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THE ANAEROBIC INTERACTION OF FERROCYTOCHROME *c* WITH THE "FERRIC" AND "OXYGENATED" FORMS OF PURIFIED CYTOCHROME *c* OXIDASE

XVI. CYTOCHROME OXIDASE AND ITS DERIVATIVES*

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SUMMARY

1. The anaerobic interaction of ferrocytochrome *c* with "ferric" cytochrome *c* oxidase (EC 1.9.3.1) in the absence of ascorbate or ferro/ferricyanide cannot be interpreted as the reaction of a uniform ferric oxidase. The increase of the α-band at 605 mμ precedes that of the γ-band at 444 mμ and the γ/α ratio increases over a period of several minutes.

2. The biphasic form of the reaction is far less noticeable in the reaction of "oxygenated" oxidase with ferrocytochrome *c*. A similar difference has been previously found in the reduction by dithionite.

3. A spectrophotometric method is developed which allows calculations of the concentrations of ferrous and ferric cytochromes *a*, a_3 and *c* at various times after mixing oxidase and ferrocytochrome *c*. This is based on the extinction coefficients of cytochrome *c* and those of ferro- and ferricytochromes *a* and a_3 (refs. 1 and 2) at 605, 550 and 444 mμ.

4. With ferric oxidase, rapid reduction of ferricytochrome *a* is followed by a far slower reduction of ferricytochrome a_3 by ferrocytochromes *c* and *a*. No rapid equilibrium is established. Two equivalents of ferrocytochrome *c* are required for the reduction of one ferricytochrome *a* and two for that of ferricytochrome a_3 (4 per 2 moles haem *a*). The additional electron acceptor is probably the intrinsic copper of the oxidase.

5. With oxygenated oxidase, initial rapid conversion of the ferryl form of cytochrome a_3 is suggested by the fact that initially more than two equivalents of ferrocytochrome *c* are oxidized per mole of haem *a*. The ferricytochrome thus formed (a_{3x}^{3+}, ref. 2) is spectroscopically similar to or identical with ferricytochrome a_3^{3+}, but differs from it by its far more rapid reduction by ferrocytochromes *c* and *a*.

6. Ferricytochrome a_{3x}^{3+} probably plays a larger role in the enzymic reaction of the oxidase than does the ferricytochrome known as a_3^{3+} present in the ferric oxidase preparation.

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INTRODUCTION

The problem of the interaction of cytochrome *c* oxidase with cytochrome *c* has not yet been satisfactorily solved (see ref. 2). The classical theory of Keilin and Hartree postulated oxidation of ferrocytochrome *c* by ferricytochrome *a*; ferrocytochrome *a* was in turn oxidized by ferricytochrome a_3, the product of autoxidation of ferrocytochrome a_3. However, the reaction of ferrocytochrome *a* with ferricytochrome a_3 present in the "ferric" form of purified oxidase is quite slow[3–6] Nevertheless, GIBSON AND WHARTON[5] have postulated ferricytochrome a_3 as the primary product of the autoxidation of ferrous cytochrome oxidase. In contrast, OKUNUKI[7] and WAINIO *et al.*[8] have claimed that cytochrome *c* is required for the autoxidation of a uniform "cytochrome *a*", although purified cytochrome oxidase free from cytochrome *c* is rapidly autoxidizable[4,5,9]. Recently HORIO AND OHKAWA[10] have concluded from results on the anaerobic titration of purified cytochrome oxidase in the presence of ascorbate that it is necessary to assume three forms of haem *a* (*a*-1, *a*-2 and *a*-3) in cytochrome oxidase and that the results could not be interpreted on the basis of two cytochromes *a* and a_3 with the extinction coefficients assumed for them by VANNESTE[1].

There is still an unresolved contradiction in the stoichiometry of the reaction of cytochrome oxidase with cytochrome *c*. VAN GELDER and co-workers[11–13] found that NADH + phenazine methosulphate reduce two atoms of oxidase copper as well as the two ferric haematins *a* of oxidase. MINNAERT[14] and SLATER *et al.*[15], measuring the alterations of $A_{605\,m\mu}$ only, concluded from the slope of the curve log c^{2+}/c^{3+} *versus* log a^{2+}/a^{3+} that one molecule of cytochrome *c* was able to reduce two haem *a* groups of cytochrome *a* in the presence of ferro/ferricyanide. It has already been pointed out by LEMBERG[2] that this interpretation is unlikely, even if the copper of the oxidase does not react with ferrocytochrome *c*. Earlier findings[16] suggested this to be so. However, cupric copper in ionic, organic-chelated and protein-bound form is an effective oxidant of ferrocytochrome *c* (ref. 17), and later experiments of BEINERT AND PALMER[18] have shown the reduction of the copper of the oxidase is greatly accelerated by cytochrome *c*; these authors postulate that the intrinsic copper of the oxidase, but not extrinsic copper reacted with ferrocytochrome *c*.

The findings of WILLIAMS *et al.*[19] as well as those of the present paper show that two equivalents of ferrocytochrome *c* are required to reduce one equivalent of ferric haem *a* in the oxidase and it appears likely that the second electron acceptor is the copper of the oxidase, although this remains to be proved. In the present paper the same experimental approach is used as by WILLIAMS *et al* [19] and some of their data are used, but the results are calculated without some of the assumptions made in the previous paper and without the use of the Slater–Minnaert relation to calculate concentrations of ferro- and ferricytochrome *a* from those of ferro- and ferricytochrome *c*. By measuring the absorption at 444 mμ as well as those at 605 and 550 mμ at various times after anaerobic mixing of ferric oxidase with ferrocytochrome *c*, it is now shown that a second oxidation equivalent, probably the copper of the oxidase, reacts with ferrocytochrome *c*, both in the rapid reaction with ferricytochrome *a* and in the slow reaction with ferrocytochrome a_3

Similar experiments are carried out with "oxygenated" oxidase, showing a clear-cut kinetic difference between it and ferric oxidase in the reaction with ferrocytochrome *c*.

METHODS

Cytochrome oxidase was prepared by the simplified Okunuki–Yonetani method as previously described[20], using Emasol 1130 (1 %) in 0.1 M phosphate buffer (pH 7.4) as solvent in the later stages of the preparation. Attempts to replace cholate altogether by Emasol have been unsuccessful. Ferric oxidase was used as obtained in the purification. Oxygenated oxidase and ferrocytochrome *c* were freed from sodium dithionite and products of its autoxidation by Sephadex filtration as previously described[19]. The reaction was carried out in evacuated optical Thunberg tubes containing about 3 ml of the oxidase solution in the bottom part and 0.15 ml of a solution of ferrocytochrome *c* in the stopper. No correction was applied for the dilution of the oxidase solution by the addition of the cytochrome *c* as it was balanced by the loss of volume during the exhaustion of the tube. During the 2-min evacuation by a high vacuum pump, the vessel was rocked by a Vortex shaker.

Optical measurements

Optical measurements were carried out with a Perkin–Elmer "350" recording spectrophotometer, or the Cary 14R spectrophotometer. The time-course of the interaction of ferric oxidase with ferrocytochrome *c* was followed at 605 mμ and 550 mμ in the Cary spectrophotometer (Fig. 1) in two experiments. An increase of turbidity

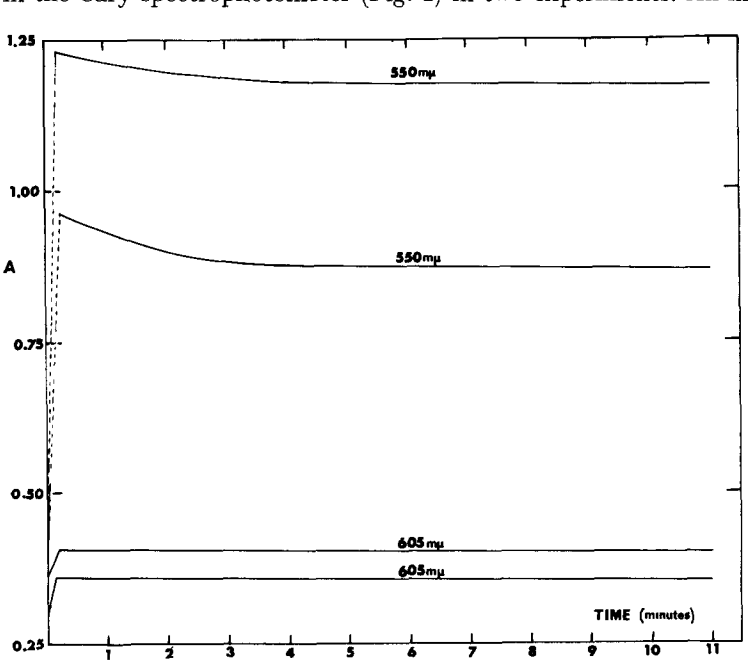

Fig 1 Time-course interaction of ferric oxidase with ferrocytochrome *c* measured in a Cary 14R spectrophotometer Increases at 550 and 605 mμ after anaerobic addition of ferrocytochrome *c* to a solution of ferric oxidase in an optical Thunberg tube The rise at 605 mμ represents practically only the rapid reduction of ferricytochrome *a* since ferrocytochrome a_3 contributes little to the 605-mμ absorption The rise at 550 mμ represents that due to the addition of ferrocytochrome *minus* the decrease caused by its partial oxidation by ferricytochromes *a* (rapid) and ferricytochrome a_3 (slow) Concentrations of cytochromes upper 550-mμ curve, Expt C87, [*c*]. 38 μM, [*a*]· 9 0 μM; lower 550-mμ curve, Expt C89, [*c*]. 25 μM, [*a*]· 6.6 μM; lower 605-mμ curve, Expt C88, [*c*]· 27 μM, [*a*]· 7 3 μM; upper 605-mμ curve, Expt C90, [*c*]: 24 μM, [*a*]. 6 2 μM

(usually < 0.10 absorbance) occurred during the evacuation and all absorption values were therefore corrected by subtracting the absorbance at 650 mμ. The $A_{605\,m\mu} - A_{650\,m\mu}$ values are converted to absolute absorbance values at 605 mμ by multiplication with 1.095 (23/21). In the experiments with oxygenated oxidase $A_{605\,m\mu}$ for ferric oxidase was obtained by dividing $A_{605\,m\mu}$ of the oxygenated oxidase by 1.1. Total concentration of haem *a* was calculated from $A_{605\,m\mu} - A_{650\,m\mu}$ in the dithionite-reduced solution using $\varepsilon_{mM} = 21$. The concentration of haem *a* thus formed also agreed with those calculated from $A_{605\,m\mu}$ (Fe^{2+}–Fe^{3+}), $\varepsilon_{mM} = 12$, or from $A_{418\,m\mu}$ of ferric oxidase after evacuation ($\varepsilon_{mM} = 100$). Total cytochrome *c* was calculated, using $\varepsilon_{mM} = 28.5$ from $A_{550\,m\mu}$ after final addition of dithionite, after subtraction of the $A_{550\,m\mu}$ that is due to absorbance of cytochrome oxidase. This is practically the same for ferrous, ferric and oxygenated oxidase and can thus be obtained from the curves of ferric or oxygenated oxidase after evacuation before addition of ferrocytochrome *c*. The amount of ferricytochrome *c* (c^{3+}) present in the solutions after addition of ferrocytochrome *c* is calculated from $A_{550\,m\mu}$ (dithionite-reduced *minus* mixed), using ε_{mM} 550 mμ (c^{2+}–c^{3+}) = 21. For the calculation of c^{3+}, the amount of cytochrome *c* oxidized by the oxidase, this must be corrected for the presence of some c^{3+} present in the ferrocytochrome *c* solution due to incomplete reduction (usually below 5 %). The absorption of cytochrome *c* at 605 mμ is negligible, but to find the absorption at 444 mμ due to oxidase, it is necessary to subtract the contributions of ferric (ε_{mM} 444 mμ = 16.75) and ferrous (ε_{mM} 444 mμ = 7.5) cytochrome *c*. If the measurements at 605 and 444 mμ are calculated with the known extinction coefficients of ferrous and ferric oxidase ($a + a_3$) (see Table I) it is found that the measurements at these wavelengths give quite different percentages of reduction. The correct percentages of reduction of

TABLE I

ASSUMED ε_{mM} OF CYTOCHROMES *a* AND a_3

	$Fe^{2+}a$	$Fe^{2+}a_3$	$Fe^{3+}a$	$Fe^{3+}a_3$
ε_{mM} 605 mμ	35.0	9.0	16.0	4.2
ε_{mM} 444 mμ	113.0	125.0	56.0	13.0

cytochromes *a* and a_3 can, however, be obtained by using extinction coefficients for ferro- and ferricytochromes *a* and a_3 such as those suggested by VANNESTE[1]. For the calculations summarized in Tables III and V we have assumed the values of Table I, which differ from those of VANNESTE only by assuming a slightly lower ε_{mM} 605 mμ for a^{2+} (35.0 rather than 39.5: see ref. 2). While these values may still require some minor adjustments they agree reasonably well with most of the properties of cytochrome oxidase. For the calculation of concentrations of a^{2+}, $a_3{}^{2+}$, a^{3+} and $a_3{}^{3+}$ we have used the following formulae based on these extinction:

$$[a^{2+}] = -2.59a_1 + 60.40a_2 - 0.09r_1 - 23.17r_2$$
$$[a_3{}^{2+}] = +10.25a_1 - 30.74a + 0.18r_1 - 2.91r_2$$
$$[a^{3+}] = +2.59a_1 - 60.40a_2 - 2.59r_1 + 60.40r_2$$
$$[a_3{}^{3+}] = -10.25a_1 + 30.74a_2 + 10.25r_1 - 30.74r_2$$

where $a_1 = A_{444\,m\mu}$ (exp.), $a_2 = A_{605\,m\mu}$ (exp.), $r_1 = A_{444\,m\mu}$ (red.), $r_2 = A_{605\,m\mu}$ (red.).

We have used the same way to calculate the percentage reductions of ferricytochromes *a* and a_{3x} (see DISCUSSION and refs. 2 and 19), in the product of action of ferrocytochrome *c* on oxygenated oxidase. This is based on the rapid conversion by ferrocytochrome *c* (half-time less than 50 msec)[9] of oxygenated oxidase into a compound spectroscopically indistinguishable from ferricytochrome a_3^{3+}. Nevertheless the results show that the ferric oxidase produced in this way differs from the ferric oxidase of the preparation (see below).

RESULTS

Anaerobic reaction of "ferric" oxidase with ferrocytochrome c

In Table II the increase of $A_{605\,m\mu}$ and decrease of $A_{550\,m\mu}$ during the interaction of ferric oxidase with ferrocytochrome *c* are used to calculate the $\Delta\varepsilon_{mM}$ 605 mμ per mole haem *a* as described under METHODS. The value of $\Delta\varepsilon_{mM}$ 605 mμ changes with time and also depends on the ratio of total cytochrome *c* to total cytochrome oxidase (haem *a*). The value decreases with time, particularly in experiments with low [*c*]/[*a*] ratios. This cannot be due to incompleteness of anaerobiosis. In fact, in a few experiments (not shown on the table) in which anaerobiosis was unsatisfactory, the rise of the absorption band at 605 mμ was at first very small and then increased later after the traces of O_2 left in the tube had been consumed. A comparison of $\Delta\varepsilon_{mM}$ 605 mμ thus found with that expected for cytochrome *a* alone (19) or for cytochromes $a + a_3$ (12) shows that the values found initially for low [*c*]/[*a*] ratios are about one half of those expected for cytochrome *a* and that they decrease with time, while with higher [*c*]/[*a*] ratios they are about one half of those expected for cytochromes $a + a_3$. The first may be possibly explained as due to the reaction of both ferricytochromes *a* and a_3 with ferrocytochrome *c*, but it will be seen below that this is not a correct explanation. The results with high [*c*]/[*a*] ratios certainly show that an electron acceptor additional to haem *a* must be involved. This is assumed to be the copper of the ferric oxidase, one of which is undoubtedly, the other possibly, in the cupric state[21]. The initial times of Table II in sec have been measured by stopwatch between the time of mixing the two cytochromes and the times of measuring at 605 mμ; in the experiments with the Perkin–Elmer spectrophotometer, the small time difference between measuring at 605 and 550 mμ (3.5 sec) has been neglected. Experi-

TABLE II

REDUCTION OF FERRIC OXIDASE WITH FERROCYTOCHROME *c*

$\Delta\varepsilon_{mM}$ 605 mμ calculated from $\Delta A_{605\,m\mu} - \Delta A_{550\,m\mu}$ and $\Delta\varepsilon_{mM}$ 550 mμ of cytochrome *c* (21). Expts 600–700, experiments of WILLIAMS *et al*[19] with Perkin–Elmer spectrophotometer. Expts. C1–C2, experiments of WILLIAMS *et al*[19] with Cary 14R spectrophotometer. Expts. 700–800, experiments of LEMBERG AND CUTLER[25] with Perkin–Elmer spectrophotometer Expts. C80–C90, experiments of LEMBERG AND CUTLER[25] with Cary 14R spectrophotometer.

		[*c*]/[*a*] *ratio.*	*1.1–2.1*	*2.6–3.7*	*4.0–5.4*	*6.9–7.5*
		Expt No.:	*763, 764, 674*	*655, 670, 759, C88*	*662, 678, 758, C85, C87*	*C1, C2*
$\Delta\varepsilon_{mM}$ 605 mμ	10 sec		10.3	6.8	6.9	7.4
	10 min		7.3	5.0	4.4	6.1

ments on the time course of the absorptions at 605 and 550 mμ on the Cary spectrophotometer (Fig. 1) show the absorbances are not significantly altered in 3.5 sec (less than 0.3 %).

The results of an experiment in which the absorption at 444 mμ had also been measured, in addition to those at 605 and 550 mμ, are set out in Table III.

TABLE III

REACTION OF FERRIC OXIDASE WITH FERROCYTOCHROME *c*

Reduction of cytochromes *a* and a_3 by ferrocytochrome *c* from measurements at 605, 550 and 444 mμ (see *Optical measurements*)

[c]/[haem a] ratio	*2.12*				*Reduced*
Time (min)	*1*	*2.5*	*6.5*	*19*	
$[c^{3+}]$ (μM)	12.6	14.0	14.4	14.55	
$[c^{2+}]$ (μM)	9.3	7.9	7.5	7.45	21.9
$\Delta[c^{3+}]$ (μM)*	10.2	11.6	12.10	12.15	
$A_{444\,m\mu}$ $(a + a_3 + c)$	0.93	1.01	1.03	1.12	1.34
$A_{444\,m\mu}$ $(a + a_3)$	0.658	0.727	0.736	0.823	1.18
$A_{605\,m\mu}$ $(a + a_3)$	0.175	0.173	0.170	0.160	0.215
γ/α ratio	3.76	4.20	4.33	5.15	5.50
Percent reduction of *a*	76	72	68	50	
Percent reduction of a_3	19	34	38	61	
$\Delta[c]/\Delta$[haem *a*]	2.1	2.2	2.3	2.2	

* $\Delta[c^{3+}]$ is lower than $[c^{3+}]$ if the ferrocytochrome *c* was not completely reduced at the beginning of the experiment

The values of $A_{605\,m\mu}$ and $A_{550\,m\mu}$ at 1 min after mixing were calculated from those observed at 20 sec in the Perkin–Elmer recording spectrophotometer using the curves illustrated in Fig. 1. This makes the time coincide with that at which $A_{444\,m\mu}$ was measured. The meaning of the parameters has been explained under *Optical measurements*. The γ/α ratio of $a + a_3$ (Line 7, Table III) increases with time indicating that the reduction of ferrocytochrome a_3 is preceded by that of ferricytochrome *a*. The results on Lines 8 and 9 show that at 1 min 76 % of ferricytochrome *a* is reduced, but only 19 % of ferricytochrome a_3. Later ferricytochrome a_3 is further reduced, partly by ferrocytochrome *a* and partly by ferrocytochrome *c*. Line 10 shows that the ratio $\Delta[c]/\Delta$[haem *a*] is slightly above 2 and did not vary greatly with time. It is also seen that the reduction of ferricytochrome a_3 is not complete after 5 min, as had been assumed in the calculations of WILLIAMS *et al.*[19].

Altogether five such experiments were carried out. The γ/α ratio after 1 min averaged 4.14. In three experiments with a low $[c]/[a]$ ratio, 1.1–3.5, the reduction of cytochrome *a* averaged 72 %, that of cytochrome a_3 31 %; after 20 min the reduction of cytochrome *a* had decreased to 53 %, while that of cytochrome a_3 had risen to 67 %. In two experiments with high $[c]/[a]$ ratio, 4–5, cytochrome *a* at 1 min was 80 % reduced, cytochrome a_3 30 %; after 20 min the reduction of cytochrome *a* had decreased to 50 % while that of cytochrome a_3 had risen to 77 %. The 1-min

$\Delta[c]/\Delta$[haem *a*] ratio averaged 2.0 in the experiments with low $[c]/[a]$ ratio and 3.0 in those with high $[c]/[a]$ ratio.

Anaerobic reaction of oxygenated oxidase with ferrocytochrome c

In Table IV $\Delta\varepsilon_{mM}$ 605 mμ for haem *a* is calculated from the increase of absorption at 605 mμ and the decrease at 550 mμ, for the reaction of oxygenated oxidase as it was in Table II for the reaction with ferric oxidase. The data show that oxygenated oxidase reacts qualitatively differently from ferric oxidase. The $\Delta\varepsilon_{mM}$ 605 mμ value is, even at low $[c]/[a]$ ratios, not initially high and does not decrease with time as it does with ferric oxidase, but is lower and remains stable or even increases with time. There is no preferential reduction of ferricytochrome *a*. The $\Delta\varepsilon_{mM}$ 605 mμ value is even less than that found for ferric oxidase under conditions at which both ferricytochromes *a* and a_3 were reduced. The average of moles of cytochrome *c* required to reduce 1 mole of haem *a* (cytochromes $a + a_3$) appears to be 2.3 rather than 2.0 as for ferric oxidase.

TABLE IV

REACTION OF OXYGENATED OXIDASE WITH FERROCYTOCHROME *c*

$\Delta\varepsilon_{mM}$ 605 mμ calculated from $\Delta A_{605\,m\mu}{}^{*} - \Delta A_{550\,m\mu}$ and $\Delta\varepsilon_{mM}$ 550 mμ of cytochrome *c* (21).

	$[c]/[a]$ *ratio*	1.2–1.8	2.2–2.8	4.0–4.7	6.0–7.5
	Expt. No	*673, 761, 770*	*660, 669, 779*	*677, 772*	*663, 685*
$\Delta\varepsilon_{mM}$ 605 mμ	10 sec	4.7	5.4	5.4	5.8
	10 min	5.0	5.8	5.2	5.6

* ($A_{605\,m\mu}$ *minus* initial $A_{605\,m\mu}$)/1.1 (see METHODS).

TABLE V

REACTION OF OXYGENATED OXIDASE WITH FERROCYTOCHROME *c*

Reduction of cytochromes *a* and a_3 by ferrocytochrome *c* from measurements at 605, 550 and 444 mμ (see *Optical measurements*).

$[c]/[$*haem a*$]$ *ratio*	1.52			*Reduced*
Time (min):	*1*	*4*	*10*	
$[c^{3+}]$ (μM)*	19.7	19.0	17.0	
$[c^{2+}]$ (μM)	2.5	3.2	5.2	22.2
$A_{444\,m\mu}$ $(a + a_3 + c)$	1.40	1.44	1.48	1.82
$A_{444\,m\mu}$ $(a + a_3)$	1.05	1.10	1.15	1.65
$A_{605\,m\mu}$ $(a + a_3)$	0.205	0.200	0.200	0.306
γ/α ratio	5.12	5.49	5.58	5.41
Percent reduction of *a*	35	29	27	
Percent reduction of a_3	56	66	73	
$\Delta[c]/\Delta$ [haem *a*]	3.14	2.92	2.46	

* $\Delta[c^{3+}] = [c^{3+}]$, since ferrocytochrome *c* was completely reduced.

In Table V, the percentage reduction of cytochromes *a*, a_3 and *c* was calculated as for ferric oxidase in Table III.

In contrast to the experiments with ferric oxidase there is no large increase of the γ/α ratio with time. In altogether six experiments it rose only from 5.2 to 5.5 in 10 min. The average percentage reductions of cytochromes *a* and a_3 are somewhat variable, but the 1-min reduction of cytochrome *a* is even slightly smaller (57 %) than that of cytochrome a_3 (61 %) and only that of cytochrome a_3 increases with time (to 71 % after 10 min). In the experiment detailed in Table V, the $\Delta[c]/\Delta$ [haem *a*] ratio was higher than those found for ferric oxidase, but the average of four experiments at $[c]/[a]$ ratios below 3 was only slightly higher (2.2) than of those for ferric oxidase (2.0), and about the same (2.9) in the two experiments with $[c]/[a]$ ratios of 4–5. It did not change much with time.

DISCUSSION

Our results showing the much faster reduction of ferricytochrome *a* than of ferricytochrome a_3 (Table III) by ferrocytochrome *c* confirm those of GIBSON *et al.*[22] who found the half-times of reduction of ferricytochrome *a* below 100 msec, that of ferricytochrome a_3 about 7.5 sec. In our experiments the half-times of ferricytochrome *a* were far less than 10 sec, no apparatus being available to measure shorter times than a few sec, while the half-time of reduction of ferricytochrome a_3 varied between 4 and 19 sec.

We have used the same parameters as those used in the study of the reaction products of ferric oxidase with ferrocytochrome *c* for those of the reaction products of oxygenated oxidase. This is not strictly correct because the much faster reduction of cytochrome a_{3x}^{3+} derived from oxygenated oxidase than of cytochrome a_3^{3+} in ferric oxidase shows that the products are not identical. However, the absorption spectrum of cytochrome a_{3x}^{3+} (refs. 2 and 19) appears to be practically indistinguishable from that of cytochrome a_3^{3+} (refs. 8 and 19). The clearly biphasic kinetics of the reduction of ferric cytochrome oxidase in contrast to the practically monophasic reduction of oxygenated oxidase[3,4,19] with ferrocytochrome *c* parallels the similar difference found in the reduction by dithionite. It is also of interest that the biphasic kinetics of the dithionite reduction of ferric oxidase are established much more slowly than is the rapid shift of the Soret band of oxygenated oxidase (428 mμ) to ferric oxidase (418 mμ) by cytochrome *c* (unpublished observation).

The differentiation between cytochromes a_3^{3+} and a_{3x}^{3+} having both a Soret band at 418 mμ resolves some difficulties of various authors. Thus GIBSON and co-workers[5,23] have concluded that the primary oxidation product of cytochrome oxidase by O_2 is ferricytochrome a_3, and have explained the differences between its properties and those of the stable ferricytochrome a_3 in the oxidase preparations by postulating a "dynamic" as different from a "static" ferricytochrome a_3. Objections against this have been raised by LEMBERG and co-workers[9,24,25] but the differentiation between cytochrome a_3^{3+} and a conformationally distinct form cytochrome a_{3x}^{3+} brings the two conceptions into closer harmony. It may indeed, be argued that cytochrome a_3^{3+}, as present in ferric oxidase, is a stabilization product and that cytochrome a_{3x}^{3+}, and under certain conditions cytochrome a_{3x}^{4+}, are more important participants in the biological oxidation catalysed by cytochrome oxidase. Thus it may be advisable to

speak in the future of cytochromes a_{3A}^{3+} and a_{3B}^{3+} rather than of cytochromes a_3^{3+} and a_{3x}^{3+}.

The results of Table II and III show that equilibrium is only slowly established if ferrocytochrome *c* reacts with ferric oxidase. Kinetic rather than thermodynamic factors determine the events. This throws some doubt on the attempts to establish oxidation–reduction potentials of cytochrome oxidase and of cytochromes *a* and a_3 at least in the absence of mediators. Our results are at present not sufficiently accurate to establish the oxidation–reduction potentials from the final results after more than 20 min and these may not have a definite meaning if changes of conformation are involved. Ascorbate[10] may accelerate the reaching of equilibria, but may also have other effects. The fact that ascorbate alone reacts only very slowly with cytochrome oxidase, does not exclude the possibility that a semi-dehydroascorbate radical produced by cytochrome *c* does so. Moreover, no consideration is given to the possible interaction of ferrocytochrome *c* with the copper or other possible oxidation–reduction systems in the oxidase.

From the results with oxygenated oxidase, it appears that there is no, or only a very small, difference in the potentials of cytochromes *a* and a_{3x}.

The results of this investigation have been presented at the 12th International Conference in Coordination Chemistry in Sydney and the abstract[26] represents a preliminary publication.

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REFERENCES

1 W. H. VANNESTE, *Biochemistry*, 5 (1966) 838.
2 M. R. LEMBERG, *Physiol. Rev.*, 49 (1969) 48.
3 R LEMBERG AND M. V. GILMOUR, *Biochim Biophys. Acta*, 143 (1967) 500.
4 M R. LEMBERG, M V GILMOUR AND M E CUTLER, *Symp. Structure and Function of Cytochromes, Osaka, 1967*, University of Tokyo Press and University Park Press, Tokyo–Baltimore, 1968, p 54.
5 Q. H GIBSON AND D C. WHARTON, *Symp Structure and Function of Cytochromes, Osaka, 1967*, University of Tokyo Press and University Park Press, Tokyo–Baltimore, 1968, p 5
6 Q. H. GIBSON AND C. CREENWOOD, *J. Biol. Chem.*, 240 (1965) 2694.
7 K OKUNUKI, in M FLORKIN AND E STOTZ, *Comprehensive Biochemistry, Biological Oxidations*, Vol 14, Elsevier, Amsterdam, 1966, p 232
8 W. W. WAINIO, D. GREBNER AND H. O'FARRELL, *Symp Structure and Function of Cytochromes, Osaka, 1967*, University of Tokyo Press and University Park Press, Tokyo–Baltimore, 1968, p. 66
9 M V. GILMOUR, R LEMBERG AND B. CHANCE, *Biochim Biophys Acta*, 172 (1969) 37
10 T HORIO AND J OHKAWA, *J Biochem Tokyo*, 64 (1968) 393
11 B VAN GELDER AND E C SLATER, *Biochim Biophys Acta*, 73 (1963) 663.
12 B F VAN GELDER, *Biochim. Biophys Acta*, 118 (1966) 36.
13 B F VAN GELDER AND A O MUIJSERS, *Biochim Biophys Acta*, 118 (1966) 47
14 K MINNAERT, *Biochim Biophys Acta*, 110 (1965) 42
15 E. C SLATER, B F VAN GELDER AND K MINNAERT, *Symp Oxidases and Related Redox Systems, Amherst 1964*, Wiley, New York, 1965, p 667
16 A EHRENBERG AND T YONETANI, *Acta Chem Scand*, 15 (1961) 1071.

17 J HARRIS AND K. RITCHIE, *Ann N Y Acad Sci* , 153 (1969) 706
18 H BEINERT AND G PALMER, *J Biol Chem* , 239 (1964) 1221.
19 G R WILLIAMS, R. LEMBERG AND M E CUTLER, *Can J Biochem* , 46 (1968) 1371
20 G. E MANSLEY, J T STANBURY AND R LEMBERG *Biochim Biophys Acta*, 113 (1966) 33
21 H BEINERT, *Symp Copper in Biological Systems, New York, 1965, The Biochemistry of Copper*, Academic Press, New York, 1966, p 213
22 Q H GIBSON, C GREENWOOD, D C WHARTON AND G. PALMER, *J Biol Chem* , 240 (1965) 888.
23 C GREENWOOD AND Q H GIBSON, *J Biol Chem* , 242 (1967) 1782
24 R LEMBERG, *Discussion Remark Symp Structure and Functions of Cytochromes, Osaka, 1967*, University of Tokyo Press and University Park Press, Tokyo and Baltimore, 1968, p 16, 17
25 R LEMBERG AND M E CUTLER, in H C FREEMAN, *Proc Intern Conf on Coordination Chemistry, Sydney, 1969*, Science Press, Sydney, 1969, p 135