

Wirgiliusz Duda, Paweł A. Osmulski

STUDIES ON PARAQUAT AND GRAMOXONE INTERACTIONS WITH HEMOGLOBINS*

In this work the oxidation of human and carp hemoglobins (Hb) by Paraquat (PQ) and Gramoxone (GX) was studied. We demonstrated that the process runs through valency hybrids, mainly $\alpha^3+\beta^2+$, to met- and ferryl-Hb. The reaction is mediated by „active forms of oxygen”: H_2O_2 , $\cdot OH$ and $O_2\cdot^-$. It is characteristic, that hydrogen peroxide oxidises directly heme iron to its ferryl form with Fe^{4+} . The active forms of oxygen in the cooperation with PQ cause globin denaturation and damage of Hb - SH groups and Trp residues.

1. INTRODUCTION

Paraquat (PQ) as well as its 25% aqueous solution – Gramoxone (GX) is systemic and contact herbicide with chemical name 1,1'-dimethyl-4, 4'-bipyridinium dichloride. It is widely used compound being at the same time highly toxic not only to all green plants but also to nearly all forms of life from microorganisms to mammals. Thus, the understanding of mechanism of its toxic action seems to be a hot topic. On the cellular level and from medical point of view our knowledge of its action is nearly full. On the other hand, on the molecular level our knowledge is relatively poor. Some data show that oxygen and its free radical active forms play the most important role in the development of poisonous effects of PQ and GX. The current study was undertaken to clarify some of the molecular mechanism of PQ and GX interaction with hemoglobin and to compare the action of these two compounds.

* In this paper results presented in XII-th International Symposium on Structure and Function of Erythroid Cells, August 28–31, 1989, Berlin have been summarized.

2. MATERIALS

1. Human HbA was isolated using the method of Riggs, Bonaventura and Bonaventura [6] and purified according to Tentori et al. [10].
2. Carp Hb (Hb Cc – *Cyprinus carpio*) was isolated using the method of Lin et al. [4].
3. The proteins studied were oxidized with about two fold excess of $K_3[Fe(CN)_6]$ which was subsequently removed by a Sephadex G-25 column chromatography.
4. PQ p.a. was purchased from Sigma (U.S.A.), GX was obtained as a commercial 25% aqueous stock solution of herbicide from Agrotek (Hungary).
5. All other chemicals were of analytical grade and used without further purification.
6. Double-distilled, deionized water was used throughout the experiments.

3. METHODS

Reaction conditions:

- concentration of Hb $100 \mu\text{mol} \cdot \text{l}^{-1}$ (in respect to heme – 1.6 mg/ml of Hb),
- PQ or GX concentration 0–50 $\mu\text{g}/\text{ml}$ of Hb,
- incubation during 0–2 hrs in $0.2 \text{ mol} \cdot \text{l}^{-1}$ K-phosphate buffer, pH 6–8, at temperature 37°C .

Analytical methods:

- spectrophotometry,
- cycle measurements of absorption spectra in a UV-Vis range (SPECORD M40 Carl Zeiss Jena).
- multicomponent analysis on the basis of absorption changes at characteristic wavelengths for different Hb forms, using „Computer Kassette DATA HANDLING II zum SPECORD M40”,
- chromatography after Hb incubation with the agents, Hb forms were analysed using column chromatography with CM-Cellulose [10],
- determination of free -SH groups [1],
- tryptophan analysis [3].

4. RESULTS

OxyHb absorption spectra after the PQ or GX action showed the decrease of α and β bands (540 and 576 nm, respectively) and the increase at 500 and 630 nm (see Fig. 1, 2).

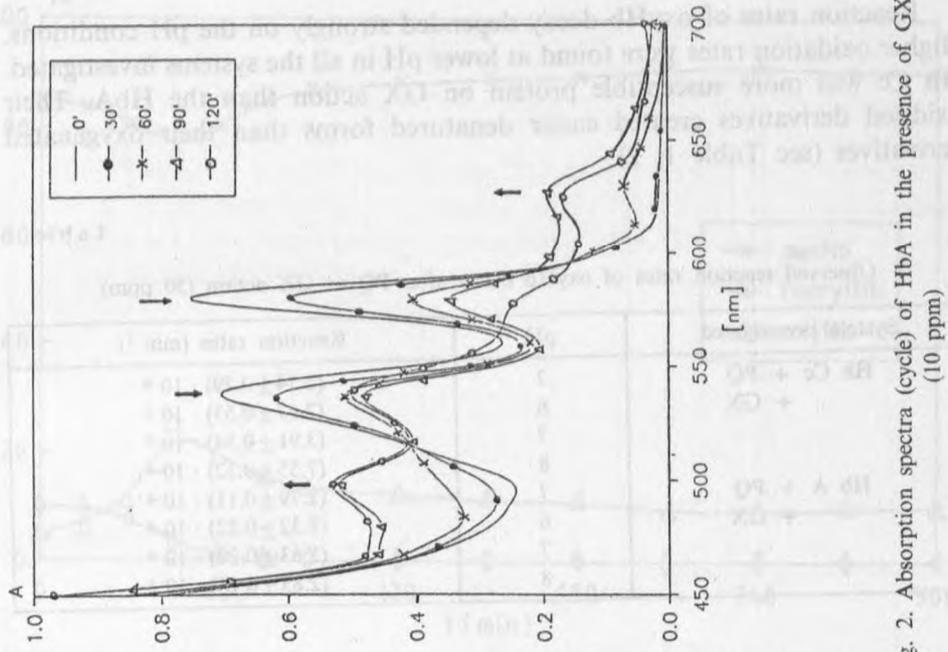


Fig. 2. Absorption spectra (cycle) of HbA in the presence of GX (10 ppm)

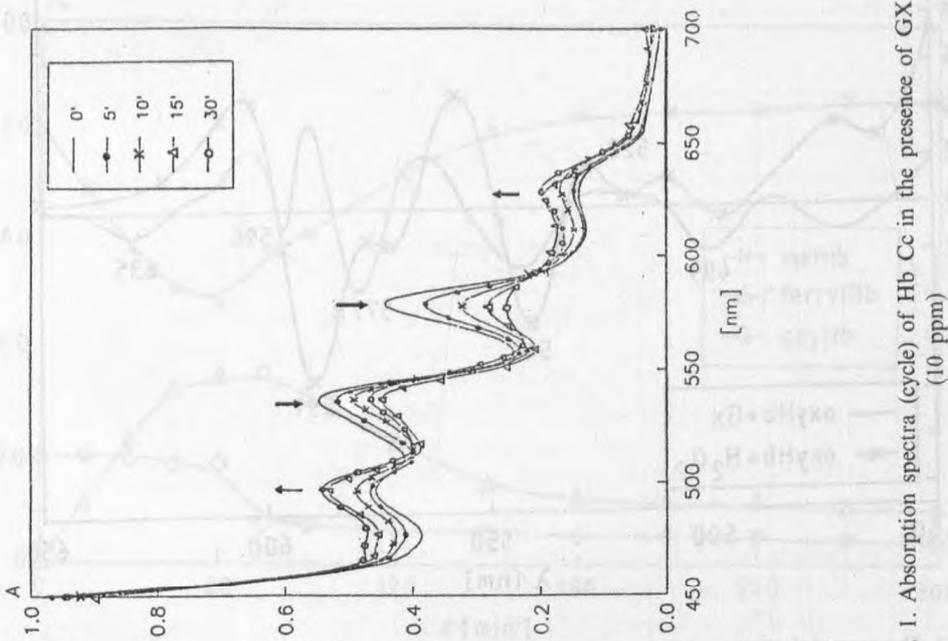


Fig. 1. Absorption spectra (cycle) of Hb Cc in the presence of GX (10 ppm)

Reaction rates of oxyHb decay depended strongly on the pH conditions. Higher oxidation rates were found at lower pH in all the systems investigated. Hb Cc was more susceptible protein on GX action than the HbA. Their oxidised derivatives created easier denatured forms than their oxygenated derivatives (see Table 1, 2).

Table 1

Observed reaction rates of oxyHb decay after PQ or GX action (50 ppm)

System investigated	pH	Reaction rates (min ⁻¹)
Hb Cc + PQ + GX	7	$(5.74 \pm 0.29) \cdot 10^{-4}$
	6	$(7.67 \pm 0.53) \cdot 10^{-3}$
	7	$(3.91 \pm 0.34) \cdot 10^{-3}$
	8	$(7.55 \pm 0.32) \cdot 10^{-4}$
Hb A + PQ + GX	7	$(2.79 \pm 0.11) \cdot 10^{-4}$
	6	$(8.32 \pm 0.22) \cdot 10^{-4}$
	7	$(5.63 \pm 0.20) \cdot 10^{-4}$
	8	$(2.83 \pm 0.15) \cdot 10^{-4}$

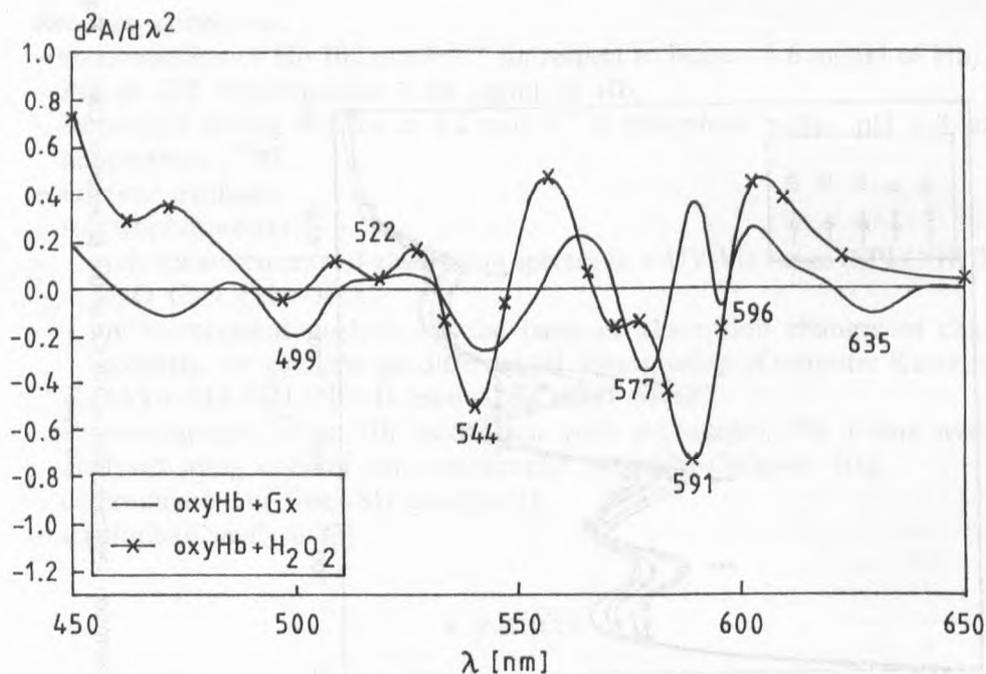


Fig. 3. The second derivatives of absorption spectra of HbA incubated with GX (50 ppm) or with H₂O₂ (5 μg/ml Hb)

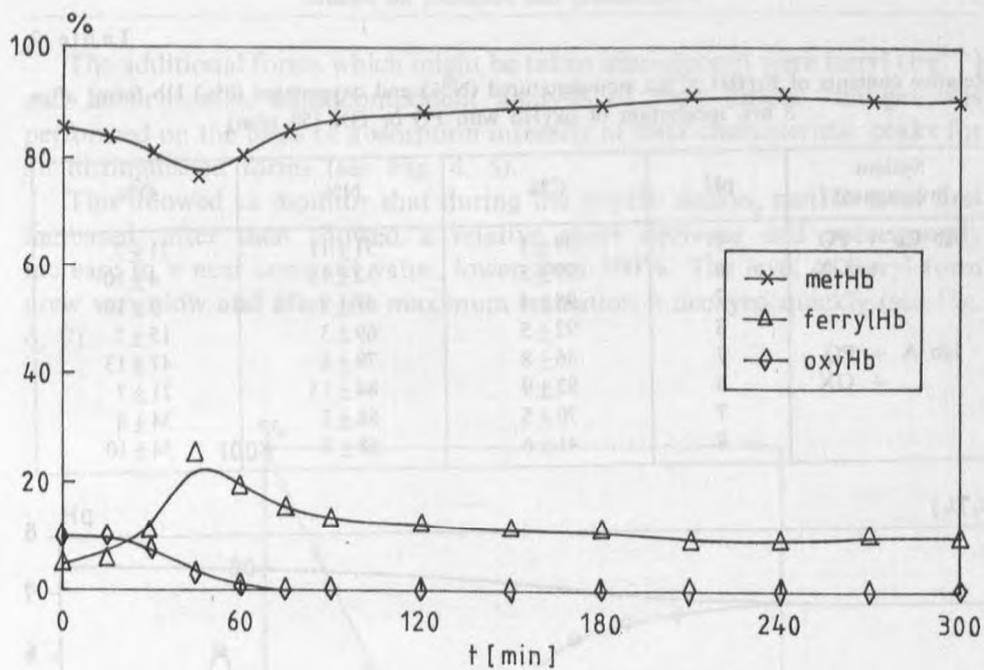


Fig. 4. Relative contents of Hb forms during PQ action (50 ppm) on Hb Cc, pH 7.0 (results of multicomponent analysis)

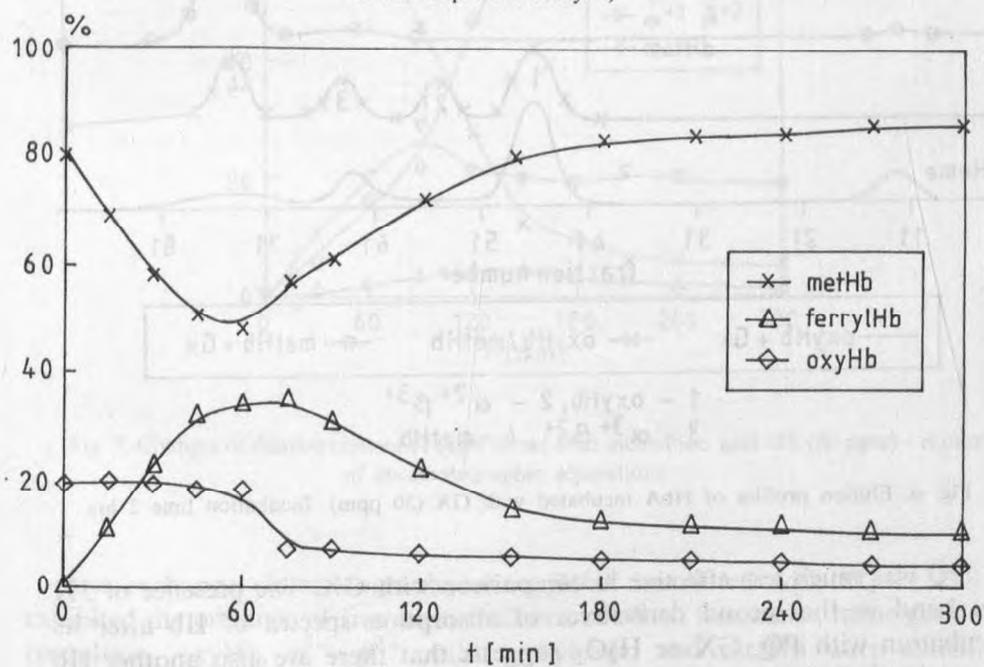


Fig. 5. Relative contents of Hb forms during GX action (50 ppm) on Hb Cc, pH 7.0 (results of multicomponent analysis)

Table 2

Relative contents of Fe(III) (C%), non-denatured (N%) and oxygenated (O%) Hb forms after 3 hrs. incubation of oxyHb with PQ or GX (50 ppm)

System investigated	pH	C%	N%	O%
Hb Cc + PQ + GX	7	89 ± 5	51 ± 11	21 ± 7
	6	99 ± 5	32 ± 13	4 ± 10
	7	98 ± 4	47 ± 9	9 ± 10
	8	92 ± 5	69 ± 3	15 ± 7
Hb A + PQ + GX	7	56 ± 8	79 ± 6	47 ± 13
	6	82 ± 9	84 ± 13	21 ± 7
	7	70 ± 5	88 ± 5	34 ± 8
	8	41 ± 6	88 ± 3	54 ± 10

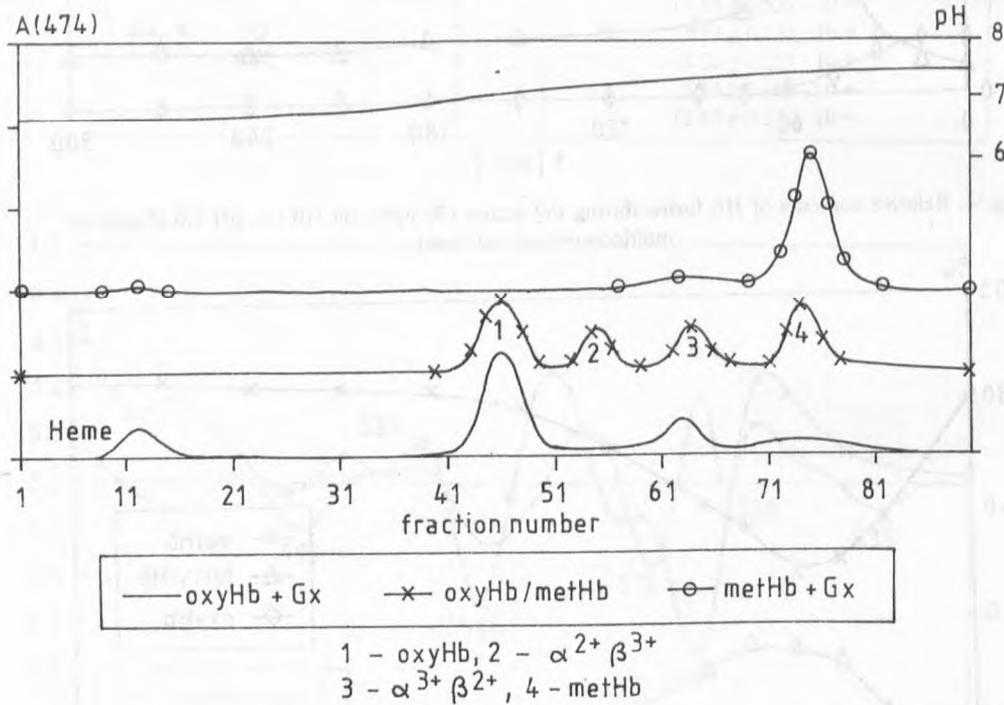


Fig. 6. Elution profiles of HbA incubated with GX (50 ppm). Incubation time 2 hrs

PQ was much less effective in comparison with GX. The presence of 591 nm band in the second derivatives of absorption spectra of Hb after its incubation with PQ, GX or H₂O₂ suggests that there are also another Hb forms in the mixture (see Fig. 3).

The additional forms which might be taken into account were ferryl (Fe^{4+}) and hemichromes. Multicomponent analysis of their kinetic changes was performed on the basis of absorption intensity at their characteristic peaks for all distinguished forms (see Fig. 4, 5).

This allowed to monitor that during the oxyHb decays, metHb level first increased, after then showed a relative short decrease and subsequently increase to a near constant value, lower than 100%. The level of ferryl form grew very slow and after the maximum transition it decayed quickly (see Fig. 6, 7).

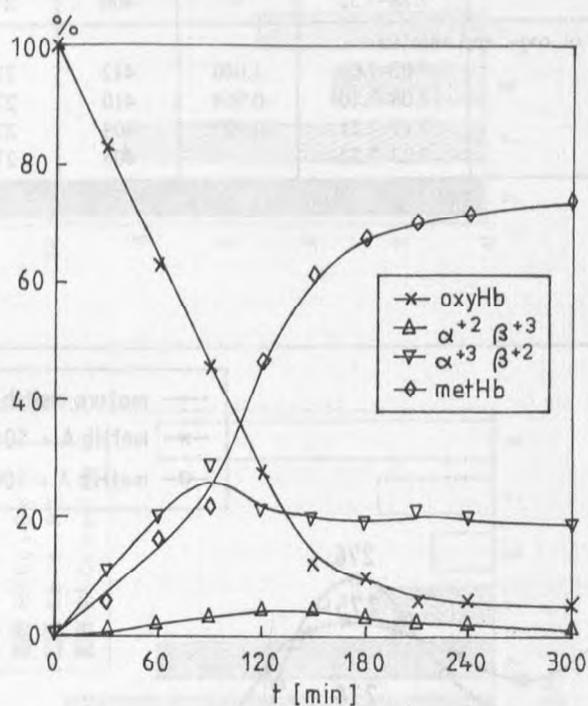


Fig. 7. Changes of relative content of HbA forms after incubation with GX (50 ppm) – results of chromatographic separations

Ion exchange chromatography of oxyHbA incubated with PQ or GX exhibited the presence of five main fractions in which free heme, heme-globin complexes, oxyHb, $\alpha^3 \beta^2$ valency hybrids, and metHb were eluted. A similar pattern of fractions was obtained after the separation of incubated metHbA (with the exception of oxyHb fraction) – see Table 3.

Table 3

Elution and spectrophotometric properties of fractions obtained after chromatographic separation of oxyHb incubated 0.5 hr. with GX, and the equimolar mixture of oxy- and metHb A

Fraction	Elution pH	α/β ratio	Soret band (nm)	UV max. (nm)	Soret/UV max. ratio
oxyHb A + 20 μg GX/ml Hb, 0.5 hr., 37°C					
globin	6.70	—	—	268	—
heme-globin complexes	6.78–6.85	1.000	414	272	2.88
oxyHb	7.02–7.04	1.042	412	275	3.57
α^3+ β^2+ hybrids	7.16–7.20	0.933	407	275	3.24
metHb	7.28–7.32	—	406	275	4.86
equimolar mixture of oxy- and metHb					
oxyHb	7.02–7.03	1.040	412	275	3.58
α^2+ β^3+ hybrids	7.08–7.10	0.964	410	275	3.93
α^3+ β^2+ hybrids	7.17–7.21	0.927	408	274	3.93
metHb	7.27–7.32	—	405	275	5.06

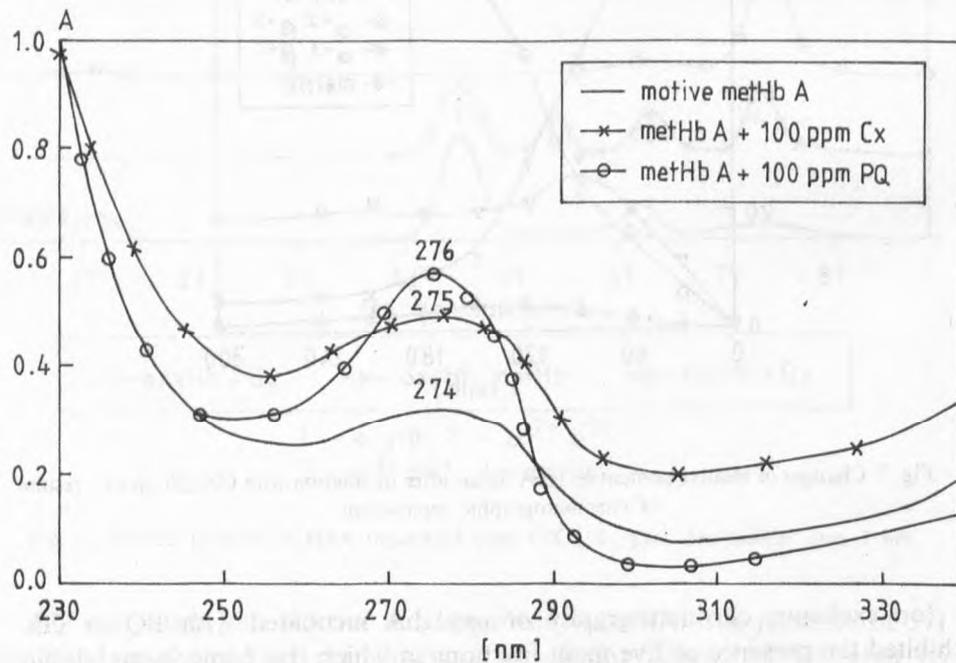


Fig. 8. Absorption spectra of Hb in a UV range after 2 hrs incubation with PQ or GX, pH 7.0

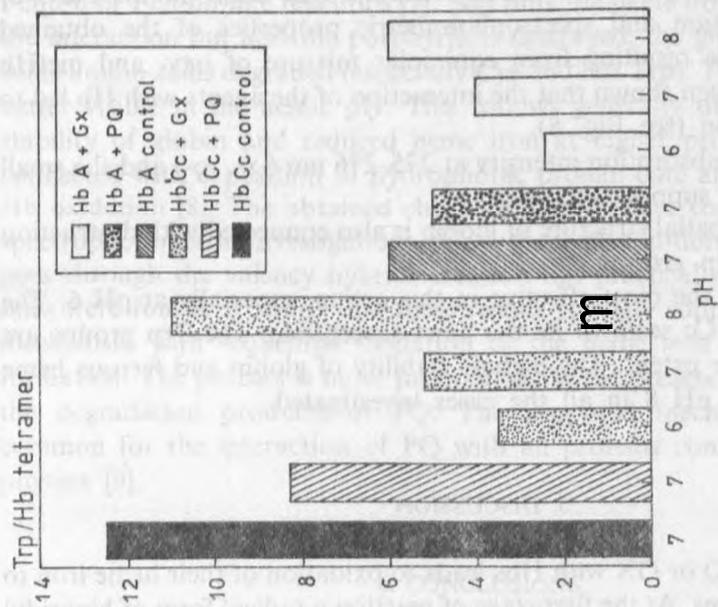


Fig. 10. Number of Trp groups per Hb tetramer after 3 hrs action of PQ or GX (50 ppm) on Hb Cc or HbA

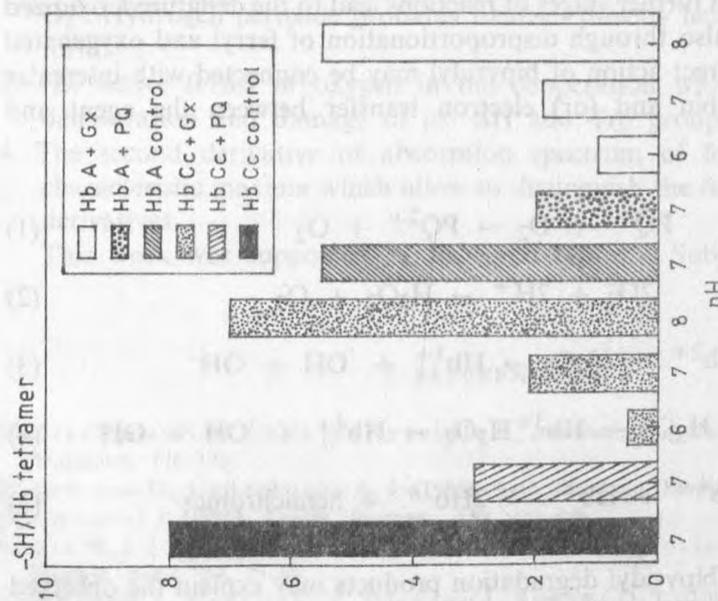


Fig. 9. Number of -SH groups per Hb tetramer after 3 hrs action of PQ or GX (50 ppm) on Hb Cc or HbA

The proper identification of eluted fractions was done on the basis of comparison of elution and spectrophotometric properties of the obtained fractions with those resulting from equimolar mixture of oxy- and metHb separation. It has been shown that the interaction of the agents with Hb led to protein denaturation (see Fig. 8).

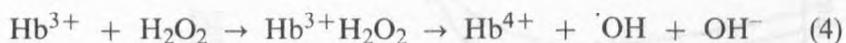
The increase of absorption intensity at 275–276 nm (-x-, -o-) and the small shift (-o-) strongly support this idea.

The change of spatial structure of globin is also connected with destruction of the -SH and Trp groups (see Fig. 9, 10).

Again, GX was the most effective in this action, especially at pH 6. The -SH groups of Hb Cc seem to be the most susceptible. The Trp groups are destroyed to smaller extent. The highest stability of globin and ferrous heme iron was found at pH 8 in all the cases investigated.

5. DISCUSSION

Interaction of PQ or GX with Hbs leads to oxidation of their heme iron to ferric and ferryl forms. At the first stage of reaction a radical form of bipyridyl is generated which after the reaction with molecular oxygen creates superoxide radicals (1). They dismutate to hydrogen peroxide which seems to be a direct reason for the late ferryl production (2–4) [9, 2]. Results of multicomponent analysis indicate that further stages of reactions lead to the denatured, oxidized derivatives of Hb, also through disproportionation of ferryl and oxygenated forms (4, 5). The direct action of bipyridyl may be connected with intercalary binding to the globin and (or) electron transfer between the agent and porphyrin.



The presence of bipyridyl degradation products may explain the observed higher efficiency of GX in the comparison with the pure PQ solution [5]. However, a certain role in the development of toxic action may be played by

ions of transition metals (iron) which also originate from Hb. They can work in Fenton or Fenton-like reactions [7]. Not only the heme iron is oxidized during the interaction but also the porphyrin is destroyed, the globin denatured and some amino acids degraded (especially Cys and less Trp). The process is always better visible in the acidic pH. This follows generally the concept of lower stability of globin and reduced heme iron at higher proton concentrations connected with exposition of hydrophobic protein core and proton-catalysed Hb oxidation [8]. The obtained chromatographic data confirm the results of spectrophotometric investigations. They show in addition that the oxidation goes through the valency hybrids creation but practically only the α^{3+} β^{2+} ones were found. It seems that these results strongly support the free radical mechanism with sequential oxidation of the heme iron through the ferryl formation. The process is more pronounced in the presence of metal ions and the degradation products of PQ. The proposed mechanism is probably common for the interaction of PQ with all proteins containing metalloporphyrins [9].

6. CONCLUSIONS

1. PQ and GX induce oxidation of Hb to metHb and ferrylHb. The process runs through the valency hybrids, mainly α^{3+} β^{2+} .
2. The reaction is mediated by the „active forms of oxygen”: H_2O_2 , $\cdot OH$ and O_2^- . Hydrogen peroxide probably oxidises directly heme iron to its ferryl form.
3. The active forms of oxygen in the cooperation with PQ cause globin denaturation and damage of its -SH and Trp groups.
4. The second derivative of absorption spectrum of ferryl form exhibits characteristic maxima which allow to distinguish the ferryl from other Hb derivatives.

This work was supported by Research Grant – Subject II.11.1.1.

7. REFERENCES

- [1] Alexander N. M. (1976), [in:] R z u c i d ł o R., *Immunologia ogólna i doświadczalna*, PWN, Warszawa, 176–178.
- [2] Beloqui O., Cederbaum A. I. (1985), *Arch. Biochem. Biophys.*, **242**, 187–196.
- [3] Chrastil J. (1986), *Analys. Biochem.*, **158**, 443–446.
- [4] Lin M. J.-J., Noble R. W., Winterhalter K. H., Di Iorio E. E. (1988), *Biochem. Biophys. Acta*, **954**, 73–81.
- [5] Nanni E. J., Angelis C. T., Dickson J., Sawyer D. T. (1981), *J. Am. Chem. Soc.*, **103**, 4268–4270.

- [6] Riggs A., Bonaventura J., Bonaventura C. (1981), [in:] Antonini E., Rossi-Bernardi L., Chiancone E. (Eds.), *Methods in Enzymology*, vol. 76, „Hemoglobins”, Academic Press, New York, 5–29.
- [7] Sadrzadeh S. M., Graf E., Panter S. S., Hallaway P. E., Eaton J. W. (1984), *J. Biol. Chem.*, **259**, 14 354–14 356.
- [8] Satoh Y., Shikama K. (1981), *J. Biol. Chem.*, **256**, 10 175–10 272.
- [9] Smith L. L. (1985), *Phil. Trans. Res. Soc. Lond.*, **B311**, 647–657.
- [10] Tentori L., Vivaldi G., Carta S., Salvati A. M., Sorcini M., Valami S. (1965), *Arch. Biochem. Biophys.*, **100**, 404–414.

Came in editorial office
„Folia biochimica et biophysica”
26.06.1991

Chair of Biophysics
University of Łódź

Wirgiliusz Duda, Paweł A. Osmulski

BADANIA INTERAKCJI PARAKWATU I GRAMOXONU Z HEMOGLOBINĄ

W pracy przedstawiono wyniki badań nad utlenianiem przez parakwat i gramoxon hemoglobiny człowieka i karpia. Jak wykazano, proces ten przebiega z wytworzeniem hybrid walencyjnych Hb głównie $\alpha^{3+} \beta^{2+}$ i ferryhemoglobiny do końcowego produktu – metHb. Ta reakcja jest wywoływana przez aktywne formy tlenu: $\cdot\text{OH}$, $\text{O}_2^{\cdot-}$ i H_2O_2 . Wykazano, iż charakterystyczne jest, że H_2O_2 bezpośrednio utlenia żelazo hemu do formy ferrylowej (tj. Fe^{4+}). Aktywne formy tlenu (rodniki) pod wpływem parakwatu lub gramoxonu powodują również, w dłuższym czasie działania, częściową denaturację globiny przy czym występuje uszkodzenie grup -SH i reszt tryptofanu.