The Stoichiometry and Absorption Spectra of Components a and a_3 in Cytochrome c Oxidase*

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ABSTRACT: The determination of carbon monoxide binding capacity is a means of evaluating the cytochrome a_3 content of both particulate-bound and soluble cytochrome c oxidase. A particulate preparation from heart muscle shows a capacity for binding carbon monoxide equal to one-half the molar content of heme a. This indicates that native cytochrome c oxidase consists of cytochromes a and a_3 in equimolar ratio.

Highly purified preparations show less carbon monoxide binding ability. From spectral evidence it is concluded that part of the cytochrome a_3 is degraded in the course of the isolation procedure. Calculations from published absorption data show that several preparations contain approximately identical quantities of cytochrome a_3. The photochemical action spectrum of carbon monoxide inhibited cytochrome c oxidase may be used as a starting point for the computation of absolute spectra of components a and a_3. Results are in general agreement with the predictions made from studies of difference spectra; *i.e.*, components a and a_3 show comparable intensities of absorption in the Soret region, but cytochrome a absorbs by far the strongest in the visible range. The method gives a γ/α ratio of 2.8 for cytochrome a, and 13 for cytochrome a_3. The spectral features revealed in this study suggest that cytochrome a is a low-spin type; cytochrome a_3 a high-spin type complex.

Two spectrally different components of the terminal respiratory enzyme, cytochrome c oxidase, were recognized as early as 1938 by Keilin and Hartree. Component a, absorbing much stronger than component a_3 in the visible part of the spectrum, is not autoxidizable and does not react with respiratory inhibitors such as carbon monoxide and cyanide. Component a_3, on the other hand, is autoxidizable and forms complexes with these inhibitors.

A precise knowledge of the composition and structure of the terminal oxidase system is a prerequisite of any attempt toward a more or less complete elucidation of the mechanism of action of this catalyst. Perhaps the most puzzling problem concerned with the composition of cytochrome c oxidase is that of the distinct character of its components, cytochromes a and a_3. The finding by Gibson and Greenwood (1963) (recently confirmed by Morrison and Horie (1964)) that the amount of CO in chemical combination with cytochrome c oxidase is much less than the total heme a content appears to be the most unambiguous argument in favor of the separate existence of cytochromes a and a_3. However, a controversy has arisen about the exact value of the carbon monoxide binding capacity and consequently about the a_3 content of cytochrome c oxidase. At first, Gibson and Greenwood (1963) working with Yonetani preparations (Yonetani, 1960, 1961) found a ratio of carbon monoxide bound to total heme a equal to one-third. Later, Morrison and Horie (1964, 1965) determined the combining ratio of their preparation (Horie and Morrison, 1963a) and reported an average value of $^1/_{3.8}$. On the basis of the similarity among the ratios of the Soret absorption of reduced preparations and carbon monoxide derivatives, it was suggested that the a_3 content of several different preparations was nearly identical. More recently, Gibson *et al.* (1965) reported a great variability in the combining ratio of Enzyme Institute preparations (Griffiths and Wharton, 1961). Values ranging from $^1/_{2.3}$ to $^1/_{4.0}$ were observed. It was assumed that the higher ratios were most representative of native cytochrome c oxidase, especially so, since an experiment with a particulate preparation indicated the presence of one carbon monoxide binding heme a/two hemes a.

The spectral properties of components a and a_3 of cytochrome c oxidase have been revealed in difference spectra (Yonetani, 1960; Lemberg *et al.*, 1964; Horie and Morrison, 1963b; Gibson and Greenwood, 1964), but no extinction coefficients have been reported since the exact concentrations of cytochromes a and a_3 were unknown. Horie and Morrison (1963b) determined spectra of their preparation with the components a and a_3 in different oxidation–reduction states. This original approach allowed the authors to point to some characteristics of the absolute spectra of a and a_3, and was recently followed by an attempt (Horie, 1964a,b) to calculate the absorption spectra themselves of the entities a and a_3 in cytochrome c oxidase.

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In recent studies, we have investigated the carbon monoxide binding capacity as a means of evaluating the a_3 content of both particle-bound and soluble cytochrome *c* oxidase. The present paper reports data which indicate that components *a* and a_3 of native cytochrome *c* oxidase occur in 1:1 stoichiometry. Also communicated are values of the difference extinction coefficients, which were obtained from measurements of the difference spectra in conjunction with a determination of the a_3 content of the preparation. Finally, a new approach is presented for a computation of absolute spectra of cytochromes *a* and a_3. Preliminary communications describing part of this work have appeared (Vanneste, 1965a; Vanneste and Vanneste, 1965).

Experimental Procedures

Preparations. Heart muscle particles bearing the respiratory chain components (Keilin–Hartree preparation), which are free of hemoglobin or myoglobin contamination, are prepared from heart muscle mince by a procedure which is essentially a hybrid of several existing methods. The mince (500 g) is washed twice with 10 l. of cold tap water and then serially with 5-l. aliquots of cold 0.1 M and 0.02 M potassium phosphate buffer, pH 7.4, until the muscle fragments have lost their pink color. The washings are performed under continuous mechanical stirring as described by King *et al.* (1962). A washing with cold deionized water concludes this step. The muscle mince is then homogenized in the cold during 6 min with 1 l. of 0.1 M phosphate buffer, pH 7.4, in a Waring Blendor (Chance, 1952a; Horie and Morrison, 1963a). The homogenate, freed of nuclei and cell debris by low speed centrifugation, is centrifuged in a Spinco preparative ultracentrifuge (King *et al.*, 1962) and the pellet obtained is washed with 1 l. of 0.1 M phosphate buffer, pH 7.4, and recentrifuged. The final Keilin–Hartree preparation sedimented by centrifugation (KH_c)[1] is suspended in 0.25 M sucrose since phosphate buffer causes coagulation and sedimentation of the particles on standing. On one occasion in this work, particles are collected by acid precipitation (KH_a) (Keilin and Hartree, 1947).

Cytochrome *c* oxidase prepared according to the method of Horie and Morrison (1963a) was kindly supplied by Dr. M. Morrison. The precipitate of the last ammonium sulfate fractionation, sent to us by air express, is dissolved in 0.1 M phosphate buffer, pH 7.4, containing 1% Tween 80. The preparation of cytochrome *c* oxidase, used in most of the work described here, is obtained in this laboratory by application of the procedure of Fowler *et al.* (1962) to Keilin–Hartree preparations instead of mitochondria. The final medium is 0.1 M Tris–HCl buffer, pH 8.0, containing 1% deoxycholate. The yield of this preparation was repeatedly found to lie between 0.55 and 0.65 μmole of purified cytochrome *c* oxidase/g of protein of the Keilin–Hartree preparation. Complete analogy was observed for all characteristics of our preparation when compared with the preparations of Fowler *et al.* (1962) or Griffiths and Wharton (1961).

Cytochrome *c* was purchased from the Sigma Chemical Co. (Type V). The concentration of cytochrome *c* is calculated from the difference in absorbance at 550 mμ between a sample of the compound reduced with dithionite and a sample of the oxidized substance using 21.1 mM^{-1} cm^{-1} as extinction coefficient (Van Gelder and Slater, 1962).

Heme a Analyses. An elegant method for simultaneous determination of cytochromes *c*, c_1, *b*, and aa_3 has been worked out by Williams (1964). We feel, however, that more exact results will be obtained if (a) the differences in extinction coefficients at the four wavelength pairs of the difference spectra (reduced minus oxidized) of cytochromes *c* and aa_3 are changed to conform to Van Gelder's and Slater's (1962, 1963) recent results from oxidation–reduction titration experiments; and if (b) the differences in extinction coefficients of the difference spectrum of cytochrome *b* at the four wavelength pairs are adjusted to fit the results of Zaugg and Rieske (1962) on the value of the molar extinction of cytochrome *b* in submitochondrial particles. The method modified in this manner (Vanneste, 1965b) is applied to determine *a*-type cytochrome in Keilin–Hartree preparations. Solubilization with sodium deoxycholate is not required when the difference spectra are determined with a spectrophotometer equipped with large end-on photomultiplier and high-intensity light source (Cary Model 14 provided with scattered transmission attachment and Sylvania, type DXL, Halogen Quartz lamp). In this case Williams' experimental procedure is followed, however, with omission of deoxycholate from the contents of the spectrophotometer cells. In the absence of deoxycholate, reduction of the Keilin–Hartree preparation can be accomplished by succinate (Chance, 1952b). The reduced minus oxidized difference spectrum is then recorded after a time lapse for exhaustion of oxygen in the sample cell.

It is customary to estimate heme in cytochrome preparations under the form of pyridine hemochrome. Williams' work (1964) indicates incomplete separation of the spectral bands of pyridine hemochromes derived from cytochrome aa_3, on the one hand, and cytochromes *b*, c_1, and *c*, on the other hand. Therefore, values for heme *a*, which are calculated from the change in ΔA from 587 to 620 mμ in the difference spectrum, pyridine ferrohemochromes minus pyridine ferrihemochromes, of Keilin–Hartree preparations, should be regarded as approximations. Obviously, these con-

[1] The following abbreviations are used: KH_c, Keilin–Hartree preparation sedimented by centrifugation; KH_a, Keilin–Hartree preparation precipitated with acid; NADH, reduced nicotinamide–adenine dinucleotide; a^{3+}, oxidized (ferric) cytochrome *a*; a^{2+}, reduced (ferrous) cytochrome *a*; a_3^{3+}, oxidized cytochrome a_3; a_3^{2+}, reduced cytochrome a_3; $a_3^{3+}CN$ and $a_3^{3+}CN$, cyanide derivatives of oxidized and reduced cytochrome a_3; $a_3^{2+}CO$, carbon monoxide complex of reduced cytochrome a_3. Also used in this paper are combined notations such as, *e.g.*, a^{2+}, a_3^{2+}, which refer to the preparation as a whole by indicating the state of the components. It should be noted that such notations do not imply a 1:1 stoichiometry of the components.

siderations do not hold for purified cytochrome *c* oxidase and Williams' differential procedure is extensively applied in this work to derive the heme *a* concentration of purified preparations. Cuvets are chilled in an ice bath while the ice-cold cytochrome sample and reagents are added and spectra are recorded within 1–2 min after addition of the last reagent. The scattered transmission attachment is used in connection with the Cary Model 14 spectrophotometer, when necessary to eliminate turbidity influences.

Heme *a* in purified cytochrome *c* oxidase is also calculated from ΔA at 605 mμ between reduced and oxidized preparation using the value, $\Delta\epsilon = 12$ mM^{-1} cm^{-1}, reported by Van Gelder and Slater (1963).

Measurement of Cytochrome c Oxidase Activity. The rate of oxygen uptake at 25° in a mixture consisting of 0.05 M phosphate buffer, pH 7.0 (Smith, 1954), 50 mM ascorbate, 0.1 mM EDTA, varying concentrations of cytochrome *c* (7–40 μM), and about 40 mμM cytochrome aa_3 is measured in the GME oxygraph. The oxygen electrode is calibrated before each experiment with buffer saturated with air at 25°. Treatment with deoxycholate precedes the activity estimation of Keilin–Hartree particles (Smith and Camerino, 1963). Activity is expressed as turnover number of cytochrome a_3 at infinite cytochrome *c* concentration (Vanneste, 1965).

Determination of Carbon Monoxide in Chemical Combination with Keilin–Hartree Preparations or Purified Cytochrome c Oxidase. Details of the apparatus and method for estimation of bound carbon monoxide will be reported elsewhere. In this method one starts with an equilibration of the preparation under both reduced and oxidized form with a similar mixture of carbon-^{14}C monoxide and nitrogen. The carbon monoxide present in both samples is then released by the action of alkaline ferricyanide, collected in special counting vials, and counted in a Packard Tricarb scintillation spectrometer using Pilot B plastic scintillator beads as phosphor. The difference in observed counting rates corresponds to chemically combined carbon monoxide. The concentration of the bound gas is calculated by applying a conversion factor obtained from simultaneous standardization experiments with hemoglobin. Conditions particular to the present work are as follows: partial pressures of carbon monoxide in the equilibration step exceed 10 mm; concentrations of dithionite used to reduce purified cytochrome *c* oxidase or Keilin–Hartree preparations during equilibration with carbon monoxide equal 2 mg/ml and 0.5 mg/ml, respectively; complete oxidation during the same operation is accomplished by making the sample 2.5 mM with respect to ferricyanide; duration of the equilibration step, 30 min. An absorption spectrum of the solution of purified cytochrome *c* oxidase in equilibrium with carbon monoxide is routinely recorded in order to check the completeness of saturation. A 2-mm light path is employed since the concentrations used are too high for a longer light path.

Spectra. Absolute absorption spectra of various forms of cytochrome *c* oxidase (a^{3+}, a_3^{3+}; a^{2+}, a_3^{2+};[2] a^{3+}, a_3^{3+}CN; a^{2+}, a_3^{2+}CN; a^{2+}, a_3^{3+}CN; a^{2+}, a_3^{2+}CO) prepared according to the procedures of Horie and Morrison (1963b) are recorded on a Cary Model 14 spectrophotometer. Appropriate algebraic combination of these absolute spectra leads to difference spectra for the Fowler preparation of the type reported by Yonetani (1960) and Lemberg *et al.* (1964) (see Table I).

TABLE I: Outline of Algebraic Procedure to Obtain Difference Spectra.

Difference Spectrum	Algebraic Combination[a]
$a^{2+} - a^{3+}$	a^{2+}, a_3^{3+}CN $- a^{3+}$, a_3^{3+}CN
$a_3^{2+} - a_3^{3+}$CN	a^{2+}, $a_3^{2+} - a^{2+}$, a_3^{3+}CN
a_3^{3+}CN $- a_3^{3+}$	a^{3+}, a_3^{3+}CN $- a^{3+}$, a_3^{3+}
$a_3^{2+} - a_3^{3+}$	$(a_3^{2+} - a_3^{3+}\text{CN}) + (a_3^{3+}\text{CN} - a_3^{3+})$
a_3^{2+}CO $- a_3^{2+}$	a^{2+}, a_3^{2+}CO $- a^{2+}$, a_3^{2+}
a_3^{2+}CN $- a_3^{2+}$	a^{2+}, a_3^{2+}CN $- a^{2+}$, a_3^{2+}

[a] The + and − signs refer to addition and subtraction of absorption curves, which is accomplished by adding or subtracting either chart readings taken every 2 mμ or previously computed values for difference spectra.

The absolute spectrum of the cytochrome a_3–carbon monoxide complex (a_3^{2+}CO) of this preparation is calculated from data on the photochemical action spectrum of the terminal oxidase (Chance, 1961) and the observed a_3 content as determined by carbon monoxide binding experiments. Further algebraic combinations as outlined in Table II are introduced to yield separate absolute spectra of the components *a* and a_3 of cytochrome *c* oxidase.

TABLE II: Outline of Algebraic Procedure to Obtain Absolute Spectra.

Absolute Spectrum	Algebraic Combination[a]
a_3^{2+}	a_3^{2+}CO $- (a_3^{2+}\text{CO} - a_3^{2+})$
a_3^{3+}	$a_3^{2+} - (a_3^{2+} - a_3^{3+})$
a^{2+}	a^{2+}, $a_3^{2+} - a_3^{2+}$
a^{3+}	a^{3+}, $a_3^{3+} - a_3^{3+}$
a_3^{2+}CN	a^{2+}, a_3^{2+}CN $- a^{2+}$
a_3^{3+}CN	a^{3+}, a_3^{3+}CN $- a^{3+}$

[a] See Table I.

[2] In order to obtain this form of the oxidase, we incubate the preparation for 20–30 min at 0° in presence of 2 mg/ml of dithionite.

TABLE III: Heme *a* Content and Carbon Monoxide Binding Capacity of Preparations of Insoluble Heart Muscle Particles and Purified Cytochrome *c* Oxidase.

Preparation	Heme *a* Concn (μM)			Concn of CO in Chemical Combination (μM)		Combining Ratio (2):(1)
	Method[a]	Mean[b] (1)	Extremes	Mean[b] (2)	Extremes	
1. Insoluble heart muscle particles (KH_c No. 644)	Pyridine hemochromogen	62 (1)		29.6 (4)	27.9–31.4	1:2.09
	Modified Williams' method (solubilized dithionite)	55.6 (3)	54.3–56.7	29.6 (4)	27.9–31.4	1:1.88
	Modified Williams' method (not solubilized succinate)	56.3 (7)	52.9–59.1	29.6 (4)	27.9–31.4	1:1.90
2. Purified cytochrome *c* oxidase (Fowler *et al.*)	Pyridine hemochromogen	77.1 (2)		31.7 (8)	27.4–36.9	1:2.43
	ΔA_{605} (reduced–oxidized)	76.7 (2)		31.7 (8)	27.4–36.9	1:2.42
3. Purified cytochrome *c* oxidase (Horie and Morrison)	(1) Pyridine hemochromogen	75.7 (2)		30.1 (2)		1:2.51
	(2) Pyridine hemochromogen	72.7 (2)		30.7 (2)		1:2.37
4. Damaged Horie and Morrison preparation	Pyridine hemochromogen	75.0 (2)		36.8[c] (2)		1:2.04
	Pyridine hemochromogen	46.6 (2)		28.0[d] (2)		1:1.66
	Pyridine hemochromogen	52.3 (2)		34.0[e] (2)		1:1.54

[a] For more details see Experimental Procedure. [b] Number of estimations in parentheses. [c] Time of equilibration with carbon monoxide: 30 min. [d] Time of equilibration with carbon monoxide: 50 min. [e] Time of equilibration with carbon monoxide: 100 min.

The carbon monoxide difference spectrum[3] of heart muscle particles is recorded on a Cary Model 14 equipped with scattered transmission attachment and high intensity light source. The contents of both sample and reference cell are first reduced with dithionite (1–2 mg) or succinate (final concentration. 13 mM) and a base line is recorded. The sample cell is then bubbled for 30 sec with a stream of carbon monoxide, whereafter the difference spectrum is recorded. A base-line correction is made as described by Chance (1953a). Two levels of protein concentration are used: 5–10 mg/ml for observations in the visible region; 1–3 mg/ml for the Soret region. Corresponding dilutions of the Keilin–Hartree preparation suspended in 0.25 M sucrose are made with 0.1 M phosphate buffer, pH 7.4.

The spectral data used for calculations of a_3 contents of different preparations (Table V) are obtained from published absorption curves. The relevant absorbance values are read after redrawing the curves and scales on translucent graph paper.

Protein Estimation. Protein concentration of the Keilin–Hartree preparation is measured with the biuret reaction (Gornall *et al.*, 1949), in the presence of 0.3% H_2O_2 (Yonetani, 1961) and 0.1% deoxycholate (Smith and Camerino, 1963).

Results

Carbon Monoxide Binding Capacity of Keilin–Hartree Preparations and Purified Cytochrome c Oxidase. In Table III are summarized the values for the carbon monoxide binding in addition to those for the heme *a* content of both heart muscle preparations and cytochrome *c* oxidase preparations. The results clearly demonstrate that not all of the *a*-type heme present in either one of these preparations is capable of combining with carbon monoxide. It is also clear that the combining ratio of the heart muscle particles differs from that of the purified preparations. When a comparison is made on the basis of equal heme *a* contents, it appears that normal cytochrome *c* oxidase preparations contain about 20% less carbon monoxide binding *a*-type heme than heart muscle particles. One Horie and Morrison preparation is seen to behave differently by showing an increased carbon monoxide binding capacity. However, the absorption spectrum of this preparation after equilibration with carbon monoxide in the presence of dithionite is markedly different from that ordinarily

[3] On the assumption that no carbon monoxide binding pigments other than cytochrome a_3 occur in the preparation, this spectrum represents the differences in absorption between the carbon monoxide derivatives and the reduced form of cytochrome a_3.

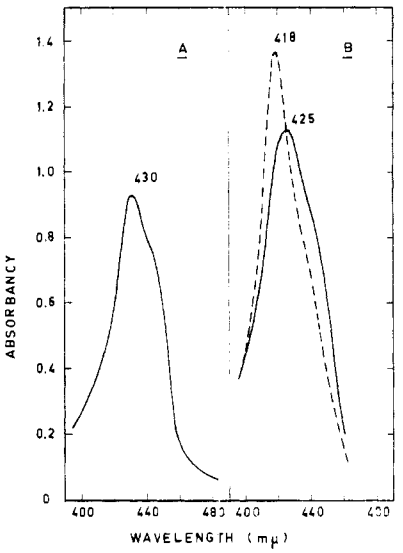

FIGURE 1: Comparison of Soret bands of CO compounds of normal (A) and damaged (B) Horie–Morrison preparations (full lines). The spectra are recorded on samples of the preparations equilibrated with low partial pressures of CO (14.1 mm (A), 12.1 mm (B)) in the course of estimation of the carbon monoxide binding capacity. Equilibration times for A and B are 30 and 100 min, respectively. The dashed line represents the Soret peak of the altered preparation recorded after keeping the (stoppered) cuvet for 3 hr at 6°. Light path: 2 mm. The medium used is 0.1 M phosphate buffer, pH 7.4, containing 1% Tween 80.

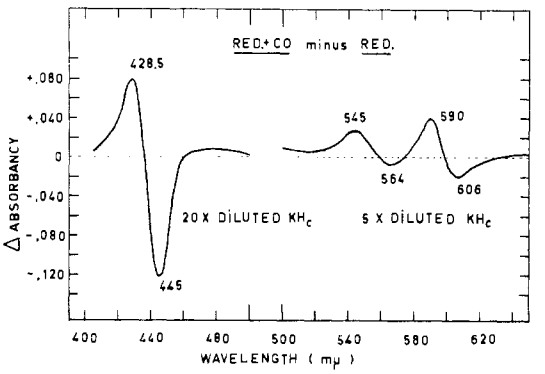

FIGURE 2: Carbon monoxide difference spectrum of Keilin–Hartree preparation recorded on a Cary spectrophotometer equipped with scattered transmission attachment and high intensity light source. Conditions are described in the experimental part of the paper.

observed (Figure 1). Also, the capacity for carbon monoxide binding increases as the exposure to carbon monoxide and dithionite in the equilibration step is prolonged. It should be noted that no such increase is ever observed with the usual Fowler and Horie–Morrison preparations. Apparently, profound alterations in the oxidase, which are at least in part induced by the medium of the carbon monoxide estimation, are causing this disparate result. The dotted spectrum included in Figure 1B shows the tendency of the changes induced by the medium, *i.e.*, a decreasing absorbancy at 444 mμ, indicative of a modification in reduced cytochrome *a*, and concomitant appearance of an absorption peak at 418 mμ. Simultaneously, cytochrome a_3 is altered to form the same final product as is evidenced by the disappearing 430-mμ band. Since the ratios of carbon monoxide bound to total heme *a* are tending to unity, it is concluded that the altered cytochromes possess carbon monoxide binding ability.

The carbon monoxide difference spectrum of heart muscle particles has been well documented as a characteristic revelation of cytochrome a_3 (Chance, 1953a,-b,c, 1957; Ball *et al.*, 1951). Figure 2 shows both visible and Soret regions of this spectrum in our Keilin–Hartree particles suspension. Taking the carbon monoxide in chemical combination as an exact measure of the a_3 content of the preparation, we find values of 136 mM^{-1} cm^{-1} and 10.1 mM^{-1} cm^{-1} as extinction coefficients for the peak to trough differences in the Soret region (428.5–445 mμ) and visible region (590–606 mμ), respectively. As expected, the carbon monoxide difference spectrum of purified cytochrome *c* oxidase is qualitatively identical with that of the particles. Extinction coefficients calculated on the same basis have values of 148 mM^{-1} cm^{-1} and 9.9 mM^{-1} cm^{-1}, respectively.

With these values in hand, it becomes possible to obtain the a_3 content of any preparation from the carbon monoxide difference spectrum. This rather fast procedure is applied in Table IV which illustrates the constancy of the a_3 to total heme *a* ratio of heart muscle preparations either freshly prepared or after storage. This ratio is always close to one-half, although more variation is observed in the a_3 to protein ratio.

With respect to Tables III and IV it should be noted, first, that the heart muscle preparations show no signs of contamination by myoglobin or hemoglobin as evidenced by our spectrophotometric test for freedom from these pigments (Vanneste, 1965a); second, that the fractional ratios cannot be explained as due to incomplete reaction of a single *a*-type cytochrome with carbon monoxide, since a three- to fivefold increase in partial pressure of equilibration does not alter the binding result. Also, the carbon monoxide difference spectrum of the particles and purified preparations remains unchanged over the range of pressures between 10 and 760 mm. Thus, all carbon monoxide in chemical combination may be considered as bound to fully saturated cytochrome a_3.

Cytochrome c Oxidase Activity of Keilin–Hartree Particles. Under the conditions described in the experimental procedure, maximal turnover rates of cytochrome a_3 are to be expected (Smith and Camerino, 1963). With a number of different Keilin–Hartree preparations values ranging from 460 to 480 sec^{-1} are obtained after extrapolation to infinite cytochrome *c* concentration by the method of Lineweaver and Burk (1934) (see also Vanneste, 1965). It is found that storage

TABLE IV: Constancy of the Cytochrome a_3 to Total Heme a Ratio of Heart Muscle Particles.

Preparation	Heme a Concn[a] (μM)	Cytochrome a_3 Concn[b] (μM)	a_3:Total Heme a	a_3:Protein (mμmoles/mg of protein)
KH_c No. 642	29.8	15.5	1:1.92	0.50
KH_c No. 643	60.5	29.6	1:2.04	0.55
KH_c No. 645	75.5	35.3	1:2.13	0.70
KH_c No. 644	56.3	29.6[c]	1:1.90	0.69
KH_c No. 644 after 3 days of storage at 0°	59.0	29.9	1:1.97	
KH_a No. 646	26.5	12.7	1:2.08	0.50

[a] Estimated following modified Williams method for simultaneous estimation of the different cytochromes. [b] Derived from the carbon monoxide difference spectrum: $\Delta A_{428.6-445}/136 \times 10^{-3}$. [c] Value obtained in the carbon monoxide binding experiment (Table III).

of the particles during 3 days at 0° or 10 days at −15° does not alter the value for the maximal turnover number at infinite concentration of cytochrome *c*. This result proves that the carbon monoxide binding experiments, though they require several hours of handling the sample at 0°, are performed with fully active preparations.

Spectra. In Figure 3 are shown the difference spectra (reduced minus oxidized) for cytochromes *a* and a_3 separately. The well-known disparity in $\Delta\gamma/\alpha$ ratios of the two cytochromes is clearly observed. We find for cytochrome *a* $\Delta\gamma/\alpha$ ratios in the range of 2.75–3.0, which is in general agreement with values of 3.1 and 3.7 reported previously (Lemberg *et al.*, 1964; Yonetani, 1960). Cytochrome a_3 is found to have a $\Delta\gamma/\alpha$ ratio between 21 and 25, which is appreciably higher than Yonetani's result, but within the range observed by Lemberg *et al.* The isosbestic points in the Soret region are well defined and lie at 436 and 461 mμ for cytochrome *a* and at 428 and 466 mμ for cytochrome a_3. Extinction coefficients of cytochrome a_3 in the difference spectrum of Figure 3 are based on measurement of the absorbance differences and of the carbon monoxide binding capacity of the preparation. Extinction coefficients of the difference spectrum of cytochrome *a* are calculated on the basis of [cytochrome *a*] = $^1/_2$[heme *a*] (see Discussion). No other difference spectra are given since all agree well with those reported in other communications.

In Figures 4–6 are compared the absolute absorption curves of a cytochrome *c* oxidase preparation with those of the components *a* and a_3 calculated by means of the algebraic methods described in Table II. The most characteristic feature apparent from these curves is the overwhelming contribution of cytochrome *a* to the visible absorption. The α- and β-bands of cytochrome *c* oxidase consist of cytochrome *a* absorption bands only slightly modified by the absorption due to cytochrome a_3. The pronounced peak at 565 mμ in the spectrum of reduced cytochrome a_3 is very likely responsible for the well-known inflection (Griffiths and Wharton, 1961) on the short wavelength side of the α-band of reduced preparations. The finding of this 565-mμ peak of cytochrome a_3 is considered further evidence for the fact that the absorption bump observed in this region with highly purified preparations is not caused by contaminating pigments such as cytochromes *b* or c_1, but is an integral part of the cytochrome *c* oxidase spectrum.

Components *a* and a_3 are seen to contribute more equally to the Soret absorption bands. This band in the oxidized preparation is composed of a peak at 414 mμ due to cytochrome a_3 and a peak at 426 mμ due to cytochrome *a*. The 12-mμ distance between the maxima of the component spectra brings about the broad character of the Soret band of oxidized preparations. Reduced cytochrome *c* oxidase, on the other hand, exhibits a much sharper Soret band due to a greater overlap of the component spectra. A sometimes hardly observable shoulder, which is not obliterated by prolonged reducing conditions, appears in all preparations on the short wavelength side of the Soret peak. This absorption is viewed much clearer in the spectrum of cytochrome *a* and its nature is discussed at length later on in this paper.

The spectral data presented in Figures 5 and 6 allow us to compute values, hitherto only guessed at, for γ/α ratios of the two cytochromes. Results for the reduced components are 2.8 for cytochrome *a* and 13 for cytochrome a_3. Especially the latter ratio is quite different from the corresponding ratio in the difference spectrum.

The absorption curves of the cyanide complexes of reduced and oxidized cytochrome a_3 are presented in Figure 7. a_3^{2+}CN exhibits an unusually strong α-band at 594 mμ and a Soret band at 441 mμ with a band ratio of 5.5. The visible spectrum of a_3^{3+}CN is strikingly similar to that of a_3^{3+}. However, the similarity does not extend into the ultraviolet region where the cyanide complex shows a broad double band of low over-all absorbance.

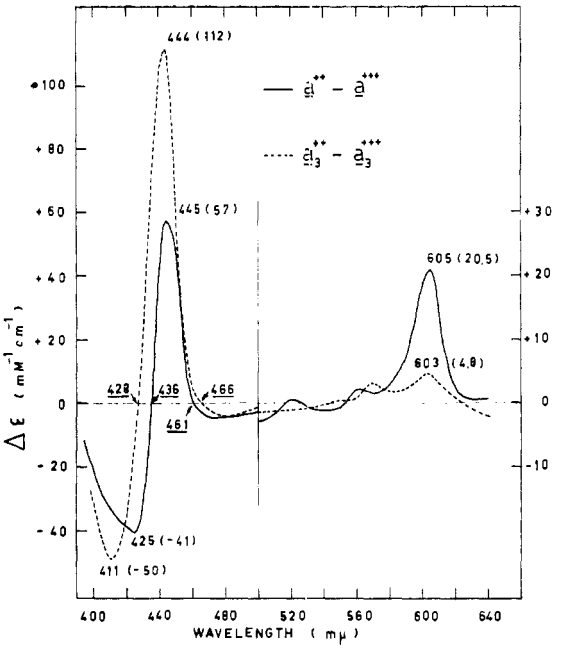

FIGURE 3: Difference spectra (reduced minus oxidized) of cytochromes *a* (———) and a_3 (-----). The procedure for obtaining these spectra is as outlined in the text and in Table I. Extinction coefficients in the spectrum of cytochrome a_3 are based on the carbon monoxide binding capacity of the preparation. Those of cytochrome *a* are based on the assumption that this cytochrome equals one-half of the total heme *a* content of the preparation. In the drawing appear the wavelengths of maxima and minima with the calculated extinction coefficents between brackets. Underlined wavelengths are the isosbestic points of the spectra of reduced and oxidized components.

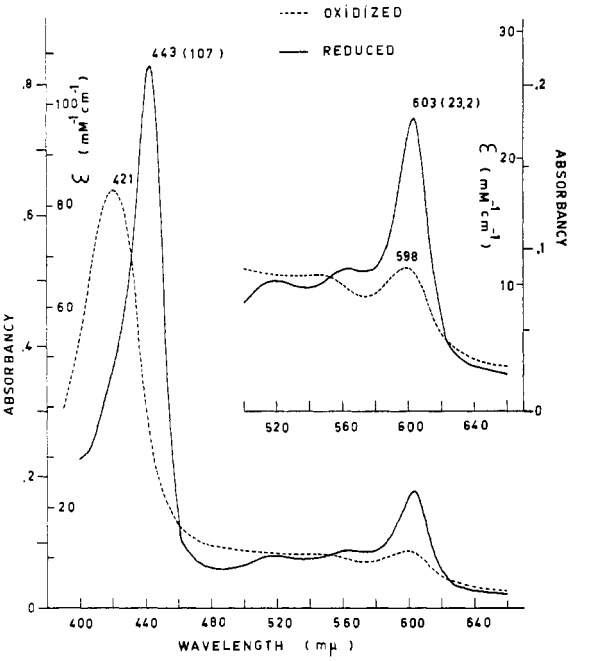

FIGURE 4: Absolute absorption spectra of reduced and oxidized cytochrome *c* oxidase preparation (Fowler *et al.* (1962)) in 0.1 M Tris-HCl buffer, pH 8.0, containing 1% sodium deoxycholate. The enzyme as prepared is considered to be oxidized. Reduction is accomplished by 2 mg/ml dithionite during 20–30 min at 0°. Extinction values are based on the total heme *a* content of the preparation. The absorbancy scale is for a solution of the oxidase, which is 7.7 μM with respect to heme *a*.

Discussion

Stoichiometry. So far all quantitative analyses of binding capacity agree on the fractional character of the combination of cytochrome *c* oxidase with carbon monoxide. This finding constitutes unequivocal chemical evidence for the point of view that two differently reactive entities, cytochromes *a* and a_3, exist. The question of their stoichiometry in the native oxidase system seems to have a straightforward answer, since heart muscle particles bearing fully active cytochrome *c* oxidase bind carbon monoxide in a ratio of 1 molecule per 2 hemes *a* (Table III and IV). As the separation of the two cytochromes, if not at all impracticable, still awaits a skillful performance, it seems reasonable to consider, for the time being, the native oxidase as a unit consisting of cytochromes *a* and a_3 in equimolar ratio. The name cytochrome aa_3 (Slater *et al.*, 1966) correctly expresses this situation, and it is proposed to adopt this nomenclature when referring to the native *a*-type cytochrome in the respiratory chain of heart muscle.

Highly purified preparations exhibit a decreased carbon monoxide binding capacity. Although the present study does not deal with a large number of preparations, our results, nevertheless, seem to indicate that fresh preparations in the completely reduced state show a fairly constant combining ratio. Incomplete reduction and possibly a variation in the amount of degraded cytochrome a_3 is offered as an explanation for Morrison's and Horie's (1964, 1965) and Gibson's *et al.* (1965) observations of an appreciable variability in binding capacity of their preparations. Lemberg *et al.* (1964) and Lemberg and Mansley (1965) have shown that dithionite reduces cytochrome *a* instantaneously, but cytochrome a_3 only slowly. The reaction times involved are variable from one preparation to the other. At a preset time, varying fractions of the total a_3 content occur in the reduced form and have acquired the ability to bind carbon monoxide.

In the case of Keilin–Hartree preparations cytochrome *a* is identified with noncarbon monoxide binding heme *a*. The question arises whether the same assumption holds for purified cytochrome *c* oxidase. The loss of carbon monoxide binding capacity during purification can be accounted for either by an alteration of part of the native cytochrome a_3 with concomitant

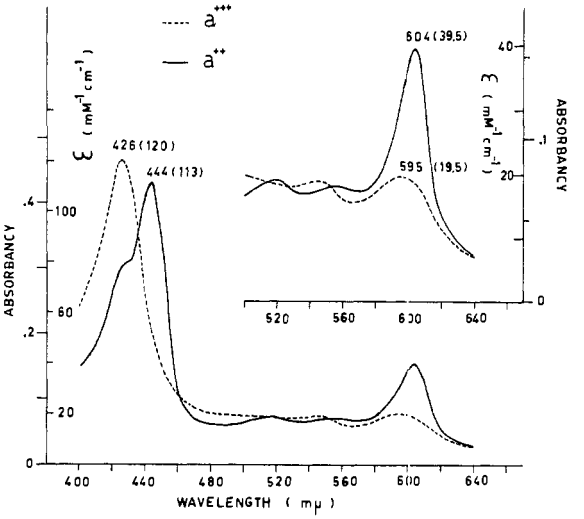

FIGURE 5: Absolute absorption spectra of the reduced and oxidized component *a* of cytochrome *c* oxidase. The procedure employed in obtaining the spectra is outlined in the text and in Table II. Absorbancy readings apply to cytochrome *a* in the preparation of Figure 4, which is 7.7 μM with respect to *total* heme *a*. Millimolar extinction coefficients are based on one-half of total heme *a* content of the preparation (*i.e.*, 3.85 μM).

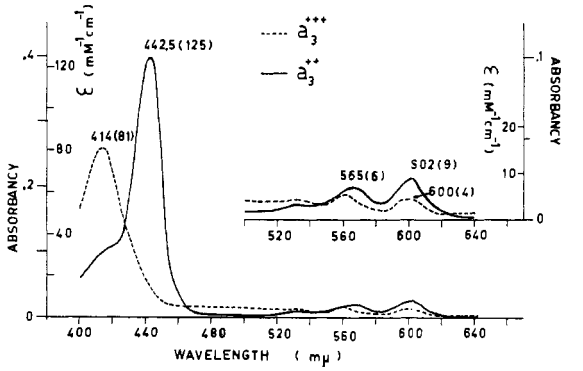

FIGURE 6: Absolute absorption spectra of the reduced and oxidized component a_3 of cytochrome *c* oxidase. The procedure employed in obtaining the spectra is outlined in the text and in Table II. Absorbancy readings are for cytochrome a_3 in the preparation of Figure 4, which is 7.7 μM with respect to *total* heme *a*. Millimolar extinction coefficients are based on the carbon monoxide binding of the preparation (*i.e.*, 3.17 μM).

loss of the carbon monoxide binding property or by the removal of some cytochrome a_3 from its counterpart, cytochrome *a*. Little evidence can be put forward to show by which of both mechanisms the purification procedure results in loss of binding ability. Attention has already been drawn to the presence of a shoulder on the short wavelength side of the Soret band of the cytochrome *c* oxidase spectrum, and the better resolved

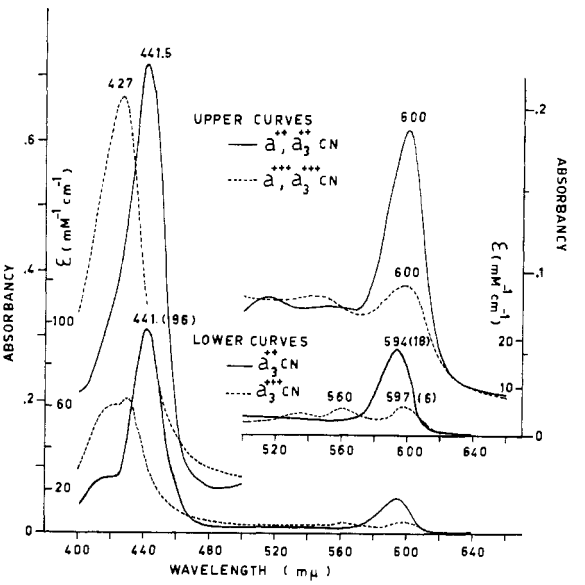

FIGURE 7: Upper curves: Absorption spectra of the cyanide derivatives of oxidized (-----) and reduced (———) cytochrome *c* oxidase preparation. Total heme *a* concentration of the solution is the same as for Figure 4 (7.7 μM). Spectra are recorded without delay after addition of 15 mM cyanide. Lower curves: Computed absolute absorption spectra of the cyanide complexes of reduced (———) and oxidized (-----) component a_3 of this cytochrome *c* oxidase preparation. The procedure used in obtaining these spectra is outlined in the text and Table II. Extinction values are based on the a_3 content (carbon monoxide binding) of the preparation.

appearance of this absorption in the spectrum of cytochrome *a* has been mentioned. Its position (about 428 mμ) drastically restricts the number of possible assignments. Cytochrome *b* fulfills the requirement of the position of Soret maximum. However, the absence of a 562 mμ absorption peak in the spectrum of cytochrome *a* makes this pigment an unlikely guess. Oxidized cytochrome *a* can be considered as well, but Lemberg's finding of a fast reduction by dithionite also excludes it from being responsible for the shoulder. The interesting possibility that a hitherto unidentified contamination product causes the 425–430 mμ shoulder seems likely, especially so since the other possibilities have been disproved. It is assumed then that cytochrome aa_3 loses part of its carbon monoxide binding capacity during the isolation procedure by a process of modification of cytochrome a_3 and that the degradation product of the cytochrome remains attached to cytochrome *a*. This decomposition product would be a heme *a* compound exhibiting in the reduced form a Soret maximum at about 428 mμ. Considering the above assumptions, it follows that in purified cytochrome *c* oxidase preparations, cytochrome *a* equals one-half the total heme *a* content.

Elliott *et al.* (1959) have studied the appearance of mitochrome (Polis and Shmuhler, 1957) in the course of ageing a Smith–Stotz (1954) cytochrome *c* oxidase

TABLE V: Carbon Monoxide Binding Capacity of Some Currently Used Purified Cytochrome *c* Oxidase Preparations.[a]

Prepn	Method for Determination of Heme *a*	Ref for CO Difference Spectrum	Moles of Heme *a* Required to Bind 1 Mole of CO (Total Heme a/a_3)
a. Wainio *et al.* (1948)	Applying 23.2[b] mM^{-1} cm^{-1} to A_{605} of reduced preparation	Figure 6 of Wainio (1955)	2.50
b. Okunuki *et al.* (1958)	Iron content given by author	Figure 2 of Okunuki *et al.* (1957)	2.75
c. Yonetani (1960)	Applying 12 mM^{-1} cm^{-1} to ΔA_{605} (reduced–oxidized prepn)	Figure 9 of Yonetani (1960)	2.69
d. Horie and Morrison (1963a)	Value given by authors	Figure 3 of Morrison and Horie (1964, 1965)	2.65
e. Lemberg *et al.* (1964) (modified Okunuki–Yonetani method)	Applying 23.2 mM^{-1} cm^{-1} to A_{605} of reduced prepn	Table VI of Lemberg *et al.* (1964)	2.23

[a] Carbon monoxide in chemical combination (= a_3 content of the preparation) is calculated by applying 148 as millimolar extinction coefficient to the peak (428.5 mμ) to trough (445 mμ) difference in the carbon monoxide difference spectrum. The value so obtained is compared with the heme concentration of the preparation. [b] This paper, Figure 4.

preparation. The newly formed substance (reduced form) was characterized by a peak at 422 mμ, and carbon monoxide was found to cause a marked increase of the absorption and a shift of 4 mμ to shorter wavelengths.

It could be argued that our preparations contain mitochrome contamination. However, several lines of evidence can be adduced to disprove this fact. First, the spectrum of the pyridine hemochrome derived from the preparations applied in the present study shows no signs of modified heme with α-peak at 575 mμ instead of 587 mμ. Second, the hypothesis of decomposition is introduced to explain a decreased carbon monoxide binding. If mitochrome is the resulting substance, no change in binding capacity should be expected. Third, the absorption maximum of the contamination product lies at 428 mμ rather than at 422 mμ. Furthermore, the spectra of aged preparations (Figure 4 of Elliott *et al.* (1959)) show a distinct shoulder at 428 mμ superimposed on the mitochrome spectrum. It is suggested, therefore, that the degradation of cytochrome *c* oxidase proceeds in steps, the pigment absorbing at 428 mμ being an intermediate and mitochrome the end product.

The establishment of a spectral parameter characteristic for cytochrome a_3 enables us to obtain approximate estimates for the cytochrome a_3 content of currently used oxidase preparations. The findings summarized in Table V are all in reasonable agreement with the results presented in this paper from direct carbon monoxide binding experiments. This suggests that the a_3 content of different preparations is fairly constant and actually lies between 45 and 36% of the total heme *a* content. It is interesting to note that two of the five preparations included in Table V (a and b) have been claimed to contain a single hemoprotein uniformly reactive with carbon monoxide.

On the basis of titrations of cytochrome *c* oxidase with NADH in the presence and absence of chelating agents, Van Gelder and Muysers (1964) and Slater *et al.* (1965) have suggested that prepared oxidase consists of one molecule each of cytochrome *a* and cytochrome a_3. However, the lack of titration data in the region where the reduction proceeds to completion seriously hampers the conclusion of these authors. Indeed, changes in slope of the titration lines are expected toward the end when the components are not present in exactly equimolar proportion. Until more information of this sort is available, no comparison can be made between the conclusion of the Dutch workers and our results from carbon monoxide binding experiments.

Spectral Properties of Cytochrome a and a_3. The derivation of difference spectra of the components *a* and a_3 separately is based on the principle that cyanide delays the reduction of ferric iron in cytochrome a_3 (Yonetani, 1960). Whereas other workers have obtained difference spectra by recording absorbance differences between an appropriate sample–reference pair, we have preferred to record absolute spectra of the cell contents and to compute the difference spectra by algebraic methods, since this procedure shortened the time spent on observations and also avoided slit-widening with accompanying decrease of resolution in the Soret region. Lemberg *et al.* (1964) have investigated the validity of the method for deducing difference spectra and have concluded that cyanide sufficiently stabilizes trivalent iron in cytochrome a_3 to

enable one to obtain approximate difference spectra.

Besides this matter of kinetic stabilization, several other interesting points should be considered in a discussion of the validity of the method. Indeed, it is assumed throughout (1) that the spectral properties of the entities *a* and a_3 comprising the same unit are independent of one another; (2) that the absorption due to chelated copper is negligible; and (3) that no other pigments besides cytochromes *a* and a_3 are present. The second and third assumptions require some comment. Gibson and Greenwood (1965) have suggested that the species responsible for the 820 mμ absorption band, tentatively identified with enzyme-bound copper, contributes to the absorption in the visible region of the spectrum. Also, evidence has been presented above, showing contamination of purified cytochrome *c* oxidase with degraded cytochrome a_3. However, the low extinction coefficient of the copper species and the relatively small extent to which degradation occurs in our preparations seems to guarantee a fair accuracy of the evaluated difference spectra.

Obviously, the newly calculated absolute spectra are approximate too, even more so since the computation was done with the photochemical action spectrum of Baker's yeast instead of heart muscle. However, this approximation is reasonable as it was shown that the spectral properties of the carbon monoxide derivatives of both cytochrome *c* oxidases are closely similar (Chance, 1953a).

The absorption spectra of cytochromes *a* and a_3, here reported, are similar in many respects to those of Horie (1964a,b). However, important differences are seen in the relative heights of the Soret bands and in the positions of some visible and Soret bands of the reduced cytochromes. The wavelength assignments of Horie, 602 mμ for α-peak of cytochrome *a*, 608 mμ for cytochrome a_3, and 441 and 443 mμ, respectively, for the Soret bands of *a* and a_3, are almost the exact opposite of our findings, which are, therefore, in better agreement with the early observations of Keilin and Hartree (1939). The validity of the assumption at the basis of Horie's calculation (*i.e.*, in cytochrome *c* oxidase preparations the spectrum of $a_3{}^{3+}$CN equals a fraction ($^1/_5$) of the spectrum of a^{3+}) can be checked now by comparing the curves of Figures 5 and 7. There seems to be a fair agreement in the visible region, which unfortunately does not extend into the near ultraviolet.

The bond type (ionic or covalent) of the complex of iron with porphyrin and fifth and sixth position ligands is reflected in the absorption spectrum of a hemoprotein. According to current concepts about relationships between bond type and spectra (Brill and Williams, 1961; Williams, 1961), the following two spectral features are pertinent to the suggestion that cytochrome *a* is a more low-spin (covalent) and cytochrome a_3 a more high-spin (ionic) iron complex. (1) Upon reduction, cytochrome a_3 shifts its Soret maximum 28 mμ to longer wavelengths, whereas this shift equals only 18 mμ in the case of cytochrome *a*. (2) The visible bands of cytochrome *a* are of high intensity but those of cytochrome a_3 are not. The latter characteristic is clearly reflected in the disparate γ/α ratios. No distinct ligand to metal charge-transfer bands, typical of high-spin iron porphyrin complexes, are observed, however, in the spectrum of ferric cytochrome a_3. This is probably due to the fact that the position of such a band (above 650 mμ) falls outside the range of our computation, the range of wavelengths for which data on the photochemical action spectrum are available. It is, perhaps, not too speculative to propose that heme *a* in cytochrome *a* has its fifth and sixth position occupied by nitrogenous ligands, *e.g.*, the imidazoles of histidine residues. Cytochrome a_3 would have ligands of lesser ligand field strength, such as the carboxylate anion and water, although the interesting possibility exists that Cu–imidazole functions as a ligand at the fifth position in cytochrome a_3. Indeed, (chelated) copper, bound to a 1-nitrogen of an imidazole ring, may decrease the σ-donor property of the 3-nitrogen as well as the π-acceptor character of the ligand as a whole, and thus change imidazole into a sufficiently weak-field ligand.

The spectral properties of the cyanide derivative of cytochrome a_3 seem to point to a more low-spin character of this complex. The nature of the broad Soret band of $a_3{}^{3+}$CN will be investigated further since it may indicate the presence of a thermal mixture of low- and high-spin complexes, similar to, *e.g.*, that observed by George *et al.* (1961) in the case of ferrimyoglobin and ferrihemoglobin hydroxides.

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