ACTA UNIVERSITATIS LODZIENSIS
FOLIA BIOCHIMICA ET BIOPHYSICA 9, 1992

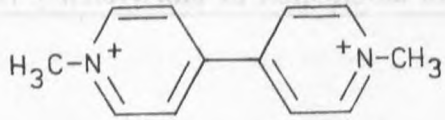
Wirgiliusz Duda

COMPARATIVE STUDIES OF PARAQUAT INTERACTIONS WITH FISH AND BOVINE OXY- AND DEOXYHEMOGLOBINS

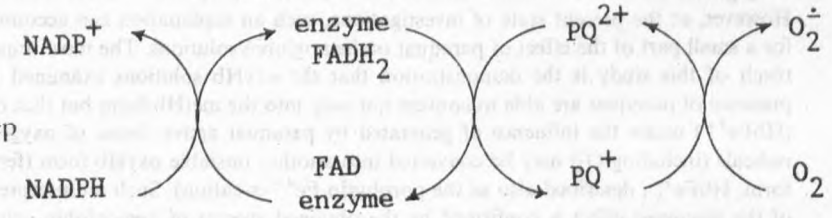
In this study it has been demonstrated that in oxyhemoglobin solutions paraquat induces a decrease in the content of this form of hemoglobin followed, after some time delay, by an increase in the metHb content. These differences have been tentatively explained till now by differences in the cooperative properties of hemoglobins (e.g. carp and bovine Hb). Carp hemoglobin exhibits the so-called Root effect while bovine hemoglobin (like other mammalian Hbs) is characterized by the so-called Bohr effect. However, at the present state of investigations, such an explanation can account only for a small part of the effect of paraquat on hemoglobin solutions. The most important result of this study is the demonstration that the oxyHb solutions examined in the presence of paraquat are able to convert not only into the metHb form but that oxyHb ($HbFe^{2+}$) under the influence of generated by paraquat active forms of oxygen and radicals (including O_2^-) may be converted into another unstable oxyHb form (ferrylHb form, $HbFe^{4+}$, described also as the porphyrin Fe^{4+}-π-cation). Such an interpretation of the discussed effect is confirmed by the obtained spectra of hemoglobin solutions, especially by spectra of oxy-, deoxy-, metHb and Raman spectra.

1. INTRODUCTION

Paraquat (PQ, 1,1'-dimethyl-4,4'-bipyridylium ion) is a powerful herbicide which shows contact and systemic activity. Michaelis and Hill [9] described in 1933 its redox properties and since that time it has been used as a „redox indicator" better known for chemists under the name of „methyl viologen". One electron reduction of the PQ molecule leads to the relatively stable cation radical formation. This process is related to the transfer of one of the pair of electrons from the bipyridylium group. As a result, the resonance form of the compound is created. These free radicals have potential ability to react with oxygen (what leads probably to generation of superoxide anion radical) with the simultaneous reoxidation of its ionic form.



Primary mechanism of Paraquat toxicity. Paraquat has the ability to undergo a one-electron reduction from the cation to form a stable blue coloured free radical in the absence of oxygen [9]. In the presence of oxygen the radical will immediately reform the cation with the concomitant production of superoxide anion ($O_2^{\cdot-}$). This reaction between paraquat radical and oxygen is so rapid that it is diffusion-limited [5]. Thus, provided there is a continuous supply of electrons to PQ, and oxygen is present, PQ will rapidly cycle from its oxidized to reduced form with the continuous production of $O_2^{\cdot-}$. Gage [6] first reported that in cells under anaerobic conditions, NADPH together with a flavoprotein could reduce PQ from its cation to radical. Under aerobic conditions the radical is reoxidized and this redox cycling continues until available NADPH is consumed.



II nd step

$$O_2^{\cdot-} + O_2^{\cdot-} \xrightarrow{2H^+} H_2O_2$$

$$\text{then} \quad H_2O_2 + O_2^{\cdot-} \longrightarrow OH^{\cdot} + OH^- + O_2$$

III rd step Reaction of H_2O_2 and/or free radicals (i.e. mainly $O_2^{\cdot-}$, $OH^{\cdot}$) with hemoglobin.

The studies of Gage [6] were extended by Baldwin et al. [1] who demonstrated that microsomal preparations from liver, lung and kidney were able to generate radicals of PQ and eventually in the presence of Hb produce H_2O_2. The production of $O_2^{\cdot-}$ and H_2O_2 may then lead to the formation of more reactive oxygen radicals, which in turn may be more toxic to the cell and function of respiratory proteins (hemoglobins) [2]. Thus the cycling of PQ

from its reduced to reoxidized form provides a plausible primary mechanism of its toxicity that is entirely analogous with the proposed mechanism of the hemoglobin toxicity of paraquat. PQ toxicity on mammalian organisms is differentiated what depends on an absorbed dose and a kind of tissue on which it acts. However, a toxic dose, regardless of the method of ingestion (per os or intravenous), causes a similar complex of toxic symptoms. The presence of PQ at a dose of 0.2 μg/ml in human serum is a lethal dose (during 24 h). For PQ doses of over 10^{-5} mol/l inhibition of mitochondrial oxidation was observed. Michael and Levis [8] reported that the most significant cumulative effects were given by the range of 10^{-5}–10^{-4} mol/l of PQ concentration which were the best visible from half an hour till two hours after the dose administration into an isolated biological system.

2. MATERIALS AND METHODS

1. Bovine HbA was isolated using the method of Rigss, Bonaventura and Bonaventura [14] and purified according to Tentori et al. [17].
2. Fish (Carp) Hb was isolated using the method of Lin et al. [7].
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.).
5. All other chemicals were of analytical grade and used without further purification, and double-distilled, deionized water was used throughout the experiments.

Reaction conditions:
- concentration of Hb 1.8 mg/ml in respect to one heme,
- paraquat concentration of 5,10, 25 and 50 μg/ml of Hb,
- incubation during 0–5 hrs in 0.2 M phosphate buffer, pH 7.0–7.1, at temperature 37° C.

Analytical methods:
- spectrophotometry,
- – measurements of absorption spectra (cycle) in a UV-Vis range (SPECORD UV-VIS and SPECORD M4O Carl Zeiss Jena),
- – Raman resonance spectra were performed thanks to kindnes of Doc. J. Twardowski from Jagiellonian University – Cracow and Prof. K. Gersonde from Rheinisch-Westfälische Technische Hochschule – Aachen.

3. RESULTS AND DISCUSSION

The influence of PQ on changes of carp (Ciprinus carpio) and bovine (Bos taurus – the Lowland Black-and-White cows) oxy- and deoxyhemoglobin was investigated. The reaction mixture contained oxy- or deoxyHb (concentration: 0.0018 ± 0.001 g Hb/ml in NaH_2PO_4 : Na_2HPO_4 buffer, pH 7.1 ± 0.02 and different doses of PQ – 5, 10, 25 and 50 ppm). The interactions of PQ with Hb were analysed spectrophotometrically where changes in the visible range of absorption spectra of Hb in the presence of PQ were compared with these of control samples without PQ. The solution of oxyHb containing PQ showed the decreasing absorption maxima at 540 and 570 nm and the parallel increasing at 500 and 630 nm what seems to be due to metHb formation (Fig. 1 and Table 1). The quantitative enhancement of the metHb level under these conditions was always observed considerably later in the relation to „the loss" of the oxy form. The degree of the disapperance of oxyHb was also positively correlated with the increase of doses of PQ. Instead, for the same doses of PQ, the metHb

Table 1

Percent decrease of A) carp oxyHb and B) bovine oxyHb induced by paraquat (25 and 50 ppm). All conditions the same as in Fig. 1

A)

	25 ppm PQ	50 ppm PQ
	X%	X%
0'	–	5.5
5'	2.0	14.5
15'	6.2	23.5
30'	9.8	34.6
1 h	14.5	45.0
2 h	24.6	55.0
3 h	34.5	62.0
4 h	37.5	67.9
5 h	39.6	–

B)

	25 ppm PQ	50 ppm PQ
	X%	X%
0'	–	4.6
5'	2.2	8.6
15'	3.3	12.8
30'	5.0	18.7
1 h	8.3	24.5
2 h	17.2	32.3
3 h	22.7	36.8
4 h	26.5	42.2
5 h	28.0	47.7

X% – Mean values for n = 20 samples, percent with respect to the control Hb = 100% oxyHb.

formation in the case of bovine Hb was time-dependent and linear in the character, contrary to the formation of fish metHb which during the same time increased exponentially (Table 2 and Fig. 2). Carp Hb form transformation

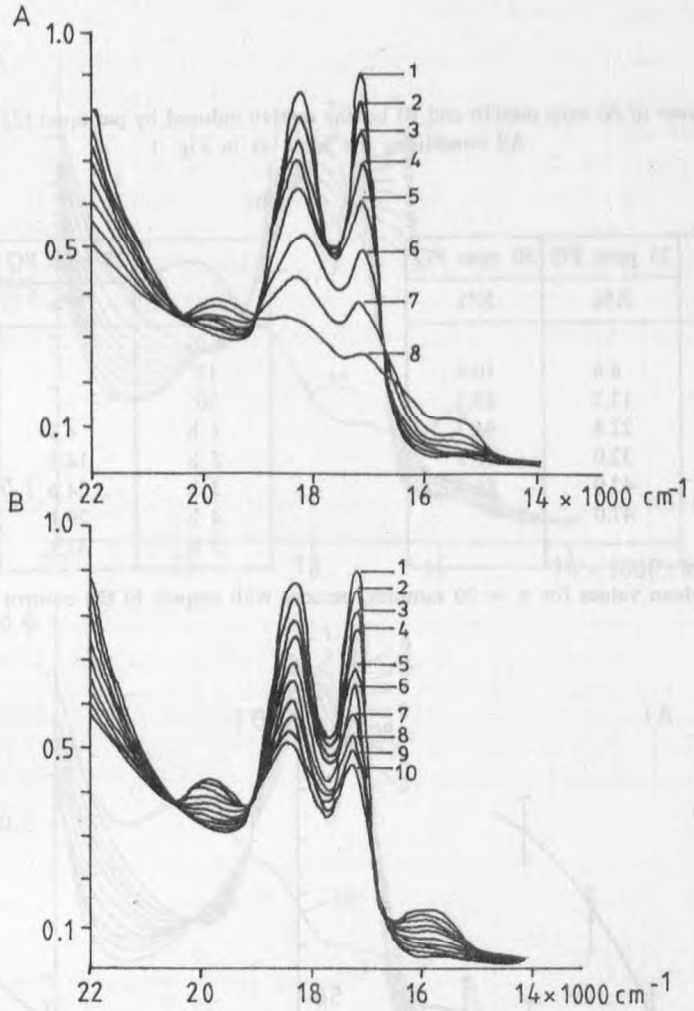


Fig. 1. Paraquat – induced changes in absorption spectra of oxyhemoglobin in the λ region of 22 000–14 000 cm^{-1}. OxyHb concentration: 0.0018 g/ml in 0.2 M phosphate buffer, pH 7.1. Paraquat concentration: 50 ppm. A) Carp oxyHb, time: 1 – 0' (oxyHb), 2 – 0' (oxyHb + PQ), 3 – 5' (oxyHb + PQ), 4 – 15' (oxyHb + PQ), 5 – 30' (oxyHb + PQ), 6 – 1 h (oxyHb + PQ), 7 – 2 h (oxyHb + PQ), 8 – 3 h (oxyHb + PQ); B) Bovine oxyHb, time: 1 – 0' (oxyHb), 2 – 0' (oxyHb + PQ), 3 – 5' (oxyHb + PQ), 4 – 15' (oxyHb + PQ), 5 – 30' (oxyHb + PQ), 6 – 1 h (oxyHb + PQ), 7 – 2 h (oxyHb + PQ), 8 – 3 h (oxyHb + PQ), 9 – 4 h (oxyHb + PQ), 10 – 5 h (oxyHb + PQ)

appeared to be more susceptible to the influence of PQ than bovine Hb. So, the more considerable level of metHb occured in solution of carp Hb than of bovine Hb. This may be explained through the existence of the differences

Table 2

Percent increase of A) carp metHb and B) bovine metHb induced by paraquat (25 and 50 ppm). All conditions the same as in Fig. 1

A)

	25 ppm PQ	50 ppm PQ
	X%	X%
0'	–	–
15'	6.6	10.8
30'	12.7	25.3
1 h	22.8	44.7
2 h	32.0	63.2
3 h	42.0	86.3
4 h	47.0	–
5 h	–	–

B)

	25 ppm PQ	50 ppm PQ
	X%	X%
0'	–	–
15'	–	–
30'	–	10.1
1 h	4.9	18.7
2 h	14.5	25.6
3 h	24.6	35.2
4 h	29.1	43.3
5 h	33.9	54.4

X% – Mean values for n = 20 samples, percent with respect to the control Hb = 100% metHb.

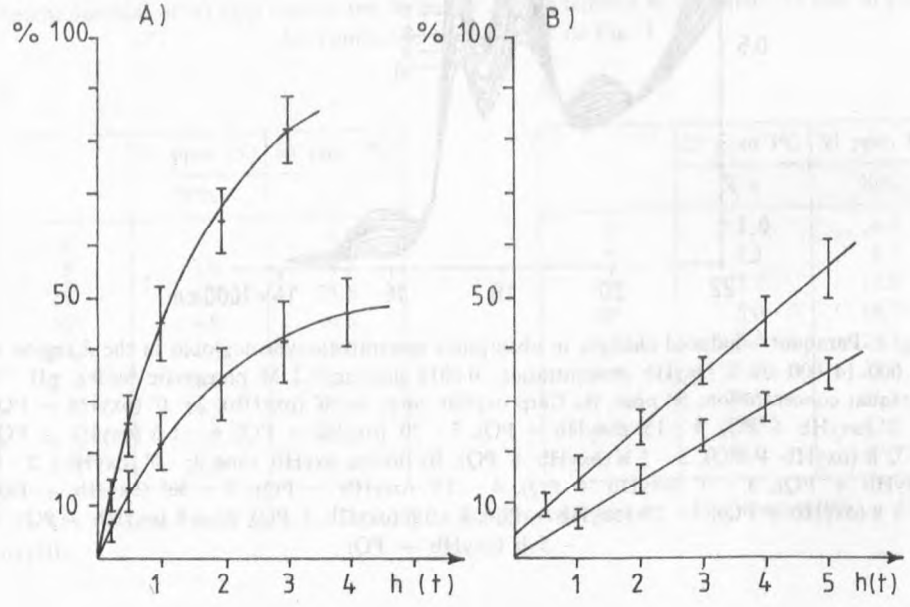


Fig. 2. Graphic representation of the increase in A) carp metHb and B) bovine metHb for a paraquat dose of 25 and 50 ppm

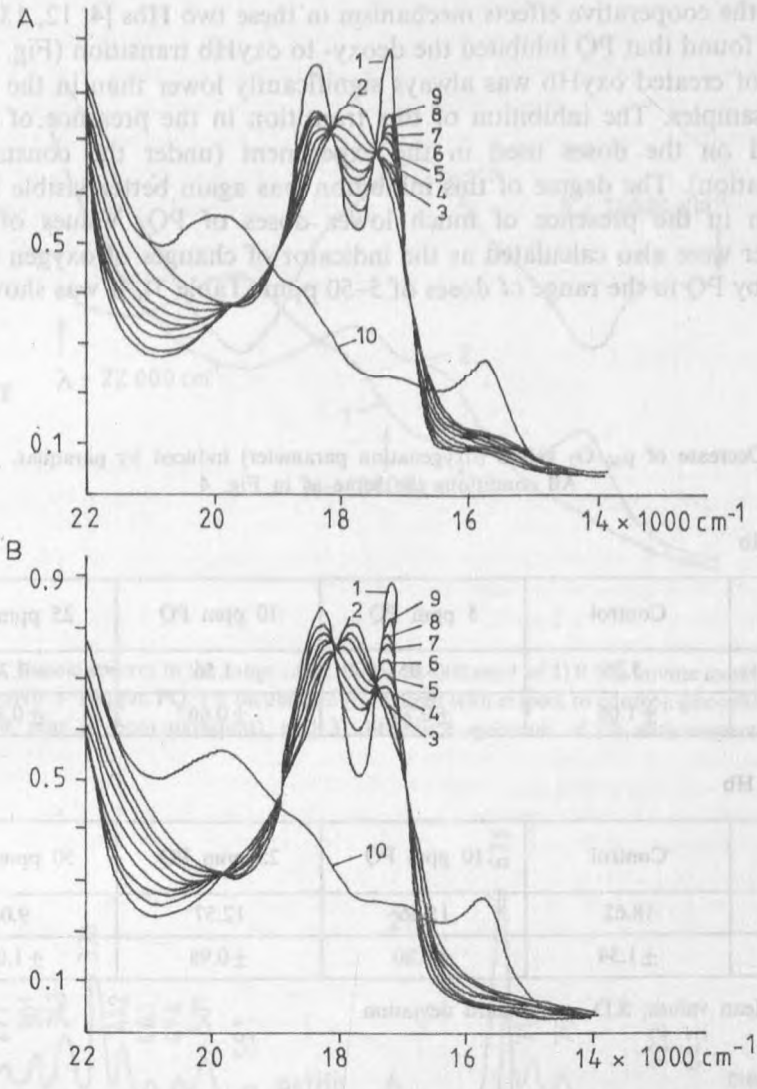


Fig. 3. Paraquat + O_2 - induced changes in absorption spectra of deoxyhemoglobin in the λ region of 22 000 – 14 000 cm^{-1}. DeoxyHb concentration: 0.0018 g/ml in 0.2 M phosphate buffer, pH 7.1. Paraquat concentration: 25 ppm. A) Carp deoxyHb: 1 – oxyHb, 2 – deoxyHb, 3 – deoxyHb + PQ, 4 – deoxyHb + PQ + 2 cm^3 O_2, 5 – deoxyHb + PQ + 4 cm^3 O_2, 6 – deoxyHb + PQ + 6 cm^3 O_2, 7 – deoxyHb + PQ + 8 cm^3 O_2, 8 – deoxyHb + PQ + 10 cm^3 O_2, 9 – deoxyHb + PQ + 12 cm^3 O_2, 10 – metHb; B) Bovine deoxyHb: 1 – oxyHb, 2 – deoxyHb, 3 – deoxyHb + PQ, 4 – deoxyHb + PQ + 5 cm^3 O_2, 5 – deoxyHb + PQ + 10 cm^3 O_2, 6 – deoxyHb + PQ + 15 cm^3 O_2, 7 – deoxyHb + PQ + 20 cm^3 O_2, 8 – deoxyHb + PQ + 25 cm^3 O_2, 9 – deoxyHb + PQ + 30 cm^3 O_2, 10 – metHb

between the cooperative effects mechanism in these two Hbs [4, 12, 13, 15]. It was also found that PQ inhibited the deoxy- to oxyHb transition (Fig. 3). The amount of created oxyHb was always significantly lower than in the case of control samples. The inhibition of this transition in the presence, of oxygen depended on the doses used in the experiment (under the constant Hb concentration). The degree of this inhibition was again better visible for fish Hb, even in the presence of much lower doses of PQ. Values of $P_{50}O_2$ parameter were also calculated as the indicator of changes of oxygen affinity induced by PQ in the range of doses of 5–50 ppm (Table 3). It was shown that

Table 3

Decrease of p_{50} O_2 values (oxygenation parameter) induced by paraquat.
All conditions the same as in Fig. 4

A) Carp Hb

p_{50} O_2 (mmHg)	Control	5 ppm PQ	10 ppm PQ	25 ppm PQ
$\bar{X}$	5.75	3.05	1.56	0.78
S.D.	±1.06	±0.75	±0.66	±0.38

B) Bovine Hb

p_{50} O_2 (mmHg)	Control	10 ppm PQ	25 ppm PQ	50 ppm PQ
$\bar{X}$	18.62	15.36	12.57	9.04
S.D.	±1.34	±0.80	±0.98	±1.01

$\bar{X}$ – Mean values; S.D. – Standard deviation.

values of this parameter increased with the increase of the dose. These results indicate that transfer of oxyHb to its met form goes through additional intermediate(s) probably ferrylHb [3, 10, 20]. As it has been shown the action of PQ on Hb solution (as well as on other hemoproteins [16, 18]) containing high amount of dissolved oxygen, is connected with the production of more active species of oxygen, especially $O_2^{\cdot}$ radicals. It is suggested that during this rather complex and complicated reaction an unstable porphyrin Fe^{4+}-π-cation (i.e. ferryl derivative) is formed [11]. Walters and Frederick [19] in their work have even hypothesised the direct oxydation of Mb (mioglobin) resulting PQ

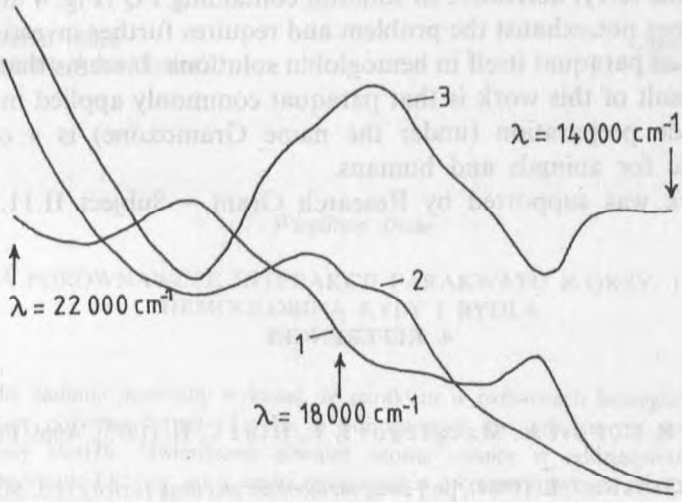


Fig. 4. Visible spectra in the range of 22 000 – 14 000 cm^{-1} of 1) 0.5% bovine metHb, 2) 0.5% bovine metHb + 10 ppm PQ, 1 h incubation (measured with respect to control: phosphate buffers, pH 7.0, plus 10 ppm paraquat), and 3) difference spectrum of (2) with respect to (1)

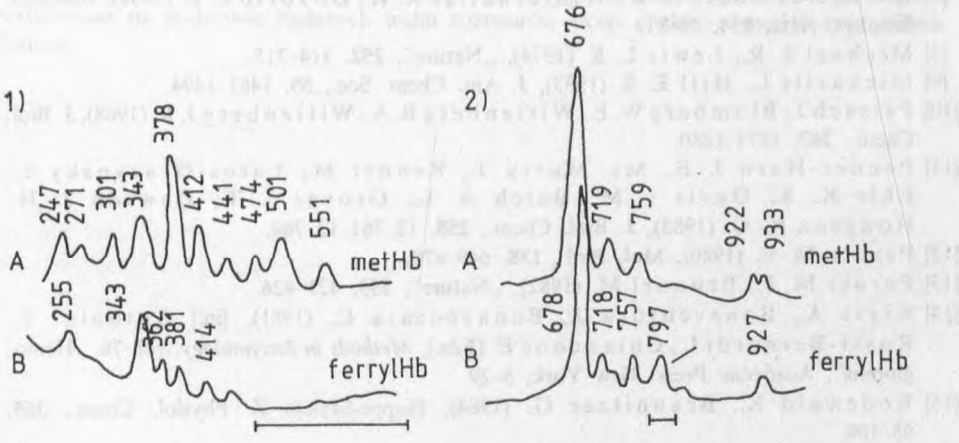


Fig. 5. Resonance Raman spectra of A) metHb and B) ferrylHb, using 406.7 nm excitation in the 250 to 600 cm^{-1} (1) and 600 to 1000 cm^{-1} frequency region (2). All conditions the same as in Fig. 3. characteristic region of ferrylHb

action as a transfer reaction $MbFe^{2+} \rightarrow MbFe^{4+}$ although the details of mechanism of this reaction remains to be explained. On the other hand our pilot studies using Raman spectroscopy methods demonstrate evidences of the existence of the ferryl derivative in solution containing PQ (Fig. 4 and Fig. 5). This study does not exhaust the problem and requires further investigations of the behavior of paraquat itself in hemoglobin solutions. It seems that the more important result of this work is that paraquat commonly applied in solutions as a herbicide preparation (under the name Gramoxone) is a compound severely toxic for animals and humans.

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

4. REFERENCES

[1] Baldwin R. C., Pasi A., Macgregor J. T., Hine C. H. (1975), Appl. Pharmac., **32**, 298–304.
[2] Bus J. S., Gibson J. E. (1984), [in:] *Drug metabolism and drug toxicity* (Ed. Mitchell J. R., Horning M. G.), Raven Press, New York, 21–32.
[3] Campbell J. R., Clark R. J. H., Clore G. M., Lane A. (1980), Inorg. Chim. Acta, **46**, 77–84.
[4] Donnell S., Mandaro R., Schuster T., Arnone A. (1979), J. Biol. Chem., **254**, 12 204–12 208.
[5] Farrington J. A., Ebert M., Land E. J., Fletcher K. (1973), Biochim. Biophys. Acta, **314**, 372–381.
[6] Gage J. C. (1968), J. Biochem., **109**, 757–761.
[7] Lin M. J.-J., Noble R. W., Winterhalter K. H., Di Iorio E. E. (1988), Biochem. Biophys. Acta, **954**, 73–81.
[8] Michael S. R., Lewis L. S. (1974), „Nature", **252**, 314–315.
[9] Michaelis L., Hill E. S. (1933), J. Am. Chem. Soc., **55**, 1481–1494.
[10] Peisach J., Blumberg W. E., Wittenberg B. A., Wittenberg J. B. (1968), J. Biol. Chem., **243**, 1871–1880.
[11] Penner-Harn J. E., Mc Murry T., Renner M., Latos-Grazynsky L., Eble K. S., Davis I. M., Balch A. L., Groves J. T., Dawson J. H., Hodgson K. O. (1983), J. Biol. Chem., **258**, 12 761–12 764.
[12] Perutz M. F. (1980), Mol. Biol., **138**, 649–670.
[13] Perutz M. F., Brunori M. (1982), „Nature", **299**, 421–426.
[14] Rigss 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.
[15] Rodewald K., Braunitzer G. (1984), Hoppe-Seylers Z. Physiol. Chem., **365**, 95–104.
[16] Sitter A. J., Reczek C. M., Ternner J. (1985), Biochim. Biophys. Acta, **828**, 229–235.
[17] Tentori L., Vivaldi G., Carta S., Salvati A. M., Sorcini M., Valami S. (1965), Arch. Biochem. Biophys., **100**, 404–414.

[18] Terner J., Sitter A. J., Reczek C. M. (1985), Biochim. Biophys. Acta, **828**, 73–80.
[19] Walters F. C., Frederick B. (1983), FEBS Lett., **163**, 2–4.
[20] Wittenberg B. A. (1972), J. Biol. Chem., **247**, 4008–4013.

Came in editorial office
„Folia biochimica et biophysica"
25.06.1991

Chair of Biophysics
University of Łódź

Wirgiliusz Duda

BADANIA PORÓWNAWCZE INTERAKCJI PARAKWATU Z OKSY- I DEOKSY-HEMOGLOBINĄ RYBY I BYDŁA

Wykonane badania pozwoliły wykazać, że parakwat w roztworach hemoglobiny powoduje zmniejszenie się poziomu formy oksyHb, z jednoczesnym (w tym samym czasie) wzrostem poziomu formy metHb. Stwierdzono również istotne różnice w oddziaływaniu parakwatu z hemoglobiną karpia i krowy, co w części związane jest z kooperatywnymi właściwościami tych hemoglobin. Kooperatywność hemoglobiny karpia zachodzi zgodnie z efektem Roota, a kooperatywność hemoglobiny krowy (podobnie jak u pozostałych ssaków) opisywana jest poprzez efekt Bohra. Takie wyjaśnienie, na podstawie obecnego stanu badań, tłumaczy tylko niewielką część wpływu parakwatu na hemoglobinę. Najważniejszym wynikiem obecnych badań jest wykazanie, że oksyHb w roztworach w obecności parakwatu przekształcana jest nie tylko w formę metHb, ale również oksyHb (Fe^{2+}) może przechodzić pod wpływem aktywnych form tlenu i generowanych przez parakwat rodników, do innych „wyżej utlenionych" niestabilnych form hemoglobiny zawierającej tlen, np. do wykazanej formy ferrylHb (Fe^{4+}), opisywanej także jako porfiryna Fe^{4+}-π-kation. Taka interpretacja powyższego efektu – wpływu parakwatu na hemoglobinę jest postulowana na podstawie badanych widm roztworów oksy- deoksy- i metHb oraz widma Ramana.