The Enzymic Oxidation of Glutathione in Rat Liver Homogenates

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1. The aerobic oxidation of GSH and other thiols by rat liver homogenate is abolished either by previous dialysis or by removal of the proteins but is restored by a mixture of the protein-free filtrate and the dialysed homogenate. 2. The oxidation is prevented by previously heating the dialysed homogenate but not the protein-free filtrate and also by known inhibitors of xanthine oxidase. 3. A similar oxidation occurs with hypoxanthine in place of protein-free filtrate.

When GSH is added to rat liver homogenates and the mixture is incubated aerobically, there is a loss of GSH due to oxidation (Pinto, 1961) that is linear with time and independent of GSH concentration (Jocelyn, 1962). This paper presents evidence bearing on the nature of this oxidation. A brief account of the results has previously been given (Jocelyn, 1964).

MATERIALS AND METHODS

GSH, ergothioneine hydrochloride, glucose 6-phosphate (disodium salt), FAD and pyridoxal 5'-phosphate were obtained from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., U.K.), L-thyroxine (sodium salt), EDTA and 2,6-dichlorophenol-indophenol from British Drug Houses Ltd. (Poole, Dorset, U.K.), ascorbic acid from Roche Products Ltd. (Welwyn Garden City, Herts., U.K.), vitamin B₁₂ derivatives from Glaxo Ltd. (Greenford, Middx., U.K.) and hypoxanthine from Calbiochem (Los Angeles, Calif., U.S.A.). 2-Amino-4-hydroxy-6-formylpteridine was prepared as described by Blair (1957). Phosphate saline, liver homogenates and dialysed homogenates were prepared as described previously (Jocelyn, 1962). Protein-free filtrates were obtained from undialysed homogenates by adding 10% metaphosphoric acid (0.1 vol.) to precipitate the proteins and by adjusting the pH of the filtrate to 7.6 with 2M-NaOH. As previously described, GSH, cysteine and homocysteine were determined in metaphosphoric acid filtrates and GSSG was determined by enzymic reduction (Jocelyn, 1967). Ascorbic acid was assayed by titration with 2,6-dichlorophenol-indophenol.

RESULTS

A loss in GSH of 0.77 (s.d. 0.37) $\mu\text{mol/ml}$ is observed 30 min after the thiol is incubated aerobically at 37°C with a 10% homogenate in phosphate saline. The loss, due to oxidation to GSSG, is almost completely prevented by previous dialysis of the homogenate but largely restored (70-90%

of the original value) by adding a filtrate from which proteins have been precipitated (protein-free filtrate), although the latter also has little capacity to oxidize GSH by itself. Protein-free filtrate previously heated at 100°C is only slightly less effective than the unheated filtrate, whereas there is no oxidation when the dialysed homogenate used has been heated briefly at this temperature (Table 1). The oxidation is not inhibited by EDTA, fluoride, azide or carbon monoxide, but is inhibited by cyanide and partially at high concentration by arsenite and diethyldithiocarbamate. Almost complete inhibition is found on adding 2-amino-4-hydroxy-6-formylpteridine at low concentration (Table 2). Other thiols are also oxidized by homogenate, and the effect of dialysed homogenate and protein-free filtrate on the oxidation of cysteine, homocysteine and ascorbic acid was examined. In these experiments EDTA was added since it considerably minimizes the autoxidation of cysteine in the buffer control. With cysteine and homocysteine, both protein-free filtrate and dialysed homogenate separately induce more oxidation than is found in phosphate saline, but, as with GSH, the oxidation induced by the mixture is greater still. In contrast with these results, the oxidation of ascorbic acid by dialysed homogenate or protein-free filtrate either separately or together is not significantly greater than the oxidation in phosphate saline (Table 3). The negligible rate of loss of GSH in dialysed homogenate is not significantly increased by the addition in place of protein-free filtrate of the following substances in the final concentration given: ergothioneine hydrochloride (30 $\mu\text{g/ml}$), glycine (10mg/ml), glycyglycine (3mg/ml), glutamic acid (10mg/ml), FeSO₄ · 7H₂O (20 $\mu\text{g/ml}$), glucose 6-phosphate (disodium salt, 0.05mg/ml), L-thyroxine (sodium salt, 20 $\mu\text{g/ml}$), ascorbic acid (0.5mg/ml), AFD

(20 $\mu\text{g/ml}$), pyridoxal 5'-phosphate (50 $\mu\text{g/ml}$), vitamin B₁₂ and its 5,6-dimethylbenzimidazolylcobamide coenzyme (1 μM).

Cu²⁺ ions increase the oxidation of GSH, but this increase is considerably less than the increase found

when the same concentration of Cu²⁺ ions is added to phosphate saline (Table 1). This difference is presumably due to binding of Cu²⁺ ions by the proteins of the dialysed homogenate.

Hypoxanthine, which has no effect on GSH in buffer, increases its loss when added to dialysed homogenate and to an extent that increases with increasing hypoxanthine concentration. As in undialysed homogenate, the GSH lost is quantitatively converted into GSSG, as shown by subsequent enzymic reduction (Fig. 1).

Table 1. *Loss of GSH in various fractions prepared from rat liver homogenates*

The mixtures were prepared from fresh 20% rat liver homogenate. Dialysed homogenate was heated for 2 min and protein-free filtrate for 20 min, both at 100°C. The 20% homogenate (1.5 ml) and/or protein-free filtrate (1.3 ml) was made up to 2.8 ml with phosphate saline. Cu²⁺ was added as CuSO₄ · 5H₂O (final concn. 90 $\mu\text{g/ml}$). GSH (to give final concn. 1 $\mu\text{mol/ml}$) was added in phosphate saline (0.2 ml). The mixtures were sampled (0.75 ml), incubated at 37°C with shaking and sampled again after 30 min. Fresh metaphosphoric acid (10%; 0.075 ml) was immediately added to the samples and, after sedimentation of the precipitated proteins, filtrates were assayed for GSH. The results given are for a single typical experiment.

Medium	Loss ($\mu\text{mol/ml}$)
Phosphate saline	0.08
Undialysed homogenate*	0.99
Protein-free filtrate (PFF)	0.08
Dialysed homogenate (DH)	0.03
DH plus PFF	0.68
Heated PFF plus DH	0.56
PFF plus heated DH	0.07
Phosphate saline plus Cu ²⁺	0.60
DH plus Cu ²⁺	0.29

* No significant loss of GSH after enzymic reduction with glutathione reductase and NADPH.

DISCUSSION

The aerobic oxidation of GSH to GSSG in rat liver homogenates shows wide variations between different homogenates. This has also been observed by Pinto & Bartley (1969) who have correlated it with age and sex of their rats. The oxidation involves an interaction between diffusible and non-diffusible components. In agreement with a report by Neubert & Lehninger (1962) that mitochondrial oxidation is slow in the absence of added cytochrome c, respiratory-chain-dependent oxidation is not involved since there is no inhibition by azide or carbon monoxide.

The diffusible component of the homogenate is replaceable by hypoxanthine and, apart from arsenite and diethyldithiocarbamate, which partially inhibit at high concentration, the effective inhibitors of the oxidation are also inhibitors of xanthine oxidase, whereas the non-inhibitory substances used do not inhibit xanthine oxidase either (for references see Bray, 1962). 2-Amino-4-hydroxy-6-formylpteridine, which inhibits by 90% at 0.1 mM, is known as a specific inhibitor of xanthine

Table 2. *Effect of some substances tested as inhibitors on the loss of GSH from mixed dialysed homogenate and protein-free filtrate*

The substances (final concentrations shown) were added to dialysed homogenate (0.8 ml), protein-free filtrate (0.8 ml) and GSH (to give 2 $\mu\text{mol/ml}$), final vol. 1.8 ml. The solutions were sampled before and after incubation with shaking for 30 min at 37°C. The values for GSH lost are given as a percentage of the loss when the substance is omitted. Results are given (where appropriate) as means $\pm$ S.D. (or for two readings $\pm$ average error), with numbers of determinations, each with a different homogenate, in parentheses.

Substance added	Concn. (mM)	% loss of GSH
Sodium cyanide	2	34 $\pm$ 16 (4)
EDTA	3	95 $\pm$ 5 (2)
Carbon monoxide	—	100 $\pm$ 5 (3)
Sodium fluoride	24	97 $\pm$ 12 (2)
Sodium arsenite	8	49 $\pm$ 4 (3)
Sodium diethyldithiocarbamate	5	43 $\pm$ 37 (3)
Sodium azide*	10	101 $\pm$ 4 (2)
Sodium <i>p</i> -chloromercuribenzoate	0.2	85 (1)
2-Amino-4-hydroxy-6-formylpteridine	0.1	9 $\pm$ 4 (4)

* Sodium azide also catalyses the oxidation of GSH in phosphate saline and the results have been adjusted to allow for this.

Table 3. *Loss of cysteine, homocysteine and ascorbic acid compared with that of GSH in solutions prepared from rat liver homogenates*

The solutions, made up to 3 ml with phosphate saline, contained EDTA (3 mM) and, as indicated, dialysed homogenate (1.3 ml), protein-free filtrate (1.3 ml) and either GSH, DL-homocysteine, L-cysteine hydrochloride (all 2.5 $\mu\text{mol/ml}$) or ascorbic acid (2.85 $\mu\text{mol/ml}$). The mixture was sampled, incubated with shaking at 37°C, sampled again 30 min later and the samples were assayed for thiol.

Substrate added*	Loss of substrate after incubation ($\mu\text{mol/ml}$)			
	In phosphate saline	In protein-free filtrate	In dialysed homogenate	In protein-free filtrate and dialysed homogenate
GSH	0.12	0.07	0.05	0.94
Cysteine	0.12	0.52	0.24	1.20
Homocysteine	nil	0.28	0.39	1.75
Ascorbic acid	0.06	0.23	nil	0.17

* Oxidation of the following thiols ($\mu\text{mol/ml}$) by whole homogenate under the above conditions was: cysteamine (0.72), 2-mercaptoethanol (0.65), thioglycollate (0.69).

Fig. 1. Oxidation of GSH in mixtures of hypoxanthine and dialysed homogenate. Hypoxanthine (final concentration shown) was incubated with GSH (2 $\mu\text{mol/ml}$) in 10% dialysed homogenate (see Table 1) ($\times$, $\circ$) or phosphate saline (Δ) for 30 min at 37°C. Proteins were precipitated and GSH was assayed either directly ($\times$, Δ) or after enzymic reduction ($\circ$).

oxidase (Petering & Schmidt, 1950). These findings suggest that the oxidation involves xanthine oxidase acting on its substrate, xanthine or hypoxanthine. This reaction has been found capable of oxidizing added cysteine or sulphite (Fridovitch & Handler, 1961) via the O_2^- free-radical anions produced (Knowles, Gibson, Pick & Bray, 1969). The hypothesis is in accordance with the fact that GSH is not oxidized at all when added to a homogenate of pigeon liver, and the rate for guinea-pig liver is much lower than for rat liver (Pinto, 1961) since pigeon liver lacks xanthine oxidase and the

activity of the enzyme in guinea-pig liver is much lower than in rat liver (Richert & Westerfeld, 1951).

Free-radical oxidation would be non-specific, and the involvement of a coupling enzyme is suggested by the fact that, though various thiols are oxidized, ascorbic acid is not. Glutathione peroxidase, which is present in rat liver (Mills, 1960), will utilize thiols other than GSH and is not specific for hydrogen peroxide as oxidant (Little & O'Brien, 1968).

Further observations bearing on this interpretation are presented in the accompanying paper (Jocelyn, 1970).

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