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The Effect of some Protein Solutions on the Oxidation of Glutathione in Oxygenated Erythrocytes

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(Received 7 March 1960)

Though much is known about the oxidation *in vitro* of reduced glutathione (Barron, 1951; Isherwood, 1959), the corresponding intracellular reaction has been little studied. Meldrum (1932) aerated glucose-depleted ox erythrocytes and observed a slow fall in the concentration of glutathione which, since it was prevented by the addition of glucose, he attributed to its intracellular oxidation. Klebanoff (1957) using this system found that besides glucose, adenosine, inosine and 33 % plasma (but not bovine-serum albumin) prevented the loss of glutathione by oxidation when added to the saline in which the cells were suspended. The effect of plasma was said to be due to its supplying 'utilizable metabolites to the cell'.

Enzymes have also been described which convert glutathione into its oxidized form with or without oxygen itself. Among these are glutathione peroxidase (Mills, 1957), cytochrome *c*-cytochrome *c* oxidase (Boeri, Baltscheffsky, Bonnichsen & Gustav-Paul, 1953), glutathione-homocystine transhydrogenase (Racker, 1955*b*) and dehydroascorbic acid reductase (Hopkins & Morgan, 1936). Apart from the last these have all been found in animal

tissues but their quantitative importance as routes for the intracellular oxidation of glutathione is not known. However, in view of the speed of the metal-catalysed oxidation *in vitro* (see Isherwood, 1959) and the presence of such catalytic metals (e.g. iron and copper) in the tissues, it seems likely that the major proportion of glutathione oxidized intracellularly is oxidized by direct reaction with oxygen itself.

In the presence of glucose, net intracellular oxidation of glutathione cannot normally be observed (Meldrum, 1932; Klebanoff, 1957). This is probably due to the activity of the widely distributed enzyme, glutathione reductase which mediates the reduction of the oxidized glutathione by means of reduced triphosphopyridine nucleotide (Rall & Lehninger, 1952). Regeneration of the latter from its oxidized form requires the metabolism of glucose (see Horecker, 1951). To investigate systematically the intracellular oxidation of glutathione, it is therefore necessary to use cells depleted of glucose, thus enabling the oxidation to be followed, uncomplicated by any subsequent reduction of the oxidized glutathione formed.

This paper describes, with glucose-depleted human erythrocytes, how the rate of oxidation of glutathione in the oxygenated cells can vary according to the nature of the medium in which the cells are suspended; evidence is also presented to show the occurrence in human serum of a factor with an inhibitory effect on the oxidation.

EXPERIMENTAL

Materials. Oxidized glutathione (GSSG) was obtained from Boehringer und Söhne, Mannheim; reduced glutathione (GSH) was a gift from The Distillers Co. Ltd., Speke, Liverpool; disodium ethylenediaminetetra-acetate (EDTA) was from British Drug Houses Ltd., Poole; bovine-serum albumin, fraction V, and bovine γ -globulins, fraction II, were from Armour Biochemicals Ltd., Eastbourne; human-serum albumin, fraction V (HSA), was kindly provided by the Blood Transfusion Service, Royal Infirmary, Edinburgh. Both albumin fractions were prepared as described by Cohn *et al.* (1946).

Dialysis. Solutions were dialysed against running water at 16–18° in Visking $\frac{3}{8}$ in. cellophan tubing. Purified protein solutions were dialysed for 6–10 hr. before dilution to the required concentration. These solutions were kept at 5° and freshly made up each week.

Dialysed serum. This was prepared from human oxalated plasma. After dialysis for the desired time, NaCl (0.65 g./100 ml. of the dialysed solution) was added and dialysed plasma was converted into dialysed serum by incubating at 37° for 10 min., when a clot of fibrin formed and was squeezed from the clear solution.

Washed erythrocytes. Erythrocytes were obtained from fresh, oxalated human blood by centrifuging for 10 min. at 1000 *g* and removing the plasma and buffy coat. When pooled erythrocytes were used, the plasma was separated before mixing the cells. Erythrocytes were resuspended in 2 vol. of 0.9% sodium chloride containing EDTA (2 mM) and incubated at 37° for 15 min. The cells, separated from the sodium chloride-EDTA solution by centrifuging, were incubated for a further 15 min. with 2 vol. of fresh solution. The re-separated washed cells were then stored at 5°. After this treatment, glucose, estimated according to Folin & Wu (1920) could not be detected.

During washing, losses in GSH and GSSG (total glutathione) were often incurred (see also Martin & McIlwain, 1959). If EDTA was omitted from the 0.9% sodium chloride, these losses were much greater. Washed erythrocytes were used for the oxygenation experiments within 30 hr. of the shedding of the original blood.

Treatment of washed cells with carbon monoxide. Carbon monoxide was prepared by the action of concentrated sulphuric acid on formic acid and freed from acid fumes by bubbling through 10% sodium hydroxide solution. The gas was bubbled for 30 min. through a suspension of the washed erythrocytes in 1 vol. of sodium chloride-EDTA solution in the dark. The separated cells were kept in a stoppered container at 5°. In the experiments in which these erythrocytes were used, the corresponding ungassed cells (controls) were otherwise treated in exactly the same way.

Oxygenation of erythrocytes. Oxygen, saturated with water vapour at 23° or 37°, was bubbled through tubes con-

taining suspensions of washed erythrocytes in the required solutions at a flow rate of about 5 ml./min. Foaming was prevented by addition of 1 drop of Silicone M.S. Antifoam emulsion 'RD' (Hopkin and Williams Ltd., Chadwell Heath, Essex). The outlet of one tube was connected to the inlet of another, up to 12 oxygenations thus being performed at the same time and with the same oxygen flow rate. Tubes were immersed in water at 23° or 37° and kept in an incubator at the same temperature. Samples of the oxygenated mixtures, pipetted from the tubes at the required intervals, were centrifuged for 10 min. at 1000 *g* and the supernatant solutions were separated from the packed erythrocytes.

Reduced glutathione and total glutathione. These were estimated on haemolysates of the erythrocytes as already described (Jocelyn, 1960). All duplicate readings agreed within 20 μ moles/100 ml. of cells. The standard deviation from the mean was 6.5 μ moles/100 ml. of cells.

Oxyhaemoglobin. A Brinkmann Haemoreflexor (Kipp and Sons, Delft, Holland) was used. Measurements were made on a suspension of erythrocytes in the required solutions. Zero oxyhaemoglobin was taken as the reading with a sample of unoxygenated erythrocytes in 1 vol. of 0.9% sodium chloride solution containing sodium dithionite (1 g./100 ml.).

RESULTS

When intact human erythrocytes are oxygenated at 37°, a gradual fall in their GSH content occurs. The rate of this fall varies from one batch of cells to another and also according to the constitution of the medium in which the cells are suspended during the oxygenation. Two media which have been extensively used are 0.9% sodium chloride (saline) and a solution of human-serum albumin containing 0.75% sodium chloride to produce iso-osmotic conditions (HSA solution).

In forty-two experiments, each with a different batch of fresh, washed, pooled cells, the rates of fall of GSH in the same cells oxygenated at 37° in both these media have been noted together with the GSH content before oxygenation. The rate of fall of GSH in different cells varies between 5 and 50 μ moles of GSH/100 ml. of cells/hr. in saline and is 15–60 μ moles of GSH/hr. in HSA solution. The data for each batch of cells has been classified in one of three groups according to the order of the rate of fall in saline (Table 1). In each group, the range and mean have been included for the initial cell concentrations of GSH before oxygenation. Also included are the differences found between the GSH content of the cells oxygenated for 3 hr. in saline and the same cells oxygenated for 3 hr. in HSA solution. From this it is seen that the mean initial GSH concentration found in group 1 (with the lowest rates of fall) is significantly lower ($> 2 \times \text{s.d.}$) than that found in group 3 (with the highest rates of fall).

The data in Table 1 also show that the mean fall of GSH in HSA solution after 3 hr. of oxygenation

Table 1. A comparison of the rates of loss of reduced glutathione in different batches of human erythrocytes, oxygenated in saline or human-serum albumin solution with the corresponding initial concentrations of reduced glutathione

Figures for the rate of loss of GSH/hr. are the average from the rate of loss over the first 3 hr. of oxygenation. A different batch of cells was used for each experiment. The initial concentration of GSH is the concentration found after washing but before oxygenation. The HSA solution contained 4 g. of HSA/100 ml. of 0.75% NaCl. The experiments were all carried out as described in Fig. 1. The values given for GSH are in μ moles/100 ml. of cells.

Group	No. of experiments	Rate of loss of GSH/hr. in		Difference after 3 hr. of oxygenation between GSH of cells in saline and the same cells in HSA solution		Initial GSH concn.	
		Saline	HSA solution	Range	Mean $\pm$ S.D.	Range	Mean $\pm$ S.D.
1	17	5-16	15-43	18-113	51 $\pm$ 29	100-270	178 $\pm$ 48
2	17	17-33	25-53	10-75	43.5 $\pm$ 17	140-275	222 $\pm$ 38
3	8	34-50	45-60	8-57	34.5 $\pm$ 15.5	220-300	256 $\pm$ 24

Fig. 1. Effect of oxygenation on the GSH and total glutathione content of washed human erythrocytes, oxygenated in 0.9% NaCl solution (GSH, ●; total, ○) or in HSA solution (GSH, ▲; total, △) and upon the difference in GSH (x) between cells from these two media. A mixture of 1 vol. of the washed pooled erythrocytes (18 ml.) and 1 vol. of 0.9% NaCl or 1 vol. of HSA solution containing 4 g. of HSA/100 ml. of 0.75% NaCl was oxygenated at 37°. At intervals, 2 ml. of the mixtures were withdrawn and glutathione (GSH and total) determined on the separated cells.

is greater by 34.5-51 μ moles of GSH/100 ml. of cells than the fall for the same cells oxygenated for the same time in saline. The different rates of oxidation in these two media are illustrated for a single experiment in Fig. 1, in which the GSH concentrations of the same batch of pooled cells, oxygenated in either saline or HSA solution, have been plotted against time of oxygenation. In both media, the rate of fall of GSH does not significantly slow up until the cell concentration of GSH has reached a low value. The difference between the GSH content of the cells oxygenated in saline and HSA solution for the same time has also been plotted against time. This difference increases to a maximum value after 3 hr. of oxygenation. The increase of this

difference with time of oxygenation has also been followed in twenty-three of the experiments summarized in Table 1. In fifteen of them, a maximum difference was found after 3 hr. of oxygenation (as in Fig. 1) and in the other eight the difference was still diverging after this time (as in Fig. 5).

Different rates of fall of GSH in saline and HSA solution have also been observed with cells from single blood donors (see Fig. 3).

Effect of oxygenation on the concentration of total glutathione. To show whether the fall in the concentration of GSH in oxygenated cells can be accounted for by its oxidation to GSSG, the levels of GSH and total glutathione have been measured simultaneously at intervals during the oxygenation. The results of one experiment (Fig. 1) show that GSH is largely oxidized to GSSG but that there is also a slow fall in the concentration of total glutathione. This fall is greater for the cells oxygenated in HSA solution than for the cells oxygenated in saline. To determine the conditions under which this fall in total glutathione occurs, erythrocytes have been oxygenated in HSA solution containing increasing amounts of glucose (Table 2). Glucose at a concentration of 120 mg./100 ml. of HSA solution prevents the oxidation of GSH and maintains the level in the erythrocytes at the initial value throughout 3 hr. of oxygenation (see also Meldrum, 1932; Klebanoff, 1957). When the glucose concentration is decreased below this value, however, oxygenation of the cells then leads to a fall in the concentration of GSH and the fall is greater the smaller the concentration of glucose in the original solution. From the corresponding figures for the total glutathione in these cells the concentration of GSSG can be calculated. It is then evident that the losses observed in total glutathione during oxygenation increase in magnitude with increasing concentration of the GSSG found. After oxygenation for 3 hr., only 2-3 % of the cells are found to be haemolysed and added

Table 2. *Effect of varying concentrations of glucose on the concentrations of reduced and oxidized glutathione in washed oxygenated human erythrocytes*

The washed pooled erythrocytes (1.5 ml.) were oxygenated for 3 hr. at 37° in 0.75 % NaCl solution (1.5 ml.) containing HSA (4 g./100 ml.) and glucose as indicated. Percentage haemolysis after 3 hr. was determined as described by Klebanoff (1957) and reduced and total glutathione were estimated on haemolysates of the separated cells. Recoveries of GSSG added to the haemolysates are given as additional GSH formed/100 ml. of cells after the enzymic reduction. Loss in total glutathione is based on the content of the unincubated cells.

Glucose added (mg./100 ml. of HSA solution)	Glutathione (μmoles/100 ml. of cells)				Recovery of added GSSG (100% = 200)	Haemolysis (%)
	Reduced	Total	Oxidized	Total lost		
0	10	115	105	60	190	2.5
40	45	137	92	38	192	2
80	95	152	57	23	197	2
120	140	160	20	15	200	2
200	135	160	25	15	195	2.5
Unincubated cells	137	175	38	—	190	—

Table 3. *Effect of incubation on the concentration of total glutathione of previously oxygenated, washed, human erythrocytes*

Washed pooled erythrocytes, after oxygenation in 1 vol. of 0.9 % NaCl at 37° for 11 hr., were incubated at 37° without oxygenation under the conditions shown. Samples were taken at the stated intervals for estimation of reduced or total glutathione.

Conditions	Glutathione (μmoles/100 ml. of cells)				
	Before incubation		After 1 hr. Total	After 3 hr. Total	After 5 hr. Total
	Reduced	Total			
Unxygenated cells	190	220	—	—	—
Oxygenated cells alone	55	172	130	100	82
Oxygenated cells with 1 vol. of 0.9 % NaCl	—	—	125	97	87
Oxygenated cells with 1 vol. of 0.75 % NaCl containing HSA (4 g./100 ml.)	—	—	130	102	80

GSSG is still quantitatively recovered from the haemolysates (see Jocelyn, 1960).

The effect of incubating cells, previously oxygenated to produce a high concentration of GSSG, has also been examined (Table 3). The cells have been kept at 37° without further oxygenation and the concentrations of total glutathione determined at intervals over 5 hr. There is a steady fall in the concentration of total glutathione over this period and the rate of fall is unaffected whether the cells are incubated alone or suspended in either saline or HSA solutions.

Mechanism of the effect of albumin. The difference between the GSH concentration of the same cells oxygenated in saline and in HSA solution has been chiefly studied at a concentration of 4 g. of HSA/100 ml. of solution. Decreasing this amount to 0.6–0.8 g./100 ml., however, does not affect the GSH difference and below this value the difference declines roughly linearly with decreasing concentration of HSA (Fig. 2). This result has been obtained with five batches of cells and with periods of oxygenation varying between 2 and 4 hr. If the cells in saline or HSA solution are treated with nitrogen instead of oxygen, there is no fall in the GSH concentration in either medium (Fig. 2).

Fig. 2. Influence of HSA on the level of GSH in washed pooled human erythrocytes treated in HSA solutions with oxygen for 4 hr. (●) or 2.5 hr. (▲) or with nitrogen for 2.5 hr. (△). Values after 2.5 hr. were obtained with one batch of pooled cells and values after 4 hr. with another batch. Oxygen or nitrogen at the same flow rate was bubbled through the washed erythrocytes in 1 vol. of the solution at 37° for the times stated. GSH was estimated on the separated cells: the mean of duplicate readings is given for the 2.5 hr. values.

When cells are oxygenated with saline, Krebs-Ringer or Krebs-Ringer-phosphate solutions, made up as described by Umbreit, Burris & Stauffer (1949), there are no differences between the rates of oxidation of GSH in these solutions or between the amount of increase when HSA is added (Table 4). On the other hand, the rate of oxidation of GSH in cells oxygenated in either saline or HSA solution is considerably increased when cupric ions are added to the solutions before oxygenation.

A comparison of the effect at various concentrations of HSA and iodine-oxidized HSA on the rates of oxidation of GSH in oxygenated erythrocytes shows that there are no significant differences between the two substances (Table 5).

Is oxyhaemoglobin involved in the oxidation? Oxyhaemoglobin concentrations, measured by reflectance in the intact cells after oxygenation with HSA solutions, decrease with increasing concentration of HSA in the solution used (Table 6). To test whether the effect of albumin on the oxidation of GSH might involve oxyhaemoglobin, the rates of this oxidation in oxygenated cells pretreated with carbon monoxide have been compared with the rates for the same untreated cells. The results from three experiments in which both sets of cells have been oxygenated in saline and HSA solution are shown in Table 7. Treatment with carbon monoxide increases the rate of oxidation over that found with the untreated cells, in both solutions by a factor of 1.2–1.4, and there is consequently a similar increase in the difference between the GSH of cells oxygenated in the two media. Treatment of erythrocytes with carbon monoxide is known to increase the concentration of GSH well above that found for the corresponding untreated cells (Numata, 1940). This increase, measured before oxygenation, is to about 1.2–1.5 times the concentration in the untreated cells, i.e. about the same amount as the subsequent increase in the rate of oxidation. On oxygenating the carbon monoxide-treated cells, there is a decrease (30–35%) in the measured percentage saturation with carbon monoxide which is complete after 2 hr. of oxygenation, nevertheless the rates of oxidation in the treated cells do not decrease during this period.

The effect of some other protein solutions on the oxidation. A comparison has been made between the rates of oxidation of GSH in the same cells oxygenated with saline, HSA solution, bovine-serum albumin solution and bovine γ -globulin solution, all present at the same concentration in 0.75% sodium chloride. The results of four experiments are summarized in Table 8. In three experiments, the rate with bovine-serum albumin solution is not significantly different from the rate with

Table 4. *Influence of the composition of the saline medium, with or without human-serum albumin, on the oxidation of reduced glutathione in oxygenated washed human erythrocytes*

HSA concentrations were 4 g./100 ml. of the saline solution. Krebs-Ringer and Krebs-Ringer-phosphate solutions were made up as described by Umbreit, Burris & Stauffer (1949) but where HSA was added the 0.9% NaCl prescribed was replaced by 0.75% NaCl. The washed pooled erythrocytes were oxygenated with 1 vol. of the solution for 3 hr. at 37° and the cell GSH concentration subsequently estimated.

Medium	GSH (μ moles/100 ml. of cells)	
	Alone	With albumin
0.9% NaCl	150	130
Krebs-Ringer solution	155	132
Krebs-Ringer-phosphate solution	157	130
0.9% NaCl + CuSO ₄ (0.016 mm)	110	92

Table 5. *Comparison of the effects of untreated and iodinated human-serum-albumin solutions on the oxidation of reduced glutathione in oxygenated washed human erythrocytes*

Iodinated HSA was prepared according to Hughes & Straessle (1950). The proteins were dissolved in 0.75% NaCl to give the concentrations shown and the washed pooled erythrocytes oxygenated for 3 hr. at 37° with 1 vol. of these protein solutions. GSH was estimated on haemolysates of the separated cells.

Protein concentration (g./100 ml. of solution)	GSH (μ moles/100 ml. of cells) in	
	HSA solution	Iodinated HSA solution
0	120	120
0.8	96	90
1.6	90	87
2.4	84	88
3.2	90	90
4.0	88	92

Table 6. *Comparative oxyhaemoglobin concentrations of washed human erythrocytes oxygenated in human-serum-albumin solutions*

The HSA was dissolved in 0.75% NaCl solution to give the concentrations shown. After oxygenation of the washed pooled erythrocytes at 37° for 1.5 hr. with 1 vol. of the solution, oxyhaemoglobin was measured as described in the text, the oxyhaemoglobin concentration of the cells oxygenated with 0.75% NaCl alone being taken as 100.

HSA concentration (g./100 ml. of solution)	Oxyhaemoglobin
0	100
0.8	92
1.6	90
2.4	84
3.2	78
4.0	69

Table 8. *Rate of oxidation of reduced glutathione in washed human erythrocytes, oxygenated in different protein solutions*

The protein solutions each contained 4 g. of the protein in 100 ml. of 0.75% NaCl. Washed, pooled cells were oxygenated at 37° in 1 vol. of the solution and GSH was then determined on the separated cells.

GSH oxidized/hr. (μ moles/100 ml. of cells)
Average over 3 hr.

Solution	Expt. 1	Expt. 2	Expt. 3	Expt. 4
0.9% NaCl	16	23	35	25
Bovine-serum albumin	16	32	33	29
HSA	29	48	53	44
Bovine-serum γ -globulins	—	48	53	40

Fig. 3. Rate of oxidation of GSH in washed human erythrocytes oxygenated at 37° in the following media: ●, 0.9% NaCl; ▲, 0.75% NaCl containing HSA (4 g./100 ml.); ■, human dialysed serum containing NaCl (0.65 g./100 ml.), dialysis time, 24 hr.; □, the same dialysed serum previously heated at 70° for 1 min.; ▢, dialysed serum-NaCl, dialysis time, 44 hr. The erythrocytes used to obtain the data plotted in A and B were from the same batch of fresh, oxalated blood taken from a single donor. They were used 30 hr. after separating the plasma for A and 24 hr. later for B. The separated plasma from this same blood was used to prepare the dialysed serum used in A, B and C. The cells used for C were washed pooled erythrocytes and the dialysed serum (stored at 5°) was 24 hr. older than when used for B. The solution (1 vol.) was oxygenated at 37° with 1 vol. of the erythrocytes. At the stated times, samples of the mixtures were withdrawn and GSH determined in duplicate on the separated cells. Values given represent the mean of the two readings. For C, each oxygenation was also duplicated and the results are therefore the mean of four readings.

Table 9. *Relation between length of dialysis and heat treatment of human dialysed serum and its effect on the oxidation of reduced glutathione in washed human erythrocytes*

Washed pooled cells were oxygenated at 37° in 1 vol. of dialysed serum (prepared from fresh, pooled, oxalated plasma and containing 0.65 g. of NaCl/100 ml.) or the corresponding heated dialysed serum or 0.75% NaCl containing HSA (4 g./100 ml.). After oxygenation for the times shown, GSH was estimated on haemolysates of the separated cells. Each experiment relates to a different batch of dialysed serum and erythrocytes.

Expt. no.	Treatment of dialysed serum		GSH (μ moles/100 ml. of cells) after oxygenation of cells with		Time of oxygenation (hr.)
	Period of dialysis (hr.)	Time of heating at 70° (min.)	Dialysed serum	HSA solution	
1	3	0	200	127	2
		20	205		
		30	175		
		40	137		
2	8	0	135	80	2
		3	125		
		10	95		
		15	70		
3	20	0	200	145	2.25
		3	152		
4	24	0	190	165	2
		0.5	160		

Dialysed serum (from pooled plasma; dialysis time, 20 hr.) remains active for at least 5 days after dialysis if stored at 5° and for 2–3 days if kept at 20°; plasma stored at –15° for 6 months and then dialysed yields an active dialysed serum.

In Fig. 4 the effect of diluting active dialysed serum (dialysis time, 5 hr.) with HSA solution, on the concentration of GSH in erythrocytes after various periods of oxygenation, is illustrated. Measured after the first 2 hr. of oxygenation, dialysed serum must be diluted more than four times before there is any significant fall in the GSH concentration from that found in the dialysed serum alone. After 3 or 4 hr. of oxygenation, when the difference between the GSH concentrations of the cells oxygenated in these two media has more than doubled, a fall in the concentration of GSH below that found in dialysed serum alone begins when the dialysed serum–HSA solution still contains 50–60 % of dialysed serum.

Fig. 4. Effect of diluting human dialysed serum with 0.75% NaCl containing HSA (4 g./100 ml.) on the GSH concentration of washed pooled human erythrocytes, subsequently oxygenated in the mixture solution at 37° for 1 hr. (x), 2 hr. (●), 3 hr. (▲) and 4 hr. (△). Dialysed serum (dialysis time, 5 hr.) containing NaCl (0.65 g./100 ml.) was added to the HSA solution to give the required dilution of the dialysed serum: 1 vol. of each mixture was oxygenated with 1 vol. of washed erythrocytes and samples were withdrawn at the stated intervals for estimation of GSH. The mean of duplicate estimations is plotted. The dialysed serum was obtained from fresh plasma taken from a single donor.

The influence of temperature has been studied on the rate of oxidation of GSH in erythrocytes oxygenated in saline, active dialysed serum or HSA solution and the data from two experiments are illustrated in Fig. 5. The rates of oxidation of GSH at 37° are almost twice as great in the cells used in one of the experiments as the corresponding rates for the cells used in the other. On lowering the temperature from 37 to 23°, there is a substantial decrease in all the rates of oxidation. For the experiment with the lower rates at 37°, the rate for dialysed serum at 23° falls to 58 % of its former value and the rate for HSA solution to 42 %; in the other experiment, the rate for dialysed serum falls to 64 % and the rate for HSA

Fig. 5. Comparison of the rate of oxidation of GSH in washed pooled human erythrocytes oxygenated at 37° (●, ▲, ■) and 23° (○, △, □) in 0.9% NaCl (●, ○), 0.75% NaCl containing 4 g. of HSA/100 ml. (▲, △) and pooled human dialysed serum (dialysis time, 3 hr.) containing 0.65 g. of NaCl/100 ml. (■, □). The dialysed serum was obtained from fresh, pooled, oxalated plasma. The pooled washed erythrocytes were oxygenated in 1 vol. of the solution at the stated temperature, samples of the mixture were withdrawn every hour and GSH was estimated on haemolysates of the separated cells. Each graph represents a separate experiment with a different batch of erythrocytes.

Table 10. *Effect of length of dialysis and heat treatment of human dialysed serum on the comparative oxyhaemoglobin concentrations of oxygenated washed human erythrocytes*

Washed pooled erythrocytes were oxygenated at 37° in 1 vol. of the solution. Figures given were obtained after 1 hr. of oxygenation but there were no significant changes after 3 hr. The oxyhaemoglobin concentration of cells oxygenated in 0.9% NaCl was taken as 100%. The dialysed serum was obtained from fresh, pooled, oxalated plasma.

Treatment of pooled dialysed serum	Activity (see text)	Oxyhaemoglobin (%)
Dialysis for 8 hr.: unheated	Active	52
Dialysis for 8 hr.: heated for 1 min. at 70°	Active	22
Dialysis for 8 hr.: heated for 15 min. at 70°	Inactive	22
Dialysis for 30 hr.: unheated	Inactive	50

solution to 51%. Thus in both experiments the proportional decreases resulting from lowering the temperature are of the same order and are greater for HSA solution than for dialysed serum.

The rate of oxidation of GSH in cells oxygenated in saline, which at 37° is equal to that in dialysed serum (see also Fig. 3.4), also falls, on reducing the temperature to 23°, to the same extent as dialysed serum.

The oxyhaemoglobin concentration of cells after oxygenation in dialysed serum prepared in various ways has been measured (Table 10). Cells oxygenated with unheated dialysed serum whether active or inactive (in the sense defined above) show similar concentrations of oxyhaemoglobin and these are lower than those observed after oxygenation in HSA solution containing 4 g. of HSA/100 ml. (see Table 6). If the dialysed serum used has previously been heated, the oxyhaemoglobin concentrations found are much lower still and of the same order whether or not the dialysed serum remains active after the heat treatment used.

DISCUSSION

The wide variations found for the rates of oxidation of GSH in different batches of washed erythrocytes oxygenated in the same media and under the same conditions may be partly due to their different content of metals which catalyse the oxidation *in vitro*. One of these metals (see introduction) is copper, and cupric ions added to the medium do in fact increase the rate of oxidation in oxygenated cells. This effect is presumably exerted intracellularly because cupric ions are known to diffuse rapidly into human erythrocytes when added to blood either *in vivo* or *in vitro*. These ions gradually become bound in the cells because the amounts escaping when the cells are subsequently incubated with fresh plasma decrease the longer they are kept after exposure to the ions (Bush, Mahoney, Gubler, Cartwright & Wintrobe, 1956). One of the substances binding the added copper may be GSH since it reacts readily *in vitro* with cupric ions (Hopkins, 1929). If so, some of the endogenous copper found in erythrocytes (70–80 µg./100 ml. of cells) (Cartwright, 1950) may also be bound to GSH, the actual proportion of the total copper in the cells thus bound depending on the cell concentration of GSH. The higher the concentration of GSH the more catalyst for its own oxidation it would bind and this hypothesis would therefore explain the apparent paradox that the rate of oxidation of GSH tends to be greater the greater its concentration in the cells before oxygenation although the rate does not decrease during oxygenation as the concentration falls.

Variations in the rate of oxidation of GSH in

different cells may also be due, in part, to variations in their content of substrates reducing triphosphopyridine nucleotide (Jocelyn, 1960) since these take part in the reverse reaction, the reduction of GSSG, and would therefore diminish the overall rate of oxidation of GSH. Such substrates (e.g. glucose 6-phosphate) would probably remain in the cells after washing (Aten & Hevesy, 1938).

In addition to a fall in the concentration of GSH in oxygenated erythrocytes, there is also a fall in the total glutathione (GSH + GSSG) content. The rate of both these falls is greater in the cells oxygenated in HSA solution than in the same cells oxygenated in saline. In both media, however, the rate of fall in total glutathione is much smaller than the rate of fall in GSH so that the cells oxygenated in HSA solution contain more GSSG than those oxygenated in saline for the same time. The fall in total glutathione in both media is therefore probably due to a fall in the GSSG level at a rate dependent on its rate of formation from GSH. This is further shown by the fact that when the formation of GSSG is diminished by adding glucose to the oxygenated cells (see introduction), the loss of total glutathione is found to be correspondingly smaller.

Of possible explanations for this fall in GSSG, the following appear to be excluded for the reasons given: (i) haemolysis: the degree of haemolysis of the cells after oxygenation is too small to account for the observed loss; in any case it is unaffected by alterations in the glucose content of the oxygenation medium; (ii) incomplete recovery: GSSG added to the haemolysates after oxygenation is quantitatively recovered as GSH; (iii) further oxidation, e.g. to the sulphonic acid: there is a continuing loss of total glutathione on incubating cells with a high GSSG content without further oxygenation; (iv) diffusion from the cells: the loss of total glutathione on incubating cells with a high GSSG content proceeds at the same rate whether the cells are incubated alone or suspended in saline.

The most likely explanation remaining is that the fall of GSSG is due to its participation in a slow non-oxidative reaction. Such a reaction may be with the SH group of proteins (Pr) within the erythrocyte to yield mixed glutathione-protein disulphide compounds:

Evidence has been given for the occurrence of a similar reaction between protein SH groups and (as the non-protein disulphide) cystamine (Eldjarn & Pihl, 1956) or a derivative of diphenyl disulphide (Klotz, Ayers, Ho, Horowitz & Heiney, 1958). On the other hand, GSSG does not react *in vitro* with the SH group of HSA (Isles & Jocelyn, 1960).

When washed erythrocytes are treated with nitrogen in HSA solution, they show no fall in their GSH concentrations. Hence the additional fall of GSH in cells oxygenated in HSA solution over that of the same cells oxygenated in saline is due to a stimulant effect of HSA on the oxidation of GSH and not to any direct oxidation-reduction reaction between GSH and HSA.

Since it is unlikely that HSA can penetrate the erythrocyte membrane (Solomon, 1958), its stimulant effect on the oxidation of GSH is probably exerted extracellularly. A buffering action would appear to be ruled out since HSA has the same stimulant effect on the oxidation when added to Krebs-Ringer or Krebs-Ringer-phosphate solutions as when added to saline. Moreover, a buffering action would not explain the finding that bovine-serum albumin, which is chemically and antigenically similar to HSA (Boyd, 1954), does not give the effect. Bovine γ -globulins do so, however, and to the same extent as HSA so that certain structural features of the HSA molecule which are absent from bovine-serum albumin but present in bovine γ -globulins are needed for the effect to operate. The SH group cannot be one of these features since after treating HSA with iodine which oxidizes the SH group (Hughes & Straessle, 1950), the stimulant effect of the treated protein remains the same as before iodination; this contrasts with the finding (Saktor, O'Neill & Cochran, 1958) that the effect of HSA in stimulating oxidative phosphorylation in mosquito mitochondria is abolished by oxidation of the SH group with iodine.

If the rate of reaction between GSH and oxygen depended on the oxygen concentration within the cell, a plausible explanation for the stimulation by HSA could be given. This would envisage an effect on the cell membrane decreasing, below the value in saline, the gradient between the extracellular and the lower intracellular oxygen concentrations and thus increasing the latter.

The oxygen dissociation properties of oxyhaemoglobin in cells suspended in iso-osmotic saline are unlikely to be altered merely by adding HSA to the saline. Consequently, under these conditions, changes in the oxyhaemoglobin concentrations of the cells probably reflect changes in their oxygen concentrations. Cells oxygenated in HSA solutions have in fact lower oxyhaemoglobin concentrations than cells oxygenated in saline so that HSA may well have an effect on oxygen concentration gradient across the cell membrane. However, this effect would be a reduction in tension, and would not therefore account for its stimulation of the oxidation of GSH. In fact there would seem to be no relationship, not even an inverse one, between the oxyhaemoglobin concentration in the cell and the

rate of oxidation of GSH. Thus HSA exerts its maximum effect on the oxidation at a concentration of 0.6–0.8 g./100 ml., whereas its effect in decreasing the oxyhaemoglobin concentration increases linearly up to at least 4 g./100 ml. Further, with differently treated batches of dialysed serum as the media, the same oxyhaemoglobin concentrations are associated in the same cells with widely different rates of oxidation of GSH.

The possibility that oxyhaemoglobin plays some essential but not rate-limiting role in the oxidation of GSH is not supported by the observation that the oxidation goes faster when the cells have been treated with carbon monoxide. The weight of this evidence however is complicated by the fact that carbon monoxide also raises the initial concentration of GSH as determined after subsequent precipitation of the proteins. Though this fact has not been explained, it is usually considered that the concentrations found in the treated cells represent the true endogenous concentrations (Numata, 1940; Thomson & Martin, 1959), the lower concentrations found in untreated cells being then ascribed to a loss, prevented by carbon monoxide, due to some kind of interaction with haemoglobin or oxyhaemoglobin during the precipitation of the proteins. On this explanation, there is no difference in the amount of GSH actually present in either the treated or untreated intact cells and the higher oxidation rate after treatment would suggest that haemoglobin or oxyhaemoglobin actually inhibits the oxidation in the untreated cells. But it is also possible that treatment with carbon monoxide does raise the concentration of GSH in the treated cells, e.g. by releasing it from some complexed form. In that case, the higher rates of oxidation observed would be due to the increased initial concentrations of GSH in the treated cells. These increased concentrations might result in a redistribution of copper and other heavy metals within the cell so that more became bound to GSH and hence available as catalysts to increase proportionately its rate of oxidation. In favour of this explanation is the fact that in the treated cells both the rates of oxidation in saline or HSA solution and the initial concentrations of GSH are all increased by about the same fraction (0.2–0.5) of their values in the untreated cells.

From the range found in normal serum (Stewart & Dunlop, 1958) dialysed serum contains 3.6–5.2 g. of HSA/100 ml., allowing for the 10% increase in volume which occurs on dialysis. When used after 3–24 hr. dialysis, as the medium for the oxygenation of washed erythrocytes, the rate of oxidation of GSH corresponds to the rate in saline not to the rate in HSA solution so that, present in the dialysed serum, there is some factor (or factors) inhibiting the effect of HSA.

Part of this factor is probably a protein because the HSA-inhibiting property (activity) is lost after heating this 3–24 hr.-dialysed serum at 70°. These conditions are unlikely to destroy a non-protein substance especially as the unheated dialysed serum retains activity for 2–3 days at room temperatures.

It also seems probable that a slowly diffusible cofactor is required since there is no activity when the dialysed serum is prepared after more lengthy dialysis (e.g. 44 hr.) of plasma. The connexion between length of dialysis and ease of heat inactivation at 70° could then be explained by the assumption that the factor required for activity is a complex between the protein and the cofactor which must be present in the dialysed serum in adequate concentration and which slowly dissociates on dialysis. Hence the shorter the dialysis the higher would be the concentration of this factor and consequently the longer the heat treatment needed to produce inactivation.

This explanation entails that after short dialysis the factor is present in dialysed serum in excess of the amount needed for full activity. This in fact appears to be the case because dialysed serum continues to inhibit the effect of HSA when diluted with HSA solution until its concentration in the mixture has fallen to 25–60%. The actual point of dilution at which the inhibition begins to disappear probably depends on the magnitude of the stimulating effect of HSA itself. Consequently it may vary considerably both after different times of oxygenation of the same cells (as in Fig. 4) and also as between one batch of cells and another. There is also a further aspect to be considered. When dialysed serum, inactivated by long dialysis (and occasionally by heating), has been used as the medium for oxygenating cells, the rate of oxidation of GSH has usually been found to be much greater than the rate for the same cells in HSA solution (as in Fig. 3*B*). Hence in dialysed serum other proteins present may potentiate or supplement the effect of HSA on the cells so that the stimulative effect inhibited by the factor may considerably exceed that found for the same cells with HSA solution alone. Dilution of the dialysed serum with HSA will also dilute all the proteins other than HSA and this may reduce the stimulating effect as well as the concentration of inhibiting factor. The point of dilution with HSA where counteraction of the stimulating effect begins to disappear cannot therefore be used to measure factor activity.

Whether lowering the temperature affects the inhibiting activity is also uncertain. The rates of oxidation of GSH in cells oxygenated in saline and dialysed serum both fall by the same amount and therefore there is no evidence that the inhibiting is preferentially affected by temperature.

The existence in dialysed serum (and therefore serum) of a factor inhibiting the effect of HSA on the oxidation of GSH in oxygenated erythrocytes may be of some relevance in physiology. A requirement for oxyhaemoglobin has not been demonstrated so that it is possible that both the effect and also its counteraction by a serum factor would also be observed in cells containing GSH from other tissues.

In the presence of glucose, however, net oxidation of GSH cannot normally be shown, because the reduction of the GSSG formed by reduced triphosphopyridine nucleotide (TPNH) and glutathione reductase is rapid enough to maintain the glutathione of the tissues predominantly as GSH (Bhattacharya, Robson & Stewart, 1955). TPNH also predominates over its oxidized form (TPN) (Glock & McLean, 1955; Bassham, Birt, Hems & Loening, 1959) and it is likely that the rate at which it can be oxidized limits glucose metabolism by the TPN-specific pentose phosphate pathway (de Duve & Hers, 1957). Glutathione reductase is an abundant enzyme with high activity in most tissues (Rall & Lehninger, 1952) and, like the pentose phosphate pathway enzymes, is present in cells mainly in the extraparticulate fraction (Kinoshita & Masurat, 1957) so that it is likely and in some tissues (e.g. the bovine lens) probable, (Kinoshita & Masurat, 1957) that the reduction of GSSG provides an important route for the oxidation of TPNH (see also Waley, 1959*a*). The rate of this reaction would be limited by the availability of GSSG, i.e. by the rate of oxidation of GSH (the 'missing step' of Waley, 1959*b*).

The range for the rate of oxidation of GSH in washed erythrocytes oxygenated in saline (and therefore active dialysed serum) is 5–50 μ moles of GSH/hr./100 ml. of cells (Table 1). Reduction of GSSG by reduced diphosphopyridine nucleotide is negligible (Racker, 1955*a*; Francoeur & Denstedt, 1954) so that probably in the presence of glucose all the GSSG formed is converted back into GSH by TPNH. If the oxidation of TPNH is the rate-limiting reaction this would enable 1.2–12.4 μ moles of glucose (0.2–2.1 mg.)/100 ml. of cells to proceed through the pentose phosphate pathway per hour taking this pathway to involve the reduction of two molecules of TPN/molecule of glucose. The mean consumption rate of glucose in normal erythrocytes aerated in plasma at 37° is 31–43 mg./100 ml. of cells/hr. (Buffa, Maraini & Chiarle, 1959) giving a range of 0.5–6.7% of glucose metabolized to pentose phosphate. This estimate agrees with the conclusion of Coxon & Robinson (1956) that the pathway is not important quantitatively in erythrocytes. However, if the rate of oxidation of GSH in erythrocytes could be stimulated by HSA *in vivo*, then, from the data for the initial rates *in vitro* (e.g.

Fig. 1), this proportion of glucose metabolized to pentose phosphate would have to be increased at least two- to three-fold.

In other tissues (e.g. liver and spleen), much higher values have been given for glucose metabolized by this pathway (see Siperstein, 1959; Dickens, 1958). In these tissues the concentration of GSH is also higher than in erythrocytes (see Jocelyn, 1959) and, for rat and rabbit liver, its rate of oxidation in saline (and hence possible stimulation by HSA) is over 10 times as fast (Hopkins & Elliott, 1932). Accordingly, there would seem to be the prospect that human-serum albumin and a serum factor, acting antagonistically on the rate of oxidation of glutathione, might form part of a general mechanism for regulating extracellularly the tissue utilization of glucose along the pentose phosphate pathway.

SUMMARY

1. When intact washed human erythrocytes are oxygenated, e.g. in saline (0.9 % sodium chloride solution), glutathione is converted into its oxidized form. Some of this is then slowly lost by an unknown route.

2. The rate of oxidation of glutathione in oxygenated erythrocytes is greater when the oxygenation is carried out in human-serum-albumin or bovine- γ -globulin solutions than when it is carried out in bovine-serum-albumin or saline solutions.

3. In human-serum-albumin solutions, the maximum stimulant effect is obtained at a protein concentration of 0.6–0.8 g./100 ml. and this is unaffected by previous oxidation of the albumin thiol group.

4. The rate of oxidation of glutathione is unrelated to the oxyhaemoglobin level of the cells during the oxygenation.

5. The rate of oxidation of glutathione in erythrocytes oxygenated either in saline or in human-serum-albumin solution is increased as a result of treating the cells with carbon monoxide.

6. The rate of oxidation of glutathione in erythrocytes oxygenated in dialysed serum, prepared from human plasma after dialysis for 3–24 hr., is similar to the rate for the same cells oxygenated in saline.

7. If this dialysed serum is preheated at 70° or prepared after dialysis of plasma for e.g. 44 hr., the rate is then similar to that found for the same cells oxygenated in human-serum-albumin solution. The time of heating required at 70°, to change the rate of oxidation in the same cells from that in saline to that in human-serum-albumin solutions, increases as the time of dialysis decreased.

8. The rate of oxidation of glutathione in oxygenated erythrocytes is reduced by lowering the temperature by 14°, more so when the oxygenation

is carried out in human-serum-albumin solution than when it is carried out in saline or dialysed serum.

9. The effect of progressively diluting dialysed serum with human-serum-albumin solution on the rate of oxidation of glutathione in erythrocytes oxygenated in the dialysed serum-albumin mixtures has been studied.

10. The evidence given suggests that human serum contains a heat-labile protein and a slowly diffusible substance which together inhibit the stimulant effect of human-serum albumin on the oxidation of glutathione in erythrocytes.

11. The mechanism of this inhibition and its possible role in metabolism are discussed.

I am grateful to my chief, Dr C. P. Stewart, and to my colleagues, especially Dr R. Kapeller-Adler, for helpful discussion and criticism of the typescript of this paper.

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The Sulphatase of Ox Liver

7. THE INTRACELLULAR DISTRIBUTION OF SULPHATASES A AND B*

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(Received 29 February 1960)

Previous papers in this series (Roy, 1953a, b, 1954a, 1956) have shown that ox liver contains three arylsulphatases, sulphatases A, B and C, which differ both in their general properties and in their intracellular localizations. Sulphatase C apparently occurs only in the microsomes (Roy, 1956, 1958), whereas sulphatases A and B appear in several cell fractions. It was originally suggested that they occurred predominantly in the mitochondria and microsomes of rat liver (Roy, 1954b), but Viala & Gianetto (1955) showed that their distribution was consistent with their arising from the lysosomes, a conclusion that was supported by subsequent work in other species (Roy, 1958). However, these investigations all suffered from the disadvantage that sulphatases A and B were determined as one unit, the two individual enzymes not being separately determined. It is obvious that such studies could give misleading results and that important differences in the localizations of the two enzymes might not be detected. Unfortunately, the separate determination of sulphatases A and B in a mixture is a matter of some difficulty, especially when the situation is further complicated by the simultaneous presence of sulphatase C.

* Part 6: Roy (1957).

This paper describes the results of an investigation into the intracellular distribution of sulphatases A and B in ox liver. This species was chosen for study as in it the differences between these two enzymes are greater than those between the corresponding enzymes of the rat (Dodgson, Spencer & Thomas, 1955) and, further, they can readily be separated by their different solubilities in water, sulphatase A being insoluble (Roy, 1953b), whereas sulphatase B is soluble (Roy, 1954a).

A preliminary account of this work was presented at the Canberra meeting of the Australian Biochemical Society in January 1960.

EXPERIMENTAL

Cell-fractionation and general techniques. The samples of ox liver were obtained at the slaughter house within a few minutes of the death of the animal and were immediately placed in 0.25 M-sucrose at 0° for transport to the laboratory. Further treatment was commenced within 30 min. of death.

The mitochondria and microsomes were isolated from a 10% (w/v) dispersion of ox liver in 0.25 M-sucrose by the standard technique of Hogeboom (1955), as described by Roy (1958) except that the mitochondria were washed only once. The particles were extracted by suspending them in the appropriate solvent at 0° (1 ml./g. of liver) and then