Effect of Methylene Blue on Glutamate and Reduced Glutathione of Rabbit Erythrocytes

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Methylene Blue injected into rabbits resulted in increased glutamate, reduced glutathione and pyrrolid-2-one-5-carboxylate in erythrocytes. The increase in reduced glutathione was probably due to increased glutamate changing the feedback control of glutathione on γ -glutamylcysteine synthetase.

Erythrocyte GSH* is synthesized in two enzymic steps:

The first reaction is catalysed by γ -glutamylcysteine synthetase (EC 6.3.2.2), and the second by GSH synthetase (EC 6.3.2.3) (Minnich *et al.*, 1971). Some γ -glutamylcysteine may be split by γ -glutamylcyclotransferase (EC 2.3.2.4) to pyrrolid-2-one-5-carboxylate and cysteine (Palekar *et al.*, 1974). Because its synthesis is controlled, erythrocyte GSH normally is maintained at a relatively constant concentration. Two drugs, Methylene Blue and diaminodiphenylsulphone, disrupt control mechanisms, allowing GSH to increase (Bockris & Smith, 1962; Degowin *et al.*, 1966). Both drugs decrease the Michaelis constant (K_m) of GSH synthetase for γ -glutamylcysteine (Paniker & Beutler, 1972) and this appears to provide a possible explanation for the GSH increase.

Alternatively non-enzymic changes in the erythrocyte might be important. To test aspects of that possibility, we measured the substrates required for GSH synthesis in rabbits during treatment with Methylene Blue.

Materials and Methods

New Zealand rabbits weighing 2.3–3.0 kg were used. Blood was collected from the marginal ear vein.

Amino acid concentrations were determined on 0.6M-HClO₄ extracts of packed erythrocytes. We determined glycine by degrading it to formaldehyde (Sardesai & Provideo, 1970), glutamate by using glutamate dehydrogenase (Bernt & Bergmeyer, 1963) and cysteine by the noradenochrome method (Roston, 1968). ATP was measured by the luciferin/

luciferase method (Beutler, 1971), haemoglobin as the cyanmethaemoglobin derivative (Henry, 1974), and GSH as the yellow anion of the 5,5'-dithiobis-(2-nitrobenzoic acid) derivative (Beutler, 1971).

Pyrrolid-2-one-5-carboxylate was analysed with glutamate dehydrogenase (Bernt & Bergmeyer, 1963) after conversion into glutamate by acid hydrolysis (Hagenfeldt *et al.*, 1974). For this, packed erythrocytes were mixed with an equal volume of 0.6M-HClO₄; then 4 ml of supernatant obtained by centrifugation was mixed with 2 ml of conc. HCl. Half of the mixture was incubated for 3 h at 100°C; the remainder was stored at 4°C for 3 h and used as a blank. A standard solution of pyrrolid-2-one-5-carboxylate was treated similarly.

We tested the significance of the effects of Methylene Blue on the various metabolites measured by analysis of variance (Sokal & Rohlf, 1969).

Results

Three rabbits were injected with Methylene Blue (5 mg/kg) intraperitoneally twice daily; three additional rabbits injected with 0.9% NaCl served as controls. Day 0 was the first day of injection. Preliminary studies had indicated that excessive blood loss would increase erythrocyte GSH, so we measured those compounds (glutamate, pyrrolid-2-one-5-carboxylate, cysteine and glycine) requiring relatively large amounts of blood only on days 1, 3 and 5 of the experiment. GSH and ATP were measured on days 1–5 inclusive.

At 24 h after the first injection, erythrocyte glutamate (Fig. 1a) was significantly higher ($P < 0.05$) in the treated animals and continued to rise (0.24 $\mu\text{mol/day per g}$ of haemoglobin) for the remainder of the experiment. After a 1-day delay, erythrocyte GSH (Fig. 1b) increased linearly (1.34 $\mu\text{mol/day per g}$ of haemoglobin). Neither erythrocyte GSH nor glutamate changed significantly in control rabbits. Pyrrolid-2-one-5-carboxylate (Fig. 1c) did not differ

* Abbreviation: GSH, reduced glutathione.

Fig. 1. Effect on rabbit erythrocyte glutamate (a), GSH (b) and pyrrolid-2-one-5-carboxylate (c) of injecting Methylene Blue (●) or 0.9% NaCl (○)

Results are means for three rabbits. Test values were significantly higher than control values ($P < 0.05$) (a) for days 1-5, (b) for days 2-5 and (c) for day 5.

between control and treated rabbits until day 5, when the pyrrolid-2-one-5-carboxylate in the treated animals was nearly twice that of the control group. Packed cell volume decreased from 39.9 to 33.9%, but there was no significant difference between control and treated rabbits. Erythrocyte glycine, cysteine and ATP did not change significantly ($P > 0.05$) during the experiment.

Discussion

Paniker & Beutler (1972) have emphasized the role of enzyme kinetics to explain the increase in erythrocyte GSH when Methylene Blue is administered to rabbits. For changes in enzyme kinetics to be effective, they must involve the rate-limiting or controlling enzyme. Thus, to evaluate the proposed explanation of the changes observed with Methylene Blue, one must know the rate-limiting enzyme in GSH synthesis. Two observations suggest that it is GSH synthetase: (1) it is lower in activity than γ -glutamylcysteine synthetase when both are measured *in vitro* under optimum conditions (Smith, 1974); (2) increased GSH in patients with myeloproliferative disorder correlates with increased GSH synthetase activity (Blume *et al.*, 1974).

But the evidence that γ -glutamylcysteine synthetase is the rate-limiting enzyme and is controlled by a classical feedback mechanism is much more convincing.

(1) γ -Glutamylcysteine synthetase is inhibited by GSH (Jackson, 1969). The inhibition is non-allosteric and involves the binding of GSH to the glutamate-binding site of the enzyme and to another enzyme site (Richman & Meister, 1975).

(2) Although GSH synthetase deficiency does result in markedly decreased GSH concentrations it

also increases γ -glutamylcysteine, and the γ -glutamylcysteine is converted by γ -glutamylcyclotransferase into pyrrolid-2-one-5-carboxylate. The lack of feedback inhibition is apparently responsible for overproduction of γ -glutamylcysteine (Wellner *et al.*, 1974).

(3) The overall biosynthesis of GSH in membrane-free haemolysates under physiological conditions mainly depends on γ -glutamylcysteine formation (Heinle *et al.*, 1976). Thus it appears that the kinetic change observed in GSH synthetase during Methylene Blue administration probably is unimportant as a mediator in changing GSH concentrations.

Our results suggest that changes in substrate concentration (particularly of glutamate) may be more important primarily than enzymic changes in controlling GSH. Increased erythrocyte glutamate can increase GSH by changing control processes. GSH regulates its own synthesis by inhibiting γ -glutamylcysteine synthetase. That inhibition is non-allosteric and involves competition between glutamate and GSH for a common binding site (Richman & Meister, 1975). If glutamate increases, γ -glutamylcysteine synthetase activity, and presumably GSH synthesis, should increase. As the GSH increases, it should eventually inhibit γ -glutamylcysteine synthetase again at the higher glutamate concentration. Thus the Methylene Blue-induced increase in erythrocyte GSH could be explained by the drug's effect on glutamate concentration.

We cannot explain how Methylene Blue increases erythrocyte glutamate. Labelled glutamate is incorporated very slowly into erythrocyte GSH during incubation *in vitro* because dicarboxylic amino acids enter the cells slowly. Glutamine and 2-oxoglutarate may cross the erythrocyte membrane and be converted into glutamate (Miller & Horiuchi, 1962; Sass,

1963). However, *in vivo* glutamate can be shifted rapidly into and out of erythrocytes (Aoki *et al.*, 1972).

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