4. Sodium 2-naphthylamine *N*-glucosiduronate was isolated from urine of rabbits dosed with 2-naphthylamine and detected in urine of rats dosed with the amine.

5. The 2-naphthylamine *N*-glucosiduronate was shown by infrared absorption to have a structure similar to 2-naphthylamine *N*-glucoside.

6. Urine of rats and rabbits dosed with 2-naphthylamine contains an acid-labile derivative of 2-amino-1-naphthylsulphuric acid. This compound is not the sulphuric ester of 1-hydroxy-2-naphthylsulphamic acid, which was synthesized, but is 2-amino-1-naphthylsulphuric acid *N*-glucosiduronic acid.

We thank Miss E. Engels for assistance with part of this work and Miss M. Biggs for injecting the animals. The analyses were carried out in the micro-analytical laboratory, Organic Chemistry Department, Imperial College of Science and Technology, by Mr F. H. Oliver.

This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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Glutathione Metabolism

2. THE OXIDATION AND REDUCTION OF GLUTATHIONE IN INTACT ERYTHROCYTES*

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(*Received* 2 *August* 1956)

The sulphydryl–disulphide oxidation–reduction system is involved in a large number of biological processes. The metabolic activity of many of these processes is dependent, to a large extent, on the level of oxidation of the sulphur constituent. The glutathione system (GSH $\rightleftharpoons$ GSSG), as the chief soluble sulphydryl–disulphide component of the cell, is thought to play an important role in the regulation of the oxidation–reduction equilibrium of the cell by virtue chiefly of its effect on other sulphydryl–disulphide systems (see Barron, 1951). Thus reduced glutathione has been found to activate or protect sulphydryl enzymes or coenzymes under a variety of conditions, whereas a number of reactions dependent on an intact disulphide group are inhibited by this substance. Conversely, oxidized glutathione is inhibitory to many sulphydryl systems. The relative proportion of reduced to oxidized glutathione within the cell, as well as the factors which control this steady state, may therefore have an important influence on the regulation of intracellular metabolism.

The oxidation and reduction of glutathione by cell-free tissue preparations has been extensively investigated (see Vennesland & Conn, 1954). Less is known, however, of the factors which control the SS–SH equilibrium within the intact cell. As an approach to this problem, an investigation of the oxidation and reduction of glutathione in the intact erythrocyte was undertaken.

The exposure of intact erythrocytes to oxygen leads to a fall in the reduced glutathione level (Meldrum, 1932). In addition, the glyoxalase activity of the cells is decreased (Jowett & Quastel, 1933). Since reduced glutathione is a specific co-factor in the glyoxalase system (Lohmann, 1932), it was suggested (Jowett & Quastel, 1933) that the

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inhibition of glyoxalase activity by oxygen is a result of the oxidation of glutathione. The inhibition of glyoxalase activity by oxygen is prevented by the addition of glucose (Jowett & Quastel, 1933). The fall in reduced glutathione level is similarly prevented (Meldrum, 1932), which suggests that, in the intact erythrocyte, the glyoxalase activity is limited by the reduced glutathione level and therefore may be used for an investigation of the factors affecting the oxidation and reduction of glutathione in the intact cell. A study of the properties of the glyoxalase system of intact and lysed erythrocytes has been previously reported (Klebanoff, 1956).

METHODS

Collection and preparation of erythrocytes. Human blood was collected from the antecubital fossa with a syringe containing one drop of heparin (Connaught, 1000 units/ml.), care being taken to avoid haemolysis. The blood was immediately centrifuged at 3000 *g* for 10 min. and the plasma and buffy coat were removed. The erythrocytes were washed three times with 0·16 M-NaCl, and diluted with saline to double the packed cell volume. The cells were freshly prepared once a week and stored at 5°.

Estimation of glyoxalase activity. The glyoxalase activity of intact erythrocytes was determined manometrically as previously described (Klebanoff, 1956). The flasks were gassed with either CO_2+N_2 (5:95) or CO_2+O_2 (5:95). The intact erythrocyte proved to be ideal for the measurement of glyoxalase activity in the presence of O_2, since under the conditions employed (0·05 ml. of the erythrocyte preparation in a total volume of 2·0 ml.), the O_2 uptake and the CO_2 release in the absence of methylglyoxal were negligible. The results reported are representative of a number of experiments.

Estimation of haemolysis. Since, in the absence of added glutathione, lysis of the erythrocytes results in an almost complete loss of glyoxalase activity (Jowett & Quastel, 1933), the degree of haemolysis was determined after the completion of each glyoxalase estimation. The contents of the Warburg vessels were centrifuged at 3000 *g* for 10 min. A volume (1·2 ml.) of the supernatant solution was added to 4·8 ml. of 0·1 M-Na_2CO_3, the whole mixed and the intensity of colour determined in an EEL portable colorimeter (Evans Electroselenium Ltd., Harlow, Essex) with a Chance OGR 1 green filter. The percentage of haemolysis was estimated by comparison with a standard curve.

Chemical estimation of reduced glutathione. A volume (1·0 ml.) of the erythrocyte preparation, the reactants under investigation and 0·16 M-NaCl to a final volume of 1·5 ml. were placed in duplicate Thunberg tubes. The tubes were evacuated and filled with gas as indicated. The reagents were mixed in a mechanical shaker at room temperature for a specified period and the glutathione level was estimated by the cyanide–nitroprusside method of Grunert & Phillips (1951).

RESULTS

Effect of plasma

The inhibitory effect of O_2 on the glyoxalase system of intact erythrocytes became progressively more pronounced on storage of the cells at 5° for periods up to 2 weeks. Further, cells well washed with saline were more susceptible to the inhibitory effect of O_2 than were poorly washed or unwashed cells. The glyoxalase activity of aerated cells supplemented with small amounts of plasma was greater than that of cells incubated with O_2 in the absence of plasma (Fig. 1). The protective effect of plasma could be more readily demonstrated by the use of cells stored at 4° for several days, as a result of the more pronounced inhibition of glyoxalase activity by O_2 after storage. The effect of plasma was maximal at concentrations as low as 1·25 % of the total flask contents, a further increase in plasma concentration to 15 % producing no further protection. At concentrations higher than 15 %, however, the protective effect of plasma appeared to be less complete. The glyoxalase activity as indicated by the evolution of CO_2 fell, the effect being more pronounced as the plasma concentration was increased. The inhibition of glyoxalase activity was not

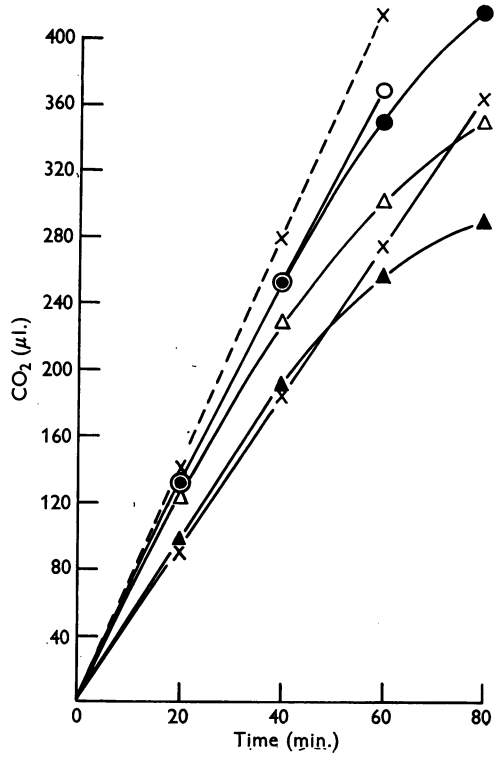

Fig. 1. Effect of plasma on the glyoxalase activity of aerated erythrocytes. The main vessel contained 0·05 ml. of the erythrocyte preparation, 0·4 ml. of 0·2 M-$NaHCO_3$ and plasma as follows: none (×); 0·025–0·3 ml. (○); 0·4 ml. (●); 0·6 ml. (△); 1·2 ml. (▲); and 0·16 M-NaCl in a final volume of 2·0 ml. The side arm contained 0·2 ml. of 1 % methylglyoxal. The gas phase was either CO_2+N_2 (5:95), – – –, or CO_2+O_2 (5:95),——.

associated with a corresponding increase in haemolysis.

The exposure of erythrocytes to O_2 resulted also in a fall in reduced glutathione level which was prevented by the addition of plasma (Table 1).

Effect of glucose

The protective effect of plasma was unaltered by previous exposure to 60° for periods up to 60 min., indicating that the factor or factors responsible were heat-stable. A protein-free ultrafiltrate of fresh human plasma also protected the glyoxalase system from the inhibitory effect of O_2, although not to the same extent as did fresh whole plasma. Thus plasma components of low molecular weight were implicated. Glucose increased the glyoxalase activity of aerated erythrocytes in concentrations as low as 10^{-5} M, the effect reaching a maximum at a concentration of approximately 10^{-3} M (Table 2, Expt. *A*). The effect of glucose was less evident at concentrations above 10^{-2} M, whereas a distinct inhibition of glyoxalase activity resulted from the presence of glucose at a concentration of 16×10^{-2} M. However, glucose in concentrations greater than 10^{-2} M also produced haemolysis of the erythrocytes. The decline in glyoxalase activity and the haemolysis were completely prevented by an increase in the concentration of the NaCl from isotonic to hypertonic levels (Table 2, Expt. *B*). Furthermore, the protective effect of glucose on the glyoxalase activity of the aerated cells was again evident.

Mannose, fructose and, to a lesser extent, galactose and maltose were also found to protect the glyoxalase system of intact erythrocytes from the inhibitory effect of O_2 although they were less effective than glucose in this regard. Lactose, sucrose and D- and L-arabinose had no effect. The stimulatory effect of the various sugars on the glyoxalase activity of aerated cells was not additive with that of plasma, nor did a combination of the sugars give a greater activity than glucose alone. The stimulation produced by plasma was greater than that produced by an equivalent concentration of glucose. The addition of glucose, mannose, fructose, galactose and maltose to aerated erythrocytes also resulted in an increase in the reduced glutathione level (Meldrum, 1932; see also Table 1).

Effect of adenosine and inosine

The metabolism of adenosine by the intact erythrocyte is associated with an increase in the phosphate exchange (Prankerd & Altman, 1954) and in the glucose uptake (Gabrio, Hennessey, Thomasson & Finch, 1955), and leads to the expulsion of sodium by the cell (Harris & Prankerd, 1955) as well as to the maintenance of the intracellular potassium level (Gabrio, Donohue & Finch, 1955). Adenosine, in concentrations as low as 10^{-5} M, also produced a distinct increase in the glyoxalase activity of aerated cells (Table 3). Inosine produced a stimulatory effect similar in magnitude to that of adenosine. The effect of adenosine at maximal concentrations was not additive with that of glucose at maximal concentrations, although a summation of

Table 1. *Effect of various substances on the reduced glutathione (GSH) level of erythrocytes*

Procedure as described in Methods, with supplements and gas phase as indicated. Incubation period, 6 hr. Figures for GSH are mg./100 ml. of cell suspension in saline (1:1, v/v).

Supplement (final concn.)	Gas phase	GSH (mg. %)
None	$CO_2 + N_2$ (5:95)	30·0
None	N_2	29·0
None	$CO_2 + O_2$ (5:95)	19·0
None	O_2	9·0
Plasma (33%)	O_2	36·0
Glucose (5×10^{-3} M)	O_2	35·0
Adenosine ($1{\cdot}7 \times 10^{-3}$ M)	O_2	34·0
Inosine ($1{\cdot}7 \times 10^{-3}$ M)	O_2	34·0
Bovine albumin (25 mg.–2 g./100 ml.)	O_2	9·0–12·0

Table 2. *Effect of glucose on the glyoxalase activity of aerated erythrocytes*

The main vessel contained 0·05 ml. of the erythrocyte preparation, 0·4 ml. of 0·2 M-$NaHCO_3$, and NaCl and glucose at the final concentrations indicated. The side arm contained 0·2 ml. of 1% methylglyoxal. The final volume was 2·0 ml. and the gas phase $CO_2 + O_2$ (5:95). Erythrocyte preparation was 2 days old.

Expt.	Glucose (M)	Sodium chloride (M)	Tonicity	Glyoxalase activity: µl. of CO_2/60 min.	Glyoxalase activity: Difference (%)	Haemolysis (%)
A	—	$10{\cdot}8 \times 10^{-2}$	Isotonic	290	—	6
	$3{\cdot}3 \times 10^{-4}$	$10{\cdot}8 \times 10^{-2}$	Isotonic	350	+21	6
	$3{\cdot}3 \times 10^{-3}$	$10{\cdot}8 \times 10^{-2}$	Isotonic	348	+20	6
	$1{\cdot}6 \times 10^{-2}$	$10{\cdot}0 \times 10^{-2}$	Isotonic	331	+14	15
	$8{\cdot}2 \times 10^{-2}$	$6{\cdot}8 \times 10^{-2}$	Isotonic	300	+ 3	35
	$16{\cdot}5 \times 10^{-2}$	$2{\cdot}8 \times 10^{-2}$	Isotonic	232	−20	80
B	$16{\cdot}5 \times 10^{-2}$	$3{\cdot}7 \times 10^{-2}$	Hypertonic	306	+ 6	42
	$16{\cdot}5 \times 10^{-2}$	$6{\cdot}2 \times 10^{-2}$	Hypertonic	350	+21	14
	$16{\cdot}5 \times 10^{-2}$	$11{\cdot}2 \times 10^{-2}$	Hypertonic	384	+32	5

effects was evident when submaximal concentrations of each were used. D-Ribose and adenine were without effect on the glyoxalase system. The fall in reduced glutathione level which results from the aeration of erythrocytes was also prevented by the addition of adenosine or inosine (Table 1).

Effect of albumin

A number of tissue preparations are known to exist in a better functional state *in vitro* in the presence of serum proteins. Thus the surviving rat diaphragm has a higher rate of glucose uptake when incubated in a solution containing 3 % of bovine albumin than in the absence of albumin (Bornstein & Park, 1953). As can be seen in Table 4, the addition of bovine albumin (Armour), in concentrations as low as 25 mg. %, produced an increase in the glyoxalase activity of the aerated cells. An increase in reduced glutathione level was not observed under comparable conditions (Table 1). An increase in glyoxalase activity was produced by serum albumin in the presence of optimum concentrations of glucose or adenosine, whereas no further effect was observed in the presence of optimum concentrations of plasma. As with large concentrations of plasma (Fig. 1), albumin in concentrations greater than 1 g. % produced an inhibition of glyoxalase activity (Fig. 2).

Table 3. *Effect of adenosine and inosine on the glyoxalase activity of aerated erythrocytes*

Conditions as in Table 2 except for the addition of supplements as indicated. Erythrocyte preparation was 3 days old. Gas phase $CO_2 + O_2$ (5:95).

	Glyoxalase activity	
Supplement	μl. of CO_2/60 min.	Difference (%)
None	205	—
Adenosine (10^{-5} M)	245	+19
Adenosine (10^{-4} M)	270	+31
Adenosine (10^{-3} M)	266	+30
Inosine (10^{-5} M)	240	+17
Inosine (10^{-4} M)	265	+29
Inosine (10^{-3} M)	268	+31

Table 4. *Effect of bovine albumin on the glyoxalase activity of aerated erythrocytes*

Conditions as in Table 2 except for the addition of supplements as indicated. Erythrocyte preparation was 2 days old. Gas phase $CO_2 + O_2$ (5:95).

	Glyoxalase activity	
Supplement	μl. of CO_2/60 min.	Difference (%)
None	305	—
Albumin (25 mg. %)	338	+11
Albumin (100 mg. %)	346	+13
Albumin (250 mg. %)	375	+23
Albumin (1 g. %)	376	+24

The decrease in glyoxalase activity in the presence of large concentrations of plasma (Fig. 1) or bovine albumin (Fig. 2) was observed under both aerobic and anaerobic conditions and was not associated with a decrease in the reduced glutathione level of the cells. The further addition of methylglyoxal to the 'inhibited' preparation led to a return of activity (Fig. 2), suggesting that the fall in CO_2 output is not due to an inhibition of the enzyme system but to a decrease in the substrate concentration. This is supported by further evidence. The pre-incubation of the erythrocyte preparation with large concentrations of plasma for periods varying from 10 to 60 min. did not alter the response of the cells to the subsequent addition of methylglyoxal. The fall in CO_2 output always occurred after a lag period of similar length. This is contrary to the expected response if an inhibitory

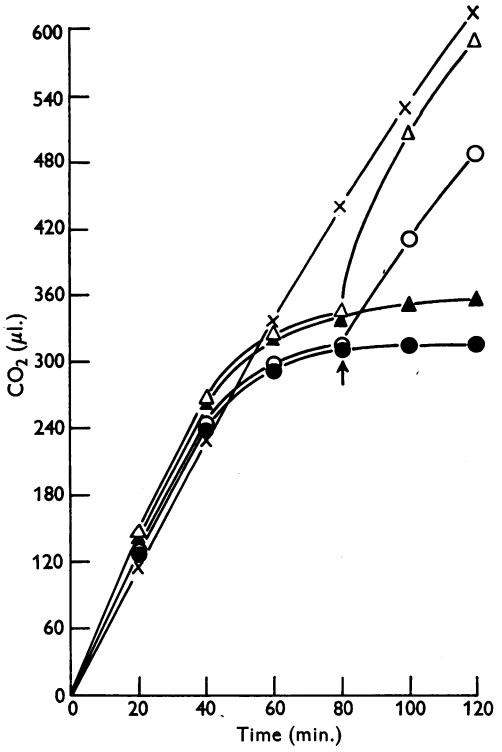

Fig. 2. Effect of methylglyoxal on the 'inhibition' of the glyoxalase activity by plasma and bovine albumin. The main vessel of double-side-arm Warburg flasks contained 0·05 ml. of the erythrocyte preparation, 0·4 ml. of 0·2 M-$NaHCO_3$, 1·2 ml. of plasma (△, ▲), 0·8 ml. of bovine albumin (0·1 g./ml.) (○, ●) and 0·16 M-NaCl in a final volume of 2·0 ml. One side arm contained 0·2 ml. of 1 % methylglyoxal, which was added at zero time. The other side arm contained 0·2 ml. of 0·16 M-NaCl (×, ●, ▲) or 0·2 ml. of 1 % methylglyoxal (○, △) which was added at the arrow. The gas phase was $CO_2 + N_2$ (5:95).

principle had been in contact with the erythrocytes during the pre-incubation period. Furthermore, the the level of CO_2 output at which the decline in glyoxalase activity occurred was found to vary directly with the methylglyoxal concentration. The formation of a complex between methylglyoxal and various chemical groups, e.g., sulphydryl groups (Schubert, 1935) of the plasma proteins, may account for the decrease in glyoxalase activity.

Effect of glucosamine

Harpur & Quastel (1949) demonstrated the phosphorylation of glucosamine by ATP in acetone-dried brain extracts. Glucose and fructose appeared to compete with glucosamine for phosphorylation by this tissue, with glucose having the greatest affinity. Glucosamine 6-phosphate formed under the influence of hexokinase was found to inhibit the glucokinase and glycolysis of *Schistosoma mansoni* (Bueding & MacKinnon, 1955) and to inhibit competitively the glucose 6-phosphate dehydrogenase system of yeast (Glaser & Brown, 1955). The intracellular transfer of glucosamine in the eviscerated and nephrectomized rabbit is under the influence of insulin and is associated with a substantial decrease in the transfer rate of glucose (Wick, Drury, Nakada, Barnet & Morita, 1955). Glucosamine similarly inhibits the uptake of glucose by the isolated rat diaphragm (Nakada, Morita & Wick, 1955).

In view of the inhibitory effect of glucosamine on glucose metabolism, this substance was tested on the glyoxalase system of intact erythrocytes. Glucosamine hydrochloride (Eastman), neutralized with NaOH, at concentrations of $0{\cdot}4$–$1{\cdot}6 \times 10^{-2}$M produced an inhibition of glyoxalase activity although haemolysis was negligible (Table 5). An increase in the concentration of glucosamine (4–8×10^{-2}M) produced considerable haemolysis, which was almost completely prevented by an increase in the NaCl concentration from isotonic to sufficiently hypertonic levels. The addition of NaCl, however, had little or no effect on the inhibition of glyoxalase activity. The further addition of methylglyoxal or sodium bicarbonate to the inhibited preparation did not alter the glyoxalase activity.

The inhibition of the glyoxalase activity by glucosamine was partially reversed by the addition of plasma, glucose, adenosine or inosine (Table 6). Glucose, at concentrations greater than $1{\cdot}6 \times 10^{-2}$M, caused a further decrease in glyoxalase activity associated with an increase in haemolysis. The

Table 5. *Effect of glucosamine on the glyoxalase activity of aerated erythrocytes*

Conditions as in Table 2, except for the addition of glucosamine and NaCl as indicated. Blood was 1 day old. Gas phase $CO_2 + O_2$ (5:95).

Expt.	Glucosamine (M)	Sodium chloride (M)	Tonicity	Glyoxalase activity: µl. of CO_2/60 min.	Glyoxalase activity: Difference (%)	Haemolysis (%)
A	—	$10{\cdot}8 \times 10^{-2}$	Isotonic	346	—	3
	$0{\cdot}4 \times 10^{-2}$	$10{\cdot}8 \times 10^{-2}$	Isotonic	325	− 6	4
	$0{\cdot}8 \times 10^{-2}$	$10{\cdot}8 \times 10^{-2}$	Isotonic	289	−16	6
	$1{\cdot}6 \times 10^{-2}$	$9{\cdot}2 \times 10^{-2}$	Isotonic	236	−32	7
	$4{\cdot}0 \times 10^{-2}$	$7{\cdot}6 \times 10^{-2}$	Isotonic	139	−60	55
	$8{\cdot}0 \times 10^{-2}$	$2{\cdot}8 \times 10^{-2}$	Isotonic	81	−77	73
B	$8{\cdot}0 \times 10^{-2}$	$5{\cdot}3 \times 10^{-2}$	Hypertonic	80	−78	60
	$8{\cdot}0 \times 10^{-2}$	$7{\cdot}8 \times 10^{-2}$	Hypertonic	86	−75	25
	$8{\cdot}0 \times 10^{-2}$	$12{\cdot}8 \times 10^{-2}$	Hypertonic	85	−75	6

Table 6. *Effect of glucose, adenosine and inosine on the inhibition of glyoxalase activity by glucosamine*

Conditions as in Table 2, except for the addition of glucosamine and supplements as indicated. Glucosamine was added to a final concn. of $1{\cdot}6 \times 10^{-2}$M in all cases except the first, the control, in which no glucosamine was present. Erythrocyte preparation was 2 days old. Gas phase $CO_2 + O_2$ (5:95).

Sodium chloride (M)	Glucosamine	Supplement	Tonicity	Glyoxalase activity: µl. of CO_2/60 min.	Glyoxalase activity: Difference (%)	Haemolysis (%)
$10{\cdot}8 \times 10^{-2}$	—	—	Isotonic	276	—	8
$9{\cdot}2 \times 10^{-2}$	+	—	Isotonic	177	−36	19
$7{\cdot}6 \times 10^{-2}$	+	Plasma (10%)	Isotonic	213	−23	20
$8{\cdot}4 \times 10^{-2}$	+	Glucose ($0{\cdot}16 \times 10^{-2}$M)	Isotonic	210	−24	22
$5{\cdot}2 \times 10^{-2}$	+	Glucose (8×10^{-2}M)	Isotonic	154	−44	88
$1{\cdot}2 \times 10^{-2}$	+	Glucose (16×10^{-2}M)	Isotonic	104	−62	95
$11{\cdot}2 \times 10^{-2}$	+	Glucose (16×10^{-2}M)	Hypertonic	225	−18	15
$20{\cdot}4 \times 10^{-2}$	+	—	Hypertonic	180	−35	6
$9{\cdot}2 \times 10^{-2}$	+	Adenosine (10^{-2}M)	Isotonic	203	−26	36
$9{\cdot}2 \times 10^{-2}$	+	Inosine (10^{-2}M)	Isotonic	207	−25	34

Table 7. *Effect of glucosamine on the reduced glutathione (GSH) level of erythrocytes*

Conditions as in Table 1, except that the tonicity of the control and of the experimental tubes was maintained at the same level by the use of hypertonic NaCl solutions. Figures for GSH are mg./100 ml. of cell suspension in saline (1:1, v/v).

Supplement	Gas phase	GSH (mg. %)
None	N_2	29·3
Glucosamine (0·06 M)	N_2	28·2
Glucosamine (0·15 M)	N_2	23·7
Glucosamine (0·3 M)	N_2	15·0
None	O_2	16·5
Glucosamine (0·15 M)	O_2	11·7
Glucose (5×10^{-3} M)	O_2	32·4
Glucose (5×10^{-3} M) + glucosamine (0·15 M)	O_2	19·5
Adenosine ($1·7 \times 10^{-3}$ M)	O_2	32·7
Adenosine ($1·7 \times 10^{-3}$ M) + glucosamine (0·15 M)	O_2	24·0
Inosine ($1·7 \times 10^{-3}$ M)	O_2	36·0
Inosine ($1·7 \times 10^{-3}$ M) + glucosamine (0·15 M)	O_2	25·5

haemolytic effect of glucose was prevented by the addition of NaCl. However, even under these conditions, glucose at a concentration 10 times that of glucosamine did not completely prevent the inhibition of glyoxalase activity. A decrease in the reduced glutathione level of the erythrocyte was also observed after incubation with glucosamine (Table 7).

Effect of sodium arsenate

Prankerd & Altman (1954) observed that sodium arsenate had an inhibitory effect on the ^{32}P exchange of erythrocytes in the presence of adenosine or of additional glucose. In the present study, sodium arsenate was found also to inhibit the glyoxalase activity of aerated erythrocytes, both in the absence of an added substrate and in the presence of added glucose or adenosine (Table 8). Haemolysis was negligible at all sodium arsenate concentrations employed. Sodium arsenate also produced a fall in the reduced glutathione level under comparable conditions (Table 9).

Table 8. *Effect of sodium arsenate on the glyoxalase activity of aerated erythrocytes*

Conditions as in Table 2, except for the addition of supplements as indicated. Erythrocyte preparation was 3 days old. Gas phase $CO_2 + O_2$ (5:95).

Supplement	Glyoxalase activity: µl. of CO_2/60 min.	Glyoxalase activity: Difference (%)	Haemolysis (%)
None	279	—	5
Sodium arsenate ($0·16 \times 10^{-2}$ M)	277	—	3
Sodium arsenate ($0·4 \times 10^{-2}$ M)	264	− 5	4
Sodium arsenate ($1·6 \times 10^{-2}$ M)	231	− 17	6
Sodium arsenate (4×10^{-2} M)	188	− 33	3
Sodium arsenate ($10·8 \times 10^{-2}$ M)	123	− 56	5
None	254	—	7
Sodium arsenate (8×10^{-2} M)	146	− 43	4
Plasma (10%)	324	—	5
Plasma (10%) + sodium arsenate (8×10^{-2} M)	223	− 31	5
Glucose (10^{-4} M)	297	—	5
Glucose (10^{-4} M) + sodium arsenate (8×10^{-2} M)	197	− 34	5
Adenosine (10^{-4} M)	297	—	5
Adenosine (10^{-4} M) + sodium arsenate (8×10^{-2} M)	186	− 37	4

Table 9. *Effect of sodium arsenate on the reduced glutathione (GSH) level of erythrocytes*

Conditions as in Table 1. Figures for GSH are mg./100 ml. of cell suspension in saline (1:1, v/v).

Supplement	Gas phase	GSH (mg. %)
None	N_2	28·5
Sodium arsenate ($2·7 \times 10^{-2}$ M)	N_2	10·5
None	O_2	15·0
Sodium arsenate ($2·7 \times 10^{-2}$ M)	O_2	3·0
Plasma (17%)	O_2	28·5
Plasma (17%) + sodium arsenate ($2·7 \times 10^{-2}$ M)	O_2	6·0
Glucose (5×10^{-3} M)	O_2	24·0
Glucose (5×10^{-3} M) + sodium arsenate ($2·7 \times 10^{-2}$ M)	O_2	12·0
Adenosine ($1·7 \times 10^{-3}$ M)	O_2	25·5
Adenosine ($1·7 \times 10^{-3}$ M) + sodium arsenate ($2·7 \times 10^{-2}$ M)	O_2	4·5
Inosine ($1·7 \times 10^{-3}$ M)	O_2	27·0
Inosine ($1·7 \times 10^{-3}$ M) + sodium arsenate ($2·7 \times 10^{-2}$ M)	O_2	3·0

DISCUSSION

It has been amply demonstrated that mechanisms exist in mammalian tissues which are capable of both the oxidation and reduction of glutathione (see Vennesland & Conn, 1954). The processes which lead to a reduction of oxidized glutathione are predominant *in vivo*, as indicated by the presence of glutathione largely in its reduced form (see Bhattacharya, Robson & Stewart, 1955). In the erythrocyte, the maintenance of glutathione in its reduced form appears to be dependent largely on the normal metabolic activity of the cell. Plasma, which is highly effective in maintaining the reduced glutathione level (Table 1) and the glyoxalase activity (Fig. 1) of aerated erythrocytes, appears to act largely by supplying utilizable metabolites to the cell. In addition the serum proteins may exert a stabilizing influence on the glyoxalase system.

The chief metabolite of the erythrocyte is glucose, and this substance, as first indicated by Meldrum (1932) and Jowett & Quastel (1933), is extremely active in maintaining the glyoxalase activity (Table 2) and the reduced glutathione level (Table 1) of aerated cells. An effect on the glyoxalase system is evident on the addition of glucose in concentrations well within the physiological range, suggesting that this mechanism for the maintenance of glutathione in its reduced form may be operative also *in vivo*. The glutathione reductase system, which catalyses the reduction of oxidized glutathione by reduced TPN and, to a lesser extent, by reduced DPN (Francoeur & Denstedt, 1954; Racker, 1955), is present in the erythrocyte (Meldrum & Tarr, 1934; Rall & Lehninger, 1952; Francoeur & Denstedt, 1954; Collier & McRae, 1955). The oxidation of glucose and glucose metabolites may therefore be linked with the reduction of glutathione by this system. In addition, the metabolism of glucose is required for the synthesis of glutathione (Krahl, 1953). Although glutathione can be synthesized by mature erythrocytes (Dimant, Landsberg & London, 1955; Elder & Mortensen, 1956), in the present experiments the absence of glutathione precursors and the short-term nature of most of the experiments would suggest that synthesis is not an important factor.

In addition to glucose and other sugars, adenosine and its deaminated derivative inosine are also effective in the maintenance of the glyoxalase activity (Table 3) and the reduced glutathione level (Table 1) of aerated erythrocytes. The metabolism of adenosine and inosine by the erythrocyte appears to be initiated by their phosphorylysis to form ribose phosphates (Dische, 1938, 1951). A purified nucleotide phosphorylase prepared from erythrocytes has been found to be active only with inosine or guanosine as substrate, which suggests that adenosine must first be converted into inosine (Gabrio & Huennekens, 1955). The experiments of Prankerd & Altman (1954) suggest that the ribose phosphate formed is converted into glyceraldehyde 3-phosphate, from which the depleted stores of 2:3-diphosphoglyceric acid are replenished. Rubinstein, Kashket & Denstedt (1956) have observed the accumulation of this last substance, as well as of lactic acid, on the storage of human cells with adenosine or inosine. The metabolism of adenosine leads also to the formation of fructose 1:6-diphosphate and hexose monophosphate (Dische, 1938, 1951). The subsequent oxidation of glucose 6-phosphate via the triphosphopyridine nucleotide-(TPN)-dependent glucose 6-phosphate dehydrogenase system, coupled with the reduction of oxidized glutathione (Francoeur & Denstedt, 1954; Collier & McRae, 1955), may be responsible for the observed effect on the reduced glutathione level (Table 1) and on the glyoxalase activity (Table 3).

Glucosamine was found to decrease the glyoxalase activity (Table 5) and the reduced glutathione level (Table 7) of aerated erythrocytes. Since the metabolism of glucose appears to be intimately involved in the maintenance of glutathione in its reduced form, it would seem logical to assume that this effect of glucosamine is associated with its inhibitory effect on glucose metabolism (Wick *et al.* 1955; Nakada *et al.* 1955). The production of an inhibition of glyoxalase activity in the absence of glucose, either added or endogenous, indicates that the effect of glucosamine is not necessarily a result of an inhibition of the initial phosphorylation of glucose. It is of interest in this regard that glucosamine 6-phosphate inhibits the glycolysis system of *Schistosoma mansoni* (Bueding & MacKinnon, 1955) and the glucose 6-phosphate dehydrogenase system of yeast (Glaser & Brown, 1955).

Glucose has been in general use in the preservation of erythrocytes since the observation by DeGowin, Harris & Plass (1939) that the haemolysis of stored blood was considerably delayed by the addition of this substance. Similarly, adenosine and inosine have been found in recent years to increase the survival time of stored erythrocytes (Donohue, Finch & Gabrio, 1956; Rubinstein *et al.* 1956). The mechanism by which these substances stabilize the erythrocyte during storage is not fully known. The maintenance of adenosine triphosphate levels by glucose (see Denstedt, 1953) and by adenosine or inosine (Gabrio, Finch & Huennekens, 1956), suggests that the production of adenosine triphosphate may be required for the maintenance of the intact cell structure. However, another factor may be operative. It has been suggested by a number of investigators (Fegler, 1952; Benesch & Benesch, 1954; Flanagan, Beutler, Dern & Alving, 1955; Sheets, Hamilton & DeGowin, 1956) that substances

containing sulphydryl groups, including reduced glutathione, play an important role in the maintenance of the intact erythrocyte structure. In this regard it is of interest that the increased rate of spontaneous haemolysis on the exposure of erythrocytes to oxygen (Fishman & Hunter, 1951) has been correlated with a corresponding decrease in reduced glutathione level (Fegler, 1952). The glyoxalase activity of erythrocytes stored in citrate at 5° remains normal for about 20 days and then decreases rapidly, reaching zero after 30–40 days of storage (Alivisatos, 1949). The glyoxalase activity is maintained for a much longer period when a citrate–glucose medium is employed. Since the enzyme moiety of the glyoxalase system is relatively stable on storage (Keilin & Wang, 1947), variations in reduced glutathione level are probably involved. The protective effect of glucose, adenosine and inosine on stored erythrocytes may therefore be related, in part at least, to the ability of these substances to maintain the reduced glutathione level of the cells.

SUMMARY

1. The factors affecting the oxidation and reduction of glutathione in mature mammalian erythrocytes have been investigated.

2. The glyoxalase activity of aerated erythrocytes is increased by plasma in low concentrations, by glucose, by adenosine and inosine and by bovine albumin.

3. Glucosamine and sodium arsenate, on the other hand, produce a fall in glyoxalase activity.

4. In all instances except that of albumin a corresponding alteration of reduced glutathione level was produced by the addition of each of these materials.

5. Plasma and serum albumin in high concentrations produce, under the conditions employed, a decrease in glyoxalase activity which is overcome by the further addition of methylglyoxal, suggesting that the fall in activity is due to a decrease in substrate concentration.

6. The importance of glucose, adenosine and inosine in the maintenance of the reduced glutathione level is discussed in relation to the storage of erythrocytes.

I should like to acknowledge with gratitude the help and encouragement of Professor J. A. Dauphinee and to thank the Banting Research Foundation for a grant-in-aid toward the expense of this investigation.

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