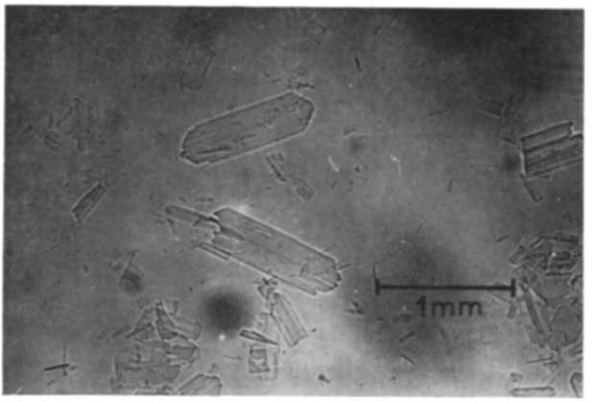

Fig. 1. Crystals of D-mannitol dehydrogenase. Recrystallized enzyme from the glucose-grown cells of *Leuconostoc mesenteroides*.

advantage to be able to prepare the crystalline enzyme from glucose medium by very convenient purification steps.

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Electrophoresis of glutathione reductase from human red blood cells

Glutathione reductase from human red blood cells can be separated into two distinct isozymes (GR I, GR II) by high-voltage electrophoresis. Cases of partial deficiency of the enzyme are frequently associated with a hemolytic, non-spherocytic anemia[1] or pancytopenia[2]. In one family[2] with this genetically determined polymorphism oligophrenia and neurological disorders were found (spastic signs of the Babinski group and pathologically altered electroencephalograms). Simultaneous occurrence of partial deficiency of glutathione reductase with hemoglobin C disease[3] and β-thalassemia minor (G. W. LÖHR, unpublished results) have been observed. Occasionally we have noticed the development of acute leukemia in cases of partial deficiency of glutathione reductase (G. W. LÖHR, unpublished results). Frequently

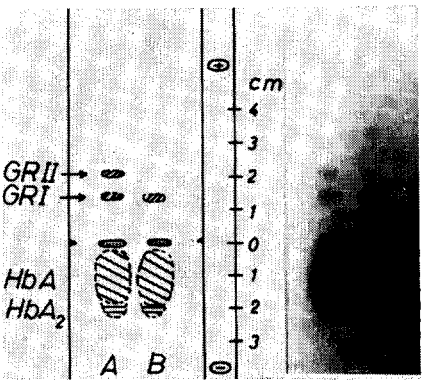

Fig. 1. Electrophoretic patterns of glutathione reductase isozymes, photographed in ultraviolet light. (A) Normal enzyme; (B) a case of partial deficiency of glutathione reductase. The zone at the origin does not represent glutathione reductase as it appears with and without oxidized glutathione in the staining solution.

hematological disorders in cases of partial deficiency of the enzyme are minor and will be apparent only after application of certain drugs[1] (chloroquine, salicylazosulfapyridine, nitrofurantoin, sulfonamids, phenylbutazone, phenacitin, dicumarine, and chloramphenicol). In different cases of partial deficiency of glutathione reductase from human red blood cells we only found a single isozyme band (see Fig. 1).

In normals the activity of glutathione reductase[2] is 11.7 ± 2.5 units/10^{11} erythrocytes. It is diminished to about 5–7 units in persons with partial deficiency of the enzyme[1,2]. Until now it has not been known whether the partial deficiency of glutathione reductase itself is responsible for the hematological and neurological disorders. Preliminary genetic studies[2,4] would lead one to suppose that the mode of inheritance is autosomally incompletely dominant. In crude hemolysates the K_m value of glutathione reductase for oxidized glutathione (GSSG) at pH 7.5 and 25° is about $6.4 \cdot 10^{-5}$ M. It is altered to $1.4 \cdot 10^{-4}$ M in cases of partial deficiency of glutathione reductase. The K_m for NADPH with both enzymes is about $1.6 \cdot 10^{-5}$ M. The pH optimum is shifted from pH 6.8 to 6.4. In 2000-fold enriched glutathione reductase preparations[5] the pH optimum was at pH 6.8 for the normal and 6.4 for the altered enzyme, whereas the corresponding K_m values for GSSG were about $4.2 \cdot 10^{-5}$ M and $9.4 \cdot 10^{-5}$ M, respectively. The K_m for NADPH in both enzymes is about $1.1 \cdot 10^{-5}$ M.

The following method was used for the separation of glutathione reductase from human red blood cells.

Preparation of hemolysates: red blood cells (3 times washed with 0.9% NaCl), 1.00 ml; destilled water, 0.67 ml; digitonin (saturated solution), 0.30 ml; MgK_2EDTA (0.2 M), 0.02 ml; 2-mercaptoethanol ($2.0 \cdot 10^{-4}$ M), 0.01 ml.

The mixture is rapidly frozen and thawed twice for complete hemolysis and is centrifuged for 20 min at 40 000 $\times$ g. The hemoglobin concentration in the supernatant is about 12 g/100 ml. Electrophoresis is performed on cellulose acetate in a gel form ('Cellogel', Chemetron, Milano, Italy). This has already been used in investigations on lactate dehydrogenase[6] and glucose-6-phosphate dehydrogenase[7] electrophoresis.

Cellogel strips are incubated for 1 h in $3 \cdot 10^{-2}$ M phosphate buffer (pH 6.2) containing $2 \cdot 10^{-6}$ M 2-mercaptoethanol and $4 \cdot 10^{-4}$ M MgK_2EDTA. This incubation

is repeated twice for 10 min with fresh buffer. The samples (about 3 μl) are applied on the porous surface of the strips (0.25 mm × 40 mm × 170 mm) in the form of thin streaks 5 mm long at the origin, 60 mm from the anodic end, using a micropipette. Within 1 min the samples are absorbed on the gel. Linen wicks with cellophan covers are used.

Applying a concentration gradient the cathodic chamber is filled with $4.5 \cdot 10^{-2}$ M phosphate buffer (pH 6.2). The anodic chamber contains $1.5 \cdot 10^{-2}$ M phosphate buffer (pH 6.2). The concentration of 2-mercaptoethanol and MgK_2EDTA corresponds to the amount mentioned above. The run usually takes 100 min at 4–5 mA/strip, at about 1000 V and $-4°$.

Staining procedure: the staining solution, which must be prepared freshly, consists of 0.3 M phosphate buffer (pH 7.1), $8.5 \cdot 10^{-4}$ M NADPH, and $1.9 \cdot 10^{-3}$ M GSSG. A filter paper is soaked with 1 ml of this solution. The strip is placed with its porous surface on the moist paper. After incubation for 10 min at 37° the result can be observed under ultraviolet light. In zones of glutathione reductase the bright fluorescence of NADPH is extinguished. At these locations no absorbance of fluorescence will appear if no GSSG is present in the staining solution.

Replacing NADPH by NADH ($2.0 \cdot 10^{-3}$ M NADH, $1.9 \cdot 10^{-3}$ M GSSG, 0.3 M phosphate buffer, pH 6.2) the same separation of glutathione reductase in the same location can be observed.

After elution of the isozymes from the gel strips, using triethanolamine buffer (pH 7.5) activity[8] can be determined in a spectrophotometric assay: $3.0 \cdot 10^{-7}$ M NADPH, $5.1 \cdot 10^{-6}$ M GSSG, eluates used as enzymes, pH 7.5, d = 1 cm, vol. = 1 ml, t = 25°, λ = 366 nm. About 65% of the activity of glutathione reductase is found in position GR I and about 35% in position GR II. In different cases of partial deficiency of glutathione reductase we found all the activity in position I. In white blood cells and platelets there is only one isozyme. Its position after electrophoresis is behind the GR I zone from red blood cells.

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