BBA 45538

AZIDE INHIBITION OF MITOCHONDRIAL ELECTRON TRANSPORT

II. SPECTRAL CHANGES INDUCED BY AZIDE

D. F. WILSON

Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa. (U.S.A.)

(Received September 5th, 1966)

SUMMARY

Azide inhibition of coupled mitochondrial transport is accompanied by spectral changes which indicate that the cytochrome a_3 is oxidized and cytochrome *a* reduced. The cytochrome *a* α absorption band is shifted to shorter wavelengths in the azide-inhibited system. This shift in the α absorption band can be reversed by conditions leading to reduction of cytochrome a_3 such as uncouplers and anaerobiosis, or terminal inhibitors such as sulfide, cyanide or CO.

Titrations of the azide-induced spectral changes indicate the binding of one azide molecule in the complex, and that the dissociation constant is experimentally indistinguishable from the uncompetitive inhibitor constants for inhibition of State 3 respiration. The azide inhibition is postulated to involve the formation of a reduced cytochrome *a* azide compound which is unstable in the presence of reduced cytochrome a_3.

INTRODUCTION

The spectral properties of the cytochrome components of the respiratory chain of mitochondria have been extensively examined by CHANCE AND co-workers[1–8] both at room temperature and liquid nitrogen temperatures. The increased resolution afforded by the trapped steady-state technique[3–5] at liquid nitrogen temperatures provided evidence that the positions of the absorption maxima of cytochrome *b* are dependent on the energy state of pigeon-heart mitochondria. A form of reduced cytochrome *b* with absorption maxima at 424 mμ and 555 mμ was present under conditions of high energy (ATP or the inhibited state, *i.e.*, State 6) and a form with maxima at 430 mμ and 559 mμ under conditions of low energy (uncouplers, *etc.*).

Cytochrome *b* is also distinguished from the other cytochromes because the respiratory inhibitor, antimycin A, reacts with the respiratory chain to form a spectrally distinct form of the reduced cytochrome *b* (ref. 9) despite the failure of native cytochrome *b* to form recognizable compounds with cyanide, CO and other heme-binding agents. These properties are strong evidence for a dual role of cytochrome *b* in electron transport and energy conservation reactions. The cytochrome

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole.

oxidase ($a + a_3$) spectrum has been found to be affected by the high to low energy transition[7], but this has not been studied in detail.

The observation of a spectrally different form of reduced cytochrome *a* in azide-inhibited mitochondria[10,11] and the evidence for an increased rate of oxidation of this form in the presence of uncouplers of oxidative phosphorylation[10,11] give added interest to the azide inhibition. Therefore, a thorough study of the spectral changes during azide inhibition was undertaken in order to determine the characteristics of this cytochrome and to clarify its relationship to electron transport and oxidative phosphorylation.

METHODS AND MATERIALS

The methods and materials have been previously described[11].

RESULTS

The spectral changes at 445 mμ minus 455 mμ

The affinity of azide for the heme groups of hemoproteins and the spectral changes which accompany the formation of heme-azide compounds suggested the possibility of spectral changes caused by the azide inhibition. A tracing from the double-beam spectrophotometer (Fig. 1) indicates the changes observed at 445–455 mμ in a typical experiment. The mitochondria were suspended in the assay medium and respiration was started with succinate. Mineral oil was then added to cover completely the surface of the cuvette and the mitochondria were allowed to exhaust the oxygen by State 4 respiration. After anaerobiosis the spectrophotometer was reset and the sensitivity increased 2-fold. 400 μM ADP was then introduced through the oil surface

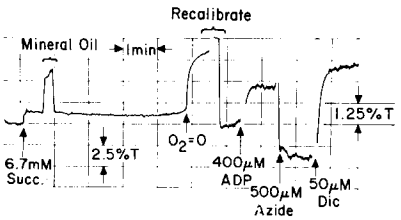


Fig. 1. The effect of ADP, azide and dicoumarol on the 445–455-mμ absorbance of anaerobic mitochondria. The respiration of a mitochondrial suspension (2.2 mg/ml) in a MST medium (pH 7.2) containing 200 μM EDTA, 10 mM orthophosphate, 70 μM Mg^{2+} and 3 μM rotenone was initiated by adding 6.7 mM succinate. Mineral oil was added to completely cover the reaction chamber to a depth of 3 mm and the State 4 respiration (48 μM O_2/min) was allowed to exhaust the oxygen. After anaerobiosis the spectrophotometer gain was increased 2-fold and the instrument rezeroed.

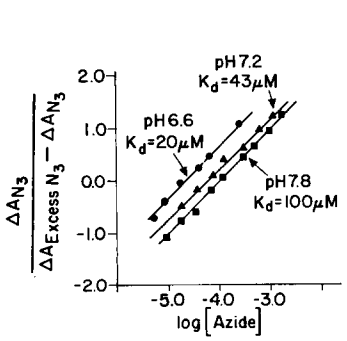
causing an apparent increase in reduction of cytochrome a_3 as differentiated from cytochrome *a* by the absence of a corresponding absorbance change at 605–575 mμ. This increased reduction of cytochrome a_3 is an expression of energy control of the reduction of a_3 since it could be observed with ADP + P_i, uncouplers or divalent cations It is not due to residual oxygen or oxygen diffusion because under conditions of equal steady-state oxygen uptake in the presence of azide and uncoupler, the change in absorbance at 445–460 mμ was rapid and complete on exhaustion of the

oxygen in solution. A rapid reduction of a_3 was also observed when 1 mM Mg^{2+} was present in the medium although this concentration of Mg^{2+} did not stimulate State 4 respiration.

When azide was added after the ADP an apparent oxidation was observed which, although not shown here, was stable for several minutes. This stability was observed for tightly coupled mitochondria in Mg^{2+}-free media or with Mg^{2+} concentrations less than the EDTA concentration, but not in media containing 1 mM Mg^{2+}. Oxygen introduced with the azide (about 2 μM) in this particular experiment can account for the initial oxidation but not the stability of the change since the addition of equal amounts of oxygen in the absence of azide causes only a transient oxidation and reduction (< 10 sec). Dicoumarol was added and the azide effect was reversed; the absorbance returning to slightly above that prior to the azide addition. This overshoot was not reproducible and the return was usually to the pre-azide level (Fig. 4). The concentration of azide used here would have inhibited the State 3 respiration to slightly less than State 4 respiration.

Titration of the azide-induced spectral change with azide

Several experiments were suggested by the tracing shown in Fig. 1, the first of which was the dependence of the decrease in absorbance at 445–455 mμ on the azide concentration. As may be seen in Fig. 2, the loss in absorbance can be titrated with azide to obtain apparent dissociation constants for an azide complex. The logarithm of the calculated ratio $\Delta A_{N_3}/(\Delta A_{\text{excess } N_3} - \Delta A_{N_3})$ is a linear function of the logarithm of azide concentration as shown in Fig. 2. The slopes of the lines are approximately one, indicating the binding of a single azide molecule in the complex. In the particular experiments shown, the apparent dissociation constant varied from 20 μM at pH 6.6 to 100 μM at pH 7.8. The absolute values of these dissociation constants at a given



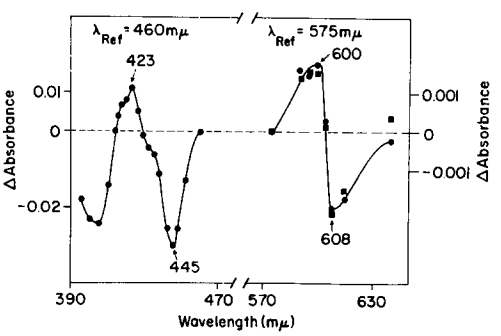


Fig. 2. Determination of the dissociation constant for the azide complex by titration of the absorbance change at 445–455 mμ. Assay conditions were the same as for Fig. 1, except that the protein concn. was 3.8 mg protein/ml. The mitochondria were anaerobic in the presence of 10 mM succinate and 400 μM ADP. The ordinate is the logarithm of the ratio of the absorbance change on addition of a known amount of azide to the difference between that value and the maximum absorbance change obtainable by azide addition under these conditions.

Fig. 3. The wavelength dependence of the spectral change induced by the addition of azide to anaerobic mitochondria in the presence of ADP and P_i. The effective bandwidths of the spectrophotometer beams were between 0.8 and 1.5 mμ. The experimental conditions were the same as for Fig. 1, except that the protein concentration was 3.3 mg protein/ml and no mineral oil covering was used for the assay chamber. ●, absorbance change on addition of azide. ■, the opposite sign change on subsequent addition of 50 μM dicoumarol.

pH vary somewhat from experiment to experiment, but the relative values as a function of pH remain the same. The magnitude of the decrease in absorbance (445–455 mμ) which can be obtained on addition of azide is approximately one-half of the increase in absorbance in the oxidized mitochondria (State 2) to anaerobic (+ADP).

The wavelength dependence of the azide-induced spectral change at room temperature

The wavelength dependence of the azide-induced spectral change is shown in Fig. 3, and was determined by adding a constant amount (500 μM) of azide to anaerobic mitochondria at pH 7.2 and varying the wavelength pair of the double-beam spectrophotometer. Each point represents a separate measurement. The absorbance change in the Soret region was with respect to a reference wavelength of 460 mμ while that in the α region was for a different mitochondrial preparation and for a reference wavelength of 575 mμ. In the Soret region the minimum at 445 mμ and the maximum at 418 mμ are typical of the oxidized—reduced spectrum of cytochrome oxidase. The distortions of the spectrum at 435 mμ and 423 mμ are probably due to small changes in the spectra of cytochromes *b* and c_1. The changes in the α region can best be interpreted as a blue shift of this absorption peak of cytochrome *a* of less than 8 mμ. The absolute magnitude of the changes measured in the α region were very small, so advantage was taken of the uncoupler reversal of the azide-induced change. The changes were measured on addition of azide; 50 μM dicoumarol was added next and the absorbance again measured. Although these two measurements did not give identical values, they were only slightly different. In Fig. 3 the changes are superimposed to allow direct comparison although their algebraic sum is approx. 0.

The dependence of the reversal of the azide-induced spectral change on uncoupler concentration

The loss of the azide-induced spectral change at 445–455 mμ caused by uncouplers was a function of the concentration of the added uncoupler as shown in Fig. 4. In this experiment, the mitochondria were allowed to become anaerobic in the presence of ADP; then 500 μM azide was added. Approx. 1 min later, concentrations of the uncoupler carbonylcyanide *m*-chlorophenylhydrazone (CCCP) were added which varied from 0.04 μM to 0.17 μM. The rate of loss of the azide-induced spectral change clearly increased with increasing uncoupler concentration but the transition had no simple kinetic order with respect to time and could not be evaluated

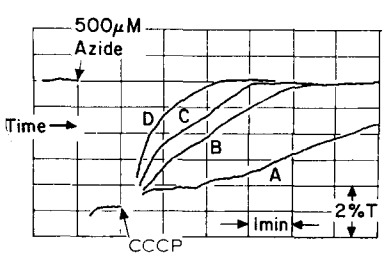


Fig. 4. The effect of CCCP on the azide-induced spectral change at 445–455 mμ. The reaction medium was the same as for Fig. 1, except that the mitochondrial suspension contained 1.6 mg protein/ml. The final CCCP concn. (mμM) were 41.6, 83.4, 125 and 167 for Curves A, B, C, and D, respectively.

quantitatively. This reversal of the azide-induced spectral change would be measured at concentrations of the uncouplers normally used for partial uncoupling; for example, comparable rates for the reversal were observed for 2.2 μM 4-octyl-2,6-dinitrophenol, 2 μM dicoumarol, 13 μM 2,4-dinitrophenol and 0.07 μM CCCP. These values have the same relationship as those reported for the release of azide inhibition of State 3 respiration by uncouplers as measured by steady-state oxygen consumption[10,11].

The dependence of the uncoupler reversal of the azide-induced spectral change on azide concentration

The azide concentration dependence of the uncoupler-induced change in Fig. 1 was measured by adding varying amount of azide to the anaerobic mitochondria with excess ADP and adding a constant amount of uncoupler. Table I is for 0.084 μM CCCP at pH 7.2. The rate of absorbance change could only be estimated (Fig. 4), but when these rates were estimated for azide concentrations between 41 and 1500 μM and corrected for the dissociation constant of the azide complex (Fig. 2) they were independent of the azide concentration. Thus, the uncouplers react not with the azide complex itself, but with a form of the energy-coupled electron transfer system which makes possible the azide spectral shift. The binding of the azide does not noticeably influence the uncoupler reaction.

TABLE I

THE EFFECT OF AZIDE CONCENTRATION ON THE UNCOUPLER-DEPENDENT LOSS OF THE AZIDE-INDUCED SPECTRAL CHANGE

The rat-liver mitochondria were 1.6 mg protein/ml in MST medium (pH 7.2). Succinate and orthophosphate at 10 mM were present and 500 μM ADP was added after anaerobiosis. The uncoupler was 0.084 μM CCCP and the measuring wavelengths 445–455 mμ.

Azide concn. (μM)	*Rate (arbitrary units/min)*	*Correction factor from dissociation constant*	*Corrected rate (arbitrary units/min)*
41	1.5	0.24	6.2
83	3.1	0.4	7.7
125	2.9	0.53	5.5
500	4.5	0.8	5.6
1000	4.9	0.89	5.5
1500	5.7	0.95	6.0

The trapped steady-state spectra

The spectral characteristics of the mitochondria at liquid nitrogen temperatures were examined using the trapped steady-state technique[3]. The upper curve of Fig. 5 is a difference spectrum of mitochondria reduced by succinate and anaerobiosis *minus* mitochondria in State 2. The typical low temperature cytochrome spectra are evident with maxima or shoulders at 601 mμ, 559 mμ, 548 mμ in the visible region representing cytochromes $a + a_3$, b, c_1 and c, respectively, and at 443 mμ and 419 mμ in the Soret region, representing cytochromes $a + a_3$ and $c + c_1$, respectively. In the middle curve, the dramatic change due to azide is readily apparent. The α peak of cytochrome a has shifted from 601 and 595 mμ and is greatly sharpened. In the Soret region, the

smooth maximum at 443 mμ of cytochromes $a + a_3$ has been replaced by double maxima at 446 and 437 mμ of much reduced total absorbance. These changes are more evident in the lower curve of Fig. 5, which is a directly recorded difference spectrum in which azide is also present in the anaerobic sample. The blue shift of

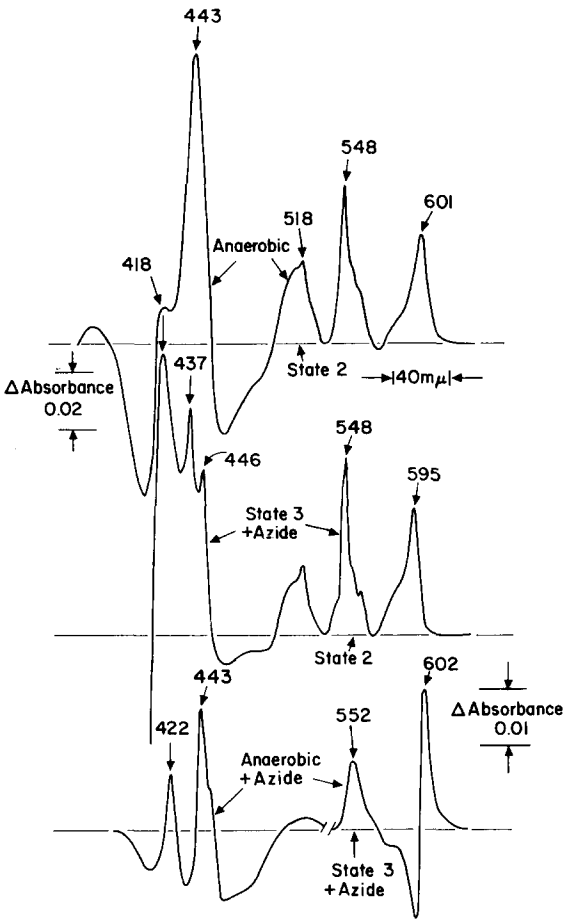


Fig. 5. The liquid nitrogen temperature spectra of rat-liver mitochondria during azide inhibition. The mitochondria were suspended at 7.7 mg protein/ml in MST medium (pH 7.2) containing 10 mM orthophosphate, 2 mM ADP and 5 μM rotenone. Upper curve: anaerobic mitochondria in the presence of 15 mM succinate and 10 μM 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB) *minus* aerobic mitochondria containing TTFB and no succinate. Middle curve: aerobic mitochondria in the presence of 15 mM succinate, 10 μM TTFB and 500 μM azide *minus* aerobic mitochondria in the presence of TTFB but absence of azide and succinate. Lower curve: anaerobic mitochondria in the presence of 15 mM succinate, 10 μM TTFB and 500 μM azide *minus* aerobic mitochondria in the presence of 15 mM succinate, 10 μM TTFB and 500 μM azide.

the α peak appears as a minimum at 595 mμ and a maximum at 602 mμ. The Soret region is characterized by the appearance of absorption bands with a maximum at 443 mμ and at 422 mμ. The 443-mμ band is due to a reduction of cytochrome a_3 while the 422-mμ band is variable in intensity depending on the experimental conditions and is due in part to cytochromes *b* and b_5 reduction after anaerobiosis. The

uncouplers change the rate at which the fully reduced spectrum is attained but not the spectrum itself. The blue shift in the α maximum is also reversed by adding inhibitors which form reduced cytochrome a_3 compounds such as sulfide, cyanide and CO.

The question was then asked if the form of cytochrome *a* which was observed in the presence of azide was a mandatory intermediate in electron transfer or if uncouplers could stimulate respiration without the formation of the a_{596}. Cytochrome a_{596} was found to be the dominant form of reduced cytochrome *a* present whether the respiration was occurring in the presence of 50 μM dicoumarol, 1 μM CCCP, or 2 % cholate. This observation is consistent with the previous data indicating that the increased rate of oxidation of reduced cytochrome *a* in the presence of uncouplers was due to their ability to stimulate the oxidation of cytochrome a_{596}. Cytochrome a_{596} is characterized by the sharp α peak and a double Soret peak with nearly equal maxima at 447 mμ and 438 mμ. This is the low temperature counterpart of the room temperature reduced—oxidized difference spectrum as published by CHANCE AND WILLIAMS[12] where the Soret peak had a single maximum at 444 mμ and the α peak was at 602 mμ. In the present work the room temperature difference spectrum had an α band at 602 mμ and a Soret band at 442 mμ with an asymmetry on the long-wavelength side. The ratio of the Soret absorbance to that of the α band was between 4 and 5.

The possibility that the double Soret maximum was an artifact introduced by cytochrome *b* reduction was eliminated by using uncouplers to keep the cytocrome *b* oxidized in the steady state[11]. The trapped steady-state spectrum of the aerobic steady state in the presence of 800 μM azide *minus* the aerobic steady state in the absence of azide clearly shows the cytochrome a_{596}.

Identification of the cytochrome responsible for the Soret maxima at 447 mμ and 438 mμ

The identification of the split Soret maximum as a reduced form of cytochrome *a* and not a reduced cytochrome a_3–azide compound was made by comparison of its spectrum with that shown in Fig. 6. The latter is a liquid nitrogen temperature spectrum of a suspension of sperm from the sea urchin *Lytichinus pictus* in aerobic sea water *versus* the sperm frozen 20 sec after the addition of dithionite. The double Soret maximum at 447 and 438 mμ is clearly visible although no azide is present.

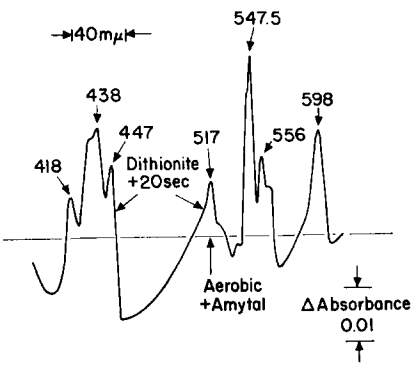


Fig. 6. The liquid nitrogen temperature spectrum of sperm of the sea urchin, *Letichinus pictus*. An aerobic sperm suspension at 15.5 mg protein/ml in sea water containing 3 mM Amytal *minus* the same suspension frozen 20 sec after the addition of dithionite.

If more time is allowed after dithionite addition before freezing the sperm (more than 2 min) the cytochrome a_3 becomes reduced and a single Soret maximum of greater intensity is observed with a maximum at 445 mμ and a shoulder at 439 mμ (ref. 13). The sperm were used for this experiment because their cytochrome oxidase is spectrally very similar to mammalian cytochrome oxidase and yet the cytochrome a is reduced much more rapidly on addition of dithionite than is cytochrome a_3 (ref. 13). It is, therefore, possible to obtain a difference spectrum for cytochrome a without interference by cytochrome a_3 or the use of artificial inhibitors of cytochrome oxidase.

Recently R. LEMBERG AND M. GILMOUR (personal communcation) using isolated soluble cytochrome oxidase have noted a splitting of the Soret maximum of cytochrome a at liquid nitrogen temperatures when cytochrome a is reduced and a_3 oxidized.

DISCUSSION

The inhibition of coupled mitochondrial electron transport by azide has previously been shown to cause a crossover between cytochromes a and a_3 (refs. 10, 11) indicating an inhibition of the reduction of a_3. This inhibition, as measured by the State 3 steady-state oxygen uptake, is released by uncouplers of oxidative phosphorylation to the extent that the azide concentration for half-maximal inhibition is at least 10-fold greater for the uncoupled than for the coupled system.

Spectral observations made after anaerobiosis indicated that in the presence of ADP and P_i, the addition of azide causes an oxidation of cytochrome a_3 with the rest of the cytochromes remaining reduced. In agreement with the release of azide inhibition by uncouplers, the uncouplers reverse the azide-induced spectral change causing a return to the fully reduced spectrum. Titrations of the induced spectral change with azide can best be interpreted as the reversible formation of an azide compound containing one azide molecule and having a dissociation constant indistinguishable from the inhibitor constant for State 3 respiration.

It is unlikely that azide forms a reduced cytochrome a_3–azide compound since the Soret maxima at 447 and 438 mμ ($-190°$) are due to reduced cytochrome a alone, and no other absorption bands are observed in the Soret region which can be attributed to a reduced from of a_3. For such a mechanism of inhibition a competition between azide and CO would also be expected and this is not observed[14]. The possibility that azide forms a complex with oxidized cytochrome a_3 cannot be excluded on spectral grounds but it is difficult on that basis to explain the effects of the addition of ADP and orthophosphate on the azide inhibition. In as much as the addition of ADP and orthophosphate to State 4 mitochondria causes an increased steady-state reduction of cytochromes a and a_3 (refs. 1, 15), an inhibitior (azide) reacting with oxidized cytochrome a_3 would not be expected to cause reversed control of respiration by inhibiting State 3 more than State 4.

The rather unexpected control of cytochrome a_3 reduction at anaerobiosis by ADP in low-Mg^{2+} media would suggest that this is expression of the reaction inhibited by azide. It would, therefore, not be surprising to find a State 4 to 3 crossover between cytochromes a and a_3 in mitochondria from other sources since this would require only a slight change in relative reaction rates. The crossover between cytochrome a_3 and oxygen postulated by RAMIREZ[16] for intact lobster-heart muscle is not

consistent with the data here and would require a completely different mechanism. In view of the difficulty in differentiating cytochrome *a* from a_3 at the wavelength used by RAMIREZ (445 mμ), it is possible that the spectral changes observed by RAMIREZ were also consistent with a crossover between cytochrome *a* and oxygen.

One possible site of azide inhibition which is consistent with the available data is the formation of a reduced cytochrome *a*–azide compound. If this were the case, the Soret absorption bands in the difference spectra would be those of cytochrome *a* and the α band at 595 mμ would be displaced by the formation of the azide compound. Reduction of cytochrome a_3 would then be postulated to change the structure of cytochrome *a* to the extent that azide would be replaced by a 'native' ligand. Uncouplers would act by stimulating transfer of electrons directly from the cytochrome *a*–azide compound to cytochrome a_3, a reaction which is normally very slow. Cytochrome *a* has been regarded as unreactive toward heme-binding agents, but this must no longer be considered a serious argument against the formation of a cytochrome *a*–azide compound. LEMBERG[17] has observed that in the 'oxygenated' compound of OKUNUKI[18] the cytochromes *a* and a_3 are spectrally indistinguishable, having a common Soret band at 428 mμ, and that both must be active in forming the 'oxygenated' compound.

It is interesting to note that many hemoproteins have been reported to have split α and β absorption maxima at liquid nitrogen temperatures[19,20] but the Soret absorption maximum is normally single. Cytochrome *a* is most unusual in having a single α maximum and a split Soret maximum. No similar splitting has been reported for isolated heme *a* or other formyl-substituted hemes. CLEZY AND MORELL[21,22] found two Soret bands in the room temperature spectra of ether solutions of hemin chlorides but not for the corresponding hemochromes.

The greater splitting observed for cytochrome *a* than for cytochrome a_3 (Figs. 5B, C) although the heme groups are identical[23] would suggest that the splitting is the result of protein–heme interaction rather than being a property of the heme itself. Such an interpretation assumes that the Soret absorption of hemochromes is the summation of contributions from two separate electronic transitions which normally have very similar energies. In the case of cytochrome *a* the protein causes separation of the transition energies, possibly by distortion of the porphyrin plane or by providing a non-polar environment or both.

ACKNOWLEDGEMENTS

The author is grateful to Prof. B. CHANCE for his guidance and encouragement and to Dr. T. YONETANI and Prof. R. LEMBERG for many stimulating discussions and helpful suggestions. This work was supported by U.S. Public Health Service research grants GM 12202 and 5-F2-GM 18,154.

REFERENCES

1 B. CHANCE AND G. R. WILLIAMS, *Advan. Enzymol.*, 17 (1956) 65.
2 B. CHANCE, *Proc. Intern. Congr. Biochem., 3rd, Brussels, 1955*, Academic Press, New York, 1956, p. 300.
3 B. CHANCE AND E. L. SPENCER, Jr., *Discussions Faraday Soc.*, 27 (1959) 200.
4 R. W. ESTABROOK, in J. E. FALK, R. LEMBERG AND R. K. MORTON, *Haematin Enzymes*, Vol. 2, Pergamon Press, New York, 1961, p. 436.

5 B. CHANCE, *Federation Proc.*, 22 (1963) 404.
6 B. CHANCE AND B. SCHOENER, *J. Biol. Chem.*, 241 (1966) 4567.
7 B. CHANCE, C. P. LEE AND B. SCHOENER, *J. Biol. Chem.*, 241 (1966) 4574.
8 B. CHANCE AND B. SCHOENER, *J. Biol. Chem.*, 241 (1966) 4577.
9 B. CHANCE, *J. Biol. Chem.*, 233 (1958) 1223.
10 D. F. WILSON AND B. CHANCE, *Biochem. Biophys. Res. Commun.*, 23 (1966) 751.
11 D. F. WILSON AND B. CHANCE, *Biochim. Biophys. Acta*, 131 (1967) 421.
12 B. CHANCE AND G. R. WILLIAMS, *J. Biol. Chem.*, 221 (1956) 477.
13 D. F. WILSON AND D. EPEL, in preparation.
14 R. J. WINZLER, *J. Cellular Comp. Physiol.*, 21 (1943) 229.
15 B. CHANCE AND G. R. WILLIAMS, *J. Biol. Chem.*, 217 (1955) 409.
16 J. RAMIREZ, *Biochim. Biophys. Acta*, 88 (1964) 648.
17 M. R. LEMBERG, in B. CHANCE, R. W. ESTABROOK AND T. YONETANI, *Hemes and Hemoproteins*, Academic Press, New York, 1966, p. 477.
18 I. SEKUZU, S. TAKEMORI, T. YONETANI AND K. OKUNUKI, *J. Biochem.*, 46 (1959) 43.
19 D. KEILIN AND E. F. HARTREE, *Nature*, 164 (1949) 254.
20 J. KEILIN, in B. CHANCE, R. W. ESTABROOK AND T. YONETANI, *Hemes and Hemoproteins*, Academic Press, New York, 1966, p. 173.
21 D. B. MORELL AND P. S. CLEZY, *Biochim. Biophys. Acta*, 71 (1963) 157.
22 P. S. CLEZY AND D. B. MORELL, *Biochim. Biophys. Acta*, 71 (1963) 165.
23 D. B. MORELL, J. BARRETT AND P. S. CLEZY, *Biochem. J.*, 78 (1961) 739.