GLUCOSE METABOLISM IN HUMAN ERYTHROCYTES FROM NORMAL AND FAVA BEAN-SENSITIVE SUBJECTS *

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Many studies during the last decade have been concerned with the biochemical properties underlying various states of the human red blood cell, including its maturation, aging, and some abnormal conditions.

A considerable amount of work has been devoted particularly to the study of erythrocyte metabolism in the fava bean- and drug-induced hemolytic anemias. Observations were first made by Beutler and his co-workers (1, 2) concerning the instability and decreased content of reduced glutathione (GSH) in red cells in drug-induced anemia. Carson (3) and Gross (4) and their colleagues, and Sansone and Segni (5, 6) demonstrated glucose-6-phosphate dehydrogenase (G6PD) deficiency in these cells. Further work has since been done in an attempt to find a link between the genetically determined metabolic defect and the hemolytic phenomenon.

Such studies have been concerned mainly with the activity and properties of various enzymes, and some work in this field has been done also in our laboratory (7-10). In the present paper a somewhat different approach is described. Glucose metabolism in fava bean-sensitive versus normal erythrocyte suspensions has been followed by quantitatively estimating the different metabolites that arise from its consumption *in vitro*. On these transformations, as well as on triose phosphate oxidation by hemolyzed red cells, the action of methylene blue (Mb) has also been studied.

METHODS

Subjects. Blood was obtained from seven men and two women from Sardinia, five of whom belong to the same

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family. Two of them had experienced acute hemolytic anemia (24 and 16 months ago, respectively) after fava bean ingestion. In all of them red cell G6PD was lacking, reduced glutathione was decreased, the Beutler test (2) was positive, and the reticulocyte count was normal. Normal subjects of both sexes and of approximately corresponding ages were used as controls.

Preparation of red blood cell suspensions. The blood was collected by venipuncture, and heparin added. After centrifugation the plasma and buffy coat were removed by suction, the red blood cells (RBC) were washed thrice with buffered isotonic KCl, pH 7.4, and then made up to volume. The packed cell volume was determined on this suspension by centrifuging in a hematocrit tube for 60 minutes at $1,200 \times G$. RBC suspensions were never completely free from leukocytes. The contamination, however, was always less than 0.05 per cent.

Hemolyzed preparations. These were prepared by treating the washed RBC with 2 vol of ice-cold water. The ghosts were removed by centrifuging for 30 minutes at $30,000 \times G$. The supernatant fluid was then diluted as required. All manipulations were performed at $+2^\circ C$.

Incubation and extraction. In the experiments concerning glucose metabolism in intact RBC, each reaction vessel contained, per milliliter: 10 μ moles of glucose; 30 μ moles of sodium phosphate buffer, pH 7.4; 0.43 ml RBC; and where required, 0.038 μ mole of Mb (unless otherwise stated). After 10 minutes' preincubation at $37^\circ C$ the reaction was started by adding glucose and was incubated at the same temperature with shaking at 80 cycles per minute. At the desired intervals 2.0-ml aliquots of the mixture were added to a centrifuge tube containing 4.0 ml of ice-cold 0.15 M KCl in 0.005 M NaF and spun down at once at $+2^\circ C$. The supernatant fluid was gently aspirated, and the RBC were washed twice with the KCl-NaF solution, then hemolyzed with 4 vol of cold water, and extracted with trichloroacetic acid (10 per cent final concentration). The clear fluid obtained by filtration was neutralized with NaOH and made up to volume.

For the experiments with fructose-1,6-diphosphate (F-1,6P) the incubation mixture contained per milliliter: 3 μ moles of F-1,6P; 25 μ moles of pH 7.4 glycylglycine buffer; 15 μ moles of nicotinic amide; 50 μ moles of Na_2AsO_4 ; 3 μ moles of $MgSO_4$; 0.10 μ mole of DPN; 0.1 ml of hemolyzed cells corresponding to 0.03 ml of RBC; and Mb at the required concentration. After incubation in a water bath at $37^\circ C$, 2.0-ml aliquots were

TABLE I
Metabolite concentrations in erythrocytes from normal and favic human subjects *

Subjects	Glucose-6-phosphate	Fructose-6-phosphate	Fructose-1,6-diphosphate	Heptose	Pentose-5-phosphate	Nucleotide pentose	Triose phosphate	Pyruvic acid	Lactic acid
Normal	8.3 ± 0.7	3.6 ± 0.4	12.5 ± 1.1	2.4 ± 0.4	1.8 ± 0.3	380.8 ± 18.6	11.5 ± 2.0	9.3 ± 1.0	84.0 ± 7.8
Favic	7.9 ± 0.6	3.8 ± 0.4	11.8 ± 1.2	2.1 ± 0.5	2.2 ± 0.5	396.4 ± 20.3	12.2 ± 2.3	9.8 ± 1.2	76.2 ± 8.1

* Values reported represent means ± SE of 10 determinations on normal subjects and 12 determinations on favic subjects. All values in $\mu\text{moles}/100\text{ ml RBC}$ (packed cell volume).

removed, extracted with trichloroacetic acid (10 per cent final concentration), filtered, and neutralized with NaOH.

Chemical determinations. Glucose was determined by means of glucose oxidase (11); glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) by the spectrophotometric technique (12) without and with added phosphohexose isomerase; and F-1,6P and triosephosphate (TP) according to Slater (13) with and without added aldolase. Heptose was estimated by the cysteine- H_2SO_4 procedure (14); lactic acid by the Barker method (15); and total pentose and pentose-5-phosphate by a modified Bial's reaction (16).

Pyruvic acid was determined by means of lactate dehydrogenase (17), whereby the quantitative oxidation of DPNH was followed spectrophotometrically and lactate formation colorimetrically. The mixture contained per milliliter: 0.5 ml of neutralized trichloroacetic acid extract (diluted if required); 0.1 ml of 0.001 M DPNH; 0.39 ml of 0.25 M glycylglycine buffer, pH 7.6; 0.01 ml

of lactate dehydrogenase. The mixture was either prepared in a spectrophotometer cuvet and the decrease in absorbance at 340 $m\mu$ recorded, or incubated at 37° C for 1 hour and lactic acid determined thereafter by the usual method. Results were the same.

For nucleotide estimation the acid extract was adsorbed on Norite A. Pentose was redetermined on the filtrate by the orcinol reaction and the values for nucleotide pentose obtained by difference.

Calculations. For experiments with intact cells the results are expressed in micromoles per milliliter of the extracellular and intracellular water. The latter was assumed to be 65 per cent of the packed cell volume (18). The data reported are the means of nine experiments on favic subjects and eight on controls. Six experiments were performed on hemolysates from normal and favic subjects. The mean values reported correspond to 1 ml of intact red blood cells.

Reagents. Glucose was a product of Merck and Co.;

FIG. 1. GLUCOSE UPTAKE BY SUSPENSIONS OF RED BLOOD CELLS FROM NORMAL AND FAVA BEAN-SENSITIVE SUBJECTS. Assay mixture as in Table II.

FIG. 2. FREE GLUCOSE ACCUMULATION IN RED BLOOD CELLS FROM NORMAL AND FAVA BEAN-SENSITIVE SUBJECTS. Assay mixture as in Table II. In experiments with Mb its final concentration was 3.8×10^{-5} M.

F-1,6P (sodium salt) and DPN were purchased from the Sigma Chemical Co. All enzymes used for spectrophotometric determination were products of Biochemica Boehringer.

RESULTS

Concentration of metabolites. The data concerning concentrations of some metabolites ($\pm$ SE) in normal and fava RBC are reported in Table I. No significant difference is found between the two groups with respect to these compounds, which,

determined throughout incubation, account for about 100 per cent of the carbon transformed.

Glucose metabolism by suspensions of red blood cells. Glucose uptake by normal and fava RBC suspensions at increasing concentrations of Methylene blue is shown in Figure 1. In the absence of Mb, glucose disappearance from the medium is only slightly decreased in experiments with abnormal as compared with normal cells. When the dye is added, glucose uptake by the normal RBC increases progressively, as described by Brin and Yonemoto (19), whereas in the fava cells a "paradoxical" inhibition is found.

On the other hand, if we turn to the intracellular glucose concentration (see Figure 2) we can see that, whereas Mb is ineffective, the initial accumulation rate of the free sugar is considerably higher in fava cells: at 30 minutes' incubation the amount is increased about fourfold. In this figure and in Tables II-VI Mb concentration was 3.8×10^{-5} M.

In striking contrast to free glucose, the concentration of G6P and F6P never increased during incubation; on the contrary, a depletion with respect to the values at zero time was often observed. Glucose phosphorylation seems therefore to be the limiting step in glucose utilization by RBC suspensions.

The data concerning free ¹ and nucleotide pen-

¹ In experiments in which both total pentose and pentose-5-phosphate were determined, the values have always been equal; the former was omitted thereafter.

TABLE II
Pentose phosphate and heptose synthesis in normal erythrocytes *

Incubation time	Pentose-5-phosphate		Nucleotide pentose†		Heptose	
	Without Mb	With Mb†	Without Mb	With Mb†	Without Mb	With Mb†
$\mu\text{moles/ml intracellular water}\S$						
10 min	0.28	0.70	0	0	0.01	0.07
30 min	0.42	1.36	0	0	0.04	0.22
1 hr	0.65	1.70	0.11	0.20	0.04	0.33
2 hr	0.78	2.80	0.25	0.29	0.02	0.45
4 hr	1.52	3.92	0.35	0.40	0	0.66
6 hr	1.65	4.12	0.52	0.54	0	0.72

* The incubation mixture contained per ml: 10 μmoles of glucose; 30 μmoles of phosphate buffer, pH 7.4; 0.43 ml of RBC (packed cell volume). The reaction vessel was incubated at 37° C with shaking. A 10-minute preincubation was performed before glucose addition, in order to insure temperature equilibration. Values reported are means of at least 6 experiments.

† Final concentration, 3.8×10^{-5} M.

‡ Nucleotide pentose synthesis was statistically significant: $p < 0.05$ at 1 hour and < 0.001 afterward.

§ Intracellular water was assumed to be 64 per cent of the packed cell volume.

TABLE III
Pentose phosphate and heptose synthesis in favic erythrocytes *

Incubation time	Pentose 5-phosphate		Nucleotide pentose		Heptose	
	Without Mb	With Mb	Without Mb	With Mb	Without Mb	With Mb
<i>μmoles/ml intracellular water</i>						
10 min	0.27	0.31	0	0	0	0
30 min	0.40	0.48	0	0	0.03	0.03
1 hr	0.68	0.73	0.10	0.12	0.03	0.03
2 hr	0.88	0.95	0.22	0.20	0.03	0.04
4 hr	1.48	1.50	0.42	0.45	0	0.03
6 hr	1.87	2.00	0.58	0.61	0	0

* Conditions and other indications are as in Table II.

tose, as well as heptulose synthesis, are summarized in Tables II and III.

No difference can be detected between the two groups of subjects in the absence of Mb. The formation of pentose and heptulose, which takes place in the normal erythrocyte mainly through the oxidative pathway (20), proceeds at a normal rate in the sensitive cells; the impaired function of the oxidative shunt is apparently compensated completely in this respect, as previously demonstrated (9, 10), by the high transketolase (TK) and transaldolase (TA) activities, which bring about the synthesis of the same compounds by means of nonoxidative reactions. This mechanism, however, is unaffected by Mb, which actually stimulates pentose and heptulose formation in normal, but not in favic, red blood cells.

In Table IV the pattern of F-1,6P and TP concentration is reported. Two features seem of interest: 1) In the absence of Mb both sugars accumulate in the cells during the first hour of incubation, and disappear thereafter; 2) when the dye is added, F-1,6P and TP invariably become

undetectable. No explanation is offered so far for point 1; point 2 is discussed below.

Pyruvic and lactic acid have been determined both in the cells and in the supernatant fluid of the reaction mixture. The values obtained are shown in Tables V and VI.

The time course of pyruvate synthesis is similar in normal and favic cells and is accelerated by Mb. At all incubation times after 10 minutes, extracellular pyruvate is in higher concentration than is intracellular pyruvate. Lactic acid behaves in a similar way, although the concentration difference is smaller and delayed in time. Lactate synthesis in favic RBC is slightly lower than in normal cells. The different concentration of both compounds in the extracellular and intracellular water still awaits a full explanation.

As a check of our follow-up of glucose fate during erythrocyte glycolysis we have reported the carbon recovery values in Table VII. The values shown are the ratios between the sum of carbon microatoms recovered at each incubation time as F-1,6P, TP, pentose, heptulose, pyru-

TABLE IV
Fructose-1,6-diphosphate and triose phosphate synthesis in normal and favic erythrocytes *

Incubation time	Normal subjects				Favic subjects			
	Fructose-1,6-diphosphate		Triose phosphate		Fructose-1,6-diphosphate		Triose phosphate	
	Without Mb	With Mb	Without Mb	With Mb	Without Mb	With Mb	Without Mb	With Mb
<i>μmoles/ml intracellular water</i>								
10 min	0.09	0.02	0.16	0.06	0.09	0	0.15	0
30 min	0.25	0.03	0.73	0.08	0.29	0	0.52	0
1 hr	0.39	0.04	0.83	0.08	0.39	0.02	0.89	0
2 hr	0.25	0.01	0.42	0	0.30	0	0.73	0.03
4 hr	0	0	0.03	0	0.09	0	0.02	0
6 hr	0	0	0	0	0	0	0	0

* Conditions and other indications are as in Table II.

TABLE V
Pyruvate synthesis in normal and fava erythrocytes *

Incubation time	Normal subjects				Favic subjects			
	Extracellular pyruvate		Intracellular pyruvate		Extracellular pyruvate		Intracellular pyruvate	
	Without Mb	With Mb	Without Mb	With Mb	Without Mb	With Mb	Without Mb	With Mb
	$\mu\text{moles/ml}$		$\mu\text{moles/ml}$		$\mu\text{moles/ml}$		$\mu\text{moles/ml}$	
10 min	0.03	0.28	0.02	0.04	0.03	0.30	0.02	0.03
30 min	0.08	0.46	0.04	0.10	0.07	0.52	0.03	0.09
1 hr	0.22	0.75	0.07	0.13	0.19	0.63	0.08	0.12
2 hr	0.30	0.70	0.10	0.15	0.31	0.75	0.11	0.16
4 hr	0.45	1.40	0.13	0.20	0.43	1.32	0.12	0.19
6 hr	0.66	1.65	0.14	0.22	0.64	1.56	0.15	0.21

* Conditions and other indications as in Table II.

vic and lactic acid, and the glucose carbon microatoms disappeared at the same time.

It is clearly seen that in the absence of Mb, in normal as well as in favic RBC, the compounds determined account for the whole of the glucose uptake; there is no "carbon leakage." Addition of the dye, by stimulating the oxidative pathway, brings about CO_2 evolution in normal cells, and the carbon recovery values are thus lowered; the behavior of abnormal cells remains unaffected because of the G6PD deficiency.

F-1,6P metabolism in hemolysates. The action described above of Mb on F-1,6P and TP synthesis, as well as the pyruvate to lactate ratios, suggested a possible effect of the dye on TP oxidation. This was tested on hemolyzed cells, which were incubated with F-1,6P in an arsenolytic system; at given times the diester consumption and the TP, pyruvate, and lactate formed were estimated.

The figures obtained were linear with time in each experiment. In Figure 3 the 60-minute

values concerning normal and favic cases are plotted as a function of Mb concentration, varying from 1.2 to 12×10^{-5} M. It is clearly seen that in normal hemolysates the dye enhances F-1,6P breakdown as well as pyruvate and lactate formation. In favic hemolysates the amount of F-1,6P metabolized is decreased. At a low concentration Mb addition results in a slight activation of TP conversion into pyruvate and lactate, F-1,6P consumption being unaffected. At higher concentrations an inhibitory effect is observed.

DISCUSSION

The data presented seem to give some insight into the different characteristic features of glucose metabolism in favic red blood cells as compared with normal cells. Whereas in the absence of Mb only a slight decrease in the sugar consumption is found, addition of the dye results in definitely different patterns in the two kinds of cells. In normal RBC glucose uptake increases as a function of Mb concentration, whereas in favic

TABLE VI
Lactate synthesis in normal and fava erythrocytes *

Incubation time	Normal subjects				Favic subjects			
	Extracellular lactate		Intracellular lactate		Extracellular lactate		Intracellular lactate	
	Without Mb	With Mb	Without Mb	With Mb	Without Mb	With Mb	Without Mb	With Mb
	$\mu\text{moles/ml}$		$\mu\text{moles/ml}$		$\mu\text{moles/ml}$		$\mu\text{moles/ml}$	
10 min	0.30	0.51	0.44	0.56	0.32	0.37	0.24	0.26
30 min	1.06	1.22	1.21	1.40	1.38	1.00	0.87	0.78
1 hr	2.41	3.02	1.58	1.62	1.75	1.75	1.11	0.87
2 hr	5.29	5.53	1.72	1.66	4.25	4.18	1.57	1.42
4 hr	11.25	12.52	2.78	2.90	8.50	8.25	2.16	2.01
6 hr	14.65	15.31	5.21	5.47	13.50	12.75	4.30	4.48

* Conditions and other indications as in Table II.

RBC not only the usual catalytic activity fails, but an inhibitory effect appears. This has been described by Tarlov (21) in RBC from primaquine-sensitive subjects.

These findings may be interpreted fairly simply. It has been well known for many years that Mb stimulates oxygen uptake by the red cell (22, 23). It can now be stated that in normal cells Mb acts as an activator of the reoxidation of reduced triphosphopyridine nucleotide, formed by G6PD and 6-phosphogluconic dehydrogenase activities; this leads to an increased oxidative formation of pentulose phosphate, which in turn is recycled to G6P via TK and TA (see reference 24). Incomplete recovery of added carbon (owing to CO_2 evolution) and higher concentrations of some metabolites (like pentose phosphate and heptulose phosphate) are consistent with this interpretation, which is in agreement with findings of other authors (19, 25).

In favic cells the catalytic action of Mb fails, owing to lack of G6PD, and neither the synthesis of pentose and heptulose is enhanced, nor does the carbon leakage as CO_2 become apparent.²

² After this paper had been submitted a report appeared (J. Lab. clin. Med. 1961, 58, 715) in which experiments with erythrocytes from primaquine-sensitive subjects are described. In these cells also, pentose fails to accumulate in the presence of glucose and Mb.

TABLE VII

Recovery of added carbon during glucose metabolism in RBC suspensions from normal and favic subjects *

Incubation time	Per cent carbon recovery†			
	Normal subjects		Favic subjects	
	Without Mb	With Mb	Without Mb	With Mb
10 min	104	86	105	98
30 min	99	82	102	98
1 hr	103	85	99	98
2 hr	105	80	101	99
4 hr	96	79	101	99
6 hr	98	76	104	96

* Conditions and other indications as in Table II.

† Per cent ratio between microatoms of carbon recovered in the metabolites determined and microatoms of carbon in the glucose, which has disappeared.

These results could be approximately anticipated on the grounds of previous knowledge.

Further consideration of the experimental data suggests that a coenzyme-linked activation is exerted by Mb not only at the G6P, but also at the triose phosphate level, and this holds true for favic as well as for normal cells. This effect could explain the higher removal rate of F-1,6P and TP (which are assumed to be in aldolase and TP isomerase equilibrium, respectively) in intact erythrocytes, and has been reproduced clearly in a soluble hemolyzed system, where the reoxidation

FIG. 3. EFFECT OF METHYLENE BLUE ON FRUCTOSE-1,6-DIPHOSPHATE BREAKDOWN IN HEMOLYSATES FROM NORMAL AND FAVA BEAN-SENSITIVE RED BLOOD CELLS. The assay mixture contained per ml: 3 μ moles of F-1,6P, 25 μ moles of pH 7.4 glycylglycine buffer, 15 μ moles of nicotinic amide, 50 μ moles of Na_2AsO_4 , 3 μ moles of MgSO_4 , 0.10 μ mole of DPN, 0.1 ml of hemolyzed cells corresponding to 0.03 ml of RBC, and Mb at the concentration indicated. Lact. refers to lactic acid and Pyr. to pyruvic acid. Left, normal RBC. Right, fava bean-sensitive RBC. The curve marked F-1,6-P represents breakdown values.

FIG. 4. PATHWAYS OF DIPHOSPHOPYRIDINE NUCLEOTIDE OXIDATION AND REDUCTION IN THE RED CELL. GR: glutathione reductase; GAPD, phosphoglyceraldehyde dehydrogenase; LD, lactate dehydrogenase; MR, methemoglobin reductase. The last is the only Mb-sensitive system.

of the dehydrogenase-formed DPNH was the limiting step for TP metabolism. Three systems are known to oxidize DPNH in RBC: 1) lactic dehydrogenase, 2) glutathione reductase, and 3) methemoglobin reductase (see Figure 4). The last is better defined as a diaphorase which may use DPNH or TPNH as hydrogen donor and methemoglobin, cytochrome C or O_2 as acceptor (26). Besides, methemoglobin reductase differs from the other two in that it is Mb-sensitive, and this could explain the action of the dye. It may be recalled here that Stumpf (27) observed in plant tissues that Mb could replace pyruvate or acetic aldehyde as a DPN-coupled diaphorase activator during F-1,6P breakdown, whereas Wendel (28) reported in 1929 the Mb catalysis of lactate oxidation to pyruvate in RBC. As may be seen in Tables V and VI and Figure 3, we have found accordingly that the effect of Mb appears to be much more pronounced when pyruvate rather than lactate accumulation is considered. Furthermore, other experiments (29) have indicated that DPNH and TPNH oxidation, like TP oxidation, undergoes a concentration-dependent stimulation by Mb.

The evidence presented supports the conclusion that failure of F-1,6P and TP to accumulate in the erythrocytes during glucose consumption in the presence of Mb is due to the ability of the latter to act as a carrier between DPNH and O_2 . Contrary to the G6PD-linked effect, this action is also found in cells from fava subjects, at a concentration of 1.2×10^{-5} M; however, it is less

pronounced, presumably because of a reduced diaphorase activity (8). As to the inhibition observed at higher dye concentrations, no explanation can be offered at the moment; we can only note that it is analogous to the effect exhibited on glucose uptake.

The free glucose concentration in the erythrocyte is a function both of entry rate and of utilization rate. The finding of a higher intracellular glucose concentration in fava cells as compared with the normal is leading us to a closer consideration of the sugar transport process, which might well be involved in the peculiar abnormality of fava RBC. Studies are now in progress in our laboratory concerning the entry pattern of various metabolizable and nonmetabolizable monosaccharides in normal and fava red cells.

SUMMARY

1. Glucose metabolism in intact normal and fava red cells has been studied by means of estimation of intracellular and extracellular concentrations of various metabolites during incubation.
2. In normal cells the addition of methylene blue brings about a change in the metabolite pattern, characteristic of a high operation rate of the hexose monophosphate oxidation pathway. This fails in the fava cells where, on the contrary, some inhibition of glucose uptake appears at high concentrations of dye.
3. Experiments with hemolyzed cells have shown that methylene blue increases the rate of breakdown of triose phosphate in fava as well as in normal erythrocytes. This explains some findings on intact cells, and the evidence presented points to a stimulated reoxidation of reduced diphosphopyridine nucleotide as the mechanism of this effect of the dye.
4. In spite of lower glucose consumption, the intracellular glucose concentration throughout incubation is always higher in fava than in normal cells.
5. The results are discussed in relation to the present knowledge of fava cell metabolism and the action of methylene blue.

REFERENCES

1. Beutler, E., Dern, R. J., Flanagan, C. L., and Alving, A. S. The hemolytic effect of primaquine. VII.

- Biochemical studies of drug-sensitive erythrocytes. *J. Lab. clin. Med.* 1955, **45**, 286.
2. Beutler, E. In vitro studies of the stability of red cell glutathione: A new test for drug sensitivity (abstract). *J. clin. Invest.* 1956, **35**, 690.
3. Carson, P. E., Flanagan, C. L., Ickes, C. E., and Alving, A. S. Enzymatic deficiency in primaquine-sensitive erythrocytes. *Science* 1956, **124**, 484.
4. Gross, R. T., Hurwitz, R. E., and Marks, P. A. An hereditary enzymatic defect in erythrocyte metabolism: Glucose-6-phosphate dehydrogenase deficiency. *J. clin. Invest.* 1958, **37**, 1176.
5. Sansone, G., and Segni, G. Prime determinazioni del glutatione (GSH) ematico nel favismo. *Boll. Soc. ital. Biol. sper.* 1956, **32**, 456.
6. Sansone, G., and Segni, G. Nuovi aspetti dell'alterato biochimismo degli eritrociti di favici: Assenza pressoché completa della glucoso-6-P deidrogenasi. *Boll. Soc. ital. Biol. sper.* 1958, **34**, 327.
7. 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.* 1960, **36**, 1215.
8. Bonsignore, A., Fornaini, G., Segni, G., and Fantoni, A. Glutathione-reductase and methemoglobin-reductase in erythrocytes of human subjects with a case history of favism. *Ital. J. Biochem.* 1960, **9**, 345.
9. Bonsignore, A., Fornaini, G., Segni, G., and Fantoni, A. Biosynthesis of pyridine coenzymes in erythrocytes from subjects with favism history. *Ital. J. Biochem.* 1961, **10**, 212.
10. Bonsignore, A., Fornaini, G., Segni, G., and Seitun, A. Transketolase and transaldolase reactions in the erythrocytes of human subjects with favism history. *Biochem. biophys. Res. Com.* 1961, **4**, 147.
11. Kaplan, N. O. Enzymatic determination of free sugars in *Methods in Enzymology*, S. P. Colowick and N. O. Kaplan, Eds. New York, Academic Press, 1957, vol. 3, p. 107.
12. Horecker, B. L., and Wood, W. A. D-glucose-6-phosphate. *Ibid.*, p. 152.
13. Slater, E. C. Spectrophotometric determination of fructose-1:6-diphosphate, hexosemonophosphates, adenosinetriphosphate and adenosinediphosphate. *Biochem. J.* 1953, **53**, 157.
14. Dische, Z. Qualitative and quantitative colorimetric determination of heptoses. *J. biol. Chem.* 1953, **204**, 983.
15. Barker, S. B. Preparation and colorimetric determination of lactic acid in *Methods in Enzymology*, S. P. Colowick and N. O. Kaplan, Eds. New York, Academic Press, 1957, vol. 3, p. 241.
16. Bonsignore, A., Conte, G., and Orunesu, M. Determination of pentose-5-phosphate in the presence of other pentoses. *G. Biochim.* 1952, **1**, 383.
17. Meister, A. Crystalline lactate dehydrogenase in *Biochemical Preparations*, E. G. Ball, Ed. New York, Wiley, 1952, vol. 2, p. 18.
18. Wintrobe, M. M. *Clinical Hematology*, 4th ed. Philadelphia, Lea & Febiger, 1956.
19. Brin, M., and Yonemoto, R. H. Stimulation of the glucose oxidative pathway in human erythrocytes by methylene blue. *J. biol. Chem.* 1958, **230**, 307.
20. Hiatt, H. H., and Laureau, J. Studies of ribose metabolism. VIII. Pathways of ribose biosynthesis *in vivo* and *in vitro* in rat, mouse, and human tissues. *J. biol. Chem.* 1960, **235**, 1241.
21. Tarlov, A. R. quoted by Carson, P. E. Glucose-6-phosphate dehydrogenase deficiency in hemolytic anemia. *Fed. Proc.* 1960, **19**, 995.
22. Harrop, G. A., Jr., and Barron, E. S. G. Studies on blood cell metabolism. I. The effect of methylene blue and other dyes upon the oxygen consumption of mammalian and avian erythrocytes. *J. exp. Med.* 1928, **48**, 207.
23. Moruzzi, G. Sul problema della posizione della glicolisi rispetto alla ossidazione del glucosio. Azione del monoiodacetato sodico sulla catalisi da blu di metilene. *Arch. Sci. biol. (Bologna)* 1936, **22**, 1.
24. Bonsignore, A. Carbohydrate interconversion reactions and their significance in intermediary metabolism. Lecture held at the Quintas Jornadas Bioquimicas Latinas, Barcelona, May 1959.
25. Szeinberg, A., and Marks, P. A. Substances stimulating glucose catabolism by the oxidative reactions of the pentose phosphate pathway in human erythrocytes. *J. clin. Invest.* 1961, **40**, 914.
26. Huennekens, F. M., Caffrey, R. W., Basford, R. E., and Gabrio, B. W. Erythrocyte metabolism. IV. Isolation and properties of methemoglobin reductase. *J. biol. Chem.* 1957, **227**, 261.
27. Stumpf, P. K. Carbohydrate metabolism in higher plants. III. Breakdown of fructose diphosphate by pea extracts. *J. biol. Chem.* 1950, **182**, 261.
28. Wendel, W. B. *Proc. Soc. exp. Biol. (N. Y.)* 1929, **26**, 865, quoted by Pranker, T. A. *J. in The Red Cell*. Oxford, Blackwell, 1961, p. 81.
29. Leoncini, G., and Fornaini, G. Unpublished results.