A Negative Correlation between Oxygen Uptake and Glutathione Oxidation in Rat Liver Homogenates

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GSH added to rat liver homogenates inhibited respiration and increased GSSG formation approximately proportionally to the amount of GSH added; the effect was increased by added magnesium chloride. Added NADPH and citrate decreased GSSG formation and increased respiration; 6.0 mM-nicotinamide prevented GSSG formation and increased respiration. There was a negative correlation between GSSG formation and oxygen uptake. It is suggested that the decrease in oxygen uptake is mainly due to GSSG concentration and also that *in vivo* a GSH–GSSG steady state occurs.

Both thiols and disulphides inhibit respiration of isolated cells, tissue preparations and whole animals. Decreases in respiration caused by thiols have been observed in yeast (Bhuvaneshwaran, Sreenivasan & Rege, 1964), in Ehrlich ascites cells (Eaton & Scala, 1964), in chick-embryo intestine (Kirmann, 1964), in slices of guinea-pig cerebral cortex (McIlwain, 1959) and in the whole rat (Betz, Lelièvre & Smoliar, 1967). Inhibition of oxygen uptake by disulphides is known to occur in Yoshida hepatoma ascites cells and in the mitochondria of rat tissues (Lelièvre, 1963, 1965; Skrede, Bremer & Eldjarn, 1965). Interference of thiols and disulphides with phosphate metabolism (McIlwain, 1959; Lelièvre, 1963; Skrede *et al.* 1965) has also been reported. The effects of thiols and disulphides may be interdependent owing to interconversion by oxidoreduction, since some tissue preparations preferentially maintain one of the forms of glutathione. Rat liver homogenates oxidize GSH and pigeon liver homogenates maintain GSH and reduce GSSG (Pinto, 1961).

A negative correlation between the GSSG formation from added GSH and the oxygen uptake was found when GSH was added to rat liver homogenates incubated under aerobic conditions. It is suggested that the main form of glutathione responsible for the inhibition of respiration is the disulphide rather than the thiol and also that in intact liver an oxidoreduction steady state of the glutathione is maintained through glutathione peroxidase- and glutathione reductase-catalysed reactions.

MATERIALS AND METHODS

Materials. GSH, GSSG and NADPH were supplied by C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. All the other reagents used were obtained from

British Drug Houses Ltd., Poole, Dorset, and were of analytical grade or the purest available.

Animals. Male Wistar rats used were from the University of Sheffield Animal House colony and weighed 180–250 g. They were fed on Oxoid diet 86 (Herbert C. Styles Ltd., Bewdley, Wores.). The pigeons used were young adult animals.

Preparation of homogenates. Rats were killed by stunning and decapitation and pigeons by decapitation. In both cases the liver was removed and cooled in 0.25 M-sucrose at 0°, dried between filter papers and minced in a chilled Fischer mincer (Jouan, Paris, France). The minced liver was weighed and homogenized in 4 vol. of ice-cold 0.25 M-sucrose at 600 rev./min. in a chilled Potter–Elvehjem homogenizer. This homogenate was diluted with an equal volume of 0.154 M-KCl and used as such.

Incubations. Unless otherwise stated, the incubation mixture was: 3.0 ml. of homogenate, 0.7 ml. of 0.1 M-potassium phosphate buffer, pH 7.4, and water to the final volume of 4.0 ml. In some of the experiments (Table 1) tris-HCl buffer, pH 7.4, replaced the phosphate buffer. Sometimes MgCl₂, NADPH, nicotinamide and citrate were also added to the Warburg flasks. Glutathione was always added from the side arm after 10 min. preliminary incubation. The centre wells contained 0.2 ml. of 4 M-NaOH and filter paper. Incubations were performed for 2 hr. in Warburg manometers at 30° with O₂ as the gas phase. Deproteinization and centrifugation were done as described by Pinto & Bartley (1969).

GSH and GSSG determinations. Measurements of both forms of glutathione were made as previously described (Pinto, 1961; Pinto & Bartley, 1969). It should be pointed out that the method used to measure GSH actually measures non-protein thiols and not only GSH. The experiments in the Tables are typical of many. Each individual experiment quoted was carried out with a single animal.

RESULTS

Effect of GSH concentration (Table 1, Expt. 1). In rat liver homogenates the decrement in oxygen uptake was proportional to the concentration of

Table 1. *Effects of GSH and of Mg²⁺ on the oxygen uptake and the GSSG formation in rat and pigeon liver homogenates*

All values are changes/Warburg vessel/2hr. The volume of the incubation mixture was 4.0 ml. For full experimental details see the text.

Expt. no.	Animal	GSH added (mM)	MgCl ₂ added (mM)	O ₂ uptake (μmoles)	GSSG formation (μmoles)	GSH disappearance (μmoles)	Decrease in O ₂ uptake (μmoles)	Increase in GSSG formation (μmoles)	Increase in GSH disappearance (μmoles)
1	Rat	0	0	38.0	1.1	2.5	—	—	—
		1.5	0	35.7	1.8	3.8	2.3	0.7	1.3
		6.0	0	28.8	2.7	6.0	9.2	1.6	3.5
		0	0	30.3*	1.1	2.5	—	—	—
		6.0	0	13.4*	3.3	8.4	16.9	2.2	5.9
2	Rat	0	0	40.0	1.0	2.1	—	—	—
		0	1.0	37.7	1.0	2.1	2.3	0	0
		0	2.5	31.3	0.95	2.1	9.7	-0.05	0
		2.25	0	33.0	1.9	4.0	—	—	—
		2.25	1.0	26.8	2.4	5.4	6.2	0.5	1.4
		2.25	2.5	20.0	3.1	6.5	13.0	1.2	2.5
3	Rat	2.75	0	11.6*	2.9	—	—	—	—
		2.75	2.5	11.2*	3.0	—	—	—	—
4	Pigeon	0	0	58.0	0	—	—	—	—
		0	2.5	50.5	0	—	—	—	—
		2.5	0	56.0	-0.1	—	—	—	—
		2.5	2.5	49.5	-0.1	—	—	—	—

* Tris-HCl buffer used instead of phosphate buffer.

GSH added, although GSSG formation or GSH disappearance was increased. GSH oxidation was about 30% higher when tris was used as buffer instead of phosphate. This agrees with the higher peroxidation found in tris buffer compared with phosphate buffer in both mitochondrial and microsomal preparations (Christophersen, 1968a). When tris was the buffer the disappearance of GSH was about 25% higher than the formation of GSSG, suggesting that some oxidation through haemoprotein-catalysed reactions might occur to give the sulphonic acid (Little & O'Brien, 1968). The increase in GSH oxidation and the decrease in oxygen uptake were almost proportional. In pigeon liver homogenates no GSSG formation occurred on adding GSH, and also inhibition of respiration was very slight (Table 1, Expt. 4). In general, these experiments show a negative correlation between the GSSG formed and the oxygen uptake.

Effect of Mg²⁺ concentration (Table 1, Expts. 2 and 3). In rat liver homogenates containing phosphate buffer the decrement in oxygen uptake was approximately proportional to the increase of Mg²⁺ concentration added (Expt. 2). The formation of GSSG from 2.25 mM-GSH added (9.0 μmoles) or the disappearance of GSH was practically proportional to the concentration of added magnesium chloride in the homogenate with phosphate buffer (Expt. 2), but this effect was not observed in tris-HCl buffer (Expt. 3).

Table 2. *Effect of GSSG on the oxygen uptake in rat liver homogenates*

The values are changes/Warburg vessel/2hr. The volume of the incubation mixture was 4.0 ml. For full experimental details see the text.

Expt. no.	GSH added (mM)	GSSG added (mM)	O ₂ uptake (μmoles)	Decrease in uptake (μmoles)
1	0	0	35.2	—
	0	1.0	30.7	4.5
	0	2.0	25.6	9.6
2	0	0	25.0	—
	2.5	0	17.2	7.8
	0	1.25	20.0	5.0

Effect of GSSG concentration on the oxygen uptake (Table 2, Expt. 1). When 1 mM-GSSG (4 μmoles) was added to rat liver homogenates, it produced a decrease of 4.5 μmoles in the oxygen uptake, and 2 mM-GSSG produced approximately twice that decrease (9.6 μmoles). Thus both these experiments and those in Table 1 suggest that GSSG is responsible, at least in part, for the inhibition of respiration.

Comparison of the inhibitions of respiration by GSH and GSSG. (Table 2, Expt. 2). In rat liver homogenates the fall in the oxygen uptake due to

Table 3. *Effects of NADPH, nicotinamide, citrate, NADPH + citrate, and nicotinamide + citrate on the GSSG formation and the oxygen uptake in rat liver homogenates*

The values are changes/Warburg vessel/2hr. The volume of the incubation mixture was 4.0ml. For full experimental details see the text.

Expt. no.	GSH added (mm)	MgCl ₂ added (mm)	NADPH added (mm)	Nicotinamide added (mm)	Citrate added (mm)	O ₂ uptake (μmoles)	GSSG formation (μmoles)	Increase O ₂ uptake (μmoles)	Decrease in GSSG formation (μmoles)
1	0	0	0	0	0	34.6	0.9	—	—
	0	0	0.5	0	0	38.0	0.5	3.4	0.4
	2.75	0	0	0	0	30.0	1.3	—	—
	2.75	0	0.5	0	0	34.7	0.7	4.7	0.6
2	0	0	0	0	0	21.9	0.7	—	—
	0	0	0.25	0	0	29.0	0.5	7.1	0.2
	0	0	0	0	1.25	37.5	0.6	15.6	0.1
	0	0	0.25	0	1.25	41.5	0.4	19.6	0.3
	2.75	0	0	0	0	17.0	1.4	—	—
	2.75	0	0.25	0	0	26.5	1.0	9.5	0.4
	2.75	0	0	0	1.25	37.0	1.0	20.0	0.4
	2.75	0	0.25	0	1.25	43.0	0.7	26.0	0.7
3	0	0	0	0	0	38.9	0.9	—	—
	0	0	0	6.0	0	48.8	0	9.9	0.9
	2.5	0	0	0	0	26.4	1.6	—	—
	2.5	0	0	6.0	0	41.2	0.1	14.8	1.5
4	0	2.5	0	0	0	29.0	1.1	—	—
	0	2.5	0	6.0	0	38.0	0	9.0	1.1
	0	2.5	0	6.0	1.25	49.0	0	20.0	1.1
	0	2.5	0	6.0	5.0	47.0	0	18.0	1.1
	2.5	2.5	0	0	0	19.5	3.0	—	—
	2.5	2.5	0	6.0	0	34.5	0	15.0	3.0
	2.5	2.5	0	6.0	1.25	47.0	0	27.5	3.0
	2.5	2.5	0	6.0	5.0	50.0	0.1	30.5	2.9

2.5mm-GSH added was 7.8 μmoles and that due to 1.25mm-GSSG was 5.0 μmoles. Thus even if all the added GSH had been converted into GSSG the diminution of oxygen uptake could not have exceeded 5.0 μmoles. This suggests that some other cause than the presence of GSSG may be also responsible for the inhibition of respiration in rat liver homogenates.

Influence of NADPH on the oxidation of GSH and the oxygen uptake in rat liver homogenates (Table 3, Expts. 1 and 2). Expt. 1 shows that 0.5mm-NADPH decreased GSSG formation from 2.75mm-GSH added by 0.6 μmole and increased oxygen uptake by 4.7 μmoles. When only endogenous GSH was present (about 0.5mm) the decrease of GSSG formed was 0.4 μmole and the increase in the oxygen uptake was 3.4 μmoles. When NADPH was added (Table 3, Expt. 1) the decrease in the oxygen uptake produced by the added GSH was less (3.3 μmoles compared with 4.6 μmoles). Expt. 2 shows that 0.25mm-NADPH decreased by 0.4 μmole the GSSG formed from 2.75mm-GSH and increased the oxygen uptake by 9.5 μmoles. NADPH (0.25mm) + citrate (1.25mm) decreased by 0.7 μmole the formation of GSSG and abolished the inhibition of oxygen uptake

by added GSH. Since NADPH is required for GSSG reduction by glutathione reductase (Rall & Lehninger, 1952), NADPH antagonizes GSSG formation and therefore the inhibition of respiration brought about by GSSG is diminished. Also it compensates in part for any decrease of NADPH concentration in the homogenates that may occur during incubation (Bassham, Birt, Hems & Loening, 1959).

Influence of nicotinamide on the oxidation of GSH and on oxygen uptake in rat liver homogenates (Table 3, Expts. 3 and 4). Expt. 3 shows that 6mm-nicotinamide abolished the formation of GSSG from both endogenous and added GSH and increased the oxygen uptake by 9.9 μmoles and 14.8 μmoles respectively. The nicotinamide also protected against the inhibition of oxygen uptake by the added GSH. With no nicotinamide the decrease in the oxygen uptake by added GSH (2.5mm) was 12.5 μmoles, whereas it was 7.6 μmoles in the presence of nicotinamide. The oxygen uptake when both nicotinamide and GSH were added to the homogenates was higher than the oxygen uptake of the homogenate alone.

When Mg²⁺ (2.5mm) was present (Expt. 4),

nicotinamide abolished GSSG formation and diminished the inhibiting effect of added 2.5 mM-GSH on respiration. In the absence and in the presence of nicotinamide the decrement in oxygen uptake due to GSH was 9.5 μ moles and 3.5 μ moles respectively. Since it is known that nicotinamide protects against nicotinamide-nucleotide cleavage (Zatman, Kaplan, Colowick & Ciotti, 1954), the effect of nicotinamide was probably due to the maintenance of nicotinamide-nucleotide concentration.

Influence of citrate and of nicotinamide + citrate on GSSG formation and oxygen uptake in rat liver homogenates (Table 3, Expts. 2 and 4). Expt. 2 shows that 1.25 mM-citrate decreased the formation of GSSG from 10 μ moles of GSH added by 0.4 μ mole (1.0 compared with 1.4 μ moles), and increased the oxygen uptake by 20.0 μ moles. It also abolished the inhibition by added GSH (2.5 mM) of the oxygen uptake. The oxygen uptake of the homogenate in the presence of GSH and citrate was 37 μ moles, whereas that of the homogenate with GSH alone was 17 μ moles. Expt. 4 shows that 6 mM-nicotinamide plus 1.25 mM- or 5 mM-citrate virtually abolished both the formation of GSSG and the inhibition of oxygen uptake.

DISCUSSION

Influence of the preparation of the homogenates and incubation mixtures. Christophersen (1966) failed to maintain GSH in rat liver homogenates by adding nicotinamide (25 mM). Thus the differences in the experimental conditions used for the experiments reported here are probably responsible for the maintenance of GSH in rat liver homogenates in the presence of such a low concentration of nicotinamide as 6 mM. Homogenization in 0.25 M-sucrose and incubations in 0.25 M-sucrose-0.154 M-potassium chloride (1:1, v/v) instead of 0.154 M-potassium chloride possibly protects the tissue against peroxidation or increases the protection by nicotinamide against nicotinamide-nucleotide cleavage or both (Zatman *et al.* 1954). In mitochondria the swelling, lysis and lipid peroxidation that occur in potassium chloride do not occur in sucrose (Hunter, Gebicki, Hoffsten, Weinstein & Scott, 1963). It is also known that potassium chloride increases the thiol-disulphide inhibition of respiration in brain-cortex slices (McIlwain, 1959).

Inhibition of respiration and GSSG formation. In general the experiments show a negative correlation between GSSG formation and oxygen uptake, although only in the absence of citrate was the inverse proportionality at all precise. The experiments with rat liver homogenates suggest that GSSG rather than GSH is responsible for the inhibition of respiration. Other supporting evidence

is given by the experiments with pigeon liver where GSSG was not formed and no decrease in oxygen uptake occurred. GSH may also affect the oxygen uptake, since added GSSG (1.25 mM) produced a fall in the oxygen uptake smaller than that with 2.5 mM-GSH. The inhibition of respiration by GSSG is possibly due to mixed-disulphide formation with some structural proteins, enzymes and non-protein thiols. Possible sensitive substances may be dehydrogenases involved in electron-transport mechanisms (both mitochondrial and microsomal), CoA, lipoic acid, lipoamide dehydrogenase (Schwartz, Lee & Stesney, 1962) and any dithiol involved in oxidative phosphorylation (Fluharty & Sanadi, 1960).

Possible oxidoreduction steady state of glutathione in intact rat liver. The effect of nicotinamide in rat liver homogenates shows that prevention of destruction of nicotinamide nucleotides and maintenance of their reduced state eliminates the formation of GSSG and so maintains almost all the glutathione in the reduced form as also happens in the intact liver (Bhattacharya, Robson & Stewart, 1955). The very high GSH/GSSG concentration ratio that occurs *in vivo* may be maintained by an oxidoreduction steady state through glutathione peroxidase- and glutathione reductase-catalysed reactions (Pinto & Bartley, 1969). Although net GSH oxidation is not observed in pigeon liver homogenates, an oxidoreduction steady-state maintenance of the GSH appears to be theoretically possible in this case also, since GSH peroxidase is present in pigeon liver. Unpublished work (R. E. Pinto & W. Bartley) shows that its activity is about one-half of that in adult male rats (Pinto & Bartley, 1968, 1969). The substrates for GSH peroxidase are hydrogen peroxide (Mills, 1960) and fatty acid hydroperoxides (Little & O'Brien, 1968). Lipid peroxides are formed aerobically in rat liver homogenates apparently without need for NADPH (Wills, 1966; Christophersen, 1966) and non-enzymically in microsomes and mitochondria (Christophersen, 1968a). However, in microsomes lipid peroxidation also increases with added NADPH (Hochtein & Ernster, 1963; May, Poyer & McCay, 1965) and NADPH is also required for desaturation of fatty acids (Brenner & Peluffo, 1966; Mohrhauer, Christiansen, Gran, Deubig & Holman, 1967). The latter reaction may produce substrates for peroxidation and therefore may be connected with the peroxide formation stimulated by NADPH (see Scheme 1). Since reduction of GSSG to GSH requires NADPH (Rall & Lehninger, 1952), a lowering of NADPH concentration will decrease the rate of GSSG reduction. A lowered concentration of NADPH will also decrease the rate of GSH oxidation by those peroxides that require NADPH for their formation. This will have no effect on

Scheme 1. Outline of the oxidoreduction steady state of glutathione. $R\cdot O\cdot OH$ and $R\cdot OH$ represent lipid or fatty acid polyunsaturated hydroperoxides and their products of reduction through glutathione reductase respectively. Reaction (1) represents glutathione reductase, reaction (2) represents glutathione peroxidase and reaction (3) represents NADP-dependent dehydrogenases.

that fraction of GSH oxidation that is due to hydrogen peroxide or peroxides formed by a route not requiring participation of NADPH (see Scheme 1). Thus the disappearance of NADPH from the homogenates ought to abolish GSSG reduction but should only lower the rate of GSH oxidation. The result expected would be a formation of GSSG and a fall in oxygen uptake since peroxides that require NADPH for their formation should no longer occur. Since GSSG oxidizes NADPH this suggests an interpretation of the inhibition of oxygen uptake by GSSG other than the mixed-disulphide formation. The alcohols formed by reaction of hydroperoxides through rat liver GSH peroxidase may be metabolized by β -oxidation (Christophersen, 1968b). If this is so the cleavage of nicotinamide nucleotides must have the effects of decreasing both the formation of peroxides and the oxidation of the alcohols formed from these peroxides. Scheme 1 also suggests that the contribution to oxygen uptake given by GSH oxidation *in vivo* may be more important than that obtained by calculating the oxygen corresponding to the GSSG formation in the homogenates.

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