Effect of Oxidized Glutathione on some Enzymes of Erythrocytes and its Relation to Erythrocytic Enzyme Activity and Electrophoretic Mobility

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1. Oxidized glutathione reacts or interacts with some erythrocytic enzymes (glucose 6-phosphate dehydrogenase, EC 1.1.1.49, aspartate aminotransferase, EC 2.6.1.10) but not with some others (lactate dehydrogenase, EC 1.1.1.27). 2. GSSG does not diminish the activity of any of these enzymes and is therefore not responsible for the decreased enzyme activities associated with older erythrocytes. 3. It may be that the reaction of aspartate aminotransferase with GSSG is the cause for the more rapid anodic electrophoretic mobility of this enzyme derived from human erythrocytes when compared with the mobility of the same enzyme from other human tissues. 4. A reinterpretation of some related, previously published, data with regard to the electrophoretic mobility of the above-mentioned enzymes from young and old erythrocytes is presented.

Haemoglobin and some enzymes (e.g. G6PD,* acid phosphatases) derived from stored human blood have increased electrophoretic mobilities when compared with those of the same protein derived from fresh blood (Huisman & Dozy, 1962; Fildes & Parr, 1963; Hopkinson, Spencer & Harris, 1963). Similarly, old erythrocytes have a greater proportion of the electrophoretically more rapidly moving haemoglobin (A₃) than the younger cells (Kunkel & Bearn, 1957). Since all these proteins are contained in the same changing cellular environment (namely the stored or aging erythrocyte) the same or similar reactions may account for an altered electrophoretic mobility.

It has been suggested that the presence of GSSG in erythrocytes and its increase in older cells as a consequence of a diminished concentration of NADPH₂ (with a concomitant decrease in glutathione-reductase activity) may lead to the formation of mixed disulphides between haemoglobin and GSSG (Muller, 1961) and between enzymes and GSSG (Walter, 1963; Bottini & Modiano, 1964). Indeed, Bottini & Modiano (1964) have shown that erythrocyte acid phosphatases react with GSSG *in vitro* and that this reaction results in a diminished acid-phosphatase activity.

An important corollary is whether (Bottini & Modiano, 1964) the formation of such mixed disulphides *in vivo* is responsible for the decreased

enzyme activities observed for a number of erythrocytic enzymes in older cells (Marks, Johnson & Hirschberg, 1958; Walter & Selby, 1966). It is the purpose of the present paper to show that these two events are independent phenomena. Further, we suggest that the reaction between some enzymes and GSSG that occurs without loss of enzyme activity (e.g. aspartate aminotransferase; see below) may account for the increased electrophoretic mobility reported for these enzymes derived from human erythrocytes as compared with the same enzyme derived from other human tissues (Nisselbaum, 1965).

METHODS

Normal human or horse blood was collected by using EDTA as anticoagulant. Cells from 10ml. of blood were washed six times with 0.9% NaCl solution, care being taken to remove the buffy coat. The erythrocytes were lysed in 2vol. of water, and the stroma was removed by centrifugation at 13000g for 30min. at 20°. Studies on the storage effect were carried out on lysates from blood cells kept at 4° for 3–4 weeks and compared with lysates from fresh blood from the same individual. For horses, blood from different animals was compared. In one experiment lysates from an individual with 50% reticulocytes were obtained and the electrophoretic mobilities of the three enzymes indicated below compared with those of the same three enzymes in lysates from a normal blood population.

In experiments on the reaction of G6PD with GSSG, 2ml. of haemolysate was incubated at 37° for 2hr. with 33 μmoles of GSSG (adjusted to pH 7.4) and 2.6 μmoles of

* Abbreviation: G6PD, glucose 6-phosphate dehydrogenase.

NADP in a total volume of 4 ml. Portions were taken out at the start of the reaction. Fractions were dialysed in the cold against 0.05 M-tris-HCl buffer, pH 8, which also contained NADP (10 μ M). When the reactions of aspartate aminotransferase and lactate dehydrogenase with GSSG were studied, the lysate was adjusted to pH 7.4 with 0.1 M-tris-HCl buffer. Preliminary experiments indicated that the omission of NADP or incubation of lysate in the presence of phosphate or tris buffer, or both, led to a rapid loss of G6PD activity.

We also noticed that, in some of the experiments, incubation results in an increased G6PD activity in the untreated fresh or stored haemolysates. This increase in activity is accompanied by the precipitation of stromal material during incubation that is then removed before enzyme assay. In the experimental tubes (i.e. GSSG-treated lysates) no such stromal precipitate appears on incubation and no increase in enzyme activity is observed. For a discussion and references on the relationship between stroma, incubation and G6PD activity see Mellbye & Scott (1964).

Enzymes (G6PD, aspartate aminotransferase, lactate dehydrogenase) were assayed by standard spectrophotometric methods (Walter, Selby & Francisco, 1965; Wróblewski & LaDue, 1955) in fresh and stored lysates and in fresh GSSG-treated lysates (at zero time and after 2 hr. incubation; see above). Haemoglobin concentration was determined on a suitable sample by measuring its extinction at 540 m μ . One enzyme unit is defined as that amount of enzyme required to give ΔE_{340} 0.001/min./ml. of lysate. Specific enzyme activities are expressed as enzyme units/haemoglobin extinction.

Electrophoresis of lysates on starch gel followed by specific staining for G6PD, aspartate aminotransferase or lactate dehydrogenase was carried out as described by Walter *et al.* (1965). G6PD from horse erythrocytes was subjected to electrophoresis in a gel containing 0.05 M-tris-HCl buffer (pH 8.8)-2.7 mM-EDTA-20 μ M-NADP. In each case 10 enzyme units (100 units for lactate dehydrogenase) were applied to Whatman no. 17 paper strips and these strips inserted into the gel. Electrophoresis was carried out at 4°.

RESULTS AND DISCUSSION

The electrophoresis of haemolysates followed by specific staining for certain enzymes sometimes affords a simple and convenient method for a study of possible interactions between an enzyme and reagents that, if a reaction occurs, alter the enzyme's electrophoretic mobility (Fildes & Parr, 1963; Bottini & Modiano, 1964; Walter *et al.* 1965). Thus we found (Fig. 1) that the increased electrophoretic mobility reported by Fildes & Parr (1963) for G6PD derived from stored erythrocytes (especially those stored in EDTA) could be reversed by dialysis. The storage effect could be simulated by treating a fresh haemolysate with GSSG *in vitro* and subsequently dialysing (Fig. 1). The same treatments with regard to pH and buffer, but without GSSG, do not have any effect on the electrophoretic properties of this enzyme. No

Fig. 1. Relative electrophoretic mobilities (on horizontal starch gel in 0.01 M-phosphate buffer at 6 v/cm. for 17 hr.) of G6PD in lysates from fresh and stored human erythrocytes and from lysates treated with GSSG. Details are given in the text.

appreciable alteration of specific enzyme activity accompanied either storage (Mellbye & Scott, 1964) or the 'reaction' of G6PD with GSSG followed by dialysis (Table 1). It is likely from these results that the interaction between GSSG and G6PD *in vitro* under the conditions used, and whatever (e.g. GSSG or some other reagent) causes the altered electrophoretic mobility of this enzyme on storage of erythrocytes *in vitro*, is not due to mixed-disulphide formation or other covalent reaction but rather to an adsorption phenomenon.

When a haemolysate is treated with GSSG *in vitro*, the haemolysate subjected to electrophoresis and the gel specifically stained for aspartate aminotransferase, an increased anodic electrophoretic mobility for this enzyme is apparent that cannot be reversed by dialysis (Fig. 2). This may indicate that mixed-disulphide formation between aspartate aminotransferase and GSSG has taken place. Further, no change in specific enzyme activity is apparent as a consequence of this reaction (Table 1). It should be mentioned that aspartate aminotransferase does not display a consistently reproducible increase in electrophoretic mobility on storage *in vitro* (at least not within a 3-4-week storage period). The specific enzyme activity of aspartate aminotransferase remains constant on storage of erythrocytes during this time.

The isoenzymes of human-erythrocyte lactate dehydrogenase and of horse-erythrocyte G6PD had unchanged electrophoretic mobilities after GSSG treatment and unaltered specific enzyme activities.

It has been reported (Walter *et al.* 1965) that these same enzymes (human-erythrocyte lactate dehydrogenase and horse-erythrocyte G6PD) show identical electrophoretic mobilities from the cells most resistant to hypo-osmotic lysis (i.e. the youngest cells) and those from cells that are less

Table 1. *Specific enzyme activities of aspartate aminotransferase in lysates from fresh and stored human erythrocytes and of aspartate aminotransferase and G6PD in lysates from fresh treated erythrocytes with GSSG*

Specific enzyme activities are expressed as enzyme units/haemoglobin extinction. Enzyme activities were assayed after dialysis. Details are given in the text.

Enzyme	Lysate from	Enzyme activity	
		At zero time	After 2 hr. incubation
Aspartate aminotransferase	Fresh cells (control)	10.3	10.7
	Fresh cells plus GSSG	12.1	11.1
	Stored cells	12.2	—
G6PD	Fresh cells (control)	20	21.9
	Fresh cells plus GSSG	19.8	18.6

Fig. 2. Relative electrophoretic mobilities (on horizontal starch gel in 0.01 M-phosphate buffer at 6 v/cm. for 17 hr.) of aspartate aminotransferase in control and GSSG-treated lysates from human erythrocytes. Details are given in the text.

resistant (i.e. older cells). Human-erythrocyte aspartate aminotransferase and G6PD derived from the less resistant cells have a greater anodic electrophoretic mobility than the same enzymes from the most resistant cells. Because the possibility existed (Walter *et al.* 1965) that enzymes from residual quantities of leucocytes, which are rather resistant to hypo-osmotic lysis, contaminated the lysates from the youngest erythrocytes, the experiment described below was conducted. The results of this investigation force a reinterpretation of these previously published data.

A rare human blood sample containing 50% reticulocytes was obtained. Haemolysates were subjected to electrophoresis and the electrophoretic mobilities of G6PD, aspartate aminotransferase and lactate dehydrogenase were compared with those of the same enzymes in haemolysates from a normal blood population. No difference

was observed in the electrophoretic mobility of any of the enzymes in these very young and older (normal) cells. The conclusion must thus be reached that the 'altered' (increased) electrophoretic mobility for aspartate aminotransferase and G6PD previously reported to occur as a function of erythrocyte age (Walter *et al.* 1965) is most probably due to a difference between the mobilities of these enzymes from erythrocytes and those from resistant leucocytes contaminating the youngest erythrocyte fractions.

Since the very same enzymes that react with GSSG *in vitro* (human-erythrocyte aspartate aminotransferase and G6PD) are the ones that show a greater electrophoretic mobility when derived from erythrocytes, it is possible that they also react or interact with GSSG in the erythrocyte *in vivo*. Such a reaction, which gives rise, for example, to an aspartate aminotransferase with unaltered enzyme activity (Table 1) but greater electrophoretic mobility, would explain the difference in electrophoretic mobility between human-erythrocyte aspartate aminotransferase and aspartate aminotransferase from other human tissues reported by Nisselbaum (1965). This may also apply to some other enzymes.

Since both aspartate aminotransferase and G6PD are enzymes with markedly lower activities in older erythrocytes (Marks *et al.* 1958; Walter & Selby, 1966), it follows that the reaction or interaction of these enzymes with GSSG (which occurs without loss of enzyme activity; Table 1) cannot be the reaction responsible for the lowered enzyme activities in erythrocytes aged *in vivo*. Though this reaction may account for the decreased enzyme activities of some enzymes (e.g. acid phosphatases; Bottini & Modiano, 1964), it appears that for G6PD (human and horse) and human aspartate aminotransferase they are independent phenomena. The reaction of an enzyme with GSSG depends on readily available and reactive thiol groups. The

decrease in activity of an enzyme that has reacted with GSSG is a function of the location and relation of such thiol groups to the enzyme active site (Holbrook & Pfeleiderer, 1965; Cecil & Thomas, 1965). Thus the finding of decreased enzyme activities in old erythrocytes cannot be ascribed to this (perhaps not to any) single event. It may be a consequence of enzyme catabolism or interaction or reaction of enzymes with a number of different reagents (of which GSSG is just one) present in the erythrocyte that result in an inhibition or loss of enzyme active sites.

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