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EFFECT OF FACTORS OF FAVISM ON THE PROTEIN AND LIPID COMPONENTS OF RAT ERYTHROCYTE MEMBRANE

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Erythrocytes prepared from riboflavin- and tocopherol-deficient (RT^-) and from control rats were used to investigate the mechanism of oxidative hemolysis by the factors of favism. RT^- erythrocytes have a defense system against the oxidative stress which is blocked either where regeneration of GSH occurs or the scavenging of the radicals from the membrane is prevented. The oxidative factors used were isouramil, divicine and diamide. When RT^- erythrocytes were treated with isouramil, GSH decreased to undetectable levels and was not regenerated. Complete hemolysis occurred, but no oxidation of SH groups of membrane proteins or formation of spectrin polymers was detected. A similar effect was observed with diamide. However, SH groups of membrane proteins were completely oxidized and spectrin polymers were formed. Extensive lipid peroxidation was also detected together with a 30% fall in the arachidonic acid level. Control erythrocytes treated with either isouramil or diamide were not hemolyzed. When treated with isouramil, after a fall in the first few minutes, the GSH level was completely regenerated after 20 min. Incubation with diamide caused extensive oxidation of SH groups of membrane proteins and formation of spectrin polymers. No lipid peroxidation was detected after treatment with isouramil, but the same decrease of arachidonic acid occurred as in RT^- erythrocytes. These results support the hypothesis that oxidative hemolysis by the factors of favism is caused by uncontrolled peroxidation of membrane lipids.

Introduction

Several hypotheses have been proposed to explain the mechanism by which the erythrocyte hemolyzes when exposed to oxidative stress in vivo or in vitro. Experimental evidence has pointed to changes in hemoglobin or to membrane alterations. Hemoglobin appears to be the main site of damage when various oxidative drugs are used [1–3], with Heinz body formation and lysis of the erythrocyte. Under other oxidative conditions, the membrane appears to be the target of injury leading to hemolysis [4].

According to many authors, the uncontrolled oxidation of polyunsatured fatty acid residues, with formation of toxic hydroperoxides and other secondary products damaging the fine structure of the membrane, would cause the rupture of the cell [5–8]. Results obtained with tocopherol-deficient rats [9] demonstrated, however, that, when the erythrocytes are incubated in a hypertonic solution of GSH, lipid peroxidation is concurrent and does not precede the hemolysis.

Another hypothesis to explain the rupture of the erythrocytes in oxidative hemolysis is the increased rigidity of the cell membrane, caused by the formation of intermolecular disulfide bridges between protein molecules [10]. If this hypothesis is correct, GSH regeneration would be the main event protecting the cell from oxidative stress.

GSH, in fact, represents both the protection of the sulfide (SH) groups of proteins and the substrate for the detoxifying action of glutathione peroxidase.

Favism is a hemolytic disease occurring in glucose-6-phosphate dehydrogenase deficient individuals as a consequence of the ingestion of faba beans. The aglycones, isouramil and divicine, of the glycosides, convicine and vicine, isolated from faba beans have been recognized as the main active factors responsible for the hemolytic crisis [11].

In previous papers [12–14], we have shown that the erythrocyte of riboflavin- and tocopherol-deficient rats when treated in vitro with isouramil or divicine mimicked the response of the erythrocyte of subjects with favism. These erythrocytes are characterized by a lack of ability to regenerate GSH due to the riboflavin deficit and to scavenge the toxic radicals originating from membrane lipids due to tocopherol deficiency.

We have used the RT^- erythrocytes to study whether, and in which sequence, alterations of fatty acids and/or of proteins of the membrane are involved in the in vitro hemolysis caused by the factors of favism.

Materials and Methods

β-Glycosidase (EC 3.2.1.21, spec. act. 1000 units/mg material) from sweet almonds was purchased from Miles; vicine from Roth, reduced glutathione (GSH) and dithiothreitol from Sigma; butylhydroxytoluene and sodium dodecyl sulfate (SDS) from C. Erba – Italy; 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and thiobarbituric acid from Merck and 1,1,3,3-tetramethoxypropene from Fluka A.G.

Convicine was prepared from fresh faba beans by Dr. G. Zaza and diamide was synthesized by Dr. A. Gambacorta.

Animals. Male albino Sprague-Dawley rats (60–70 g) were kept in wire steel cages under controlled humidity and temperature and on a 12 h-12 h light-dark cycle. They were fed a tocopherol-free diet for 3 months and subsequently a tocopherol- and riboflavin-free diet for two more months or until the glutathione reductase (EC 1.6.4.2) activity of the erythrocytes was less than 10% of that of control animals. Control rats were fed for the same length of time the same diet to which tocopherol and riboflavin were added. The erythrocytes from rats in the double deficiency are defined as RT^-.

Preparation of isouramil and divicine. Convicine and vicine were dissolved at 10 mM in a phosphate-saline buffer (120 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 16.5 mM NaH_2PO_4/Na_2HPO_4 (pH 6.0), 5 mM glucose) at 60°C for 1 min. The solution was then cooled to 37°C and β-glycosidase added (500 units/mg convicine or vicine). The samples were incubated under nitrogen for 20 min at 37°C. Under these conditions, the hydrolysis of the glycoside was complete as assayed by glucose determination. The 10 mM supersaturated stock solution of convicine remains stable at the concentrations used (0–5 mM) during incubations of up to 1 h.

Treatment of blood. blood was taken by heart puncture in the presence of heparin, and erythrocytes were sedimented for 10 min at $1500 \times g$, at 0°C. Supernatant and buffy coat were aspirated and the erythrocytes washed three times with 4 vol. isotonic phosphate-saline buffer (pH 7.4) and then resuspended to a hematocrit value of 50% in the absence or in the presence of 0 to 5 mM isouramil, divicine or diamide. Incubations were carried out at 37°C under aerobic conditions for 0 to 60 min.

Preparation of erythrocyte ghosts. Ghosts were prepared from erythrocytes by hypotonic lysis of washed cells at 0–4°C in 30 vol. 10 mM Tris-HCl buffer (pH 7.4) followed by centrifugation for 30 min at $27000 \times g$ at 2°C with three washings [15].

The protein content of the ghosts was determined according to Lowry et al. [16].

GSH determination. GSH was assayed according to Beutler [17] on the supernatant, after precipitation of the proteins of the erythrocytes with metaphosphoric acid. The SH groups of GSH were reacted with DTNB and the product was assayed spectrophotometrically at 412 nm.

Assay of glutathione reductase. Glutathione reductase activity was determined according to Tillotson and Sauberlich [18], by measuring the rate of decrease of the absorbance of NADPH at 340 nm and 37°C with and without FAD addition

during 10 min, and is quoted as the coefficient of activity.

Hemolysis. The percentage of spontaneous hemolysis in the different samples of blood was determined after 1 h and 4 h incubation at 20°C in phosphate-saline buffer (pH 7.4) by dividing their extinction value at 410 nm in isotonic buffer by that of the completely hemolyzed samples in H_2O $\times 100$ [19]. The degree of hemolysis after incubation with the different compounds for 60 min was determined by measuring the concentration of hemoglobin in the supernatant after centrifugation of the erythrocytes [20].

Analysis of lipids. Lipids were extracted from the ghosts with chloroform/methanol (2 : 1, v/v) in the presence of butylhydroxytoluene. The samples were dried by evaporation under N_2 and methyl esters of fatty acids were then formed using 13 mg/ml of diphenylhydrazine in acidic methanol. Methyl esters of fatty acid and diphenylhydrazones were extracted with hexane. The hexane was evaporated and residues were dissolved in chloroform [21]. Aldehydes were eliminated as diphenylhydrazones by thin-layer silica gel chromatography. Methyl esters were recovered in chloroform, and analyzed on a 13% diethylene glycol succinate column with N_2 as carrier (Perkin Elmer Gas chromatographer F-30).

Lipid peroxides were measured as malonaldehyde by the modified 2-thiobarbiturate test [9]. To an aliquot of the erythrocyte suspension, trichloroacetic acid and thiobarbituric acid were added to a final concentration of 3.0% and 0.34%, respectively. The mixture was heated at 50–60°C for 15 min, cooled in ice and centrifuged 10 min at $500 \times g$. The absorbance of the supernatant was measured at 534 nm. The standard curve was prepared using malonaldehyde prepared from 1,1,3,3-tetramethoxypropane [22].

Polyacrylamide gel electrophoresis. The proteins of the ghosts solubilized in 2% SDS were fractionated by slab SDS-polyacrylamide-gel electrophoresis in 5% acrylamide in the absence or in the presence of dithiothreitol according mainly to the method of Laemmli [23].

Sulfide groups of membrane proteins. The SH groups of membrane proteins were assayed by measuring the increase of the absorbance at 412 nm, 1 h after the addition of 0.1 mM DTNB in 5 mM phosphate buffer (pH 8.0) to the suspension of washed ghosts solubilized in 1% SDS [24].

Results

Characteristics of the erythrocytes deprived of riboflavin and tocopherol

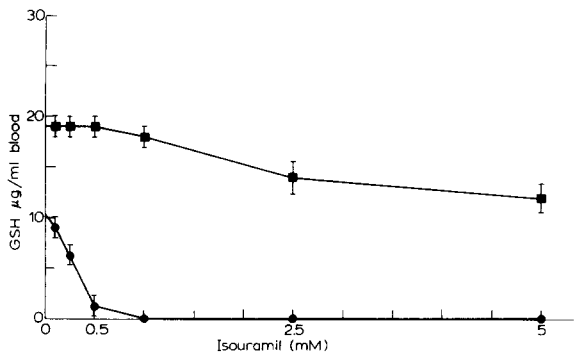
The GSH reductase activity of the RT^- erythrocytes was always less than 10% of control erythrocytes (taken as 100%). The RT^- erythrocytes after 1 h incubation in isotonic buffer were only 9% hemolyzed, but after 4 h they were 80% hemolyzed. However, control erythrocytes under the same conditions showed only a small hemolysis of 7% even after 4 h.

Fig. 1 shows the change in the amount of GSH (μg/ml blood) of the RT^- erythrocytes and of their controls, as a function of the concentration of isouramil added (0–5 mM). The amount of GSH of the control erythrocytes (19.0 μg/ml) remained constant at concentrations of 0.25–1 mM isouramil, and decreased to 12.0 μg/ml at 5 mM isouramil. In the RT^- erythrocytes, however, the amount of GSH was lower (10.5 μg/ml) even before isouramil treatment, decreased to 1.2 μg/ml at 0.5 mM isouramil and became undetectable at 1 mM isouramil.

In Fig. 2, the short-time effect (3–30 min) of 1 mM isouramil on GSH concentration of the RT^- erythrocytes is compared with that of controls. In the control erythrocytes, after a transient decrease in the first 3 min of incubation (from 19.0 μg to 14 μg/ml), the GSH concentration increased again and reached the starting level after 30 min. In RT^- erythrocytes after a sharp drop in the first 3 min of incubation, the GSH concentration continued to decrease and reached undetectable values after 30 min.

Effect of isouramil on SH groups of erythrocytes membrane proteins and on their electrophoretic pattern

In Table I the responses of intracellular GSH, SH groups of membrane proteins and the presence of membrane protein polymers of control and RT^- erythrocytes incubated for 60 min with 5 mM isouramil or diamide are reported. Whereas control erythrocytes treated with isouramil showed only a small drop in GSH concentration but no



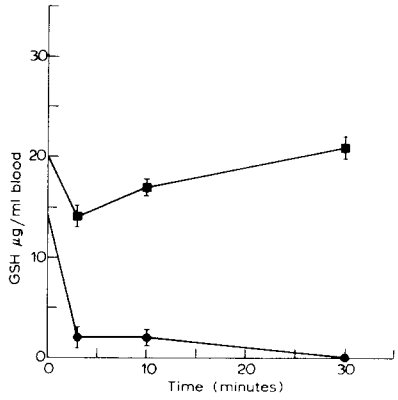


Fig. 1. Effect of increasing concentration (0–5 mM) of isouramil on the GSH content of control erythrocytes (■) and RT^- erythrocytes (●). Data represent the averages ± S.E. of at least four determinations. Incubation time was 60 min at 37°C. Same results were obtained at the same concentrations of divicine.

Fig. 2. Short time effect of 1 mM isouramil on the GSH concentration of control erythrocytes (■) and RT^- erythrocytes (●). Data represent the averages ± S.E. of at least four determinations.

modifications of the other parameters, RT^- erythrocytes showed a 20% drop in the SH groups of membrane proteins, a decrease to undetectable levels of GSH and complete hemolysis. Diamide, however, caused a drop of the SH groups to 30% of the initial value and of GSH concentration to an undetectable level in control erythrocytes, but no hemolysis occurred; the two parameters decreased to zero with complete hemolysis in RT^- erythrocytes treated with diamide.

In Fig. 3, the electrophoretic patterns of membrane proteins after incubation of the erythrocytes with and without isouramil or diamide are shown. 5 mM isouramil after 1 h incubation in the presence of oxygen did not cause any modification in control or in RT^- erythrocytes. Incubation of

TABLE I

EFFECT OF ISOURAMIL AND DIAMIDE ON GSH, PROTEIN STRUCTURE AND HEMOLYSIS OF RT^- AND CONTROL (C) ERYTHROCYTES

For determination of GSH see Fig. 1. The SH groups of membrane proteins were assayed by measuring the increase of the absorbance at 412 nm, 1 h after the addition of 0.1 mM DTNB in 5 mM phosphate buffer (pH 8.0) to the suspension of washed ghosts solubilized in 1% SDS. Hemolysis after 60 min incubation at 37°C with isouramil (5 mM) and diamide (5 mM) was determined by measuring the concentration of hemoglobin in the supernatant after centrifugation of the erythrocytes [20]. (0 = no Hb in the supernatant; 100 = all Hb in the supernatant). The figures represent the average value ± S.D. of at least four determinations.

Erythrocytes	GSH	SH groups	Protein oligomers	Hemolysis (%)
C	100 [a]	100 [c]	–	6
C + diamide	0	30 ± 4.2	+	7
C + isouramil	78 ± 8.2	100 [c]	–	7
RT^-	100 [b]	100 [c]	–	9
RT^- + diamide	0	0	+	100
RT^- + isouramil	0	80 ± 8.3	–	100

[a] 100% = 19.0 ± 1.3 µg/ml.
[b] 100% = 10.5 ± 1.2 µg/ml.
[c] 100% = 5.8 ± 0.5 µmol/mg protein of ghosts.

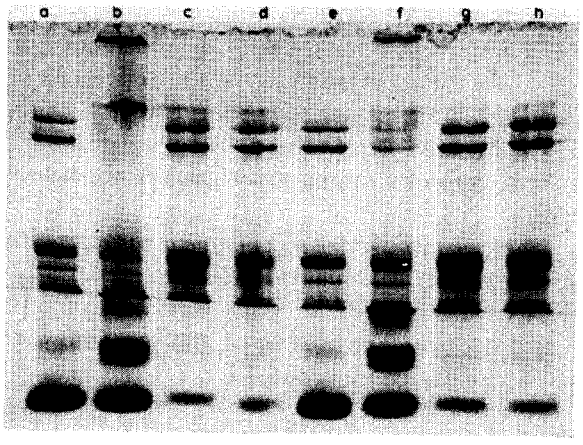


Fig. 3. Gel electrophoresis of membrane proteins. Erythrocytes were incubated with isouramil (5 mM) and diamide (5 mM) for 6 min at 37°C. Ghosts were prepared from washed and hemolyzed erythrocytes dissolved in 2% SDS and fractioned by SDS-polyacrylamide slab gel electrophoresis (5% acrylamide) in the absence or in the presence of dithiothreitol according mainly to the method of Laemmli [21]. Gels were stained with Coomassie blue. (a) RT^- incubated with no additives; (b) RT^- with diamide; (c) RT^- with isouramil; (d) RT^- with isouramil + dithiothreitol; (e) C with no additives; (f) C with diamide; (g) C with isouramil; (h) C with isouramil + dithiothreitol. Same results obtained with isouramil were obtained with 5 mM divicine.

erythrocytes with 5 mM diamide for 1 h under aerobic conditions resulted in the complete disappearance of monomeric spectrin and in the formation of oligomers in both kinds of erythrocytes. All aggregates disappeared in the presence of dithiothreitol.

TABLE III

EFFECT OF ISOURAMIL AND DIAMIDE ON LIPID PEROXIDATION IN CONTROL (C) AND RT^- ERYTHROCYTES

Lipid peroxides formed were measured in the trichloroacetic acid supernatant of the erythrocytes at 532 nm after reaction with thiobarbituric acid according to Brownlee et al. [9]. Data represent the means ± S.D. of at least four determinations.

Treatment and incubation time (min)	Malonaldehyde (nmol/g Hb)	
	Control	RT^-
0	3.63 ± 0.04	3.91 ± 0.02
60	4.00 ± 0.03	4.08 ± 0.02
60 + isouramil (5 mM)	4.62 ± 0.02	109.99 ± 1.09
60 + diamide (5 mM)	4.37 ± 0.05	87.32 ± 1.23

Effect of isouramil on the lipid moiety of the erythrocyte membrane

Table II shows the fatty acid composition of phospholipids prepared from ghosts of control and RT^- erythrocytes before and after incubation with 5 mM isouramil. Arachidonic acid was the only fatty acid exhibiting a marked decrease under the effect of isouramil in both control and RT^-

TABLE II

CONTENT OF THE MAJOR FATTY ACIDS OF THE ERYTHROCYTE GHOSTS BEFORE AND AFTER INCUBATION WITH ISOURAMIL

Lipids were extracted with chloroform/methanol and aldehydes were eliminated as diphenylhydrazones. Methyl esters of fatty acids in chloroform were analyzed on a 13% diethyleneglycol succinate column with N_2 as a carrier. − and + indicate that incubation was carried out without or with 5 mM isouramil. Data represent the average ± S.D. of three determinations, on ghosts prepared from pooled erythrocytes of four rats. Results identical to those obtained with 5 mM isouramil were obtained with 5 mM divicine.

Fatty acid	Content (weight %)			
	Control		RT^-	
	−	+	−	+
18:0	20.4 ± 0.5	20.6 ± 0.5	18.1 ± 0.4	21.1 ± 0.6
18:1	11.4 ± 0.3	14.5 ± 0.4	18.3 ± 0.4	21.0 ± 0.6
18:2	4.0 ± 0.2	4.2 ± 0.2	2.7 ± 0.2	3.0 ± 0.1
18:3	0.2 ± 0.02	0.4 ± 0.08	0.1 ± 0.04	0.1 ± 0.05
20:4	33.4 ± 1.2	20.5 ± 0.5	24.4 ± 0.8	16.1 ± 0.4

erythrocytes. This decrease was of the same order of magnitude for the two preparations (34.4% in control and 38.0% in RT^- erythrocytes); however, the basal level of arachidonic acid of the RT^- erythrocytes was lower than that in control erythrocytes.

Lipid peroxidation, as shown by malonaldehyde formation (expressed as nmol malonaldehyde formed per g hemoglobin in Table III), was dramatically increased in RT^- erythrocytes incubated with isouramil, but was unmodified in controls although the isouramil caused the same arachidonic decrease. The diamide treatment had a similar but less pronounced effect on lipid peroxides formation.

Discussion

In a previous paper [12], we showed that erythrocytes depleted of riboflavin when incubated with isouramil or divicine did not hemolyze even when GSH concentration had fallen to undetectable levels. However, hemolysis occurred when the erythrocytes incubated with isouramil or divicine were also depleted in tocopherol [13].

In this paper we present data indicating that the peroxidation of polyunsaturated fatty acids of the membrane represents the main event causing the hemolysis.

As a result of incubation with isouramil, a significant decrease of arachidonic acid of the erythrocytes membrane occurred in both control and RT^- erythrocytes. However, only RT^- erythrocytes (where the decrease of arachidonic acid was accompanied by a great production of malonaldehide) were seen to be hemolyzed. This result indicates that it is not lipid peroxidation per se that causes the hemolysis, but the lack of protection of the erythrocyte from peroxyl radicals formed during the oxidation of arachidonic acid and not destroyed or eliminated by the combined action of vitamin E and glutathione peroxidase.

The hypothesis of Johnson et al. [10], that the presence of polypeptide aggregates (resulting in decreased deformability) can be the cause of chronic hemolysis, is not supported by our results.

RT^- erythrocytes treated with isouramil hemolyze without any oxidation of SH groups or formation of spectrin polymers. Moreover, whereas the same erythrocytes treated with diamide hemolyze and show extensive oxidation of the SH groups of their membrane proteins with formation of aggregates, the same modifications of the proteins occur also in control erythrocytes which do not hemolyze.

These last results are in agreement with the data of Haest et al. [25] obtained in human erythrocytes treated with diamide in which formation of protein aggregates and decreased deformability are not accompanied by hemolysis.

A major manifestation of the action of oxidative drugs in vivo and in vitro is the formation of methemoglobin and the appearance of Heinz bodies. These bodies represent a product of hemoglobin denaturation resulting from the oxidation of its SH groups with concurrent formation of a mixed glutathione disulfide and loss of the heme group [26]. In our experiments, in all the cases in which there was hemolysis, the formation of Heinz bodies was the only modification of proteins common to all the situations in which the erythrocytes hemolyzed. With our data, however, it is not possible to establish whether the hemolysis is the consequence of the interaction of Heinz bodies with the stroma. Evidence has been obtained that enzymatically generated superoxide and H_2O_2 also caused hemolysis in circumstances in which formation of Heinz bodies was blocked [26].

From our results, it is possible to conclude that, while gross modifications of the proteins of the erythrocyte membrane are not involved in the in vitro hemolysis caused by the factors of favism, modifications of the fatty acid moiety of the membrane when the propagation of peroxyl radicals is not blocked play a substantial role. The interaction between the products of oxidation of hemoglobin, mainly Heinz bodies, and the cell membrane which has become more fragile as a result of the oxidative processes may represent the final event causing the hemolysis.

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References

1 Goldberg, B. and Stern, A. (1977) Arch. Biochem. Biophys. 178, 218–225

2 Hopkins, J. and Tudhope, G.R. (1974) Br. J. Clin. Pharmacol. 1, 191–195

3 Peisach, J., Blumberg, W.E. and Rachmilewitz, E.A. (1975) Biochim. Biophys. Acta 393, 404–418

4 Trotta, R.J., Sullivan, S.G. and Stern, A. (1981) Biochim. Biophys. Acta 679, 230–237

5 Barker, M.O. and Brin, M. (1975) Arch. Biochem. Biophys. 166, 32–40

6 Chow, C.K. (1979) Am. Clin. Nutr. 32, 1066–1081

7 Logani, M.K. and Davies, R.E. (1980) Lipids 15, 485–495

8 McLay, P.B. and King, M.M. (1981) in vitamin E (Machlin, L.J., ed.), pp. 289–317, Marcel Dekker, New York, Basel

9 Brownlee, N.R., Huttner, J.J., Panganamala, R.V. and Cornwell, D.G. (1977) J. Lipid Res. 18, 635–644

10 Johnson, G.J., Allen, D.W., Cadman, S., Fairbanks, V.F., White, J.G., Lampkin, B.C. and Kaplan, M.E. (1979) N. Engl. J. Med. 301, 522–527

11 Mager, J., Chevion, M. and Glaser, G. (1980) in Toxic Constituents of Plant Foodstuffs. Favism (Liener, I.E., ed.), pp. 265–294, Academic Press, New York

12 D'Aquino, M., Gaetani, S. and Spadoni, M.A. (1979) Nutr. Rep. Int. 20, 1–9

13 D'Aquino, M., Gaetani, S. and Spadoni, M.A. (1981) in Il problema del favismo in vista del potenziamento della produzione e del consumo della fava e dei suoi derivati, pp. 31–37, CNR, Roma, Italia

14 Arese, P., Bosia, A., Naitana, A., Gaetani, S., D'Aquino, M. and Gaetani, G.F. (1981) in The Red Cell (Brewer, ed.) Vol. 55, pp. 725–746, Alan R. Liss, New York

15 Liu, S.C., Fairbanks, G. and Palek, J. (1977) Biochemistry 16, 4066–4074

16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275

17 Beutler, E. (1975) in Red Cell Metabolism. A Manual of Biochemical Methods, pp. 103, Grune and Stratton, New York

18 Tillotson, J.A. and Sauberlich, H.E. (1971) J. Nutr. 101, 1459–1466

19 Draper, H.H. and Csallany, A.S. (1969) J. Nutr. 98, 380–386

20 Van Kampen, E.J. and Zijlstra, W.G. (1961) Clin. Chim. Acta 6, 538–542

21 Katz, I. and Keeny, M. (1966) J. Lipid Res. 7, 170–174

22 Philpot, J.St.L. (1963) Radiation Res. Suppl. 3, 55–70

23 Laemmli, U.K. (1970) Nature 227, 680–685

24 Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21–32

25 Haest, C.W.M., Kamp, D., Plasa, G. and Deuticke, B. (1977) Biochim. Biophys. Acta 469, 226–230

26 Kellog, E.W., III and Fridovich, I. (1977) J. Biol. Chem. 252, 6721–6728