Biochimica et Biophysica Acta, 333 (1974) 421–429

BBA 46713

BIOCHEMICAL AND BIOPHYSICAL STUDIES ON CYTOCHROME *c* OXIDASE

XIV. THE REACTION WITH CYTOCHROME *c* AS STUDIED BY PULSE RADIOLYSIS

K. J. H. VAN BUUREN[a, b], B. F. VAN GELDER[a], J. WILTING[b] and R. BRAAMS[b]

[a]*Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, Amsterdam (The Netherlands) and* [b]*Physical Laboratory, State University of Utrecht, Sorbonnelaan 4, Utrecht (The Netherlands)*

(Received September 25th, 1973)

SUMMARY

1. The reduction of cytochrome *c* oxidase by hydrated electrons was studied in the absence and presence of cytochrome *c*.

2. Hydrated electrons do not readily reduce the heme of cytochrome *c* oxidase. This observation supports our previous conclusion that heme *a* is not directly exposed to the solvent.

3. In a mixture of cytochrome *c* and cytochrome *c* oxidase, cytochrome *c* is first reduced by hydrated electrons ($k = 4 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ at 22 °C and pH 7.2) after which it transfers electrons to cytochrome *c* oxidase with a rate constant of $6 \cdot 10^{7}\ M^{-1} \cdot s^{-1}$ at 22 °C and pH 7.2.

4. It was found that two equivalents of cytochrome *c* are oxidized initially per equivalent of heme *a* reduced, showing that one electron is accepted by a second electron acceptor, probably one of the copper atoms of cytochrome *c* oxidase.

5. After the initial reduction, redistribution of electrons takes place until an equilibrium is reached similar to that found in redox experiments of Tiesjema, R. H., Muijsers, A. O. and Van Gelder, B. F. (1973) Biochim. Biophys. Acta 305, 19–28.

INTRODUCTION

The steady-state kinetics for the oxidation of ferrocytochrome *c* by cytochrome *c* oxidase have been studied extensively [1–7], but such studies supply only a limited amount of information concerning the mechanism of action of the enzyme. A more appropriate way to clarify the individual steps of the reaction is by study of the binding reaction of the enzyme with its substrates, the so-called pre-steady state or transient kinetics. A problem in such study of the transient kinetics of the oxidation of ferrocytochrome *c* by cytochrome *c* oxidase [8–10], is that the first step is usually complete within a few milliseconds. Although there has been extensive development of rapid-mixing apparatus since the introduction of such methods by Hartridge and

Roughton [11], the time needed for mixing (1–3 ms) remains a limitation of the method. Such a time is often too long for an accurate description of the events in the reaction of cytochrome *c* with cytochrome *c* oxidase. Recently, however, perturbation and relaxation methods have been developed [12, 13] which permit the study of reactions in the microsecond range.

In previous papers [14, 15], we have described the use of the pulse-radiolysis technique for study of the reduction mechanism of cytochrome *c*, and have reported that the hemoprotein is reduced by hydrated electrons, rapidly (5–20 μs) and in high yield. Since it is now shown in this paper that the heme *a* of cytochrome *c* oxidase is hardly at all reduced directly by hydrated electrons, the reduction of cytochrome *c* oxidase by cytochrome *c* can be studied with the pulse-radiolysis technique at a 100-fold higher time resolution than with the flow methods. By this means, more accurate kinetic data can be collected. Moreover, pulse radiolysis has a further advantage over the stopped-flow method in that anaerobic conditions can be easier fulfilled. This considerably simplifies study of the kinetics of reduction of cytochrome *c* oxidase. There are, however, also some difficulties. The rather high reactivity of the hydrated electrons with ions [16] has forced us to work at low ionic strength. Although cytochrome *c* oxidase usually aggregates [17, 18] at low salt concentrations we found that it remains stable in solution after preincubation with a soya-bean phospholipid, Asolectin, at least during the time of the experiment.

The present paper reports results concerning the transient kinetics of the reaction of cytochrome *c* with cytochrome *c* oxidase at low ionic strength.

MATERIALS AND METHODS

Enzymes

Cytochrome *c* was isolated according to the method of Margoliash and Walasek [19]. The final preparation, chromatographed on Amberlite CG 50 (particle diameter 40–60 μm) to remove modified material [20], was exhaustively deionised on mixed-bed columns, and was then lyophilyzed and stored at −20 °C. Handled in this way the enzyme shows full enzymic activity and a 30–50% higher rate of reaction with hydrated electrons than the commercially available lyophilyzed preparations (unpublished observation). The concentration of cytochrome *c* was calculated [21] using either a $\Delta A_{550\ nm}$ (red minus ox) of 21 $mM^{-1} \cdot cm^{-1}$ or a $\Delta A_{416\ nm}$ (red minus ox) of 57 $mM^{-1} \cdot cm^{-1}$.

Cytochrome *c* oxidase was isolated from heart-muscle preparations generally according to the method of Fowler et al. [22]. It was further purified by cholate–$(NH_4)_2SO_4$ fractionations as described by MacLennan and Tzagoloff [23]. The procedure for the isolation has been described in detail by Van Buuren [24]. The final preparation was dissolved in 50 mM Tris–sulphate buffer (pH 8.0) and after centrifugation at 200 000 × *g* for 1 h it was stored in small quantities at 77 °K. These preparations meet the specifications previously described [7]. The concentration of cytochrome *c* oxidase (per 2 heme *a*) was calculated [25] using either a $\Delta A_{605\ nm}$ (red minus ox) of 24 $mM^{-1} \cdot cm^{-1}$ or a $\Delta A_{444\ nm}$ (red minus ox) of 160 $mM^{-1} \cdot cm^{-1}$. After each experiment the total concentration of cytochrome *c* and cytochrome *c* oxidase in the reaction mixture was determined by difference spectroscopy, using a Cary-17 recording spectrophotometer. The observed absorbance changes (red minus

ox) were corrected for the mutual contribution of the cytochromes by means of the following set of simultaneous equations [26]:

$$\Delta A_{605\ nm}\ (\text{cytochrome } c \text{ oxidase}) = \Delta A_{605\ nm} + 0.06\ \Delta A_{550\ nm}$$
$$\Delta A_{550\ nm}\ (\text{cytochrome } c) = \Delta A_{550\ nm} + 0.10\ \Delta A_{605\ nm}$$

or

$$\Delta A_{444\ nm}\ (\text{cytochrome } c \text{ oxidase}) = \Delta A_{444\ nm} + 0.52\ \Delta A_{550\ nm}$$
$$\Delta A_{416\ nm}\ (\text{cytochrome } c) = \Delta A_{416\ nm} + 1.35\ \Delta A_{605\ nm}$$

The error introduced in these calculations is less than 1%.

In these pulse-radiolysis experiments the rapid changes in transmittance had to be measured at relatively large slit width (bandwidths of 1.5–6 nm). Therefore, the absorbance coefficients were corrected appropriately in calculating concentrations. Except for the absorbance coefficient of the α band of cytochrome *c*, these corrections were small.

Pulse radiolysis

For the pulse-radiolysis experiments the enzyme was diluted to 100–150 μM with a solution mixture containing 2.5 mg Asolectine/ml, 10 mM phosphate buffer (pH 7.0) and 0.25 M sucrose. It was then centrifuged at 200 000 $\times g$ for 10 min. The clear supernatant was diluted to the appropriate concentration in the matrix solution, which consisted of a deaerated sodium phosphate buffer (5 mM, pH 7.2) in triple distilled water. Methanol (40 mM) was always present for effective scavenging of the OH radicals [27] produced during the irradiation. After addition of the enzyme(s) to the matrix solution the last traces of oxygen were removed by bubbling with highly purified argon for 10–20 min. The silica reaction cell was filled anaerobically using a method originally described by Christensen et al. [28].

Apparatus

Hydrated electrons at 0.2–2.0 μM concentrations were produced in the matrix solution by a pulsed beam of high-energy electrons (1.8 MeV) from a 2 MV Van de Graaff accelerator (High Voltage Engineering (Europe) N.V.). The beam dimensions were 20 mm $\times$ 20 mm, and the pulse time was 0.55 μs. The concentration of the hydrated electrons was determined [29] either spectrophotometrically using an $A_{650\ nm}$ of 14.1 $mM^{-1} \cdot cm^{-1}$ (at $\Delta\lambda$ of 6 nm) or by measuring the net charge of electrons captured by the aluminium cell holder. In the latter case the charge was standardized by measuring the concentration of hydrated electrons in the solvent spectrophotometrically. The temperature during the measurements was monitored continuously with a Philips Chromel-Alumel thermocouple connected to a Philips GM 6020 Voltmeter. The fast optical spectrometry was performed with a system consisting of a Bausch and Lomb grating monochromator (1350 rulings per mm), a 1 P28 photomultiplier, a pulse amplifier, and a Tektronix 545 A oscilloscope fitted with a G or D plug-in unit. Ultraviolet light was filtered (Corning CS 3-74) in order to avoid photolytic effects of the cell contents and to eliminate higher-order light. Both the photomultiplier tube and the cell were protected against excessive continuous illumination by a shutter between the high-intensity Xenon arc (450 W) and the measuring cell.

Chemicals

Asolectin (Associated Concentrated Inc., New York) sols (50 mg/ml) were prepared according to the method of Wharton and Griffiths [30] and stored at 0–5 °C. The chemicals used were of Analar grade and were purchased mainly from Baker Chemicals.

RESULTS AND DISCUSSION

Fig. 1 shows the decay of hydrated electrons measured at 650 nm in the absence and presence of the hemoproteins. The reaction is pseudo-first order and the half-time in the matrix solution (5 mM phosphate buffer (pH 7.2), 20 μg Asolectine per ml, 2.5 mM sucrose, and 40 mM methanol) is 6.9 μs. In the presence of 1.8 μM cytochrome *c* oxidase the half-time decreases to 3.7 μs, indicating that a portion of the hydrated electrons have reacted with the enzyme. The fraction of hydrated electrons (α) that react with cytochrome *c* oxidase is calculated from the equation [31]:

$$\alpha = 1 - \frac{t_{\frac{1}{2}}\,(\text{cytochrome+matrix})}{t_{\frac{1}{2}}\,(\text{matrix})}, \text{ in which } t_{\frac{1}{2}} \text{ represents half-time.}$$

Of the 2.45 μM hydrated electrons produced, then, 1.15 μM react with cytochrome *c* oxidase, while the rest are dissipated in the matrix solution. To investigate whether the reaction of hydrated electrons with cytochrome *c* oxidase leads to reduction of the heme, we followed the change in transmittance at 444 nm, the absorbance maximum of the γ-band of cytochrome *c* oxidase. This is shown in the inset of Fig. 1,

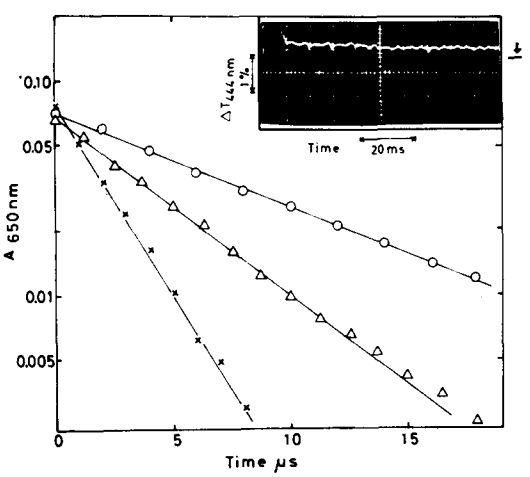

Fig. 1. Decay tracings of hydrated electrons in the presence and absence of cytochromes. ○—○, matrix solution (5 mM phosphate buffer (pH 7.2), 2.5 mM sucrose, 40 mM methanol and 20 μg Asolectin per ml); △—△, matrix solution plus 1.15 μM cytochrome *c* oxidase; ×—×, matrix solution, 1.15 μM cytochrome *c* oxidase plus 10.4 μM cytochrome *c*. Pulse dose: 19.0, 19.5 and 19.5 nC, respectively; temp., 23–24 °C. $\Delta\,\lambda$, 6 nm. The inset shows the reduction of heme *a* by hydrated electrons. Pulse dose, 21 nC; temp., 22 °C; $\Delta\,\lambda$, 1.5 nm. The arrow indicates the final level of transmittance measured 2–5 s after the pulse with a Philips digital multimeter connected to the photomultiplier.

with the time axis in ms. From the small changes in transmittance it can be calculated that only 14 nM heme *a* is reduced, corresponding to a yield of less than 3%.

Upon addition of 10.4 μM cytochrome *c* to the solution of cytochrome *c* oxidase, the half-time decreases to 1.7 μs. It is calculated that in this experiment 0.52 μM of hydrated electrons have been consumed by cytochrome *c* oxidase, 0.58 μM by the matrix solution, while the remaining 1.35 μM have reacted with cytochrome *c*. From the change in absorbance at 550 nm (not shown) it could be calculated that in this particular experiment 1.08 μM cytochrome *c* was reduced and thus, of the hydrated electrons that have reacted with cytochrome *c*, 80% are used for the reduction of heme *c*. This yield corresponds with that found in the absence of cytochrome *c* oxidase (unpublished). In this context it is interesting to note that although the distribution of hydrated electrons reacting with cytochrome *c* and cytochrome *c* oxidase is very dependent on their respective concentrations, the yield of reduction of the hemes was found to be rather constant.

Since hydrated electrons are very reactive with heme [15] the low yield of reduction of heme *a* indicates that the heme is masked by the protein moiety. That the heme of cytochrome *c* oxidase is not directly exposed to the solvent is in accordance with our earlier results obtained from ligand binding studies [24, 26, 32–34]. In cytochrome *c* where also the heme is nearly completely surrounded by the polypeptide chain [35], a specific electron transfer pathway from the surface of the enzyme to the heme via certain amino-acid side chains has been proposed [30, 36, 37]. The rapid and effective reduction of the heme iron by hydrated electrons can be explained in the same way. The observation that hydrated electrons do not reduce heme *a* effectively might indicate that in cytochrome *c* oxidase no such electron-transfer pathway exists. However, reduction of heme *a* by cytochrome *c* is fast ($> 10^7\ M^{-1} \cdot s^{-1}$ at pH 7.4, 25 °C) and effective [8] and since direct heme *c*-heme *a* interaction is unlikely, it is proposed that in cytochrome *c* oxidase an electron-transfer pathway is also present.

However, an explanation is needed for the difference in efficiency of heme *a* reduction when hydrated electrons or ferrocytochrome *c* are used as electron donors. It has been proposed that the interaction between cytochrome *c* and cytochrome *c* oxidase is mainly electrostatic [38–40] with the negative charge on the oxidase and the positive charge on cytochrome *c*. It is consistent with the above results that the entrance to the electron path in the oxidase is surrounded by negative charges, which will attract cytochrome *c* and repulse the hydrated electron and thus decrease the reactivity towards this site. Support for electrostatic interaction between the two cytochromes is the observation that on increasing the phosphate or cacodylate buffer concentration from 5 to 150 mM the second-order rate constant of the reaction of ferrocytochrome *c* with cytochrome *c* oxidase decreases to 1% of the value at 5 mM (unpublished).

The fact that hydrated electrons rapidly reduce the heme of cytochrome *c* [14, 15, 41, 42] ($t_{\frac{1}{2}} = 1$–5 μs), whereas this is not the case for cytochrome *c* oxidase, has been used for the study of the reaction of reduced cytochrome *c* with cytochrome *c* oxidase under anaerobic conditions. In Fig. 2A the transmission was monitored at 416 nm, the Soret band of ferrocytochrome *c*. The reaction is fast, reduction being complete in about 10 μs. As can be seen from Fig. 2B the reduction by hydrated electrons is monophasic and first order, with a half-time of 2.0 μs. From this value

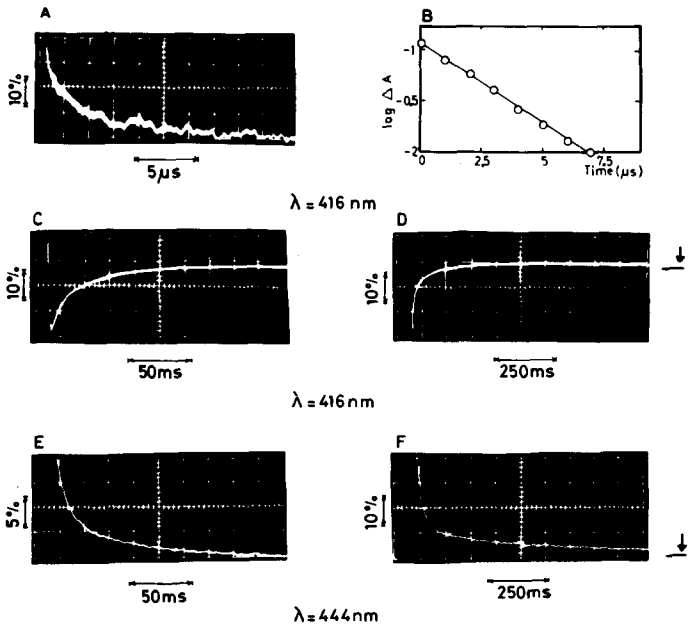

Fig. 2. Changes in transmittance of the hemes after irradiation of a mixture of 5.7 μM cytochrome *c* and 1.15 μM cytochrome *c* oxidase with primary electrons. (A) Reduction of cytochrome *c* by hydrated electrons. Pulse dose, 19 nC; temp., 23 °C; $\Delta\lambda$, 3 nm. (B) First-order plot for the reduction of cytochrome *c*, calculated from A. (C) Oxidation of cytochrome *c* by cytochrome *c* oxidase at 0.2-s sweep. Pulse dose, 22 nC ; temp., 22.5 °C; $\Delta\lambda$, 3 nm. (D) Oxidation of cytochrome *c* by cytochrome *c* oxidase at 1-s sweep. Pulse dose, 21 nC; temp., 22 °C; $\Delta\lambda$, 3 nm. (E) Reduction of cytochrome *c* oxidase by cytochrome *c* at 0.2-s sweep. Pulse dose, 16.5 nC; temp., 22 °C; $\Delta\lambda$, 1.5 nm. (F) Reduction of cytochrome *c* oxidase by cytochrome *c* at 1-s sweep. Pulse dose, 16.0 nC; temp., 22 °C; $\Delta\lambda$, 1.5 nm. The arrows in Figs D and F indicate the final level of transmittance measured as described in Fig. 1.

a second-order rate constant for the reaction can be calculated. The resulting value of $4 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ at 24 °C and pH 7.2 is in excellent agreement with the value reported earlier [15].

Figs 2C and 2D show the rate of oxidation of reduced cytochrome *c* by cytochrome *c* oxidase, measured at the Soret band of ferrocytochrome *c* at two different time spans. The sharp decrease in transmittance at 416 nm as shown in Fig. 2C represents the rapid reduction of cytochrome *c*, which is then followed by its much slower re-oxidation. The second-order rate constant calculated from the initial rate, has a value of $5.4 \cdot 10^{7}\ M^{-1} \cdot s^{-1}$ (22 °C, pH 7.2) similar to the highest value reported by Gibson et al. [8].

The tracings of Figs 2E and 2F illustrate the rate of reduction of cytochrome *c* oxidase by cytochrome *c* at two different time spans. Since the time courses for the initial part of the reaction for the oxidation of cytochrome *c* and for the reduction of heme *a* are similar (cf. Figs 2C and 2E), no indications for intermediates in the reaction are present. This is also seen from the fact that the second-order rate constants for the oxidation of cytochrome *c* and reduction of cytochrome *c* oxidase, as calculated from initial rates, have almost the same value ($5.4 \cdot 10^{7}\ M^{-1} \cdot s^{-1}$ and $6.0 \cdot 10^{7}\ M^{-1} \cdot s^{-1}$, respectively).

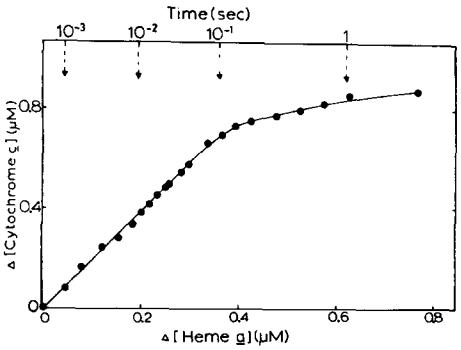

Fig. 3. Relationship between the concentration of cytochrome *c* oxidized (measured at 416 nm) and heme *a* reduced (measured at 444 nm) at selected times after pulses of 20 ± 1 nC. Concentrations of cytochrome *c* and cytochrome *c* oxidase were 5.8 and 1.20 μM, respectively; all other conditions are as described in Fig. 2.

Under the conditions used cytochrome *c* oxidation is virtually complete after 150 ms (Fig. 2D). As seen in Fig. 2F this is not the case for the reduction of heme *a*; after 150 ms a progressive decrease in transmittance is still observed. This second phase is complete after 1–2 s, and is probably to be explained by redistribution of electrons to a more favourable thermodynamic equilibrium. Whether such equilibration takes place inside one molecule, or is intermolecular, is still under investigation. It should be noted that the rate of this reaction is too slow to be of importance for the catalytic function of the enzyme.

In order to study the redistribution phenomenon more closely we determined, from tracings such as are shown in Fig. 2, the amount of cytochrome *c* oxidized at selected times and the amount of heme *a* reduced. In Fig. 3 the amounts of ferrocytochrome *c* oxidized are plotted against the simultaneous amounts of heme *a* reduced. The initial part of the graph is a straight line, with a slope of 1.9. This slope indicates that two equivalents of cytochrome *c* are oxidized for each equivalent of heme *a* that is reduced. From this finding we conclude, in accordance with Gibson et al. [8] (see also ref. 9) that concomitant with the reduction of heme *a* another group in cytochrome *c* oxidase takes up an electron. It is probable that copper is this second electron acceptor, since Beinert and collaborators [43–45] have observed that the reduction of enzymically active copper detectable by EPR is complete within 10 ms. Whether heme and copper are reduced simultaneously or consecutively can not be decided from these experiments. Work to elucidate this is now in progress.

At a later phase of the reaction, as shown in Fig. 3, the line of the graph curves, the ratio of cytochrome *c* oxidized to heme *a* reduced, decreasing to a value of 1.1. This decrease indicates a transfer of electrons to heme *a*. A possible source for the reducing equivalents might be the hydrated electrons that initially have been dissipated in the protein. Since hardly any changes in the redox state of the cytochromes (K. J. H. van Buuren, unpublished, and Fig. 1) are observed at times longer than 100 ms after the pulse, it is unlikely that the hemes are reduced by these electrons. We therefore ascribe the slow changes in absorbance to a transfer of electrons from the second electron acceptor to the heme; most likely an adjustment towards a thermodynamic equilibrium.

That most electrons should finally reach heme *a* is not surprising. It has been found by Muijsers et al. [46] and Tiesjema et al. [47] for the purified enzyme in the presence of cytochrome *c* that the redox potential of one of the hemes is 80 mV higher than that of the copper and 120 mV higher than that of the other heme. The potential at the final stage of the pulse radiolysis experiment, calculated from the degree of reduction of cytochrome *c* oxidase, was about 340 mV. From Fig. 3 of ref. 47 it was determined that at this potential only a minor fraction, 5–10% of the copper, would be reduced, so that at equilibrium most of the electrons are located on the heme *a*. This result corresponds well with the results of the pulse-radiolysis experiment, where the final ratio of ferrocytochrome *c* oxidized over heme *a* reduced is 1.1, indicating that about 10% of the reduction equivalents remain on the copper.

ACKNOWLEDGEMENTS

We wish to thank Professor Dr E. C. Slater and Dr H. Nauta for their interest and valuable discussion. Our thanks also go to Dr G. Casteleijn and Mr I. A. Raap for their help with the Van de Graaff accelerator and to Mrs P. M. C. Harthoorn and Mr J. A. H. Hüttmann for their assistance with the enzyme isolations. This work was supported in part by grants from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under auspices of the Foundation for Chemical Research (S.O.N.) and for Biophysics. One of us (K. J. H. V. B.) wishes to thank Z.W.O. for a long-term fellowship at the Universities of Amsterdam and Utrecht.

REFERENCES

1 Slater, E. C. (1949) Biochem. J. 44, 305–318
2 Smith, L. and Conrad, H. (1956) Arch. Biochem. Biophys. 63, 403–413
3 Minnaert, K. (1961) Biochim. Biophys. Acta 50, 23–34
4 Smith, L. and Camerino, P. W. (1963) Biochemistry 2, 1428–1432
5 Nicholls, P. (1964) Arch. Biochem. Biophys. 106, 25–48
6 Yonetani, T. and Ray, G. S. (1965) J. Biol. Chem. 240, 3392–3398
7 Van Buuren, K. J. H., Van Gelder, B. F. and Eggelte, T. A (1971) Biochim. Biophys. Acta 234, 468–480
8 Gibson, Q. H., Greenwood, C., Wharton, D. C. and Palmer, G. (1965) J. Biol. Chem. 240, 888–894
9 Andréasson, L.-E., Malmström, B. G., Strömberg, C. and Vänngård, T. (1972) FEBS Lett. 28, 297–301
10 Antonini, E., Brunori, M., Greenwood, C. and Wilson, M. T. (1973) Biochem. Soc. Trans. 1, 34–35
11 Hartridge, H. and Roughton, F. J. W. (1923) Proc. R. Soc. London Ser. A 104, 376–394
12 Eigen, M. and Hammes, G. G. (1963) Adv. Enzymol. 25, 1–38
13 Rapid Mixing and Sampling Techniques in Biochemistry (1964) (Chance, B., Eisenhardt, R. H., Gibson, Q. H. and Lonberg-Holm, K. K., eds) Academic Press, New York
14 Wilting, J., Nauta, H. and Braams, R. (1971) FEBS Lett. 16, 147–151
15 Wilting, J., Braams, R., Nauta, H. and Van Buuren, K. J. H. (1972) Biochim. Biophys. Acta 283, 543–547
16 Dorfman, L. M. and Matheson, M. S. (1965) in Progress in Reaction Kinetics, Vol. III (Porter, G., Jennings, K. R. and Stevens, B., eds), pp. 237–301
17 Jacobs, E. E., Andrews, E. C., Cunningham, W. and Crane, F. L. (1966) Biochem. Biophys. Res. Commun. 25, 87–95

18 Sun, F. F., Prezbindowski, K., Crane, F. L. and Jacobs, E. E. (1968) Biochim. Biophys. Acta 153, 804–818
19 Margoliash, E. and Walasek, O. F. (1967) in Methods in Enzymology, Vol. X (Estabrook, R. W. and Pullman, M. E., eds), pp. 339–348, Academic Press, New York
20 Margoliash, E., Ferguson-Miller, S., Tulloss, J., Kang, C. H., Feinberg, B., Brautigan, D. L. and Morrison, M. (1973) Biochemistry, in the press
21 Van Gelder, B. F. and Slater, E. C. (1962) Biochim. Biophys. Acta 58, 593–595
22 Fowler, L. R., Richardson, S. H. and Hatefi, Y. (1962) Biochim. Biophys. Acta 64, 170–173
23 MacLennan, D. H. and Tzagoloff, A. (1965) Biochim. Biophys. Acta 96, 166–168
24 Van Buuren, K. J. H. (1972) Binding of Cyanide to Cytochrome aa_3, Ph.D. Thesis, University of Amsterdam, Gerja, Waarland
25 Van Gelder, B. F. (1966) Biochim. Biophys. Acta 118, 36–46
26 Muijsers, A. O. (1973) Conformations and Properties of Cytochrome *c* oxidase, Ph.D. Thesis, University of Amsterdam, Gerja, Waarland
27 Anbar, M., Meyerstein, D. and Neta, P. (1966) J. Phys. Chem. 70, 2660–2662
28 Christensen, H. C., Nilsson, G., Pagsberg, P. and Nielsen, S. O. (1969) Rev. Sci. Instrum. 40, 786–789
29 Gordon, S. and Hart, E. J. (1964) J. Am. Chem. Soc. 86, 5343–5344
30 Wharton, D. C. and Griffiths, D. E. (1962) Arch. Biochem. Biophys. 96, 103–114
31 Henglein, A., Schnabel, W. and Wendenburg, J. (1969) Einführung in die Strahlenchemie, pp. 172–173, Verlag Chemie, GMBH, Weinheim/Bergstr
32 Van Buuren, K. J. H., Zuurendonk, P. F., Van Gelder, B. F. and Muijsers, A. O. (1972) Biochim. Biophys. Acta 256, 243–257
33 Van Buuren, K. J. H., Nicholls, P. and Van Gelder, B. F. (1972) Biochim. Biophys. Acta 256, 258–276
34 Wever, R., Muijsers, A. O. and Van Gelder, B. F. (1973) Biochim. Biophys. Acta 325, 8–15
35 Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O. B., Samson, L., Cooper, A. and Margoliash, E. (1971) J. Biol. Chem. 246, 1511–1535
36 Winfield, M. E. (1965) J. Mol. Biol. 12, 600–611
37 Takano, T., Kallai, O. B., Swanson, R. and Dickerson, R. E. (1973) J. Biol. Chem. 248, 5234–5255
38 Wainio, W. W., Eichel, B. and Gould, A. (1960) J. Biol. Chem. 235, 1521–1525
39 Davies, H. C., Smith, L. and Wassermann, A. R. (1964) Biochim. Biophys. Acta 85, 238–246
40 Person, P., Fine, A. S., Mora, P. T. and Zipper, H. (1964) J. Biol. Chem. 240, 3159–3164
41 Pecht, I. and Faraggi, M. (1972) Proc. Natl. Acad. Sci. U.S. 69, 902–906
42 Wilting, J., Van Buuren, K. J. H., Nauta, H. and Braams, R. (1972) Abstr. 8th Meet. Fed. Eur. Biochem. Soc., North Holland, Amsterdam, Abstr. no. 591
43 Beinert, H. and Palmer, G. (1964) J. Biol. Chem. 239, 1221–1227
44 Beinert, H. and Palmer, G. (1965) in Oxidases and Related Redox Systems (King, T. E., Mason, H. S. and Morrison, M., eds), Vol. II, pp. 567–590, John Wiley and Sons, New York
45 Van Gelder, B. F. and Beinert, H. (1969) Biochim. Biophys. Acta 189, 1–24
46 Muijsers, A. O., Tiesjema, R. H., Henderson, R. W. and Van Gelder, B. F. (1972) Biochim. Biophys. Acta 267, 216–221
47 Tiesjema, R. H., Muijsers, A. O. and Van Gelder, B. F. (1973) Biochim. Biophys. Acta 305, 19–28