Table 2. Amino acid analysis of cytosine deaminase

| | n | | n |
|---|---|---|---|
| Asp | 37 | Met | 1 |
| Thr | 19 | Ileu | 15 |
| Ser | 22 | Leu | 19 |
| Glu | 27 | Tyr | 5 |
| Pro | 10 | Phe | 13 |
| Gly | 36 | Lys | 22 |
| Ala | 28 | His | 7 |
| Cys | 1 | Arg | 13 |
| Val | 31 | $NH_3$ | 8 |
| | | Trp | (2)* |

* Determined by the method of Barman and Koshland[6].

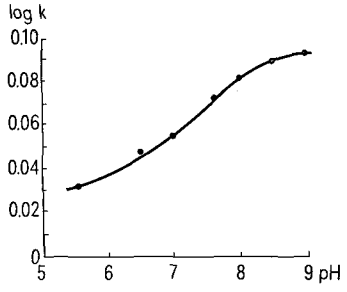


Photo-oxidation with methylene blue. Plot of log k against pH.

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with beef lung lactate dehydrogenase, where the amino acid analysis of a portion of the enzyme showed the cysteine residue concerned to be in a very hydrophobic region[9].

Substrate protection experiments, using cytosine as substrate and mercuric acetate, showed that the cysteine residue was at the active site (11.9% inhibition in the presence of the substrate, compared with 54.1% in the control). Titration of enzyme solution with p-chloromercuribenzoate as described by Ronca et al.[8] showed only one reactive –SH group present in the enzyme; the amino acid analysis showed only one cysteine residue present in the enzyme. The reaction of cyanate with this enzyme was probably with a thiol group. The inhibition was much less at pH 8.5 than at pH 7.0, which is in agreement with the observations of Stark[10] who studied the reaction of cysteine with cyanate; only carbamyl derivatives of amines are stable at both pH 7.0 and 8.5[11].

In photo-oxidation studies, 70% inhibition was observed with methylene blue ($1 \times 10^{-1}$ mg/ml) in 30 min, while 39% inhibition was observed with rose bengal ($1.6 \times 10^{-3}$ mg/ml). It is known that rose bengal is much more specific for histidine than methylene blue[12]. It seems likely that there is a histidine residue at the active site; again there is no reaction with alkylating agents (table 1). From data on the photo-oxidation of the enzyme by methylene blue at various pHs, the log of the rate of inactivation was plotted against pH[13].. The plot (figure) suggested that a residue with an ionizing group which has a pH of 7.1 is involved in the catalytic process; this would suggest histidine. From a series of experiments using phenylmercuric acetate, and photo-oxidation using methylene blue, it was concluded that 2 different groups were being modified by these reagents.

The inactivation of the enzyme by glyoxal may indicate that an arginine residue plays an important role. Interestingly, the enzyme is not inactivated by phenylglyoxal, indicating that the size of the reagent is probably important. These studies show that there is a cysteine, a histidine and possibly an arginine residue at the active site. This enzyme is the subject of further studies at these laboratoires.

## A highly active glutathione peroxidase in red blood cells of normal and acatalasemic mice[1]

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*Summary.* The activities of catalase (E.C.1.11.1.6) and glutathione peroxidase (E.C.1.11.1.9) were compared in red blood cells from humans, ducks and normal and acatalasemic mice. In the cells from both strains of mice, an equally high activity of GSH-Px was found which could be inhibited completely by iodoacetate but was not sensitive to N-ethylmaleimide.

In comparative studies on catalase activities in red blood cells from various species, the duck (no activity) and the primates (high activity) are considered the extremes[3], whereas these red cells show a reversed distribution of glutathione peroxidase (GSH-Px). By selection and crossing of mouse mutants, Feinstein[4] was able to obtain red cells with normal or very low catalase activity within the same species. The question arose whether the red cells from the acatalasemic mice developed an adaptation in $H_2O_2$ removal by an increased GSH-Px activity.

*Materials and methods.* Human blood was provided by the hospital blood bank. Ordinary farm ducks were bled from the wing vein. The normal ($Cs^a$) and acatalasemic ($Cs^b$)

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Activities of catalase and glutathione peroxidase in erythrocytes[a]

| | Red cells from human | duck | Mice[b] Normal | Acatalasemic |
|---|---|---|---|---|
| Catalase activity [$sec^{-1}$ $g^{-1}$ ml] | 354 ± 13 (18)[c] | 1 ± 0.1 (6)[d] | 174 ± 6.4 (9) | 13 ± 0.3 (6) |
| GSH-Px activity [μmoles NADPH/(min × g Hb)] | 22 ± 2.5 (6)[e] | 88 ± 8.5 (3) | 297 ± 11.5 (3) | 305 ± 8.7 (3) |
| After NEM [mM] | 3 ± 2 (3) [5] | 13 ± 0.6 (3) [15] | 266 ± 14.3 (3) [15] | 317 ± 14.5 (3) [15] |
| After IA [mM] | 3 ± 0.4 (3) [1] | 20 ± 0.9 (3) [5] | 3 ± 1.8 (3) [5] | 6 ± 0.8 (3) [5] |

[a] Means ± SEM (number of determinations). [b] Each experiment with pooled blood from 3 mice. [c] From 6 individuals. [d] From 2 ducks. [e] From 2 individuals.

mice were progenies from the colony of Dr Feinstein at the Argonne National Laboratory, Argonne, Illinois[4]; their blood was withdrawn by heart puncture under ether anaesthesia. Red cells were washed 3 times in isotonic NaCl, and the packed cells from the last wash were either incubated with inhibitors or hemolyzed with water under vigorous shaking. These hemolysates were prepared with the following red cell concentrations (v/v) for the enzyme assays: a) catalase: human, 10%; duck, 20%; normal mice, 15%; acatalasemic mice, 30%; b) GSH-Px: human and duck, 10%; mice, 5%. In the inhibition experiments, 1 vol. of red cells was incubated in 4 vol. of 0.9% NaCl in 0.01 M phosphate buffer containing the inhibitor. The incubation was carried out at 37 °C for 15 min in a shaking water bath, and subsequently the red cells were washed once in inhibitor-free saline. Thereafter hemolysates were prepared as above. Catalase activity was determined by the oxygen evolution which was recorded with a micro $pO_2$ electrode in a closed reaction vessel[5]. The activity of the glutathione peroxidase was measured by the coupled test of Paglia and Valentine[6] which was slightly modified: first, after mixing the water–hemolysate with an equal volume of twice concentrated Drabkin's reagent, the resulting solution was cleared from the ghosts by centrifugation at 10,000 × g for 10 min; second, the final volume of the assay was scaled down to 0.615 ml to allow the use of 0.5 ml cuvettes. Hemoglobin[7] was estimated in the hemolysate for the catalase assay and in the hemolysate–Drabkin solution for the GSH-Px assay. The specific activities were calculated by relating the first order rate constant of the catalase reaction or the oxidation of NADPH in the coupled test to the hemoglobin concentration. Thus the activity of catalase was expressed in $sec^{-1}$ $g^{-1}$ ml, whereas for GSH-peroxidase 1 unit/g Hb equals the consumption of 1 μmole NADPH/(min × g Hb).

*Results and discussion.* The specific activities of both enzymes in red cells from humans and ducks are consistent with those reported in the literature[3]. Despite the almost complete absence of catalase, the duck erythrocytes are adequately protected against oxidative damage by their relatively more active glutathione peroxidase, i.e. an activity level of the latter of 100 units/g Hb would be expected to compensate sufficiently for a total lack of catalase. Given the fact that the 2 types of mice take an intermediate position between man and duck with regard to blood catalase activity, their GSH-Px activity far exceeded that of the duck enzyme and was one order of magnitude higher than the human enzyme (table). Both strains of mice proved to have an equally active glutathione peroxidase, which supports the view that catalase might be less important for the in vivo removal of hydrogen peroxide from erythrocytes than GSH-Px[8].

The preincubation of human red cells in 5 mM N-ethylmaleimide (NEM) or 1 mM iodoacetate (IA) reduced the GSH-Px activity by more than 80% to values similar to those reported by Paglia and Valentine[6]. Both inhibitor concentrations had to be raised in order to yield a comparable effect in duck erythrocytes. But the same concentration of IA as used for the duck was much more effective in the mouse red cells, inhibiting their activity to less than 2% of the control. In contrast to iodacetate NEM in a concentration highly effective in duck cells did not cause any inhibition at all in mouse cells. Because of increasing hemolysis in the preincubation, the erythrocytes could not be exposed to a NEM concentration beyond 15 mM.

In order to rule out a possible permeability barrier for NEM in mouse red cells, a 20% hemolysate was prepared in water containing 15 mM NEM and incubated under the same conditions. After the incubation, this lysate was diluted to 5% with water and the GSH-Px activity was estimated. Again no inactivation of the enzyme was found when mouse red cells had been treated in this way. As the results for untreated or inhibited red cells from humans or ducks show, a failure in the method is unlikely to account for the high activity of glutathione peroxidase in mouse erythrocytes and its absence of NEM sensitivity. Furthermore this high activity is not a common feature for rodents, since red cells from rats fed with a diet not deficient in vitamin E and selenium showed an average activity of 18 units/g Hb[9]. But for glutathione peroxidase from rat liver, an inhibition pattern has been described which is the reverse of that found in mouse blood, i.e. iodoacetate induces none and NEM 64% inhibition[10]. Purification studies could reveal whether the various GSH peroxidases are isoenzymes and how specifically the inhibitors affect them.

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