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BIOCHEMICAL AND BIOPHYSICAL STUDIES ON CYTOCHROME *c* OXIDASE

XIII. EFFECT OF CHOLATE ON THE ENZYMIC ACTIVITY

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SUMMARY

1. Cholate is a mixed-type inhibitor of the enzymic activity of cytochrome *c* oxidase. The rate equations for mixed-type inhibition of the enzyme have been derived, based on Minnaert's Mechanism IV (1961, Biochim. Biophys. Acta 50, 23–34). The K_i of cholate for the free enzyme (E) and for the complexes of the enzyme with cytochrome *c* (ES and EP) was determined, being 125 and 190 μM, respectively.

2. Comparison of the properties of cholate and the intrinsic inhibitor of cytochrome *c* oxidase (Van Buuren et al. (1971) Biochim. Biophys. Acta 243, 468–480) with respect to their type of inhibition and their affinity for enzyme, reveals that they are identical.

INTRODUCTION

In a previous paper [1] we measured the activity of cytochrome *c* oxidase polarographically using ascorbate plus cytochrome *c* as electron donor. It was found that ascorbate does not interfere with the cytochrome *c* oxidase reaction neither with the isolated nor with the particulate enzyme. In such polarographic assays the rate of oxygen uptake is not always proportional to the enzyme concentration. This is due to the rather slow reaction of ascorbate with cytochrome *c*, which will be rate-limiting at high enzyme concentration. The rates at infinite ascorbate and/or cytochrome *c* concentration were found to be proportional to the concentration of particle-bound enzyme, but not to the concentration of isolated cytochrome *c* oxidase. This was thought to be due to the presence of an intrinsic inhibitor in the isolated cytochrome *c* oxidase preparation.

Since the surface-active agents needed for the solubilization of cytochrome *c* oxidase are inhibitors of the biological activity of the enzyme [2–6], we suggested that the bile salts used in our isolation procedure cause the intrinsic inhibition of the cytochrome *c* oxidase activity. At that time, however, the data on the inhibitory effect of cholate and deoxycholate on the enzymic activity [2–6] were too qualitative

for a definite conclusion on the nature of the intrinsic inhibitor in cytochrome *c* oxidase. We have, therefore, investigated the effects of cholate and deoxycholate on the activity of cytochrome *c* oxidase, and since it was found that our preparations contain a negligible amount of deoxycholate we focused our attention on the effects of cholate.

Part of this work has been published in Van Buuren's thesis [7].

MATERIALS AND METHODS

Enzyme preparations

Cytochrome *c* was isolated from horse heart according to the method of Margoliash and Walasek [8]. Ferrocytochrome *c* was prepared by Sephadex G-25 gel filtration as described by Horton [9], and stored in solutions of 1–3 mM at −20 °C. The concentration of cytochrome *c* was determined [10] from difference spectra (red minus ox) using a $\Delta A_{550\ nm}$ of 21 $mM^{-1}\ cm^{-1}$.

Cytochrome *c* oxidase was isolated from beef-heart muscle preparation by modification of the method of Fowler [11]. The enzyme was further purified by two cholate–ammonium sulphate fractionations as described by MacLennan and Tzagoloff [12]. The details of the procedure have been described by Van Buuren [7]. The final preparations were stored in small quantities at 77 °K and meet the specifications mentioned before [7, 13]. The concentration of cytochrome *c* oxidase was determined [14] from difference spectra (red minus ox) using a $\Delta A_{605\ nm}$ of 24 $mM^{-1}cm^{-1}$.

To decrease the cholate content of the cytochrome *c* oxidase preparations, the concentrated enzyme solutions (0.45–0.65 mM) were diluted with 20–30 vol. ice-cold 50 mM Tris–sulphate buffer (pH 8.0). Cytochrome *c* oxidase was precipitated from about 40% saturated ammonium sulphate solutions and the enzyme was collected by centrifugation at 30 000 × *g* for 10 min. The precipitate was dissolved in a minimal amount of 50 mM Tris–sulphate buffer (pH 8.0).

The activity of cytochrome *c* oxidase was determined either spectrophotometrically or polarographically as described before [15].

Determination of cholate and deoxycholate in cytochrome c oxidase

The bile-acid content of the preparations was determined by the method of Mosbach et al. [16] with some slight modifications. The procedure was as follows: samples of cytochrome *c* oxidase (2–20 nmoles heme *a*) were mixed with 10 µl 1 M HCl. After standing for 5–10 min at room temperature, the bile acids were extracted with two 5-ml portions of boiling ethanol. After centrifugation at 2000 × *g* for 10 min the supernatants were combined and the ethanol was evaporated. The residue was dissolved in 5 ml 65% H_2SO_4 and heated in a waterbath at 60 °C for 15 min. Cholate concentration was determined from $A_{320\ nm}$ and deoxycholate from $A_{385\ nm}$. The absorbances were corrected for the mutual contribution of the bile acids with the following equations:

$$A_{320\ nm}\ (\text{cholate}) = A_{320\ nm} - 0.13\ A_{385\ nm}$$

$$A_{385\ nm}\ (\text{deoxycholate}) = A_{385\ nm} - 0.20\ A_{320\ nm}$$

Cholic and deoxycholic acid standards (50–500 nmoles) were run with all determina-

tions. The bile acids have been crystallized two times from hot ethanol after treatment with active charcoal [7].

RESULTS

Bile-acid content of cytochrome c oxidase preparations

The concentration of deoxycholate in the concentrated enzyme solutions (0.45–0.65 mM) was found to be less than 2.4 mM. Yet the cholate concentration can be very high: up to 250 mM, i.e. about 400 moles cholate per mole cytochrome *c* oxidase. Thus if one of the bile salts causes the intrinsic inhibition of isolated cytochrome *c* oxidase preparations, cholate is most likely the candidate.

In a previous paper [1] we have derived rate equations, based on Minnaert's Mechanism IV [17] for the steady-state kinetics of cytochrome *c* oxidase in the polarographic/manometric system. According to Eqn 7 of that paper [1],

$$\frac{e}{v} = \frac{e}{k_3[AH_2][S+P]} + \frac{k_{-1}+k_2}{k_2}\left\{1+\frac{ae}{K_i}\right\}\left\{\frac{1}{k_1[S+P]}+\frac{1}{k_{-1}}\right\} \tag{1}$$

the lines for various cytochrome *c* oxidase concentrations in a Lineweaver–Burk plot intersect in the second quadrant at:

$$\frac{1}{[S+P]}, \quad \frac{e}{v} = -\frac{k_{-1}+k_2}{k_{-1}} \cdot \frac{k_1 k_3 a[AH_2]}{(k_{-1}+k_2)k_3 a[AH_2]+k_1 k_2 K_i},$$
$$\frac{k_{-1}+k_2}{k_{-1}} \cdot \frac{k_1 K_i}{(k_{-1}+k_2)k_3 a[AH_2] + k_1 k_2 K_i} \tag{2}$$

where *e* indicates the total concentration of cytochrome *c* oxidase; S, ferrocytochrome *c*; P, ferricytochrome *c*; AH_2, ascorbate; *a*, moles intrinsic inhibitor per mole cytochrome *c* oxidase; k_1, k_{-1}, k_2, the rate constants of Minnaert's Mechanism IV and k_3, the second-order rate constant for the reduction of ferricytochrome *c* by ascorbate.

From Eqn 2 it can be calculated that the distance of the point of intersection to the ordinate (1/[S+P]) becomes smaller upon decreasing the molar ratio of intrinsic inhibitor to cytochrome *c* oxidase. Thus, if cholate is the intrinsic inhibitor, a decrease in cholate content of the enzyme would move the point of intersection of lines at different enzyme concentrations towards the ordinate. We have, therefore, tried to decrease the cholate content of isolated cytochrome *c* oxidase by ammonium sulphate fractionation of a solution without extra added detergent. The fractionation, as described in Materials and Methods, decreased the cholate content of concentrated enzyme solutions from 200–250 mM to 35–60 mM, corresponding to a 4–6-fold decrease in molar ratio of cholate to cytochrome *c* oxidase (60–100). A further decrease in the ratio to 2–10 moles cholate per mole of cytochrome *c* oxidase could be achieved by Sephadex G-25 gel filtrations or repeated ammonium sulphate fractionations, but these procedures are not recommended since they tend to decrease the solubility and activity of the enzyme.

With a "low-cholate" preparation containing 68 moles cholate per mole cytochrome *c* oxidase the point of intersection for lines with various enzyme concentrations in a Lineweaver–Burk-type plot was determined. As can be seen in Fig. 1, the lines intersect at or very close to the ordinate. Fig. 3B of ref. 1 shows that at a high

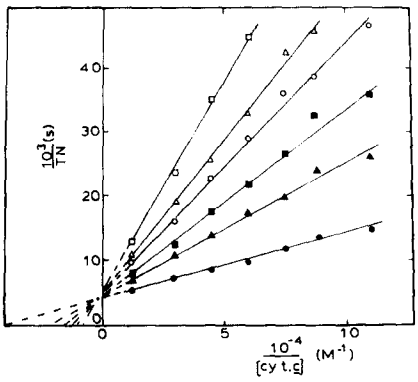

Fig. 1. Effect of cytochrome *c* and cytochrome *c* oxidase concentration on the enzymic activity. The rates, determined polarographically, are corrected for auto-oxidation of the electron donor. The assay medium consisted of 100 mM phosphate buffer (pH 7.4), 0.5 % Tween 80, 1 mM EDTA, 7–30 μM ferrocytochrome *c* and 30 mM ascorbate. Cytochrome *c* oxidase was reprecipitated from 40 % ammonium sulphate solution as described in Methods and Materials and contained 68 moles of cholate per mole cytochrome *c* oxidase. Temp. 25 °C. Cytochrome *c* oxidase: ●–●, 50 nM; ▲–▲, 100 nM; ■–■, 150 nM; ○–○, 200 nM; △–△, 275 nM and □–□, 350 nM.

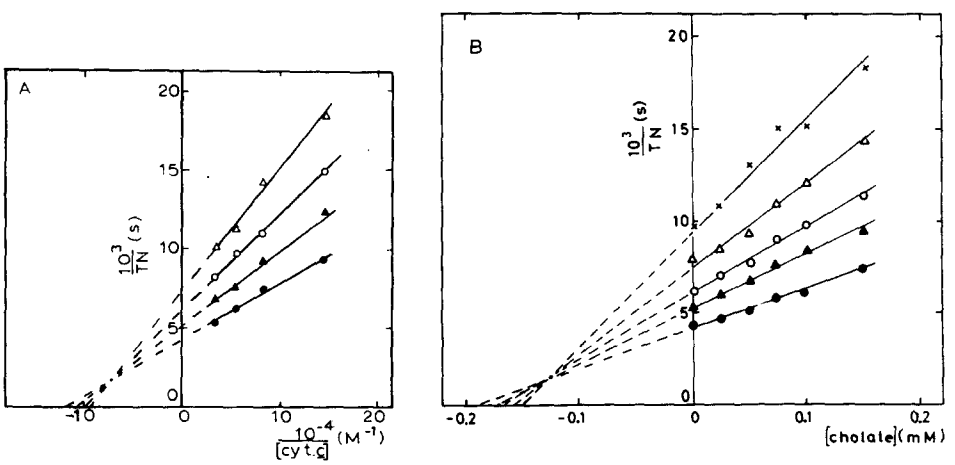

Fig. 2. Inhibition of the cytochrome *c* oxidase activity by cholate. The rates are spectrophotometrically determined. The enzyme was preincubated with cholate for 1 h in 0.2 % Asolectin, 10 mM phosphate buffer (pH 7.4), 0.25 M sucrose and 0.5 % Tween 80 at 0 °C. The assay medium as in Fig. 1, without ascorbate. In the preincubation mixture the same cholate concentration was present as during the assay. Cytochrome *c* oxidation was initiated by addition of cytochrome *c* oxidase to a final concentration of 5 nM. The turnover number (TN) is expressed as moles cytochrome *c* oxidized per mole cytochrome *c* oxidase per s. Temp., 25 °C. A. Lineweaver–Burk plot. Cholate: ●–●, no addition; ▲–▲, 50 μM; ○–○, 100 μM and △–△, 150 μM. B. Dixon plot. The points are obtained from the experiment described in Fig. 2A. Cytochrome *c*: ●–●, infinite (obtained by extrapolation of the lines in Fig. 2A); ▲–▲, 30 μM; ○–○, 18 μM; △–△, 12 μM and ×–×, 7.2 μM.

ratio (320) of cholate to cytochrome *c* oxidase, the point of intersection lies in the second quadrant but further away from the ordinate. From these observations that fit the relationship of the distance of the point of intersection to the ordinate and the ratio of intrinsic inhibitor over cytochrome *c* oxidase calculated above, it seems likely that cholate causes the intrinsic inhibition of the enzymic activity in isolated cytochrome *c* oxidase preparations.

Inhibition of cytochrome c oxidase by cholate

A more quantitative approach is obtained when the properties of cholate and the intrinsic inhibitor are compared with respect to their type of inhibition and their affinity for cytochrome *c* oxidase. The effect of cholate on the cytochrome *c* oxidase activity was measured spectrophotometrically since the very low concentrations needed for this assay contain a negligible amount of cholate with respect to the amounts of cholate added. The Lineweaver–Burk-type plot of Fig. 2A shows that cholate is a mixed-type inhibitor, in agreement with the type of inhibition found for the intrinsic inhibitor.

The general formulation of Minnaert's Mechanism IV [17] is

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow[(O_2)]{k_2} EP \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} E+P \tag{3}$$

where E, S and P represent cytochrome *c* oxidase, ferro- and ferricytochrome *c*, respectively. The rate equation for the oxidation of ferrocytochrome *c* calculated for Mechanism IV may be written (see also ref. 1, Eqn 2):

$$\frac{e}{v_0} = \frac{k_{-1}+k_2}{k_2}\left[\frac{1}{k_1[S+P]} + \frac{1}{k_{-1}}\right]\left[1+\frac{[P]_0}{[S]_0}\right] \tag{4}$$

where v_0 is the initial rate and $[P]_0/[S]_0$ the initial ratio of ferri- to ferrocytochrome *c*. Since the cytochrome *c* used in our spectrophotometric assay had been reduced for more than 96%, this ratio is close to zero and, therefore, the last term of Eqn 4 can be omitted.

A mixed-type inhibition in this mechanism demands that the affinity of E for the inhibitor differs from those of ES and EP but that the affinities of the ES and EP complex are rather similar. If ES and EP differ in their affinity to cyrochrome *c* oxidase it can be calculated that the lines in the first-order plots of the cytochrome *c* oxidation will be curved. Since straight lines are observed we can write:

$$\begin{aligned} &E+I \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} EI, \qquad \frac{k_{-4}}{k_4} = K_\alpha \\ &ES+I \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} EIS \quad \text{and} \quad EP+I \underset{k_5}{\overset{k_{-5}}{\rightleftharpoons}} EIP, \qquad \frac{k_{-5}}{k_5} = K_\beta \end{aligned} \tag{5}$$

where I represents the inhibitor, K_α and K_β are dissociation constants for the inhibitor. The steady-state solution of (3) and (5) yields Eqn 6,

$$\frac{e}{v_0} = \left\{\frac{k_{-1}+k_2}{k_2}\right\}\left\{\frac{1+\dfrac{i}{K_\alpha}}{k_1[S+P]} + \frac{1+\dfrac{i}{K_\beta}}{k_{-1}}\right\}, \tag{6}$$

where i is the total inhibitor concentration.

Eqn 6 predicts straight lines in a Dixon plot (e/v versus i) with slopes depending on the substrate concentration with a point of intersection in the second quadrant. The coordinates of this point are,

$$i, \frac{e}{v_0} = -K_\alpha, \qquad \left\{\frac{k_{-1}+k_2}{k_{-1}k_2}\right\}\left\{1-\frac{K_\alpha}{K_\beta}\right\} \tag{7}$$

Thus K_α can be directly obtained from the i-coordinate of the point of intersection. Fig. 2B which is a Dixon transform of the data of Fig. 2A, shows that the straight lines intersect in the second quadrant and thus fulfil the demands of Eqn 6. The K_α determined from the point of intersection was found to be 125 μM. At infinite cytochrome c concentration the $1/[S+P]$ term in Eqn 6 is zero and the line at infinite substrate concentration will intersect the abscissa at $i = -K_\beta$. This intersection point in Fig. 2B yields a value of 190 μM for the dissociation constant of cholate with the cytochrome c complexed enzyme.

If cholate and the intrinsic inhibitor in cytochrome c oxidase preparation are identical, the latter must also show a mixed-type inhibition of the enzymic activity. Ref. 1, Fig. 5, shows that this is indeed the case. Therefore, the inversed rate equation for the O_2 uptake at infinite ascorbate concentration is:

$$\left(\frac{e}{v}\right)_{AH_2\to\infty} = \left\{\frac{k_{-1}+k_2}{k_2}\right\}\left\{\frac{1+\frac{ae}{K_\alpha}}{k_1[S+P]} + \frac{1+\frac{ae}{K_\beta}}{k_{-1}}\right\} \tag{8}$$

This equation predicts a point of intersection for lines with different enzyme concentrations in the second quadrant with coordinates:

$$i, \left(\frac{e}{v}\right)_{AH_2\to\infty} = -\frac{K_\alpha}{a}, \qquad \left\{\frac{k_{-1}+k_2}{k_{-1}k_2}\right\}\left\{1-\frac{K_\alpha}{K_\beta}\right\} \tag{9}$$

Thus K_α/a can be determined from the coordinate of the point of intersection of the lines in ref. 1, Fig. 5. The value is found to be 0.32 mM. Analogous to the determination of K_β the value for K_β/a is determined from the point of intersection of the line at infinite cytochrome c concentration with the abscissa, and is about 0.54 mM.

From these data and the values for K_α and K_β determined from Fig. 2B, the molar ratio (a) of cholate to cytochrome c oxidase in our preparation used in ref. 1, Fig. 5, was calculated to be 370 and 350, respectively. These calculated values for the ratio correspond well with the value found by analytical determinations of cholate and enzyme that yielded a ratio of $320\pm5\,\%$ for our preparation used in ref. 1, Fig. 5.

The inhibition pattern for deoxycholate is identical to that of cholate and determinations analogous to those described above revealed that the values for K_α and K_β are 0.08 and 0.2 mM, respectively. These relatively large inhibitor constants and the low deoxycholate content of our cytochrome c oxidase preparations exclude the possibility that deoxycholate causes the intrinsic inhibition in our preparations.

DISCUSSION

We have found in this study that the type of inhibition of cholate and the intrinsic inhibitor of the cytochrome *c* oxidase preparation are the same. Moreover, it was shown that the calculated and experimentally determined ratios of the intrinsic inhibitor and cholate to the enzyme are similar. These findings and the fact that the intrinsic inhibition decreases upon lowering the cholate content of the enzyme preparations, clearly demonstrate that in our preparations cholate is the intrinsic inhibitor.

Soluble cytochrome *c* oxidase preparations can be obtained only by the use of surface-active agents such as cholate [18, 19], deoxycholate [11], dehydrocholate [20], Triton X 100 and X 114 [21] and other detergents. These surfactants are firmly bound to the protein and, as shown in Table I, the various cytochrome *c* oxidase preparations listed all contain bile salts at the final stage of their purification. The molar ratio of detergent to enzyme of most preparations is rather constant, except for that of the dialysed preparation of Yonetani [19]. The cholate content of this preparation is comparable with that of our "low-cholate" enzyme, prepared by reprecipitation of the solubilized enzyme from 40 % saturated ammonium sulphate solutions.

Because most purified cytochrome *c* oxidase preparations contain detergents one must be aware of the fact that the properties of the enzyme are mostly studied in the presence of an intrinsic inhibitor of the enzymic activity. For the study of steady-state kinetics in the spectrophotometric or polarographic system, where rather low concentrations of cytochrome *c* oxidase are used, the effect of the intrinsic detergent will be small. But, when large amounts of enzyme are used, as for instance in pre-steady-state kinetics, the high detergent concentration may interfere with the reaction under investigation. Therefore, in this type of experiments the concentration of cholate should be kept as low as possible.

It is no use trying to replace cholate by deoxycholate, Triton X 100, Triton X 114 or sodium dodecylsulphate since all these detergents were found to inhibit the

TABLE I

DETERGENT CONTENT OF CYTOCHROME *c* OXIDASE PREPARATIONS*

Authors	Ref.	Molar ratio of bile salt to cytochrome *c* oxidase
Okunuki et al., Takemori et al.	24, 25	240
Yonetani	19	40**
Griffiths and Wharton	26	480
Horie and Morrison	27	"high"
Wainio et al.	22	440***–2200
Van Buuren and Van Gelder	this paper	70†–380

* After Wainio, refs 22, 23.
** Based on a mol. wt of 200 000.
*** After chromatography on Amberlite XE 98.
† After ammonium sulphate fractionation.

rate of cytochrome *c* oxidation (unpublished). On the other hand, detergents that are not capable of extracting cytochrome *c* oxidase from the membrane, such as Tween 80 and Emasol 1130 were found to stabilize the enzyme at low cholate concentration between 0.1–5 % without inhibiting its enzymic activity [3, 28]. Unfortunately, these non-ionic detergents can only stabilize cytochrome *c* oxidase at room temperature for a period of 4–6 h, and since Muijsers et al. [29] have shown that the spectra of cholate-treated cytochrome *c* oxidase is less affected on aging than that of Tween 80-treated enzyme, the use of 0.5 % (w/v) cholate is preferred when very slow reactions of the enzyme, e.g. with ligands, must be studied.

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REFERENCES

1 Van Buuren, K. J. H., Van Gelder, B. F. and Eggelte, T. A. (1971) Biochim. Biophys. Acta 234, 468–480
2 Smith, L. (1955) J. Biol. Chem. 215, 833–846
3 Yonetani, T. (1959) J. Biochem. Tokyo 46, 917–924
4 Kremzner, L. T. and Wainio, W. W. (1961) Biochim. Biophys. Acta 52, 208–209
5 Cooperstein, S. J. (1963) J. Biol. Chem. 238, 3750–3756
6 Orii, Y. and Okunuki, K. (1965) J. Biochem. Tokyo 58, 561–568
7 Van Buuren, K. J. H. (1972) Binding of Cyanide to Cytochrome aa_3, Ph. D. Thesis, University of Amsterdam, Gerja, Waarland
8 Margoliash, E. and Walasek, O. F. (1967) in Methods in Enzymology, (Estabrook, R. W. and Pullman, M. E., eds) Vol X., pp. 339–348, Academic Press, New York
9 Horton, A. A. (1968) Anal. Biochem. 23, 334–335
10 Van Gelder, B. F. and Slater, E. C. (1962) Biochim. Biophys. Acta 58, 593–595
11 Fowler, L. R., Richardson, S. H. and Hatefi, Y. (1962) Biochim. Biophys. Acta 64, 170–173
12 MacLennan, D. H. and Tzagoloff, A. (1965) Biochim. Biophys. Acta 96, 166–168
13 Van Buuren, K. J. H., Nicholls, P. and Van Gelder, B. F. (1972) Biochim. Biophys. Acta 256, 258–276
14 Van Gelder, B. F. (1966) Biochim. Biophys. Acta 118, 36–46
15 Van Buuren, K. J. H., Zuurendonk, P. F., Van Gelder, B. F. and Muijsers, A. O. (1972) Biochim. Biophys. Acta 256, 243–257
16 Mosbach, E. H., Kalinsky, H. J., Halpern, E. and Kendall, F. E. (1954) Arch. Biochem. Biophys. 51, 402–410
17 Minnaert, K. (1961) Biochim. Biophys. Acta 50, 23–34
18 Yakushiji, E. and Okunuki, K. (1940) Proc. Imp. Acad. (Japan) 16, 299–302
19 Yonetani, T. (1961) J. Biol. Chem. 236, 1680–1688
20 Wainio, W. W., Cooperstein, S. J., Kollen, S. and Eichel, B. (1948) J. Biol. Chem. 173, 145–152
21 Sun, F. F., Prezbindowski, K. G., Crane, F. L. and Jacobs, E. E. (1968) Biochim. Biophys. Acta 153, 804–818
22 Wainio, W. W. (1964) J. Biol. Chem. 239, 1402–1405
23 Wainio, W. W. (1970) in The Mammalian Mitochondrial Respiratory Chain, pp. 282–330, Academic Press, New York
24 Okunuki, K., Sekuzu, I., Yonetani, T. and Takemori, S. (1958) J. Biochem. Tokyo 45, 847–854

25 Takemori, S., Sekuzu, I. and Okunuki, K. (1961) Biochim. Biophys. Acta 51, 464–472
26 Griffiths, D. E. and Wharton, D. C. (1961) J. Biol. Chem. 236, 1850–1856
27 Horie, S. and Morrison, M. (1963) J. Biol. Chem. 238, 1855–1860
28 Van Gelder, B. F. (1966) Heem *a* en Koper in Cytochroom *c* Oxidase, Ph. D. Thesis, University of Amsterdam, Hollandia Offset, Amsterdam
29 Muijsers, A. O., Tiesjema, R. H. and Van Gelder, B. F. (1971) Biochim. Biophys. Acta 234, 481–492