BBA 45835

STUDIES OF THE HEME COMPONENTS OF CYTOCHROME *c* OXIDASE BY EPR SPECTROSCOPY

BOB F. VAN GELDER* AND HELMUT BEINERT

Institute for Enzyme Research, University of Wisconsin, Madison, Wisc. 53706 (U.S.A.)

(Received March 18th, 1969)

SUMMARY

Cytochrome *c* oxidase was studied by electron paramagnetic resonance (EPR) spectroscopy at different states of oxidation in the presence or absence of specific ligands. The EPR signals observed are described, and an attempt is made to quantitatively evaluate and assign them to components of cytochrome *c* oxidase. According to this only cytochrome *a* and part of the copper were detected by EPR in the oxidized form of the enzyme. The reduced form was devoid of significant signals. At intermediate states of oxidation, cytochrome a_3 was manifest in its ferric form by a signal typical of high-spin ferric iron. This indicates that in the oxidized form of the enzyme, ferricytochrome a_3 interacted with another component, *viz.* another molecule of ferricytochrome a_3 or the EPR-undetectable fraction of the copper, so as to result in a decreased paramagnetism of the system. Strong ligands such as cyanide or azide prevent the intermediate appearance of cytochrome a_3 in the high-spin state; in the presence of azide, a low-spin ferric intermediate was detected. The changes in absorbance at 605 mμ and of the EPR signals during complete titrations of the enzyme with NADH and phenazine methosulfate or dithionite have been measured. Whereas the absorbance at 605 mμ measured at room temperature appeared to decrease linearly with added reductant, the EPR-detectable copper was not reduced initially except in the presence of cyanide. Cytochrome *a* was reduced in a linear fashion, while the EPR-detectable form of ferricytochrome a_3 accumulated and was reduced after cytochrome *a*. In the presence of azide, an EPR-detectable low-spin form of ferricytochrome a_3 accumulated, which was not readily reducible. The high-spin form of ferricytochrome a_3 was also seen on rapid ($>$ 10 msec) reduction of cytochrome *c* oxidase by ferricytochrome *c* or dithionite; it was not seen ($>$ 10 msec) on rapid reoxidation of fully reduced cytochrome *c* oxidase.

These findings support the concept of the separate existence of cytochromes *a* and a_3, and indicate that cytochrome *a* and the EPR-undetectable copper are titrated first, at which point ferricytochrome a_3 becomes detectable by EPR. The EPR-detectable copper and cytochrome a_3 are titrated thereafter.

* Present address: Laboratorium voor Biochemie, B.C.P. Jansen Instituut, Universiteit van Amsterdam, Plantage Muidergracht 12, Amsterdam, The Netherlands.

INTRODUCTION

Studies of cytochrome *c* oxidase by EPR spectroscopy have so far been limited to the copper components of the enzyme. Weak absorptions, typical of heme or iron compounds, have, however, been observed in oxidized* cytochrome *c* oxidase preparations[1,2]. With improved instrumentation applied to highly concentrated samples, it has become feasible to study these signals in more detail. This paper describes properties of the EPR signals of the heme components of cytochrome *c* oxidase and the conditions under which they may be observed. The most significant finding is that not all ferric heme components are represented in the signals of the oxidized enzyme, but such heme signals do appear at intermediate oxidation states, indicating changes in molecular structure during oxidation–reduction. Preliminary accounts of this work have been presented[3,4].

EXPERIMENTAL

Materials

Throughout this paper concentrations of cytochrome *c* oxidase are expressed in terms of concentration of "total" heme, disregarding any difference between the cytochrome components present. Cytochrome *c* oxidase was prepared from beef heart mitochondria by the method of FOWLER *et al.*[5], as modified by MACLENNAN AND TZAGOLOFF[6]. In the final step the enzyme was taken up in 0.1 M potassium phosphate (pH 7.2) so that an approximately millimolar concentration was obtained. Enzyme so prepared contained between 8 and 11 μmoles heme per g protein and 12–16 μmoles copper. When necessary, the enzyme was diluted with 0.1 M phosphate (pH 7.2). The concentration of total heme was calculated from the $\Delta A_{605\ m\mu}$ (reduced–oxidized) using a molar absorbance of $12.0 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ (ref. 7). Cytochrome *c* monomer was prepared from beef heart by the method of MARGOLIASH AND WALASEK[8] and was reduced by $Na_2S_2O_4$. The dithionite was removed by dialysis against 1 mM phosphate (pH 7.2) for approx. 12 h. NADH was obtained either from the Sigma Chemical Co. or P. L. Biochemicals. Fresh stock solutions in 0.2 M Tris base were prepared daily and were diluted to the desired concentration with water or with 0.1 M Tris chloride (pH 8.1) when more than 10-fold dilutions were needed. The concentration was determined from the total absorbance at 340 mμ, using a molar absorbance of $6.22 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ (*cf.* ref. 9). Phenazine methosulfate, obtained from Sigma, was dissolved in water and protected from light. Stock solutions of 1 % concentration were kept frozen for several weeks. Dilutions of 0.01 % were prepared fresh daily. Dithionite was obtained from British Drug Houses and was assayed[10] as 85 % dithionite (determined by Dr. W. H. Orme-Johnson in our laboratory).

Meat by-products were obtained from Oscar Mayer and Co., Madison, Wisc.

Methods

Titrations

The titrations with NADH and phenazine methosulfate were carried out in the manner described by VAN GELDER[7] and VAN GELDER AND SLATER[11] as modified for EPR spectroscopic observation (*cf.* ref. 12). Prepurified grade N_2 was purged using a

* The term "oxidized" refers throughout this paper to the untreated enzyme as isolated in the presence of O_2 and not to a chemically oxidized form.

train of 5 bottles containing alkaline pyrogallol, and in some of the experiments by passage through a tube containing copper at 200°. The O_2 content was found[13] to be 8 ppm and 0.3 ppm (as determined by Dr. W. H. Orme-Johnson), respectively, following these treatments. In the titrations with dithionite, a special technique, using solid dithionite diluted with KCl was used[14], which will be described in a separate publication[15].

Rapid reactions

A rapid mixing–freezing apparatus was used based on the principles of BRAY[16]. The ram of this apparatus was driven by an electric motor and incorporated many features of a system previously described[17]. The system used here was improved in that reaction times in excess of 350 msec were produced by stopping the ram and restarting it after a suitable interval rather than by passing the mixed reactants through a delay line. This "double-push" system will be described elsewhere[18]. It has the advantage that very long reaction times can be chosen with the same expenditure of material and with conditions being unchanged. In the old system[17], the pressure built up in a long delay line sets an upper limit to possible reaction times. Also, a syringe and mixing chamber system with a 1:5 instead of the usual 1:1 volume ratio was used to allow higher protein concentrations with the corresponding increase in the ratio of signal-to-noise.

Optical spectroscopy

It was desirable to follow the progress of the titrations independently by optical absorbance measurements. This was particularly important as VAN GELDER[7] and VAN GELDER AND MUIJSERS[19] had obtained linear titration curves from such measurements, while BEINERT[20] observed by EPR spectroscopy that copper is not reduced in the initial part of the titration. Since it appeared essential that optical measurements be made on the same samples that were also examined by EPR spectroscopy and at the same state, except for possible differences due to the temperature change, a suitable attachment to the regular sample compartment of the Cary spectrophotometer Model 14 was constructed[21]. This attachment rigidly holds the lower portion (6 cm) of the sample tubes, leaving a slit (15 mm × 3 mm) for the light to pass through the sample. The device can be adjusted in a direction parallel and perpendicular to the light beam. The optimal position is established once and thereafter only the tubes are exchanged. The most essential feature is complete absence of play in the whole mechanism. The curvature of the surface of the 4-mm bore tubes used is sufficiently small as compared with the width of the light beam, that no difficulties were observed. The tubes had an apparent average light path of 3.8 mm. Although it would have been useful to obtain complete spectra, at the enzyme concentrations required for EPR spectroscopy of the heme components, absorbances were too high (3–5.5 at 605 mμ) to do so efficiently. A series of neutral density screens was used in the reference path in order to measure these absorbances. With a high intensity lamp, slit widths were held at 0.1–0.2 mm by varying the amplifier gain ("slit control") and the voltage supplied to the dynodes of the photomultiplier. When measurements were made in the 600-mμ region, a red filter (No. 85U Harrison (RD-5)) was inserted in the light beam in order to reduce stray light. Under these conditions Beer's law was followed up to total absorbance values of at least 5.

EPR spectroscopy

This was carried out largely with the apparatus previously described[17,22–24]. The

following features were important for increasing the signal-to-noise ratio with the weak heme signals to be expected. A special sample Dewar was used which accommodates tubes having an outer diameter of up to 5.2 mm (inner diameter approx. 4 mm), which in our system leads to a 1.75-fold increase in signal intensity over signals from tubes of 3-mm inner diameter. Since the heme signals are very sensitive to temperature, it was essential to operate at the lowest convenient temperature and to maintain this temperature within $\pm 0.5°$ for extended periods. This was achieved by the gas flow and temperature control system recently described[25].

The spectra recorded and shown in this paper represent the first derivatives of the absorption curves. None of the signals that were observed were saturated with the power available (350 mW) at the lowest routine operating temperature (80°K), except for the signal due to "inactive" copper (*cf.* ref. 26). We took advantage of this property by purposely saturating this signal so that it would make only a minor contribution to the copper signal of the enzyme (*cf.* refs. 23, 27). Copper signals were routinely observed at 91 mW, a modulation amplitude of 6 gauss and a scanning rate of 250 gauss per min and heme signals at 27 mW, a modulation amplitude of 20 gauss, a scanning rate of 1000 gauss/min, both at a time constant of 0.5 sec and at 80–82°K. The spectra at 20–40°K were recorded at 0.67 mW. The microwave frequency was at or close to 9250 MHz. When field markers (in gauss) are used in the figures, the specific frequency used is given. Note that the values indicated at the top of the figures are not to be taken as true g values; for the purpose of quick orientation they merely indicate on the scale of g values prominent points in the spectra, whose positions in the field were actually measured. The g values mentioned in the text represent the best preliminary estimates of true g values which we could make from the spectra obtained without reconstructing spectra by computation.

Evaluation of data

Errors involved in all procedures used here will be discussed in a separate section below. For a quantitative comparison of the copper signals, the maximum excursions of the derivative curve were measured. For intercomparison of heme signals of the same kind, only the low field lines ($g > 2.5$), which are sharper than those at high field were used. Since these lines partially overlap, and in some cases are also interfered with by the much more pronounced copper signal, their relative heights were estimated with respect to an assumed base line. This base line was derived from the spectrum of the completely oxidized and reduced samples, since in the former only the signals at $g = 4.3$, $g = 3$ and the copper signal and in the latter only that at $g = 4.3$ and that of a fraction of the copper, but no heme signal, are present. The signals at $g = 6$ can be measured with relatively high accuracy and precision, whereas measurements of the signals at $g = 3$ and $g = 2.6$ may suffer from considerable error, as will be more extensively discussed below.

For the determination of the absolute number of unpaired electrons represented in the signals the following standards were used: CuEDTA for cupric copper and as primary standard for all signals; in addition metmyoglobin fluoride in comparison with high-spin ferric signals ($g_\perp = 6$) and metmyoglobin azide in comparison with low-spin ferric signals (3 g values centered around $g = 2$). Since at the temperature of liquid N_2 CuEDTA is partially saturated at powers exceeding 25 mW[27], the spectra intended for double integration of cytochrome *c* oxidase and CuEDTA were recorded at 9 mW so that the "inactive" copper is largely (approx. 75 %) included in this mea-

surement. From a comparison of integrations made at high and low power, we estimate that in the preparations used approx. 20 % of the copper signal seen under nonsaturating conditions is contributed by "inactive"[23,26,27] copper. Signals of the high-spin ferric heme compounds have the g values $g_\perp \approx 6$; $g_{||} = 2$ (ref. 28). Because of the strong copper signals, it is not possible to see the $g_{||}$ portion of the high-spin ferric heme signals or exact details of the center line ($g \approx 2.2$) of the low-spin ferric heme signals in cytochrome *c* oxidase. Accurate integrations of the complete heme signals of cytochrome *c* oxidase are therefore impossible.

We have attempted to arrive at estimates of total integrated intensities of the signals by measurements on the resolved components at low field alone and by using appropriate calculations. VÄNNGÅRD[29] has treated the problem of arriving at the total integrated intensity from measurements on a single hyperfine line in the $g_{||}$ region in the case of axial symmetry ($g_x = g_y$). We have attempted to use this treatment for integrating the signals of low-spin ferric hemes. Although in the case at hand the absence of nuclear hyperfine interaction simplifies the treatment, the low-spin heme signals are typical of a rhombic environment ($g_x \neq g_y \neq g_z$) to which Vänngård's formula does not immediately apply. We therefore tried to use an average of the g values at higher field $\left(\sqrt{\tfrac{1}{2}(g_x^2 + g_y^2)}\right)$ instead of the $g_\perp$ value of the axial case. To test the validity of this approach we applied it to the signals obtained from azide derivatives of methemoglobin and metmyoglobin and from P_{450}. The values for the concentrations of these compounds arrived at in this fashion deviated by less than $\pm$ 20 % from those determined by spectrophotometry or by double integration of the complete signals performed in the conventional fashion.

The approach outlined by Vänngård is, however, not applicable in the case of the signal of the high-spin ferric heme when the intensity would have to be calculated from the part of the signal at $g_\perp$. In this instance we derived an empirical formula from a comparison of the doubly integrated area of a CuEDTA standard with the doubly integrated area of the low field portion of the signals from acid metmyoglobin, metmyoglobin fluoride and methemoglobin fluoride the concentrations of which had been determined spectrophotometrically. The partial double integration of these signals was carried out from the low field end to the point of intersection of the derivative line with the base line. According to preliminary computer simulations of spectra of this type, this partial area increases relative to the total area with increasing peak width of the low field ($g_\perp$) portion of the derivative curve. An appropriate correction was therefore applied to the double integral of the low field area*.

No effort was made to evaluate the signals at $g = 4.3$. These signals were very variable from preparation to preparation and did not respond to oxidation–reduction in a consistent way. It was also found, after part of this work was completed, that the quartz tubes used gradually developed signals at $g = 4.3$. This iron contamination could only be removed by brief treatment with HF. In view of these observations and the relatively low intensity of that portion of the $g = 4.3$ signals, which can be attributed to the enzyme itself, we doubt that this signal represents a significant constituent of the enzyme. Rather it may be the counterpart of the "inactive" copper, *i.e.* unspecifically bound ferric iron, possibly derived from heme iron of the enzyme.

* The partial integrated area changes approximately with $B^{-0.37}$, where B is the distance (in gauss) from the derivative maximum point at low field to the intersection of the curve with the base line, *i.e.*, to the point to which the partial integration is carried out.

Consideration of errors and final presentation of data

Since in procedures of the kind described here a number of sources of errors influence the final values, it is useful to consider together the various sources and their relative weight. We are mainly concerned with the titrations, which form the major part of this paper.

By weighing the micropipets before and after use, it was found that only 98 % of the relatively viscous cytochrome *c* oxidase solutions was in fact delivered into the titration tubes. This systematic error was largely compensated for by a similar error in depositing the NADH solution in the sidearm of the tubes. The loss of water on evacuation is not easily controlled and may thus vary in a set of tubes used in an experiment. The tubes used were calibrated and matched for equal volume and resonance frequency. Since, after mixing in the upper part and freezing in the lower end of the tubes, all the water remaining in the system rapidly distilled in the vacuum to the cooled lower part, a correction for volume differences due to unequal water loss on evacuation could be derived from measurements of the final liquid columns after thawing. Through the known volume of the tubes and this correction factor, the initial concentrations of the reactants added could be related to the concentrations at the time of mixing and those determined on the sample by optical and EPR spectroscopy. The standard error of these measurements related to the volume is $\pm$ 2 %.

The precision of the absorbance measurements depends on the voltage supplied to the dynodes of the photomultiplier tube. The error therefore increases with samples of high absorbance (reduced). We estimate that the uncertainty of the values rises from $\pm$ 2 % of the total absorbance for the more oxidized samples to $\pm$ 5 % for the reduced samples.

The measurements of the EPR signals vary widely in accuracy and precision, depending on the line widths, intensities and mutual interferences. The estimated standard error of the measurements does not exceed $\pm$ 2 % for the copper signal, $\pm$ 4 % for the high-spin ferric signals and $\pm$ 10 % for the signals at $g = 3$, 2.9 and 2.6 and is generally less for the more intense signals. However, in comparing a series of tubes, drifts in instrument components and small nonrandom variations in positioning and temperature caused by minor differences in the shape of the tube, are additional sources of error. We therefore measured in one consecutive series those samples which were known or expected to exhibit signals of similar intensities, such as the copper signals, in the first part of the titrations. Also, signals were not only measured repeatedly, but for such measurements the same tube was inserted in the cavity 2 or 3 times alternately with other tubes. With these precautions and considering the mentioned standard errors, we think that we have thus reduced the uncertainty in the comparison of signals within a series of tubes so as not to exceed $\pm$ 10 % for copper, $\pm$ 20 % for the high-spin ferric signal and $\pm$ 40 % for the low-spin ferric signals. The determination of absolute concentrations for the various components derived from double integrations or related procedures described above is inevitably less accurate than are comparisons among signals of the same types. From intercomparison of a series of integrations, we estimate that the true copper concentrations lie within $\pm$ 10 % of the values reported here, but the approximations used to calculate heme concentrations from the EPR signals might lead to values that differ from the true values by as much as a factor of 2 or possibly even more. A comparison, however, of the values for the concentrations of individual heme components and total heme, which we calculated

from EPR data, with values known from chemical and spectrophotometric determinations suggest that the limits set by a factor of 2 are probably not exceeded.

The sources of error considered thus far could not have led to the scatter of points which we actually observed in some instances. No doubt the most serious error may be introduced by traces of O_2, which remain in or reenter the gas space of the titration tubes. Here the measurements of different parameters that were made were the best safeguard for recognizing and discarding measurements where such interference had occurred. As mentioned above, we found that the copper signal does not change during the initial phase of the titration in the absence of inhibitors. This could have been due to interference by O_2 or preferential reduction of other components in the enzyme. This could be decided by measuring the optical absorbance and the appearance of the EPR signals at $g = 6$ and 2.6, which are very readily produced on even fractional reduction. We were thus able to eliminate from consideration those samples in which a synopsis of all measurements indicated technical failure. Most of the final curves describing concentration changes of copper or low-spin ferric heme components derived from EPR signal amplitudes and total heme from spectrophotometry were obtained through a least-squares fit to a straight line (and in one case to a polynomial in using a computer program). In some instances, when the slope of a curve was obvious or the overall uncertainty of the values did not appear to warrant such treatment, the curves were fitted by hand. Details are found in the legends to the figures.

Since the enzyme and NADH concentrations varied slightly from experiment to experiment, we have normalized all values to a unit heme basis so that reducing equiv added per heme are plotted on the abscissa, and the components observed (optically or by EPR), per heme present, are plotted on the ordinate.

RESULTS

EPR signals of cytochrome c oxidase

The properties of the copper signal of this enzyme have been described and discussed previously[20,23,26,27]. In the enzyme preparations obtained in our laboratory (and also in other preparations which we have had the opportunity to examine), one dominant heme signal, typical of low-spin ferric heme compounds (*cf.* ref. 30), with g values at 3.0, 2 and 1.5 (Fig. 1A), is observed. The central line is obscured by the large copper signal, and the high field line, being very broad, is easily overlooked. Usually a signal is also found at $g = 4.3$. This signal is weak and of doubtful significance, as discussed above (see *Methods*). In many preparations there is also found a weak but relatively sharp signal at $g = 6$. This signal is very small in fresh preparations which have undergone a minimum of handling. A signal with $g = 6$ and $g = 2.0$ is typical of high-spin ferric heme compounds[28,30–32], but the signal seen in oxidized cytochrome *c* oxidase corresponds to an insignificant quantity in relation to the concentration of heme components in this enzyme. On partial reduction with the reducing agents which we tested (NADH and phenazine methosulfate, ascorbate, ascorbate and cytochrome *c* and cytochrome *c*), while the copper signal and the heme signal at $g = 3$ decrease, a significant signal appears at $g = 6$ (Fig. 1B). Concomitantly a new signal of low-spin ferric heme appears with g values at 2.6, 2.2 and 1.87, exhibiting relatively sharp lines. This species can be demonstrated more clearly when the signal of the inactive cop-

per[23,26,27] is saturated (*cf.* Fig. 2) by high microwave power. Both signals disappear again as titration progresses (see below). However, the two types of signal which appear on partial reduction do not belong to the same species as their intensities are not proportional. This can be seen by comparing Figs. 1B (approx. $^1/_2$ reduced) and 1C (approx. $^3/_4$ reduced) in which the high-spin signal decreases while the low-spin signal increases noticeably on further reduction.

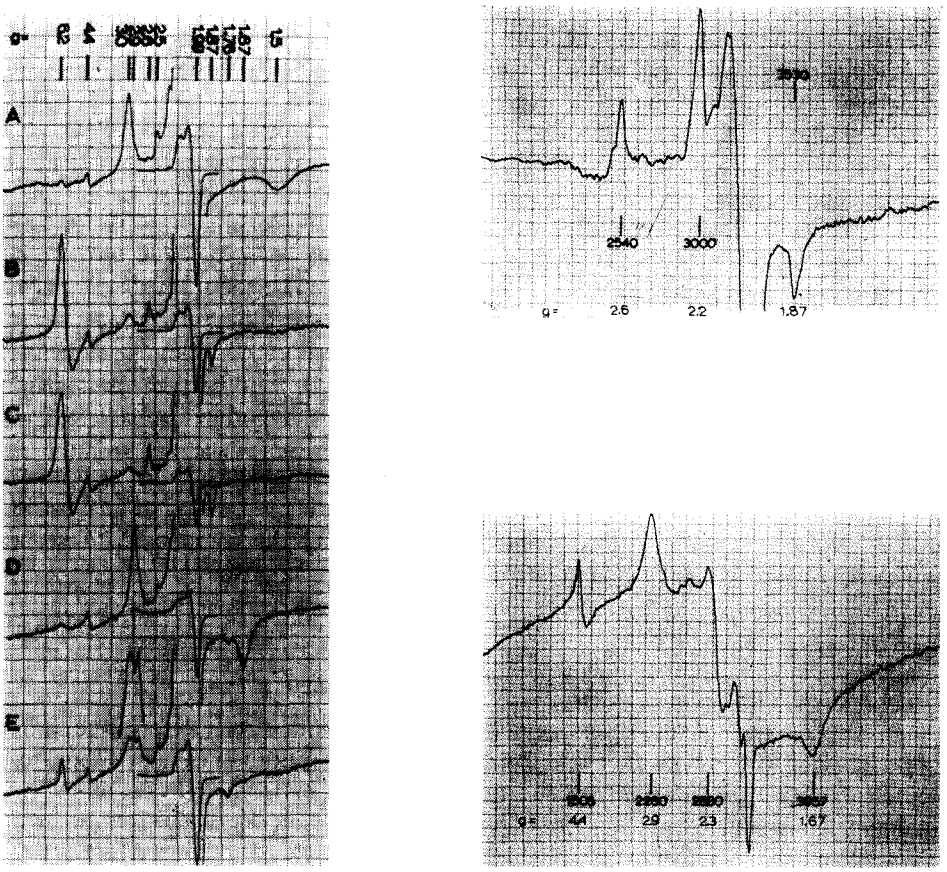


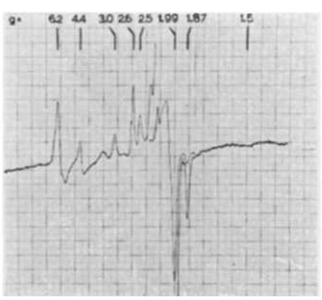
Fig. 1. EPR spectra (first derivative) of approx. 0.5 mM cytochrome *c* oxidase, under various conditions. A, isolated enzyme; B, approx. 50% reduced with 1.5 mM ascorbate under anaerobic conditions; C, approx. 70% reduced anaerobically with ascorbate; D, approx. 50% reduced anaerobically with ascorbate in the presence of 0.1 M sodium azide; and E, as D but only approx. 20% reduced. The conditions for EPR spectroscopy were those described as standard for observation of heme signals in the text. The temperature was 81° K. The copper signal was recorded under the same conditions, except for a 20-fold diminished amplifier gain. In E the enlarged signal at $g = 3$ was recorded at a 4-fold increased amplifier gain.

Fig. 2. EPR spectrum of cytochrome *c* oxidase approx. 70% reduced anaerobically with NADH and phenazine methosulfate showing the low-spin ferric signal of an intermediate arising in the absence of inhibitors. The conditions of EPR spectroscopy were standard, except for a microwave power of 330 mW. The field markers are in gauss: the frequency was 9250 MHz.

Fig. 3. EPR spectrum of cytochrome *c* oxidase approx. 50% reduced anaerobically with NADH and phenazine methosulfate in the presence of 0.1 M sodium azide, showing the low-spin ferric signal of an intermediate arising in the presence of azide. The conditions of EPR spectroscopy were those of Fig. 2, except that the frequency was 9242 MHz and the temperature 85°K.

When potential heme ligands are added to cytochrome *c* oxidase, certain changes or entirely new signals have been observed, as will be more extensively discussed below. The best defined of the new signals is that appearing on addition of azide. It appears at $g = 2.9$, 2.2 and 1.67 and is again best demonstrated when the signal of inactive copper is saturated (*cf.* Fig. 3). Although this signal is similar to that present in the oxidized form of the enzyme, it does not represent the same heme component. This is clearly apparent from the difference in *g* values which is particularly pronounced for the high field components (*cf.* Figs. 1A and 1D). At temperatures higher than that of liquid N_2 the signal of the azide complex is also sharper than that of the low-spin heme of the oxidized enzyme. It should be emphasized, however, that when azide is added to the oxidized enzyme, the signal just described, which is typical of the azide complex, does not appear. Only on reduction, as the low-spin heme signal ($g = 3.0$) disappears, does the signal typical of the azide compound appear, just as had been observed for the signal at $g = 6$ in the absence of azide. In fact, if the signal at $g = 6$ is produced first by partial reduction of the enzyme in the absence of extra ligands and azide is then added in increments from a sidearm without changing the oxidation state of the enzyme, the gradual disappearance of the signals at $g = 6$ and $g = 2.6$ and the appearance of the signal at $g = 2.9$, 2.2 and 1.67 can be followed.

As can be seen on the high field side (Fig. 1D), in the presence of azide apparently two new signals appear on partial reduction. The high field wing of the major one is located at $g = 1.67$ and that of the minor one at $g = 1.76$. On the low field side the minor peak is not resolved from the major one. However, the low field counterpart of the $g = 1.76$ signal can be clearly seen at the beginning of reduction (Fig. 1E). Here the signal of the low-spin component of the oxidized enzyme at $g = 3.0$ is seen together with the developing azide signal, which at low reductant concentrations appears to be exclusively the type of the minor signal seen at more advanced reduction with



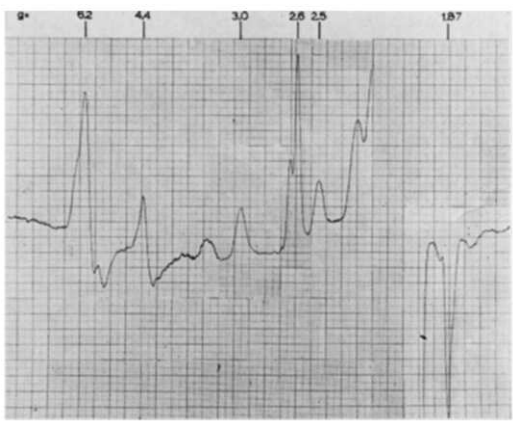


Fig. 4. EPR spectrum of cytochrome *c* oxidase approx. 70% reduced anaerobically with ascorbate. The conditions of EPR spectroscopy were: microwave power, 0.67 mW; modulation amplitude, 12 gauss; scanning rate, 1000 gauss/min; time constant, 0.5 sec; temperature, 23°K. The copper signal was recorded at a 6.4-fold reduced amplifier gain.

Fig. 5. EPR spectrum as in Fig. 4 but on an expanded scale, omitting the high field portion and copper signal. The conditions of EPR spectroscopy were those of Fig. 4, except for a scanning rate of 500 gauss/min and a temperature of 41° K.

$g = 1.76$ at high field. As seen in Fig. 1E, the low field peak of this minor signal is located at $g = 2.85$.

After the work that forms the essence of this paper had been completed, we had the opportunity to examine samples of cytochrome *c* oxidase of different oxidation states at 20–40°K. No principally new observations were made at these temperatures; however, certain features in the EPR spectra which were recognizable at the temperature of liquid N_2 but not sufficiently clear to deserve discussion, stood out clearly at the lower temperatures. Figs. 4 and 5 show such low temperature spectra of an approx. 70 % reduced sample. The two figures differ mainly in the width of the scan used which is related to the resolution. As a comparison of the corresponding spectra at the temperature of liquid N_2 (Fig. 1) shows the following features are emphasized by the 20–40°K spectra; several of the signals are split which could indicate either more complex underlying structures or heterogeneity of species, *viz.*, the signals at $g = 6$, $g = 3$, $g = 2.6$ and $g = 1.87$. The shape of the signal at $g = 6$ cannot be simply explained by a splitting of $g_\perp$ into g_x and g_y as observed with a number of heme compounds[30,33,34]. Similarly the extra peaks observed at $g \approx 3.4$, $g \approx 2.65$, $g \approx 1.89$ and $g \approx 1.80$ must be due to heterogeneity of species, probably to structures similar to those giving the main signals. Since the new components resolved at 20–40°K are minor components, they have not been separately considered in the quantitative evaluation of our titration and kinetic data but have been counted as part of the related major signals. This does not apply to the signal at $g = 3.4$, as this signal is largely lost in the background at the temperature of liquid N_2. If is interesting to note that the splitting of the signal at $g = 6$ is more pronounced in the presence of F^- and can be readily recognized at the temperature of liquid N_2 when F^- has been added. Since splitting is observed even in the absence of F^-, particularly at very low temperature, the hyperfine structure of fluorine is not responsible for the splitting, although it may modify it to some extent.

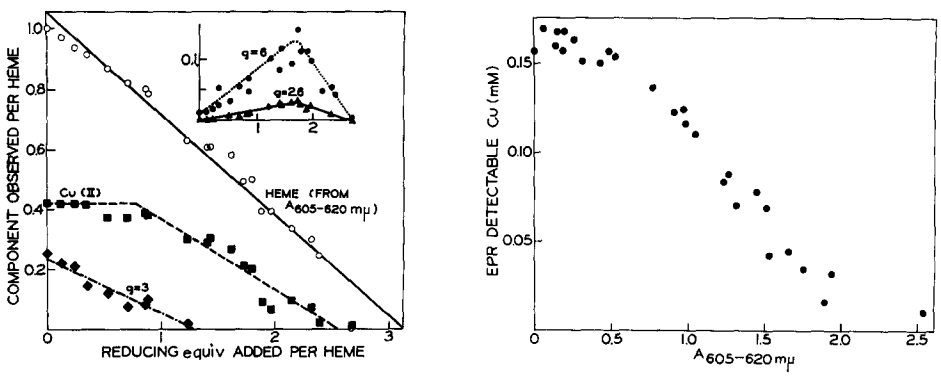


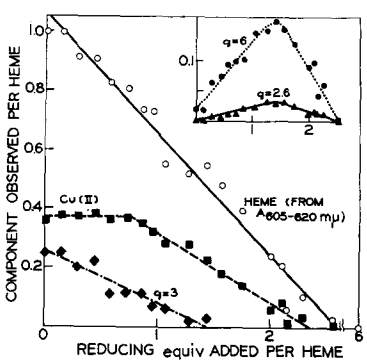
Fig. 6. Anaerobic titration of 0.400 mM cytochrome *c* oxidase with NADH in the presence of phenazine methosulfate. The conditions of measurement are those given in the text. The main figure shows the behavior of the components detectable in the oxidized form of the enzyme; the inset shows the intermediates. Note that the inset uses a 2-fold expanded ordinate and a 2-fold contracted abscissa. The curves for the oxidized form of heme (from $A_{605\,m\mu} - A_{620\,m\mu}$) and $g = 3$ were obtained by a fit to a straight line and that for Cu(II) by a fit to two straight-line portions.

Fig. 7. Plot of concentration of Cu(II) calculated from the signal observed in the titration experiment of Fig. 6 against the difference of the absorbances measured at 605 and 620 mμ, respectively. The values given are those actually observed and corrected for differences in volumes but not normalized. Note that the effective light path was 3.8 mm.

Titrations with NADH and phenazine methosulfate without inhibitor

Fig. 6 shows the course of a titration of cytochrome *c* oxidase with NADH and phenazine methosulfate in the absence of an inhibitor. In this experiment complete reduction of all measurable components was achieved with 2.5–3 reducing equiv/heme. With most preparations this value fell between 2.0 and 2.5 equiv. The most striking features are: (1) The low-spin signal at $g = 3.0$, 2 and 1.5 disappears with addition of somewhat less than 1.5 equiv/heme. (2) The copper signal does not significantly decrease until at least 0.5 equiv is added, and the curve describing its reduction assumes a linear slope only after addition of 1 equiv. This cannot be due to O_2 in the system because the optical absorbance as well as other signals increase, while the signal at $g = 3$ decreases linearly. (3) New EPR signals, which were described above, appear immediately after the first addition of the reducing agent. Within the limits of error of our quantitative evaluation, the two signals taken together represent an amount of heme approaching, or in some instances equivalent to, that represented in the initial heme signal ($g = 3.0$, 2, 1.5). It must be emphasized that these signals are typical of ferric, not ferrous, heme species. The appearance of these signals is, therefore, apparently an indirect effect of reduction occurring in the cytochrome *c* oxidase unit. (4) The notion that the signals which appear represent components of the oxidase that have not taken up reducing equiv is supported by the fact that they reach maximal intensity after addition of approx. 1.5 equiv and then disappear completely together with the signals typical of oxidized enzyme, indicating complete reduction of all components.

The delay in the reduction of copper has been consistently observed in more than 20 different titrations with different enzyme preparations. It is most convincingly demonstrated when the intensity of the EPR copper signal is plotted against absorbance at room temperature (Fig. 7) regardless of the NADH concentration. This plot includes more points than that of Fig. 6, which could be included since interference by O_2 does not appear to influence the relationship considered here.



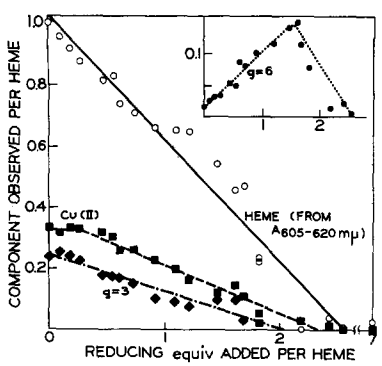


Fig. 8. Anaerobic titration of 0.430 mM cytochrome *c* oxidase in the presence of 67 mM Na EDTA, otherwise identical with that described in Fig. 6. The evaluation of data was analogous.

Fig. 9. Anaerobic titration of 0.340 mM cytochrome *c* oxidase in the presence of 0.1 M KF. The enzyme had been left standing with fluoride for 16 h before the titration. Conditions and evaluation are otherwise analogous to those described for the experiment of Fig. 6.

Titrations in the presence of inhibitors or ligands

EDTA. There was no significant difference between the titration in the absence and presence of 67 mM EDTA (Fig. 8). Any apparent differences can be explained by the normal scatter observed in different experiments.

Fluoride. When the enzyme has been exposed to 0.1 M KF for more than 20 h at 0°, the signal of low-spin ferric iron at $g = 2.6$, 2.2 and 1.87 does not appear on partial reduction. The usual low-spin ferric signal at $g = 3.0$ of the oxidized form is split into two overlapping signals with peaks at $g = 3.0$ and 3.1. When the enzyme is exposed to fluoride for a few hours only, the signal at $g = 2.6$, 2.2 and 1.87 does appear and the $g = 3$ peak is not split, as is the case in the absence of fluoride. In the presence of fluoride the signal at $g = 6$ shows a more pronounced structure, as was mentioned above. There appears to be a difference in the onset of the decrease of the copper signal in the curves of Figs. 6 and 8 as compared with that of Fig. 9. We are not certain whether this difference is significant. The signal at $g = 3$, however, seems to disappear only at larger reductant concentrations. This could be due to errors in the measurement of this relatively broad signal introduced by the mentioned splitting of the peak in the presence of fluoride. Otherwise no significant differences were seen in the presence of fluoride (Fig. 9).

Azide. In the presence of 0.1 M azide, the signals show the changes described above, *i.e.* no high-spin signal appears, but a new low-spin signal does and the course of the titration is radically changed (Fig. 10), as expected from previous spectrophotometric observations[35,36].

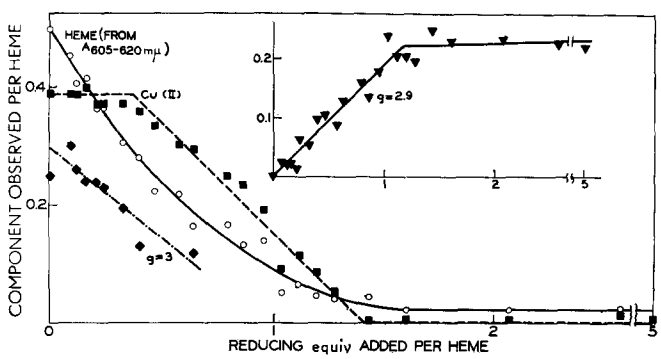
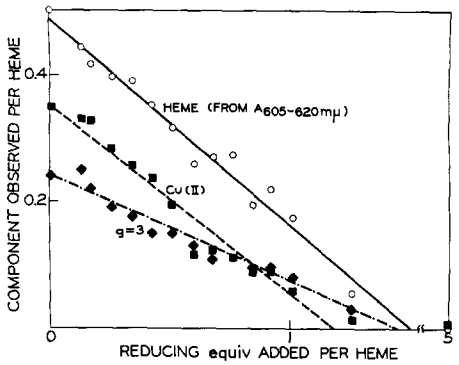


Fig. 10. Anaerobic titration of 0.434 mM cytochrome *c* oxidase in the presence of 0.1 M sodium azide. Conditions and evaluation were otherwise analogous to those of Fig. 6, except that the curve for oxidized heme was obtained by a least-squares fit to a polynomial using a computer. The curve for the signal at $g = 3$ was fitted to a straight line and that for the signal at $g = 2.9$ was fitted to two straight-line portions by a least-squares method. Note that for convenience the scale of the ordinate on the inset is equal to that of the ordinate on the main graph, *i.e.* one half the scale of the ordinate of the other insets.

The signals of all detectable components, except those at $g = 2.9$, 2.2 and 1.67, disappear with 1.5 equiv of reductant per heme. The remaining signal persists, *i.e.* the corresponding heme component is not reduced, with as much as 5 equiv/heme. Higher NADH concentrations were not tried, but the intermediate is reduced with an excess of dithionite. The delay in the reduction of the EPR-detectable copper is still observable but is not as pronounced as in the absence of azide.

Cyanide. With cyanide the reduction of all detectable components is complete between 1.0 and 1.5 equiv/heme. The reduction of the detectable copper proceeds without delay and is linear (Fig. 11). The signals seen at intermediate oxidation states are those present in the oxidized enzyme but with diminished intensities.



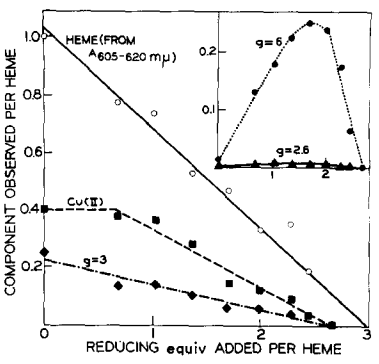


Fig. 11. Anaerobic titration of 0.450 mM cytochrome *c* oxidase in the presence of 0.1 M KCN. Conditions and evaluation were otherwise analogous to those of Fig. 6.

Fig. 12. Anaerobic titration of 0.400 mM cytochrome *c* oxidase with solid sodium dithionite as described in the text. The enzyme was left with dithionite for 30 min before freezing. Conditions and evaluation were otherwise analogous to those of Fig. 6.

Titration with dithionite

With dithionite approx. 2.5–3.0 equiv/heme were required for complete reduction of all detectable components. The types and shapes of signals that appeared at intermediate oxidation states were the same as those seen with NADH and phenazine methosulfate. The intensities of the signals at $g = 6$ were particularly high with dithionite, and those of the low-spin species at $g = 2.6$ were low. We observed that, with dithionite, equilibrium was not readily reached. Changes in signal intensity persisted up to 1 h at room temperature. In particular the signals at $g = 6$ almost doubled between 10 and 40 min after addition of the reductant along the ascending branch of the curve (Fig. 12) describing the intensity of the $g = 6$ signal. In the same time interval there was also a large increase in absorbance at 605 mμ, whereas the values for copper and the signal at $g = 3$ decreased only slightly. The values plotted in Fig. 12 are those observed after a total of 40 min.

Kinetic studies

After the properties of the signals, which were observed at intermediate oxidation states, had been studied, it became important to learn whether these intermediate states would also occur at short reaction times.

For this purpose cytochrome *c* oxidase was reduced by reduced cytochrome *c* or dithionite in the rapid mixing apparatus which allows observations to be made approx. 10 msec after mixing. Although this time interval still exceeds the turnover time of the enzyme by 1–2 orders of magnitude, it is nevertheless 5 orders of magnitude smaller than the time elapsing between mixing and freezing in the titration experiments. Figs. 13 and 14 show experiments on the rapid anaerobic reduction of cyto-

chrome *c* oxidase by excess dithionite and cytochrome *c*, respectively. In either case the intermediate high- and low-spin signals apppear, as they did in the titrations. As expected, reduction with dithionite is finally more extensive. The failure to observe complete reduction of copper and the low-spin heme ($g = 3$) with dithionite after several seconds may be due to the admixture of O_2 during the rapid mixing and freezing process. It is interesting to note that in the experiments with cytochrome *c*, the intensity of the signal at $g = 6$ which is formed, is inversely related to the concentration of the reductant. As expected, the cytochrome *c* oxidase components are less extensively reduced at the lower cytochrome *c* concentration.

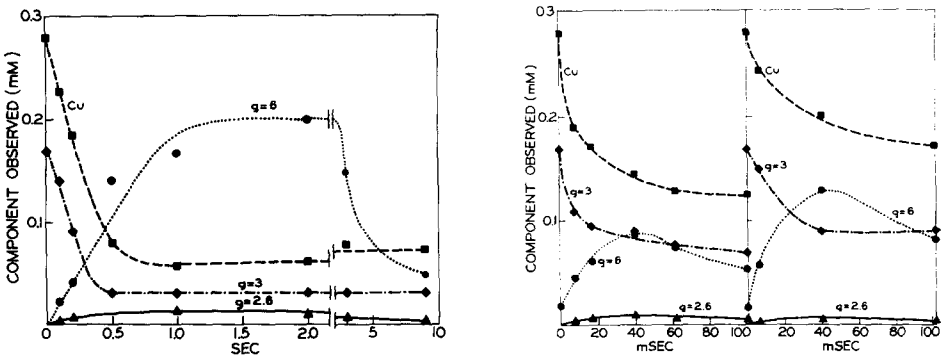


Fig. 13. Time-course of reduction of 0.683 mM cytochrome *c* oxidase by 14.5 mM sodium dithionite (concentrations after mixing), mixed with the rapid mixing–freezing apparatus at 22°. Dithionite was dissolved anaerobically in a tonometer[23] in 1.5 M sucrose containing 35 mg of Tris-free base per ml. The values plotted on the ordinate are actual concentrations calculated form the signals and corrected but not normalized as for Figs. 6 and 8–12.

Fig. 14. Time-course of reduction of 0.683 mM cytochrome *c* oxidase by 1.08 mM (right) or 1.57 mM (left) cytochrome *c* (concentrations after mixing), mixed with the rapid mixing–freezing apparatus at 22°. The evaluation is analogous to that of the experiment of Fig. 13.

The incomplete reduction of the oxidase by cytochrome *c* under the conditions used here (cytochrome *c*/cytochrome *c* oxidase heme ratios of 2.3 and 1.6) appears to be due to the establishment of an equilibrium[37]. When, for instance, cytochrome oxidase was mixed with cytochrome *c* in an evacuated tube as used in the titrations, at a cytochrome *c*/cytochrome oxidase heme ratio of 1.4, only 58 % of the copper signal and 50 % of the low-spin heme signal at $g = 3.0$ disappeared. Under these conditions interference by O_2 is minimized. The requirement for high concentrations of oxidase for EPR spectroscopy and the solubility of cytochrome *c* set limits to the ratio of cytochrome *c* to cytochrome *c* oxidase that can be achieved in such experiments.

Kinetic studies of the reoxidation of reduced cytochrome *c* oxidase were also carried out with the rapid freezing technique, although it is known that the reoxidation rate of all optically observable components of the enzyme is too fast to be followed by this technique[38,39]. These experiments are summarized in Table I. No signals of the intermediate high- and low-spin ferric forms were seen when secondary reduction by any excess of reductant left in the reduced enzyme was avoided. Ascorbate, which in the absence of added cytochrome *c* is able to reduce the oxidase within a few minutes, is the reductant of choice for such experiments. Similarly, when ascorbate-reduced enzyme was mixed with an equal quantity of oxidized enzyme, but with the exclusion of

TABLE I

EPR SIGNALS ON REACTION OF REDUCED CYTOCHROME *c* OXIDASE WITH OXIDIZED OXIDASE OR O_2

Anaerobic solutions mixed from syringe*		*Signals** observed (mm) after reaction time*											
		8 msec			*100 msec*			*1000 msec*			*60 000 msec*		
A	*B*	*Cu*	*g* = 6	*g* = 3	*Cu*	*g* = 6	*g* = 3	*Cu*	*g* = 6	*g* = 3	*Cu*	*g* = 6	*g* = 3
Oxidized enzyme	Sucrose	120	2	10									
Reduced enzyme	Sucrose	47	2	6	44	0	3	48	0	3			
Oxidized enzyme	Reduced enzyme	150	2	10	124	2	9	143	3	8			
Oxidized enzyme	Sucrose + ascorbate	154	3	10	152	2.5	9	154	3	9	108	10	7

* 0.25 M sucrose was used to provide adequate viscosity. Reduced enzyme was prepared in a tonometer with a sidearm by a technique previously described[23]. 3 mg of potassium ascorbate were used per ml of 0.75 mM enzyme solution. The sucrose solution containing ascorbate was prepared in an analogous manner.

** The standard conditions of EPR spectroscopy were used (see *Methods*). The maximal signal at $g = 6$ under conditions corresponding to those used here and referring to the mixtures of enzyme with sucrose would be approx. 30 mm. Note that enzyme concentrations, and therefore signal intensities, are potentially doubled when enzyme is mixed with enzyme, as in one series above.

O_2, the signals of the high- and low-spin intermediates were not seen, indicating that the electron carriers in different molecules do not interact rapidly, if at all. What we call molecules here is of course ill-defined; what we mean is the smallest independent structural unit, not the minimal functional unit, since minimal functional units which are embedded in one and the same larger structural unit might well interact.

DISCUSSION

A number of features of cytochrome *c* oxidase have not yet found a satisfactory explanation. Those that we consider particularly relevant to the present discussion are the following.

(1) All evidence points to the presence of at least two distinct functional heme components in the active enzyme; nevertheless only one type of heme can be isolated from the protein.

(2) The quantity of cupric copper accounted for in the EPR spectrum of the enzyme is only approx. 40 % of that found by chemical analysis; nevertheless, in anaerobic reductive titrations the number of reducing equivalents taken up by the oxidase corresponds to its total heme and copper content.

(3) The cupric copper which is detected in the enzyme by EPR spectroscopy is not significantly reduced with the first 0.5–1 reducing equiv that are taken up.

(4) Although light absorption spectra at room and liquid N_2 temperature and measurements of magnetic susceptibility at room temperature indicate that one of the heme components of the native oxidized enzyme is of the low-spin and one of the high-spin type, low temperature EPR spectra show only a low-spin heme component.

Although for all these points more or less plausible explanations have been suggested from time to time, experimental support for such explanations has generally been lacking. The experiments reported in this paper provide clear-cut answers to some of the questions, altough they admittedly raise a number of new questions.

We consider the demonstration by EPR spectroscopy, that a heme component of the low-spin and one of the high-spin type are present in cytochrome *c* oxidase in approximately equal quantities, as the most compelling direct experimental evidence so far brought forth on the presence of two distinct heme components in the enzyme. Since, as it appears from our experiments, these two components show different behavior during oxidation–reduction, at equilibrium as well as under conditions of rapid reaction, they cannot be an equilibrium mixture of high- and low-spin forms of a single heme component. Although light absorption spectra had suggested the presence of two different heme components (*cf.* ref. 40), EPR spectra have the advantage that the significant bands of high- and low-spin components are widely separated, whereas their optical bands overlap, and that the theoretical explanation of EPR spectra is considerably more straightforward than that of light absorption spectra.

The reason that a significant EPR spectrum of the high-spin component of cytochrome *c* oxidase has not previously been observed is that, at best, traces of it are detectable in the native enzyme. We had, however, previously observed that a significant high-spin signal may appear on denaturation of the enzyme without concomitant loss in the intensity of the low-spin signal. Since all the copper of the enzyme simultaneously appears as the cupric form in the EPR spectrum[27], we had suggested that the unexplained features of both cytochrome a_3 and the EPR- undetectable copper

may have the same explanation, namely interaction of paramagnetic components (see below) in the active oxidized form of the enzyme, strong enough to cancel or broaden the signals. We may be dealing with heme–heme and copper–copper or with copper–heme interactions. In the former case, the smallest cytochrome *c* oxidase unit would have to contain every component in duplicate, as the EPR spectra do not indicate that the EPR-detectable species are involved in strong magnetic interactions. If the interaction of paramagnetic components was indeed the explanation for the "latency" of part of the heme and copper in the oxidized form of the active enzyme, this interaction obviously breaks down on denaturation. The experiments reported here indicate that the interaction between the "latent" components also breaks down as soon as the first 0.5 or 1 reducing equiv/heme is taken up by the enzyme. As obviously the "uptake of 0.5 equiv/heme" has at best reality in a statistical sense, and a molecular weight of cytochrome *c* oxidase is not known with certainty, it will aid the discussion at this point to introduce the concept of a minimal cytochrome *c* oxidase unit which consists of 2 heme and 2 copper components, *i.e.* cytochromes *a* and a_3* and the EPR-detectable and undetectable copper. We consider this as the minimal functional unit of cytochrome *c* oxidase. The nature of a minimal structural unit is not known with certainty and may vary between different types of preparations. For a unit of the size of the minimal functional unit, then 0.5 equiv/heme would mean 1 equiv/unit. We found during titrations in the absence of inhibitors that 2–2.5 equiv/heme or 4–5 equiv/unit were taken up by the electron acceptors of the enzyme. Since small amounts of reducible contaminants, such as nonheme iron, extraneous copper originally not associated with the enzyme or *b*- and *c*-type cytochromes may be present, and considering the overall accuracy of the methods used, we feel that consumption of 2 equiv/heme is probably correct. Since, at what we assume is 2 equiv/heme, all EPR signals have disappeared and the optical spectra indicate complete reduction of all units, we can conclude that the EPR signal of the high-spin ferric form is not present either before uptake of any electrons or after uptake of 2 equiv/heme, *i.e.* units that have taken up 4 electrons cannot have the high-spin ferric signal. Consequently, the appearance of this signal on partial reduction (*e.g.* 1 equiv/heme) cannot be due to a mixture of units that have taken up 4 electrons and fully oxidized units. It follows that the high-spin ferric signal is a characteristic property only of partly reduced units, which have taken up between 1 and 3 electrons, and is not simply a property of any cytochrome *c* oxidase preparation whose overall oxidation state is statistically, intermediate between the oxidized and reduced states. This is supported by experiments on the reoxidation of reduced cytochrome *c* oxidase, which will be discussed below.

Keeping these considerations in mind, we will now discuss the titrations performed under various conditions.

Titrations in the absence of inhibitors

The striking features are that the low-spin heme signal at $g = 3$, which we attribute to cytochrome *a*, and the light absorption at 605 mμ disappear in an approximately linear fashion, whereas the EPR-detectable copper disappears only after a lag

* We follow here the original definition of KEILIN AND HARTREE[53], namely that cytochrome a_3 is the component of the oxidase that reacts with CN^- and cytochrome *a* that which does not react with CN^-.

(*cf.* Figs. 6 and 7). The low-spin heme has disappeared at approx. 1.25 equiv/heme, whereas reduction of all components requires twice as many equiv. The high-spin signal is maximally developed between 1.5 to 2 equiv/heme, when the copper is half reduced. The possibility that the low-spin signal is converted into the high-spin signal cannot be completely ruled out from the available data, since the error in measuring the low-spin signal and the calculation, by integration, of total quantities of components represented is rather high.

However, the mode of the decline of the low-spin signal and that of the rise of the high-spin signal as observed in Figs. 6 and 8, for instance, appear to preclude the notion that the latter originates from the former. We must also consider that if there were conversion of one component from a high- to a low-spin species, there may be superimposed on such a conversion, to some extent, reduction of either species. The observation that on denaturation the high-spin signal appears without decline of the signal at $g = 3$ would also speak against any interconversion of the species responsible for these signals.

Together with the high-spin signal, another low-spin signal ($g = 2.6$, 2.2 and 1.87) appears and disappears on complete reduction. This signal reaches maximal intensity at slightly higher concentrations of reductant than does the high-spin signal. This is not clearly seen in the titration plots but is obvious from a comparison of Figs. 1B and 1C, which show the typical behavior of the two intermediate signals in relation to each other. At no point, however, does the intermediate low-spin ferric heme signal represent an amount of material close to that represented by any of the other observed signals. The fact that the intensity of this signal is not proportional to that of the high-spin signal precludes the possibility that both signals could arise from the same component originating from a thermally balanced mixture of spin states, a phenomenon discussed frequently in recent publications[30,41,42]. However, other equilibria between related forms of the same component (*e.g.* due to pH) could be involved and it appears quite possible that both signals which appear at intermediate oxidation states may be due to forms of cytochrome a_3. Since, obviously, in any single minimal unit cytochrome a_3 would be either high or low spin, the question of the homogeneity of the preparations has to be considered in this context (*cf.* refs. 39, 43), and it is entirely possible that differences may exist in the units making up a preparation. However, an alternative possibility might be that as electrons are taken up by a cytochrome *c* oxidase unit, cytochrome a_3 changes from a high- to a low-spin form, *e.g.* after uptake of 1 or 2 electrons per unit, the high-spin state would prevail and the low-spin state after uptake of 2 or 3 electrons, respectively.

Summarizing now our tentative conclusions as to how the titration proceeds, *i.e.* in which components the reducing equiv are sequentially deposited, the following is suggested. The first two reducing equiv/unit (1 equiv/heme) are obviously not used to any great extent to reduce cytochrome a_3 or EPR-detectable copper; these components are rather reduced by the third and fourth equiv introduced. Cytochrome *a* is reduced with the first 2 equiv added, but since there is only 1 cytochrome *a* per unit, one of the first 2 electrons introduced must be deposited elsewhere. Although no direct evidence can be brought to bear on this point, we suggest that the component reduced concomitantly with cytochrome *a* is the EPR-undetectable copper.

The simplest model involving only the minimal cytochrome *c* oxidase unit would therefore demand that the EPR-undetectable copper and cytochrome a_3 inter-

act, so as to abolish the EPR signals expected from these paramagnetic components. As the first one or two electrons enter the unit, this copper is reduced, the copper–heme interaction ceases and cytochrome a_3 becomes detectable. After most of this copper and cytochrome *a* are reduced, further electrons are used to reduce the EPR-detectable copper and cytochrome a_3. It is difficult to reconcile with this simple model the linear increase in absorbance at 605 mμ observed here and in previous work[7,11,19] during such titrations, which would indicate that at least the heme components are reduced without preference. It must be kept in mind, however, that the EPR spectra were taken at low temperature, whereas the optical spectra refer to room temperature. It appears possible that on freezing, a redistribution of electrons within units may take place, leading to the phenomena just discussed. In addition the simple model does not explain other subtleties of the titration behavior. However, in view of the uncertainties in some quantitative aspects and the possibility of heterogeneity in the preparation, which could partly be due to aggregation phenomena, it does not appear useful at this time to propose more elaborate models which would have to involve units of larger size than the minimal 4 component unit considered above.

It is noteworthy that EHRNENBERG AND VANNESTE[44] and EHRENBERG[55] recently found in titrations similar to those reported here that the magnetic susceptibility measured at room temperature did not decline in a linear fashion during reduction but increased to a maximum between 50 and 75 % reduction. This is in good agreement with our observations on the appearance of a high-spin ferric form during reduction. In order to explain either observation one would have to invoke for the oxidized forms of the enzyme a sufficiently strong interaction of that component, which on reduction gives rise to the high-spin ferric signal or to increased paramagnetism, with another component in the system so as to reduce the total paramagnetism.

Titrations with EDTA or fluoride

Fluoride is a relatively weak ligand of cytochrome *c* oxidase and does not therefore greatly influence the course of the titration as observed by EPR spectroscopy. That there is a clear-cut effect of fluoride on the optical absorption has been shown previously[19]. We are not certain that the earlier decrease of the copper signals detected by EPR and the longer persistance of the signal at $g = 3$ (*cf.* Fig. 9) as compared with a titration without added ligands (*cf.* Fig. 6), is significant. When the enzyme is exposed to fluoride for many hours, the signal of the low-spin intermediate does not appear, but an extra peak appears at $g = 3.1$ in the oxidized state. Whether the absence of one and the presence of the other signal are related, *e.g.* that the component producing the $g = 2.6$, 2.2 and 1.87 signal in the absence of fluoride now appears in the oxidized state at $g = 3.1$ (other g values are not certain), is not clear. EDTA does not appear to have any influence on the course of the titration according to the present experiments.

Titrations with cyanide or azide

These ions are known to be strong heme ligands, which rarely allow high-spin states of ferric iron to exist (*cf.* refs. 45, 46). Consequently, no high-spin forms were seen with either ligand. In the presence of cyanide, in fact, no signals other than those present in the oxidized enzyme ever appear, *i.e.* on titration one simply follows the disappearance of the signals. It is significant that this disappearance is linear for both

cytochrome *a* and copper and that all detectable components are reduced with approx. 1–1.5 equiv/heme, *i.e.* half the reducing equiv needed in the absence of cyanide. This indicates that 2 of the 4 electron acceptors per cytochrome *c* oxidase unit are not reduced, and these 2 acceptors must be cytochrome a_3 and the EPR-undetectable copper. Presumably both react with cyanide. If cyanide reacts in this manner and, as one would expect, abolishes the interaction between these two components, one may then ask why no EPR signals appear. Cupric copper may of course be reduced by cyanide which would explain the absence of a copper signal, but a low-spin heme signal may be expected. Since we did not see any increase in the signal at $g = 3$, the region where a low-spin signal should be observed, and since whatever signal is present at $g = 3$ disappears readily on titration with just over 1 equiv/heme, we can only suspect that the low-spin signal of ferricytochrome a_3 cyanide may be very broad and might be detectable only at temperatures below those used in our work ($> 15°K$).

Although it is often thought that cyanide and azide act very similarly, our experiments certainly show great differences between the effects of these two ligands. With azide a total of 1.5 equiv/heme is needed for the reduction of cytochrome *a* and the EPR-detectable copper. While these components are reduced, a new low-spin ferric signal appears with $g = 2.9$, 2.2 and 1.67. Its mode of appearance in the titration in the presence of azide is similar to the appearance of the high-spin ferric signal in the absence of strong ligands; *i.e.* it is typical of that of an intermediate, but the low-spin signal appearing in the presence of azide does not disappear on continued addition of reductant except at very high levels. In the following experiment it can be demonstrated that the low-spin signal appearing in the presence of azide is due to the same component, which gives rise to the high-spin signal at $g = 6$ at intermediate oxidation states when azide is absent. When the signal at $g = 6$ is produced in the absence of azide and small increments of azide are added anaerobically from a side arm, one can observe the gradual fading of the signal at $g = 6$ and the increase of those at $g = 2.9$, 2.2 and 1.67. That the signal at $g = 2.9$ is not the azide compound of the component represented in the signal at $g = 3$ in the oxidized enzyme, namely cytochrome *a*, can be seen from the simultaneous existence of the signals at $g = 2.9$ and $g = 3$ at saturating azide but low reductant concentrations. The situation is somewhat complicated in this instance by an additional minor signal which also arises at low reductant concentrations (*cf.* RESULTS and Fig. 1E). We conclude from these experiments that the low-spin ferric signal at $g = 2.9$, 2.2 and 1.67 represents the azide form of cytochrome a_3. As seen in Fig. 10, this form is not readily reduced. The lag in the reduction of the EPR-detectable copper is not as pronounced in the presence of azide as it is in its absence.

Titration with dithionite

It has been observed by others in spectrophotometric studies that dithionite reduces cytochrome *a* much faster than cytochrome a_3 (refs. 47, 48). In the titration with dithionite electron donors and acceptors apparently equilibrated rather slowly and at equilibrium, large high-spin ferric signals are observed on partial reduction with dithionite, while very little of the intermediate low-spin form ($g = 2.6$, 2.2 and 1.87) is seen. Complete reduction of cytochrome *a* and copper required approx. 2.5 equiv per heme.

Kinetics of reduction and reoxidation

Thus far we have only considered conditions when the enzyme was exposed to the reductant for several minutes or longer so that equilibrium between electron donors and acceptors should have been reached.

In order to ascertain whether the intermediate forms of cytochrome *c* oxidase observed under these conditions may be significant species in catalysis, it was necessary to study the time dependence of their formation. GIBSON *et al.*[49] have shown that reduction of cytochrome *a* by cytochrome *c* largely occurs within 10 msec after mixing but that reduction of cytochrome a_3 may take seconds. Our results shown in Fig. 14, are not incompatible with this. Copper and cytochrome *a* are rapidly reduced. At the higher cytochrome *c* concentration the rates of their reduction are almost equal. The high-spin heme signal and the minor low-spin signal ($g = 2.6$, 2.2 and 1.87) are rapidly formed on reduction, reach their maximum at 40 msec but disappear more slowly. Although a detailed comparison between the spectrophotometric experiments of GIBSON *et al.*[49] and those reported here is probably not feasible because of the different conditions, the order of events observed by the two techniques is certainly in agreement.

Judging from these experiments we would expect that significant concentrations of the high-spin ferric intermediate should also be present under steady state conditions. Formation of an intermediate species in the presence of azide has recently attracted considerable interest[50–52]. Although the experiments reported here furnish no direct evidence for the identity of such a species, it is very possible that our observation of a distinct low-spin ferric intermediate in the presence of azide has considerable bearing on this question.

Reduction with dithionite proceeds more slowly but is finally more extensive (Fig. 13); again intermediates are formed rapidly. Within the limits set by the resolution of our rapid freezing technique, the components represented by the signals at $g = 6$ and $g = 2.6$, 2.2 and 1.87 qualify, therefore, as catalytically significant species in the reduction of cytochrome *c* oxidase.

According to GIBSON AND GREENWOOD[38] the kinetic analysis of the oxidation of reduced cytochrome *c* oxidase by O_2, even the slowest rate ($1 \cdot 10^3$ sec^{-1}), namely that for the cytochrome *a* component, is still beyond the resolution of our rapid reaction technique. It was therefore predicted (Q. H. GIBSON, personal communication) that the intermediate high- and low-spin forms should not be observed during reoxidation, and this was indeed verified in our experiments. Even under conditions when there was insufficient O_2 for complete reoxidation of all the electron carriers in cytochrome *c* oxidase, no intermediate forms were seen. It may be recalled that we pointed out earlier in the discussion that it is not a sufficient condition for the appearance of the intermediate forms that a preparation be at a state of partial reduction, statistically speaking; it is necessary that individual cytochrome *c* oxidase units be partly reduced. Therefore, when reduced cytochrome *c* oxidase is mixed with an amount of O_2 insufficient for complete reoxidation, a mixture of completely reduced and completely oxidized units is obtained within the time interval preceding our first observation (approx. 10 msec), whereas on reduction of oxidized cytochrome *c* oxidase partly reduced units are formed. In confirmation of the findings in the reoxidation experiments, we did not observe any intermediates when oxidized and reduced cytochrome *c* oxidase were mixed. Only after seconds high-spin signals arose gradually,

particularly in the presence of traces of electron carriers such as phenazine methosulfate or cytochrome *c*, which could mediate oxidoreduction between cytochrome *c* oxidase units, or when the reduced enzyme contained a slight excess of reductant.

Interaction of paramagnetic components

We have repeatedly referred to magnetic interactions as the reason for the fact that no EPR signals were observed for two cytochrome *c* oxidase components, *viz.* cytochrome a_3 and part of the copper. Three principal possibilities have to be considered here: firstly an increase in the electron spin relaxation rate to the extent that signals are broadened beyond detection ("lifetime broadening"); secondly, the static component of magnetic dipolar interaction between neighboring electron spins could bring about a similar broadening; and thirdly, antiferromagnetic coupling of electron spins could occur. In this last instance, not only is detection by EPR spectroscopy interfered with but paramagnetism is decreased. In the first and second instance, bulk magnetic susceptibility should still be measurable.

In the case at hand, namely the absence of EPR signals from components of cytochrome *c* oxidase which can be expected to be paramagnetic, rapid electron spin relaxation is not likely to be the explanation because even at the temperature of liquid He these signals are not seen. At this temperature the relaxation rate should have decreased sufficiently, so that signals might be detected*. Concerning the possibility of antiferromagnetic interaction, it must be noted that measurements of the magnetic susceptibility of cytochrome *c* oxidase at room temperature indicate a substantial contribution by the a_3 component[1], and as shown more recently[44,55], paramagnetism increases as the enzyme is partially (50–75%) reduced and decreases again on more extensive reduction.

Although the EPR measurements were made at low temperatures and the mentioned measurements of paramagnetic susceptibility at room temperature, it would appear on the basis of these findings that antiferromagnetic interaction of the iron in cytochrome a_3 with another component is responsible for the diminished paramagnetism and the lack of expected EPR signals in the oxidized form of the enzyme. Such interaction could take place between two hemes of cytochrome a_3 or between the EPR-undetectable copper and one of these hemes. The spin states resulting from such interaction and consequently the diminution of paramagnetism depend on a number of factors, the magnitude of which is unknown and therefore cannot be predicted without additional information.

ACKNOWLEDGMENTS

This work was supported in part by the National Institutes of Health by Grant GM-12394, Research Career Program award GM-K6-12,442 to H.B. both from the

* Recently, it has been found[54] that when two neighboring paramagnetic species are present in a structure which restricts their motion (*e.g.*, in a protein in solution) and their relaxation parameters, T_1 and T_2, the correlation time, τ_c, and the resonance frequency fall into a certain range of combinations of these values, EPR signal intensities may be diminished without significant broadening. This is an important condition which might have a bearing on the apparent "latency" of signals as observed here. This possibility deserves further exploration, although it appears that even in this instance the signal intensity should not show the simple dependence on the reciprocal of the absolute temperature, characteristic of EPR signals of noninteracting paramagnetic species with moderate relaxation times.

National Institute of General Medical Sciences, fellowship Grant 1F05 TW-1137 and a grant from the Research Committee of the Graduate School, University of Wisconsin, to B. F. Van G. We are deeply indebted to a number of colleagues for their substantial contributions to this work: to Dr. Richard H. Sands for stimulating our interest in the heme spectra of cytochrome *c* oxidase, to Drs. Tore Vänngård and A. Ehrenberg for their advice and active collaboration in the quantitative evaluations, to Dr. Gilda Harris for critical discussions, to Dr. W. H. Orme-Johnson for his advice in anaerobic techniques and his active collaboration in the application of his titration method using solid dithionite, to Dr. W. H. Orme-Johnson as well as to Dr. A. Ehrenberg for their critical review of the manuscript, to Mr. R. E. Hansen for advice and assistance with EPR, optical and rapid reaction techniques and to Miss Sabine Schweidler for preparations of the enzyme. We are indebted to Drs. B. Ganguli and I. C. Gunsalus for a sample of their soluble preparation P_{450} from *Pseudomonas putida*.

REFERENCES

1 A. EHRENBERG AND T. YONETANI, *Acta Chem. Scand.*, 15 (1961) 1071.
2 H. BEINERT AND G. PALMER, *Advances in Enzymology*, Vol. 27, Wiley, New York, 1965, p. 105.
3 B. F. VAN GELDER, W. H. ORME-JOHNSON, R. E. HANSEN AND H. BEINERT, *Proc. Natl. Acad. Sci. U.S.*, 58 (1967) 1073.
4 H. BEINERT, B. F. VAN GELDER AND R. E. HANSEN, in K. OKUNUKI, M. D. KAMEN AND I. SEKUZU, *Structure and Function of Cytochromes*, University of Tokyo Press, Tokyo, 1968, p. 141.
5 L. R. FOWLER, S. H. RICHARDSON AND Y. HATEFI, *Biochim. Biophys. Acta*, 64 (1962) 170.
6 D. H. MACLENNAN AND A. TZAGOLOFF, *Biochim. Biophys. Acta*, 96 (1965) 166.
7 B. F. VAN GELDER, *Biochim. Biophys. Acta*, 118 (1966) 36.
8 E. MARGOLIASH AND O. F. WALASEK, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 339.
9 B. F. VAN GELDER AND E. C. SLATER, *Biochim. Biophys. Acta*, 58 (1962) 593.
10 F. SOLYMOSI AND A. VARGA, *Acta Chim. Acad. Sci. Hung.*, 20 (1959) 399.
11 B. F. VAN GELDER AND E. C. SLATER, *Biochim. Biophys. Acta*, 73 (1963) 663.
12 K. V. RAJAGOPALAN, P. HANDLER, G. PALMER AND H. BEINERT, *J. Biol. Chem.*, 243 (1968) 3784.
13 P. W. SWEETSER, *Anal. Chem.*, 39 (1967) 979.
14 J. C. M. TSIBRIS, R. L. TSAI, I. C. GUNSALUS, W. H. ORME-JOHNSON, R. E. HANSEN AND H. BEINERT, *Proc. Natl. Acad. Sci. U.S.*, 59 (1968) 959.
15 W. H. ORME-JOHNSON AND H. BEINERT, *Anal. Biochem.*, in the press.
16 R. C. BRAY, *Biochem. J.*, 81 (1961) 189.
17 R. E. HANSEN AND H. BEINERT, *Anal. Chem.*, 38 (1966) 484.
18 R. E. HANSEN, in preparation.
19 B. F. VAN GELDER AND A. O. MUIJSERS, *Biochim. Biophys. Acta*, 118 (1966) 47.
20 H. BEINERT, in J. PEISACH, P. AISEN AND W. E. BLUMBERG, *Biochemistry of Copper*, Academic Press, New York, 1966, p. 213.
21 R. E. HANSEN, B. F. VAN GELDER AND H. BEINERT, in preparation.
22 H. BEINERT AND B. KOK, *Biochim. Biophys. Acta*, 88 (1964) 278.
23 H. BEINERT AND G. PALMER, *J. Biol. Chem.*, 239 (1964) 1221.
24 G. PALMER, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 594.
25 R. E. HANSEN, T. T. KALAL AND H. BEINERT, *Anal. Biochem.*, 20 (1967) 40.
26 H. BEINERT, D. E. GRIFFITHS, D. C. WHARTON AND R. H. SANDS, *J. Biol. Chem.*, 237 (1962) 2337.
27 H. BEINERT AND G. PALMER, in T. E. KING, H. S. MASON AND M. MORRISON, *Oxidases and Related Redox Systems*, Vols. 1 and 2, John Wiley and Sons, New York, 1965, p. 567.
28 J. S. GRIFFITHS, *Proc. Roy. Soc. London, Ser. A*, 235 (1956) 23.
29 T. VÄNNGÅRD, in A. EHRENBERG, B. G. MALMSTRÖM AND T. VÄNNGÅRD, *Magnetic Resonance in Biological Systems*, Vol. 9, Pergamon Press, Oxford, 1967, p. 213.
30 A. EHRENBERG, *Arkiv Kemi*, 19 (1962) 119.
31 J. E. BENNETT AND D. J. E. INGRAM, *Nature*, 177 (1956) 275.
32 D. J. E. INGRAM, J. F. GIBSON AND M. F. PERUTZ, *Nature*, 178 (1956) 906.
33 H. WATARI, J.-J. HWANG, K. KIMURA AND K. MURASE, *Biochim. Biophys. Acta*, 120 (1966) 131.
34 W. E. BLUMBERG, J. PEISACH, B. A. WITTENBERG AND J. B. WITTENBERG, *J. Biol. Chem.*, 243 (1968) 1854.

35 A. O. MUIJSERS, E. C. SLATER AND K. J. H. VAN BUUREN, in K. OKUNUKI, M. D. KAMEN AND I. SEKUZU, *Structure and Function of Cytochromes*, University of Tokyo Press, Tokyo, 1968, p. 129.
36 A. O. MUIJSERS, B. F. VAN GELDER AND E. C. SLATER, in B. CHANCE, R. ESTABROOK AND T. YONETANI, *Hemes and Hemoproteins*, Academic Press, New York, 1966, p. 467.
37 K. MINNAERT, *Biochim. Biophys. Acta*, 110 (1965) 42.
38 Q. H. GIBSON AND C. GREENWOOD, *J. Biol. Chem.*, 240 (1965) 2694.
39 Q. H. GIBSON AND D. C. WHARTON, in K. OKUNUKI, M. D. KAMEN AND I. SEKUZU, *Structure and Function of Cytochromes*, University of Tokyo Press, Tokyo, 1968, p. 5.
40 T. E. KING, H. S. MASON AND M. MORRISON, *Oxidases and Related Redox Systems*, Vol. 2, Wiley, New York, 1965.
41 P. GEORGE, J. BEETLESTONE AND J. S. GRIFFITH, *Rev. Mod. Phys.*, 36 (1964) 441.
42 I. IIZUKA AND M. KOTANI, *Biochim. Biophys. Acta.*, 154 (1968) 417.
43 Q. H. GIBSON, G. PALMER AND D. C. WHARTON, *J. Biol. Chem.*, 240 (1965) 915.
44 A. EHRENBERG AND W. VANNESTE, in *19th Colloq. Ges. Biol. Chem., Mosbach, 1968*, Springer, New York, 1968, p. 127.
45 J. E. FALK, R. LEMBERG AND R. K. MORTON, *Heamatin Enzymes*, Pergamon Press, Oxford, 1961.
46 B. CHANCE, R. ESTABROOK AND T. YONETANI, *Hemes and Hemoproteins*, Academic Press, New York, 1966.
47 R. LEMBERG AND G. E. MANSLEY, *Biochim. Biophys. Acta*, 118 (1966) 19.
48 M. R. LEMBERG, M. V. GILMOUR AND M. E. CUTLER, in K. OKUNUKI, M. D. KAMEN AND I. SEKUZU, *Structure and Function of Cytochromes*, University of Tokyo Press, Tokyo, 1968, p. 54.
49 Q. H. GIBSON, C. GREENWOOD, D. C. WHARTON AND G. PALMER, *J. Biol. Chem.*, 240 (1965) 888.
50 D. F. WILSON AND B. CHANCE, *Biochim. Biophys. Acta*, 131 (1967) 421.
51 M. V. GILMOUR, D. F. WILSON AND R. LEMBERG, *Biochim. Biophys. Acta*, 143 (1967) 487.
52 P. NICHOLLS AND H. K. KIMELBERG, *Biochim. Biophys. Acta*, 162 (1968) 11.
53 D. KEILIN AND E. F. HARTREE, *Proc. Roy. Soc., London, Ser. B*, 127 (1939) 167.
54 M. COHN, J. S. LEIGH AND J. S. TAYLOR, *Proc. Natl. Acad. Sci., U.S.*, in the press.
55 A. EHRENBERG, *3rd Intern. Conf. Magnetic Resonance Biol. Systems, Warrenton, Va., 1968.*