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Biochem. J. (1961) **79**, 43

Comparison of the Aerobic and Anaerobic Reduction of Oxidized Glutathione in Liver Homogenates

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(*Received* 3 *August* 1960)

Since the glutathione of animal tissues, other than blood, is predominantly in the reduced form (Bhattacharya, Robson & Stewart, 1955; Martin & McIlwain, 1959), the rate of reduction of oxidized glutathione in respiring tissues must be rapid enough to counteract the enzymic (Ames & Elvehjem, 1945, 1946) and non-enzymic (Barron, 1951) oxidation of reduced glutathione. In most studies of reduction of oxidized glutathione, non-respiring glutathione-reductase preparation (Rall & Lehninger, 1952; Van Heyningen & Pirie, 1953; McIlwain & Tresize, 1957) or anaerobic conditions (cf. Vennesland & Conn, 1954) have been employed. Hitherto few investigators have compared the aerobic and anaerobic reduction of oxidized glutathione in tissue preparations capable of respiration. Martin & McIlwain (1959) found little difference in the aerobic and anaerobic rates of reduction of endogenous oxidized glutathione in slices of guinea-pig cerebral cortex. In avocade mitochondria supplemented with citrate and triphosphopyridine nucleotide (cf. Vennesland & Conn, 1954) the aerobic and anaerobic rates of reduction of oxidized glutathione were approximately equal. In the present investigation the aerobic and anaerobic rates of reduction of added oxidized glutathione in liver homogenates have been compared under a variety of conditions.

MATERIALS AND METHODS

Glutathione. Reduced glutathione (GSH) was obtained from The Distillers Co. Ltd. Oxidized glutathione (GSSG) was prepared from GSH by a modification of the method of Pirie (1931) without added Cu^{2+} ions. To a solution of GSH (2%, w/v) was added an equivalent amount of 3% (w/v) H_2O_2, and the solution kept in a refrigerator at 2° for 12 hr. Oxidation at this stage was incomplete and the residual SH-groups were estimated potentiometrically. More H_2O_2 was added, a little less than was necessary to complete the reaction, and the solution was stored for a further 12 hr. at 2°. The purpose of this procedure was to avoid side reactions. The final product always contained a small percentage of GSH. It was considered preferable to use a solution of GSSG containing a small percentage of SH-groups rather than one containing side products of oxidation. With this method of preparation the total (GSSG + GSH) present in the final solution was equivalent to not less than 95% of the original GSH.

Sodium glucose 6-phosphate. This was prepared from the barium salt (British Drug Houses Ltd.) by ion exchange with Dowex 50.

Suspension media. 'Phosphate saline' contained 100 vol. of 0·155M-KCl, 10 vol. of 0·1M-potassium phosphate buffer, pH 7·4, and 1 vol. of 0·1M-$MgCl_2$. 'Bicarbonate saline' was a modification of the saline of Krebs & Henseleit (1932). The stock solution consisted of 100 ml. of 18% (w/v) KCl, 80 ml. of 1·15% (w/v) KCl, 15·4 ml. of 0·2M-KH_2PO_4, 20 ml. of 3·82% (w/v) $MgSO_4,7H_2O$, 60 ml. of 1·3% (w/v) $NaHCO_3$ and 1960 ml. of water. Before use 16 vol. of 1·3% (w/v) $NaHCO_3$ were added to 100 vol. of the stock solution, and the mixture was gassed with N_2+CO_2 (95:5) at 0° for 15 min. Phosphate saline was used in all experiments unless stated otherwise.

Preparation of homogenates. Immediately after removal from the animal the liver was cooled in 0·9% KCl at 0°, dried with filter paper and minced in a chilled Fischer mincer (Jouan, Paris). The minced tissue was weighed and homogenized with 8–30 vol. of ice-cold suspension medium in a chilled stainless Potter–Elvehjem homogenizer.

Incubations. These were carried out in Warburg apparatus at 30°. Vessels containing phosphate saline were gassed with O_2 or N_2 and those containing bicarbonate saline were gassed with O_2+CO_2 (95:5) or N_2+CO_2 (95:5).

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A stick of yellow phosphorus was placed in the centre well of vessels gassed with N_2 or N_2+CO_2. The centre wells of vessels gassed with O_2 contained 0·2 ml. of 2N-NaOH and filter paper. The incubation mixture consisted of 3·0 ml. of homogenate, and substrates and saline to make the final volume up to 4·0 ml. The concentrations of GSSG and GSH solutions added were usually 0·05–0·10M and the oxidized triphosphopyridine nucleotide (TPN^+) solution was 1·5 mM. In most experiments other substrates were added as 0·1–0·2 ml. of a 0·1–0·2M-solution. Only GSH and GSSG were added to the side arm. The vessels were kept at 0° after the addition of the homogenate, before gassing and incubation. The contents of the side arm were added immediately before incubation. At the end of the incubation the vessels were cooled in ice, and the reaction was stopped by adding 1·0 ml. of 10% (w/v) metaphosphoric acid. Control to estimate endogenous GSH consisted of a mixture of 1 ml. of 10% (w/v) metaphosphoric acid, 1 ml. of saline and 3 ml. of homogenate. The mixture to estimate initial GSH consisted of 1 ml. of 10% (w/v) metaphosphoric acid, 3 ml. of homogenate, GSH solution and saline to make the final volume up to 5 ml. The vessels with both controls were kept at 0°. Estimations of GSH were done at the same time for both incubated and unincubated mixtures. We considered it better to estimate initial GSH rather than determine its value by summing up endogenous and added GSH.

Estimation of reduced glutathione. The precipitated proteins were removed by centrifuging at 20 000*g* at 2° for 15 min. in the refrigerated International centrifuge. A portion (0·25–0·5 ml.) of the supernatant was diluted to 4·3 ml. with water (final pH 1·5) and titrated potentiometrically against 0·1M-$HgCl_2$ by a modification of the method of Cecil (1955). The apparatus used was the same as that described by Cecil (1950). The method described by Cecil (1955) for cysteine did not give sharp end points with GSH but these were obtained with the following modifications.

The gold wire of the electrode was fused at one end to form a ball about 0·6 mm. in diameter. The electrode was prepared by heating the gold (cf. Cecil, 1955) and dipping it for 40 sec. into mercury. Although end points were sharp with aqueous GSH solutions, unsatisfactory end points were obtained with deproteinized solutions. This was apparently due to failure of the mercury–thiol electrode to form spontaneously in deproteinized solutions (cf. Cecil, 1955). The difficulty was overcome by prior immersion of the electrodes in aqueous GSH (approx. 0·2 mM). This ensured that there was a layer of $(GS)_2Hg$ on the surface of the electrode before the titration was started. The immersion was continued until a steady potential had been reached (approx. 100 mV versus the control reference electrode), after which a little $HgCl_2$, equivalent to about 15% of the GSH present, was collected. Electrodes prepared in this way could be used for about 20 titrations provided that the concentration of free Hg^{2+} ions was kept low. After prolonged use of the electrode the initial potential rose and the end point became less sharp.

If the titration is continued beyond the equivalence point, corresponding to $(GS)_2Hg$, a second equivalence point is reached, corresponding to $(GS)_2Hg_2$. The first end point was always used as a more reliable measure of GSH (cf. Stricks & Kolthoff, 1953). Care must be taken in titrating small amounts of GSH because the inflexion between the two end points is small.

The error of the method was about 1% with 1·0 μmole and 5% with 0·2 μmole of GSH. Recoveries of added GSH from manometer-flask contents containing pigeon-liver homogenate (approx. 75 mg. dry wt./flask) were 90, 98 and 100% with concentrations of GSH of 0·5, 2·5 and 5 mM respectively.

Estimation of oxidized glutathione. This was carried out in aqueous solution according to Cecil (1955).

RESULTS

Aerobic disappearance of added reduced glutathione in fresh and boiled liver homogenates

GSH is oxidized non-enzymically by molecular oxygen (Barron, 1951) and by certain substances which may be present in biological systems. The oxidation of GSH is also accelerated by certain tissue preparations (Ames & Elvehjem, 1945, 1946).

Therefore before measuring the reduction of GSSG in liver homogenates the fate of added GSH was investigated (Table 1). There was no disappearance of GSH incubated aerobically with fresh pigeon-liver homogenate, but with boiled pigeon-liver homogenate 15–17% of the initial GSH disappeared after 60 min. The percentage of GSH disappearing on incubation with the boiled homogenate was independent of the concentration of tissue or GSH. These findings suggest that there is a mechanism in fresh pigeon-liver homogenate for the reduction of GSSG which is more rapid than the non-enzymic oxidation of GSH.

On incubation with fresh rat-liver homogenate (Table 1) 36% of the initial GSH disappeared in 60 min., whereas with boiled rat-liver homogenate only 18% of the initial GSH disappeared in 60 min. Thus, in contrast with pigeon-liver homogenate, the disappearance of GSH is twice as rapid in fresh as in boiled rat-liver homogenate. It is probable that the rapid removal of GSH is a property of rat-liver homogenate and not of the intact tissue since Bhattacharya *et al.* (1955) found that glutathione in rat liver is predominantly in the reduced form. This idea is supported by the observation (Expt. 3, Table 1) that the rate of disappearance of GSH increased with incubation time. The rate of removal of GSH between 0 and 60 min. was almost twice as rapid as the rate in the first 20 min. It seems therefore that the capacity of rat liver to oxidize GSH appears on homogenization and increases during incubation.

Comparison of the aerobic and anaerobic reduction of oxidized glutathione in homogenates of pigeon, rat and guinea-pig liver

The anaerobic reduction of GSSG is approximately linear for 20 min. in homogenates of rat, pigeon and guinea-pig liver (Table 2) but falls off after longer incubation in homogenates of both rat

Table 1. *Aerobic removal of reduced glutathione in fresh and boiled liver homogenates*

Vessels contained GSH as indicated below, 3·0 ml. of homogenate, and phosphate saline to a total volume of 4·0 ml. 'Boiled' homogenate was heated for 15 min. at 100°.

Animal	Dry wt. of tissue (mg.)	Homogenate	GSH added (μmoles)	Endogenous GSH (μmoles)	Initial GSH (μmoles)	Incubation time (min.)	Final GSH (μmoles)	GSH removed (μmoles)	GSH removed (%)
Pigeon	96·0	Fresh	6·0	1·8	7·8	10	7·8	0	0
						20	7·8	0	0
						40	7·8	0	0
						80	7·8	0	0
		Boiled	6·0	1·8	7·8	80	6·3	1·5	19
Pigeon	38·5	Fresh	20·0	0·7	21·0	60	20·9	0·1	0
		Boiled	20·0	0·6	20·4	10	19·5	0·9	4
						25	18·7	1·7	8
						60	17·3	3·1	15
			5·0	—	5·8	10	5·4	0·4	7
						25	5·1	0·7	12
						60	4·8	1·0	17
Rat	140·0	Fresh	7·0	2·0	9·2	10	9·0	0·2	2
						20	8·6	0·6	7
						60	6·0	3·2	36
		Boiled	7·0	1·8	9·1	60	7·5	1·6	17

Table 2. *Time course of the aerobic and anaerobic reduction of oxidized glutathione in homogenates of pigeon, rat and guinea-pig liver*

Vessels contained 0·2 ml. of GSSG (approx. 0·05 or 0·025M, containing GSSG + GSH as indicated below), 3·0 ml. of homogenate, and phosphate saline to a total vol. of 4·0 ml.

Expt. no.	Animal	Dry wt. of tissue (mg.)	GSSG added (μmoles)	GSH added (μmoles)	Endogenous GSH (μmoles)	Initial GSH (μmoles)	Incubation time (min.)	Final GSH (μmoles) N_2	Final GSH (μmoles) O_2	GSH formed (μmoles) N_2	GSH formed (μmoles) O_2
1	Pigeon	50·5	9·0	0·4	0·8	1·2	10	1·9	2·0	0·7	0·8
							25	3·2	3·2	2·0	2·0
							70	5·2	4·5	4·0	3·3
2	Pigeon	39·0	9·0	0·4	0·6	1·1	10	1·7	1·6	0·6	0·5
							25	2·1	2·0	1·0	0·9
							60	2·4	1·9	1·3	0·8
3	Rat	140·0	3·9	2·2	2·0	4·4	10	5·2	4·9	0·8	0·5
							27	6·8	3·8	2·4	−0·6
							55	8·8	1·4	4·4	−3·0
4	Rat	145·2	3·9	2·2	1·8	4·0	10	5·0	4·5	1·0	0·5
							20	5·9	3·5	1·9	−0·5
							60	7·2	1·0	3·2	−3·0
5	Rat	71·5	3·6	1·6	0·7	3·1	10	3·3	—	0·2	—
							20	3·6	—	0·5	—
							40	4·2	—	1·1	—
		133·3	—	—	1·3	3·9	10	5·1	—	1·2	—
							20	6·6	—	2·7	—
							40	8·5	—	4·6	—
6	Guinea pig	80·8	3·6	1·8	1·5	3·6	10	4·2	4·1	0·6	0·5
							20	4·8	3·8	1·2	0·2
		150·5	—	—	2·9	4·8	10	6·2	6·2	1·4	1·4
							20	7·3	6·2	2·5	1·4

and pigeon liver. Homogenate equivalent to about 50, 150 and 100 mg. dry wt. of tissue/flask was required respectively for pigeon, rat and guinea-pig liver, in order to obtain approximately equal anaerobic rates of reduction of GSSG (about 1·0 μmole of GSH/10 min.). It was difficult to assess absolutely the relative rates of reduction of GSSG in homogenates of the three tissues as this was not proportional to tissue concentration. In preliminary experiments the rates of reduction of GSSG in homogenates of pigeon, rat and guinea-pig liver prepared in 8 vol. of phosphate saline were respectively about four, six and three times the rates in homogenates prepared in 16 vol. of the saline.

Aerobically the pattern of reduction of GSSG differed widely in homogenates of pigeon, rat and guinea-pig liver (Table 2). In pigeon-liver homogenate the aerobic rate of GSSG reduction from 0 to 25 min. was about the same as (Tables 2 and 4) or slightly less than (Table 3) the anaerobic rate. With incubation periods greater than 20 min. the aerobic rate of reduction of GSSG was invariably less than the anaerobic rate (Tables 2 and 4). In rat-liver homogenate (Table 2) a small aerobic reduction of GSSG (about half of the anaerobic rate) was observed in the first 10 min. but with longer periods of incubation the removal of GSH was so rapid that even the endogenous GSH disappeared (cf. Hopkins & Elliot, 1931). In guinea-pig-liver homogenate (Expt. 6, Table 2) the aerobic rate of GSSG reduction was the same as the anaerobic rate up to 10 min. but with longer incubation GSH ceased to accumulate (150 mg. dry wt. of tissue/flask) or disappeared (80 mg. dry wt. of tissue/flask).

In all experiments therefore with all three tissues the anaerobic rate of reduction of GSSG is greater than or equal to the aerobic rate. The difference between the aerobic and anaerobic rate of reduction of GSSG is much smaller in pigeon-liver homogenate than in rat-liver or guinea-pig-liver homogenate in which GSH disappears on prolonged aerobic incubation.

Since GSH is stable under aerobic conditions in pigeon-liver homogenate (Table 1) this tissue was

Table 3. *Effect of concentration of oxidized glutathione on its aerobic and anaerobic reduction in pigeon-liver homogenates*

Vessels contained 0·1–0·4 ml. of approx. 0·05 M-GSSG (containing GSSG + GSH as indicated below), 3·0 ml. of homogenate, and phosphate saline to a total vol. of 4·0 ml.

Dry wt. of tissue (mg.)	Gas	GSSG added (μmoles)	GSH added (μmoles)	Endogenous GSH (μmole)	Initial GSH (μmoles)	Incubation time (min.)	Final GSH (μmoles)	GSH formed (μmoles)
35·7	N_2	3·6	2·1	0·7	2·8	10	3·6	0·8
						20	4·3	1·5
						50	6·3	3·5
		7·2	4·2	0·7	4·9	10	5·6	0·7
						20	6·2	1·3
						50	8·4	3·5
		14·4	8·4	0·7	9·0	10	10·0	1·0
						20	10·5	1·5
						50	12·8	3·8
40·8	N_2	3·6	1·8	0·7	2·5	12	3·7	1·2
						20	4·2	1·7
						40	4·9	2·4
	O_2	3·6	1·8	0·7	2·5	12	3·7	1·2
						20	3·9	1·4
						40	3·9	1·4
		7·2	3·6	0·7	4·1	12	5·0	0·9
						20	5·2	1·1
						40	5·1	1·0
41·5	N_2	7·2	3·6	0·8	4·5	5	5·3	0·8
						10	5·9	1·4
						20	7·3	2·8
	O_2	7·2	3·6	0·8	4·5	5	5·3	0·8
						10	6·2	1·7
						20	6·4	1·9
		14·4	7·2	0·8	7·8	5	8·6	0·8
						10	9·3	1·5
						20	9·1	1·3

used in subsequent experiments. The standard incubation period was usually 10 min. (Tables 5–7) because the rate of reduction of GSSG was approximately linear during this incubation period (Tables 2 and 3).

Effect of concentration of oxidized glutathione on its aerobic and anaerobic rate of reduction in pigeon-liver homogenates

The anaerobic rate of reduction of GSSG was not affected by increasing the amount of GSSG added per flask from 3·6 to 14·4 μmoles (Table 3). Aerobically the rate of reduction of GSSG was decreased by raising the concentration of GSSG. The reason for this effect is not clear. Perhaps the GSSG solution contained some impurity which under the action of oxygen forms an inhibitor of GSSG reduction. Alternatively the inhibition of GSSG reduction with increasing GSSG concentration may be associated with mitochondrial swelling produced by the GSH in the preparation (Lehninger & Schneider, 1959). Since increasing the GSSG concentration did not increase the rate of GSSG reduction, it is to be concluded that under the conditions summarized in Table 3 the concentration of GSSG is not rate-limiting. This is consistent with the finding of McIlwain & Tresize (1957) that a GSSG concentration of 0·2 mM is required for maximum glutathione-reductase activity in cerebral extracts. The lowest GSSG concentration used in the present experiments was 0·9 mM.

Effect of oxidized triphosphopyridine nucleotide on the reduction of oxidized glutathione in pigeon-liver homogenates

Addition of 0·0375 μmole of TPN^+/ml. (Table 4) increased both the aerobic and anaerobic rate of reduction of GSSG two- to three-fold. In the presence of added TPN^+ the aerobic rate of GSSG reduction was the same as the anaerobic rate for incubation periods up to 70 min. These observations indicate that the endogenous TPN^+ in the homogenate is rate-limiting for GSSG reduction.

Both the aerobic and anaerobic rates of GSSG reduction increased in a non-linear fashion with TPN^+ concentration up to 0·11 μmole of added TPN^+/ml. (Table 5). The increase in the rate of GSSG reduction was about threefold with 0·0375, fourfold with 0·075 and 4·5-fold with 0·11 μmole of added TPN^+/ml.

When up to 0·11 μmole of TPN^+/ml. was added the aerobic and anaerobic rates of GSSG reduction were the same. When the TPN^+ concentration was raised above 0·11 μmole/ml. there was little change in the anaerobic rate of GSSG reduction, but the aerobic rate continued to increase. This resulted in a more rapid aerobic than anaerobic reduction of GSSG at high TPN^+ concentrations (Table 5). The TPN^+ concentration (about 0·11 mM) required to obtain maximum anaerobic rates of reduction of GSSG (Table 5, Expt. 1) is much higher than the concentration of reduced triphosphopyridine nucleotide (TPNH) (0·02 mM) found by McIlwain & Tresize (1957) to give maximum rates of GSSG reduction in brain extracts. This may be due to the fact that in liver homogenate the rate of reduction of TPN^+ limits the reduction of GSSG and possibly due to the destruction of added TPN^+.

Effect of citrate, fumarate and glucose 6-phosphate on the reduction of oxidized glutathione in pigeon-liver homogenates

When fumarate or citrate was added to the homogenate the rate of GSSG reduction was increased to about four times the endogenous rate (Table 6).

Table 4. *Effect of oxidized triphosphopyridine nucleotide on the time course of reduction of oxidized glutathione in pigeon-liver homogenates*

Vessels contained 0·2 ml. of approx. 0·05 M-GSSG (containing 9·0 μmoles of GSSG and 0·5 μmole of GSH), 3·0 ml. of homogenate; 0·1 ml. of 1·5 mM-TPN^+ was added, and phosphate saline to a total vol. of 4·0 ml.

Dry wt. of tissue (mg.)	TPN^+ added (μmole)	Endogenous GSH (μmole)	Initial GSH (μmoles)	Incubation time (min.)	Final GSH (μmoles)		GSH formed (μmoles)	
					N_2	O_2	N_2	O_2
50·5	Nil	0·8	1·2	10	1·9	2·0	0·7	0·8
	Nil			25	3·2	3·2	2·0	2·0
	Nil			70	5·2	4·5	4·0	3·3
	0·15			10	3·5	3·5	2·3	2·3
	0·15			25	5·4	5·6	4·2	4·4
	0·15			70	8·3	8·4	7·1	7·2
39·0	Nil	0·6	1·1	10	1·7	1·6	0·6	0·5
	Nil			25	2·1	2·0	1·0	0·9
	Nil			60	2·4	1·9	1·3	0·8
	0·15			10	2·8	2·8	1·7	1·7
	0·15			25	3·5	3·6	2·4	2·5
	0·15			60	4·0	3·8	2·9	2·7

Added TPN^+ (0·0375 mM) stimulated GSSG reduction three- to four-fold (Table 6). When both citrate and TPN^+ were added, GSSG reduction was stimulated 11-fold and 14-fold in Expts. 1 and 2 (Table 6) respectively, and in both experiments the stimulation by citrate plus TPN^+ was about equal to the product (stimulation by citrate alone × stimulation by TPN^+ alone). Glucose 6-phosphate (Table 6, Expt. 2) stimulated GSSG reduction 1·5 times and in the presence of added TPN^+ stimulated GSSG reduction sevenfold. The stimulation of GSSG reduction by glucose 6-phosphate plus TPN^+ was equal to the product (stimulation by TPN^+ alone × stimulation by glucose 6-phosphate alone). Thus the stimulatory effects of added TPN^+ and hydrogen donors are complementary, indicating that both factors are rate-limiting for the endogenous reduction of GSSG in the homogenate. Citrate and fumarate are more than twice as effective as glucose 6-phosphate as hydrogen donors for the reduction of GSSG in pigeon-liver homogenate.

In Expt. 1 (Table 6) the aerobic rates of GSSG reduction with and without added citrate and fumarate were less than the anaerobic rates. However, when 0·15–0·45 μmole of TPN^+ was added (Tables 4–6), with or without additional substrate, the aerobic and anaerobic rates of reduction of GSSG were equal. This observation suggests that the aerobic rate of reduction of GSSG without added TPN^+ is lower than the anaerobic rate, owing to a more rapid breakdown of the coenzyme under aerobic conditions.

The maximum observed rate of GSSG reduction was 140 μmoles of GSSG/g. fresh wt. of tissue/hr., which is of the same order as the rate observed by

Table 5. *Effect of oxidized triphosphopyridine nucleotide concentration on the rate of reduction of oxidized glutathione in pigeon-liver homogenates*

In Expt. 1 the homogenate was prepared in phosphate saline and in Expt. 2 in bicarbonate saline. Vessels contained 0·2 ml. of approx. 0·05 M-GSSG (9·0 μmoles of GSSG + 0·5 μmole of GSH), 3·0 ml. of homogenate, 0·1–0·8 ml. of 1·5 mM-TPN^+, and phosphate (Expt. 1) or bicarbonate (Expt. 2) saline to a total vol. of 4·0 ml. Incubation time, 10 min. For further details see experimental section.

Dry wt. of tissue (mg.)	TNP^+ added (μmoles)	Endogenous GSH (μmoles)	Initial GSH (μmoles)	Final GSH (μmoles)		GSH formed (μmoles)	
				N_2	O_2	N_2	O_2
46·7	Nil	0·9	1·4	2·4	2·2	1·0	0·8
	0·15			4·4	4·4	3·0	3·0
	0·30			5·3	5·3	3·9	3·9
	0·45			6·0	5·8	4·6	4·4
	0·75			5·7	6·2	4·3	4·8
				N_2+CO_2	O_2+CO_2	N_2+CO_2	O_2+CO_2
73·2	Nil	1·3	1·9	4·4	4·4	2·5	2·5
	0·15			9·7	9·3	7·8	7·4
	1·20			9·9	12·2	8·0	10·3

Table 6. *Effect of citrate, fumarate and glucose 6-phosphate on the reduction of oxidized glutathione in pigeon-liver homogenates*

Vessels contained 0·2 ml. of approx. 0·05 M-GSSG (9·0 μmoles of GSSG + 0·5 μmole of GSH), 3·0 ml. of homogenate, 0·2 ml. of 0·1 M additional substrate; 0·1 ml. of 1·5 mM-TPN^+ was added and phosphate saline to a total volume of 4·0 ml. Incubation time, 10 min.

Expt. no.	Dry wt. of tissue (mg.)	Additional substrate	TPN^+ added (μmole)	Endogenous GSH (μmole)	Initial GSH (μmoles)	Final GSH (μmoles)		GSH formed (μmoles)	
						N_2	O_2	N_2	O_2
1	47·5	Nil	Nil	0·9	1·7	2·4	2·3	0·7	0·6
		Citrate	Nil			4·4	4·0	2·7	2·3
		Fumarate	Nil			4·4	4·0	2·7	2·3
		Nil	0·15			3·8	3·8	2·1	2·1
		Citrate	0·15			9·4	9·4	7·7	7·7
2	40·5	Nil	Nil	0·8	1·1	1·6	1·6	0·5	0·5
		Citrate	Nil			2·8	2·8	1·7	1·7
		Glucose 6-phosphate	Nil			1·9	1·8	0·8	0·7
		Nil	0·15			3·0	2·9	1·9	1·8
		Citrate	0·15			8·0	7·9	6·9	6·8
		Glucose 6-phosphate	0·15			4·8	4·6	3·7	3·5

Table 7. *Effect of pyruvate and fumarate on the reduction of oxidized glutathione in pigeon-liver homogenates in bicarbonate saline*

Vessels contained 0·2 ml. of approx. 0·05 M-GSSG (9·0 μmoles of GSSG + 0·5 μmole of GSH), 3·0 ml. of homogenate, 0·2 ml. of 0·1 M additional substrate as indicated below (except in Expt. 2, in which 0·2 ml. of 0·5 M-pyruvate was added), 0·1 ml. of 1·5 mM-TPN^+, and saline to a total volume of 4·0 ml. Incubation time, 10 min.

Expt. no.	Dry wt. of tissue (mg.)	Additional substrate	TPN^+ added (μmoles)	Endogenous GSH (μmole)	Initial GSH (μmole)	Final GSH (μmoles) $N_2 + CO_2$	Final GSH (μmoles) $O_2 + CO_2$	GSH formed (μmoles) $N_2 + CO_2$	GSH formed (μmoles) $O_2 + CO_2$
1	73·2	Nil	Nil	1·3	1·9	4·4	4·4	2·5	2·5
		Pyruvate	Nil			3·3	4·4	1·4	2·5
		Fumarate	Nil			7·0	6·9	5·1	5·0
		Nil	0·15			9·7	9·3	7·8	7·4
		Pyruvate	0·15			9·2	10·4	7·3	8·5
2	64·5	Nil	Nil	1·2	1·8	4·5	4·4	2·7	2·6
		Pyruvate	Nil			2·5	2·6	0·7	0·8
		Fumarate	Nil			6·7	6·5	4·9	4·7
		Pyruvate + fumarate	Nil			3·1	4·2	1·3	2·4
		Nil	0·15			9·7	9·8	7·9	8·0
		Pyruvate	0·15			6·6	6·6	4·8	4·8
3	35·4	Nil	Nil	0·4	0·6	1·1	1·0	0·5	0·4
		Pyruvate	—	—	0·7	0·9	1·0	0·2	0·3

McIlwain & Tresize (1957) for cerebral tissue (250–300 μmoles of GSSG/g. fresh wt. of tissue/hr.). Higher rates could probably be obtained by increasing the concentration of added TPN^+.

Effect of pyruvate and fumarate on the reduction of oxidized glutathione in pigeon-liver homogenates in bicarbonate saline

In order to ascertain if a mechanism other than coupling to TPN-linked oxidation (see Discussion) is involved in the aerobic reduction of GSSG, experiments were carried out under conditions similar to those used by Krebs (1954) and L. Rathbone & H. A. Krebs (unpublished work, 1959) in studies of malate formation. Homogenates were prepared in bicarbonate saline (for details see Materials and Methods section), manometers were gassed with oxygen–carbon dioxide or nitrogen–carbon dioxide and the reduction of GSSG was studied in the presence of high concentrations (25 mM) of pyruvate (Table 7, Expt. 2) similar to those employed by L. Rathbone & H. A. Krebs (unpublished work, 1959). No stimulation of GSSG reduction by oxygen was, however, observed.

Pyruvate at low concentrations (5 mM) inhibited the anaerobic reduction of GSSG by about 50 %, but had no effect under aerobic conditions (Table 7). Under the same conditions fumarate (5 mM) stimulated both the aerobic and anaerobic reduction of GSSG about twofold (Table 7). When pyruvate and TPN^+ were added together the anaerobic reduction of GSSG was inhibited by only 7 %. With higher concentrations of pyruvate (25 mM), similar to those used by L. Rathbone & H. A. Krebs (unpublished work, 1959), both the aerobic and the anaerobic reduction of GSSG were inhibited by about 70 % (Table 7, Expt. 2). When pyruvate (25 mM) and TPN^+ were added together the reduction of GSSG aerobically and anaerobically was about 40 % less than with TPN^+ alone. With pyruvate (25 mM) and fumarate (5 mM) the aerobic reduction of GSSG was restored to its endogenous rate and the anaerobic reduction of GSSG was increased to about half of its endogenous rate.

Thus fumarate partially counteracts the inhibition of GSSG reduction by pyruvate, being more effective under aerobic than anaerobic conditions. Alternatively pyruvate may be considered to reverse the stimulation of GSSG reduction by fumarate.

It is possible that the reductive carboxylation of pyruvate catalysed by malic enzyme (Ochoa, Mehler & Kornberg, 1948) competes with glutathione reductase for TPNH and hence inhibits the reduction of GSSG. Why low concentrations (5 mM) of pyruvate inhibit GSSG reduction anaerobically but not aerobically is not clear. Perhaps pyruvate is rapidly metabolized under aerobic conditions by pathways other than the malic enzyme reaction, and hence does not compete with GSSG for TPNH. When a large excess of pyruvate is present aerobically it could participate in the malic enzyme reaction as well as being metabolized by other pathways and hence compete with GSSG for TPNH. The finding that fumarate partially counteracts the inhibitory action of pyruvate supports these ideas, since fumarate is a precursor of malate, which would be expected to inhibit the reductive carboxylation of pyruvate by mass action.

DISCUSSION

The striking differences in the stability of GSH and the rate of reduction of GSSG in homogenates of pigeon, rat and guinea-pig liver (Tables 1 and 2) does not necessarily reflect the behaviour of GSSG in the initial tissue. This is suggested by the fact that the glutathione of a variety of intact tissues was found by Bhattacharya *et al.* (1955) to be predominantly in the reduced form.

Thus in the intact respiring tissue the rate of reduction of GSSG must be rapid enough to counteract the oxidation of GSH. The nature of the changes which increase the rate of oxidation of GSH and decrease the rate of reduction of GSSG, or both, on homogenization of rat liver require further study. Pigeon-liver homogenate maintained both endogenous and added GSH in the reduced form when incubated aerobically for periods of more than 1 hr. (Table 1) and was therefore assumed to be more similar to the intact tissue than the rat-liver or guinea-pig-liver homogenate. Hence pigeon-liver homogenate was chosen for further investigations.

When GSH was incubated with boiled tissue at 30° it was oxidized at the rate of about 17 %/hr. (Table 1). The concentration of endogenous GSH in pigeon-liver homogenate is about $4{\cdot}6 \pm 0{\cdot}4$ μ-moles/g. fresh wt. of tissue/hr. (12 expts.) and therefore about 0·8 μmole (17 %) of the endogenous GSH could be oxidized non-enzymically/g. fresh wt. of tissue/hr. The initial rates of reduction of added GSSG in pigeon-liver homogenate were $13{\cdot}8 \pm 5{\cdot}0$ μmoles of GSSG/g. fresh wt. of tissue/hr. (9 expts.), which is high enough to maintain the endogenous GSH in the reduced form if we assume there is no enzymic GSH oxidation. However, enzymic oxidation possibly exists and more than 0·8 μmole of GSH is oxidized. By supplementing pigeon-liver homogenate with TPN^+ and citrate, rates of reduction as high as 140 μmoles of GSSG/g. fresh wt. of tissue/hr. were obtained. Krebs (1954) observed that the formation of malate from pyruvate and carbon dioxide in pigeon-liver homogenates is more rapid under aerobic than under anaerobic conditions. Since coupling of the reductive carboxylation of pyruvate to TPN-linked oxidations should be just as effective anaerobically as aerobically, Krebs suggested that some mechanism other than simple coupled oxidation–reduction was involved in the aerobic formation of malate. He proposed that aerobically, when the ratio adenosine triphosphate/adenosine diphosphate (ATP/ADP) was high, TPNH could be formed by a reversal of the first step of oxidative phosphorylation according to the equation:

$$\text{Dihydroflavoprotein} + TPN^+ + ATP \rightarrow \text{Flavoprotein} + TPNH + ADP + \text{phosphate} + H^+$$

If such a mechanism were to act in pigeon liver one would expect TPN-linked reductions generally to be more rapid aerobically than anaerobically. However, under most conditions the aerobic and anaerobic rates of reduction of GSSG were approximately equal. It is interesting, though, that added DPN^+ at concentrations above 0·11 mM has a stimulating effect on GSSG reduction, higher under aerobic than anaerobic conditions (Table 5). This suggests an effect of oxygen on GSSG reduction, perhaps by a conversion of TPN^+ into TPNH by a mechanism other than TPN-linked oxidations.

In the presence of 5 mM-pyruvate the aerobic reduction of GSSG was more rapid than the anaerobic reduction (Table 7), but this may be possibly due to an inhibition of the anaerobic rate by pyruvate and not to a stimulation by oxygen. The aerobic rate of reduction of GSSG was up to 30 % higher than the anaerobic rate when large quantities of TPN^+ (more than 0·45 μmole/flask) were added (Table 5). However, both these effects were small in comparison with the large (up to ten-fold) difference between the aerobic and anaerobic formation of malate from pyruvate and carbon dioxide (Krebs, 1954; L. Rathbone & H. A. Krebs, unpublished work, 1959). It is possible that the low value for GSSG reduction by oxygen that was observed is related to swelling of homogenate mitochondria caused by GSH added or formed from the added GSSG (Lehninger & Schneider, 1959).

Although both GSSG reduction and reductive carboxylation of pyruvate are higher in aerobic than in anaerobic conditions, we cannot say whether they have the same origin.

SUMMARY

1. There was no disappearance of reduced glutathione incubated aerobically with pigeon-liver homogenate, but with rat-liver homogenate 36 % of the initial reduced glutathione disappeared in 1 hr. at 30°. On incubation with boiled rat-liver or pigeon-liver homogenate 17 % of the initial reduced glutathione disappeared in 1 hr.

2. In homogenates of rat and guinea-pig liver the anaerobic rate of reduction of oxidized glutathione was more rapid than the aerobic rate. The aerobic reduction of oxidized glutathione in the first 10 min. was followed by reoxidation of the reduced glutathione formed. In pigeon-liver homogenates the initial (0–20 min.) aerobic and anaerobic rates of reduction of oxidized glutathione were approximately equal.

3. Addition of oxidized triphosphopyridine nucleotide (final concentration up to 0·11 mM) to

pigeon-liver homogenates stimulated the aerobic and anaerobic rates of reduction of oxidized glutathione to the same degree. At concentrations of oxidized triphosphopyridine nucleotide above 0·11 mM the anaerobic reduction of oxidized glutathione was lower than the aerobic reduction.

4. Citrate, fumarate and glucose 6-phosphate stimulated oxidized glutathione reduction in pigeon-liver homogenates. The addition of oxidized triphosphopyridine nucleotide (0·04 mM) further stimulated the reduction.

5. In pigeon-liver homogenates incubated under 5% carbon dioxide, 5 mM-pyruvate inhibited the anaerobic reduction of oxidized glutathione by about 50% but had no effect aerobically. Higher concentrations (25 mM) of pyruvate inhibited both the aerobic and anaerobic reduction of oxidized glutathione by about 70%. Added fumarate and oxidized triphosphopyridine nucleotide both partially counteracted the inhibition of oxidized glutathione reduction due to pyruvate. Fumarate was more effective in aerobic conditions.

6. The significance of these findings is discussed in relation to the mechanism of maintenance of glutathione in the reduced form in intact tissue.

The author would like to thank Professor Sir Hans Krebs, F.R.S., for suggesting this problem and for his encouragement, advice and criticism, Dr R. G. Kulka for his constant help as supervisor, Dr W. Bartley for valuable discussions and Dr R. Cecil for instruction in SH-group analysis. He would also like to thank the Calouste Gulbenkian Foundation for a fellowship and The Distillers Co. Ltd. (Biochemicals) for a gift of GSH. This work was aided by a grant from the Rockefeller Foundation.

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Biochem. J. (1961) **79**, 51

The Pectic Enzymes of *Aspergillus niger*

1. THE PRODUCTION OF ACTIVE MIXTURES OF PECTIC ENZYMES

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(*Received* 29 *September* 1960)

Aspergillus niger, like many other fungi, produces enzymes which attack pectic substances. It is probably true to say that none of these enzymes has been obtained in a pure state from a filamentous fungus, although Patel & Phaff (1959) produced preparations of a single pectic enzyme from the yeast *Saccharomyces fragilis*. Some work has been published on the mode of action of mould enzymes (Jansen & MacDonnell, 1945; Saito, Yasuji & Marumo, 1954; Ozawa & Okamoto, 1954; Ayres, Dingle, Phipps, Reid & Solomons, 1952; Saito, 1955), but almost invariably crude preparations were used, often obtained from commercial material of unknown provenance; where defined culture methods were employed the activities reported were very low (Saito, 1955) in comparison with those of the commercial preparations.

The entire field of pectic enzymes is extremely confused; almost as many systems of nomenclature exist as authors who have dealt with this subject. A recent review by Demain & Phaff (1957) has brought a welcome clarification and we have adopted their system of nomenclature. Basically this scheme divides pectic enzymes into poly-