## G-6PD loading of G-6PD-deficient erythrocytes

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*Summary.* G-6PD-deficient erythrocytes were loaded during hypotonic hemolysis with G-6PD extracted from yeast. It was shown that enzyme was really trapped into red blood cells and remained functionally active.

There have been many recent reports on the enzyme loading of erythrocytes during hypotonic hemolysis[1-7]. During hypotonic shock the red cell membrane pores open, which causes the hemoglobin to leave the cells and allows enzymes to enter, they remain when tonicity is adjusted to normal. Adriaenssens et al.[3] also demonstrated the possibility of modifying the metabolic state of erythrocytes with a genetic defect by means of exogenous enzyme trapping.

This study deals with the loading of erythrocytes from a subject with a total deficiency of glucose-6 phosphate dehydrogenase (G-6PD), using G-6PD extracted from yeast. We also estimated the reduced glutathione (GSH) content of the resealed red cells after incubation with acetylphenylhydrazine.

*Materials and methods.* Venous blood anticoagulated with acid-citrate-dextrose (ACD) was obtained from a subject with G-6PD-deficiency, Mediterranean type ($Gd^{Med}$).

G-6PD extracted from yeast with specific activity 350 units/mg, was purchased from Boehringer Mannhein Corp. The enzyme suspension in ammonium sulfate was dialyzed against 0.90% NaCl pH 7.4 at 4 °C. The enzyme was diluted with NaCl 0.90% solution to obtain a final enzyme concentration of 33 IU/ml (1 IU is equal to the quantitiy of enzyme necessary to reduce 1 µmole of NADP/min at 25 °C). The loading procedure was carried out by the method of Ihler et al.[1]. The sample of whole blood was centrifuged at 2500 rpm for 10 min, plasma and buffy-coat were removed by aspiration, and red cells were washed 4 times with cold (4 °C) 0.90% NaCl solution. 0.2 ml of packed cells (hematocrit about 90%) were mixed with 2 ml of enzyme solution and incubated for 5 min at room temperature. Then 9 ml of NaCl solutions varying from 0.10% to 0.90% were added, and after 60 sec of hypotonic hemolysis, sufficient hypertonic NaCl was added to restore isotonicity, and the cells were allowed to reseal for 30 min at room temperature. After centrifuging at 2500 rpm for 10 min, the supernatant was collected to determine hemolysis, and the resealed red cells were centrifuged in a saline solution 5 times, and then diluted with isotonic solution to a hematocrit of about 50%. The corpuscular hemoglobin content after the last wash varied from 3.8 to 31.5 pg per cell according to different solution tonicity. The time-course estimation of G-6PD uptake was obtained by mixing packed cells with enzyme solution in NaCl 0.20% for different periods of time and resealing erythrocytes, using the same method.

Hemolysis was determined by absorbance at 540 nm using physiological saline as a control. The percentage of hemolysis was calculated by comparing every reading with that of the supernatant obtained from the red cells hemolyzed in distilled water. Assay of G-6PD was measured according to the method recommended by W.H.O. study group[8] and modified by Beutler[9]. The glutathione stability test was carried out according to Beutler's method[10,11].

*Results and discussion.* The results are shown in the table. G-6PD incorporation into red cells occurs only when a hypotonic medium is added to the erythrocyte-enzyme suspension. When the final NaCl concentration was 0.225%, with a hemolysis of 85%, the concentration of the intracellular enzyme obtained was 25% of the concentration of the extracellular enzyme. Similar results were obtained by Ihler et al.[1] for β-glucosidase (29%) and for β-galactosidase (24%), and by Updike et al.[5] for asparaginase (20%). When the NaCl concentration was more than 0.30%, no significant enzyme activity was seen in the erythrocytes. At a fixed hemolysis time of 60 sec, varying the NaCl concentration in medium, enzyme uptake paralleled the hemoglobin release from 0.225% to 0.40% NaCl concentration (figure 1). The lower enzyme incorporation for NaCl at a concentration of less than 0.225%, may be the result of a different resistance of the red cells to withstand hypotonicity.

Figure 2 shows the graphs of the time-course of G-6PD uptake and of hemoglobin release. The entry of enzyme parallels the exit of hemoglobin for 60 sec after the beginning of hemolysis, when there is maximum enzyme uptake. If hemolysis lasts more than 60 sec, G-6PD uptake progressively decreases owing to red cell resealing inability. The mean cell volume of the enzyme-loaded cells had increased from 92 to 113 µm³.

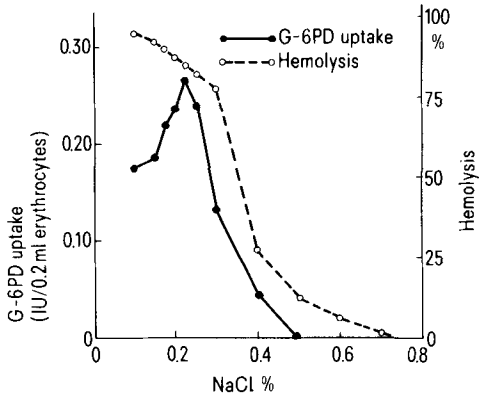


Fig. 1. Effect of NaCl concentration on G-6PD uptake by G-6PD-deficient erythrocytes during hypotonic hemolysis for 60 sec.

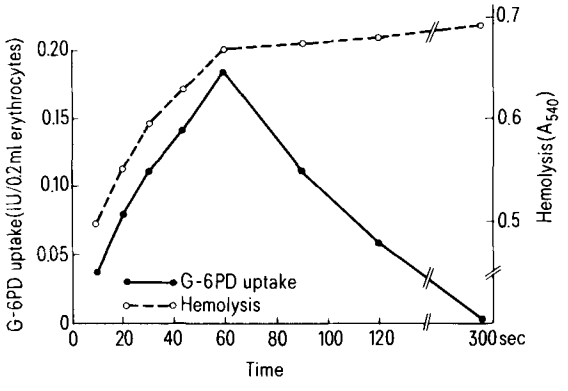


Fig. 2. Time-course of G-6PD uptake by G-6PD-deficient erythrocytes during hypotonic hemolysis in NaCl 0.20%.

G-6PD uptake by G-6PD-deficient erythrocytes after hypotonic hemolysis

| NaCl (%) | 0.100 | 0.150 | 0.175 | 0.200 | 0.225 | 0.250 | 0.300 | 0.400 | 0.900 |
|---|---|---|---|---|---|---|---|---|---|
| G-6PD activity (IU/ml) | | | | | | | | | |
| Added | 33 | 33 | 33 | 33 | 33 | 33 | 33 | 33 | 33 |
| Recovered | 4.45 (14%) | 5.67 (17%) | 6.72 (20%) | 7.28 (22%) | 8.15 (25%) | 7.35 (22%) | 3.30 (10%) | 1.10 (3%) | 0 0 |
| Hemolysis (%) | 95 | 92 | 90 | 87 | 85 | 82 | 78 | 27 | 0 |

Some experimental data support the hypothesis that the added enzyme is really trapped into the cells. There is no enzyme activity when the loading procedure is carried out at NaCl concentrations between 0.40 and 0.90% where negligible or no hemolysis occurs. The loaded enzyme is not washed out by 5 centrifugations of the cells, but is released only when erythrocytes are lysed.

Additional evidence of enzyme trapping comes from the behavior of GSH in loaded red cells after incubation with acetylphenylhydrazine. The percent fall of GSH in deficient cells after incubation with acetylphenylhydrazine amounted to 64%, while the value for G-6PD loaded erythrocytes was 44%.

Thus we conclude that, in accordance with what was shown in previous reports with other enzymes[1-7], G-6PD also enters through the red cell membrane pores during hypotonic hemolysis, remains inside when tonicity is corrected to normal value, and is functionally active in reducing glutathione.

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## Lipid composition of *Microsporum gypseum*

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*Summary.* The lipid composition of *Microsporum gypseum* has been studied. The lipids amounted to 10.1% and phospholipids to 1.1% of the mycelial dry weight. Phosphatidyl choline, phosphatidyl serine and phosphatidyl ethanolamine were the major components, while lysophosphatidyl choline, and phosphatidyl inositol were present in smaller quantities. Neutral lipids consisted of monoglycerides, diglycerides, triglycerides, free and esterified cholesterol.

Lipids are vital constituents of biological membranes and have been involved in allergic responses[2]. The fatty acids of some dermatophytes, a group of fungi causing superficial infections, have been investigated[3,4], whereas other lipid classes have received limited attention. Among the classes of lipids, phospholipids of the genus *Trichophyton* have recently been examined[5,6] while no such report is available on the genus *Microsporum*. Therefore, it was of interest to conduct a quantitative study on the lipid classes of *M. gypseum. Materials and methods. M. gypseum* isolated from a human case of dermatophytosis were grown at room temperature in Sabouraud's dextrose broth, pH 5.4–5.6. Cells were grown as stationary cultures for 4 weeks. Mycelia were harvested after autoclaving by filtration. The washed cells were dried at 50 °C to constant weight. Extraction and purification of lipids were done as described previously[7]. Total lipids were quantitated gravimetrically. The purified lipids were fractionated into phospholipids and neutral lipids by silicic acid column chromatography. The phospholipid classes were analysed by 2 dimensional TLC[8] and quantitated as described[9,10]. The separation, isolation, characterization and quantitation of neutral lipids were done as detailed earlier[11]. Alkaline hydrolysis was carried out as reported previously[7].

*Results and discussion.* The total chloroform-methanol soluble fraction of *M. gypseum* amounted to 10.1% and phospholipids to 1.1% of the mycelial dry weight. The total phospholipids of *M. gypseum* gave 8 components on TLC with iodine vapors. The 5 major components were identified as lysophosphatidyl choline, phosphatidyl choline, phosphatidyl inositol, phosphatidyl serine and phosphatidyl ethanolamine. The presence of these phospholipids was confirmed by cochromatography of the isolated lipids in different solvent systems. Identification was further substantiated by paper chromatography of the water-soluble products obtained after mild alkaline hydrolysis of these components along with authentic standards. In addition, 3 more phospholipid fractions were present only in minor amounts and were not characterized. It is evident from table 1 that phosphatidyl choline, phosphatidyl serine and phosphatidyl ethanolamine are the major phosphatides of *M. gypseum.* Chromatography of the neutral lipids revealed the presence of free and esterified cholesterol, triglycerides, diglycerides and monoglycerides. It is clear from table 2 that triglycerides represent a major fraction of the quantitated neutral lipids.

Almost all of the major classes of lipids were present in *M. gypseum,* but the values for total lipids and phospholi-