The Effect of Flavin Coenzymes on the Activity of Erythrocyte Enzymes

The recent letter from GLATZLE et al.[1] regarding the effect of flavin-adenine-dinucleotide (FAD) on red cell glutathione reductase activity has lead us to re-examine the effect of both FAD and of flavin-adenine-mononucleotide (FMN) on the activity of many different enzymes in hemolysates prepared from normal subjects. We have found that when added to normal hemolysates prior to NADPH or GSSG as little as 0.020 μM FAD strongly stimulates the activity of glutathione reductase. Activity of this enzyme was stimulated when either NADPH or NADH served as substrate. NADH-methemoglobin reductase is also strongly activated by FAD, activity as measured with the methemoglobin-ferrocyanide complex[2] but only slightly when dichloroindophenol[3] is used as an acceptor. No stimulation of hexokinase, phosphoglucose isomerase, phosphofructokinase, aldolase, triosphosphatase isomerase, phosphoglycerate kinase, pyruvate kinase, glucose-6-phosphate dehydrogenase, phosphogluconic dehydrogenase, phosphoglucomutase, or NADPH-methemoglobin reductase could be observed.

The mechanisms by which FAD produced stimulation of glutathione reductase and of NADH-methemoglobin reductase appeared to be different. In the case of glutathione reductase, incubation of the enzyme with FAD produced essentially irreversible stimulation of enzyme activity: dialysis or subsequent dilution of the enzyme-FAD mixture resulted in no loss of enzymatic activity. The administration of 5 mg riboflavin daily to normal subjects resulted in a marked increase of glutathione reductase activity within 2 days. In contrast, although even 0.001 μM FAD stimulated red cell NADH-methemoglobin reductase activity approximately 2-fold, it was necessary for FAD to be present at an adequate concentration in the assay system. If the mixture of hemolysate and FAD was diluted so that the FAD concentration dropped to an ineffective level, the stimulation which was readily observed when FAD was present in the assay system could no longer be detected. The addition of FAD to a hemolysate from a patient who has severe congenital methemoglobinemia due to NADH-diaphorase deficiency produced slight stimulation of the residual enzyme activity, but the degree of stimulation was no greater than that observed with normal samples. Administration of riboflavin to normal subjects failed to affect the activity of the enzyme in vivo.

It is of interest that of all of the red cell enzymes studied only the two which are concerned with the oxidation of nicotinamide-adenine-dinucleotides require flavin compounds. This is consistent with the classical role of flavins in other tissues as coenzymes for the oxidation of reduced pyridine nucleotides[4].

Zusammenfassung. Aktivitätsmessung von 14 Erythrozytenenzymen normaler Personen mit und ohne Hinzufügung der Flavinenzyme Flavinmononukleotid (FMN) und Flavinadenindinukleotid (FAD). Keines der untersuchten Enzyme wurde durch FMN aktiviert, während jedoch FAD die Glutathione Reduktase und die NADH Methämoglobin Reduktase stimulierte.

E. BEUTLER

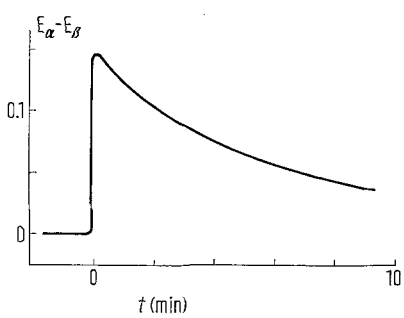
City of Hope Medical Center,
Department of Hematology,
Duarte (California 91010, USA), 2 May 1969.

[1] D. GLATZLE, F. WEBER and O. WISS, Experientia *24*, 1122 (1968).
[2] E. HEGESH, N. CALMANOVICI and M. AVRON, J. Lab. clin. Med. *72*, 339 (1968).
[3] E. M. SCOTT, J. clin. Invest. *39*, 1176 (1960).
[4] This work was made possible by a supporting fund established in the name of the Barry T. Leithead Research Fellowship, and was supported, in part, by Public Health Service Grant No. HE 07449 from the National Heart Institute, National Institutes of Health. I thank E. WISE and A. MEUL for their technical assistance.

Difference in Iron Release by α-Ferritin and β-Ferritin

Ferritin, a well defined iron storage protein, is readily fractionated by electrophoresis into α ($\sim$80%), β ($\sim$15%), γ-, δ- and ε-Isoferritins[1]. α- and β-ferritin was isolated from commercial horse spleen ferritin by polyacrylamide gel electrophoresis (5%, pH 6.6) by eluting ferritin from appropriate segments of the gel. In some experiments ferritin was purified by ultracentrifugation ($2^1/_2$ h, 50,000 rpm, Beckman Type 50 Ti rotor); the sticky precipitate only was then used. The optical density of ferritin in the range of 600–400 nm is almost entirely due to the (FeOOH) core as indicated by comparison of the spectra of ferritin and apoferritin. The iron to protein ratio of α- and β-ferritin was compared by measuring the absorption at 420 nm and by protein determination according to LOWRY[2]; no significant differences between α- and β-ferritin were found.

α- and β-fractions were dialysed for 2 days against 3 changes of 10^3 volumes of distilled water. The solutions were adjusted to give an optical density of 1.0 against water at 420 nm and a difference of less than 0.005. 2 ml of a solution of α-ferritin were added to the sample cuvette of a Beckman-DB spectrophotometer and the same quantity of a solution of β-ferritin to the reference cuvette. To each cuvette 0.5 ml of acetate buffer (1 ml sodium acetate brought to pH 5.2 with 99% acetic acid) containing 6% sodium bisulfite was added; the recorder was started immediately. The wavelength remained constant. A typical curve is shown in the Figure.



(E_α–E_β) means the difference in optical density of α-ferritin sample and β-ferritin sample at 420 nm. At time $t = 0$ the sodium bisulfite was added to the samples.

[1] W. RICHTER and F. WALKER, Biochemistry, Wash. *6*, 2871 (1967).
[2] O. H. LOWRY, N. J. ROSENBROUGH, A. L. FARR and R. J. RANDALL, J. biol. Chem. *193*, 265 (1951).