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The Function of Subcellular Fractions in the Oxidation of Glutathione in Rat Liver Homogenate

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1. The aerobic loss of GSH added to the supernatant fraction from rat liver is much increased by including the microsome fraction, which both inhibits the concurrent reduction of the GSSG formed and also augments the net oxidation rate. 2. Oxidation occurs with a mixture of dialysed supernatant and a protein-free filtrate; the latter is replaceable by hypoxanthine and the former by xanthine oxidase, whereas fractions lacking this enzyme give no oxidation. 3. In all these instances augmentation occurs with microsomes, with fractions having urate oxidase activity and with the purified enzyme; uric acid and microsomes alone also support the oxidation. 4. Evidence implicating additional protein factors is discussed. 5. It is suggested that GSH oxidation by homogenate is linked through glutathione peroxidase to the reaction of endogenous substrate with supernatant xanthine oxidase and of the uric acid formed with peroxisomal urate oxidase.

The evidence previously presented that GSH oxidation by rat liver homogenate may depend on xanthine oxidase acting on endogenous xanthine or hypoxanthine (Jocelyn, 1970) appeared incompatible with a report that the oxidation is prevented by removal of microsomes (Christophersen, 1966), since xanthine oxidase is exclusively a supernatant enzyme (Villela, Mitidieri & Affonso, 1955).

The further investigations reported in this paper have resolved the discrepancy and elucidated the role of the subcellular components.

MATERIALS AND METHODS

Xanthine oxidase (xanthine–oxygen oxidoreductase, EC 1.2.3.2) was obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.) and urate oxidase (urate–oxygen oxidoreductase, EC 1.7.3.3) from L. Light and Co. Ltd. (Colnbrook, Bucks., U.K.). The buffer used unless otherwise specified was 0.5M-tris–HCl containing EDTA (10mM).

Liver preparations. A 20% rat liver homogenate was prepared in 0.25M-sucrose. Mitochondria and microsomes were separated from the supernatant as described by Schneider & Hogeboom (1950) and used as suspensions containing particles from 1g of liver/ml of sucrose. Ribosomes or submitochondrial particles released by adding 3.3% (w/v) sodium deoxycholate (0.1ml) to 1ml of suspension were sedimented at 104000*g*, and after washing resuspended in 1ml of sucrose. Deoxycholate supernatants were dialysed against buffer, pH7, at 0°C.

Dialysed supernatant was prepared from supernatant by dialysis against 0.25M-sucrose. Fractions 1 and 2 were obtained by adding acetone at −5°C to the dialysed supernatant. Fraction 1 is the fraction precipitated when the solution is made 30% (v/v) with respect to acetone, and fraction 2 that precipitated at 40% (v/v) acetone. Both fractions were dissolved in 0.1M-buffer, pH7. The protein content of the fractions was measured by the biuret method (Gear, 1965). Protein-free filtrate was obtained from the supernatant by heating for 5min at 100°C, then centrifuging off the precipitated proteins.

Assays. Glutathione peroxidase (glutathione–hydrogen peroxide oxidoreductase, EC 1.11.1.9) was assayed as described by Pinto & Bartley (1969), except that the assay was by loss of GSH, the initial GSH was eightfold less, the protein fourfold less and the assay time extended to 30s. Xanthine oxidase was assayed as described by Avis, Bergel & Bray (1955) and urate oxidase by incubating the protein fraction with 1μmol of uric acid (final vol. in 0.02M-sodium phosphate buffer, pH7.4, 1.2ml) for 15min at 37°C and measuring the decrease in uric acid after deproteinizing with $HClO_4$. Uric acid was determined with urate oxidase (Plesner & Kalckar, 1956) and GSH as previously described (Jocelyn, 1970).

RESULTS

Aerobic and anaerobic changes in GSH concentration in rat liver homogenate and fractions derived from it have been studied with an added GSH+GSSG mixture. The results (Table 1) show that the mean observed aerobic loss with rat liver supernatant fraction is much smaller than with the corresponding homogenate, but greatly increases either if microsomes are added or after preincubation with microsomes and removal of the latter before the incubation with GSH+GSSG.

Table 1. *Changes in GSH concentration after incubation of fractions derived from rat liver with a mixture of GSH and GSSG*

To whole homogenate or derived supernatant (1 ml) and 0.25 M-sucrose or microsome suspension (0.25 ml) was added buffer, pH 7.4 (0.5 ml) and a mixture of GSH (8 mM) and GSSG (4 mM) (0.25 ml). The mixture was sampled, incubated in air or under N_2 at 37°C with shaking for 30 min, then deproteinized with 0.1 ml of metaphosphoric acid. Preincubation of supernatant with microsomes was performed by mixing supernatant with 0.25 vol. of microsome suspension and, after 20 min at 37°C, removing the microsomes by centrifuging. Values, expressed as losses (−) or gains (+) of GSH, are means ± S.D. from five assays each with a different homogenate.

Fraction	Change in concn. of GSH (μmol/ml)		
	Aerobic	Anaerobic	Net oxidation
(1) Homogenate	−0.71 ± 0.51	+0.04 ± 0.05	−0.75 ± 0.51
(2) Supernatant*	−0.08 ± 0.47	+0.49 ± 0.25	−0.57 ± 0.30
(3) Supernatant plus microsomes	−0.87 ± 0.61	+0.08 ± 0.19	−0.95 ± 0.53
(4) Supernatant preincubated with microsomes	−0.57 ± 0.30	+0.07 ± 0.12	−0.64 ± 0.24
(3)–(2)	−0.80 ± 0.55	−0.41 ± 0.19	−0.38 ± 0.28

* Uric acid, 0.28 ± 0.10 μmol/ml, formed aerobically.

Table 2. *Comparison of GSH oxidation by the supernatant and by fractions derived from it*

Dialysed supernatant and protein-free filtrate were each added to give the same concentration as present in the original homogenate. The compositions of the incubation mixtures were as described in Table 1. Values are given as means ± S.D. (or, for two assays, ± average error) with the numbers of determinations in parentheses.

Fraction from homogenate	Change in concn. of GSH (μmol/ml)	
	Aerobic	Net oxidation
Supernatant	+0.12 ± 0.05 (2)	0.40 ± 0.00 (2)
Dialysed supernatant	+0.05 ± 0.00 (2)	0.00 ± 0.00 (2)
Dialysed supernatant plus protein-free filtrate	−0.52 ± 0.07 (3)	0.43 ± 0.05 (3)

Anaerobically, however, with supernatant alone there is an appreciable mean reduction of GSSG to GSH, whereas with added microsomes there is none. The result is that, though the mean oxidation values with homogenate or supernatant with or without microsomes are all of the same order, there is nevertheless a consistent increase in the net oxidation if microsomes are added directly to supernatant with GSH + GSSG, but not if they are added and removed after a preliminary incubation without these substrates. Similar results have been obtained with supernatant prepared from liver homogenized in phosphate saline buffer (Jocelyn, 1970).

A protein-free filtrate prepared from the supernatant by heat precipitation of protein and combined with dialysed supernatant gives much higher aerobic losses of added GSH than the original supernatant but the same net oxidation (Table 2), showing that the filtrate prepared in this way has lost the capacity to reduce GSSG when added to the dialysed supernatant. In this system aerobic loss therefore measures net oxidation directly. The increase by microsomes is still found and the effect of varying separately the concentration of each of these components in the presence of the others is shown in Fig. 1. The maximum extent of oxidation is achieved at low concentrations of dialysed supernatant or microsomes, whereas when protein-free filtrate is varied the extent increases continuously with increasing amounts.

The oxidation with protein-free filtrate and dialysed supernatant increases with increasing temperature or pH. The proportion by which this oxidation is augmented by microsomes does not change appreciably with temperature (Fig. 2*a*), but increasing pH gradually diminishes the augmentation (Fig. 2*b*).

As with dialysed whole homogenate (Jocelyn, 1970), hypoxanthine mixed with dialysed supernatant in place of protein-free filtrate increases the oxidation of GSH. This is augmented by microsomes, although hypoxanthine alone with microsomes has little effect on the oxidation (Fig. 3*a*). Similar results have been obtained with xanthine.

If uric acid is used instead, however, there is little oxidation with dialysed supernatant, but an increase with microsomes only that is augmented

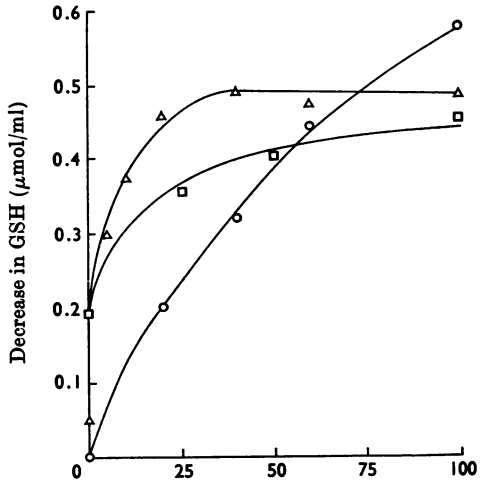

Fig. 1. Effect of varying separately the concentration of each component on GSH oxidation in a mixture of protein-free filtrate, dialysed supernatant and microsomes. GSH (1 μmol) was added to buffer, pH 7 (0.2 ml), and the components (final vol. 1 ml). The mixtures were incubated with shaking for 30 min at 37°C. The same homogenate was used to prepare each component and the weight of liver from which the amount of each component used was derived is as given: ○, protein-free filtrate varied (dialysed supernatant, 0.20 mg; microsomes, 100 mg); □, microsomes varied (dialysed supernatant, 60 mg; protein-free filtrate, 60 mg); △, dialysed supernatant varied (protein-free filtrate, 60 mg; microsomes, 100 mg).

by dialysed supernatant (Fig. 3b). Fractions containing different glutathione peroxidase and xanthine oxidase activities were substituted for dialysed supernatant in the oxidation (Table 3). The oxidation with added protein-free filtrate was inhibited on addition of a fraction prepared from dialysed supernatant but lacking xanthine oxidase activity; conversely purified xanthine oxidase (from milk) at the same activity as found in dialysed supernatant gave with protein-free filtrate a considerable oxidation of GSH.

Increased oxidation occurs on mixing a fraction with high xanthine oxidase but low glutathione peroxidase activity with one having high glutathione peroxidase but no xanthine oxidase activity. Augmentation of the oxidation of GSH is also obtained on addition of various other fractions in place of microsomes to dialysed supernatant and protein-free filtrate. The effect of mitochondria is equivalent to that of microsomes, and in each case the particles sedimenting after treatment with deoxycholate catalyse the oxidation whereas the corresponding supernatants (after dialysis to remove deoxycholate) do not. Effective fractions all have urate oxidase activity (Table 4). The oxidation obtained with a mixture of either protein-free filtrate or hypoxanthine and xanthine oxidase is augmented on adding purified urate oxidase, though this has no effect by itself. If dialysed supernatant of the same activity is substituted for xanthine oxidase, not only is the oxidation greater (see Table 3), but the augmentation by urate oxidase is also increased (Table 5).

DISCUSSION

Christophersen (1966) observed little or no oxidation of GSH by the supernatant from rat liver, but no allowance was made for concurrent reduction of the GSSG formed. In the absence of oxidation, aerobic and anaerobic rates of reduction of GSSG are the same (Pinto, 1961), and hence the true extent of oxidation is found by adding to the observed aerobic change in GSH any rise occurring anaerobically in the presence of GSSG. Calculated in this way, the extent of oxidation with supernatant is only a little less than for the homogenate.

An anaerobic rise that occurs with supernatant but not with whole homogenate is due to microsomal consumption of endogenous $NADP^+$ and NADPH (Jacobson & Kaplan, 1957), since the rise is also abolished in supernatant by preincubation with microsomes. Apart from this effect, microsomes do increase the oxidation of GSH by supernatant.

It has been suggested that oxidation is linked to a mechanism that prevents microsomal lipid peroxidation (Christophersen, 1966), but the amount of oxidation is unaffected by the presence of EDTA at a concentration that inhibits such peroxidation (Ottolenghi, 1959). Above a minimum amount the oxidation is also independent of microsome concentration.

The following evidence suggests instead that the oxidation depends on the concerted action of xanthine oxidase present in the supernatant on endogenous hypoxanthine or xanthine and of urate oxidase present in the particulate fractions on the uric acid produced.

(i) Oxidation of GSH with dialysed supernatant proceeds when xanthine or hypoxanthine replaces protein-free filtrate and is still augmented by microsomes. The amount of oxidation is limited by the amount of hypoxanthine or protein-free filtrate added.

(ii) If xanthine oxidase replaces dialysed supernatant, oxidation proceeds with either hypoxanthine or protein-free filtrate and again the microsomal augmentation occurs; conversely the oxidation with protein-free filtrate does not occur

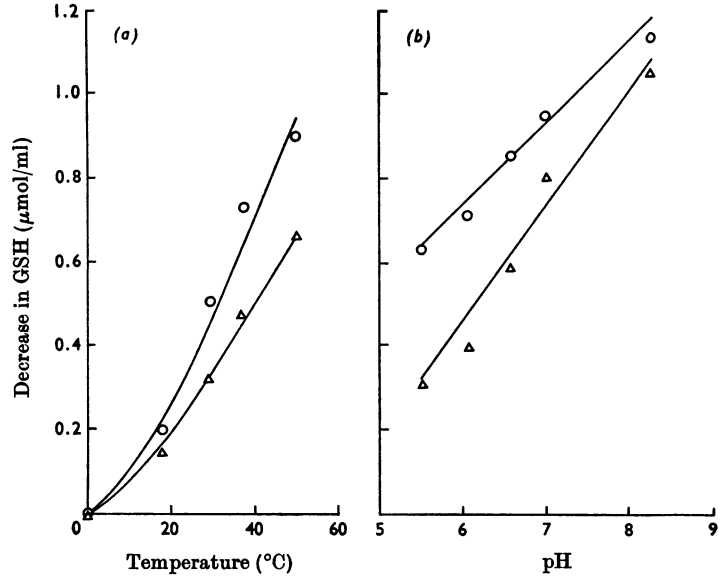

Fig. 2. Effect of temperature (*a*) and pH (*b*) on the oxidation of GSH by dialysed supernatant and protein-free filtrate with (○) or without (△) added microsomes. GSH (1 μmol) was added to 0.2 ml of buffer, pH 7 (*a*), or 0.5 M-phosphate buffer containing EDTA (10 mM) at the appropriate pH (*b*), then mixed with dialysed supernatant and either sucrose or microsomes (0.1 ml), final vol. 1 ml. The solutions were incubated with shaking either at 37°C (*b*) or at the temperatures shown (*a*). A different homogenate was used for each experiment.

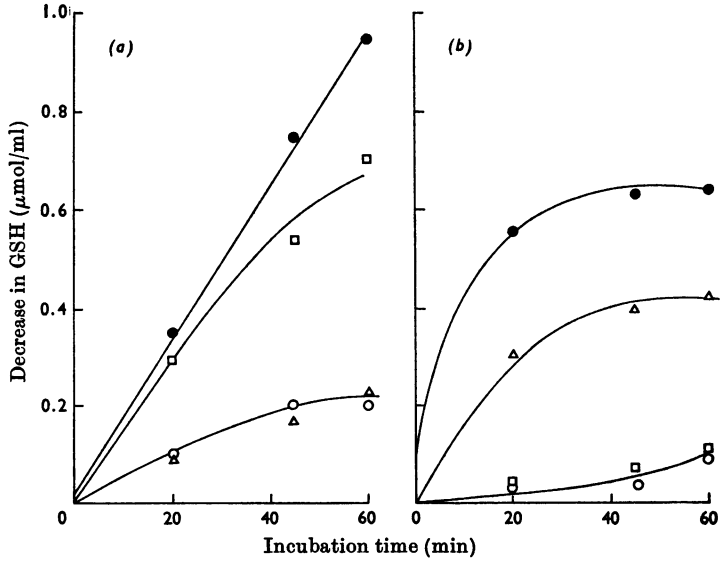

Fig. 3. Comparison of the effects of hypoxanthine (*a*) and uric acid (*b*) on GSH oxidation when added alone (○), with dialysed supernatant (□), with microsomes (△) or with dialysed supernatant plus microsomes (●). The purines (1 mM in 10 mM-Na_2CO_3) (0.5 ml) were added as indicated to dialysed supernatant (0.1 ml), microsomes (0.1 ml), 0.5 M-tris–HCl buffer, pH 7, containing 10 mM-EDTA (0.2 ml) and GSH (1 μmol), final vol. 1 ml. Samples were assayed for GSH before and after incubation with shaking at 37°C.

Table 3. *Glutathione peroxidase and xanthine oxidase activities of protein fractions and their effects on the oxidation of GSH when mixed with protein-free filtrate*

Protein fractions were obtained as described in the Materials and Methods section. To the protein fraction (made up to 0.3ml) and protein-free filtrate (0.5ml) was added GSH (1μmol) in buffer, pH7 (0.2ml), and the mixtures were incubated with shaking at 37°C for 45min. Oxidation occurring when the protein was omitted [0.21±0.08 (3) μmol/ml] has been subtracted. Results are expressed as a percentage of the corresponding values obtained with 0.1ml of dialysed 20% supernatant [protein, 0.95±0.2 (2) mg; xanthine oxidase, 0.019±0.003 (2) international units; glutathione peroxidase, 1.96±0.32 (2) units (as defined by Pinto & Bartley, 1969); oxidation, 0.56±0.08 (3) μmol of GSH/ml]. Where appropriate, values are given as means±S.D. (or, for two assays, ±average error), with the numbers of determinations in parentheses.

		Activity		
Protein	Amount added (mg)	Glutathione peroxidase	Xanthine oxidase	GSH oxidation
Fraction 1	0.5	40±10 (2)	125±30 (2)	64±34 (3)
Fraction 2	1.5	125±40 (2)	0	Negative* (3)
Fractions 1 and 2	0.5 and 1.5	165±50 (2)	125±30 (2)	120±30 (2)
Xanthine oxidase	0.025	0	100	72±19 (4)
Xanthine oxidase plus fraction 2	—	125	100	121±15 (2)

* Oxidation less than when the fraction is omitted.

Table 4. *Augmentation of the oxidation of GSH by dialysed supernatant and protein-free supernatant with fractions derived from subcellular particles*

Each fraction (0.1ml) was added to a mixture of protein-free filtrate, dialysed supernatant (from 20% homogenate, 0.1ml), buffer, pH7 (0.2ml), and GSH (1μmol; 0.1ml). The mixtures were incubated with shaking at 37°C for 45min. The extra oxidation of GSH found with the added fraction is expressed as a percentage of the extra oxidation [0.27±0.06 (3) μmol/ml] with 0.1ml of added microsomes containing 0.89±0.06 (2) mg of protein. Urate oxidase activity is expressed as a percentage of the activity of 0.1ml of microsomes (0.04 international unit at 37°C and pH7.4). Where appropriate, values are given as means±S.D. (or, for two assays, ±average error), with the numbers of determinations in parentheses.

Fraction	Protein added (mg)	Extra oxidation	Urate oxidase activity
Mitochondria	0.64±0.09 (3)	102±15 (3)	70
Submitochondrial fraction	0.16±0.01 (2)	85±12 (2)	32
Mitochondrial deoxycholate supernatant	—	3 (1)	0
Ribosomes	0.15±0.01 (2)	100±20 (3)	54
Microsomal deoxycholate supernatant	—	12±21 (3)	0
Microsomal deoxycholate supernatant and ribosomes	—	110±20 (3)	—

Table 5. *Oxidation of GSH with urate oxidase, xanthine oxidase or dialysed supernatant*

Urate oxidase (0.004 international unit at pH7), xanthine oxidase (0.02 international unit) or dialysed supernatant (0.1ml) were added to buffer, pH7 (0.2ml), and 0.5ml of sucrose, hypoxanthine (1mM, in sucrose) or protein-free filtrate. The mixture (final vol. 1ml) was incubated with GSH (1μmol) for 45min at 37°C, then deproteinized with perchloric acid. The results given are for a single typical experiment.

	Decrease in GSH (μmol/ml) after adding to protein fraction		
Protein fraction	Protein-free filtrate	Hypoxanthine	Sucrose
Xanthine oxidase	0.20	0.22	0.07
Urate oxidase	—	0.00	0.04
Xanthine oxidase and urate oxidase	0.27	0.40	0.00
Dialysed supernatant	0.54	0.47	—
Dialysed supernatant and urate oxidase	0.91	0.89	—

with a dialysed supernatant fraction lacking xanthine oxidase activity.

(iii) Oxidation of GSH occurs in the presence of uric acid when microsomes with urate oxidase activity are the only protein fraction, but not if they are replaced by dialysed supernatant, which lacks this enzyme. Uric acid is formed when supernatant is incubated in the absence of microsomes.

(iv) Urate oxidase, which itself has no effect on GSH oxidation, augments that occurring with xanthine oxidase and hypoxanthine or protein-free filtrate. Other subcellular fractions with urate oxidase activity have the same effect, whereas those without it are ineffective.

Urate oxidase activity in these fractions is due to the peroxisomes, since these contain all the intracellular urate oxidase (de Duve & Baudhuin, 1966) and they are partitioned between the mitochondria and microsomes in the standard methods of preparation (Thomson & Mikuta, 1954).

The oxidation of GSH in rat liver has also been correlated with the activity of glutathione peroxidase (Pinto & Bartley, 1969). Several observations support its involvement in the present system. Dialysed supernatant gives more oxidation both with protein-free filtrate and with hypoxanthine than does purified xanthine oxidase of equivalent activity. A dialysed supernatant fraction of high glutathione peroxidase activity but lacking xanthine oxidase also augments the oxidation by xanthine oxidase itself or by a supernatant fraction with high xanthine oxidase and low glutathione peroxidase activities. Similarly, the oxidation occurring with uric acid and microsomes is increased by dialysed supernatant, whereas urate oxidase added with protein-free filtrate or hypoxanthine augments the oxidation much more than when added to xanthine oxidase. Moreover microsomal augmentation is probably not limited by urate oxidase, since it decreases with pH whereas the activity of the enzyme increases up to pH 9.25 (Keilin & Hartree, 1935). Unlike xanthine oxidase, urate oxidase produces hydrogen peroxide (Schein, Podner & Novikoff, 1951) without forming oxidizing radicals (Fridovitch & Handler, 1961). A model system linking GSH oxidation with hydrogen peroxide continuously produced by enzyme action (glucose and glucose oxidase) through glutathione peroxidase has already been described (Hochstein & Utley, 1968).

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