Furthermore, the results obtained in this work with PMSF suggest, that β-esterase differs from other *Drosophila* esterases, not only in its substrate specificity.

Another important implication of this work is the necessity of very cautious application of PMSF in biochemical studies of *Drosophila* esterases. This agent is most widely used as an inhibitor of proteases in various preparative and analytical manipulations with proteins and enzymes, in pariticular. As was shown here, the use of PMSF to inhibit protease activity during the purification of esterases of *Drosophila* and most probably of other insects may be misleading for the interpretation of the results.

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Time course study of the changes in blood glutathione induced by acute ethanol intoxication in the rat

V. Fernández[1], N. Fernández, A. Valenzuela and L.A. Videla

Unidad de Bioquímica, División de Ciencias Médicas Occidente, Facultad de Medicina, Universidad de Chile, Casilla No. 10455, Correo Central, Santiago (Chile), and Laboratorio de Bioquímica, INTA, Universidad de Chile, Santiago (Chile), September 20, 1982

Summary. Acute ethanol treatment of rats (5 g/kg) has a biphasic effect on the glutathione content of the erythrocyte. After 3 h of intoxication there is a diminution in total GSH equivalents, followed by a recovery to basal values 6 h after treatment. The decrease of total GSH equivalents is mainly due to a diminution of the oxidized form of the tripeptide. Concomitantly a marked increase in the plasma level of glutathione was found at 3 h, followed by a diminution to values obtained at time zero.

Previous studies by our group[2,3] and others[4] have shown that both acute and chronic ethanol administration to rats were able to induce a marked diminution of the levels of reduced glutathione (GSH) in the liver, and a moderate decrease in kidney. Liver GSH depletion induced by acute ethanol ingestion is partially due to a conversion of GSH into glutathione disulfide (GSSG) in the tissue, which accounts for 20% of the total decrease in GSH[5]. In this condition, the biliary excretion of GSH and GSSG is decreased, while the levels of both metabolites were found to be enhanced in blood plasma[5]. This increase in the plasma content of glutathione induced by ethanol was suggested not to be a consequence of a release from the erythrocyte, since total GSH equivalents ($GSH+2\,GSSG=GSH_T$) in red blood cells were found to be comparable at zero and 6 h of intoxication[5]. Despite this observation, the glutathione status of the erythrocyte did seem to be altered by acute ethanol ingestion, as its GSH content was elevated and that of GSSG was reduced after 6 h of treatment[5]. This indicates a change in the redox state of the glutathione couple, the GSH/GSSG ratio being markedly enhanced[5]. The studies presented here were undertaken in order to clarify the changes induced by acute ethanol ingestion in the glutathione status of the erythrocyte during the period of intoxication of 6 h, for comparison with liver and kidney[2,3]. The time courses of changes in GSH and GSSG levels in plasma and whole blood were also determined under the same conditions.

Materials and methods. Male Wistar rats (División de Ciencias Médicas Occidente, Facultad de Medicina, Universidad de Chile) weighing 150-200 g were fasted overnight (16 h) prior to the treatment. Animals receiving ethanol were intubated with 5 g of ethanol/kg b.wt as a 40% w/v solution in saline. Control animals received isocaloric amounts of glucose 8.75 g/kg b.wt p.o. as a 50% w/v solution. All rats were kept in a warm environment (25-28 °C) during the period of intoxication. The time course study of changes in erythrocyte, plasma and whole blood GSH and GSSG was carried out 1, 2, 3, 4, 5, and 6 h after treatment, on blood samples obtained by cardiac puncture with heparinized syringes, from animals under light ether anesthesia.

For determination of glutathione in plasma and erythrocytes, the blood samples were centrifuged at 2500×g for 10 min at 4 °C. Erythrocytes were washed once with cold 154 mM NaCl and then resuspended in a 1:1 ratio with 154 mM NaCl. Plasma, erythrocyte and whole blood samples were deproteinized with 1 N $HClO_4$ and neutralized with 1.75 M K_3PO_4 prior to the determination of GSH and GSSG as described by Bernt and Bergmeyer[6]. Proteins were determined according to Lowry et al.[7], and hemoglobin was measured as described by Drabkin and Austin[8]. All

Changes in the content of total GSH equivalents (GSH_T) in red blood cells and whole blood from rats given a single dose of ethanol

Period of treatment	0 h GSH_T (nmoles/mg hemoglobin)[a]	3 h	Effect (%)	p
Whole blood	8.11±0.63 (11)[b]	10.09±0.37 (4)	+24.4	<0.02
Red blood cells	7.51±0.30 (12)	5.56±0.41 (10)	−25.8	<0.002

[a] GSH and GSSG were determined enzymatically in erythrocytes and whole blood samples from rats given 5 g of ethanol/kg, and were expressed as total GSH equivalents ($GSH_T=GSH+2\,GSSG$). [b] Number of animals used.

reagents used were obtained from Sigma (St. Louis). Results are expressed as means ± SEM and the significance of the differences between mean values was assessed by Student's t-test for unpaired data.

Results. Data presented in figure 1 show the time course of the changes in GSH_T equivalents (GSH + 2 GSSG) of erythrocytes from rats subjected to acute ethanol intoxication. It can be observed that the GSH_T content of erythrocytes progressively decreased, reaching a minimal value after 3 h of treatment. When the GSH_T content of erythrocyte and whole blood samples following 3 h of intoxication are compared to basal values found in control animals at time zero a 25.8% decrease was observed in the former and 24.4% increase was evidenced in the latter (table). From 3- to 6 h of intoxication, GSH_T levels returned to basal values (fig. 1).

Figure 2 shows the time course of the differences between the levels of GSH, GSSG and GSH_T in erythrocytes from rats after 1–6 h of treatment with ethanol and those found in control animals at time zero (Δ GSH, Δ GSSG and Δ GSH_T respectively). It can be seen that after 3 h of ethanol treatment Δ GSSG and Δ GSH_T were markedly decreased, while Δ GSH was diminished to a lesser extent. From 3- to 6 h of intoxication Δ GSH_T returned to basal values, while Δ GSSG remained lower than the baseline and Δ GSH increased above it (fig. 2).

Total GSH equivalents in plasma increased after acute ethanol ingestion, reaching a maximum after 3 h of intoxication (fig. 3). After this time, GSH_T was found to decline, to reach a level at 6 h which was still higher than basal values (fig. 3).

Discussion. The results reported above show that acute ethanol administration to fasted rats induced a biphasic effect on the content of glutathiones of the erythrocyte. First there was a diminution in GSH_T levels which was maximal after 3 h of ethanol treatment, followed by a recovery to basal levels of GSH_T at 6 h.

The decrease in the GSH_T content of the red blood cell by ethanol was observed concomitantly with an increase in the concentration of GSH_T in plasma, which was found to peak at 3 h of treatment. These findings are suggesting that ethanol-induced reduction in red blood cell GSH_T levels could be related to a translocation process from the erythrocyte into blood plasma following 3 h of intoxication. The decrease in erythrocyte GSH_T levels after ethanol treatment was mainly due to a fall in the concentration of GSSG at 3 h of intoxication, the levels of GSH being less markedly diminished. In fact, the decrease in GSSG accounts for 90% of the reduction of GSH_T at this time. Thus, ethanol ingestion seems to be decreasing the content of glutathione of the erythrocyte mainly because of a loss of GSSG, which is in agreement with the general view that glutathione released from cells occurs in its oxidized form[4,10]. Furthermore, it has recently been reported that the addition of ethanol to red blood cell membranes from normal rats induces an increase in their fluidity[12]. Thus, a single oral dose of ethanol could produce a similar fluidizing effect in the erythrocyte membrane in vivo, leading to an enhanced GSSG release into plasma and a reduction in its intracellular content. Although these changes induced by ethanol in red blood cell GSH_T content should not alter the GSH_T

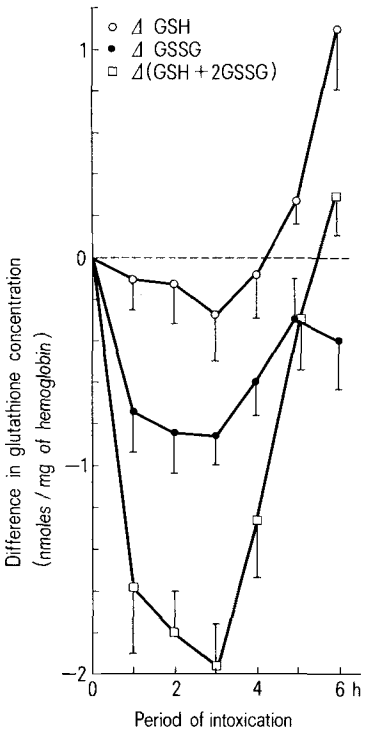

Figure 2. Time course of the differences between the levels of GSH, GSSG and GSH_T in erythrocyte from rats, after acute ethanol treatment, and those found in control animals. Each point represents the mean ± SEM for 10–12 animals.

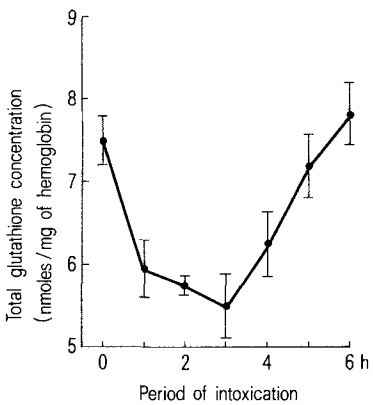

Figure 1. Time course of ethanol-induced changes in GSH_T equivalents (GSH + 2 GSSG) content in erythrocytes from rats, after acute ethanol treatment. Each point represents the mean ± SEM for 10–12 animals.

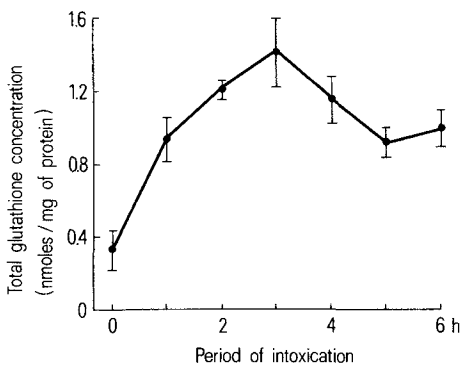

Figure 3. Time course of ethanol-induced changes on the plasmatic levels of GSH_T equivalents in rats, after acute ethanol treatment. Each point represents the mean ± SEM for 10–12 animals.

content in whole blood, it increased by 24% after 3 h of treatment. This could conceivably be due to a release of glutathione from tissues such as liver[2,3] and kidney[3], which have been shown to exhibit a moderate diminution in cellular glutathione (10–15% and 22% respectively) at this experimental time[2,3].

Following 3–6 h of the administration of ethanol the levels of GSH_T in the erythrocytes increased, reaching basal values at 6 h. This was due to a marked increase in the concentrations of GSH within the red blood cell, which reached a level above control values. GSSG levels were also enhanced, but they remained lower than levels found at time zero. The recovery of GSH levels in the erythrocyte could be related to an active resynthesis of the tripeptide, since γ-glutamyl-cysteine synthetase, the first enzyme involved in this process, is markedly inhibited by physiological levels of GSH[11]. Furthermore, GSSG is also able to inhibit hepatic γ-glutamyl-cysteine synthetase[11]. Thus, the decrease in the erythrocyte levels of GSH and GSSG observed after 3 h of ethanol intoxication could activate this enzyme by de-inhibition. The basal values of GSH_T observed in red blood cells after 6 h of ethanol intoxication were found concomitantly with an increased content (209%) of GSH_T in plasma. These findings indicate either that the erythrocyte is still releasing glutathione to the plasma and/or that another tissue is contributing to this effect. Although previous studies have indicated that the liver is maximally depleted of GSH after 6 h of ethanol ingestion[2,5], pointing to this organ as the main source of plasma GSH_T, further studies are needed to clarify this.

It is concluded that acute ethanol ingestion diminishes the glutathione levels of the erythrocyte, as is found in the liver tissue. However, this effect of ethanol appears earlier in the erythrocyte than in the liver and seems to be due to a translocation of glutathione from the red blood cell into the plasma, mainly as GSSG. Both an enhanced release of GSSG and an increased GSH synthesis in the erythrocyte could conceivably explain the change in the redox state of the glutathione couple previously observed after 6 h of ethanol ingestion, represented by an increase in the GSH/GSSG ratio[5].

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Subcellular distribution of mannophosphoinositides in *Mycobacterium smegmatis* during growth

N. Penumarti[1] and G.K. Khuller[2]

Department of Biochemistry, Postgraduate Institute of Medical Education and Research, Chandigarh-160012 (India), November 26, 1982

Summary. Subcellular distribution of total phospholipids and mannophosphoinositides (mannosides) was examined in *Mycobacterium smegmatis* ATCC 607 during its transition from the early exponential to the stationary phase of growth. There was relatively more of these substances in the cell membrane than in the cell wall, and the total amount increased with the age of the culture. Among individual mannosides, dimannophosphoinositides (dimannosides) were distributed equally in the cell wall and membrane. However, hexamannophosphoinositides (hexamannosides) were more predominant in the cell membrane, and the level increased with the age of the culture.

A number of bacterial genera belonging to the class Actinomycetales have the unique characteristic of possessing mannose-containing phospholipids called mannophosphoinositides[3]. These phospholipids have been known to possess antigenic characteristics in mycobacteria[4] and nocardia[5]. The cellular distribution of these components in mycobacteria is unclear[6, 7]. The present investigation was undertaken in an attempt to ascertain the cellular distribution of these phospholipids in mycobacteria. Changes, if any, in localization of these mannosides within mycobacterial organelles were also studied with respect to age of the culture.

Materials and methods. Mycobacterium smegmatis ATCC 607 was obtained from the American Type Culture Collection, and maintained as described earlier[8]. Cells in early exponential (2 days), mid-exponential (4 days) and early stationary (6 days) phases were harvested by filtration, washed thoroughly with normal saline (0.85% sodium chloride) and dried at 60 °C. Extraction of lipids and phosphorus estimation were carried out according to standard procedures[9]. Mannosides were resolved on silica gel H plates using the solvent system chloroform-methanol-water, 10:5:1 (v/v/v)[10]. Areas from the TLC plate corresponding to particular phosphatides were scraped off into Packard vials containing scintillation fluid and radioactivity was monitored in a Packard Liquid Scintillation counter[8].

Isolation of cell wall and cell membrane fractions. Mycobacterium smegmatis ATCC 607 cells, grown in Youman's medium containing ^{32}P-orthophosphate (500 µCi/100 ml of medium), were harvested after 2, 4 and 6 days of growth. A 25% suspension of the cells of a particular age was prepared in 0.01 M phosphate buffer (pH 7.0). Cells were sonicated