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THE *b*-TYPE CYTOCHROMES OF BOVINE HEART MITOCHONDRIA: ABSORPTION SPECTRA, ENZYMATIC PROPERTIES, AND DISTRIBUTION IN THE ELECTRON TRANSFER COMPLEXES

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SUMMARY

1. Three *b*-type cytochromes ($b_{557.5}$, b_{560}, and $b_{562.5}$), *plus* a chromophore with an absorption peak at 558 nm at 77 °K, have been found to be associated with the electron transport system of bovine heart mitochondria. The reduced *minus* oxidized spectra of these components at 77 °K, as well as that of cytochrome c_1, have been recorded with a wavelength accuracy of ± 0.1 nm and presented to the nearest 0.5 nm. All the major α and β absorption peaks of cytochromes $b_{557.5}$, b_{560}, $b_{562.5}$, c_1 and c have been shown by fourth derivative analysis to be present in the dithionite-reduced *minus* oxidized spectra of mitochondria and submitochondrial particles.

2. The distribution of the above components has been studied in the four electron transfer complexes of the respiratory chain. Cytochromes b_{560}, $b_{562.5}$ and c_1, as well as chromophore-558, were found to fractionate into Complex III (reduced ubiquinone–cytochrome *c* reductase), whereas cytochrome $b_{557.5}$ was found in Complex II (succinate–ubiquinone reductase).

3. Cytochrome b_{560} was readily reduced by NADH or succinate, but $b_{562.5}$ was not reduced by substrates unless the preparation was treated with antimycin A. In antimycin-treated preparations pre-reduction of c_1 with ascorbate inhibited the subsequent reduction of $b_{562.5}$ by substrates. These results indicate that b_{560} and $b_{562.5}$ correspond, respectively, to b_K and b_T previously described by Chance *et al.*[14] (1970, *Proc. Natl. Acad. Sci. U.S.* 66, 1175–1182).

4. Similar to b_{560}, chromophore-558 can be reduced by substrates in the absence or presence of antimycin A. However, in antimycin-treated preparations, pre-reduction of c_1 inhibits its subsequent reduction by substrates. This property is similar to that of $b_{562.5}$.

5. Cytochrome $b_{557.5}$, which occurs in Complex II, appears to have a low mid-point potential. It can be reduced with dithionite and oxidized by fumarate or

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Abbreviations: ETP, non-phosphorylating electron transport particle preparation; ETP_H, phosphorylating electron transport particle preparation; TMPD, tetramethylphenylenediamine; Complexes I, II, III, I–III and II–III, respectively, preparations of NADH–ubiquinone reductase, succinate–ubiquinone reductase, reduced ubiquinone–cytochrome *c* reductase, NADH–cytochrome *c* reductase, and succinate–cytochrome *c* reductase.

ubiquinone. CO treatment of dithionite-reduced $b_{557.5}$ neither modified the spectrum of this cytochrome nor diminished the extent of $b_{557.5}$ reoxidation by fumarate.

6. Antimycin A treatment does not appear to alter the spectra of the above cytochromes. However, small amounts ($< 4\%$) of ethanol or methanol, which are usually added to particles as solvent for antimycin A, have a pronounced effect on the α peaks of cytochrome c_1. The spectrum of cytochrome c_1 at 77 °K as modified by 3 % (v/v) ethanol is shown.

INTRODUCTION

Multiple forms of cytochrome *b* have been discovered in various electron transport systems[1-9]. Recently, Slater *et al*[3] and Chance and his colleagues[4,5] have observed two types of cytochrome *b* in the mitochondrial respiratory chain, the behavior of which differ in energized and nonenergized membranes. These cytochromes were designated b_K (Keilin-type cytochrome *b*) and b_T (transducing cytochrome *b*) by the latter group. The reason for these designations was that b_K was found to be readily reduced in mitochondria upon addition of substrates, thus corresponding to the cytochrome *b* first discovered by Keilin in 1925, whereas b_T reduction required membrane energization by ATP. Wilson and Dutton[5] found that ATP energization of mitochondria also resulted in an apparent redox potential change of b_T from $E_m = -35$ mV to $E_m = +245$ mV. Rieske[10] showed that antimycin A treatment increased the extent of total cytochrome *b* reduction in Complex III preparations provided cytochrome c_1 was in the oxidized state. Similar results were reported by Wilson *et al.*[11] on a mitochondrial preparation with succinate–cytochrome *c* reductase activity, and Erecinska *et al.*[12] showed that in antimycin A and ascorbate–tetramethylphenylenediamine (TMPD)-treated particles, the reduction of b_T by substrate followed the reoxidation of cytochrome c_1.

Our preliminary studies[6] showed that the inner membranes of beef heart mitochondria contained three distinct *b*-type cytochromes. Two of these, b_{560}* and $b_{562.5}$**, appeared to be similar to b_K and b_T, respectively and were found to fractionate into Complex III. In these preparations, b_{560} was readily reduced by substrate, but the substrate reduction of $b_{562.5}$ occurred only in antimycin-treated particles. The third *b*-type cytochrome, $b_{557.5}$, which did not appear to be reducible by substrates, was found in Complex II. The present paper is a detailed study of the spectral characteristics of cytochromes b_{560}, $b_{562.5}$, $b_{557.5}$ and c_1 in the isolated electron transfer complexes, submitochondrial particles (ETP) and in intact mitochondria. Data will also be shown regarding the possible existence of a new, reducible chromophore in Complex III, having at 77 °K a cytochrome-like peak at 558 nm.

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* This cytochrome was previously designated $b_{559.5}$ (ref 6) However, as will be seen below, its α_{max} appears to be at 560 nm

** The *b*-type cytochromes will be identified in this manuscript by subscripts, each designating in nm the absorption maximum of the major α peak of that cytochrome at 77 °K At room temperature, the approximate α_{max} of $b_{557.5}$, b_{560}, and $b_{562.5}$ are, respectively, at 560–561, 562–563, and 565–566 nm.

MATERIALS AND METHODS

Heavy beef heart mitochondria, cytochrome *c*-depleted heavy beef heart mitochondria, non-phosphorylating electron transport particle preparation (ETP), phosphorylating electron transport particle preparation (ETP_H), partially purified cytochrome c_1, and Complexes II, III, and I–III were prepared as indicated elsewhere[6] Complex II–III was reconstituted from preparations of Complex II and Complex III as before[13]. Cytochrome *c*, type III, was procured from Sigma Chemical Co., NADH from P-L Biochemicals, and TMPD from Eastman Organic Chemicals. Antimycin A (blastmycin) was a gift from Kanegafuchi Chemical Industrial Co., Japan.

Absorption spectra were measured with a single beam spectrophotometer (consisting of a DC voltage stabilized tungsten iodide lamp, a Cary Model 14 monochromator with motor drive, a sample compartment with a vertical light path, an EMI 9558 phototube directly beneath the sample and a logarithmic amplifier to measure the phototube current) on line to a PDP 8/I digital computer with a 12 bit analog-to-digital converter[6,14]. The wavelength accuracy was calibrated to 0.1 nm with a mercury lamp. The spectral bandwidth was less than 0 3 nm. Wavelength maxima of absorption bands are reported to the nearest 0.5 nm. Absorbance values (representing the average of 256 readings) were taken and stored in the computer every 0.1 nm as the monochromator scanned (at 2 5 nm/s) through the spectral region of interest. Each spectral curve presented is the sum of two independent spectra measured on the same sample If a pair of such spectra showed differences other than random noise they were discarded. The procedure of adding pairs of independent spectra provides some additional suppression of random noise and insures that the spectra do not include spurious, nonreproducible changes.

The spectral data, digitized every 0.1 nm, are stored in blocks of 1000 points at specific locations in the memory core of the computer. These blocks of spectral data may be read out into an X–Y recorder or into a data storage device as individual curves or as differences between any pair of spectral curves. Absolute absorption spectra are obtained as the difference between the spectral curves measured on the sample and on a reference blank. The spectral data can be subjected also to higher derivative analysis by the computer.

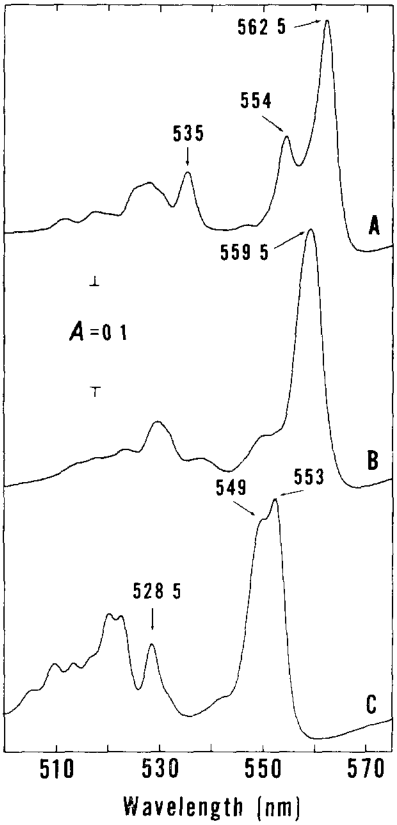
Fourth derivative analysis of the absorption spectra have been used to identify the components of the complex spectra[6,15]. The fourth derivative curves were obtained by four sequential differentiations of the absorption spectra with respect to wavelenght with finite differentiating intervals of 1.0, 1.1, 1.2 and 1.4 nm, respectively. The fourth derivative curve of a symmetrical absorption band has a maximum at the same wavelength position as the absorption band, but the width of the curve in the fourth derivative is much narrower so that greater resolution of the individual components can be achieved. It should be emphasized that these curves are used only to indicate the wavelength maxima of the component absorption bands and not the relative amounts of the individual absorbing species. No further physical significance should be attached to the fourth derivative curves. An evaluation of the fourth derivative analysis including artifacts which may be generated has been presented previously[16,17].

Samples of the various mitochondrial preparations (0.5 ml) were placed in a vertical cylindrical cuvette (14.3 mm diameter) and frozen to liquid N_2 temperature in a cylindrical Dewar with optical windows on the bottom[14]. Substrates, chemical reductants, and inhibitors (5–20 μl) were added to the sample at room temperature before freezing. For details, see the legends to figures. No attempt was made to minimize light scatter in the frozen samples. Reproducibility of preparation of the frozen sample is such that a flat line showing only random noise is obtained as the difference spectrum between two independent samples of the same material.

RESULTS

Spectral characteristics of the b-type cytochromes of beef heart mitochondria

Three *b*-type cytochromes were found in the inner membranes of mitochondria.



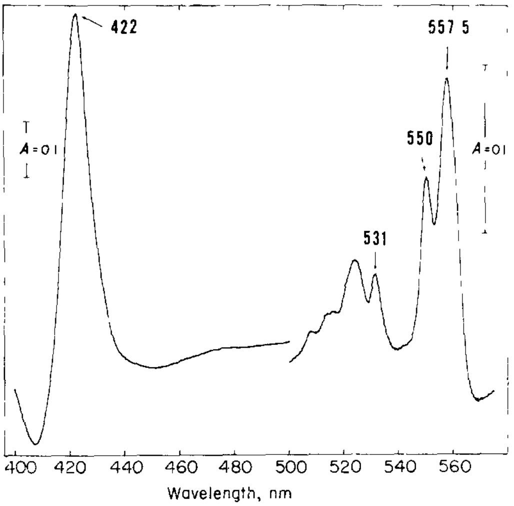


Fig. 1 Spectra of cytochromes $b_{562.5}$, b_{560} and c_1 of Complex I–III. Trace 1A ($b_{562.5}$) - Sample A Complex I–III at 3.27 mg protein/ml of 80 mM potassium phosphate, pH 7.4, treated at room temperature with 10 mM sodium azide, 80 μM TMPD, and 10 mM sodium ascorbate followed after 1-min intervals each with addition of 0.3 mM NADH and solid dithionite before freezing in liquid N_2. Reference A: same, except that dithionite addition was omitted Trace 1B (b_{560}) - Sample B. Complex I–III (3.27 mg protein/ml) treated as above with azide, ascorbate–TMPD and NADH Reference B: same as Sample B, except that NADH was omitted. Trace 1C (c_1) - cytochrome c_1 (partially purified from Complex III) at 0.76 mg protein/ml of 40 mM potassium phosphate, pH 7.4, treated with 10 mM sodium ascorbate and 80 μM TMPD

Fig 2. Reduced *minus* oxidized spectrum of cytochrome $b_{557.5}$ in Complex II. Comples II at 1.76 mg protein/ml of 40 mM potassium phosphate, pH 7.4, was treated with a small amount of dithionite in order to reduce its succinate dehydrogenase and minor ($\approx$ 10 %) Complex III contaminant, and its spectrum was recorded. This spectrum was then subtracted from that of Complex II fully reduced with dithionite

Their spectral characteristics (reduced *minus* oxidized) at 77 °K, together with those of cytochrome c_1, are shown in Figs 1 and 2. In accordance with their enzymatic properties described above and elsewhere[6], Trace A in Fig. 1 ($b_{562.5}$) is comparable to the spectrum of b_T and Trace B (b_{560}) to that of b_K (ref. 18). It is seen that the former cytochrome shows two prominent α peaks, one at 562.5 nm and another at 554 nm, and several β peaks among which the first peak at 535 nm is rather diagnostic of the reduced form of this cytochrome. The 535-nm peak is clearly discernible even in ETP and mitochondria (see Fig. 5A and 5B below) The latter cytochrome exhibits only a single prominent α peak at 77 °K, which has an absorption maximum at 560 nm. Sato *et al* [18] have assigned the α_{max} position of b_{560} (b_K) to 558 nm. However, our spectra of Complex III and ETP indicate that the α peak of b_{560} is, indeed, very close to 560 nm. Fig. 1C shows the spectrum of partially purified cytochrome c_1, which was prepared after chaotrope-induced resolution of Complex III (ref. 20). It was found to be identical to the spectrum of particle-bound cytochrome c_1 (Fig. 6A).

Fig. 2 shows the reduced *minus* oxidized spectrum of the third *b*-type cytochrome we have found in the inner membranes of beef heart mitochondria. This cytochrome exhibits at 77 °K two prominent α peaks, one at 557.5 nm and another at 550 nm, a sharp β peak at 531 nm, a broad β peak at about 523 nm, and a Soret band at 422 nm.

Localization of the b-type cytochromes in the electron transfer complexes

During resolution of the respiratory chain into the four electron transfer complexes, cytochromes b_{560}, $b_{562.5}$ and c_1 were found to fractionate into Complex III, whereas cytochrome $b_{557.5}$ was found exclusively in Complex II in amounts comparable to the concentration (moles/g protein) of succinate dehydrogenase. When Complex III was isolated according to the procedure of Rieske[21], rather than the original procedure of Hatefi *et al.*[22], some $b_{557.5}$ was also found in this preparation. The amount of $b_{557.5}$ present in the Rieske-type Complex III preparation was small, however, and was comparable to the succinate dehydrogenase contamination of this modified preparation of Complex III. Purified preparations of cytochrome oxidase (Complex IV) were essentially devoid of contamination with *b*- and *c*-type cytochromes and the cytochrome contamination of Complex I ($<$ 0.05 nmole $b+c_1$/mg protein) appeared to be due to contamination ($<$ 0.5 %) by Complex III.

Spectral characteristics of cytochromes b_{560}, $b_{562.5}$ and c_1

When binary complexes I–III or II–III were used in the absence of antimycin A, b_{560} and c_1 could be reduced with NADH in I–III or with succinate in II–III. The reductions afforded by these substrates were qualitatively the same In the presence of antimycin A, substrates (NADH or succinate) reduced both b_{560}, and $b_{562.5}$ and, because of the virtual absence of cytochrome oxidase in these preparations, the leak through the antimycin block also resulted in the reduction of c_1. Fig. 3 shows the spectra of NADH-treated Complex I–III in the absence (A) and presence (B) of antimycin A. Traces C and D are the fourth derivative spectra of A and B, respectively. Trace A shows the combined spectra of reduced *minus* oxidized b_{560}, and c_1 (see Trace C for positions of the individual absorption peaks of b_{560} and c_1), and Trace B includes the spectrum of reduced *minus* oxidized $b_{562.5}$ as well. In Fig. 3B, cytochrome $b_{562.5}$ is responsible for the increased peak heights at 559–560 nm and 553.5 nm, the

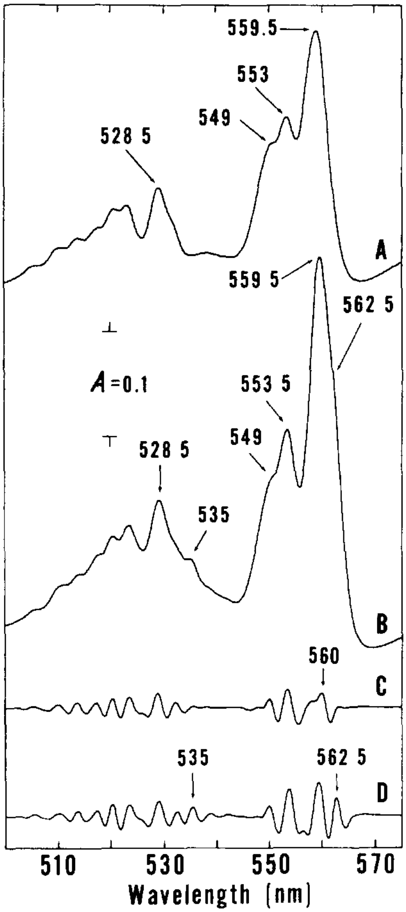


Fig. 3. Spectra of NADH-reduced *minus* oxidized Complex I–III in the absence (A) and presence (B) of antimycin A. Trace 3A: Complex I–III (3.27 mg protein/ml of 80 mM potassium phosphate, pH 7.4) was treated with 10 mM sodium azide and 0.3 mM NADH. Trace 3B: same as (3A), except that Complex I–III was first treated with 15 nmoles antimycin A. Reference materials in (3A) and (3B) were the same, except that NADH was omitted. Traces C and D are, respectively, the fourth derivatives of Traces A and B.

shoulder on the long wavelength side of 560 nm, and the appearance of the 535-nm peak (see Trace D for positions of the individual absorption peaks of $b_{562.5}$, b_{560} and c_1). As expected, treatment of Complex I–III or Complex III with dithionite in the presence or absence of antimycin A resulted in the reduction of b_{560}, $b_{562.5}$ and c_1. Cytochrome c_1 in these particles is readily and completely reduced with ascorbate *plus* TMPD (Fig. 6A), and cytochrome b_{560} is slowly reduced by ascorbate–TMPD. The latter reduction is considerably slower in the presence of antimycin A, thus suggesting that it occurs by way of c_1.

Titration of Complex I–III with graded amounts of NADH showed that, in the absence of antimycin A, cytochrome c_1 was reduced first, and only after about 60 % reduction of c_1 did the reduction of b_{560} begin (Fig. 4A). In the presence of

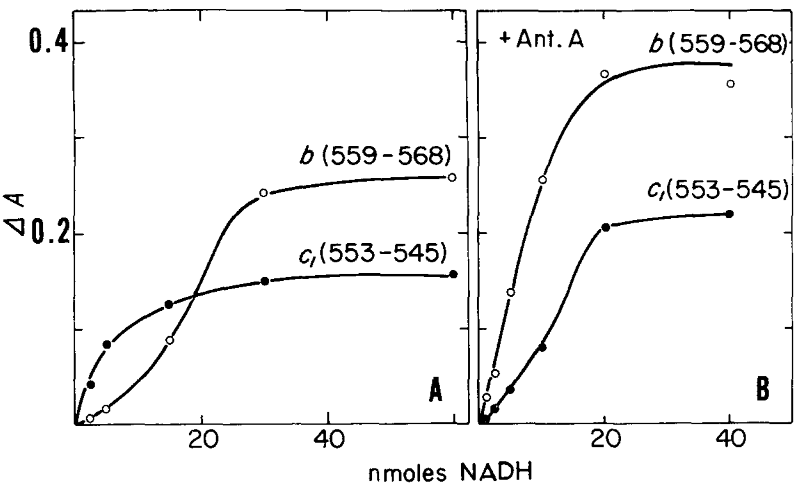


Fig 4 Reduction of cytochromes b_{560}, $b_{562.5}$ and c_1 upon addition of graded amounts of NADH to Complex I–III in the absence (A) and presence (B) of antimycin A. Complex I–III (3.16 mg protein/ml of 40 mM potassium phosphate, pH 7.4) was treated with 20 mM sodium azide, 20 μM antimycin A in (B) only, and NADH as shown. References were the same prior to NADH addition. The wavelength pair 553–545 nm in (A) is a measure of c_1 reduction, and in (B) is a measure of c_1 *plus* $b_{562.5}$ (α_2) reduction. The wavelength pair 559–568 nm in (A) is a measure of b_{560} reduction, and in (B) is a measure of b_{560} *plus* $b_{562.5}$ reduction.

antimycin A, however, b_{560} and $b_{562.5}$ were reduced prior to c_1 (Fig. 4B). In the latter figure, the increased absorption at 559 nm is due to the reduction of both b_{560} and $b_{562.5}$, and that at 553 *minus* 545 nm is due to the appearance of the α_2 band of ferrocytochrome $b_{562.5}$ at 554 nm, which adds onto the α_1 band of ferrocytochrome c_1 at 553 nm. According to this figure, the contribution of the former to the α_1 band of c_1 at 553 *minus* 545 nm is approx. 25 % at 77 °K. In agreement with indications from Rieske[10] and the subsequent work of Wilson *et al.*[11] on a succinate–cytochrome *c* reductase particle, it was also found that in antimycin-treated Complex I–III prior reduction of c_1 by ascorbate–TMPD prevented the rapid reduction of $b_{562.5}$ by NADH★.

The behavior of cytochrome $b_{557.5}$ toward substrates was completely different from b_{560} and $b_{562.5}$. Cytochrome $b_{557.5}$ of Complex II, ETP or mitochondria was reducible by dithionite, but reduced $b_{557.5}$ could not be generated upon addition of substrates. Other studies★★ have shown, however, that dithionite-reduced $b_{557.5}$ could be oxidized by fumarate or ubiquinone, thus indicating electronic communication between $b_{557.5}$ and the respiratory chain, but unfavorable thermodynamics for its reduction by succinate.

Having recognized three *b*-type cytochromes★★★ with distinctly different spec-

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★ Actually, the oxidoreduction state of c_1 in various particles treated with antimycin A affects the kinetics of the reduction of both $b_{562.5}$ and b_{560} by substrates (Hatefi and Davis, unpublished).

★★ Hatefi and Davis, unpublished.

★★★ It is important to point out in this context that in rat heart mitochondria[23], rat liver mitochondria[23] and beef heart submitochondrial particles[24], the concentration (nmoles/mg protein) of cytochrome *b* and cytochrome c_1 were found to be, respectively, 0.75 and 0.2; 0.3 and 0.07, 0.85 and 0.27.

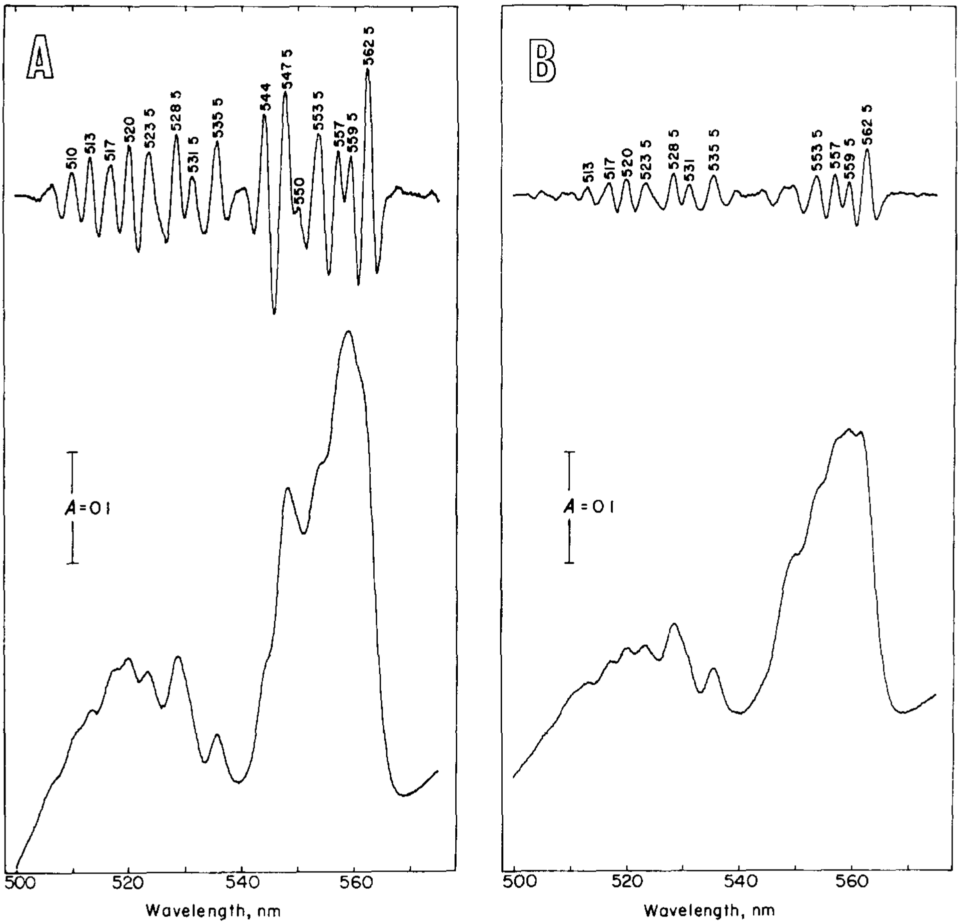

Fig. 5. Dithionite-reduced *minus* oxidized spectra of ETP_H (A) at 7.5 mg protein/ml, and cytochrome *c*-depleted heavy beef heart mitochondria (B) at 7.6 mg protein/ml. The medium was 0.25 M sucrose containing 50 mM Tris–HCl, pH 7.8. The fourth derivative spectra *plus* the peak positions in nm are shown at the top of each pannel.

tral and enzymatic properties in the electron transfer complexes derived from the respiratory chain, it was important to establish the presence of these cytochromes in mitochondria and ETP. It was also necessary to ascertain that $b_{557.5}$, which had not been previously recognized by others and which appeared to be nonreducible by substrates, was not a denatured form of the other *b*-type cytochromes. These studies were crucial, since various manipulations and the use of bile salts for isolation of the electron transfer complexes might have altered the spectral characteristics and the enzymatic properties of these cytochromes.

First, the 77 °K spectra of dithionite-reduced ETP (A) and cytochrome *c*-depleted heavy beef heart mitochondria (B) are shown in Fig. 5. The fourth derivatives of these spectra, which serve to resolve overlapping peaks into separate maxima, are also shown. It is seen that (a) the fourth derivative spectra are capable of resolving peaks that are separated by as little as 2 nm, and (b) essentially all the α and the major β peaks of cytochromes $b_{562.5}$, b_{560}, $b_{557.5}$, c_1 and c, are clearly discernible in these spectra. These results show that the isolation procedures of the electron transfer complexes have not altered the spectral properties of these cytochromes and establish the presence of $b_{557.5}$ (see the 557- and 531-nm peaks in both fourth derivative spectra) in the mitochondrial inner membranes. Furthermore, since all the fourth derivative peaks in Fig. 5A and 5B can be accounted for on the basis of the α and β peaks of $b_{562.5}$, b_{560}, $b_{557.5}$, c_1 and c, the possible existence of comparable amounts of additional *b*- and *c*-type cytochromes in beef heart mitochondria becomes rather doubtful. A possible exception is chromophore-558 (see below), which might make a small contribution in these spectra to the α_1 band of $b_{557.5}$.

Second, the possible denaturation of b_{557} during preparation of Complex II was checked by the reactivity of this cytochrome with CO. This is particularly important, since preparations of Complex II contain, as reported earlier^{6,25}, varying amounts (10–20 %) of denatured Complex III. Thus, a preparation of Complex II was reduced with dithionite, and its spectrum before and after addition of fumarate was recorded. The difference of these two spectra was identical to the spectrum of $b_{557.5}$ shown in Fig. 2. A second sample of the same preparation of Complex II was similarly reduced with dithionite. It was then treated with CO before the addition of fumarate. Spectra recorded at each stage showed that CO treatment did cause some loss of the overall absorbancy, but that it had not diminished the extent of the fumarate-oxidizable cytochrome *b*. These results confirmed that Complex II preparations contain, in addition to some denatured impurities, a new species of cytochrome *b* (Fig. 2) which does not react with CO and is capable of reducing fumarate.

Effect of antimycin A on the spectral characteristics of cytochromes c_1, b_{560} and $b_{562.5}$

Cytochrome c_1. Addition of antimycin A has no detectable effect on the spectrum of ferrocytochrome c_1. However, methanol or ethanol, in which the antimycin A is usually dissolved and added to the medium, was found to have pronounced effects on the spectrum of cytochrome c_1. Thus, as seen in Fig. 6 (Trace C), even 1–3 % ethanol (v/v) altered significantly the absorbancy of the α_2 band (549 nm) of ascorbate–TMPD-reduced cytochrome c_1, and caused a blue shift of the α_1 band by about 1 nm. A similar effect, but considerably less pronounced, was observed when either alcohol was added to ferrocytochrome *c*. Because of this distortion effect, the addition of alcohols to samples treated with antimycin A was avoided in all the experiments reported here. An ethanolic solution ($\approx$ 20 μl) of antimycin A was first added to the cuvette and the ethanol was allowed to evaporate completely, leaving antimycin A as a film on the walls of the cuvette. Then, the enzyme suspension was added and stirred for several min in the cuvette until antimycin A was taken up by the enzyme. However, cytochromes b_{560} and $b_{562.5}$ did not show spectral changes in the presence of methanol or ethanol (< 4 %).

Cytochrome b_{560}. The spectra of NADH-reduced cytochrome b_{560} in the absence (A) and presence (B) of antimycin A, as well as their fourth derivatives (C and D, respectively) are shown in Fig. 7. It is seen that as compared to 7A, the α band of 7B shows a slight red shift, and a half bandwidth thinning of 1.5–2.0 nm, which seems to occur mainly on the short wavelength side of the α peak. The fourth derivative spectra show clearly that the α band of 7A consists of two components at about

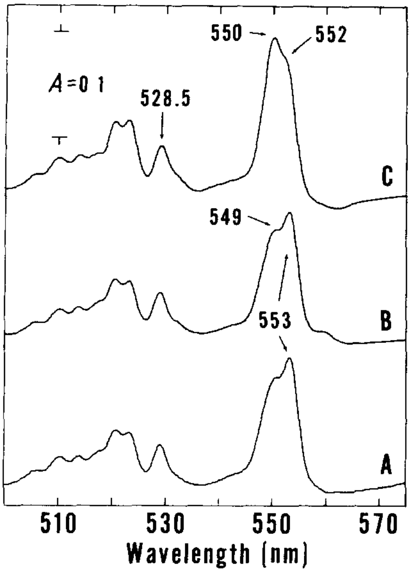

Fig. 6. Effects of antimycin A and ethanol on the reduced *minus* oxidized spectrum of cytochrome c_1. Cytochrome c_1 in Complex I–III (3.38 mg protein/ml of 80 mM potassium phosphate, pH 7.4, containing 10 mM sodium azide) was reduced with 10 mM sodium ascorbate *plus* 80 μM TMPD at room temperature and immediately frozen in liquid N_2 (A). In (B) 25 nmoles antimycin A (in the absence of ethanol), and in (C) 2.85 % (v/v) ethanol were added.

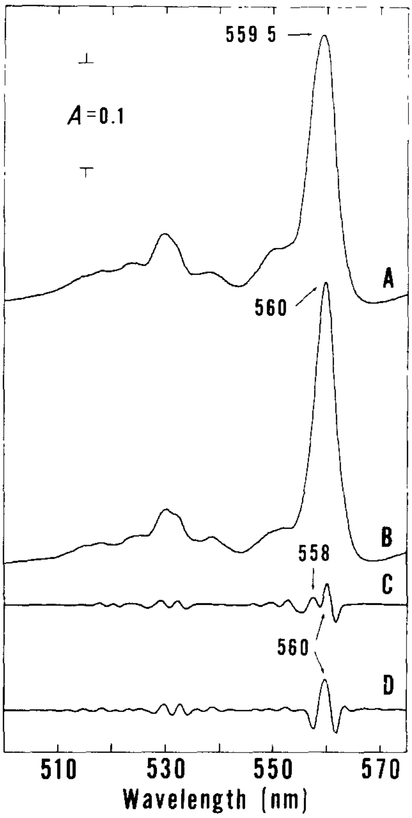

Fig. 7. Spectral properties of cytochrome b_{560} in Complex I–III in the absence (A) and presence (B) of antimycin A. Trace 7A: Complex I–III (3.27 mg protein/ml of 80 mM potassium phosphate, pH 7.4) was treated with 10 mM sodium azide, 10 mM sodium ascorbate and 80 μM TMPD at room temperature. The mixture was frozen in liquid N_2 and its spectrum was recorded to be used as "reference". The mixture was then thawed, treated with 0.3 mM NADH, and after 1.5 min frozen again in liquid N_2. The spectrum of this material was recorded and used as "sample". Trace 7A is "sample" *minus* "reference". Trace 7B: all conditions were the same as in 7A, except that Complex I–III was first treated with 15 nmoles of antimycin A. Trace 7C and 7D are the fourth derivative spectra of 7A and 7B, respectively

560 nm and 558 nm, whereas the α band of 7B shows a single component at about 560 nm. These results suggest that, in contrast to 7B, the α band of 7A includes contribution by a chromophore with a peak at about 558 nm.

Cytochrome $b_{562.5}$. The spectra of dithionite-reduced cytochrome $b_{562.5}$ in the absence (A) and presence (B) of antimycin A and their fourth derivative spectra (C and D, respectively), are shown in Fig 8. As seen in the legend of Fig. 8A, Complex I–III was treated with ascorbate–TMPD to reduce c_1 followed by NADH to

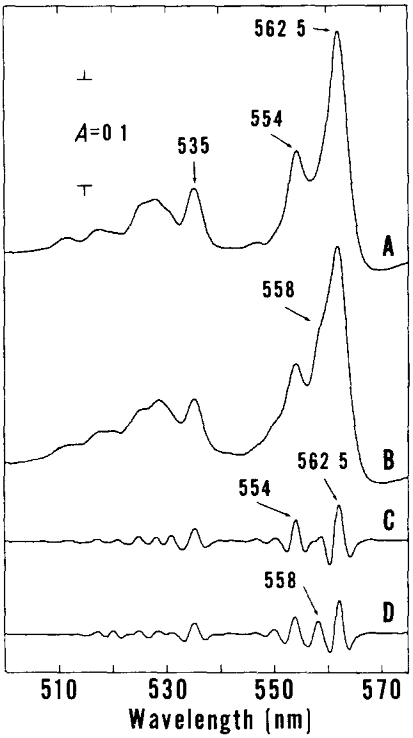

Fig 8 Spectral properties of cytochrome $b_{562\ 5}$ in Complex I–III in the absence (A) and presence (B) of antimycin A. Trace 8A. 7A "sample" treated with dithionite *minus* 7A "sample". Trace 8B 7B "sample" treated with dithionite *minus* 7B "sample". Trace 8C and 8D are the fourth derivative spectra of 8A and 8B, respectively

reduce b_{560}. The spectrum of the material was recorded, then dithionite was added to reduce $b_{562\ 5}$. Subtraction of the spectrum in the absence of dithionite from that in its presence resulted in the spectrum of dithionite-reduced $b_{562.5}$. In the experiment of Fig. 8B, where antimycin A was present, again a similar procedure was followed, because prior reduction of c_1 in antimycin-treated Complex I–III inhibited the reduction of $b_{562\ 5}$ by NADH (see refs 10–12). Therefore, subsequent addition of dithionite allowed us to obtain a spectrum of reduced $b_{562\ 5}$ under similar conditions to 8A, except for the presence of antimycin A. The spectrum of NADH-reduced $b_{562.5}$ (*i e.* without prior reduction of c_1) in antimycin-treated Complex I–III was essentially the same as 8B. Once again, it is seen that 8A and 8B differ by the apparent presence of an additional chromophore in 8B with a peak at approx. 558 nm (compare fourth derivatives 8C and 8D). The reason that this chromophore does not appear in 8A is simply that the difference spectra leading to 8A subtract its contribution.

DISCUSSION

Three *b*-type cytochromes with distinctly different spectral and functional characteristics have been shown to occur in the inner membranes of mitochondria from beef heart. Cytochromes b_{560} and $b_{562.5}$, together with c_1, are components of Complex III, whereas cytochrome $b_{557.5}$ occurs exclusively in Complex II. Cytochrome b_{560} was readily reduced by succinate or NADH in various particle preparations (I–III, II–III, ETP), but the substrate-induced reduction of $b_{562.5}$ in these systems required the presence of antimycin A. In the latter case, reduction of cytochrome c_1 by ascorbate *plus* TMPD prior to addition of substrate inhibited the substrate-induced reduction of $b_{562.5}$ The reduction pattern of cytochromes in Complex I–III with graded amounts of NADH was found to be consistent with their oxidation–reduction potentials as determined by others in the absence and presence of antimycin A (ref. 5). These results also showed that the wavelength pair 554–540 nm, commonly used for monitoring c_1 changes, includes at 77 °K appreciable $b_{562.5}$ contribution in antimycin-treated preparations

Regarding the spectra of cytochromes obtained under various conditions the following comments might be added:

(1) *Cytochrome c_1*

Cytochrome c_1 is water soluble when removed from membranes, and its spectrum remains unaltered after this transition. Therefore, it is doubtful that hydrophobic–hydrophilic perturbations of the medium as affected by such small amounts as 1 % methanol or ethanol could be responsible for the spectral changes reported in Fig. 6C. On the other hand, these results point to the possibility of the effect of alcohols by direct interaction with the cytochrome molecule.

The cytochrome c_1 spectrum (Fig 1C) presented here differs slightly from the spectrum published by Yu *et al.*[26] for their highly purified, crystalline preparation of cytochrome c_1. In the latter spectrum, the α region of reduced cytochrome c_1 at about 113 °K is shown to be composed of at least five peaks at about 554, 552.5, 550, 549, and 541 nm. Our spectrum of cytochrome c_1 at 77 °K, does not show the bands at about 554 and 550 nm. These additional bands as given by Yu *et al.*[26] are also nonexistent in the fourth derivatives of our spectra of cytochrome c_1.

(2) *Cytochrome $b_{557.5}$*

In this manuscript, we have provisionally designated this cytochrome as $b_{557.5}$, because designation by its α peak at room temperature (560–561 nm) would result in confusion with b_{560} (room temperature α_{max} at 562–563 nm). However, continued usage of the designation $b_{557.5}$ is not satisfactory because a *b*-type cytochrome from *Rhodospirillum rubrum* has been previously designated as cytochrome *b*-557.5 (ref. 9) The fact that $b_{557.5}$ does not appear to be reduced by substrates, but can donate electrons to fumarate (apparently through succinate dehydrogenase) is rather interesting, and recalls the earlier findings of others regarding the close association of a *b*-type cytochrome with succinate dehydrogenase preparations from strict and facultative anaerobes, such as *Corynebacterium diphtheriae* and *Propionibacterium pentosaceum*[27] It is possible, however, that the reduction of fumarate by $b_{557.5}$ in mitochondria is not just a vestige of evolution, but that $b_{557.5}$ is an entry point for an ancillary electron tributary of the respiratory chain.

(3) *Cytochromes $b_{562.5}$ and b_{560}*

We regard Spectrum 8A to be the least complicated result. Spectra 7A and 8B contain the additional 558-nm component, and 7B involves antimycin treatment, which might have caused minor differences between sample and reference. Therefore, Spectrum 8A may be considered to represent the reduced *minus* oxidized spectrum of $b_{562.5}$. As seen in Materials and Methods, this spectrum was obtained by subtracting the spectrum of Complex I–III treated with NADH *plus* ascorbate–TMPD (essentially 3A) from that treated with NADH, ascorbate–TMPD, and dithionite. The result, as shown in Fig. 8A, is essentially the same as the $b_{562.5}$ spectrum deduced by Sato *et al.*[18] from the difference between ATP-energized and nonenergized mitochondria treated with succinate. Trace 8B shows what happens when an attempt is made to deduce the spectrum of $b_{562.5}$ from antimycin-treated Complex I–III. The shoulder appearing on the short wavelength side of the α_1 peak of $b_{562.5}$ at about 558 nm does not appear to be due to modification of $b_{562.5}$ by antimycin A, because (a) the relative contribution of the 558-nm component to the spectrum of $b_{562.5}$ can be varied, and (b) this component appears to add onto the α band of b_{560} under conditions that no $b_{562.5}$ reduction takes place (see 7A). Therefore, 8B may be considered to be the reduced *minus* oxidized spectrum of $b_{562.5}$ in antimycin-treated particles *plus* the spectrum of a component (designated hereafter as chromophore-558), which is neither a part of $b_{562.5}$ nor due to modification of $b_{562.5}$ by antimycin A. As seen in 8A and 8B, the major peak positions of $b_{562.5}$ (*i.e.* 562.5, 554, 535 nm) are not altered in the presence of antimycin A

Conditions such as those used to obtain trace A of Fig 7 were considered previously to yield the reduced *minus* oxidized spectrum of b_{560} or b_K (refs 6, 18, 19). The above studies have indicated, however, that 7A does not represent the true spectrum of b_{560}. The α band of 7A is very broad for a cytochrome, and its fourth derivative (Fig. 7C) shows that it is not due to a single component*. The additional component seen by fourth derivatives analysis in the α band of 7A has a maximum at about 558 nm, and appears to be the same as chromophore-558, which in antimycin-treated particles, adds onto the α_1 band of $b_{562.5}$ (see Fig. 8B). In antimycin-treated particles, substrate-induced production of chromophore-558 is inhibited when the particles have been pretreated with ascorbate–TMPD. In other words, when c_1 is reduced in antimycin-treated particles prior to addition of substrate, the substrate-induced production of chromophore-558 and reduced $b_{562.5}$ are both inhibited. Therefore, chromophore-558 is not seen in 7B, but appears in 8B along with reduced $b_{562.5}$

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* We have attempted to isolate cytochromes b_{560} and $b_{562.5}$ in order that their spectral characteristics might be studied on purified samples Three procedures were used: (a) the method of Goldberger *et al*[28], (b) the method of Yamashita and Racker[29], both applied to Complex III, and (c) resolution of Complex III into a *b*-rich and a c_1-rich fraction with guanidine–HCl according to Rieske *et al.*[20]. Room temperature spectra of cytochrome *b* prepared according to procedures (a) and (b) agreed with published data, but method (c) yielded a preparation in which the α and β peaks had shifted toward blue At 77 °K, the spectra of preparations (a) and (b) were completely different, however, from b_{560} (Fig 7), $b_{562.5}$ (Fig. 8), or $b_{560}+b_{562.5}$ as they occur in Complex III Their fourth derivatives showed peaks at 560–561, 555, 535 and 528–530 nm. These results indicated gross spectral modifications, especially with regard to the absence of the α_1 peak of $b_{562.5}$. The presence of peaks at 555 and 535 nm in preparations (a) and (b) might be considered as suggestive evidence that the spectrum of $b_{562.5}$ shown in Fig 8A is due to two components, one responsible for the peak at 562.5 nm (lost in the above preparations) and another responsible for the peaks at 554 and 535 nm (present in the above preparations) Weak as it is, this possibility cannot be dismissed on the basis of available information.

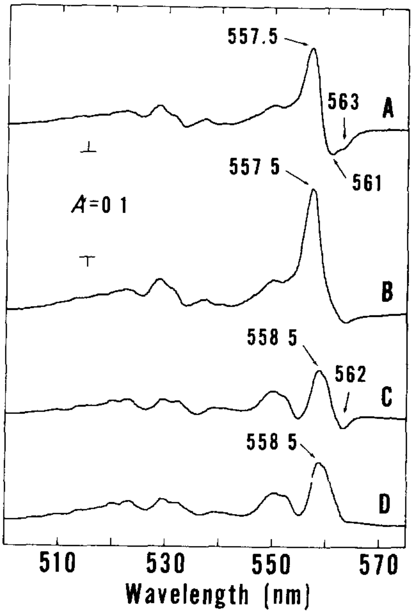

Fig 9 Difference spectra 7A *minus* 7B and 8B *minus* 8A. Trace 9A is (7A *minus* 7B), Trace 9B is (7A 1.2 *minus* 7B), trace 9C is (8B *minus* 8A), and Trace 9D is (8B 1.1 *minus* 8A).

because of subsequent treatment of the latter mixture with dithionite. As will be seen below (Fig. 9), the difference between 7A and 7B is further complicated by a trough at 561–563 nm. Both the 558-nm peak and the 563-nm trough have been assumed by Sato *et al.*[18] to be due to the effect of antimycin A on b_K (b_{560}). Our studies show that the 558-nm peak appears to be due to a separate component. However, the latter change, especially the trough at 561 nm (see Fig. 9A), might be associated with antimycin treatment At any rate, because of these differences, subtraction of 7B from 7A does not yield a reliable spectrum of chromophore-558; it yields a difference spectrum with a peak at 557.5 nm *plus* a decrease in absorbance noticeable on the long wavelength side of the 557 5-nm peak (see below and Fig. 9A)

(4) *Chromophore-558*

The questions that arise with regard to the chromophore found in association with the spectra shown in 7A and 8B might be summarized as follows: (a) Is chromophore-558 attributable to b_{560}, $b_{562.5}$ or c_1, or is it a separate entity? (b) What are the conditions under which the spectral characteristics of chromophore-558 are visualized? (c) What is the spectrum of chromophore-558? (d) What is chromophore-558 and what is its function?

With regard to question (a), it has been discussed above that chromophore-558 does not appear to be attributable to the spectral changes of cytochromes b_{560}, $b_{562.5}$ or c_1. Since this component also appears in the spectrum of b_{560} derived from submitochondrial particles prepared simply by sonication of mitochondria followed by differential centrifugation, its presence in Complex III cannot be considered as modification of a cytochrome by bile salts and the simple fractionation procedures used for isolation of Complex III. The conditions which lead to the appearance of chromophore-558 also suggest that this component is not a denatured entity, because its appearance is both substrate dependent and inhibitable. Thus, chromophore-558 appears in the spectra of appropriate particles (I–III, II–III, ETP, ETP_H) when treated with: (i) succinate or NADH, in the absence of antimycin A. This property is similar to that of b_{560}, but not $b_{562.5}$. (ii) succinate or NADH in the presence of antimycin A, provided c_1 is in the oxidized form. Pre-reduction of c_1 (or possibly a component near c_1, see Rieske in ref. 10) with ascorbate–TMPD in antimycin-treated particles inhibits the subsequent appearance of chromophore-558 upon substrate addition. This property is similar to that of $b_{562.5}$. (iii) dithionite.

We have attempted to deduce the spectrum of chromophore-558 from 7A *minus* 7B and 8B *minus* 8A. However, neither difference yields a satisfactory spectrum. The former difference shows a peak at 557.5 nm, but is complicated by the decrease in absorbance at 561–563 nm (Fig. 9A). We have tried to normalize the height of 7A α with respect to 7B before recording their difference in order to diminish the negative trough on the long wavelength side of the 557.5-nm peak in 9A, and thereby obtain a better estimate of the spectrum of chromophore-558. The result, involving multiplication of the absorbancy of 7A by a factor of 1.2, is shown in Fig. 9B. It is seen that 9B is strongly suggestive of a cytochrome with an α peak at 557.5 nm (but clearly distinct from $b_{557.5}$). However, it is especially important to note that even such a considerable normalization of 7A (20% increase) did not eradicate the negative trough at 563 nm.

The difference between 8B and 8A is shown in Fig. 9C, and, after appropriate normalization to correct for the negative trough at 562 nm, it is shown in Fig. 9D. Once again, Trace 9C shows a peak at about 558 nm, but is otherwise unsatisfactory, and Trace 9D shows the difference after normalization of 8B by a factor of 1.1. In spite of the presence of a prominent peak at about 558 nm in both 9B and 9D, and the highly suggestive nature of 9B, it should be kept in mind that these difference spectra are only attempts at deducing the spectrum of chromophore-558, and that very likely they involve exaggerated complications (*e.g.* due to spectral differences of other components in the presence and absence of antimycin A).

Trace 9B suggests that chromophore-558 might be a cytochrome occurring in Complex III at a concentration substoichiometric with respect to b_{560} and $b_{562.5}$ (assuming roughly comparable molar extinctions in the α region). This possibility is in agreement with Wikström[7] who has proposed that mitochondria contain three *b*-type cytochromes with the relative ratio of 1:4.2, of which the first is designated as b_{558} (this is a substrate-reducible species different from our $b_{557.5}$) and the second and third correspond to b_{560} and $b_{562.5}$, respectively. We feel, however, that sufficient evidence for the existence in the inner membranes of beef heart mitochondria of a fourth *b*-type cytochrome (*i.e.* in addition to $b_{557.5}$, b_{560} and $b_{562.5}$) is not yet available. Therefore, any conclusion regarding the possible nature of chromophore-558 would be premature at this time.

The substrate- and dithionite-induced production of chromophore-558, the inhibition of its appearance under conditions that $b_{562.5}$ reduction by substrates is also inhibited (*i.e.* in antimycin-treated, c_1-reduced systems) as well as other results suggest that (a) chromophore-558 is reducible, (b) its reduction is influenced by the oxidation–reduction state of other carriers, and (c) it is located on the substrate side of the antimycin block. Whether or not it is indeed a *bona fide* electron carrier and is required for electron transfer from substrates to cytochromes c_1 and c remain to be seen.

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