Electron-Paramagnetic-Resonance Studies on a Photochemically Produced Species of Horseradish Peroxidase Compound I

By ALAN R. McINTOSH and MARTIN J. STILLMAN*

Department of Chemistry, University of Western Ontario, London, Ont. N6A 5B7, Canada

(Received 20 January 1977)

Strong electron-paramagnetic-resonance signals in the $g = 2.00$ region were detected after irradiation of horseradish peroxidase Compound I at temperatures of 10 and 100 K. These signals establish the presence of new free-radical species in the peroxidase system. The new species are interpreted in terms of a haem-photosensitized oxidation of the protein's peptide groups close to the Compound I radical site. On warming to room temperature, the radicals decayed irreversibly to a species having a weak asymmetric electron-paramagnetic-resonance signal at 100 K, which could still be observed after incubation at room temperature for more than 1 h.

Horseradish peroxidase (donor- H_2O_2 oxidoreductase; EC 1.11.1.7) native enzyme has a ferric protoporphyrin IX prosthetic group and at neutral pH in aqueous solution the iron is in a mixed-spin configuration. Aqueous solutions are brown, with a Soret-band maximum of 403 nm (Keilin & Hartree, 1951; Brill & Williams, 1961; Blumberg *et al.*, 1968; Tamura, 1971; Dunford & Stillman, 1976).

Peroxidase reacts with two molar equivalents of H_2O_2 (Theorell & Ehrenberg, 1952) to form a green solution, Compound I (Chance, 1952). The Soret band broadens considerably, and has an ϵ value of 5.38×10^4 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ and a band centre of 400 nm (Schonbaum & Lo, 1972). Although this reaction and the subsequent reductions by one electron to Compound II and by a further electron back to the native enzyme have been extensively studied (see references in Dunford & Stillman, 1976), the overall electronic configuration of the iron, porphyrin and protein in Compound I has not been determined.

At present, it is generally accepted that Compound I is two oxidizing equivalents above the native enzyme and that the iron is in an oxidation state of +4 (Moss *et al.*, 1969). The other equivalent is considered to be in the form of a positive radical located close to the iron on either the porphyrin or the protein (Moss *et al.*, 1969; Aasa *et al.*, 1975). Since this is an organic free radical, it would be expected that an e.p.r.† signal should be detected. However, despite close study by Blumberg *et al.* (1968), it was not until 1975 that Aasa *et al.* were able to record a weak asymmetric signal at $g = 1.995$, which they identified as arising from the Compound I free radical. Although the signal was only 1% of that calculated for the protein concentration used, their claim that this

signal was due to the Compound I species was substantiated by titrating the peroxidase with H_2O_2 and monitoring the e.p.r. signal intensity at $g = 1.995$.

Stillman *et al.* (1975*a,b*) reported a photochemical reaction of Compound I that occurred both in frozen glasses between 5 and 100 K and in solution at room temperature. The identity of the product of this reaction was not determined, although the absorption spectra in the region 250–800 nm that were recorded at 10 K suggested that the electronic configuration of the iron porphyrin moiety of the new species was similar to that of peroxidase Compound II. The present paper reports the e.p.r. spectral changes observed after irradiation of peroxidase Compound I as a frozen glass at 10 and 100 K.

Experimental

Horseradish peroxidase was purchased from Boehringer Mannheim G.m.b.H., St. Laurent, Que., Canada, as a freeze-dried powder (batch no. 7315105) and dissolved in triple-distilled water. The purity number (A_{403}/A_{280}) for each of these solutions was in excess of 3.2. Glycerol used to prepare the low-temperature glasses was from Fisher Scientific Co., Montreal, Que., Canada. Solutions of peroxidase were made up in aq. 50% (v/v) glycerol with concentrations that gave an A_{403} of between 0.7 and 1.1 in a 1 mm-path-length cell. From Schonbaum & Lo's (1972) value of $\epsilon_{403} = 1.02 \times 10^5$ litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$, the concentrations of these solutions were between 70 and 105 μM . H_2O_2 was added in 1 μl portions to give a 1.1:1.0 molar ratio for H_2O_2 :peroxidase (Roman & Dunford, 1972). The green solutions were stirred rapidly after addition of the H_2O_2 and then syringed into a quartz tube. The tube was plunged into liquid N_2 within 20 s of preparation both to arrest the decay

* To whom reprint requests should be addressed.

† Abbreviation: e.p.r., electron paramagnetic resonance.

of the Compound I and to form a glass. A second portion of the Compound I formed was placed in a 1 mm cuvette and the absorption spectrum recorded repeatedly with a Cary 118 spectrophotometer over a period of 1 h at room temperature (20°C). The observed decay was exactly as reported previously (Schonbaum & Lo, 1972; Stillman *et al.*, 1975b).

Samples of Compound I were also prepared in dim light at -5°C to investigate the effect of light on the concentration of Compound I formed. In all these experiments, the e.p.r. signals were identical with those observed with solutions made at room temperature in the light, with the single exception that the intensity of the weak signal observed at $g = 2.00$ before irradiation (Fig. 1a) was diminished at both 10 and 100 K.

E.p.r. spectra were recorded on a Varian E12 X-band 100 kHz spectrometer coupled with a Fabri-Tek model 1072 instrument computer and a Varian model E257 gas-cooled cryostat for measuring spectra at 100 K. The temperature of 10 K was maintained with an Air Products model LTD-3-110 liquid-helium dewar and transfer system. A 100 mJ Photochemical Research Associates model 610 flash lamp and a 1000 W Hanovia Hg/Xe continuous lamp with a water filter were used to irradiate the sample in the cavity. Corning CS-051 and CS-053 filters were used to ensure that only light of wavelengths greater than 360 nm illuminated the sample.

Saturation studies were carried out by varying the microwave power over a wide range. It was determined that no significant power saturation occurred at 5 mW at 10 K and 10 mW at 100 K. The modulation amplitude used was 0.5 mT and this amplitude did not overmodulate any of the observed lines.

Results

Studies at 100 K

The e.p.r. spectrum of Compound I at 100 K before irradiation (Fig. 1a), referred to as the dark spectrum subsequently, shows the weak $g = 2.005$ band observed previously at temperatures above 30 K (Blumberg *et al.*, 1968; Aasa *et al.*, 1975) and assigned by these workers as an impurity. On irradiation with either the flash lamp or the continuous Hg/Xe lamp, a new and much more intense signal appears as in Fig. 1(b). The signal intensity is dependent on the time of irradiation with the 1000 W lamp (Fig. 3a) up to a maximum value observed after 60 min irradiation. Further light has little effect, until finally a slow decrease in intensity is observed. The effect of the light on the sample was also determined by subtracting spectra that were recorded before and after irradiation. Difference spectra were recorded at intervals in the formation of the new species by using both flash and continuous lamps (Fig. 1c)

Fig. 1. E.p.r. signal detected from a frozen solution of horseradish peroxidase Compound I at 100 K in the $g = 2.0$ region

All spectra are presented on the same magnetic-field scale, and all irradiations were performed in the e.p.r. cavity. (a) The e.p.r. spectrum recorded for a 105 μM solution of Compound I in aq. 50% (v/v) glycerol at 100 K before irradiation. The spectrometer settings were: 100 kHz modulation; modulation amplitude, 0.5 mT; microwave power, 10 mW; amplifier gain settings for the dark spectrum, 5×10^3 . (b) The e.p.r. spectrum at 100 K for a 105 μM solution of Compound I after 12 min of irradiation in the frozen state with a 1000 W Hg/Xe lamp with filters to absorb light wavelengths below 360 nm. Spectrometer settings were as for (a), except for the amplifier gain, which was 0.8×10^3 . (c) Difference spectra computed by subtracting the e.p.r. signals recorded before and after irradiation of the Compound I between several cumulative illumination times, showing the development of the $g = 2.00$ signal with increasing exposure time to the Hg/Xe lamp (all conditions were as for a). ○, E.p.r. spectrum for a sample irradiated for 50 s minus the spectrum recorded after 40 s, showing the increase caused by 10 s of light. △, Similar subtraction from the 2 min spectrum of the 50 s spectrum.

and represent the changes in the e.p.r. caused solely by the illumination of the sample within the e.p.r. cavity at 100 K.

Fig. 2. Effect on the e.p.r. spectrum of thawing the photolysed sample

E.p.r. spectrum at 100K showing the effect of thawing at -40°C for 5 min on an irradiated sample ($\circ$). The second trace (Δ) shows the effect of 5 min further irradiation of this sample at 100K and is the difference spectrum calculated by subtracting the spectrum recorded after thawing from the spectrum recorded after further irradiation for 5 min. The spectrum recorded before thawing was similar to that shown in Fig. 1(b).

This new e.p.r. signal is developing at 100K with $g = 2.0025 \pm 0.0005$ and a linewidth of about 1.2 mT, and is observed to have a doublet splitting of 1.6 ± 0.1 mT. At stages throughout the photolysis the sample was removed from the cavity and the colour of the peroxidase was inspected. A brown image of the optical port of the cavity slowly developed at the centre of the tube, with the dark-green Compound I colour above and below it. The absorption spectrum of this brown species has been reported by Stillman *et al.* (1975a,b). Unless the sample was irradiated or warmed-up, the e.p.r. signal remained constant; however, after storing the tube for several months at 77K in the dark some loss in e.p.r.-signal intensity was observed (about 5%).

The e.p.r. spectrum recorded after the sample had been warmed to the softening point of the glycerol (about -40°C) for 2 min and then cooled to 100K showed a loss in intensity of the symmetrical signal in the $g = 2.0$ region. This new spectrum had an asymmetrical band envelope, as shown in Fig. 2; however, the difference spectrum recorded after a further irradiation at 100K showed the same pattern of bands observed after irradiation of a sample that had not been thawed. The action of the light produced the same species even after thawing.

When the e.p.r.-signal intensity reached a maximum, the tube was warmed to room temperature and

shaken. During the next 1 h, the sample was rapidly cooled to 100K and an e.p.r. spectrum recorded; it was then warmed back to room temperature for some minutes and the cycle repeated. The slow decay of the asymmetric radical signal is apparent in the plot shown in Fig. 3(b).

Studies at 10K

Irradiations with the Hg/Xe lamp were also carried out at 10K. Before irradiation, a weak dark signal around $g = 2.00$ was seen as in Fig. 4(a). Spectra were recorded at 10K after fixed intervals of light as at 100K, and a similar growth of an intense spectrum was observed as shown in Fig. 4(b). One important difference in the 10K spectrum was the observation of a pronounced shoulder on the low-field central line for total irradiation times less than about 1 min. Furthermore, the difference spectra recorded at 10K during the first 1 min of irradiation were very different from the 100K spectra, as shown in Fig. 4(c). This spectrum can be interpreted as representing at least one radical with $g = 2.0025 \pm 0.0005$ and a linewidth of 2.0 ± 0.2 mT and possibly with some structure.

A most significant observation was that the signal intensity was observed to decay by approx. 10% at 10K after only a few minutes in the dark after the irradiation. A greater decay in the intensity was observed when a sample with about 2 min of irradiation at 10K was warmed rapidly to 77K by plunging the e.p.r. sample tube into liquid N_2 and then cooled back down to 10K after about 1 min at 77K. The difference spectrum of the decaying radicals is given in Fig. 5(a), and it appears to be quite similar to the initial irradiation spectrum of Fig. 4(c). It is noted that the spectrum observed after warming to 77K and cooling to 10K shown in Fig. 5(b) appears to be very similar to the spectra obtained from irradiation at 100K. The doublet spectrum of Fig. 5(b) exhibits two partially resolved central lines with a hyperfine splitting of 1.6 ± 0.1 mT as for the spectra found in the 100K irradiations.

Discussion

The observation of the symmetric signals at $g = 2.0025 \pm 0.0005$ suggests that the photochemical product contains free radicals. These new species cannot be Compound II, as no signal has been detected for Compound II made chemically (Blumberg *et al.*, 1968), and an e.p.r. signal would not be expected for the Fe(IV) porphyrin electronic configuration currently accepted for Compound II (Moss *et al.*, 1969; Dolphin & Felton, 1974). The absorption spectrum of the photochemical product at 10K resembles that of a low-spin iron porphyrin

Fig. 3. Changes in the e.p.r.-signal intensity recorded at 100 K in the region $g = 2.00$ as a function of irradiation time in the e.p.r. cavity and time at room temperature

(a) Increasing intensity as the length of time of irradiation increases. The light source was the filtered 1000 W Hg/Xe lamp. (b) Decrease in signal intensity recorded after the sample had been held at room temperature for 0, 3, 12 and 60 min. The sample was cooled to 100 K before the spectrum was recorded after each warming period. The lines joining the points are for clarity only and have no theoretical significance.

with the α - and β -bands coalesced in the 530–540 nm region and the Soret band at 418 nm (Stillman *et al.*, 1975b). This almost unperturbed absorption spectrum suggests that a radical is not located close to the porphyrin π -ring. Dolphin and co-workers (for example, Dolphin & Felton, 1974) have synthesized a considerable number of porphyrin-cation radicals, and in most cases the absorption spectrum is very much changed compared with the neutral species.

It is probable that the oxidation state of the protein as a whole for the photochemical product remains the same as for Compound I, that is, an Fe(IV) porphyrin and a cation radical. The action of light in this scheme is to sensitize a transfer of the radical to a site at least 1 nm away from the iron atom. The effect on the e.p.r. spectrum would be an increase in the intensity of the free-radical signal caused by a decrease in the effect of the dipolar broadening of the haem iron. An approximate spin concen-

tration was determined by measuring a nitroxide free-radical spectrum of known concentration. From this, we estimate that the e.p.r.-signal intensity after 12 min irradiation of Compound I at 100 K represented about 50% of the maximum theoretical value.

The currently accepted electronic configuration of peroxidase Compound I has two unpaired electrons situated on the Fe(IV) and one unpaired electron located on a nearby radical (Swartz *et al.*, 1972b; Theorell & Ehrenberg, 1952). This same configuration is suggested for cytochrome *c* peroxidase Complex ES (Yonetani *et al.*, 1966) with the radical now located some distance from the iron centre. The configuration of peroxidase Compound II is considered to have the same Fe(IV), with two unpaired electrons, but now the radical has been reduced by the substrate (Theorell & Ehrenberg, 1952; Moss *et al.*, 1969; Dunford & Stillman, 1976).

Fig. 4. E.p.r. signals detected at 10 K from a frozen solution of 100 μ M-Compound I in aq. 50% (v/v) glycerol

All spectra are presented on the same magnetic-field scale, and all irradiations were performed in the e.p.r. cavity. (a) The e.p.r. spectrum recorded before irradiation. The spectrometer settings were: 100 kHz modulation, modulation amplitude, 0.5 mT; microwave power, 5 mW; amplifier gain settings for the dark spectrum, 2.5×10^3 . (b) The e.p.r. spectrum recorded after 1 min irradiation with the filtered 1000 W Hg/Xe lamp; spectrometer settings were as for (a), except for the amplifier gain (1×10^3 , but 8×10^3 for the wings of the spectrum). (c) The e.p.r. spectrum recorded after 10 s of irradiation with the filtered 1000 W Hg/Xe lamp; spectrometer settings were as for (a).

Fig. 5. E.p.r. signals detected at 10 K from a frozen solution of 100 μ M-Compound I in aq. 50% glycerol

Both spectra are presented on the same magnetic-field scale. (a) The e.p.r. difference spectrum recorded for the radicals that decayed when the previously irradiated sample (2 min with the filtered 1000 W Hg/Xe lamp) was warmed to 77 K and then cooled back down to 10 K; spectrometer settings were as for Fig. 4(a) except for the amplifier gain, which was 4×10^3 . (b) The e.p.r. spectrum which remained after the previously irradiated sample (2 min with the filtered 1000 W Hg/Xe lamp) was warmed to 77 K and then cooled back down to 10 K; spectrometer settings were as for Fig. 4(a) except for the amplifier gain (1×10^3 , but 8×10^3 for the wings of the spectrum).

At various times, each of these species has been well studied by using e.p.r. spectroscopy (Yonetani *et al.*, 1966; Blumberg *et al.*, 1968; Aasa *et al.*, 1975). The results from these studies may be summarized as follows. Neither peroxidase Compound I nor Compound II exhibit a distinct e.p.r. spectrum characteristic of either a radical or the iron above 30 K. Compound I was found to exhibit a weak signal at $g = 1.995$ below 20 K which was assigned by Aasa *et al.* (1975) as arising from a free radical which was coupled to a fast-relaxing metal ion. Finally, in Complex ES of cytochrome *c* peroxidase

Yonetani *et al.* (1966) observed an intense narrow signal at $g = 2.00$, which they assigned as the spectrum due to the free radical located on an aromatic amino acid residue of the protein. None of these workers has observed a spectrum that could be characterized as arising from the paramagnetic Fe(IV) species.

Most biological entities containing iron reveal a large range of g factors in the cases where paramagnetism is observable. For instance, low-spin Fe(III) complexes have a range of g factors between 1.4 and 3.1, whereas high-spin Fe(II) complexes have a range

between 2.0 and 9.7 (Swartz *et al.*, 1972a). Fe(II) e.p.r. spectra are also observable, but only at temperatures near 20K where a relatively large g anisotropy is demonstrated as well.

It is highly unlikely that the e.p.r. spectra presented here with a g factor of 2.0025 ± 0.0005 could be caused by a paramagnetic iron species, but they could reasonably arise from an organic free radical. These spectra also exhibit relatively narrow line widths for species in the frozen state, and it is pertinent to discuss further the effect of dipolar broadening on the spectrum of the radical species.

The Fe(IV) atom with its two unpaired electrons will have a strong dipole-dipole interaction with any nearby radical in the non-photolysed Compound I. This dipolar interaction is a source of anisotropic broadening of e.p.r. lines which could account for the virtual lack of an e.p.r. spectrum for non-photolysed Compound I. If, however, the radical site is moved a short distance away from the paramagnetic Fe(IV), then the dipolar interaction would be dramatically diminished. If an Fe(III) species had been produced photochemically, we feel that it would have given a readily observable e.p.r. spectrum, particularly at 10K.

It is suggested that the new radicals observed at 10 and 100K could be derived from sites in the protein of Compound I, at least 1 nm away from the haem iron. To account for the formation of the observed spectra, it is reasonable to speculate that the primary photoact at 10K is the transfer of an electron or hydrogen atom from part of the nearby peptide to the radical centre, close to the haem iron in Compound I. At 10K, it is probable that this transfer is achieved by means of a quantum-mechanical tunnelling process which could operate over distances up to about 4 nm.

When the photochemistry was carried out at 10K it was found that there was a new and much sharper signal superimposed on the broad signal at 100K, and that this signal decayed quite rapidly when left for a few minutes at 10K or when the sample temperature was raised to 77K. The resultant signal was identical with that produced at 100K. It is suggested that there is at least one unstable (Figs. 4c and 5a) precursor to the doublet-spectrum species (Fig. 5b). Likely candidates for a precursor are cysteine, histidine, methionine, tyrosine, glutamine and tryptophan (Van der Vorst, 1969; Valrant & Van der Vorst, 1972; Lion & Van der Vorst, 1974).

One explanation for the appearance of the doublet-spectrum species is the transfer of a hydrogen atom from a glycine link of the peptide to the unstable precursor radical at both 10K and at 100K. A $\cdot\text{CH}$ radical fragment would thus be formed on glycine with no peptide-chain breakage, which would account for the doublet spectra observed at both

temperatures. The weak wings of the spectra (Figs. 4b and 5b) may be due to a residual portion of the precursor radical which remains even at 77 and 100K. One good candidate for forming both radical spectra of Figs. 5(a) and 5(b) would be a histidine-glycine linkage in the peptide undergoing the photochemical reactions. For instance, electron irradiation of histidine-glycine dipeptides at 150 and 300K in the solid state yields similar spectra (Panin *et al.*, 1972). However, another amino acid linked to glycine could conceivably result in similar spectra (Van de Vorst, 1969; Lion & Van der Vorst, 1974). The doublet spectrum could also be caused by a tyrosine free radical formed by loss of one hydrogen atom next to the aromatic ring (Sjöberg *et al.*, 1977).

If the new photochemical species had the electronic configuration of an Fe(IV) porphyrin and an amino acid radical, we would expect to observe in the electronic absorption spectrum bands similar in intensity and wavelength to that of Compound II. The electronic configuration of this species would then be similar to that proposed for the cytochrome *c* peroxidase-peroxide complex (Yonetani *et al.*, 1966). In the cytochrome *c* peroxidase Complex ES a symmetrical e.p.r. signal is observed at $g = 2.00$, and in the absorption spectrum the α -band of the porphyrin is observed at 561 nm, the β -band at 529 nm and the Soret band at 419 nm (Yonetani & Ray, 1965).

Absorption bands in the new peroxidase species are observed at 546, 528 and 416 nm, similar in position to those of both Compound II and the cytochrome *c* peroxidase Complex ES (Stillman *et al.*, 1975a,b). The very low wavelength values of the band centres for the α - and β -bands appear to be a feature of the high oxidation state and low spin of the Fe(IV); for examples of the wavelengths of bands observed for peroxidase and its complexes see Brill & Williams (1961) and Dunford & Stillman (1976). It has been suggested from analysis of magnetic-circular-dichroism spectra that the α -band of Compound I occurs at about 554 nm (Stillman *et al.*, 1976), again a considerably lower wavelength than found for Fe(III) and Fe(II) complexes. Finally, if this photochemically produced species is still two oxidizing equivalents above the charge on the native enzyme, reaction may proceed much more rapidly to form Compound II than from Compound I that has not been irradiated. It is possible that this accounts for the greatly enhanced decay rate of Compound I to the native enzyme after irradiation of an aqueous solution at room temperature compared with the decay of a solution left in the dark (Stillman *et al.*, 1975b).

Note Added in Proof (Received 26 July 1977)

The results of a similar study have recently been reported by Chu *et al.* (1977).

We thank Professor J. R. Bolton for use of his e.p.r. spectrometer and the National Research Council of Canada for financial support.

References

- Aasa, R., Vanngard, T. & Dunford, H. B. (1975) *Biochim. Biophys. Acta* **391**, 259–264
- Blumberg, W. E., Peisach, J., Wittenberg, B. A. & Wittenberg, J. B. (1968) *J. Biol. Chem.* **243**, 1854–1862
- Brill, A. & Williams, R. J. P. (1961) *Biochem. J.* **78**, 253–262
- Chance, B. (1952) *Arch. Biochem. Biophys.* **41**, 404–415
- Chu, M., Dunford, H. B. & Job, D. (1977) *Biochem. Biophys. Res. Commun.* **74**, 159–164
- Dolphin, D. & Felton, R. H. (1974) *Acc. Chem. Res.* **7**, 26–32
- Dunford, H. B. & Stillman, J. S. (1976) *Coord. Chem. Rev.* **19**, 187–251
- Keilin, D. & Hartree, E. F. (1951) *Biochem. J.* **49**, 88–104
- Lion, Y. & Van der Vorst, A. (1974) *Radiat. Environ. Biophys.* **11**, 239–245
- Moss, T. A., Ehrenberg, A. & Bearden, A. J. (1969) *Biochemistry* **8**, 4159–4162
- Panin, V. I., Ponomareva-Stepnaya, M. A., Molodov, L. A. & Vsatyi, A. F. (1972) *Biofizika* **17**, 594–598
- Roman, R. & Dunford, H. B. (1972) *Biochemistry* **11**, 2076–2082
- Schonbaum, G. R. & Lo, S. (1972) *J. Biol. Chem.* **247**, 3353–3360
- Sjöberg, B. M., Reichard, P., Gräslund, A. & Ehrenberg, A. (1977) *J. Biol. Chem.* **252**, 536–541
- Stillman, J. S., Stillman, M. J. & Dunford, H. B. (1975a) *Biochem. Biophys. Res. Commun.* **63**, 32–35
- Stillman, J. S., Stillman, M. J. & Dunford, H. B. (1975b) *Biochemistry* **14**, 3183–3188
- Stillman, M. J., Hollebone, B. R. & Stillman, J. S. (1976) *Biochem. Biophys. Res. Commun.* **12**, 554–559
- Swartz, H. M., Bolton, J. R. & Borg, D. C. (eds.) (1972a) *Biological Applications of Electron Spin Resonance*, p. 24, Wiley-Interscience, New York
- Swartz, H. M., Bolton, J. R. & Borg, D. C. (eds.) (1972b) *Biological Applications of Electron Spin Resonance*, pp. 309–313, Wiley-Interscience, New York
- Tamura, M. (1971) *Biochim. Biophys. Acta* **243**, 239–248
- Theorell, H. & Ehrenberg, A. (1952) *Arch. Biochem. Biophys.* **41**, 442–461
- Valrant, P. & Van der Vorst, A. (1972) *Biofizika* **17**, 965–970
- Van der Vorst, A. (1969) *Bull. Soc. R. Sci. Liège* **38**, 557–585
- Yonetani, T. & Ray, G. S. (1965) *J. Biol. Chem.* **240**, 4503–4508
- Yonetani, T., Schleyer, H. & Ehrenberg, A. (1966) *J. Biol. Chem.* **241**, 3240–3243