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The Transport of Oxidized Glutathione from the Erythrocytes of Various Species in the Presence of Chromate

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1. Erythrocytes from normal and glucose 6-phosphate dehydrogenase-deficient humans were subjected to hydrogen peroxide diffusion to oxidize the GSH. Studies were carried out in the presence and absence of chromate to inhibit glutathione reductase and with or without the addition of glucose. 2. The GSH content of erythrocytes from other species was oxidized by subjecting them to hydrogen peroxide diffusion in the presence of chromate and glucose. 3. Chromate (1·3mM) inhibited glutathione reductase by about 80%, whereas glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, hexokinase, phosphofructokinase and pyruvate kinase were not inhibited. 4. The GSSG formed was transported from the erythrocytes to the medium. 5. The transport rate of GSSG from glucose 6-phosphate dehydrogenase-deficient erythrocytes subjected to hydrogen peroxide diffusion in the presence of chromate was comparable with that from normal and glucose 6-phosphate dehydrogenase-deficient erythrocytes. 6. The rate of transport of GSSG from erythrocytes of various species studied could be ranked: pigeon > rabbit > rat > donkey > man > dog > horse > sheep > chicken > fish.

We have demonstrated the existence of a system in human erythrocytes that actively transports GSSG from inside the erythrocytes to the surrounding media (Srivastava & Beutler, 1967, 1969). The existence of this mechanism can be demonstrated in glucose 6-phosphate dehydrogenase-deficient erythrocytes subjected to hydrogen peroxide diffusion either in the presence or in the absence of glucose. However, since it is not possible to achieve appreciable concentrations of GSSG in normal erythrocytes in the presence of glucose, it was possible to demonstrate GSSG transport in normal erythrocytes only when glucose was excluded from the suspending media. Since GSSG transport is an active process that depends on the presence of energy, transport of GSSG from normal erythrocytes ceased when endogenous high-energy compounds such as ATP were exhausted (Srivastava & Beutler, 1969; Beutler & Srivastava, 1968).

To explore in greater detail the transport of GSSG from erythrocytes in the steady state, under conditions in which endogenous substrate concentrations could be maintained by the presence of glucose in the suspending medium, it was necessary to inhibit the reduction of GSSG to GSH. This was achieved by adding sodium chromate to the system, since chromate is known to inhibit glutathione reductase activity of intact erythrocytes by approx. 80% (Koutras, Hattori, Schneider, Ebaugh & Valentine, 1964). When glutathione reductase was thus inhibited, GSSG concentrations rose in normal erythrocytes exposed to peroxide, even when glucose was present in the suspending medium. In this way GSSG transport could be studied in the presence of substrate.

MATERIALS AND METHODS

Reagents. GSH, GSSG, tris, dipotassium glucose 6-phosphate, 6-phosphogluconate, NAD^+, NADH, $NADP^+$, NADPH, ATP, ADP, fructose 6-phosphate, phosphoenolpyruvate, triose phosphate isomerase (rabbit), α-glycerophosphate dehydrogenase (rabbit) and lactate dehydrogenase (rabbit) were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.; glucose 6-phosphate dehydrogenase (yeast) 6-phosphogluconate dehydrogenase (yeast) and glutathione reductase (yeast) were purchased from Calbiochem, Los Angeles, Calif., U.S.A.

Blood from rats, rabbits, pigeons and chickens was drawn from the heart under mild ether anaesthesia into heparin. Blood from normal subjects, glucose 6-phosphate dehydrogenase-deficient subjects, horses, dogs, donkeys and sheep was drawn from the vein. Blood from fish was drawn by cardiac puncture without anaesthesia. The blood was centrifuged at 1000*g* for 30min. at 4°. The plasma and buffy coat were removed and the erythrocytes were washed twice with phosphate–NaCl (1 part of 0·15M-potassium phosphate buffer, pH 7·4 + 9 parts of 0·145M-NaCl).

Samples (1ml.) of washed erythrocytes were suspended in 3·0vol. of phosphate–NaCl and placed in the main compartments of Warburg flasks. Unless otherwise indicated, 14mM-glucose and 1·3mM-Na_2CrO_4 were incorporated into the phosphate–NaCl. The centre wells each contained 0·2ml. of 30% (v/v) H_2O_2 solution. The flasks were agitated in a Dubnoff shaker at 37° at 100oscillations/min. for 2 and 4hr. or as specified in each experiment. At the end of the experiments the contents of the main compartment of each flask were centrifuged at 1000*g* for 30min. at 4°. The supernatant medium was removed and a 25% haemolysate was made in water. Samples (1ml.) of haemolysate and of supernatant media were taken for GSSG determination with glutathione reductase and NADPH (Srivastava & Beutler, 1968), and 0·5ml. for the determination of GSH by the 5,5′-dithiobis-(2-nitrobenzoic acid) method (Beutler, Duron & Kelly, 1963). Initial GSSG concentrations were also determined in some cases enzymically with 2·5ml. of erythrocytes. ATP was determined by the firefly method (Beutler & Duron, 1965; Beutler & Mathai, 1967).

Enzymic assay. The enzymes in the erythrocytes were assayed by following the change in E_{340} with a light-path of 1cm. and a final cuvette volume of 1·0ml., by using a multisample selector recording Gilford model 2000 spectrophotometer. A 1:20 haemolysate of washed erythrocytes was prepared in a solution containing (final concentrations) 10μM-$NADP^+$, 7mM-β-mercaptoethanol and 2·7mM EDTA, final pH7·0. This haemolysate was used for the assays of all enzymes except glutathione reductase, for which a 1:20 haemolysate in water was employed. 1M-Tris–HCl buffer, pH8·0 at 25° was used in all assays.

Enzyme determinations. The activities of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.43) were determined in a three-cuvette system at 25° by using a modification (World Health Organization Scientific Group, 1967) of the method of Glock & McLean (1953).

Hexokinase (EC 2.7.1.1) activity was assayed with 50μl. of haemolysate (Beutler & Teeple, 1969).

Glutathione reductase (EC 1.6.4.2) was assayed with 10μl. of haemolysate (Beutler, 1969).

Phosphofructokinase (EC 2.7.1.11) activity was assayed in a 1ml. system containing (final concentrations and amounts) 50mM-tris–HCl buffer, pH 7·8, 2mM-ATP, 5mM-$MgCl_2$, 2mM-fructose 6-phosphate, 0·2mM-NADH, 0·01mM-dithioerythritol, 0·75ml. of auxiliary enzyme solution and 10μl. of haemolysate. The auxiliary enzyme solution was prepared by adding 50 units each of fructose diphosphate aldolase, triose phosphate isomerase and α-glycerophosphate dehydrogenase and making up to 1ml. with saturated $(NH_4)_2SO_4$. This enzyme suspension was diluted ten-fold, just before use, with 0·1M-tris–HCl buffer, pH8·0, containing 2·0mg. of bovine serum albumin/ml.

Pyruvate kinase (EC 2.7.1.40) was assayed in a 1ml. system containing (final concentrations and amounts) 0·1M-tris–HCl buffer, pH7·8, 0·1M-KCl, 10mM-$MgCl_2$, 0·1mM-NADH, 0·4mM-ADP, 6 units of lactate dehydrogenase, 1·5mM-phosphoenolpyruvate and 20μl. of haemolysate.

RESULTS

Transport of GSSG from human erythrocytes. Washed erythrocytes from normal subjects and subjects deficient in glucose 6-phosphate dehydrogenase were subjected to hydrogen peroxide diffusion

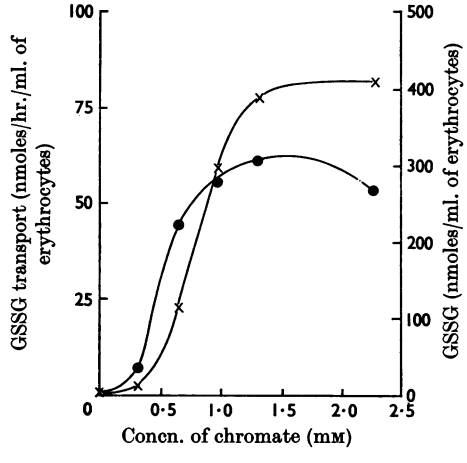

Fig. 1. Optimum concentration of chromate for the transport of GSSG from normal erythrocytes. Twice-washed erythrocytes were subjected to H_2O_2 diffusion in the presence of glucose (14mM) and various amounts of chromate as described in the text. At the end of the incubation period GSSG was determined in the erythrocytes and the incubation medium. ×, GSSG in erythrocytes; ●, GSSG transport into medium.

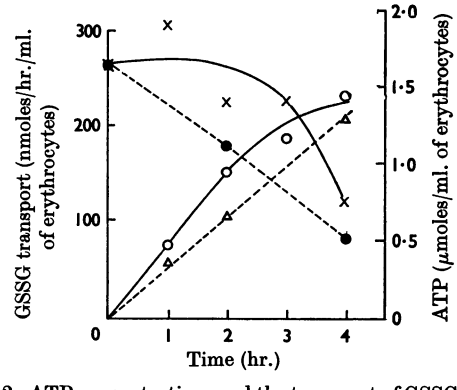

Fig. 2. ATP concentrations and the transport of GSSG from normal erythrocytes subjected to H_2O_2 diffusion in the presence and absence of glucose and chromate. Twice-washed erythrocytes were subjected to H_2O_2 diffusion in the absence of chromate and glucose and in the presence of chromate (1·3mM) and glucose (14mM) as described in the text. The contents of tubes were centrifuged after various time-intervals, and the GSSG concentration of the medium and the ATP concentration of the erythrocytes were determined as described in the text. ----, In the absence of chromate and glucose: △, GSSG in the medium; ●, ATP in the erythrocytes. ——, In the presence of chromate and glucose: ○, GSSG in the medium; ×, ATP in the erythrocytes.

in the presence or absence of chromate, with or without glucose added to the incubation medium. Samples were taken for the assay of hexokinase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphofructokinase, pyruvate kinase and glutathione reductase activities. A final concentration of 1·3mM-chromate in the incubation medium was found to be optimum with respect to the rate of GSSG transport (Fig. 1). Under identical conditions in the absence of chromate it has already been established that the efflux of GSSG from the erythrocytes is due to active transport (Srivastava & Beutler, 1969). In the presence of glucose this concentration of chromate inhibited glutathione reductase activity by about 80%, but the other enzymes assayed were not markedly affected; in the absence of glucose the inhibition of glutathione reductase was only about 35%. Hexokinase was significantly inhibited in the erythrocytes incubated with chromate in the absence of glucose, but in the presence of glucose there was no inhibition. In subsequent experiments 1·3mM-chromate was incorporated in the incubation medium. The transport rate of GSSG from normal erythrocytes subjected to hydrogen peroxide diffusion in the presence of chromate and glucose was linear for 2hr., thereafter showing a modest decrease in rate (Fig. 2). The ATP concentration of normal erythrocytes subjected to hydrogen peroxide diffusion with chromate and glucose fell very slowly for about 3hr. and then significantly. However, in normal erythrocytes subjected to hydrogen peroxide diffusion in the absence of chromate and glucose the rate of fall of the ATP concentration was significant throughout the period of incubation (Fig. 2).

The rates of GSSG transport from normal and glucose 6-phosphate dehydrogenase-deficient erythrocytes in the presence and absence of chromate are compared in Table 1. In the presence of glucose and absence of chromate GSSG transport could be demonstrated only in glucose 6-phosphate dehydrogenase-deficient erythrocytes, since normal erythrocytes had the capacity to maintain their glutathione in the reduced state. In the presence of chromate the transport rate of GSSG from glucose 6-phosphate dehydrogenase-deficient erythrocytes was comparable with that from normal erythrocytes. The recovery of total glutathione, i.e. GSH + GSSG, in the erythrocytes and incubation

Table 1. *Effect of chromate on the transport of GSSG from normal and glucose 6-phosphate dehydrogenase-deficient human erythrocytes*

Washed erythrocytes (1ml.) were added to 3·0ml. of phosphate–NaCl with or without the addition of chromate (1·3mM) or glucose (14mM) in the main compartment of a Warburg flask; 0·2ml. of 30% H_2O_2 was placed in the centre well of the flask. The contents of the flasks were agitated at 37° in a Dubnoff shaker at 100 oscillations/min. for 2 or 4hr. At the end of incubation period the contents of each flask were centrifuged, and GSH and GSSG were determined as described in the text. The results are expressed as means ± S.E.M. of the numbers of determinations indicated.

					Before incubation		After incubation			
		Additions								GSSG
Blood sample	No. of samples analysed	Chromate	Glucose	Incubation time (hr.)	GSH (nmoles/ml. of erythrocytes)	GSSG (nmoles/ml. of erythrocytes)	GSH (nmoles/ml. of erythrocytes)	GSSG (nmoles/ml. of erythrocytes)	GSSG (nmoles/3ml. of medium)	transport (nmoles/hr./ml. of erythrocytes)
Normal	25	−	−	4	2062 ± 71	< 15	771 ± 76	475 ± 19	190 ± 7	47·5 ± 2
	6	−	+	4	2231 ± 126	< 15	2211 ± 78	< 5	< 5	< 5
	12	+	+	2	2086 ± 82	< 15	638 ± 28	369 ± 36	133 ± 6	66·5 ± 3
	6	+	−	2	2166 ± 139	< 15	376 ± 64	555 ± 90	145 ± 10	72 ± 5
Glucose 6-phosphate dehydrogenase-deficient	9	−	−	4	1529 ± 62	< 15	450 ± 82	327 ± 38	205 ± 19	51 ± 4
	13	−	+	4	1418 ± 82	< 15	829 ± 109	105 ± 28	245 ± 13	61 ± 3
	7	+	+	2	1350 ± 124	< 15	150 ± 38	500 ± 48	137 ± 9	68 ± 4
	4	+	−	2	1540 ± 41	< 15	97 ± 36	388 ± 76	143 ± 24	71 ± 12

Table 2. *Transport of GSSG from the erythrocytes of various species*

Washed erythrocytes (1ml.) were incubated with 3·0ml. of phosphate–NaCl containing chromate (1·3mM) and glucose (14mM) in the main compartment of a Warburg flask; 0·2ml. of 30% H_2O_2 was placed in the centre well. The contents of the flasks were agitated at 37° in a Dubnoff shaker for 2hr. At the end of the incubation period the contents of flasks were centrifuged, and GSH and GSSG were determined as described in the text. The results are expressed as means ± S.E.M. of the numbers of determinations indicated.

		Before incubation		After incubation				
Species	No. of samples analysed	GSH (nmoles/ml. of erythrocytes)	GSSG (nmoles/ml. of erythrocytes)	GSH (nmoles/ml. of erythrocytes)	GSSG (nmoles/ml. of erythrocytes)	GSSG (nmoles/3ml. of medium)	Recovery of total glutathione (%)	GSSG transport (nmoles/hr./ml. of erythrocytes)
Chicken	9	2617 ±121	15·2 ±1·2	1354 ±157	774 ±35	57 ±5	114	28·5 ±2·5
Dog	5	1808 ±155	<5	157 ±45	640 ±61	120 ±7	92	60 ±3·5
Donkey	4	2867 ±159	—	258 ±96	901 ±22	136 ±15	81	68 ±7·5
Fish (trout)	5	2739 ±202	<5	1220 ±211	302 ±77	37 ±14	69	18·5 ±7·0
Horse	4	2008 ±181	—	287 ±24	813 ±49	112 ±7	106	56 ±3·7
Human	12	2086 ±82	<15	638 ±28	369 ±36	133 ±6	78	66·5 ±3
Pigeon	7	4208 ±357	49 ±5	1101 ±251	825 ±12	774 ±51	100	387 ±25·5
Rabbit	10	2662 ±126	6 ±0·7	680 ±44	324 ±36	198 ±11	64	90 ±5·5
Rat	20	1990 ±54	6 ±2·6	94 ±12	17 ±5	158 ±7	22	79 ±3·5
Sheep	4	2257 ±65	<5	144 ±36	572 ±32	69 ±2	63	34·5 ±1·0

medium at the end of the incubation period was about 100% in the absence of chromate and about 70–80% in the presence of chromate.

Transport of GSSG from the erythrocytes of various species. The results of studies carried out on the erythrocytes of various species are presented in Table 2. The rate of transport was greatest from pigeon erythrocytes, but this could not be attributed to the fact that these erythrocytes are nucleated, since chicken erythrocytes were found to manifest one of the lowest rates of transport. The species studied could be ranked: pigeon > rabbit > rat > donkey > man > dog > horse > sheep > chicken > fish.

Rat erythrocytes were unique in that little or no GSSG was found in them even after being subjected to hydrogen peroxide diffusion. The recovery of total glutathione from these erythrocytes was less than 25%.

DISCUSSION

The erythrocyte membrane is known to be involved in active ion transport, but until recently was believed to be impermeable to glutathione. The demonstration that an active transport system for glutathione exists (Srivastava & Beutler, 1969; Beutler & Srivastava, 1968) may provide an explanation for the fate of glutathione, which is known to be continuously synthesized in the erythrocyte, having a half-life of 2–3 days (Hochberg, Rigbi & Dimant, 1964; Boivin & Galand, 1965).

The transport of GSSG from erythrocytes requires energy (Srivastava & Beutler, 1969). In our earlier studies of GSSG transport, no glucose was added to the incubation medium when erythrocytes were subjected to hydrogen peroxide diffusion so that GSSG could accumulate. But under these circumstances the availability of an endogenous substrate may have frequently been the limiting factor in the rate of GSSG transport. Quantitative studies of the transport system were therefore not feasible. In the present investigation advantage was taken of the inhibition of glutathione reductase by chromate (Koutras *et al.* 1964).

An 80% inhibition of glutathione reductase was achieved without inhibition of other erythrocyte enzymes, a degree of inhibition sufficient to permit accumulation of GSSG in erythrocytes when these

were treated with hydrogen peroxide. This makes it possible to study GSSG transport in erythrocytes with normal glucose 6-phosphate dehydrogenase activity from man and other species, even in the presence of exogenous substrate.

The transport rate of GSSG from erythrocytes of various species does not correlate with the presence or absence of a nucleus. Thus the transport rate of GSSG from pigeon erythrocytes was the highest among all of the species studied, but nucleated erythrocytes from fish and chickens had the lowest rate of GSSG transport. The pigeon was also found to have the largest amount of GSH and GSSG in erythrocytes, but the exact relationship of transport rate of GSSG from the erythrocytes of various species to their metabolism and overall physiological function is not clearly understood at the present time.

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