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COMPONENTS OF CYTOCHROME *c* OXIDASE DETECTABLE BY EPR SPECTROSCOPY

CHARLES R HARTZELL* and HELMUT BEINERT

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

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SUMMARY

A procedure for the preparation from frozen beef heart mitochondria of cytochrome *c* oxidase (EC 1 9 3 1) of high heme (⩽ 14 μmoles/mg protein) and low extraneous copper (⩽ 1 1 atoms Cu/mole heme) and low lipid (⩽ 0 05 g phospholipid/g protein) content is described EPR signals observed with the enzyme between 6 and 100 °K at various states of oxidation and at different conditions of pH and presence of solutes are described in detail The quantities of paramagnetic species represented by these signals are estimated Under no conditions does the sum of the EPR detectable species represent more than approx 50 % of the potentially paramagnetic components of the enzyme Comparisons are made to the corresponding signals as observed in whole tissue, mitochondria and submitochondrial particles from a number of species The assignment of the observed signals to known components of cytochrome *c* oxidase is discussed briefly

INTRODUCTION

This paper is the first in a series of papers describing the oxidation–reduction behavior of the EPR detectable components of cytochrome *c* oxidase (EC 1 9 3 1) under equilibrium and rapid reaction conditions Since a systematic description of the EPR signals of this enzyme must precede any reports on changes during oxidation–reduction, we will in the present publication describe the EPR spectra observed at ⩾ 6 °K under a number of conditions with preparations of purified cytochrome *c* oxidase and subcellular particles The heme spectra of the enzyme are sensitive to a number of environmental conditions, which usually are not controlled rigorously or even reported, such as the presence of dissolved gases or other solutes Therefore, an unexpectedly large number of different types and sub-types of EPR signals can be observed from what may be only two (copper and cytochrome *a*) or perhaps at the most three (copper and cytochromes *a* and a_3) components in cytochrome *c* oxidase

* Present address Department of Biochemistry The Pennsylvania State University, University Park, Pa 16802 U S A

Abbreviations PMS, phenazine methosulfate, PCMS, *p*-chloromercuriphenylsulfonate

It was then an obvious task of the present work to study the qualitative and quantitative relationships between these signals and to sort out the apparently trivial from the essential effects

MATERIALS AND METHODS

Triton X-114 was obtained from Rohm and Haas, Co , (Philadelphia, Pa) Tween 20, Tween 80, phenazine methosulfate, and cytochrome *c* (Type VI) from Sigma Chemical Co , St Louis, Mo Cytochrome *c* was purified by the procedure of Margoliash and Walasek [1] NADH was purchased from PL Laboratories, Milwaukee, Wisc Cytochrome *c* oxidase was purified from beef heart mitochondria The present method was developed with the aim of producing a preparation of high heme, low inactive copper and low lipid content, of high activity and low viscosity so that concentrated solutions ($\geqslant$ 1 mM), as required for EPR and Mossbauer spectroscopy and for magnetic susceptibility measurements, could be easily and quantitatively handled In part the method combines procedures previously applied by Sun et al [2] and Yonetani [3] Mitochondrial paste in 0 25 M sucrose, 0 01 M Tris–HCl, pH 7 4, obtained as described [4] was diluted by one half with the same medium and either used directly or stored frozen at −20 °C The fresh or frozen paste was diluted by one half again with a solution containing 1 mM histidine HCl, 10 mM disodium dihydrogen ethylenediaminetetraacetate, and 0 02 M Tris–HCl, pH 7 4 Potassium phosphate may be substituted for Tris–HCl with no change in procedure Following dilution, the material was homogenized in a Waring Blendor alternating between low and high speed, using 30 s bursts on each speed for a total of 150 s The protein concentration was determined by the biuret method and additional histidine–EDTA–Tris medium was added, if necessary, to yield a total protein concentration of 40 mg/ml From a 20 % dispersion of Triton X-114 in water, 2 5 mg Triton X-114 per mg protein was added and solid KCl to give a final concentration of 0 2 M KCl The mixture was stirred at 4 °C for 1 h and then centrifuged at 2 °C, either in a Beckman 30 rotor at 30 000 rev /min for 100 min or in a Sorvall GSA rotor at 8 000 rev /min for 10 h The supernatant was discarded and the pellets resuspended in cold histidine–EDTA–Tris medium, and homogenized with a glass-teflon homogenizer The volume was adjusted with histidine–EDTA–Tris medium to equal the original volume of mitochondrial paste and cold ethanol was added to a final concentration of 10 % (v/v) The mixture was stirred for 1 h at 4 °C or less The solution was then centrifuged in a Sorvall GSA rotor at 13 000 rev /min for 1 h at 2 °C The supernatant usually is dark yellow and contains a considerable amount of Triton X-114 The pellet was resuspended in histidine–EDTA–Tris medium and homogenized with a glass-teflon homogenizer Freezing the material in liquid N_2 at this stage is recommended as it appears to help considerably in removing additional lipid and Triton from the preparation and in effecting solubilization The protein concentration was determined and the homogenate was diluted to 40 mg protein per ml with histidine–EDTA–Tris medium A quantity of a 20 % potassium cholate solution was then added sufficient to bring the cholate concentration to 1 5 mg per mg protein $(NH_4)_2SO_4$ was added to 10 % saturation at 4 °C The mixture was stirred 1 5 h at 4 °C and then centrifuged in a Sorvall GSA rotor at 13 000 rev /min for 45 min The residue was discarded The supernatant was brought to 25 % saturation with respect to $(NH_4)_2SO_4$, stirred

15 min at 4 °C and centrifuged at 13 000 rev /min for 30 min The grayish-pink residue was again discarded and the solution adjusted to 40 % saturation with $(NH_4)_2SO_4$ (sometimes it is necessary to adjust to 48 % saturation with $(NH_4)_2SO_4$ to achieve complete precipitation of cytochrome oxidase) After centrifugation at 13 000 rev /min in the Sorvall GSA rotor for 30 min the pellet was resuspended in Tris–EDTA solution (as histidine–EDTA–Tris medium, omitting histidine) Additional cholate and Triton X-114 may be removed by repeating the $(NH_4)_2SO_4$ fractionations between 25 and 39 % saturation Excessive fractionation without addition of further detergent such as Tween 20, Tween 80, Emasol 1130 or Emasol 4130 may yield an insoluble preparation that can be resolubilized by repeating the step involving potassium cholate and 10 % saturated $(NH_4)_2SO_4$ In this case further fractionation is required to remove the excess cholate added the second time Therefore, we have employed only one or two fractionations after the initial fractionation at 40 or 48 % saturation with $(NH_4)_2SO_4$

This preparation has yielded consistently material containing 13–14 μmoles of heme *a*, 14–15 μmoles copper and not more than 50 mg of phospholipid per g of protein At heme *a* concentrations above 100 μM no additional detergent is required and at lower concentrations of heme *a* 0 1 % non-ionic detergent (e g Tween 20) will maintain the oxidase soluble The maximal molecular activity of the oxidase reactivated with 1 % Tween 80 is typically about 11 000 min^{-1} [3]

We also verified the presence or formation of all the observed signals in two other preparations, viz , that of Fowler et al [5] as modified by McLennan and Tzagoloff [6] and that of Caughey and collaborators, which is obtained by a different procedure Unless the type of preparation used in a particular experiment is mentioned specifically, that described in this paper was used

Ferrimyoglobin was prepared according to Hapner et al [7] and standardized using an extinction coefficient of 9 23 mM^{-1} cm^{-1} at 503 nm Beef heart mitochondria were prepared as described by Blair [4] Mitochondria from other species were prepared by the method of Tyler and Gonze [8] or Chance and Hagihara [9] using 0 225 M mannitol, 0 075 M sucrose, 0 2 mM EDTA and 0 05 M Tris at pH 7 4 as the medium The EPR signals from beef heart mitochondria prepared in this medium had the same shape as those from mitochondria prepared in a sucrose medium only Sonicated particles were obtained and the freeze-clamping of fresh tissue was carried out as described [10] Heme was determined by the difference in absorbance between reduced and oxidized samples at 604 nm [11], iron and copper according to Van De Bogart and Beinert [12] and phospholipid according to Chen et al [13] All values given for concentrations of the enzyme refer to total heme concentration, not distinguishing between cytochromes *a* and a_3 Values for percentage reduction given in the text or in tables, figures, or legends refer to values calculated from the quantity of heme present and the titer determined for the reductant or oxidant used, on the assumption that two electrons are needed per heme for 100 % reduction

EPR spectroscopy was carried out with a modified Varian V4500 instrument and the spectra were evaluated as described [10, 14] Integration procedures will be discussed under Results and Discussion The microwave frequency was 9 1–9 2 GHz, except in the experiment of Fig 13 Shifts of low spin heme resonances of the order of a few gauss were observed between various samples To measure these shifts

accurately, a Varian fieldial was used, modified so that the sweep range potentiometer was optically encoded and digitized The fieldial settings were calibrated from run to run with a proton probe The frequency was continuously monitored and the field center for each sample was readjusted according to the measured frequency The modulation frequency was 8 G except for scans on whole tissue and mitochondria in the low ($g=6$) field region and for all spectra at $g < 2$, when 10–12 G were used The scanning rate was 200–400 G min except for Figs 9 and 13, when it was 1000 and 2500 G min, respectively The time constant was 0 5 s throughout

Unless mentioned otherwise, the EPR spectra were recorded at 13 °K In the standard Varian V-4531 rectangular cavity, which we are using, and at 13 °K saturation of the copper signal and the low spin heme signal at $g=2$ 6, 2 2, 1 86 of cytochrome *c* oxidase sets in between 0 1 and 0 3 mW, of the low spin heme signal at $g=3$, 2 2, 1 5 and the high spin heme signal between 3 and 10 mW We routinely used 0 27 mW for recording copper signals and 2 7 mW for heme signals Corrections were applied where necessary

RESULTS AND DISCUSSION

Copper

The signal in cytochrome oxidase observed at $g = 2$ 03 and $g = 2$ 17, and generally attributed to copper in the enzyme has been described repeatedly [15–20] The superimposed signals of low spin hemes at low temperature and of the species with broad copper hfs (spacing 184 G), which has been called "inactive copper" [17] make a quantitative evaluation of the copper signal difficult The quantity of the inactive copper varies widely from preparation to preparation and even more so between types of preparations (cf spectra in refs 15, 17–22) This variability supports the conclusion that the copper represented by this signal is adventitious Fortunately, this species is not reduced by cytochrome *c* or NADH and phenazine methosulfate within the time usually required in titrations (5–15 min), so that its signal can be isolated and used for corrections Accounting for the adventitious copper and minimizing heme signal interference by working at liquid N_2 temperature with the lowest feasible microwave power, double integrations of the signal, which is thought to represent the "endogenous copper" signal of the enzyme, yield values ranging between 30 and 45 %, most frequently 35–40 %, of the values found for total heme It is preferable to use heme content as the reference since the value for total copper depends on the amount of inactive copper present Integrations of the copper signal at temperatures between 4 and 150 °K yield, within error, the same percentage of copper represented in the signal At higher temperatures the signal of the inactive copper predominates

Low spin heme

In the oxidized form of the enzyme there is only one major signal in addition to the copper signal, the low spin ferric heme signal with resonances at $g=3$, 2 2 and 1 5 Fig 1 shows the behavior of the $g=3$ resonance in an anaerobic titration of cytochrome *c* oxidase with NADH and phenazine methosulfate It is clear that there are two species present which have slightly different apparent midpoint potentials According to the effects of pH on the low spin heme signal illustrated in Fig 2, these species are likely to be different ionic forms of the enzyme The species at pH 5 5 in

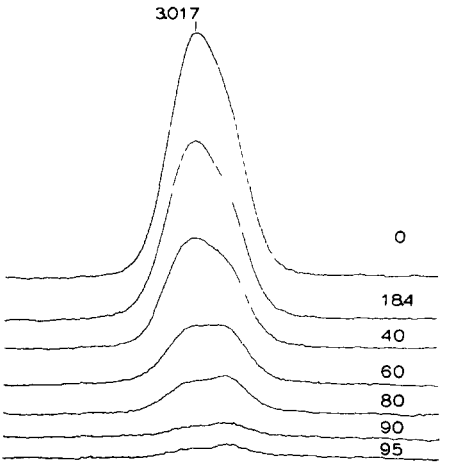

Fig 1 EPR spectra of the low spin heme recorded during titration of 0 75 mM cytochrome *c* oxidase with NADH and phenazine methosulfate at pH 7 4 Percent reduction calculated from the amount of reductant added, is given in the figure For conditions of EPR spectroscopy see Materials and Methods The peaks of the two components are seperated by 25 G

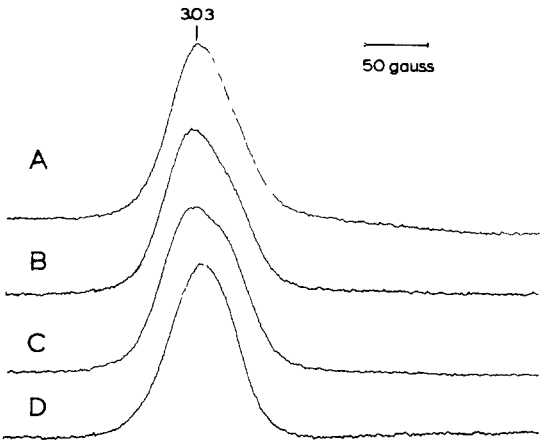

Fig 2 EPR spectra of the low spin heme of 0 42 mM cytochrome *c* oxidase at different pH Enzyme was deionized on a column of the mixed ion exchanger, Rexyn 300 (Fisher Scientific Co) and the pH was then adjusted to 5 5 with acetic acid (A), 7 3 with potassium phosphate (B), 8 0 with Tris–acetate (C) and 9 8 with Tris base (D)

Fig 2 was obtained in phosphate and in acetate buffer and that at pH 9 8 in Tris and in glycine buffer, indicating that it is the pH rather than the anions which bring about the shifts of the resonance at $g=3$ Anion effects may, however, be superimposed With glycine, for instance, the signal at $g=3$ loses approx 75 % of its intensity, while a strong signal at $g=2\,6$, 2 2 and 1 86 (see below) appears

An about 30-G shift of the $g=3$ signal during reductive titration [23] could be traced to the presence of chloride in the dithionite–KCl mixture used as titrant This effect is illustrated in Fig 3 Concentrations of KCl in the physiologically occurring

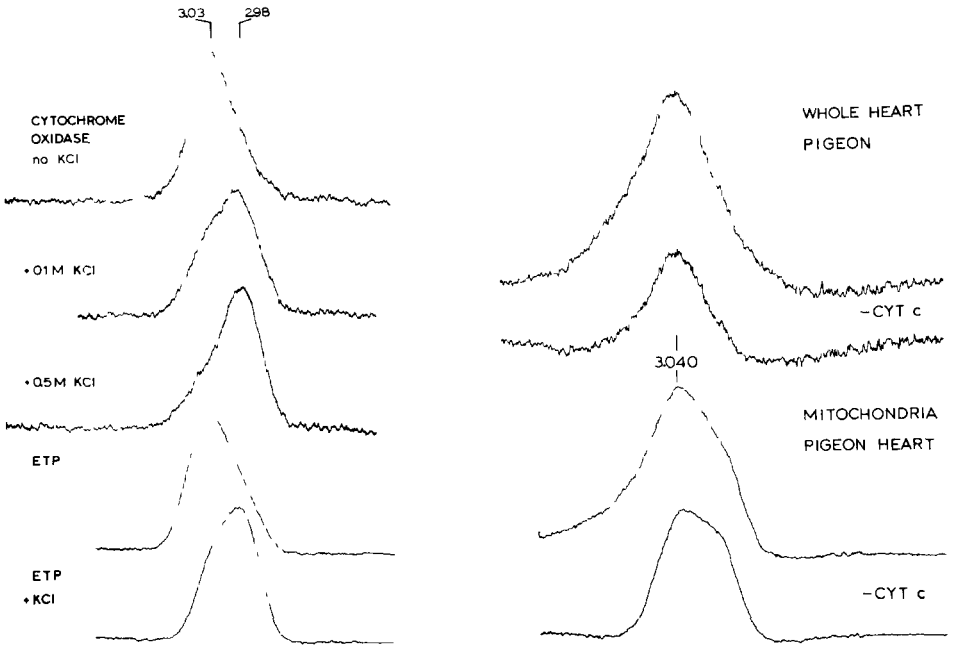

Fig 3 EPR spectra of the low spin heme of 0 1 mM cytochrome *c* oxidase and ETP in the presence of chloride The chloride concentration in ETP is not known accurately ETP depleted of cytochrome *c* by washing with KCl solution was suspended at 40 mg of protein per ml in 0 25 M sucrose, From ETP thus obtained the bottom spectrum was recorded After several washings with sucrose the upper spectrum labeled ETP was obtained

Fig 4 EPR spectra of the low spin heme of cytochrome *c* oxidase in whole pigeon heart and pigeon heart mitochondria For preparations see Materials and Methods The mitochondria were suspended in mannitol–sucrose at 50 mg protein per ml and were aerated before freezing The spectra shown are averages of 9 (whole heart) and 4 (mitochondria) spectra, respectively, accumulated by a C-1024 computer of average transients The lower spectra of each pair are corrected for the superimposed resonance of cytochrome *c* Since the cytochrome *c* spectrum appears to be somewhat different in the presence of oxidase the spectrum used for this correction was obtained by subtraction of a spectrum of oxidase from that of oxidase in the presence of cytochrome *c* To account for unpredictable shifts in *g*-value (cf Tables I and II) and the unknown intensity of the cytochrome *c* signal in particles or tissue the height and position of the cytochrome *c* signal were matched to the experimental spectrum on the low field side where the $g = 3$ signal of cytochrome oxidase has no intensity The success of the correction was judged by the appearance of the baseline on both sides of the $g = 3$ signal after subtraction of the cytochrome *c* signal

range have a distinct effect This shift is also observed with particles (cf Fig 3) It was of interest, at what stage of tissue sample preparation signal shape changes are introduced Fig 4 shows a comparison of the resonances at $g=3$ seen with rapidly frozen fresh pigeon heart and from mitochondria prepared from pigeon heart in mannitol–sucrose medium [8, 9] Both signals are shown before (upper curve of each pair) and after subtraction of a signal for cytochrome *c* It is obvious that the signals in vivo do not have the shape of the signals obtained from isolated mitochondria The $g=3$ signals in mitochondria differ depending on many factors including the animal source, the presence of sucrose, chloride and dissolved gases.

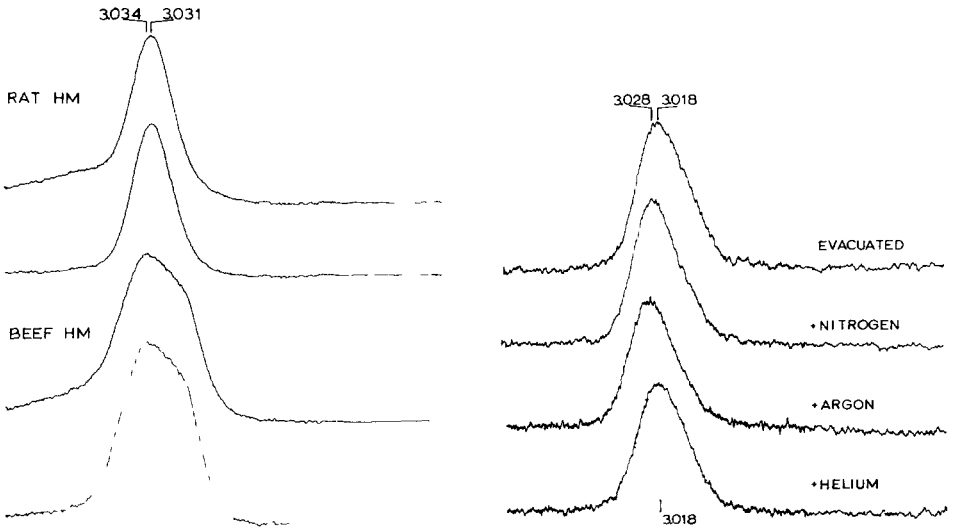

Fig 5 EPR spectra of low spin heme of cytochrome *c* oxidase in rat and beef heart mitochondria The conditions were and the presentation is as given in Fig 4

Fig 6 EPR spectra of the low spin heme of cytochrome *c* oxidase in the presence of various gases, as indicated The enzyme, 0 1 mM was subjected to several cycles of vacuum and exposure to the respective gas at atmospheric pressure

The $g=3$ signals of mitochondria isolated in mannitol–sucrose showed essentially two types of shapes the broad asymmetric signal as found in beef and pigeon heart mitochondria and the narrow type as found in rat heart mitochondria (Fig 5) Heart mitochondria of the rat, rabbit, monkey and *Candida utilis* showed similar signal shapes

Fig 6 shows effects of gases on the shape and g-values of the $g=3$ signal With the preparation described above O_2, N_2, CO and Ar produce very similar shifts whereas He has little effect With the modified preparation of Fowler et al [5, 6], we observed no shift with Ar and CO, but the effects of N_2 and O_2 are similar to those found with our preparation In order to avoid autoreduction in the presence of CO, a quantity of ferricyanide stoichiometric to the heme concentration was added to the enzymes, when CO was added There is no evidence that the oxidized form of the enzyme binds oxygen strongly, however, the association of gases with the enzyme may produce some minor conformational effects which are communicated to the heme It is of interest in this context that exposure to air of the deep frozen, evacuated sample does not change the shape of the resonance to that observed in the presence of air

There is also a dependence of the shifts of the resonances on enzyme concentration This is observed particularly when coupled with the effects of the gases At approx 1 mM heme concentration the EPR spectra have the appearance of those of evacuated samples

With so many factors affecting shape and width of the signal, measurements of peak height are fraught with error Particularly in particle preparations, when the signal to noise ratio is poor and a cytochrome *c* signal of unknown intensity is super-

imposed on the $g=3$ signal of the oxidase, the possibility of error in the interpretation of changes in signal height is considerable

Although we have not studied ion effects systematically, Table I showing the field positions of the low spin heme resonances under various conditions contains some entries concerning cyanide and azide No effect was observed with 0 1 M fluoride The low field resonance of a heme *a*–cyanide complex in cytochrome *c* oxidase has been described by DerVartanian et al [24] Table II lists values for the field positions of peaks in particle preparations and whole heart

The changes in signal shape and field position, which were described for the low field resonance, are reflected also in the center and high field resonance of the low spin heme As expected, the resonance at $g=1$ 5 shifts in the direction opposite to that at $g=3$ In titrations, the two species observed in the signal at $g=3$ (cf Fig 1) can be recognized similarly in the resonance at $g=1$ 5 (Fig 7) and in the center resonance at $g=2$ 2 (Fig 8) As shown in Fig 9 judgements concerning the degree of reduction from the amplitude of the center line can be very misleading The reasons for this are that there may be more than one species, so that the signal broadens rather than increases in height, and that the low field portion of the much more

TABLE I

FIELD POSITIONS OF THE LOW AND HIGH FIELD PEAKS OF THE MAJOR LOW SPIN FERRIC HEME SIGNAL OF CYTOCHROME *c* OXIDASE

Heme concentration in enzyme (μM)	Additions		Gas atmosphere	Field position of peak on *g*-factor scale at	
	Substance	Concentration (mM)		Low field	High field
100			Air, N_2 O_2, CO	3 028	1 46
100			Vacuum	3 019	1 47
100			He	3 019	
100			Ar	3 030	
100	Sucrose	250	Air	3 020	
100	Mannitol	250	Air	3 028	
100	KCl	1350	Air	2 984	1 50
75	KCN	9	Air	3 047	
73	NaN_3 sample 80 % reduced with NADH+PMS reoxidized with ferricyanide	29	Vacuum	3 037	
73	NaN_3 Sample 80 % reduced with NADH+PMS	29	Vacuum	2 902 2 766 (weak)	
476*			Air	3 016	
967**			Air	3 028	

* Preparation according to Fowler et al [5] and McLennan and Tzagoloff [6]
** Preparation of Caughey

TABLE II

FIELD POSITIONS OF THE LOW FIELD PEAK OF THE MAJOR LOW SPIN FERRIC HEME SIGNAL OF CYTOCHROME *c* OXIDASE IN WHOLE HEART, MITOCHONDRIA AND ETP

Type of heart preparation	Species	Medium	Additions	Field position on *g*-factor scale
ETP depleted of cytochrome *c*	Beef	0 25 M sucrose	KCl	3 006
ETP depleted of cytochrome *c* with extra washing	Beef	0 25 M sucrose	—	3 038
Mitochondria	Beef	0 1 M KCl	KCl	3 018
Mitochondria	Beef	0 25 M sucrose	—	3 034
Mitochondria	Beef	0 225 M mannitol–0 075 M sucrose	—	3 035
Mitochondria	Rabbit	mannitol–sucrose	—	3 053
Mitochondria	Rat	mannitol–sucrose	—	3 031
Mitochondria	Guinea pig	mannitol–sucrose	—	3 043
Mitochondria	Pigeon	mannitol–sucrose	—	3 040
Whole tissue	Pigeon		—	3 040

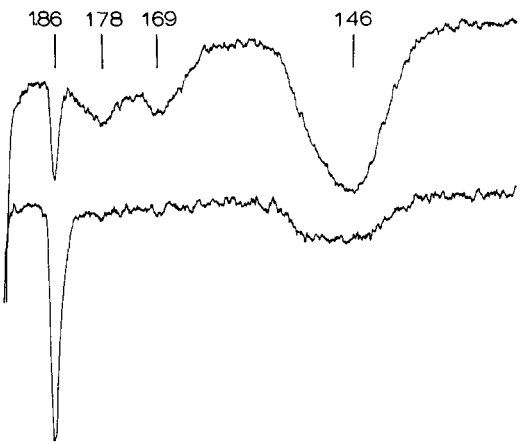

Fig 7 EPR spectra in the high field region ($g = 1\,9$ to $1\,4$) of oxidized (upper curve) and 50 % reduced oxidase, showing the high field resonances of the minor and major low spin heme components and two unidentified signals The enzyme was 0 75 mM and in vacuo

intense copper signal is partly superimposed on the center resonance of the low spin heme It will thus depend on the degree of reduction of the copper and any shift of the $g=2\ 2$ resonance relative to the copper, whether the signal at $g=2\ 2$ will be observed fully or obscured This is illustrated in Fig 9 Fig 9A represents a spectrum of the oxidized enzyme in the presence of air at pH 6 6 The resonance at $g=2\ 2$ barely is recognized When the sample is evacuated, a resonance at $g=2\ 2$ representing two species appears (Fig 9B) When this sample is 12 % reduced with dithionite (Fig 9C),

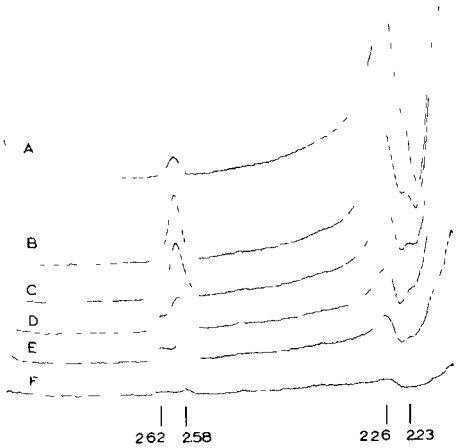

Fig 8 EPR spectra of cytochrome *c* oxidase in the center region of the field (g = 3 to 2 2) showing the low field resonance of the minor low spin heme and the center resonance of the major low spin heme The experiment was that of Fig 1 The degree of reduction was A, 0 %, B, 18 4 %, C, 40 %, D, 60 %, E, 80 % and F, 95 %

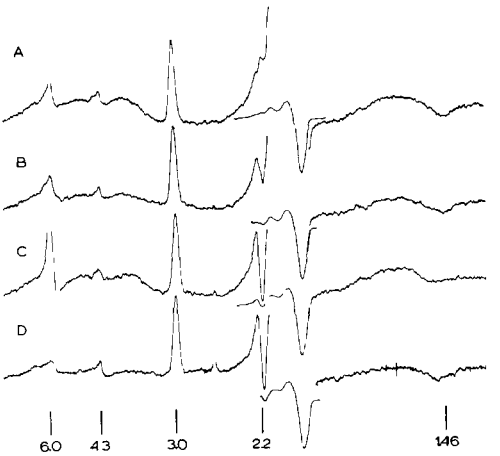

Fig 9 Effect of pH and state of oxidation on the EPR spectra of cytochrome *c* oxidase with special reference to the center resonance (g = 2 2) of the major low spin heme The enzyme 0 1 mM was deionized (cf legend to Fig 2) and the pH was adjusted to either 6 6 with potassium phosphate or to 9 8 with Tris base For reduction dithionite was used A, oxidized pH 6 6, frozen when exposed to air, B as A but frozen while evacuated, C, pH 6 6, 12 % reduced frozen as B, D, pH 9 8, oxidized, frozen while evacuated The insert in the center was recorded at one-tenth of the amplification used for the rest of the spectrum

the signal at $g=2.2$, which one would now expect to be somewhat smaller, apparently triples in size. That reduction did indeed take place can be recognized from the appearance of the signal at $g=6$, (cf. ref. 25). Finally, Fig. 9D shows a spectrum of the same enzyme in the oxidized state in vacuo at pH 9.8. Note the large difference in size of the $g=2.2$ signal between this sample and those at pH 6.6 in air or in vacuo.

The absolute intensity of the major low spin ferric signal of the enzyme is difficult to determine, as the signal spans over 2000 G. From partial integrations of the low field resonance* we obtained values ranging between 30 and 40 % of the total heme present. In another approach we integrated the two major signals of the oxidized form, viz., the copper and the low spin heme signal, together over the whole field from $g=3$ to $g=1.5$. The usefulness of this approach mainly depends on the quality of the baseline. In these integrations we found intensities accounting for 60–100 % of what one would expect if one heme and one copper out of two of each were represented in the spectra. An integration value of the copper signal obtained at a temperature sufficiently high, so that there is no interference from heme, can be subtracted from the value of the combined integration. Both approaches indicate that on the average only 30–40 % of the chemically determined value** for both the copper and the heme are represented in the EPR spectrum.

A second species of low spin ferric heme signal has been observed on partial reduction of the enzyme [25]. This signal occurs at $g=2.6$, 2.2 and 1.86 (cf. ref. 26). Occasionally a signal with these g-values is seen in apparently oxidized or resting preparations, particularly at pH $>$ 8. The signal intensity in the partly reduced enzyme depends on pH in a similar manner. As will be discussed below, the signal at $g=2.6$, 2.2 and 1.86 may represent a form in equilibrium with the high spin species ($g_\perp = 6$, $g_\parallel = 2$) which also appears on partial reduction and shows the opposite pH dependence. It is shown in Fig. 8 or Table I that the signal of the minor low spin species is split into two signals as reduction proceeds. However, in our experiments, the low spin species with $g=2.6$, 2.2 and 1.86 accounted for maximally 5 % (pH 7.4) to 10 % (pH 9) of the main low spin species ($g=3$, 2.2, 1.5), i.e. 1.5–4 % of the total heme and therefore, probably does not deserve much attention at this stage. However, in glycine buffer at pH 9, this signal can account for up to 15 % of the total heme. The field positions of the resonances of the minor low spin heme and of the center resonance of the major low spin heme are shown in Table III.

In addition to the signals of the oxidized form described thus far, resonances at $g=1.694$ and 1.785 are also seen in a number of preparations (cf. Fig. 7). Curiously, these resonances have been found in some samples, but not in others, derived from the same original batch of enzyme and to our knowledge treated identically. Autoreduction of the enzyme may account for this observation. These resonances have only been seen as a pair at the same intensity ratio. No resonances at lower field possibly related to them have been detected. If these resonances were high field lines of low spin heme signals, they would account for more material than the minor low spin species at $g=2.6$, 2.2 and 1.86. We have seen these resonances in our own and the modified Fowler preparations but not in a single preparation of Caughey.

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* Unpublished procedure of Dr. T. Vanngård.

** With consideration of inactive copper as pointed out.

TABLE III

FIELD POSITIONS OF PEAKS OF THE MAJOR AND MINOR LOW SPIN FERRIC HEME SIGNALS OF CYTOCHROME *c* OXIDASE

Heme concentration in enzyme* (μM)	pH	Major low spin heme, peak of center resonance on *g*-factor scale	Minor low spin heme resonance on *g*-factor scale at	
			Low field	High field
640	7 4		2 594	1 858
100	7 4	2 264, 2 228		
100	9 3		2 608	1 819
100	9 3		2 580, 2 622	1 841

* All samples were oxidized, except for the last one which was 42 % reduced with dithionite All samples were frozen in vacuo

High spin heme

In addition to the two dominant signal types of the oxidized form, viz , the copper and low spin ferric heme signals, there is a third major type of signal observed on partial reduction of the enzyme, namely a high spin ferric heme signal, more precisely, a number of such signals, depending on conditions Resolution of the various species requires spectroscopy $\leqslant$ 15 °K Fig 10 shows typical spectra observed during titration of cytochrome oxidase to 40 % reduction with NADH in the presence of phenazine methosulfate Table IV lists the *g*-values corresponding to the peaks exhibited by the various species that are observed After the maximal signal intensity is reached, as represented in Fig 10E, the signal merely decreases but no new species are seen except for a very small residual signal with $g \approx 6$ in the reduced form There is always a weak signal in the oxidized form (cf Fig 10A) which varies in intensity from preparation to preparation and usually represents of the order of approx 1 % of the total heme (see below) This may be due to denatured material or partial

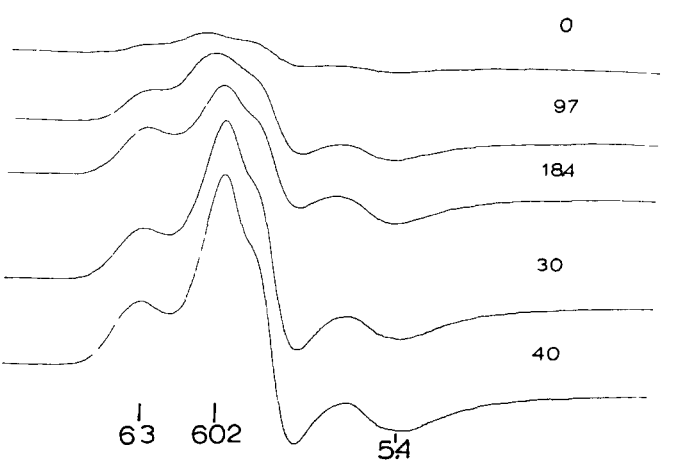

Fig 10 EPR spectra of high spin heme recorded during titration of cytochrome *c* oxidase The experiment was that of Figs 1 and 8

TABLE IV

FIELD POSITIONS OF PEAKS OF HIGH SPIN FERRIC HEME SIGNALS OF CYTOCHROME *c* OXIDASE IN PURIFIED PREPARATIONS AND PARTICLES

Heme concentration in enzyme	Reductant	Oxidant	Time of reaction	Gas atmosphere	Spectral type of figure	Field position of peak or shoulder on *g*-factor scale*					
						Axial component			Rhombic component		Zero crossing
						Low field	Shoulder	High field	Low field	High field	
(μM)	(μM)	(μM)	(s or min)		(Fig No.)	↑	↑	↓	↑	↓	
70	Cytochrome *c* 84	—	0.1 s	N_2	11A	6.07		5.75			5.85
70	Cytochrome *c* 70	—	2 min	N_2	10D, 10E	6.02_5	5.90	5.73	6.29	5.40	5.82
50	$Na_2S_2O_4$ 50	$K_3Fe(CN)_6$ 6000	0.5 s	N_2	11C	6.03	5.89		6.34	5.43_5	5.76
375	$Na_2S_2O_4$ 3000	O_2	1 min	Air	11B	6.09	5.95_5	5.70			5.85
77	NADH 80 +PMS	$K_3Fe(CN)_6$ 200	10 min	CO	12A	6.00	5.89		6.53	5.24	5.85
Pigeon heart mitochondria	Endogenous	O_2	10 min	Air	12C	6.02		5.82_5	6.82_5	4.99_5	5.85
Pigeon heart mitochondria	Endogenous	O_2	20 min	Air	12B	6.02	5.88	5.75_5	6.82_5 6.47_5	5.33_5 4.99_5	5.83

* Arrows indicate whether the respective peaks are positive or negative derivative peaks

reduction, as most of this signal is eliminated on ferricyanide addition The small $g=6$ signal is composed of contributions from an axial and a rhombic species As reduction proceeds the signals increase, the splitting of the rhombic (broad) species increases somewhat and a sharper signal emerges in the center, slightly higher in the field (see Figs 10D and 10E, and Table IV) However, a number of other signal types at g=6 have been observed dependent mainly on the reductant or oxidant used (Note

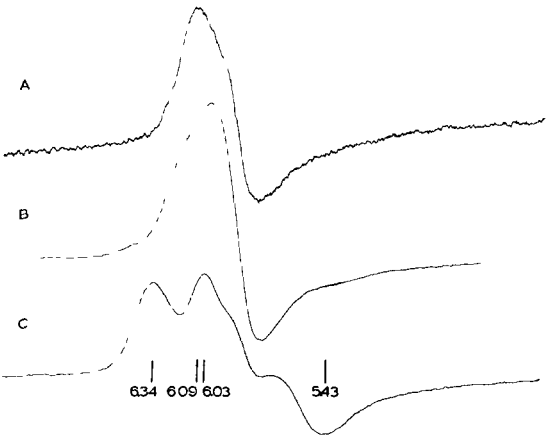

Fig 11 Different types of high spin heme resonances observed with cytochrome *c* oxidase (A) A 0 75-mM enzyme solution was saturated with oxygen and mixed in the freeze quenching apparatus with an equal volume of 10 mM dithionite solution in 20 mM Tris base The sample was frozen 1 s after mixing (B) 0 1 mM enzyme was reduced 44 % with solid dithionite in vacuo and frozen after 5 min (C) 1 4 mM enzyme anaerobically reduced with an equivalent amount of dithionite was mixed with an equal volume of 2 8 mM $K_3Fe(CN)_6$ The sample was frozen after 100 ms

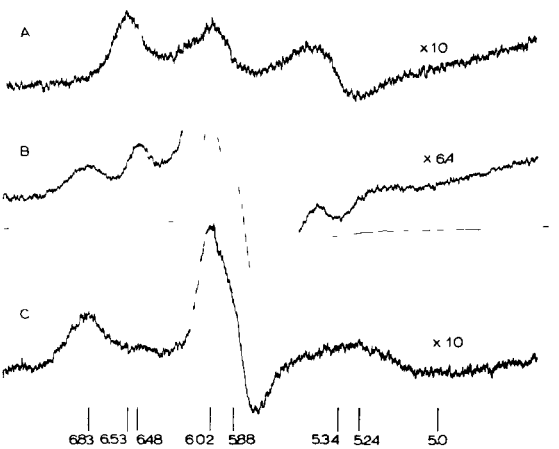

Fig 12 Different types of high spin heme resonances observed with purified cytochrome *c* oxidase and with pigeon heart mitochondria (A) 0 77 mM enzyme was reduced completely with NADH and phenazine methosulfate in the presence of CO and then reoxidized with ferricyanide (final concentration 0 2 mM) (B) Pigeon heart mitochondria, 50 mg protein per ml, were aerated and samples were frozen at different times Sample C was treated for 10 min, Sample B for approx 20 min

that partial reduction is necessary for this signal to appear at significant intensity) This is shown in Figs 11 and 12 and in Table IV Fig 11A shows the signal obtained with dithionite or cytochrome *c* as reductant at early times of reaction With dithionite this signal then develops into the type seen in Fig 11B and only after minutes a weak broad rhombic signal also appears After seconds with cytochrome *c* a strong rhombic component develops and the spectrum assumes the appearance of spectra D or E of Fig 10 The broad rhombic species is particularly evident on reoxidation of reduced cytochrome *c* oxidase with cytochrome *c* and even more so with ferricyanide (Fig 11C) As previously reported [25] the high spin signal is almost completely abolished with cyanide and azide There is always a weak signal when partly reduced preparations are exposed to CO This is shown in Fig 12A The splitting of the rhombic component is stronger in the presence of CO than in its absence (Fig 12A and Table IV) so that comparison on the basis of signal amplitude is hazardous According to recent work by Wever et al [27] this signal only arises when the reduced sample, in the presence of CO, is exposed to room light The ratio of the integrated intensity of this signal to the intensity of the $g=6$ signal in a sample reduced to the same state, but in the absence of CO, was in the range of 1 5 to 1 10 in our experiments

In particle preparations (ETP and mitochondria) the rhombic component of the high spin ferric signal usually shows a 20 % stronger splitting than in the isolated enzyme With pigeon heart mitochondria we have observed two rhombic components shown in Figs 12B and 12C The corresponding paramagnetic centers have somewhat different apparent midpoint potentials as the species with $g=6$ and that with $g=6$ 8, and 5 3 (outer wings) are present in the more reduced sample (Fig 12B) whereas all three species are seen on reoxidation (Fig 12C) Since the broad rhombic component with $g=6$ 8 and 5 0 (outer wings) has not been seen in purified preparations of cytochrome *c* oxidase we are not certain whether this signal originates from cytochrome *c* oxidase or from another heme protein It is present in fresh heart samples obtained by

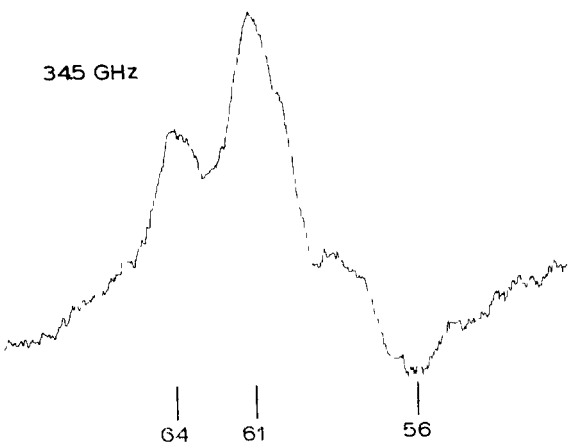

Fig 13 EPR spectrum of the high spin heme of cytochrome *c* oxidase recorded at 34 5 GHz Enzyme, 1 5 mM, was reduced with ascorbate in the presence of cytochrome *c* and partly reoxidized with ferricyanide 16 spectra were accumulated in the Nicolet 1020 signal averager and the spectra of two such runs were superimposed to produce the spectrum shown The microwave power was 30 mW, the modulation amplitude was 6 G and the temperature 13 K

freeze clamping In the preceding discussion of the high spin heme signal it has been assumed that the broad components (cf Fig 11C) are indeed due to a rhombic splitting and not to hfs That this assumption is correct, at least for the species of the type of Figs 10D or 11C, was shown by spectroscopy at 35 GHz At this frequency the measured g-values of the supposed rhombic component are sufficiently similar to those at 9 GHz (Fig 13) that any origin of the broad components from hyperfine interaction is excluded

The quantitative evaluation of these resonances is, however, particularly difficult The principle of our present evaluation was used previously [25] In view of the poor resolution obtained at 80 °K the signal at $g=6$ had been treated as that of a single species The low field peak was integrated doubly, the area was compared to that of a ferrimyoglobin standard at pH 6 0 and a correction was made for the width of the signal, as described [25] In the present work the validity of the use of ferrimyoglobin as standard was tested and account was taken of the rhombic components as will be detailed Since a quantitative comparison of the high spin ferric signals of cytochrome c oxidase with that of ferrimyoglobin would be in error if at the temperature of our measurements the population of spin states in these species were significantly different we have compared the partial double integrals of ferrimyoglobin with those of the forms of the $g=6$ signal as represented in Figs 9B, 10E and 11C at a number of temperatures between 6 and 60 °C The results, presented in Table V, show that, within the expected error of such determinations, the ratio of the integrals at 6 °K is not different from those found at higher temperatures up to 60 °K On the basis of these data it appears valid to use ferrimyoglobin as a standard at 13 °K

TABLE V

RATIO OF INTENSITY OF HIGH SPIN FERRIC HEME SIGNAL OF CYTOCHROME c OXIDASE TO THAT OF FERRIMYOGLOBIN AT DIFFERENT TEMPERATURES

Temperature (°K)	Intensity ratio		
Signal type of	Fig 9B	Fig 10E	Fig 11C
6 06	0 81	1 03	0 77
8 8	0 83	1 05	0 82
13 2	0 71	1 01	0 74
25	0 70	1 16	0 65
60	0 81	1 09	0 70

In oxidation–reduction with cytochrome c, NADH plus phenazine methosulfate and ferricyanide, the most substantial species generally encountered are those of Figs 10D, 10E and 11C, viz , a broad rhombic and a relatively narrow, nearly axial component The signals of the individual components were obtained by subtracting from each other, after suitable scaling, signals containing these two components at different ratios The original spectra and the difference spectra (at a different amplification) are shown in Fig 14 Although the two spectra chosen for this subtraction represented 40 and 95 % reduced states from the same titration of cytochrome c oxidase, the spectra of the components apparently did not have exactly the same shapes even under these closely related conditions This is evident from the extra

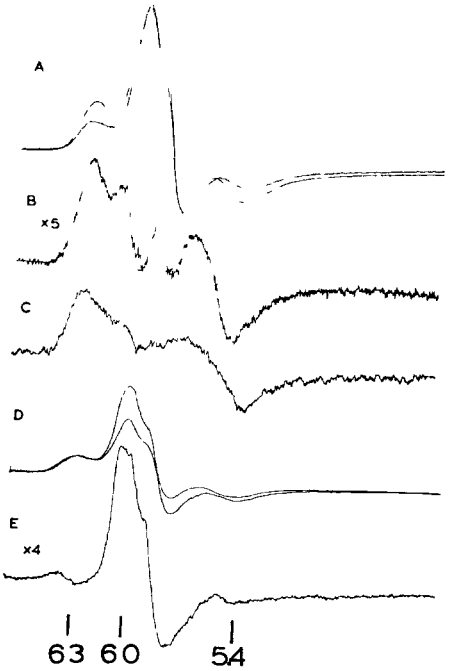

Fig 14 Difference EPR spectra showing approximations of the low field resonances of the rhombic and axial components of the high spin ferric heme signal at $g \approx 6$ The samples were those of Fig 1 marked 40 and 95 % reduced respectively The signal amplitudes were scaled approximately for these subtractions as shown in A and D The difference spectra were amplified as indicated Spectrum C was obtained in a way similar to B from samples of a different type of preparation (Caughey) The dashed lines indicate corrections applied to the spectra for purposes of integration

lines appearing in the difference spectra Fig 14C shows an additional difference spectrum obtained from another set of signals, showing that here the extra lines are present also For the integrations these lines were eliminated as indicated by the dotted lines Table VI shows a comparison of integrated signal intensities derived from these integrations vs signal heights for the axial and rhombic components, from which the relative contributions of the axial and rhombic components can be determined (cf Table VI last column)

Under optimal conditions of development the signals at $g=6$ taken together may account for as much as 40 % of the total heme in any single sample Although our determinations cannot be expected to yield absolute values better than ± 20 %, the values obtained fall within reasonable limits, considering that the two other components, viz , low spin heme and copper, yield values ranging between 30 and 50 % of the total heme or copper present

EPR signals after denaturation

It had been shown previously that on denaturation with urea in the presence of *p*-chloromercuriphenylsulfonate (PCMS) most if not all of the copper found in the enzyme by chemical analysis can be accounted for in the EPR signal [16] Under

TABLE VI

RATIO OF INTENSITIES AND PEAK HEIGHTS OF AXIAL AND RHOMBIC COMPONENTS OF HIGH SPIN FERRIC HEME SIGNAL OF CYTOCHROME *c* OXIDASE

Ratio of peak height axial/rhombic*	Ratio of intensity rhombic/axial for signal type of				Contribution of rhombic signal to total intensity of type of Figs 10D and 10E (%)
	Figs 10D and 10E (plus cytochrome *c*)	Fig 12B		Fig 12A (plus CO)	
		(inner wings)	(outer wings)		
0 5	7 65	8 80	12 5	9 6	88 5
0 75	5 10	5 85	8 35	6 4	84
1 0	3 82	4 40	6 25	4 8	79
1 5	2 55	2 93	4 17	3 2	72
2 0	1 91	2 20	3 12	2 4	65 5
2 5	1 53	1 76	2 50	1 92	60 5
3 0	1 27	1 47	2 08	1 60	56
4 0	0 955	1 10	1 56	1 20	49
5 0	0 765	0 88	1 25	0 96	43 5
6 0	0 640	0 73	1 04	0 80	39
8 0	0 477	0 55	0 78	0 60	32 5
10	0 382	0 44	0 63	0 48	27 5
15	0 255	0 29	0 42	0 32	20 5
20	0 191	0 22	0 31	0 24	16

* Peak height from low field baseline

these conditions the low spin heme signal broadens and the peak shifts to $g=2\,96$, but the intensity increases only by about 25 %. The high spin ferric signal at $g=6$ increases and broadens. An example of the quantitative relationships between the EPR detectable components in a sample of native and denatured cytochrome *c* oxidase, respectively, is shown in Table VII

Assignment of EPR signals and interpretation

Only a few comments on the assignment of the signals will be made here, namely when these directly relate to material presented in this paper

TABLE VII

EPR DETECTABLE COMPONENTS IN NATIVE VERSUS DENATURED CYTOCHROME *c* OXIDASE*

	Copper						Heme			
	Total		Active		Inactive		Low spin ($g=3$)		High spin ($g=6$)	
	μM	%	μM	%	μM	%	μM	%	μM	%
Undenatured	197	57 5	158	46	39	11 5	174	51	8	2 3
Denatured	393	115	—	—	393	115	218	64	21	6 2

* The heme concentration was 342 μM, all percentages are expressed with respect to this figure

Low spin ferric heme signals According to its kinetic behavior, we have attributed the major low spin species (g=3, 2 2, 1 5) to the component of cytochrome *c* oxidase that shows the properties generally attributed to cytochrome *a* [25, 28] An opposite view has been taken by Wilson and Leigh [29] These authors, however, attributed the low spin signal to the *a* component, when CO was present One of the reasons for this was that the appearance of the center line changed remarkably during their experiments so that they assumed the appearance of a "new signal" [29] In view of the substantial shifts that can occur in the lines of this signal during manipulations as shown above (Figs 2–9) particularly in Fig 9, we doubt whether the signal observed by these authors on addition of CO and adjustment of the oxidation state of their preparation was indeed a new signal and hence whether there is sufficient reason to propose that the bulk of the low spin signal at g=3, 2 2, 1 5 represents the *a* component in one situation and the a_3 component in another In their most recent publication Leigh et al [30] do not reiterate or otherwise refer to their previous assignment

Concerning the low spin species with signals at g=2 6, 2 2, 1 86 which we consider as a minor component, Tsudzuki and Okunuki [26] have concluded that this species and what we call the major low spin species with g=3, 2 2, 1 5 are present "in comparable amounts" This is, however, not evident from the EPR spectra published by these authors It also appears to us that they did not distinguish between the center line of the major signal, which usually is located between g=2 23 and 2 26, and the center line of the minor signal, which is slightly upfield, namely at g=2 20 Thus, in their Fig 2A [26] the signal observed at g=2 26 is part of the major low spin heme signal, whereas in their Fig 2B the line marked 2 26 is obviously part of the minor signal In our opinion, therefore, the conclusion by Tsudzuki and Okunuki that, in the oxidized enzyme, the signals at g=3 01 and 2 26 represent different hemes is incorrect

High spin ferric heme signal The proper assignment of this signal appears to us to be the key to interpreting the EPR spectroscopic experiments on cytochrome *c* oxidase Originally it had been proposed that the high spin signal at g=6 originates from the a_3 component of the enzyme [25] Then, Wilson and Leigh [29] concluded from combined potentiometric and EPR measurements at lower temperature ($\leqslant$20 °K) that this signal represents cytochrome *a* In an attempt to reconcile these and additional [28, 31] observations, the possibility was suggested [28] that both the low spin and the high spin signals seen in the EPR spectra are derived from the *a* component The high spin signal was thought to appear in those functional units in which *a* had been reoxidized by components of higher potential such as the EPR undetectable copper and the a_3 heme It was, however, not ruled out that some species of the g=6 signal, e g those appearing in the millisecond (second) range on reduction with cytochrome *c* (dithionite) may not represent part of the a_3 component In general, it is observed that on reduction the broad rhombic component only develops at longer times, past the catalytically significant period The development of the g=6 signals is also dependent on pH At pH 9 3, 7 5 and 6 06, respectively, at approximately the same oxidation state, the signal intensities are at a ratio of 1 10 30 At the same time the minor low spin component at g=2 6, 2 2, 1 87 develops at high pH, whereas this signal is absent at low pH It is thought, for this reason, that these signals may represent the same component which changes spin state as a consequence of proton

ionizations of the protein However, we do not want to imply that there is a strict quantitative relationship between disappearance of high spin and appearance of low spin signal on raising the pH According to our estimates the low spin signal at high pH does not represent the quantity of heme accounted for in the high spin signal at low pH We think, therefore, that additional (undetectable?) species participate in these equilibria

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