The Turkish Journal of Pediatrics

A Quarterly Publication

VOLUME: 3 OCTOBER 1961 NUMBER: 4

ARTICLES

THE INORGANIC PYROPHOSPHATASE ACTIVITY OF ERYTHROCYTES IN FAVISM*

Güngör Ernişli, M.D. and Faolo Brunetti, M.D.*****

In recent years, an inherited metabolic defect has been demonstrated in erythrocytes from both primaquine-sensitive and favic patients.[1]

The most important features of the erythrocyte abnormality are:

1. a marked deficiency of glucose-6-phosphate dehydrogenase, (G-6-PD),[2,3]
2. the instability of reduced glutathione, (GSH),[3,4,5,6]
3. the susceptibility of the erythrocytes to form Heinz bodies when incubated with oxidating substances like acetylphenylhydrazine (APH).[3,7,8]

Minor biochemical alterations were described in several laboratories: some enzymes of the glycolytic pathway, like aldolase,[3,9,10] glyceraldehyde-3-phosphate dehydrogenase (GAPD)[3,9] transchetolase and transaldolase[11] were investigated and reported to have

* Paper read at the Second Middle Eastern-Mediterranean Pediatric Congress, Ankara, 1961.

** Resident, Ege University Faculty of Medicine, Department of Pediatrics, İzmir, Turkey.

*** Resident, Institute of Medical Pathology and Clinical Methodology, University of Perugia, Italy.

higher activities than normal; GSH-reducatase activity was also shown to be markedly increased.[12] Most of the enzymes of the glycolytic chain were studied and found to be within the normal range.[3,9,13] A decrease of catalase activity was recently pointed out by Tarlov and Kellermeyer;[14] further, a sharp fall of this enzyme was observed during the primaquine-induced hemolytic crisis [14] along with the decrease of GSH. Hemolytic crisis is also characterized by the diminution of the erythrocyte content in ATP.[15]

As the decrease of the G-6-PD activity in the hemolysates is the most characteristic defect discovered to date, the erythrocyte abnormality is generally referred to as G-6-PD deficiency.

The enzyme defect has a racial distribution, with the highest incidence among American negroes,[16] southern Italians - especially Sardinians[1] - and Sephardic Jews.[17] The erythrocyte abnormality is responsible for the susceptibility to hemolysis induced by many drugs and some vegetable foods: Primaquine,[16,18] Atabrine,[1] quinidine,[1,19] nitrofurans,[20] Phenacetin,[1,21] sulfonamides,[1,21,22] chloramphenicol,[1,23] trinitrotoluene,[1,24] etc. are able to produce acute hemolytic crisis in subjects affected by the G-6-PD deficiency. In Italy, Israel and other Mediterranean countries, the clinical picture evoked by the erythrocyte abnormality is more often represented by favism;[1] besides fava beans, peas are very occasionally responsible for the production of acute hemolytic episodes.[1]

As the G-6-PD deficiency represents the common biochemical feature of a group of anemias resulting from diverse causes, it was considered convenient to propose the unique denomination of "Enzyme - Deficiency Hemolytic Anemias" (Larizza,[25,26] Larizza et al.[1])

Even if the mechanism of the hemolysis induced by drugs and vegetables is still basically obscure, the available information suggests that the GSH deficiency and instability play an important role in the destruction of the erythrocytes in the enzyme-deficiency hemolytic anemias.

GSH is a protective factor for the erythrocytes preventing the transformation of hemoglobin into methemoglobin and sulfhemoglobin,[27,28] and protecting the SH - containing enzymatic and stromal proteins from the action of oxidating substances. Scheuch et al. [29] recently reported that hexokinase and GAPD from normal RBC were inactivated by incubation with APH, the phenomenon being explained on the basis of the sulfydryl structure of these enzymes. Moreover, Rapoport and Scheuch,[30] from the same laboratory,

showed that inorganic pyrophosphatase of rabbit reticulocytes was equally sensitive to the oxidating action of APH: much experimental data were recorded in the literature regarding the thiol nature of this enzyme.[31]

Our present intent is to report the results of some investigations on the inorganic pyrophosphatase activity of G-6-PD deficient erythrocytes during and apart from a hemolytic episode. Data will also be presented concerning the instability of inorganic pyrophosphatase in fabic erythrocytes.*

Material and Methods

Subjects were chosen among the male patients of the Cagliari University Hospital. They were grouped as follows:

1. 8 G-6-PD deficient individuals unaffected by hemolytic crisis,
2. 11 affected by an acute crisis of favism,
3. 10 examined between 7 and 10 days after the hemolytic episode.

In all of them the pyrophosphatase content of erythrocytes was determined. Samples of blood from the patients of the first group were also used to investigate the stability of fabic pyrophosphatase in comparison with the normal enzyme. All determinations were carried out on heparinized blood.

The methods for the G-6-PD and GSH determinations and for the evaluation of the GSH stability were described elsewhere.[3,5] An assay for the inorganic pyrophosphatase determination was used based on the measurement of the inorganic phosphorus split from a Na-pyrophosphate solution, in the presence of the enzyme. The methods, both for the preparation of the hemolysate and for the enzyme assay, were described in detail in previous papers.[31,32]

A pyrophosphatase stability test was devised, incubating G-6-PD deficient blood at 37 C. with proper amounts of APH. The optimum ratio for a discrimination of normal and enzyme-deficient subjects was found to be 2 mg. of APH per 1 ml. of whole blood.

Results

As is shown in table 1, the inorganic pyrophosphatase activity of G-6-PD deficient erythrocytes is decreased in comparison with the normal average value. However, normal and pathological values are partially interspersed at the lower limits of the normal area.

* More extensive reports on this subject were published in Acta Haematol.[31,32]

Patients investigated during an acute hemolytic crisis, and particularly at its beginning, show a marked decrease of the pyrophosphatase activity; soon after the cessation of the hemolytic episode, along with the reticulocyte crisis, the enzyme content of the RBC rapidly increases up to values 3 or 4 times higher than normal. (Table 1.)

TABLE 1

VALUES OF INORGANIC PYROPHOSPHATASE ACTIVITY

(NORMAL AVERAGE VALUE: 370.9 UNITS/10^9 R. B. C.)		
Enzyme deficient subjects	Fabic patients (in crisis)	Fabic patients (after crisis)
310	380	780
362	82	960
242	112	1030
178	242	778
155	10	921
392	43	1310
164	172	730
222	116	1500
—	175	1012
—	224	1210
—	241	—
Average	Average	Average
253.1	163.3	1023.1

The incubation experiments with APH indicate that pyrophosphatase from G-6-PD deficient erythrocytes is very unstable, being easily inactivated after 1 and 2 hours at 37 C. (fig. 1) Under the same conditions, normal pyrophosphatase does not show any diminution of its values.

Discussion

The results of our present investigation indicate that inorganic pyrophosphatase of G-6-PD deficient erythrocytes 1) has a lower activity than normal and 2) is unstable in the incubation test with APH. Moreover, pyrophosphatase 3) shows a critical fall of its values when the acute hemolytic process takes place.

The striking similarity of behavior between pyrophosphatase, on the one hand, and GSH on the other, is easily understood on the basis of the experimental data which prove the sulfydryl structure of the enzyme. Inorganic pyrophosphatase is the first SH-enzyme which is shown to decrease during the fabic hemolytic crisis; other SH-enzymes, like GAPD, lactic dehydrogenase (LD),[3,9,10] fructose-6-phosphate kinase (F-6-PK)[13] and glioxalase[33] were investigated and found

to have normal activities. It seems likely that an alteration of SH-depending enzymes may play an important role in the pathogenesis of hemolysis. Until now, determinations were all carried out, necessarily, in hemolysates but it is not inconceivable that the results obtained in these experimental conditions may not reflect the intracellular metabolic situation.

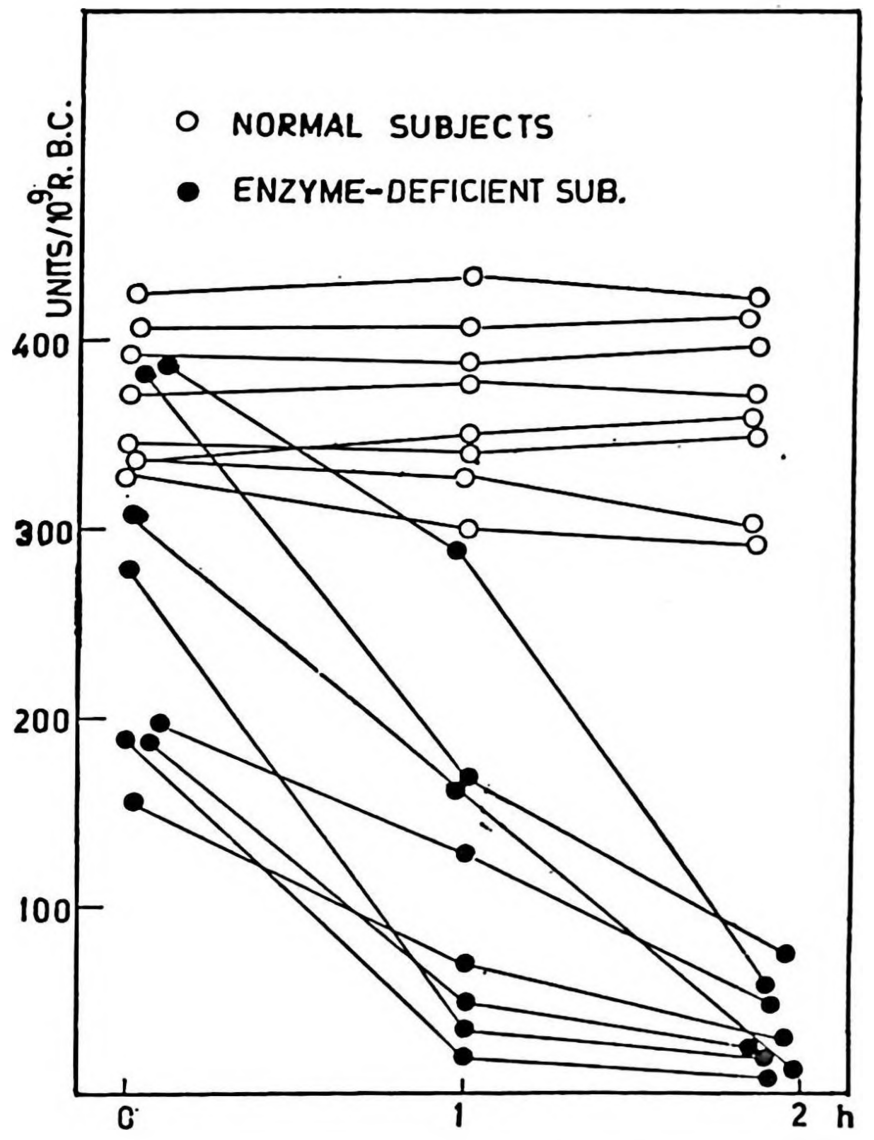

Fig. 1 — Inorganic pyrophosphatase level of erythrocytes before and after 1 and 2 hours of incubation at 37 C. with acetylphenylhydrazyne (2 mg./ml. of blood). (From Acta Haematologica.)

Many data are now available concerning the relation of SH-compounds to hemolysis. The importance of GSH in preventing the oxidation of hemoglobin and the formation of Heinz bodies was recently stressed by Allen and Jandl.[27] Rapoport et al.[29,30] testing the stability to APH of some SH-enzymes (hexokinase, GAPD, G-6-PD, pyrophosphatase), suggested the idea of a "self-stabilizing chain of enzymes" in the erythrocytes: the integrity of enzymatic sulfydryl

groups would be therefore one of the most important factors in the regulation of erythrocyte metabolism.[29] It was suggested by Aresu and Congiu[34] that the extractable stromal proteins of G-6-PD deficient erythrocytes have also a lower content of SH groups; they would also be oxidated during the hemolytic crisis of favism determining the formation of disulfide-linkages and the aggregation of normally distinguishable protein fractions.[34]

Our observation of a marked fall of the inorganic pyrophosphatase activity in patients affected by favism is a further proof of the existing interrelation between oxidation of sulfydryl groups and hemolysis. Moreover the instability of inorganic pyrophosphatase of G-6-PD deficient erythrocytes supports the idea that a primary alteration of SH-containing compounds has an important place in the fabic erythrocyte abnormality: it cannot be excluded that such instability of the free sulfydryl groups might also be the fundamental expression of the genic defect.

Summary

Inorganic pyrophosphatase activity of erythrocytes was found to be decreased in subjects affected by the glucose-6-phosphate dehydrogenase deficiency.

During the acute hemolytic episode of favism, pyrophosphatase activity shows a further critical fall.

Pyrophosphatase from glucose-6-phosphate dehydrogenase deficient erythrocytes is easily inactivated when RBC are incubated with proper amounts of acetylphenylhydrazine; such instability can be used for the detection of the glucose-6-phosphate dehydrogenase deficiency.

REFERENCES

1. Larizza, P., Brunetti, P., and Grignani, F.: Anemie Emolitiche Ensimopeniche, Haematologica 45:1, 1960.
2. Carson, P. E., Flanagan, C. L., Ickes, C. E., and Alving, A. S.: Enzymatic deficiency in primaquine-sensitive erythrocytes, Science 124:484, 1956.
3. Larizza, P., Brunetti, P., Grignani, F., and Ventura, S.: L'individualita bioenzimatica dell'eritrocito "fabico", Haematologica 43:205, 1958.
4. Beutler, E.: The glutathion instability of drug-sensitive red cells, J. Lab. and Clin. Med. 49:84, 1954.
5. Brunetti, P., and Grignani, F.: Il test di stabilita del glutatione nelle emazie dei fabici a distanza dalla crisi emolitica, Rassegna med. sarda 60:387, 1958.
6. Sansone, G., and Segni, G.: L'instabilita del glutatione ematico (GSH) nel favismo. Utilizzazione di un test selettivo. Introduzione al problema genetico. Boll. Soc. ital. biol. sper. 33:1057, 1957.

7. Aresu, G., Brunetti, P., Grignani, F., Matioli, G., and Ventura, S.: Sulla possibilita di provocare la comparsa nelle emazie dei fabici di abbondanti corpi di Heinz a distanza dalla crisi emolitica previa incubazione con acetilfenilidrazina. Rassegna med. sarda 60:365, 1958.
8. Beutler, E., Dern, R. J., and Alving, A. S.: The hemolytic effect of primaquine. VI. An in vitro test for sensitivity of erythrocytes to primaquine, J. Lab. and Clin. med. 45:40, 1955.
9. Grignani, F., and Brunetti, P.: Aldolasi, glicerinalde idefosfato-deidrogenasi e lattico-deidrogenasi eritrocitarie nel favismo, Rassegna med. sarda 60:423, 1958.
10. Schrier, S. L., Kellermeyer, R. W., Carson, P. E., Ickes, C. E., and Alving, A. S.: The hemolytic effect of primaquine. X. Aldolase and glyceraldehyde-3-phosphate dehydrogenase activity in primaquine-sensitive erythrocytes, J. Lab. and Clin. Med. 54:232, 1959.
11. Bonsignore, A., Fornaini, G., Segni, G., and Seitun, A.: Comportamento di alcuni enzimi eritrocitari di un soggetto favico nel periodo post-emolitico, Boll. Soc. ital. biol. sper. 36:1215, 1960.
12. Schrier, S., Kellermeyer, R., Carson, P., and Alving, A. S.: A second enzyme abnormality in primaquine-sensitive erythrocytes, J. Lab. and Clin. Med. 50: 951, 1957.
13. Brunetti, P., and Broccia, G.: Nuove acquisizioni in tema di bio-enzimologia del favismo ittero-emoglobinurico. Nota I. L'attivita enolasica, fruttochinasica e fosfofruttochinasica degli eritrociti. Rassegna di fisiopat. clin. e terap. 32: 202, 1960.
14. Tarlov, A. R., and Kellermeyer, R. W.: The hemolytic effect of primaquine. XI. Decreased catalase activity in primaquine-sensitive erythrocytes. J. Lab. and Clin. Med. 58:204, 1961.
15. Brunetti, P, and Grignani, F.: Il comportamento dell'ATP, dell'ADP e dell'AMP degli eritrociti nel favismo. Rassegna med. sarda 60:413, 1958.
16. Beutler, E.: The hemolytic effect of primaquine and related compounds: a review, Blood 14:103, 1959.
17. Szeinberg, A., Asher, Y., and Sheba, C.: Studies on glutathion stability in erythrocytes of cases with past history of favism or sulfa-drug-induced hemolysis, Blood 13:348, 1958.
18. Larizza, P., Brunetti, P., Grignani, F., and Ventura, S.: I fabici sono sensibili alla primachina. (Possibilita di provocare nei fabici l'insorgenza di episodi emolitici con la somministrazione di primachina). Minerva Med. 49:3769, 1958.
19. Brunetti, P., and Broccia, G.: Anemia emolitica acuta da chinidina in soggetto portatore delle stigmate bio-enzimatiche eritrocitarie proprie dei fabici. Boll. Soc. ital. biol. sper. 35:688, 1959.
20. Kimbro, E. L., Jr., Sachs, M. V., and Torbert, J. V.: Mechanism of the hemolytic anemia induced by nitrofurantoin (furadantin). Further observations on the incidence and significance of "primaquine-sensitive" red cells, Bull. Johns Hopkins Hosp. 101:245, 1957.
21. Dern, R. J., Beutler, E., and Alving, A. S.: The hemolytic effect of primaquine. V. Primaquine sensitivity as a manifestation of a multiple drug sensitivity, J. Lab. and Clin. Med. 45:30, 1955.

22. Grignani, F., Brunetti, P., and Sulis, E.: Contributo alla conoscenza del meccanismo patogenetico dell'anemia emolitica acuta da sulfamidici. Riforma Med.

23. Brunetti, P., Grignani, F., and Sulis, E.: Anemia emolitica acuta da cloramfeni colo in soggetti con eredopatie bioenzimatiche eritrocitarie, Haematologica 44:837, 1959.

24. Brunetti, P., and Grignani, F.: Genesi enzimopatica dell'anemia emolitica acuta da tritolo, Lavoro Umano 11:350, 1959.

25. Larizza, P.: Enzymopenische hamolytische Anamien, Ztschr. f. klin. Med. 156: 287, 1960.

26. Larizza, P.: Enzymopenische hamolytische Anamien. Referat zum VIII Tagung der deutschen Gesellschaft für Hamatologie, Wiesbaden 14-15. IV, 1961.

27. Allen, D. W., and Jandl, J. H.: Oxidative hemolysis and precipitation of hemoglobin. II. Role of thiols in oxidant drug action, J. Clin. Investigation 40:454, 1961.

28. Jandl, J. H., and Allen D. W.: Oxidative hemolysis and precipitation of hemoglobin: Heinz body anemias as an accelerated form of red cell aging, J. Clin. Investigation 39:1000, 1960.

29. Scheuch, D., Kahrig, C., Ockel, E., Wagenknecht, C., and Rapoport, S.: Role of glutathion and of a selfstabilizing chain of SH-enzymes and substrates in the metabolic regulation of erythrocytes, Nature, London 190: 631, 1961.

30. Rapoport, S., and Scheuch, D.: Glutathion instability and pyrophosphatase activity in reticulocytes: direct evidence for the importance of glutathion for the enzyme status in intact cells, Nature, London 186: 967, 1960.

31. Brunetti, P., Grignani, F. and Ernişli, G.: Behaviour of the erythrocyte pyrophosphatase activity in the Enzyme-Deficiency Hemolytic Anemias. I. Quantitative modifications of the enzyme, Acta Haematol., in print.

32. Brunetti, P., Grignani, F., and Ernişli, G.: Behaviour of the erythrocyte pyrophosphatase activity in the Enzyme-Deficiency Hemolytic Anemias. II. Proposal of a new test for the detection of the enzyme defect, Acta Haematol., in print.

33. Brewer, G. J.: quoted by Carson, P. E. (Federation Proc. 19: 995, 1960).

34. Aresu, G., and Congiu, L.: L'elettroforesi in fase libera delle proteine solubili, estraibili dagli stromi degli eritrociti di fabici in corso di crisi emolitica e a distanza da questa, Rassegna di fisiopat. clin. e terap. 22: 706, 1960.

35. Grignani, F., and Brunetti, P.: Weitere untersuchungen über die Eigenschaften des aktivieren den Faktors der Glucose-6-Phosphat-Dehydrogenase der menschlichen roten Blutkoerperchen bei Hämolytischen enzymopenischen Anaemien, Klin. wschr., in print.