GSSG was not due to the GSSG reductase activity of liver extracts, because under the test conditions the activity of this enzyme was very weak, and complete inhibition of the reductase by Zn^{2+} did not abolish the GSSG effect. 6. Preincubation of the enzyme preparation with GSSG in the presence of Mg^{2+} and NADP^+ before the addition of glucose 6-phosphate and NADPH much increased the GSSG effect. 7. Dialysis of liver extracts and purification of glucose 6-phosphate dehydrogenase abolished the GSSG effect, indicating the participation of a cofactor in the action of GSSG. 8. The cofactor removed by dialysis or purification is very unstable. The cofactor could be separated from glucose 6-phosphate dehydrogenase by ultrafiltration of liver homogenates. Some properties of the cofactor are described. 9. The hypothesis that GSSG exerts a fine control of the pentose phosphate cycle by counteracting the NADPH inhibition of glucose 6-phosphate dehydrogenase is discussed.

The control of the pentose phosphate cycle is not yet fully understood. The main functions of the cycle are the supply of pentose phosphate for the synthesis of nucleotides and of reducing equivalents, in the form of NADPH, for biosyntheses (e.g. of fatty acids) or, in erythrocytes, for the regeneration of haemoglobin from methaemoglobin. As these requirements are variable, regulatory mechanisms must be expected to adjust the rate of the cycle to changing needs. These are likely to operate at the initial step (the glucose 6-phosphate dehydrogenase reaction) and to take part in the control of the fate of glucose 6-phosphate, an intermediate which may form glycogen, glucose, fructose 6-phosphate or 6-phosphogluconate.

A major factor in the regulation of the glucose 6-phosphate dehydrogenase reaction is the adaptive change in the amount of this enzyme (and of 6-phosphogluconate dehydrogenase) first described by Tepperman & Tepperman (1958), Fitch *et al.*

(1959*a,b*) and Fitch & Chaikoff (1960): in response to a diet containing excess of carbohydrate and causing a deposition of fat, the amount of the two dehydrogenases in the liver may increase 5–10-fold. These adaptive changes represent a 'coarse control'. In addition a 'fine control' is expected by which prompt variations in the enzyme activities can be brought about.

These considerations led us to investigate the factors that modify the activity of hepatic glucose 6-phosphate dehydrogenase, in particular the inhibitory action of NADPH, discovered by Negelein & Haas (1935). The experiments confirm that the inhibition by NADPH is competitively reversed by NADP^+ and depends on the $[\text{NADPH}]/[\text{NADP}^+]$ ratio. The inhibition approaches 100% when the ratio reaches a value of 9. In rat liver cytoplasm the ratio of the free nucleotides is of the order of 100 (Veech *et al.*, 1969; Veech, 1968). This indicates that *in vivo* some hitherto unknown factors must overcome the inhibition by NADPH. Of many different cell

* Deceased 13 January 1974.

constituents tested only GSSG had a marked effect of this kind.

Experimental

Special substrates and crystalline enzymes were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Solutions (1 mM) of NADPH and NADP⁺ were made fresh each day. To obtain crude enzyme preparations, weighed samples of tissues from Wistar rats were homogenized with 4 vol. of ice-cold 0.1 M-potassium phosphate buffer, pH 7.4, containing 1 mM-EDTA, and centrifuged at 30000g for 20 min at 2°C. The supernatants were stored at -20°C in sealed tubes. A crude yeast extract was made by rupturing the cells of freshly crumbled baker's yeast by rapid freezing (liquid N₂) and thawing (25°C). This was once repeated and followed by centrifugation at 30000g for 20 min at 2°C. The supernatant yeast extract was also stored at -20°C.

Assay of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) activity was carried out spectrophotometrically at 24–25°C in groups of four cuvettes containing 3 ml of reactants, including (final concn.) 50 mM-Tris-HCl buffer, pH 7.4, 3.3 mM-MgCl₂, 1.33 mM-glucose 6-phosphate, various concentrations of NADP⁺ and NADPH, and samples of enzyme and tissue preparations freshly diluted with water. The amounts of tissue extracts added were the equivalent of 1–10 mg fresh weight, depending on their activity. Crystalline yeast enzymes were diluted to about the same activity as the mammalian tissues. Two of the cuvettes were used to measure the extinction changes in the presence and absence of substrate, the other two to measure the effect of added NADPH in the presence and absence of substrate. The extinction changes in the absence of substrates and added NADPH were small but became substantial usually after addition of NADPH. Corrections of the 'blanks' were important at very low NADP⁺ concentrations (less than 15 μM) when the total changes due to the dehydrogenase activity were small (0.030–0.050 extinction unit in the first 5 min).

Reactions were started by the addition of substrate, and spectrophotometer readings at 340 nm were taken during the linear period, which lasted about 10 min; later the reactions ceased to be linear mainly because of the fall in the concentration of NADP⁺. When reaction mixtures were preincubated this was carried out at 38°C in sealed polystyrene tubes. These were then cooled by shaking briefly in a water bath at 25°C before being emptied into cuvettes.

The initial concentrations of the NADP⁺ solutions used in the assays were measured separately with glucose 6-phosphate and 5 μl (0.7 unit) of crystalline yeast glucose 6-phosphate dehydrogenase, and

Tris-HCl buffer (50 mM) at pH 7.4. The reaction was usually complete within 2 min.

Amicon tissue filtrates

Amicon Centriflo membrane cones 224-CF-50A [supplied by Amicon N.V., Oosterhout (N.B.), Holland; see Blatt, 1971] were soaked for 2 h in water before use. Tissue homogenates of various concentrations (5 ml) were centrifuged in the cones for 0.5–1 h at 1000g and 4°C. Filtrates obtained were free of glucose 6-phosphate dehydrogenase and GSSG reductase (EC 1.6.4.2) and, according to the makers, contained only substances of molecular weights below 50000.

Some tissue preparations had a very high 'blank' activity, owing to the presence of endogenous substrates, and these were first removed by treating the preparation in a Minicon B-15 macro-solute concentrator (Amicon N.V.). According to the makers this removes substances of molecular weights below 15000.

Test system for 'cofactor' activity

Two pairs of cuvettes were used as for the experiments on inhibition of glucose 6-phosphate dehydrogenase by NADPH. The concentrations of NADPH (added to one pair only) were 3.5–4 times those of NADP⁺. In addition to buffer and Mg²⁺ all cuvettes contained GSSG (100 μM) and ZnCl₂ (33.3 μM). Glucose 6-phosphate was omitted from the blanks and the source of the dehydrogenase was rat liver supernatant from a 20% (w/v) homogenate, which had been dialysed for 24 h against several changes of water at 2°C. This dialysed enzyme did not deteriorate for at least 2 months at -20°C. Cuvettes contained the equivalent of 1.5–1.7 mg of original liver. Extinction changes at 340 nm were measured for 10 or 15 min at room temperature (24–26°C), with or without the addition of tissue extracts.

Conditions for comparing glutathione reductase and glucose 6-phosphate dehydrogenase activities in rat tissues

Substrate concentrations were optimal, i.e. 1.33 mM-glucose 6-phosphate and 0.33 mM-NADP⁺ in the dehydrogenase assay, and 0.33 mM-GSSG and 0.1 mM-NADPH in the reductase assay. Tris-HCl buffer (50 mM, pH 7.4) and 3.33 mM-MgCl₂ were included in the reaction mixture. Blanks without glucose 6-phosphate or GSSG were run simultaneously and the maximum linear rate of extinction changes at 340 nm was measured for up to 6 min at 25°C after the addition of tissue homogenate. Rates are expressed as μmol of substrate used/min per g fresh wt. at 25°C and pH 7.4.

Determination of GSSG

The method of Klotzsch & Bergmeyer (1963) with crystalline GSSG reductase was used.

Assays of GSH–cystine transhydrogenase (EC 1.8.4.4)

The GSH–cystine transhydrogenase reaction in rat liver preparations was assayed by coupling it with added GSSG reductase, and measuring NADPH oxidation (see States & Segal, 1969, 1973):

Two pairs of cuvettes were used for each assay and all contained 50 mM-Tris–HCl buffer, pH 7.4, 3.33 mM-MgCl₂, 0.1 mM-NADPH and 1–5 mM-GSH. One of each pair contained cystine (various amounts of a saturated solution in 50 mM-Tris–HCl buffer, pH 7.4, at room temperature), and to one pair only was added rat liver homogenate (1–2 mg fresh wt. of tissue). The total volumes were 3.0 ml. The coupled reactions were started by the addition of 10 μ l (0.9 unit) of crystalline yeast GSSG reductase, and extinction changes at 340 nm were measured every minute for 5–10 min at 25°C. At this pH there is a substantial non-enzymic reaction in the absence of liver, and this rate (constant for several minutes) was subtracted from the rate in the presence of liver. The rates in the 'blank' cuvettes in each pair (i.e. cystine omitted) were relatively small.

Results

To investigate the effect of a variety of agents on the activity of glucose 6-phosphate dehydrogenase a test system was devised to contain a suitable range of concentrations of NADP⁺ and NADPH. Since the activity of the enzyme is measured by the increase in the concentration of NADPH, the range of inhibitory concentrations was limited. A further consideration in designing the experiments was the attempt to carry out the tests at coenzyme concentrations that are of the same order as those in liver *in vivo* (about 0.07 mM-NADP⁺ and 0.30 mM-NADPH). The latter concentration was found to be about three times higher than could be used experimentally.

The main source of the enzyme was the supernatant of rat liver because this tissue is one of the major sites of the pentose phosphate cycle. To obtain a measurable reaction rate the supernatant had to be diluted at least 1000-fold. This diluted the endogenous coenzymes to negligible amounts. It also diluted the substances that bind the coenzymes. Thus whereas *in vivo* the tissue concentrations of the coenzymes measured include free and protein-bound coenzymes, the concentrations in the experiments were virtually all those of free coenzymes.

Inhibition of liver glucose 6-phosphate dehydrogenase by NADPH

Examples of the inhibition of the initial rate of the glucose 6-phosphate dehydrogenase reaction (Table 1) show that concentrations of NADPH amounting to about one-third of the total NADPH in the liver are strongly inhibitory. In general the inhibition is greater at low NADP⁺ concentrations, but no attempt has been made to analyse the kinetics of the inhibition in detail. When the ratio [NADPH]/[NADP⁺] was above 8 the inhibition was almost complete (Expt. 1). The inhibition is already known to be competitive with NADP⁺, and in the supernatants used in the present experiments K_m was 3 μ M for NADP⁺ and K_i was 7 μ M for NADPH. Glock & McLean (1953) found K_m for NADP⁺ of the rat liver enzyme to be 13 μ M. Sapag-Hagar *et al.* (1973), with different conditions of assays and different physiological states of the liver (lipogenic diet), reported K_i values of 30 μ M and K_m values for NADP⁺ of 30 μ M. When NADPH was replaced by NADH no significant inhibition was observed. Crystalline yeast glucose 6-phosphate dehydrogenase, or the enzyme in crude yeast extracts, gave similar inhibition with added NADPH (not recorded in the Tables). Since in rat liver *in vivo* the calculated ratio [free NADPH]/[NADP⁺] is of the order of 100 (Veech *et al.*, 1969), and since the absolute concentrations of free NADPH *in vivo* are of the same order as those used in the present experiments, the enzyme is expected to be almost completely inhibited *in vivo*.

Attempts to overcome the inhibition by NADPH

These considerations led to the hypothesis that there must be cell constituents that counteract the inhibitory effects of NADPH on glucose 6-phosphate dehydrogenase. Among over 100 cell constituents tested (these included amino acids, phosphates, coenzymes, B vitamins, substrates and intermediates of respiration and glycolysis, hormones, purines, nucleotides and nucleosides) only two substances were found to have significant effects, AMP and GSSG. AMP was effective only at unphysiological concentrations. Thus 1 mM was needed to diminish the inhibition from 68 to 38%. Preincubation of the enzyme with AMP did not increase the effectiveness. GSSG was the only substance effective at normal tissue concentrations (Table 2). GSH had no effect. In the absence of added NADPH there was some inhibition of glucose 6-phosphate dehydrogenase by the higher concentrations of GSSG, but the inhibitions caused by addition of NADPH were reversed by GSSG. This reversal was not due to the removal of NADPH by the GSSG reductase reaction because controls indicated that there was no measurable GSSG reductase activity when the concentration

Table 1. *Inhibition of glucose 6-phosphate dehydrogenase by NADPH in rat liver supernatant*

Details of the procedure are given in the Experimental section and in the text. Cuvettes contained (final concn.) 50 mM-Tris-HCl buffer, pH 7.4, 3.3 mM-MgCl₂, 1.33 mM-glucose 6-phosphate, various amounts of NADP⁺ and NADPH, and rat liver supernatant (about 1.25 mg of liver). Corrections were made for controls without glucose 6-phosphate. The data given in the Table are representative of six experiments.

Expt. no.	Initial NADP ⁺ concn. (μ M)	Initial NADPH concn. (μ M)	Ratio [NADPH]/[NADP ⁺]	Inhibition by NADPH (%)
1	12	26.0	2.16	52
		50.9	4.24	67
		100.0	8.33	95
		103.0	8.60	100
2	24	17.2	0.72	35
		33.3	1.39	62
		49.8	2.08	66
		96.8	4.03	87
3	104	92.0	0.89	50
		45.4	0.44	42
		21.9	0.21	26

Table 2. *Effect of GSSG on the inhibition of glucose 6-phosphate dehydrogenase by NADPH in rat liver*

Details of the assay method are given in the text and in Table 1. The NADP⁺ concentration was 19.4 μ M and the liver preparations used were from the same animal. The data have been corrected for blanks from which glucose 6-phosphate was omitted, and also for any GSSG reductase activity, which was measurable only with GSSG concentrations above 0.05 mM.

Expt. no.	Liver preparation used	GSSG concn. (mM)	10 ³ × Extinction changes in the period 1–11 min		Inhibition by NADPH (%)
			Without added NADPH	NADPH (74 μ M) added	
1	Supernatant	0	52	22	58
		0.0125	53	25	53
		0.025	52	30	42
		0.05	45	33	27
		0.10	39	35	10
		0.20	30	44	0
2	Homogenate	0	42	19	55
		0.0125	40	26	35
		0.025	36	32	11
		0.05	29	39	0
		0.10	22	42	0

of GSSG was below 0.2 mM. Further, even at the higher concentrations of GSSG the rate of NADPH removal was so slight that it did not affect significantly the concentration of NADPH during the test period. The GSSG effect was somewhat greater in the homogenate than in the supernatant. The small inhibitions of glucose 6-phosphate dehydrogenase by GSSG concentrations above 50 μ M was found to be not competitive with NADP. The inhibition is also the same in the presence of Zn²⁺ (33.3 μ M), which completely inhibits any glutathione reductase activity.

The effect of low concentrations of GSSG (0.1 mM) was substantially increased by preincubating the liver enzyme with GSSG at 25° or 38°C before the addition

of glucose 6-phosphate (Table 3). During the preincubation the presence of Mg²⁺ and NADP⁺ is essential for the GSSG effect, but the NADPH was not required at this stage (see Expts. 2 and 3 of Table 3). Thus the 'protective' effect of preincubation with GSSG was not connected with any GSSG reductase activity, as this would depend on the presence of NADPH. Further proof that GSSG reductase is not involved in the protective action of GSSG is provided by experiments in which ZnCl₂ (33.3 μ M) was added to the cuvettes (Table 4). This concentration of Zn²⁺ completely inhibits hepatic GSSG reductase (Miza & Langdon, 1962) without inhibiting glucose 6-phosphate dehydrogenase. Under these conditions 0.1 mM-GSSG,

Table 3. *Effects of preincubation of rat liver with low concentrations of GSSG*

Concentrations of added NADP⁺ were 17.3–17.5 μ M and of added NADPH were 67.5, 79.5, 64.7 and 67.0 μ M respectively in Expts. 1, 2, 3 and 4. After preincubation at 25°C in Expts. 1, 2 and 3 and at 38°C in Expt. 4 reactions were started by the addition of glucose 6-phosphate. The extinction changes have been corrected for blanks from which glucose 6-phosphate was omitted. For further details see the Experimental section.

Expt. no.	Special conditions	GSSG concn. (mM)	Preincubation time (min)	10 ³ × Extinction changes in the period 1–6 min		Inhibition by NADPH (%)
				Without added NADPH	NADPH added	
1	NADPH included during preincubation period	0	0	52	12	77
		0.033	0	50	13	74
		0.033	30	50	26	48
2	NADPH included during preincubation period	0	0	47	4	92
		0.1	0	40	13	68
		0.1	10	39	21	46
		0.1	20	36	27	25
		0.1	30	36	37	0
		0.1	40	39	48	0
3	NADPH omitted during preincubation period	0	0	46	20	57
		0.1	0	36	21	42
		0.1	10	38	25	34
		0.1	20	37	28	24
		0.1	40	36	35	3
		0.1	60	39	45	0
4	NADPH omitted during preincubation period	0	0	46	15	68
		0.1	0	37	24	35
		0.1	10	36	31	14
		0.1	15	32	35	0
		0.1	20	26	34	0

added directly to the cuvettes without preincubation, decreased inhibition by NADPH in a rat liver assay from 39% to 5%.

In contrast with GSSG, GSH had no effect when added at concentrations between 0.1 and 1 mM. At 5 mM there was a small decrease in the inhibition from 58 to 37% but this was probably due to traces of contaminating GSSG, which are difficult to exclude owing to the ready autoxidation of GHS.

Formation of GSSG in rat liver

The conversion of GSH into GSSG in animal tissues can be brought about by four different types of reactions: (1) by non-enzymic autoxidation catalysed by Fe²⁺, Cu²⁺ or Mn²⁺; (2) by non-enzymic interaction with dehydroascorbic acid; (3) by GSSG peroxidase (EC 1.11.1.9); (4) by GSH-disulphide transhydrogenases (EC 1.8.4.4, also EC 1.8.4.1, EC 1.8.4.2, EC 1.8.4.3, EC 1.8.5.1). The rate of the non-enzymic reactions is probably slight at the physiological concentrations of GSH, and *in vivo* the peroxidase reaction is probably the main source of GSSG. The rate of this reaction depends on the availability of substances which

on oxidation by O₂ can form H₂O₂, such as hypoxanthine or uric acid.

Table 5 shows that the addition of hypoxanthine, or uric acid, or DL-alanine in the presence of GSH, as expected, reverses the inhibition of glucose 6-phosphate dehydrogenase by NADPH. As the peroxidase is associated with microsomal particles, liver homogenates rather than supernatants were used as sources of enzymes. Hypoxanthine and uric acid arise continuously in animal tissues, and the concentrations of uric acid that were effective (0.033 mM) are lower than the uric acid concentrations that we find in freeze-clamped rat liver (0.07 mM).

GSH-disulphide transhydrogenases (for references see States & Segal, 1973) catalyse the reaction:

In the presence of rat liver homogenate and on addition of 50 μ M-cystine and 5 mM-GSH the rate of GSSG formation was found to average 4.7 μ mol/min per g at 25°C at pH 7.4. This rate was faster than the optimal rates of glucose 6-phosphate dehydrogenase (2.6 μ mol/min per g) or GSSG reductase (3.3 μ mol/min per g) reactions under similar conditions. As

Table 4. *Effect of GSSG on the inhibition of glucose 6-phosphate dehydrogenase in the presence of Zn²⁺*

Cuvettes contained rat liver homogenate equivalent to 1.25 mg of liver and 19 μ M-NADP⁺, with and without 72 μ M-NADPH. Corrections were made for extinction changes in controls from which glucose 6-phosphate had been omitted. GSSG reductase activity was measurable only in the controls of group (b) and for this a correction has been made. For further details see the Experimental section and Table 1.

Cuvette group	Additions	10 ³ × Extinction changes in the first 10 min		Inhibition by NADPH (%)
		Without NADPH	NADPH added	
(a)	None	51	30	41
(b)	GSSG (0.1 mM)	44	40	9
(c)	ZnCl ₂ (33.3 μ M)	46	28	39
(d)	ZnCl ₂ (33.3 μ M) and GSSG (0.033 mM)	46	34	26
(e)	ZnCl ₂ (33.3 μ M) and GSSG (0.1 mM)	44	42	5

Table 5. *Effect of the addition of H₂O₂-generating substances on the inhibition of glucose 6-phosphate dehydrogenase in the presence of GSH*

Samples of liver homogenate (1.67 mg fresh wt.) were preincubated for 15 min at 38°C with Tris-HCl buffer, pH 7.4, Mg²⁺, NADP (25 μ M) and the additions listed in the Table. After cooling to 25°C the rate of the dehydrogenase reaction was measured as described in the text. The reaction was started by the addition of 1.33 mM-glucose 6-phosphate. Corrections were made for the extinction changes in the absence of substrate.

Expt. no.	Additions and substrates for the GSSG peroxidase system in the liver	10 ³ × Extinction changes in 15 min		Inhibition by NADPH (%)
		Without added NADPH	With NADPH (69 μ M)	
1	None	53	17	68
	5 mM-GSH	42	21	50
	5 mM-GSH and 1 mM-hypoxanthine	33	39	0
	1 mM-Hypoxanthine	57	18	69
2	None	69	21	70
	1 mM-GSH	66	26	61
	1 mM-GSH and 0.33 mM-hypoxanthine	58	42	28
	1 mM-GSH and 0.167 mM-hypoxanthine	61	39	36
	1 mM-GSH and 0.33 mM-uric acid	48	59	0
	1 mM-GSH and 0.10 mM-uric acid	49	53	0
	1 mM-GSH and 0.066 mM-uric acid	49	45	8
	1 mM-GSH and 0.033 mM-uric acid	51	39	24
	0.33 mM-Uric acid and 0.33 mM-hypoxanthine	69	23	67
3	1 mM-GSH	58	23	60
	1 mM-GSH and 0.10 mM-uric acid	52	63	0
	1 mM-GSH and 2 mM-DL-alanine	48	38	21

the concentrations and availability of disulphide substrates are not known it is not possible to assess the importance of the transhydrogenase reaction *in vivo*.

Effect of dialysis and enzyme purification on the action of GSSG

Rat liver homogenate dialysed against frequent changes of water at 2°C showed no losses of either glucose 6-phosphate dehydrogenase or GSSG reductase activity, but after dialysis the addition of

GSSG no longer reversed the inhibition of glucose 6-phosphate dehydrogenase by NADPH. To obtain a complete loss of the action of GSSG the dialysis had to be continued for 24 h (Table 6). After 21 h there was still slight protection by GSSG. The GSSG effect was also lost when rat liver glucose 6-phosphate dehydrogenase was purified by the method of Bonsignore *et al.* (1970) (a procedure which also removed GSSG reductase activity). These experiments suggest that the counteraction by GSSG of the inhibition of glucose 6-phosphate by NADPH requires a cofactor.

Table 6. *Effect of the dialysis of rat liver homogenates on the GSSG reversals of NADPH inhibitions of glucose 6-phosphate dehydrogenase*

For details of the dialysis see the Experimental section. The NADP⁺ concentration was 20 μ M and that of NADPH (where added) was 70 μ M. The extinction changes have been corrected for blanks in which glucose 6-phosphate was omitted.

Liver dialysis time (h)	GSSG concn. (μ M)	10 ³ × Extinction changes in 15 min		Inhibition by NADPH (%)
		Without added NADPH	NADPH added	
0	0	61	28	54
	100	50	42	16
3	0	77	34	56
	100	62	41	34
24	0	68	33	52
	100	54	26	52

Table 7. *Presence of cofactor of GSSG effect in Amicon-cone filtrates of rat liver homogenates*

The source of glucose 6-phosphate dehydrogenase was a rat liver homogenate dialysed for 24 h against water at 2°C. The cofactor solutions were filtrates of 20% (w/v) liver homogenates, obtained by centrifugation in Amicon cones at 1000g for 1 h at 4°C. For details of the test system see the Experimental section and the text. All cuvettes contained ZnCl₂ (33.3 μ M). NADPH where added was about 3.5 times the NADP⁺ concentration. The extinction changes given below have been corrected for blanks from which glucose 6-phosphate was omitted.

Volume of Amicon-cone filtrate added to each cuvette (μ l)	GSSG concn. (μ M)	10 ³ × Extinction changes in 10 min		Inhibition by NADPH (%)
		Without added NADPH	NADPH added	
0	100	69	38	45
0	0	74	40	46
5	100	67	46	31
10	100	53	49	8
20	100	35	38	0
20	0	40	23	43

Assay of cofactor

The cofactor activity was tested with two pairs of cuvettes as described in the Experimental section for the measurement of the GSSG effect, except that the rat liver homogenate serving as a source of glucose 6-phosphate dehydrogenase was dialysed for 24 h to remove the cofactor. ZnCl₂ (33.3 μ M) was included to inhibit GSSG reductase, and GSSG (0.1 mM) was present in all cuvettes. NADPH, added to two cuvettes at concentrations about 3.5 times that of NADP⁺, gave inhibitions of glucose 6-phosphate dehydrogenase of 40–50% during the first 10 min at 25°C, and addition of cofactor solution to all four cuvettes caused a reversal of the inhibition provided that GSSG was present. Thus both GSSG and the cofactor were necessary for the reversal of the inhibition (see Table 7).

Properties of the cofactor

The cofactor proved very unstable once it had been separated from the enzyme preparation. It was present in fresh HClO₄ filtrates, but the activity

disappeared rapidly in both acid and neutralized solutions. (NH₄)₂SO₄ precipitation, heat (10 min at 60°C or 5 min at 100°C), storage at 4°C or –20°C, or evaporation to dryness *in vacuo* all caused the destruction of most of the activity. The most active cofactor solutions were obtained by centrifuging a 20% homogenate for 0.5–1 h at 1000g at 4°C in Amicon Centriflo membrane cones, which separate components below a molecular weight of 50000 from larger molecules (see Table 7). The filtrates obtained were free from glucose 6-phosphate dehydrogenase and GSSG reductase. Minicon treatment (see the Experimental section), which removes substances of molecular weights below 15000 without centrifugation, did not remove the cofactor from the homogenate, but removed it completely from the Amicon-cone filtrates.

Substantial (almost complete) reversals of the inhibition were obtained by addition of 5–10 μ l of the Amicon-cone filtrates equivalent to 1–2 mg fresh weight of liver (Table 7). Under the test conditions the amount of dialysed liver providing the enzyme was equivalent to 1.6 mg fresh weight. The

GSSG content of the Amicon-cone filtrates was negligible (less than 1% of the amount that had to be added for effectiveness). The Amicon-cone filtrates also contained an unidentified inhibitor of glucose 6-phosphate dehydrogenase, detectable when larger amounts of filtrate were used (Table 7).

The stability of the Amicon-cone filtrates was not increased by the addition of glycerol, $(\text{NH}_4)_2\text{SO}_4$, 2-mercaptoethanol, dithiothreitol or tetrahydrofolate or by changing the pH to 10.5 with glycine buffer. The most stable cofactor preparations were Amicon cone filtrates from 20% rat liver homogenates stored in small sealed tubes at room temperature. These retain measurable cofactor activity for at least 3 weeks. Attempts to concentrate cofactor solutions in a desiccator over P_2O_5 at 2°C were unsuccessful. In contrast with the instability of the cofactor after its separation from the enzymes, the starting material (liver homogenates) could be stored indefinitely at -20°C without loss of cofactor activity.

Cofactor in other tissues

The cofactor activity of various rat tissues was tested with a 24-h-dialysed rat liver homogenate as the test system. Amicon-cone filtrates were obtained from the tissues by the same procedure as was used with rat liver. With the exception of the lactating mammary gland all the tissues tested contained cofactor activities, though less than the liver (Table 8). In cardiac muscle the activity was about one-third of that of liver. It was still lower in spleen, leg muscle, kidney, lung and brain. Table 8 shows that the cofactor activity was not in proportion to the glucose 6-phosphate dehydrogenase activities or GSSG reductase activity.

Attempts to identify the cofactor

The following substances were tested for cofactor activity, with negative results: thiamin pyrophosphate, folate, FAD, tetrahydrofolate, biotin, pyridoxal phosphate, cysteine, cystine, ascorbate, 2-mercaptoethanol, insulin, glucagon, intermediates of the tricarboxylic acid cycle, ATP, UTP, GTP, CTP, cyclic AMP, CoA, acetyl-CoA and combinations of these.

Inhibition and its reversal in other tissues

In all the rat tissues tested (see Table 8) glucose 6-phosphate dehydrogenase was inhibited by NADPH, and this was reversed by GSSG except in the case of lactating mammary gland. The other rat tissues lost their sensitivity to GSSG after centrifugation of homogenates for 1 h at 1000g at 4°C in Amicon cones, but this could be partly or completely restored by addition of Amicon-cone filtrates from these homogenates.

Human tissues were more sensitive to very low concentrations of GSSG than were those of the rat. Thus preincubation for 15 min at 38°C with 3.3 μM -GSSG substantially decreased NADPH inhibition in supernatants of human liver and kidney homogenates. In contrast with mammalian tissues, the glucose 6-phosphate dehydrogenase of yeast (crude extracts or crystalline enzyme) was not affected at all by GSSG or AMP, singly or together, with or without preincubation.

Seasonal variations of the GSSG effect

The rats used in most experiments had been kept in windowed constant-temperature rooms where

Table 8. *Relative concentrations of cofactor in rat tissues*

The relative content of cofactor was assessed by determining the amounts of filtrate giving about the same effect. A dialysed rat liver preparation served as the test system. Amicon-cone filtrates of 20% rat tissue homogenate were prepared as described in the Experimental section. Cuvettes contained GSSG (100 μM) and ZnCl_2 (33.3 μM) and 10–300 μl of filtrates. The activities of glucose 6-phosphate dehydrogenase and GSSG reductase were assayed under optimal conditions in normal homogenates. The data, except for the mammary gland, are from the same rat.

Rat tissue	Enzyme activities in normal homogenates at 25°C and pH 7.4 ($\mu\text{mol/min per g}$)		Approximate concentrations of cofactor relative to that in liver (%)
	Glucose 6-phosphate dehydrogenase	GSSG reductase	
Liver	2.59	3.31	100
Heart	0.64	0.98	35
Spleen	6.40	2.58	30
Leg muscle	0.13	0.81	20
Kidney	1.54	6.33	15
Lung	2.67	1.19	15
Brain	1.59	0.73	5
Lactating mammary gland	20.10	1.46	0

the light was not regulated. Whereas the inhibitions by NADPH were of the same magnitude throughout the year the reversing effect of 0.1mM-GSSG (without preincubation) in livers of rats killed between November and January (when daylight was approx. 7.5h) was greater than in the livers obtained between May and July (when daylight was about 16.5h). In the summer months the GSSG concentration had to be raised to 0.2 or 0.3mM to give the same effect. When a group of rats were kept for 2–3 weeks in June on an artificial lighting cycle similar to the daylight periods in December, the sensitivity of GSSG increased towards the value obtained during the winter months. These phenomena may be related to the fact that rats feed mainly during the hours of darkness.

Discussion

Inhibition of glucose 6-phosphate dehydrogenase by NADPH

The glucose 6-phosphate dehydrogenase reaction has two special characteristics: first, the immediate product is a lactone, which is rapidly hydrolysed to 6-phosphogluconate. Owing to the large free-energy change of these two reactions (ΔG is estimated to be -37kJ at 25°C and pH7.0, and not much less at the metabolite concentrations in the liver; Bassham & Krause, 1969) the reaction would be expected to go virtually to completion. However, this is prevented by the second special property of the enzyme, the inhibition of the reaction by one of its products (NADPH), discovered for the yeast enzyme by Negelein & Haas (1935) and confirmed for the enzyme from other sources (Glock & McLean, 1953; Sapag-Hagar *et al.*, 1973; Bonsignore & De Flora, 1972). This inhibition is competitive with NADP^+ . By reversible inactivation of the enzyme it would establish a steady state in the glucose 6-phosphate dehydrogenases system that may be far removed from the thermodynamic equilibrium. On the basis of the concentrations of glucose 6-phosphate and 6-phosphogluconate reported by Sapag-Hagar *et al.* (1973) and the $[\text{NADPH}]/[\text{NADP}^+]$ ratio reported by Veech *et al.* (1969) the value for the mass-action ratio $[\text{glucose 6-phosphate}][\text{NADPH}]/[\text{6-phosphogluconate}][\text{NADP}^+]$ is about 90 in the liver of starved rats and about 250 in the liver of well-fed rats. The value of ΔG for the glucose 6-phosphate dehydrogenase of -37kJ (Bassham & Krause, 1969) indicates an equilibrium constant of the reaction of the order of 10^{-6} . Hence the difference between steady state and equilibrium is about 10^8 . The steady state would behave, nevertheless, like a near-equilibrium system inasmuch as withdrawal of NADPH decreases the inhibition and causes the

withdrawn NADPH to be replaced. Thus the basic control of the first step of the pentose phosphate cycle may still be considered as a matter of near-equilibrium relations.

There are uncertainties about the absolute concentrations of free NADP and free NADPH in the tissue that limit precise quantitative predictions. The concentration of free plus bound NADPH in rat liver is of the order of 0.38mM (Williamson *et al.*, 1971). Since the ratio $[\text{free NADPH}]/[\text{free NADP}^+]$ in the liver is of the order of 100 (Veech *et al.*, 1969; Gumaa *et al.*, 1971), it is probable that most of the total NADPH is in the free form; otherwise the concentration of free NADP^+ would be exceedingly low (below 0.004mM). Concentrations giving effective inhibitions in the test system were as low as $20\mu\text{M}$ (Table 1), but despite the uncertainties there can be no doubt that the inhibition by NADPH occurs under physiological conditions.

That the rate of the pentose phosphate cycle is controlled at the glucose 6-phosphate dehydrogenase step has been established for some time (Cahill *et al.*, 1958; Gumaa *et al.*, 1971). Further, since the concentration of glucose 6-phosphate exceeds by more than threefold the K_m of glucose 6-phosphate dehydrogenase, Gumaa *et al.* (1971) concluded that control is not achieved by substrate limitation, but by NADP^+ limitation. Although this is essentially correct, the position is more complex than previous authors had realized as they had not taken into account the virtually complete inhibition of hepatic glucose 6-phosphate dehydrogenase at physiological ratios of $[\text{free NADPH}]/[\text{free NADP}^+]$. The value of this ratio (about 100) is much higher than the ratio for the total NAD phosphates. The almost complete inhibition implies that control of enzyme activity is essentially a matter of de-inhibition.

Effect of GSSG

GSSG has been implicated previously in playing a role in the regulation of the activity of glucose 6-phosphate dehydrogenase. Thus the increase in the rate of this reaction during phagocytosis by macrophages (Evans & Karnovsky, 1962) has been related to the increased H_2O_2 production during phagocytosis (Iyer *et al.*, 1961; Paul & Sbarra, 1968), H_2O_2 being instrumental in killing phagocytosed bacteria. The H_2O_2 is taken to be formed by an NADPH oxidase reaction (Paul & Sbarra, 1968) and, through GSH peroxidase, would convert some GSH into GSSG. The GSSG reductase would regenerate NADP^+ from NADPH and thereby promote the glucose 6-phosphate dehydrogenase reaction (Reed, 1969). However, this mechanism cannot be responsible for the effects of GSSG in the present system, because the effects occur even when GSSG reductase is absent or completely inhibited.

The de-inhibition by GSSG is a possible, but not yet a proven, control mechanism of glucose 6-phosphate dehydrogenase *in vivo*. The difficulty in assessing the action of GSSG *in vivo* is the lack of reliable information on the tissue concentrations of GSSG (see Veech, 1968). Veech (1968) found a mean GSSG concentration in freeze-clamped liver of 0.40 mM and a mean concentration of GSH of 5.1 mM. The values for GSSG are likely to be too high; thus Wendell (1970), using *N*-ethylmaleimide to prevent oxidation of GSH, found 0.03–0.05 mM-GSSG and 3–4 mM-GSH in perfused rat hearts. Wendell (1970) calculated the intracellular concentration of GSSG in rat liver to be about 0.06 mM from the data of Güntherberg & Rapoport (1968). Concentrations of GSSG required to obtain significant de-inhibitions are of the order of 0.01–0.1 mM under the conditions used in the present experiments. Still lower values are effective after preincubation. In experiments on human liver and kidney (not recorded) even 5 μ M-GSSG reversed the NADPH inhibition.

Relative activities of glucose 6-phosphate and 6-phosphogluconate dehydrogenases

Sapag-Hagar *et al.* (1973) found that the capacity of hepatic glucose 6-phosphate dehydrogenase is about five times greater under lipogenic conditions than the capacity of 6-phosphogluconate dehydrogenase. This 'imbalance' would be expected to cause an accumulation of 6-phosphogluconate, which, however, does not occur. Moreover, the competitive inhibition of 6-phosphogluconate dehydrogenase by NADPH was greater than that for glucose 6-phosphate dehydrogenase, the K_i/K_m values being 5 for glucose 6-phosphate dehydrogenase and 1.4 for 6-phosphogluconate dehydrogenase. Sapag-Hagar *et al.* (1973) come to the tentative conclusion 'that either liver 6-phosphogluconate dehydrogenase can be markedly activated *in situ* by some as yet unrecognized factor (S factor) or there is an alternative pathway for 6-phosphogluconate in animal tissues'. In the light of the present experiments it is more likely that the adjustment of the activities of the two enzymes is achieved by control through de-inhibition of the two enzymes.

Insufficient information is available on the properties of hepatic glucose 6-phosphate dehydrogenase for a detailed analysis of the mechanism and of the kinetics of the effects of NADPH and GSSG. It is of interest that rat liver glucose 6-phosphate dehydrogenase occurs as a tetramer, dimer and monomer (Matsuda & Yugari, 1967). The human erythrocyte enzyme has similar properties, and for this enzyme it has been shown that the dimer–monomer interconversion depends on the presence of 'structural' NADP⁺ (Wrigley *et al.*, 1972; Bonsignore & De Flora, 1972).

Physiological significance

It is essential for the regulated function of the pentose phosphate cycle that the glucose 6-phosphate dehydrogenase reaction can be prevented even when there is an abundant supply of glucose 6-phosphate for either glycogen synthesis or the Embden–Meyerhof pathway. This could not be achieved by simple equilibrium relations based solely on the thermodynamic characteristics of the starting materials and the end products. The inhibition by NADPH and its relief by GSSG would allow for the regulation of the rate of the pentose phosphate cycle irrespective of the concentration of glucose 6-phosphate. The activity of glucose 6-phosphate dehydrogenase may therefore depend on the concentration of GSSG. The control of this, involving the ready interconversion of GSSG and GSH, is still an open question.

Organ differences

The fact that glucose 6-phosphate dehydrogenase of yeast and of mammary gland is insensitive to GSSG correlates with the reports that both the yeast enzyme (Yue *et al.*, 1969) and the mammary-gland enzyme (Levy, 1963) do not contain bound NADP⁺. Further, these two enzymes are inactivated in the presence of 2M-urea by dissociation to the monomers, whereas in crude rat liver the enzyme is hardly affected by urea. Both the yeast and mammary-gland enzymes, however, are inhibited by NADPH. The quantitative aspects of this inhibition and the question of de-inhibition remain to be investigated.

There may be good physiological reasons for organ differences. Unlike the liver enzyme the mammary-gland enzyme functions more or less continuously during lactation, and 'coarse' control, increasing the amount of the enzyme during lactation, is no doubt the main regulatory mechanism. In liver rapid adjustments of enzyme activity are important because fatty acid synthesis shows diurnal variations apart from variations due to nutritional factors. In growing yeast cells the requirement for glucose 6-phosphate dehydrogenase activity (in connexion with a supply of ribose phosphate and of reducing equivalents) is also expected to be steady. In erythrocytes the demand for reducing equivalents probably depends mainly on the amounts of methaemoglobin to be reduced, which are liable to be variable.

This work was supported by grants from the Medical Research Council and the U.S. Public Health Service (grant no. AM11748).

References

- Bascham, J. A. & Krause, G. H. (1969) *Biochim. Biophys. Acta*, **189**, 207–221
- Blatt, W. F. (1971) *Methods Enzymol.* **22**, 39–49

- Bonsignore, A. & De Flora, A. (1972) in *Current Topics in Cellular Regulation* (Horedecker, B. L. & Stadtman, E. R., eds.), vol. 6, pp. 21–56, Academic Press, New York and London
- Bonsignore, A., Lorenzoni, I., Cancedda, R., Nicolini, A., Damiani, G. & De Flora, A. (1970) *Ital. J. Biochem.* **19**, 165–177
- Cahill, G. F., Jr., Hastings, A. B., Ashmore, J. & Zottu, S. (1958) *J. Biol. Chem.* **230**, 125–135
- Evans, W. H. & Karnovsky, M. L. (1962) *Biochemistry* **1**, 159–166
- Fitch, W. M. & Chaikoff, I. L. (1960) *J. Biol. Chem.* **235**, 554–557
- Fitch, W. M., Hill, R. & Chaikoff, I. L. (1959a) *J. Biol. Chem.* **234**, 1048–1051
- Fitch, W. M., Hill, R. & Chaikoff, I. L. (1959b) *J. Biol. Chem.* **234**, 2811–2813
- Glock, G. E. & McLean, P. (1953) *Biochem. J.* **55**, 400–408
- Gumaa, K. A., Mclean, P. & Greenbaum, A. L. (1971) *Essays Biochem.* **7**, 39–86
- Güntherberg, H. & Rapoport, S. (1968) *Acta Biol. Med. Ger.* **20**, 559–564
- Iyer, G. Y. N., Islam, M. F. & Quastel, J. H. (1961) *Nature (London)* **192**, 535–541
- Klotzsch, H. & Bergmeyer, H.-U. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 363–366, Verlag Chemie and Academic Press, New York and London
- Levy, H. R. (1963) *J. Biol. Chem.* **238**, 775–784
- Matsuda, T. & Yugari, Y. (1967) *J. Biochem. (Tokyo)* **61**, 535–540
- Miza, C. E. & Langdon, R. G. (1962) *J. Biol. Chem.* **237**, 1589–1595
- Negelein, E. & Haas, E. (1935) *Biochem. Z.* **282**, 206–220
- Paul, B. & Sbarra, A. J. (1968) *Biochim. Biophys. Acta* **156**, 168–178
- Reed, P. W. (1969) *J. Biol. Chem.* **244**, 2459–2464
- Sapag-Hagar, M., Lagunas, R. & Sols, A. (1973) *Biochem. Biophys. Res. Commun.* **50**, 179–185
- States, B. & Segal, S. (1969) *Biochem. J.* **113**, 443–444
- States, B. & Segal, S. (1973) *Biochem. J.* **132**, 623–631
- Tepperman, J. & Tepperman, H. M. (1958) *Amer. J. Physiol.* **193**, 55–64
- Veech, R. L. (1968) D.Phil. Thesis, University of Oxford
- Veech, R. L., Eggleston, L. V. & Krebs, H. A. (1969) *Biochem. J.* **115**, 609–619
- Wendell, P. L. (1970) *Biochem. J.* **117**, 661–665
- Williamson, D. H., Mayor, F. & Veloso, D. (1971) in *Regulation of Gluconeogenesis* (Söling, H.-D. & Willms, B., eds.), pp. 92–101, Academic Press, New York and London
- Wrigley, N. G., Heather, J. V., Bonsignore, A. & De Flora, A. (1972) *J. Mol. Biol.* **68**, 483–499
- Yue, R. H., Noltmann, E. A. & Kuby, S. A. (1969) *J. Biol. Chem.* **244**, 1353–1364