Free Rad. Res. Comms., Vols. 12-13, pp. 137-146
Reprints available directly from the publisher
Photocopying permitted by license only

THE EFFECT OF FREE RADICALS INDUCED BY PARAQUAT AND COPPER ON THE *IN VITRO* DEVELOPMENT OF *Plasmodium falciparum*

ESTHER MARVA*, MORDECHAI CHEVION** and JACOB GOLENSER*

*The Departments of *Parasitology the Kuvin Centre for Tropical Diseases and **Cellular Biochemistry, The Hebrew University, P.O.B. 1172, Jerusalem, Israel*

The role of transition metals in paraquat toxicity was studied in cultures of *Plasmodium falciparum*. We showed that addition of copper led to an enhancement of the plasmodium killing, whereas addition of chelating agents, such as desferrioxamine and diethylenetriamine pentaacetic acid markedly reduced the toxic effects. Parsitized G6PD deficient erythrocytes were more sensitive than parasitized normal erythrocytes to copper and to the combination of copper and paraquat.

Abbreviations: DTPA – diethylenetriamine pentaacetic acid; DFO – desferrioxamine; G6PD – glucose-6-phosphate dehydrogenase; Hx – hypoxanthine; Paraquat – Methyl viologen, 1,1′-dimethyl-4-4′-bipyridinum dichloride, PQ^{+2}

KEY WORDS: Malaria, paraquat, transition metals, oxidant stress.

INTRODUCTION

Methyl viologen (1,1′-dimethyl-4-4′-bipyridinum dichloride, paraquat, PQ^{+2}) is a widely used herbicide affecting all green plants in the presence of light and oxygen.[1] It has been proven to be highly toxic to living cells in a variety of biological systems ranging from microorganisms to mammals.[2] Paraquat which is enzymatically reduced to the monocation radical (PQ^{+}) and reacts fast with molecular oxygen to yield the superoxide radical in a cyclic mode of action has been employed as a model for studying superoxide toxicity.[3] Many studies[3-5] showed that superoxide radical is the major causative agent responsible for this toxicity. Recent studies demonstrated an enhancement by copper or iron of the deleterious effects exerted by the superoxide radicals.[5,6] In this mechanism, paraquat metabolism[7,8] leads to the formation of hydroxyl radicals in a site-specific manner.[9]

In vitro and *in vivo* studies on the biology of plasmodia showed that a variety of oxidant stress-generating drugs[10-12] possess antimalarial activity. In this study we used cultures of *Plasmodium falciparum* as a biological system for evaluating the role of transition metals in paraquat toxicity.

The study of the mode and mechanism of parasite destruction by compounds inducing oxidant stress is necessary for the rational design of antimalarial drugs. This should include consideration of the oxidant sensitivity of infected erythrocytes and the effectiveness of oxidant drugs. In view of the growing problem of drug resistance the development of therapeutics should be vigorously pursued.

The experiments were carried out on parasites growing in normal and in G6PD-

deficient (G6PD-) cells. Malaria parasites grown in G6PD- erythrocytes, rather than in normal erythrocytes, are more sensitive to oxidant stress.[13,14]

MATERIALS AND METHODS

Experimental Design

Two experimental approaches were examined: in the first, pretreatment of erythrocytes alone, followed by infection with parasites was used, while in the second approach, treatment of the parasitized erythrocytes has been employed. Experimental systems included *in vitro* cultures of *P. falciparum* in normal and G6PD-deficient erythrocytes. The parameter for parasite development was the uptake of radioactive hypoxanthine (Hx) by the parasitized erythrocytes.

Parasites

Plasmodium falciparum (strain FCR-T3) was cultured according to the method of Trager and Jensen,[15] with some modifications as earlier described,[14] in 20 mM glucose. Cultures were synchronized by sorbitol treatment.[16]

Blood

Blood was collected from normal individuals or from G6PD-deficient (Med-) individuals and stored at 4°C. The activity of G6PD in the deficient blood was less than 3% of the normal activity. Experiments were initiated within 72 h following collection of the blood.

Pretreatment

The blood was centrifuged, plasma and buffy coat were removed and the erythrocytes were washed twice with PBS (pH 7.2). Various concentrations of paraquat, copper (as a sulfate) or combination of the two reagents, were added in 2 ml of PBS to 0.1 ml of packed erythrocytes. The cell suspensions were incubated at 37°C for 1 h with gentle shaking, washed with RPMI-1640 medium to remove remaining reagents and suspended in growth medium. Suspensions enriched in trophozoites and schizonts (mature forms) were obtained by the gelatin sedimentation technique.[17] An inoculum of parasitized erythrocytes was added to an erythrocyte suspension (5% hematocrit), so that the initial parsitemia was 1% and the contamination by untreated cells (originating from the inoculum) was less than 1.5% of the recipient cell population.

Treatment

Parasitized erythrocytes in their mature form were adjusted to 1% parasitemia (unless otherwise indicated) in red blood cell suspension (5% hematocrit). Solutions of paraquat, copper, diethylenetriamine pentaacetic acid (DTPA), desferrioxamine (DFO) or their various combinations were prepared in PBS. The reagents were added in the following order: DTPA or DFO were added first, 5 minutes later copper and immediately after that, paraquat. After an incubation period of 1 h at 37°C the cells

were washed twice with RPMI-1640 medium, dispensed into a 96-well microplate (100 μl/well) and incubated at 37°C using the candle jar method of Trager and Jensen.[15] The supernatant was replaced every day by fresh medium.

Parasite Development

Incorporation of radioactive hypoxanthine (Hx) was measured according to the method of Golenser, Casuto and Pollack[18] as follows: [^{3}H]-hypoxanthine (0.5 μCi/well, New England Nuclear), was added in 25 μl of medium to the triplicate cultures. The cells were collected by filtration on glass microfibre filters and radioactivity was counted (by a Mimaxi β Tri-Carb 4000 Packard). When necessary parasitemia was estimated in blood smears stained with Giemsa.

RESULTS

The influence of paraquat on the development of *Plasmodium falciparum*, with emphasis on the role of copper was studied by following the uptake of radioactive hypoxanthine (Hx) into parasitized erythrocytes. The incorporation of Hx in normal erythrocytes alone (without parasites) did not exceed 1–3% of the uptake by infected red blood cells. Each experiment was performed three times.

Parasitized erythrocytes (1% parasitemia), were exposed to paraquat and/or copper at the onset of the experiments. Table I shows the effect of the duration of exposure to paraquat alone (0.12 mM), to copper alone (2 μM) and to their combination on the incorporation of radioactive Hx. Inhibition of Hx uptake by either paraquat or copper was time dependent. An additive effect of the combination of the paraquat and copper was observed. In view of the results it was decided to use one hour of exposure in subsequent experiments.

Paraquat caused a dose response inhibition in the uptake of Hx that could be clearly observed already by the second day following the treatment of the parasitized erythrocytes (Figure 1A–C). Copper (2 μM) by itself, was also inhibitory causing approximately 22% inhibition (Figure 1B). The combination of copper (2 μM) and

TABLE I

The effect of duration of exposure to paraquat, copper or their combination on the development of *P. falciparum*

Treatment	Duration of Treatment		
	10′	30′	60′
Paraquat (0.12 mM)	8*	34	35
$CuSO_4$ (2 μM)	0	20	25
Paraquat (0.12 mM) and $CuSO_4$ (2 μM)	8	63	65

Parasitized erythrocytes (1% parasitemia, mature forms) were treated for various periods, after which the cultures were washed and reincubated. Hx was added 48 h later and the cells were harvested after an additional 19 hours.

*The results are expressed as percent inhibition of Hx uptake by untreated cells.

CPM of the control for 10, 30 and 60 minutes were 22335, 19935 and 17248 respectively. This is one representative experiment (out of 3).

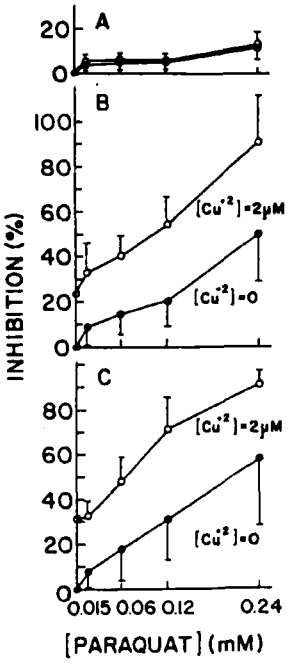

FIGURE 1 The effect of paraquat or combinations of paraquat and copper on the development of *P. falciparum*. The parasitized erythrocytes (1% parasitemia with mature forms) were treated for 1 h with paraquat ●--● or paraquat and copper O--O. Hx was added at 0–19 h (A), or 24–43 h (B) or 48–67 (C) and the cells were harvested at 19, 43 or 67 h respectively. CPM of the control at 0–19 h, 24–43 h and 48–67 were 24904, 20958 and 57340 respectively. This figure respresents combined results of three different experiments.

paraquat yielded an additive response in which the slopes of the curves with or without copper (with respect to paraquat concentration) were similar (Figure 1).

However, when parasitized erythrocytes (20% parasitemia) were exposed to paraquat and copper (for 1 h after which the culture was washed and non-treated erythrocytes were added to obtain 1% parasitemia), a synergistic effect was observed (Table II). At 19 h following the 1 h treatment, no difference was recorded in parasitemias, however, there was a difference in the distribution of the different developmental stages, between treated and non-treated parasites. In non-treated cells, 64% of the parasites were developed to the ring from, with 0.5 mM PQ only 18%, and with 2 μM $CuSO_4$ only 40%. At 43 hours following the treatment of parasitized erythrocytes 0.5 mM PQ caused about 40% reduction in parasitemia, copper alone did not cause any reduction, but their combination killed almost all of the parasites. The parasites that remained could survive later.

Figure 2 shows the dose response of copper alone on the incorporation of Hx. At 2 μM about 17% inhibition was observed, and this concentration was chosen for all further experiments. At 16 μM and above almost a total inhibition was recorded.

The effects of the chelating agents DTPA and DFO on the activity of paraquat and copper are depicted in Figures 3 and 4. Both DTPA and DFO were added 5 minutes prior to copper or paraquat, and caused a reduction in the inhibition imposed by

TABLE II

Parasitemia, stage distribution, and uptake of Hx by parasitized erythrocytes treated with paraquat, copper or their combinations

Time post treatment (H)	Paraquat [mM]	No $CuSO_4$ CPM	% inhibition	P	R	M	$CuSO_4$ (2 μM) CPM	% inhibition	P	R	M
	0	8998	0	0.4	64	36	8240	8	0.6	50	50
19	0.12	9144	−2*	0.6	50	50	6567	27	0.7	40	60
	0.25	8911	1	0.5	18	82	3695	55	0.2	19	81
	0	5680	0	1.0	10	90	5903	−4*	1.3	42	58
43	0.12	4833	15	1.0	50	50	4641	18	0.7	62	38
	0.25	4135	27	0.6	70	30	2293	60	0.2	70	30
	0	21960	0	3.8	83	17	23685	−8*	5.0	76	24
68	0.12	12654	42	4.0	62	38	11054	50	2.3	45	55
	0.25	6383	71	2.6	36	64	2697	88	0.9	30	70

P—Parasitemia (%)
R—Ring form (% young parasites)
M—"Mature Forms" (% trophozoites and schizonts)
*—Enhancement
This is one representative experiment (out of 3).

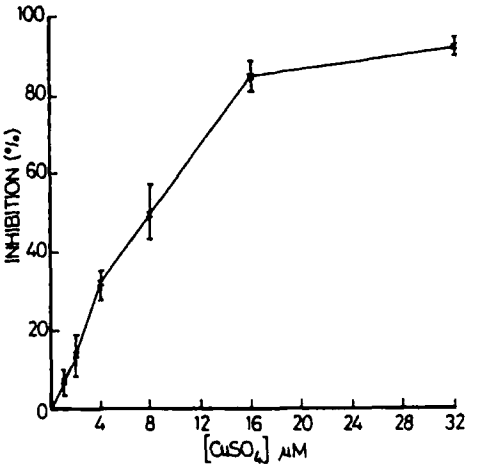

FIGURE 2 The effect of copper on the development of *P. falciparum*. Hx was added at 24 h and the cells were harvested 19 h later. See additional details in legend to Figure 1. CPM of the control was 26, 350. This figure represents the combined results of three different experiments.

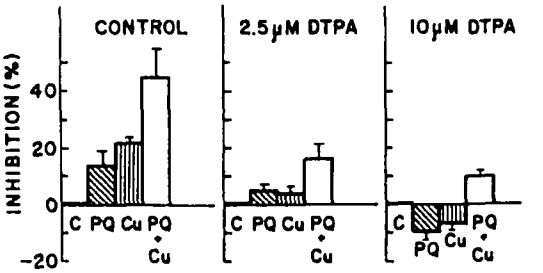

FIGURE 3 The reversal of the inhibition caused by paraquat, copper or their combination by DTPA. The parasitized erythrocytes (1% parastemia with mature forms) were treated for 1 h. Hx was added at 24 h and cells were harvested 19 h later. 0 (A), 2.5 μM (B) or 10 μM of DTPA were added. C ■ control; PQ ▨ paraquat (0.12 mM); Cu ■ copper (2 μM); PQ + CU □ paraquat (0.12 mM) and copper (2 μM). CPM of the control was 25,300. This figure represents the results of three different experiments (average ± SD).

paraquat, copper or their combination; i.e., it increased the uptake of Hx by the parasitized cells that were exposed to paraquat alone, copper alone and their combination.

Normal and G6PD-deficient parasitized erythrocytes at the trophozoite stage were exposed for 1 h to paraquat (0.5 mM), copper (2 μM) and their combination. Parasitized G6PD-deficient erythrocytes were not more sensitive to paraquat than normal infected RBCs. However, the parasitized enzyme-deficient cells were more sensitive to treatment with copper alone, and to treatment with a combination of copper and paraquat (data not shown).

We conducted another series of experiments using a different procedure – exposure of non-infected erythrocytes to oxidative challenge, and subsequently infecting the RBCs with *P. falciparum*. In these experiments, G6PD-deficient RBCs proved markedly more sensitive toward the oxidative challenge, paraquat, and in particular to copper, and their combination (Table III).

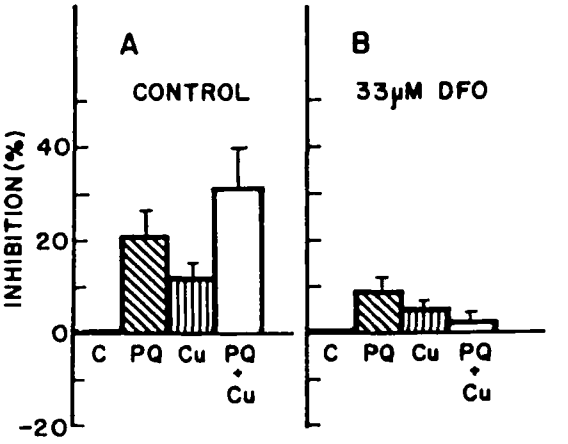

FIGURE 4 The reversal of the inhibition caused by paraquat, copper or their combination by desferrioxamine (DFO). 0 (A), 33 μM (B) of desferrioxamine. See additional details in legend to Figure 3. CPM of the control was 32, 150. This figure represents the results of three different experiments (average ± SD).

TABLE III

The effect of pretreatment of non-infected normal or G6PD(-) erythrocytes with paraquat, copper or their combination on the development of *P. falciparum* after subsequent infection of the erythrocytes

		NRBC	G6PD-
Paraquat	0.125 mM	0*	0*
	0.250 mM	0	6
	0.500 mM	9	48
Copper	2.0 μM	0	22
	2.5 μM	0	36
	3.0 μM	4	46
Paraquat + Copper	0.25 mM + 2.0 μM	13	45

*Percent inhibition of Hx uptake.

CPM of the control for NRBC and G6PD were 35230 and 32144 respectively. The experiment was performed three times and a representative one is presented.

DISCUSSION

It has been previously shown that in *E. coli* system, paraquat toxicity is enhanced by redox transition metals, Cu(II) and Fe(III).[19,20] In this study we used cultures of *Plasmodium falciparum* as a biological indicator for evaluating the role of transition metals in paraquat toxicity. It is assumed that in this system, the infected erythrocytes "provide" iron during the development of the parasite as hemoglobin is digested and a concurrent release of high levels of iron takes place within the red blood cell.[21] In this study, we have chosen to use adventitious copper as an exogenous metal in order to allow discrimination between the effects of the endogenous and added metals. Additionally, copper *per se*, is known to act as an oxidant agent on red blood cells.[22,23]

We have found that copper itself is toxic to the parasites. 16 μM of copper added to parasitized red cells killed most of the parasites. 2 μM copper inhibited the incorporation of Hx by the parasites by approximately 17%. Treatment with 2 μM copper in an oxygen depleted atmosphere caused only 2–5% inhibition (data not shown). Etkin and Eaton[24] showed that parasitized erythrocytes release H_2O_2. In the presence of copper the Fenton reaction can take place and free radicals can be generated, and may affect the non-infected cells in the culture. This conclusion is supported by the fact that pretreatment of normal red blood cells before infection with even a higher copper concentration, i.e. with 3 μM did not inhibit neither invasion nor the intracellular growth of the parasites at all. In contrast, this concentration caused 46% inhibition in pretreated G6PD(-) cells. These findings are consistent with those of Calabrese[25] who showed a markedly increased sensitivity in G6PD(-) cells which was expressed by the accumulation of copper-induced methemoglobin and decreased levels of acetylcholinesterase activity, as compared to normal red blood cells.

Inhibition of Hx uptake following treatment of parasitized erythrocytes with paraquat was dose and time dependent. The combination of copper and paraquat yielded an additive effect, whereas it was synergistic in the bacterial system.[19] The effects of paraquat and copper in a mixture of non-infected and infected cells may be directed against different cellular targets and therefore would be only additive. When we eliminated one of the targets by either treating parasitized red blood cells that were only later diluted with untreated NRBC or pretreated uninfected red blood cells before the addition of parasites, the effect of copper and paraquat was synergistic.

The role of transition metals in the enhancement of oxidant stress was further examined by the addition of the chelators diethylenetriamine pentaacetic acid (DTPA) and desferrioxamine (DFO) to the experimental system. These chelators reduced the inhibition of plasmodial growth that had been imposed by paraquat, copper or their combination. The fact that DTPA in 10 μM and DFO in 33 μM reduced PQ toxicity almost completely indicate that its activity is dependent on endogenous transition metals. The inhibitory effect of paraquat probably is mediated by the newly released yet undefined form of intracellular iron.

The effect of DTPA and DFO is in accord with the protection provided by DFO in alloxan-induced damage to plasmodia *in vivo*, in mice.[10] It could also be reconciled with long term exposure to DFO.[26] There, the chelator deprives the parasite from utilizing iron, which is essential for the parasite growth, [27,28] and by this, enhances the damage rather than provides the expected protection.

Parasitized G6PD(-) erythrocytes showed only slightly higher sensitivity toward a challenge of paraquat, than the parsitized normal erythrocytes (data not shown). Since the production of the free radicals induced by paraquat is NADPH dependent,[20] and since G6PD(-) parasitized cells have much less NADPH under oxidant stress,[29] this result is not surprising, despite the general higher sensitivity of G6PD(-) erythrocytes to oxidant stress. However, the increased sensitivity of G6PD(-) erythrocytes was expressed, as was mentioned above, in the other experiments where non-infected erythrocytes were exposed to the challenge and only subsequently were infected with *P. falciparum*. In these experiments G6PD(-) erythrocytes were markedly more sensitive toward the challengers, paraquat and in particular copper and their combination. It has long been suggested that G6PD deficiency confers some degree of protection against infection with malaria parasites.[30,31]

The results in this study suggest that the deleterious effects of paraquat are metal-dependent and occur via a free radical pathway. The synergism between paraquat and

copper substantiates this suggestion. Our understanding of the role of transition metals reducing agents and free radicals in parasite development and injury provides novel strategies in the fight against malaria.

Acknowledgements

This study was supported by grants from AID/CDR (C7-163), by research contract 1-A1 22668-NIH-NIAID and USNSF (CHG 8620149).

References

1. A.D Dodge (1971) Herbicides, paraquat, and diquat. *Endeavour*, **30**, 130–135.
2. A.P. Autor (ed.) (1977) *Biochemical Mechanisms of Paraquat Toxicity*. Academic Press, New York.
3. J.S. Bus and J.E. Gibson (1984) In: *Drug Metabolism and Drug Toxicity* (J.R. Mitchell and M.B. Horning, eds.) Raven Press, New York, pp. 21–32.
4. H.M. Hassan and I. Fridovich (1979) Intracellular production of superoxide radical and of hydrogen peroxide by redox active compounds. *Archives of Biochemistry and Biophysics*,**196**, 385–395.
5. A. Samuni, M. Chevion and G. Czapski (1981) Unusual copper-induced sensitization of the biological damage due to superoxide radicals. *Journal of Biological Chemistry*, **256**, 12632–12635.
6. A. Samuni, M. Chevion and G. Czapski (1984) Roles of copper and superoxide in the radiation-induced inactivation of T7 bacteriophage. *Radiation Research*, **99**, 562–572.
7. M.R. Montgomery (1976) Paraquat toxicity and pulmonary superoxide dismutase: an enzymatic deficiency of lung microsomes. *Research Communications in Chemical Pathology and Pharmacology*, **16**, 155–158.
8. J.A. Farrington, M. Ebert, E.J. Land and K. Fletcher (1973) Bipyridylium quaternary salts and related compounds. V. Pulse radiolysis studies of the reaction of paraquat radical with oxygen. Implications for the mode of action of bipyridyl herbicides. *Biochimica et Biophysica Acta*, **314**, 327–381.
9. A. Samuni, J. Aronovitch, D. Godinger, M. Chevion and G. Czapski (1983) On the cytotoxicity of vitamin C and metal ions: A site specific Fenton mechanism. *European Journal of Biochemistry*, **137**, 119–124.
10. I.A. Clark and N.N. Hunt (1983) Evidence for reactive oxygen intermediates causing hemolysis and parasite death in malaria. *Infection and Immunity*, **39**, 1–6.
11. H.M. Dockrell and J.H.L. Playfair (1982) Killing of blood stage murine malaria parasites by hydrogen peroxide. *Infection and Immunity*, **39**, 456–459.
12. I.A. Clark, G. Chaudhri and W.B. Cowden (1989) Some roles of free radicals in malaria. *Free Radicals in Biology and Medicine*, **6**, 315–321.
13. E.F. Roth, C. Raventos-Suarez, A. Rinaldi, R.L. Nagel (1983) Glucose-6-phosphate dehyrogenase deficiency inhibits *in vitro* growth of *Plasmodium falciparum*. *Proceedings of the National Academy of Sciences of the United States of America*, **80**, 298–299.
14. J. Golenser, J. Miller, D.T. Spira, T. Navok and M. Chevion (1983) Inhibitory effect of a fava bean component on the *in vitro* development of *Plasmodium falciparum* in normal and glucose-6-phosphate dehydrogenase-deficient erythrocytes. *Blood*, **61**, 507–510.
15. W. Trager and J.B. Jensen (1976) Human malaria parasites in continuous culture. *Science*, **193**, 673–675.
16. C. Lambros and J. Vanderberg (1979) Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *Journal of Parasitology*, **66**, 418–420.
17. J.B. Jensen (1978) Concentration from continuous culture of erythrocytes infected with trophozoites and schizonts of *Plasmodium falciparum*. *American Journal of Tropical Medicine and Hygiene*, **27**, 1274–1276.
18. J. Golenser, D. Casuto and Y. Pollack (1981) *Plasmodium falciparum: in vitro* induction of resistance to aminopterin. *Experimental Parasitology*, **52**, 371–377.
19. R. Kohen and M. Chevion (1985). Transition metals potentiate paraquat toxicity. *Free Radicals Research Communication*, **1**, 79–88.
20. P. Korbashi, R. Kohen, J. Katzhendler and M. Chevion (1986) Iron mediates paraquat toxicity in *Escherichia coli*. *Journal of Biological Chemistry*, **261**, 12472–12476.

21. S.K. Janney, J.H. Joist and C.D. Fitch (1986) Excess release of ferriheme in G6PD-deficient erythrocytes: Possible cause of hemolysis and resistance to malaria. *Blood*, **67**, 331–333.
22. E. Metz (1960) Mechanism of hemolysis by excess copper. *Clinical Research*, **17**, 32–35.
23. M. Boulard, K. Blume and E. Beutler (1975) The effect of copper on red cell enzyme activities. *Journal of Clinical Investigation*, **51**, 456–461.
24. N. Etkin and J. Eaton (1975) In: *Erythrocyte Structure and Function* (G.J. Brewer, ed.) Liss, New York, pp. 219–232.
25. E.J. Calabrese, M.S. Gary and H. Soon-Ching (1980) Low glucose-6-phosphate dehydrogenase activity in red blood cells and susceptibility to copper-induced oxidative damage. *Environmental Research*, **21**, 366–372.
26. G. Fritsch and A. Jung (1986) Carbon-14 desferrioxamine B uptake into erythrocytes infected with *Plasmodium falciparum*. *Zeitschrift für Parasitenkunde*, **72**, 709–714.
27. C. Raventos-Suarez, S. Pollack and R.L. Nagel (1982) *Plasmodium falciparum*: inhibition of *in vitro* growth by desferioxamine. *American Journal of Tropical Medicine and Hygiene*, **31**, 919–922.
28. S.J. Oppenheimer, F.D. Gibson, S.B. MacFarlane, J.B. Moody, C. Harrison, A. Spencer and O. Bunary (1986) Iron supplementation increases prevalence and effects of malaria: report on clinical studies in Papua New Guinea. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **80**, 603–612.
29. J. Miller, J. Golenser and D.T. Spira (1984) *Plasmodium falciparum*: Thiol status and growth in normal and G6PD deficient human erythrocytes. *Experimental Parasitology*, **57**, 239–247.
30. A.C. Allison and E.M. Eughui (1982) A radical interpretation of immunity to malaria parasites. *Lancet*, **11**, 1431–1433.
31. A.G. Motulsky (1960) Metabolic polymorphism and the role of infectious disease in tumour evolution. *Human Biology*, **32**, 28–63.

Accepted by Prof. G. Czapski