

The Mechanism of the Vitamin K₃ Induced Glutathione Instability in Human Red Blood Cells. The Possible Intermediary Role of Methemoglobin

Andrew Sass-Kortsak*,
Bertil Thalme, and Lars Ernster
Wenner-Gren Institute, University of Stockholm, Stockholm, Sweden

It has been known for some time that administration of vitamin K₃ to premature newborn infants may lead to the development of kernicterus. The kernicterus is probably the result of an increased rate of destruction of red blood cells, which in turn is brought about by a precipitous drop in the GSH** content of the erythrocytes, as Zinkham has shown in *in vitro* studies¹. The exact mechanism whereby vitamin K₃ produces this effect is not known.

It has been noted by others, and recently shown at this laboratory² that vitamin K₃, when incubated with red blood cells, hemolysates, or a solution of crystalline Hb, induces a rapid formation of MeHb. Vitamin K₁ does not share this effect of K₃, nor does vitamin K₁ lead to hemolytic disease in the newborn.

* On study leave from the Research Institute of the Hospital for Sick Children, Toronto, Canada.

** Abbreviations: GSH, reduced glutathione; Hb, hemoglobin; MeHb, methemoglobin; TPNH, reduced triphosphopyridine nucleotide.

Table 1. Oxidation of GSH by MeHb. Incubation at 37°C with constant shaking in tubes open to air. Final concentrations: NaCl 0.077 M; phosphate buffer, pH 7.4, 0.05 M, MeHb and GSH indicated. Total volume, 2 ml/tube.

Series No.	Initial concentration mM	MeHb reduced or GSH oxidized, μ moles/2 ml incubate			
		0 h	1 h	2 h	3 h
1	MeHb 2.0	0	0.40	0.88	1.0
	GSH 1.0	0	1.07	1.62	1.63
2	MeHb 2.0	0	1.32	1.68	—
	GSH 2.0	0	2.23	3.55	—
3	MeHb 2.0	0	1.96	2.72	2.50
	GSH 5.0	0	3.00	4.48	5.76

With this information on hand it seemed of interest to investigate whether MeHb may play an intermediary role in the loss of erythrocyte GSH, brought about by vitamin K₃.

In the first series of experiments red blood cells from freshly obtained and defibrinated adult human blood were used. The red cells were washed three times and incubated in Krebs-Ringer-phosphate solution (approx. 40 % hematocrit) at 37°C in open tubes with constant shaking. At periodic intervals aliquots were removed for the simultaneous determination of GSH and MeHb contents. GSH was determined by the method of Grunert and Phillips³. The relative amounts of MeHb were assessed in samples diluted with distilled water, by measuring the optical density at 630 and 700 $m\mu$, before and after

Table 2. Interaction between GSH, Hb and vitamin K₃-bisulfite. Incubation at 37°C with constant shaking in tubes open to air. Final concentrations: NaCl, 0.077 M; phosphate buffer, pH 7.4, 0.05 M; GSH, 1 mM; and when indicated: Hb 3.6 mM = 6.2 gm %, K₃bisulfite, 0.33 mM. Total volume, 2 ml/tube.

Additions	0 h		1 h		2 h		3 h	
	MeHb	GSH	MeHb	GSH	MeHb	GSH	MeHb	GSH
μ moles/2 ml incubation mixture								
GSH	—	2.0	—	2.0	—	1.6	—	1.4
GSH + Hb	0	1.8	0	1.9	0	1.5	0	1.6
GSH + K ₃ bisulfite	—	1.8	—	1.8	—	1.5	—	1.4
GSH + Hb + K ₃ bisulfite	0	1.8	2.9	1.2	4.6	0.8	5.5	0.6

the addition of an excess of potassium ferricyanide.

During 4 h of incubation of the washed cells, without addition of substrate, the GSH levels in the cells remained constant and formation of MeHb was not observed. When vitamin K₃bisulfite (final concentration 0.33 mM) was added, considerable amounts of MeHb were formed and the level of GSH fell to 30 % of the original. Addition of glucose in 6 mM final concentration prevented the fall of GSH level, and lessened, but did not entirely abolish, the formation of MeHb. These findings confirm some of the observations made by Zinkham¹; moreover they prove that, in addition to its detrimental effect on GSH, vitamin K₃ bisulfite induces the formation of MeHb as well.

It has been found by others that GSH can be oxidized by simple chemical interaction with MeHb⁴. It seemed that this reaction could be one way by which vitamin K₃ could bring about loss of GSH. Experiments were carried out to explore this possibility. These consisted of incubating vitamin K₃bisulfite, GSH, Hb and MeHb in various combinations and concentrations under the conditions specified in Tables 1 and 2. The Hb solution used in these experiments was prepared according to Beutler⁵, but the use of toluene was avoided, and hemolysis without undue dilution was effected by rapid freezing and thawing of the washed cells three times. MeHb was prepared by adding to the Hb solution twice the equivalent amount of solid potassium ferricyanide, followed by dialysis against large volumes of distilled water. GSH and MeHb were determined as specified above.

When GSH, in 1 mM concentration, was incubated without any addition, during 3 h the GSH concentration declined only slightly, probably due to oxidation by atmospheric oxygen. The addition of 0.33 or 3.3 mM vitamin K₃bisulfite did not lead to an acceleration of the slow autooxidation of GSH. When MeHb in 2 mM (3.4 mg %) concentration was incubated with three different concentrations of GSH, and without addition of vitamin K₃bisulfite, GSH was rapidly oxidized and MeHb was reduced (Table 1). The expected stoichiometric relation of 2 moles of GSH oxidized by 1 mole of MeHb is supported by these data. The rate of the reaction was found to depend on the concentration of both MeHb and GSH, according to first order kinetics.

Further experiments were carried out to observe the interaction between vitamin K₃bisulfite, Hb and GSH. In Table 2 it can be seen that the addition of either one of Hb or vitamin K₃bisulfite to GSH did not accelerate

the slow rate of autooxidation of GSH. Furthermore, when Hb was incubated in the presence of GSH, no MeHb was formed. However, when GSH was incubated in the presence of both Hb and vitamin K₃bisulfite, MeHb formation occurred and simultaneously the rate of oxidation of GSH was definitely increased. During 3 h of incubation, 76 % of the added Hb was oxidized and 66 % of the GSH was lost.

Another possible route by which formation of MeHb induced by vitamin K₃ may lead to glutathione instability in the red blood cell was also investigated. One may suppose that the accumulation of MeHb may lead to drainage of the existing stores of TPNH *via* the TPNH dependent MeHb reductase. Under these conditions it may be difficult for the exclusively TPNH dependent glutathione reductase to maintain glutathione in the reduced state and the GSH levels may decrease as a consequence. To explore this possibility experimental conditions were chosen to observe the effect of active intracellular MeHb reduction on the ability to maintain high levels of GSH in the erythrocyte. By exposing washed adult red blood cells to trace amounts of amylnitrite according to the method of Gibson⁶, 40–50 % of the total Hb was oxidized to MeHb. When these cells, after washing, were suspended in physiological saline containing 0.01 M phosphate buffer (pH 7.4) and incubated without the addition of substrate, the amount of preformed MeHb remained constant. The GSH levels, which were reduced somewhat by the pretreatment with amylnitrite, remained also constant during the subsequent 4 h of incubation. Addition of glucose (final concentration 10 mM) led to a gradual reduction of the preformed MeHb in the cells, disposing of about 50 % of the MeHb in 4 h. This process was markedly enhanced by further addition of catalytic amounts of methylene blue (final concentration 0.01 mM), the MeHb virtually disappearing from the cells at the end of merely 1 h of incubation. Under the latter two conditions, however, and especially when glucose and methylene blue were added together, and when there was an extremely rapid reduction of MeHb, the GSH levels did not decrease but remained stable. This suggests that at least under these experimental conditions the drainage of the TPNH stores by the MeHb reductase did not influence the maintenance of the reduced state of glutathione.

The results of these studies suggest that the formation of MeHb may play an intermediary role in the oxidation of GSH in erythrocytes exposed to vitamin K₃bisulfite.

It seems likely that this mechanism consists of direct chemical interaction between MeHb and GSH. In order to reconcile the present tentative conclusions with the often emphasized fact that methemoglobinemia and glutathione instability are not necessarily correlated *in vivo*⁷, it will be necessary to investigate more closely the mechanisms which, in the intact cell, may protect GSH from the oxidizing effect of MeHb, or may be able to replenish the losses of GSH effectively. It may well be that ultimately it will depend on the effectiveness or failure of these mechanisms whether an increased rate of formation of MeHb will lead to actual loss of GSH.

1. Zinkham, W. H. *Pediatrics* **22** (1959) 18.
2. Broberger, O., Ernster, L. and Zetterström, R. *Nature* **188** (1960) 316.
3. Grunert, R. R. and Phillips, P. H. *Arch. Biochem.* **30** (1951) 217.
4. Eggleton, P. and Fegler, G. *Quart. J. Exptl. Physiol.* **37** (1952) 163.
5. Beutler, E., Robson, M. and Buttenwieser, E. J. *Clin Invest.* **36** (1957) 617.
6. Gibson, Q. H. *Biochem. J.* **42** (1948) 13.
7. Beutler, E. *Blood* **14** (1959) 103.

Isolation of β -Fructofuranosidase from Yeast by Ion Exchange Chromatography on Diethylaminoethylcellulose

Bjørn Andersen

Universitetets fysisk-kemiske Institut,
Copenhagen, Denmark

The mechanism of the splitting of sucrose into α -D-glucopyranose and β -D-fructofuranose by invertase from yeast has been investigated by numerous workers. This has given rise to a great number of papers describing purification procedures of the enzyme. One of the most successful is that of Fischer and Kohtes¹, which using precipitation with picric acid and

acetone, followed by adsorption to Al(OH)₃, obtained a preparation containing 4–5 % N with an activity of 4 000 units/mg N.

The development of cellulose derivatives for separation of proteins by ion exchange chromatography^{2,3} has opened new possibilities for a further purification of the enzyme.

In the present work the β -fructofuranosidase from yeast has been separated on a N,N-diethylaminoethylcellulose (DEAE) column. The starting material was «Invertan.K.B.» (commercial invertase preparation, manufactured by De forenede Bryggerier, Copenhagen.) After dialysis against distilled water at 4°C for 24 h the solution was added to the column, previously equilibrated with 0.005 M phosphate buffer, pH 6.5. Elution was made with increasing concentrations of sodium chloride (0–0.25 M). A sharp separation was obtained between the active component and the other proteins in the solution. After freeze-drying the nitrogen content was determined to give a value of 11.02 %. The enzyme activity was determined according to a method described in a previous publication⁴. Converted to the units of Fisher and Kohtes the activity was 22 000 units/mg N to be compared with the 4 000 units/mg N obtained by these authors. Electrophoresis, which at the present time has only been carried out at one pH, indicates one single protein component. Determination of the molecular weight at the optimal pH (4.75), using the osmometer designed by Christiansen and Jensen⁵ gave the value 116 000. Calculation of the enzyme activity as number of substrate molecules reacting per min per molecule of enzyme gives a value of 260 000.

A more detailed description of the isolation will be given.

1. Fischer, E. H. and Kohtes, L. *Helv. Chim. Acta* **34** (1951) 1123.
2. Sober, H. A. and Petterson, E. A. *J. Am. Chem. Soc.* **76** (1954) 1711.
3. Petterson, E. A. and Sober, H. A. *J. Am. Chem. Soc.* **78** (1956) 751.
4. Andersen, B. and Bak, T. A. *Acta Chem. Scand. In press.*
5. Christiansen, J. A. and Jensen, C. E. *Acta Chem. Scand.* **7** (1953) 1247.