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

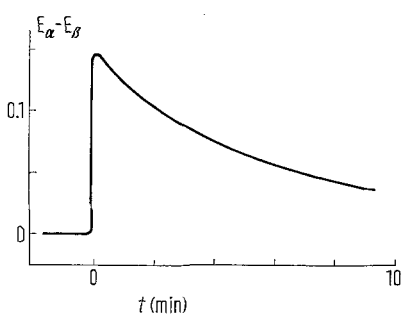
City of Hope Medical Center,
Department of Hematology,
Duarte (California 91010, USA), 2 May 1969.

[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.

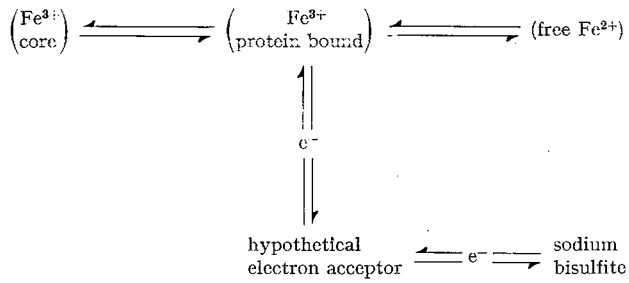


(E_α–E_β) means the difference in optical density of α-ferritin sample and β-ferritin sample at 420 nm. At time $t = 0$ the sodium bisulfite was added to the samples.

[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).

Whereas the shape of the curves was easily reproduceable, the absolute values of ΔE depended on the quality of the sodium bisulfite. The experiment shows the decoloration of β-ferritin to proceed more rapidly than that of α-ferritin.

Discussion. No difference in iron release between α- and β-ferritin would be expected if the sodium bisulfite reacted directly with the (FeOOH) core. It could be postulated, therefore, that sodium bisulfite reacts at first with a primary electron acceptor, possibly a specific electrophilic group in the protein moicty of ferritin. The overall reaction of ferritin with sodium bisulfite could then be written as



It is conceivable that the curve in the Figure is due to differences in the postulated primary electron acceptor.

One could argue that more iron release sites were available in the β-fraction due to a higher concentration of β-ferritin. This would indeed be the case if the iron content per molecule were lower in the β-ferritin than in the α-ferritin. This could be ruled out, however, by the demonstration of identical iron/protein ratios in the two molecules.

The work of JONES[3] suggests that delivery of Fe^{3+} by the (FeOOH) micelle follows first order kinetics. Hence in a hypothesis without primary electron acceptor a lower iron content of α-ferritin could not be made responsible for the slower iron release.

The observation of difference in iron release by α- and β-ferritin fits KOPP's[4] suggestion that the tendency of β-ferritin to incorporate iron may be more pronounced than that of α-ferritin. However, the biological significance of delayed iron uptake and release by α-ferritin is not understood.

Zusammenfassung. Es wurde beobachtet, dass α-Ferritin unter der Einwirkung von Natriumhydrosulfit sein Eisen langsamer abgibt als β-Ferritin. Diese Beobachtung lässt vermuten, dass das dreiwertige Eisen zunächst eine Bindung mit dem Eiweiss eingeht und erst dann durch einen primären Elektronenakzeptor reduziert wird. Ein Unterschied im primären Elektronenakzeptor zwischen α- und β-Ferritin könnte die verschieden rasche Eisenabgabe erklären.

W. NIEDERER

Institute of Medical Microbiology, University of Zürich, 8006 Zürich (Switzerland), 1 April 1969.

[3] M. N. JONES and O. D. JOHNSTON, Nature *216*, 509 (1967).
[4] R. KOPP, A. VOGT and G. MAASS, Nature *202*, 1211 (1964).

Nucleic Acid, Amino Acid, and Carbohydrate Metabolism of Nurse Cell Nucleoli in *Musca domestica*

The 15 highly polyploidisized nurse cell nuclei (NCN) in the polytroph meroistic egg follicles of *Musca domestica* (Figure 1a) develop a great number of nucleoli in the course of oogenesis. Probably the formation of these multiple nucleoli follows on principle the scheme described for the nucleolar apparatus in *Calliphora erythrocephala*[1]. The vital aspect of the NCN-nucleoli of *Musca* is demonstrated in Figure 1b by a Nomarski interference phase contrast micrograph. The NCN-nucleoli attain a maximal size on oogenetic stage 3b[2]. Their DNA-content is much lower than that of the remaining nuclear space. So ^{3}H-thymidine incorporation during s-phase of endomitotic polyploidization exhibit a labelling pattern, in which nucleoli stand out by a very low grain density[3]. Thus they are producing holes in the otherwise homogeneous high label attributed to the chromosomes (Figure 1c).

In this paper some data concerning the RNA-, protein-, and carbohydrate metabolisms of the trophocyte nucleoli will be presented.

Materials and methods. The flies were reared under standard conditions[4] at 21 °C. Females at stage 3 of oocyte development[2] were injected with 5 µl of an aqueous tracer solution containing 5 µC of ^{3}H-uridine (spec. act. 5 C/m*M*), ^{3}H-D-glucose (spec. act. 1.22 C/m*M*), or a mixture of ^{3}H-L-amino acids (valine, tyrosine, leucine, lysine, histidine in equal parts of 1 mC/ml, specific activity between 500 and 1000 mc/m*M*). After 15 or 30 min of incubation the ovaries were dissected, fixed in modified Carnoy's fluid or Gendre's fluid[5], embedded in paraplast, sectioned 10 µm thick, and prepared for autoradiography with Ilford liquid emulsion K.2 or Kodak stripping film AR10.

Results. RNA autoradiography. After 30 min of ^{3}H-uridine incorporation the NCN-nucleoli stand out from the remaining nuclear area by a stronger labelling in the autoradiographs (Figure 2a)[2, 6, 7]. After pretreatment with RNase no further labelling of nucleoli or other cellular components can be detected. By 30 min of ^{3}H-uridine incubation autoradiography demonstrates the precursor to incorporate exclusively into macromolecular RNA.

Protein autoradiography. Also by application of ^{3}H-amino acids (15–30 min), the multiple nucleoli can be identified as sites of the highest labelling in the nurse cells (Figure 2b). After 30 min the grain density of the nucleoli areas even exceeds that of the follicle epithelium, which has been shown to be the cell type with the highest protein turnover[2]. A pretreatment of the sections with RNase (Figure 2c) or diastase has no influence on the pattern and intensity of labelling compared with controls. Therefore it must be concluded that during 30 min of incubation autoradiography comprehends use of amino acids only for protein synthesis.

^{3}H-glucose autoradiography. The same pattern, with comparably high labelling intensity of the nucleoli as

[1] D. RIBBERT and K. BIER, Chromosoma, *27*, 178 (1969).
[2] K. BIER, Wilhelm Roux Arch. EntwMech. Org. *154*, 552 (1963).
[3] C. SCHOLZ, unpublished results.
[4] H. H. TREPTE, unpublished results.
[5] W. ENGELS, Acta Histochem. Suppl. 8, 323 (1968).
[6] K. BIER, J. Cell Biol. *16*, 436 (1963).
[7] K. BIER, Naturwissenschaften *51*, 418 (1964).
[8] Wir danken Herrn Prof. Dr. K. BIER für Überlassung der Figur 2a, Herrn C. SCHOLZ für die Figur 1c sowie Frau H. THIESIES für technische Assistenz.