

AN ABSTRACT OF THE THESIS OF

Morio Kuboyama for the Doctor of Philosophy in Biochemistry
(Name) (Degree) (Major)

Date thesis is presented October 26, 1964

Title MAMMALIAN HEART CYTOCHROME OXIDASE

Abstract approved

Preparation of a cytochrome c-cytochrome oxidase complex was achieved, using crystalline beef cytochrome c and purified cytochrome oxidase from beef heart muscle, or from the Keilin-Hartree preparation. Succinate oxidase was reconstituted with the complex.

A mixture of cytochrome oxidase, solubilized with non-ionic detergent, and excess cytochrome c was exposed to sonic irradiation. The cytochrome c-cytochrome oxidase complex was isolated using gel filtration of the mixture. The ratio of the concentration of cytochrome c to cytochrome oxidase in the complex was increased with the length of sonic treatment. The complex with the maximum ratio of one was separated after a 45-60 minute period of sonic irradiation.

Differential sedimentation and chromatography on Sephadex showed the complex to be an integrated entity of two components.

The complex could be formed with cytochrome oxidase and either intact cytochrome c or guanidinated cytochrome c. However, both acetylated cytochrome c and succinylated cytochrome c completely failed to form any kind of complex with cytochrome oxidase. These results indicate that the interacting force between the two components is mainly electrostatic.

Absolute and difference absorption spectra of the carbon monoxide compound of the complex showed a unique, distinct maximum at 415 m μ . On the other hand, a "free mixture" of both components exhibited no absorption peak at the same wave length in the presence of CO. The formation of this CO compound was prevented by sodium cholate. After separation by means of cation exchange resin, neither component of the complex showed the absorption maximum at 415 m μ in the presence of CO. From these and other observations, the possibility of a conformational change in the protein moiety of the cytochromes as a result of the interaction was hypothesized in reference to the electron transport mechanism.

Infrared spectrum of the complex showed no superposition of absorption bands of the two components but showed a unique band at 950 and at 1050 cm^{-1} .

The physiological activity of the complex was verified by the functional reconstitution of succinate oxidase with soluble succinate dehydrogenase, the cytochrome b-c₁ particle, and the complex. The reconstituted succinate oxidase was

inhibited by the usual respiratory inhibitors in the same manner as the Keilin-Hartree heart muscle preparation. The carbon monoxide inhibition of the reconstituted succinate oxidase was reversed by light. The behavior of the complex in the oxidation of ascorbate was the same as that of the heart muscle preparation. Addition of a catalytic amount of tetramethyl-p-phenylenediamine dramatically stimulated ascorbate oxidation.

The cytochrome oxidase (cytochrome a plus cytochrome a₃) was functionally and structurally reconstituted from cytochrome a and intact heme a. About sixty percent of the original cytochrome oxidase activity was recovered from an incubation mixture of cytochrome a, ascorbate-EDTA, cytochrome c and hematin a. Hematin a was prepared from cytochrome oxidase or directly from heart muscle mince by means of metalation of purified porphyrin a. After removal of excessive hematin a by Sephadex chromatography, the reconstituted oxidase showed spectral properties almost identical to those of the intact preparation; a slight difference was found at the α -band.

MAMMALIAN HEART CYTOCHROME OXIDASE

by

MORIO KUBOYAMA

A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

DOCTOR OF PHILOSOPHY

June 1965

APPROVED:

Professor of Chemistry

In Charge of Major

Chairman of Department of Chemistry

Dean of Graduate School

Date thesis is presented October 26, 1964

Typed by Clistie Stoddard

ACKNOWLEDGEMENT

The author wishes to express his deep appreciation to Professor Tsoo E. King for his most careful guidance and continued interest. Without his penetrating insight and generous patience, none of this work would have been done.

The author is also grateful to Dean Vernon H. Cheldelin for his kind interest and generosity.

Acknowledgement is due colleagues at Science Research Institute for their advice, especially Dr. Pat W. Camerino and Mrs. Carole M. Staneslow for their help in the preparation of the thesis.

The author is indebted to the U. S. Educational Commission in Japan and the U. S. Public Health Service for financial support during his study at Oregon State University.

TABLE OF CONTENTS

	Page
GENERAL INTRODUCTION-----	1
1. CYTOCHROME <u>c</u> -CYTOCHROME OXIDASE COMPLEX	
INTRODUCTION-----	6
EXPERIMENTAL PROCEDURES	
Materials-----	8
Methods-----	10
RESULTS	
Preparation of Cytochrome <u>c</u> -Cytochrome Oxidase Complexes and Electronic Absorption Spectra of the Complex-----	15
Infrared Spectra of the Complex and Individual Components-----	28
Integrity of the Cytochrome <u>c</u> -Cytochrome Oxidase Complex; Dissociation of the Complex-----	35
Complex Formation of Chemically Modified Cytochrome <u>c</u> with Cytochrome Oxidase-----	40
CO-compound and CN-compound of the Complex-----	41
Ascorbate Oxidation with Cytochrome <u>c</u> -Cytochrome Oxidase Complex and TMPD Stimulation-----	53
DISCUSSION-----	56
2. RECONSTITUTION OF SUCCINATE OXIDASE WITH CYTOCHROME <u>c</u> - CYTOCHROME OXIDASE COMPLEX	
INTRODUCTION-----	64
EXPERIMENTAL PROCEDURES	
Materials-----	65
RESULTS	
Reconstitution of Succinate Oxidase with Cytochrome <u>c</u> -Cytochrome Oxidase Complex, Solu- ble Succinate Dehydrogenase and Cytochrome <u>b-c</u> ₁ Particle; Turnover Number-----	66

LIST OF FIGURES

Figure	Page
1 Sephadex column chromatography of the sonically irradiated mixture of cytochrome <u>c</u> and cytochrome oxidase-----	18
2 Elution pattern of the cytochrome <u>c</u> -cytochrome oxidase complex of molar ratio 1 from a Sephadex column-----	20
3A Absorption spectra of cytochrome <u>c</u> -cytochrome oxidase complex-----	24
3B The absolute absorption spectra of the complex (1:1) in the ultraviolet region-----	25
4 Difference spectra of the cytochrome <u>c</u> -cytochrome oxidase complex-----	27
5 Infrared spectrum of cytochrome <u>c</u> -cytochrome oxidase complex-----	30
6 Infrared spectrum of cytochrome <u>c</u> -----	32
7 Infrared spectrum of cytochrome oxidase-----	34
8 Dissociation of cytochrome <u>c</u> -cytochrome oxidase complex at different ionic strengths----	39
9 CO difference spectrum of cytochrome <u>c</u> -cytochrome oxidase complex and effect of cholate	44
10 Absorption spectra of cytochrome oxidase after 50 minutes of sonic irradiation-----	46
11 Absorption spectra of cytochrome <u>c</u> separated from the cytochrome <u>c</u> -cytochrome oxidase complex-----	48
12 CO spectra: (A) The cytochrome <u>c</u> -cytochrome oxidase complex, (B) Reconstituted succinate oxidase, (C) The Keilin-Hartree heart muscle preparation-----	50
13 CN compound of the cytochrome <u>c</u> -cytochrome oxidase complex-----	52

LIST OF FIGURES (continued)

Figure	Page
14 Ascorbate oxidation with the cytochrome <u>c</u> - cytochrome oxidase complex and with the Keilin- Hartree heart muscle preparation-----	55
15 Replotted CO difference spectrum of Ball's "electron transmitter system"-----	59
16 Reconstitution of succinate oxidase with succinate cytochrome <u>c</u> reductase and cytochrome <u>c</u> -cytochrome oxidase complex-----	69
17 Effect of the concentration of the cytochrome <u>c</u> - cytochrome oxidase complex on the activity of the reconstituted succinate oxidase-----	71
18 Photo-irradiation effect on the carbon monoxide inhibited reconstituted succinate oxidase and the Keilin-Hartree heart muscle preparation-----	76
19 Optimal pH for succinate oxidase activity of the reconstituted succinate oxidase-----	81
20 Optimal pH for succinate oxidase activity of the Keilin-Hartree heart muscle preparation-----	83
21 Absorption spectra of cytochrome <u>a</u> prepara- tion-----	93
22 Reconstitution of cytochrome oxidase from cytochrome <u>a</u> and hematin <u>a</u> -----	96
23 Absolute spectra of reconstituted cytochrome oxidase-----	99
24 Effect of cyanide and carbon monoxide on abso- lute spectrum of reconstituted cytochrome oxidase-----	102

LIST OF TABLES

Table		Page
I	Properties of cytochrome oxidase preparation-----	9
II	Preparation of cytochrome <u>c</u> -cytochrome oxidase complexes-----	21
III	Formation and activity of the cytochrome <u>c</u> - cytochrome oxidase complex from chemically modified cytochrome <u>c</u> -----	41
IV	Turnover number of the reconstituted succinate oxidase-----	73
V	Inhibition of reconstituted succinate oxidase----	74
VI	Comparison of the activity of the cytochrome <u>c</u> - cytochrome oxidase complex with the free compo- nents in the reconstitution of succinate oxidase	78
VII	Systems used for "reconstitution" of cytochrome oxidase from cytochrome <u>a</u> and hematin <u>a</u> -----	94

MAMMALIAN HEART CYTOCHROME OXIDASE

GENERAL INTRODUCTION

In aerobic living systems, from plant to mammal, cellular respiration--oxygen metabolism--is one of the most important biological functions. In the chemical sense, the overall picture of this catabolic process consists of a "fuel cell" which causes a flow of four electron equivalents with resultant reduction of one molecule of oxygen.

This energy flow in the open system is phenomenologically manifested by a steady state process as the "fate" of life in general. Thus, one would expect the respiratory function to be reflected in a highly organized structural arrangement on the part of the living cell. The electron transport system in the subcellular organelle (the mitochondrion) has been postulated a priori as a sequential assembly of elementary mechanisms at the molecular level.

The terminal unit of the chain, directly connected with molecular oxygen, has been characterized as cytochrome oxidase, which may be functionally defined as the enzyme which catalyzes the oxidation of reduced cytochrome c with molecular oxygen, and which is sensitive to carbon monoxide, azide and cyanide.

Warburg recognized an iron-compound, sensitive to carbon monoxide, as a respiratory enzyme which was later called

"oxygen transferring enzyme" (87-89). Keilin and Hartree suggested that Warburg's respiratory enzyme was identical with cytochrome a₃ (cytochrome oxidase) (25-33).

These workers established the conceptual and operational vision of the terminal oxidative enzyme system. Kinetic and thermodynamic studies of the enzyme followed and yielded a wealth of information on this terminal step of the chain. The cytochrome oxidase study has been extensively reviewed by Wainio (92), Chance (10), Slater (74), Green (16), Lemberg (53), Okunuki (68), and Yonetani (99).

Slater et al. (76) recently exemplified the three lines of approach to the reaction mechanism between cytochrome c and cytochrome oxidase, in mentioning: (a) kinetic studies by Minnaert, (b) physical studies by the present author et al., and (c) thermodynamic studies by Wainio and Minnaert.

Minnaert (61, 62) formulated a mechanism to explain the kinetic anomaly of cytochrome c-cytochrome oxidase interaction by assuming the existence of a one to one stoichiometric complex between the substrate and the enzyme. However, his mechanistic prediction remained to be experimentally verified.

Wainio (91) attempted to determine an equilibrium constant for the reaction between cytochrome c and cytochrome a. Minnaert (76) also studied this aspect and proposed the equilibrium formulation which gave him a constant value.

Nonetheless, he failed to correctly explain the form of the equilibrium constant.

The awareness of danger in a kinetic approach and the importance of physical, circumstantial evidence to decipher the kinetic data have been fully discussed by King, especially in reference to the electron transport chain enzyme system (44).

With this in mind, some important features of the cytochrome oxidase will be briefly reviewed in order to depict "die Problematik" of this enzyme.

Cytochrome c-Cytochrome Oxidase Complex

The existence of some kind of complex between cytochrome c and cytochrome oxidase has been postulated by several workers (15, 20, 61, 66, 69, 77, 78).

Stotz, Altschul and Hogness (78) have proposed that a cytochrome c-cytochrome oxidase complex of the Michaelis type was formed in the aerobic oxidation of cytochrome c. However, this proposal has been seriously criticized (73). Okunuki et al. (68, 69) consider a hypothetical complex of cytochrome c and cytochrome oxidase to be an active cytochrome oxidase system. From a completely different line of attack, Gibson et al., using their rapid flow technique for the presteady and steady states analyses, have reached the conclusion that in the reaction of cytochrome c and cytochrome oxidase an

active complex is obligatorily formed (15).

On the other hand, Smith and Conrad (77) have suggested an inhibitory complex in order to explain their kinetic results. Hollocher has re-examined the Smith and Conrad data by employing a kinetic derivation which considers the reversible formation of inactive complexes between cytochrome c and cytochrome oxidase. He has reached the conclusion that the predominant species of kinetically inert complex is one to one, and has an association constant in the order of 10^5 M^{-1} (20). There can be seen a striking contradiction between these two kinetic predictions of the property of the complex; one predicts an active complex, the other predicts an inactive complex.

Reconstitution of Respiratory Chain Enzyme

The organization of the respiratory chain was first conceived by Keilin as early as 1925 (25). Indeed, the discovery of multiple cytochromes in the chain is the most clear reflection of the concept of sequential transfer of electrons. Subsequent kinetic studies have made a quantitative link between the functional manifestation and structural arrangement of the components. However, the reconstitution--that is, isolation of the fragments and reconstruction of the original assembly--has proved a most powerful tool for study of the respiratory chain system, as when Keilin and King first

succeeded in the reconstitution of succinate-oxidase (35, 36). The significance of a reconstitutive approach was fully discussed by King (38, 39, 41) and, recently, by Ernster and Lee (15). King et al. (45) further reported on the systematic fragmentation and reconstitution of succinate oxidase.

Cytochrome a and a₃

In 1939, Keilin and Hartree reported on the spectral and functional resolution of cytochrome oxidase into components designated cytochromes a and a₃ (32). Subsequent kinetic studies have centered on the question of the identity or non-identity, functional as well as structural, of cytochromes a and a₃ and have ended in polemical semantics. However, it is very difficult to visualize the functional difference without a structural differentiation of these two enzymes in such a highly organized system.

It is the purpose of this thesis to present the results of inquiries into those problems most relevant to cytochrome oxidase, mainly by means of the physical and biochemical approach. The study consists of (1) preparation of the cytochrome c-cytochrome oxidase complex and its characterization; (2) reconstitution of succinate-oxidase with the cytochrome c-cytochrome oxidase complex; (3) reconstitution of cytochrome oxidase with cytochrome a and hematin a.

1. CYTOCHROME c-CYTOCHROME OXIDASE COMPLEX (49, 50, 51)INTRODUCTION

The existence of a cytochrome c-cytochrome oxidase complex is deducible from the concept of the respiratory chain. However, the isolation of the complex has been unsuccessful. Cytochrome c is easily leached from respiratory particles in the presence of dilute salt solutions (14, 86), especially bile salts (3). This unfavorable property is further aggravated by the insolubility of cytochrome oxidase. These characteristics make the isolation and purification of this segment of the respiratory chain directly from mitochondrial preparations extremely difficult, if not impossible.

Thus, an approach by reaction of cytochrome c with cytochrome oxidase has been undertaken, since both cytochrome c and cytochrome oxidase have been purified to a stage without contamination by other respiratorily active components but with physiological activities. The recent findings show that cytochrome oxidase is an acidic protein (79), whereas cytochrome c is strongly basic and comparatively small in size (56, 58). The complex could be formed by electrostatic attraction, perhaps also being reinforced by other bindings.

Moreover, the molecular sizes and physical behaviors differ widely. Thus the complex, if indeed formed, may be separated from cytochrome c and cytochrome oxidase under

suitable conditions by means of molecular sieve chromatography (19).

This part of the thesis describes the actual separation of the cytochrome c-cytochrome oxidase complex of 1:1 molar ratio from the reaction mixture of the two components. Other forms of the complex are also found which are evidently dependent on the polymerization state of the cytochrome oxidase employed. Finally, the characteristics of the complex are presented.

EXPERIMENTAL PROCEDURES

Materials

Enzyme Preparations -- The Keilin-Hartree heart muscle preparation from beef heart was obtained as described by King (39). The purified cytochrome oxidase was prepared from beef heart muscle in principle according to the method of Okunuki et al. (67). After refractionation by ammonium sulfate, the sample was dissolved in a minimal amount of Emasol for clarification (96), and subjected to dialysis against 0.01 M sodium phosphate buffer (pH 7.4) for 12 hours at 0-4° C to remove cholate, and then dialyzed against glass-distilled water for another 12 hours. The preparation became cloudy as detergent was removed. Immediately after dialysis, the sample was lyophilized to complete dryness and kept in the desiccator at 0-4° C (98). The yield was 1.5 to 2.0 grams from 5 kgs. of fresh beef heart mince.

An alternative isolation of cytochrome oxidase from the Keilin-Hartree heart muscle preparation was developed. The sample from the Keilin-Hartree heart muscle preparation was about 20% more active than that prepared by the previous method from beef heart. The average composition and specific activity are shown in Table I. Crystalline cytochrome c was made from beef heart muscle by the methods of both Hagihara et al. (18) and Margoliash (60). The final product was

TABLE I

Properties of cytochrome oxidase preparation

Iron	9.27 μ atoms/gm
Copper	9.83 μ atoms/gm
Ratio: copper/iron	1.03
Lipid	19.4%
Protein	80.5%
Specific activity	56 electron equivalents/mole cytochrome oxidase/sec

lyophilized and kept at 0-4° C, desiccated. The chemically modified cytochrome c, i.e. acetylated-, succinylated-, and guanidinated-cytochrome c, was prepared after Takemori et al. (80).

Reagents -- Emasol No. 1130 (polyoxyethylene-sorbitan-monolaurate), a nonionic detergent, was kindly supplied by Kao Soap Company, Tokyo. Sephadex G-75 and G-200 were purchased from Pharmacia, Uppsala, Sweden. Polyvinylpyrrolidone was procured from K & K Laboratories, Inc., New York; Amberlite IRC-50 from Rohm & Haas Company; carbon monoxide of C. P. grade, from the Matheson Company, was used without further purification; and TMPD (tetramethyl-p-phenylenediamine dihydrochloride) from Eastman Organic Chemicals. L-ascorbic acid and ethylenediamine tetra-acetate were purchased from

Fisher Scientific, Fair Lawn, New Jersey, and Sigma Chemical, St. Louis, respectively.

Methods

The concentrations of cytochrome c and cytochrome oxidase (in terms of heme a) were determined spectrophotometrically according to the difference (reduced minus oxidized) of the absorbancy indices of $19.0 \times \text{mM}^{-1} \times \text{cm}^{-1}$ at 550 m μ (55) and $11.0 \times \text{mM}^{-1} \times \text{cm}^{-1}$ at 605 m μ , respectively with Cary model 11, Applied Physics Corporation, Monrovia, California, or Zeiss Spectrophotometer PMQ 11, Carl Zeiss, Oberkochen/Württ, Germany. On determination of the concentration of one cytochrome component in the presence of the other, it was observed that at a concentration as low as 3 μM of both no significant interference was detected in either the reduced or oxidized form of the purified enzyme in 0.1% Emasol 1130 (in phosphate buffer, pH 7.4). That is, both the contribution of cytochrome c to the difference in absorbancy (reduced minus oxidized) at 605 m μ and the contribution of cytochrome oxidase to the difference in absorbancy (reduced minus oxidized) at 550 m μ are negligible at the above mentioned concentration of each component. The absorbancy of oxidase reduced with dithionite was measured at least five minutes after the addition of the reagent to the sample, while the sample was kept ice cold. Reoxidation of cytochrome c by cytochrome oxidase

present during the handling of reading probes was prevented by the addition of 10^{-3} M sodium azide which did not cause any spectral change of the cytochromes. These results were confirmed by separation of the individual components in the resin chromatography. The concentrations of iron, copper, protein, and lipid were determined by the procedures as described by King et al. (43). Sonic irradiation was conducted in a Raytheon 250 W, 10 KC sonic oscillator, model DF101, with or without replacement of air in the chamber with nitrogen gas. Each two minutes of sonic exposure was followed by a one-minute interlude, during which the chamber was kept at 4° C by circulating ice water. Infrared spectroscopy was made by the KBr disc method with the apparatus of Perkin-Elmer Corporation, Norwalk, Connecticut. Carbon monoxide treatment was made by bubbling a fine stream of CO gas through the dithionite-reduced preparation retained in an ice bath (97). Ultracentrifugation was made with a Spinco preparative ultracentrifuge, model L, No. 50 rotor at $0-4^{\circ}$ C.

Sephadex Column Preparation and Application of the Sample (19) -- The Sephadex G-75 or G-200 powder was first suspended in glass-distilled water and stirred thoroughly. After sedimentation at room temperature in about 20 minutes, the supernatant was siphoned out and the procedure was repeated for complete removal of fine particles. The aggregates, if any, were removed by allowing the suspension to run

through a 30-mesh net. The decanted suspension of the hydrated Sephadex was stirred in 0.01 to 0.05 M sodium phosphate buffer (pH 7.4) containing 0.1% Emasol 1130, poured into the column tube, and allowed to sediment. The glass tubes used for the column were 1.5 cm and 4.5 cm in diameter and the column beds were made 12 cm and 30 cm high, respectively. The packing was carried out in the cold room at 4° C. When the packing was finished, fresh buffer was added to percolate through the bed. The upper surface of the bed of the column was smoothed down by stirring the uppermost layer of the bed gently and allowing the grains to settle for 30 minutes. This procedure was also repeated before each new run. The sample was sucked up into a glass dropping-pipette and was layered under the buffer on the top of the column. A flow rate was adjusted to 7.2 ml per hour for a smaller column.

R_f Value Determination -- After applying the sample to a smaller column, a 0.6 ml fraction was collected at five minute intervals with a "Technicon" Time-flow fraction collector. The position of the band in the G-200 column was determined when five ml of eluate was obtained in about 40 minutes at 4° C.

Preparation of Cytochrome Oxidase from the Keilin-Hartree Heart Muscle Preparation --

1. The Keilin-Hartree preparation from beef heart was obtained as previously and suspended in 0.1 M phosphate-borate

buffer (pH 7.4) at a final protein concentration of 20 to 25 mg/ml. Subsequent operations were carried out at 0-4° C unless otherwise stated. The pH of the protein solutions was maintained in the range of 7.0 to 7.5.

2. To the suspension a 10% sodium cholate in 0.1 M phosphate buffer (pH 7.4) was added to a final cholate concentration of 2%, and the mixture was stirred gently for an hour.

3. Solid ammonium sulfate was added to 0.25 saturation, the mixture adjusted to pH 7.0 to 7.5 with 1 N NaOH and allowed to stand overnight at 0° C.

4. About 30 minutes after making the solution to 0.40 saturation with solid ammonium sulfate, it was centrifuged at 23,000 x g for 30 minutes (Spinco L, No. 21 rotor, 15,000 r.p.m.). The precipitate was discarded.

5. Solid ammonium sulfate was further added to the supernatant to 0.55 saturation. After 30 minutes it was centrifuged at 23,000 x g for 30 minutes.

6. The precipitate was dissolved in a 0.1 M phosphate buffer (pH 7.4) containing 2% sodium cholate to about one-third the volume of heart muscle preparation, and then made to 0.30 saturation by the addition of saturated ammonium sulfate solution. The mixture was allowed to stand overnight. It was then centrifuged at 40,000 x g for 40 minutes (Spinco L, No. 21 rotor, 20,000 r.p.m.).

7. The supernatant was adjusted to 0.40 saturation by the addition of saturated ammonium sulfate solution, incubated one hour, then centrifuged at 23,000 x g for 30 minutes.

8. The precipitate was dissolved in the same cholate-phosphate buffer and then made to 0.26 saturation with saturated ammonium sulfate solution. The mixture was allowed to stand overnight, and then centrifuged at 7,000 x g for 20 minutes (Lourdes, 9RA rotor, 7,500 r.p.m.). Saturated ammonium sulfate solution was added to the supernatant to 0.33 saturation. After an hour, it was centrifuged at 7,000 x g for 20 minutes. The precipitate was dissolved in the cholate-phosphate buffer solution.

9. The above fractionation (Step 8) was repeated until the preparation was spectrophotometrically free from other components.

10. The final precipitate was dissolved in 0.05 M phosphate buffer (pH 7.4) containing 0.1% Emasol 1130 to one-thirtieth the volume of the original heart muscle preparation. The clear solution was dialyzed against a cold 0.05 M phosphate buffer containing 0.1% Emasol for 12 hours.

11. If it was necessary, the sample was lyophilized after further dialysis against cold distilled water for 12 hours.

Determination of oxygen uptake was done polarographically by a Gilson Medical Electronics oxygraph, model K, at 25° C.

RESULTS

Preparation of Cytochrome c-Cytochrome Oxidase Complexes and Electronic Absorption Spectra of the Complex

Two and eight-tenths μ moles of cytochrome oxidase lyophilized preparation was dissolved in 10 ml of cold 0.01 to 0.025 M sodium phosphate buffer (pH 7.4) containing 0.1 to 0.25% Emasol 1130 at 4° C. Subsequent operations were performed at the same temperature unless otherwise indicated. The solution was sonically irradiated as previously described for 20 minutes. Then to this solution was added 5.6 μ moles of cytochrome c lyophilized preparation. The mixture was exposed to further irradiation for another 25 minutes. Immediately afterward, 0.5 ml of the mixture was carefully layered on the top of a Sephadex column (1.5 cm in diameter and 12 cm in height) under the buffer surface which had been equilibrated with 0.01 M phosphate buffer (pH 7.4) containing 0.1% Emasol 1130 as described in the procedure. For efficiency, two to three ml of the original mixture was applied to a larger Sephadex column (4.5 cm in diameter and 30 cm in height). The column was subsequently developed with the same buffer. Sixty fractions, 0.6 ml each, were collected from a smaller Sephadex column (G-200) at a flow rate of 7.2 ml per hour with a fraction collector. During the development, the

reddish-brown colored zone was rapidly separated into two fractions. The first band, consisting of cytochrome c and cytochrome oxidase, appeared with an R_f value of approximately 0.6. The second, slow-moving band containing only cytochrome c, showed an R_f value of about 0.1. The clear separations of these two fractions were always obtained under the conditions as described above. The actual Sephadex fractionation of the cytochrome c and cytochrome oxidase mixture is shown in Fig. 1. The resultant elution pattern in Sephadex column chromatography is seen in Fig. 2. Determination of the component concentration in each fraction was made after dilution with the same buffer to the proper concentration. The ratio of cytochrome c and cytochrome oxidase was found to be unity as shown in F, G, H, and I, Table II. The absorption spectra of the complex fraction which contained cytochrome c and cytochrome oxidase in 1:1 ratio is shown in Fig. 3A and 3B. The difference spectrum of the cytochrome c and cytochrome oxidase complex is shown in Fig. 4. The absorption maxima of the reduced complex may be assigned for the components as follows:

<u>Component</u>	<u>Band</u>	<u>Absorption maximum (mμ)</u>
Cytochrome <u>c</u>	γ	415
Cytochrome oxidase	γ	443-4
Cytochrome <u>c</u>	β	523
Cytochrome <u>c</u>	α	550
Cytochrome oxidase	α	605
Cytochrome <u>c</u> and cytochrome oxidase	U. V.	271.6

Fig. 1. Sephadex column chromatography of the sonically irradiated mixture of cytochrome c and cytochrome oxidase.

A. Five minutes after the sample application.

B. Fifteen minutes after the sample application.



Fig. 1A

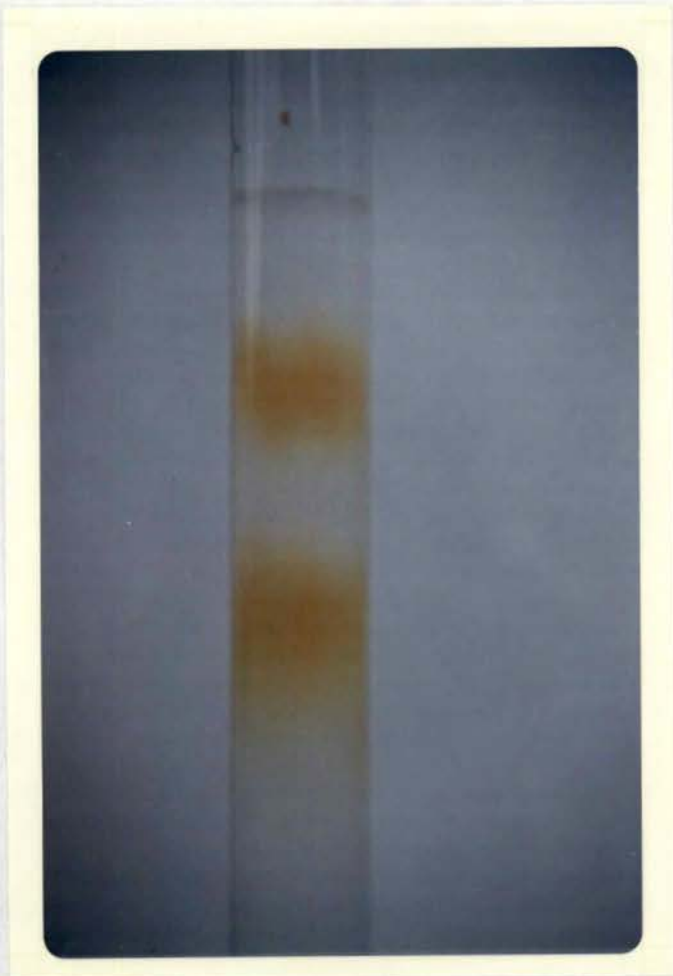


Fig. 1B

Fig. 2. Elution pattern of the cytochrome c-cytochrome oxidase complex of molar ratio 1 from a Sephadex column.

- (1) The complex.
- (2) Free cytochrome c.

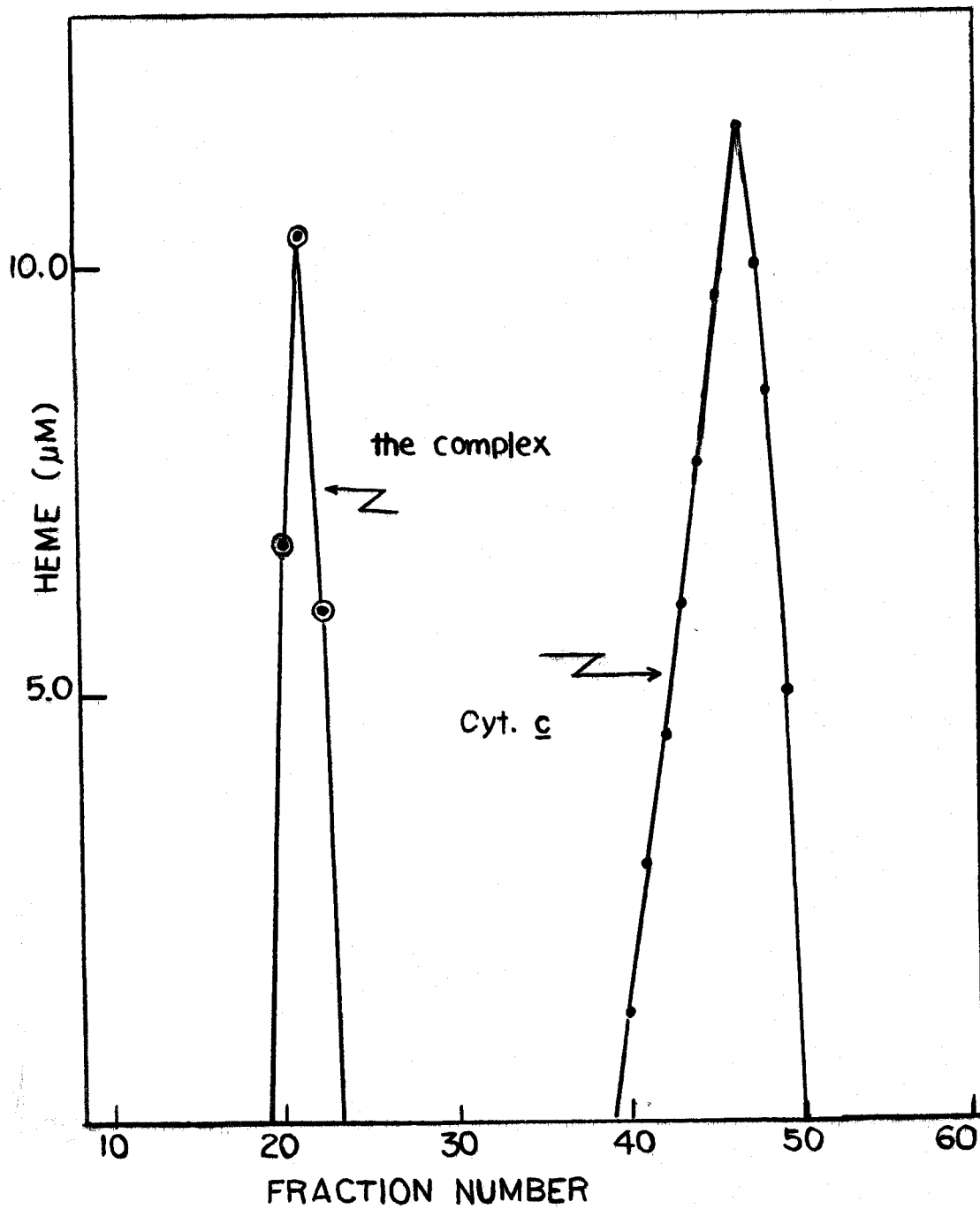


Fig. 2

TABLE II

Preparation of cytochrome c-cytochrome oxidase complexes

Exp.	Time of sonic irradiation	Fractions	Composition of complex obtained		Ratio
			Cyt. <u>c</u>	Oxidase	(Cyt. <u>c</u>)/(Oxidase)
	Min.		μM	μM	
A	0	No. 18	8.4	36.4	.22
		No. 19	6.3	33.6	.19
		No. 20	4.2	22.4	.18 (av.=.196)
B	0	Average	6.5	33.1	.196
C	30	No. 19	11.0	21.8	.51
		No. 20	13.1	25.4	.52
		No. 21	14.2	25.4	.53
		No. 22	17.4	35.5	.49
		No. 23	15.4	29.1	.53
		No. 24	6.8	13.6	.50 (av.=.52)
D	30	Average	7.3	13.6	.54
E	30	Average	16.3	31.0	.52
F	45	No. 18	5.8	6.3	.92
		No. 19	18.4	18.2	1.01
		No. 20	18.8	18.2	1.03
		---*	--	--	--
		No. 24	15.4	16.4	.94 (av.=.975)
G	45	Average	32.5	35.0	.94
H	60	Average	30.0	30.0	1.00
I	45	Average	19.0	18.2	1.04

(A) Two-tenths μmole of lyophilized cytochrome oxidase was dissolved in one ml of 0.025 M phosphate buffer (pH 7.4) containing 0.25% Emasol 1130 at 0° C. Four-tenths μmole of crystalline cytochrome c was added and homogenized with a glass hand homogenizer. Five-tenths ml of mixture was applied to Sephadex G-200 column (1.5 cm in diameter, 12 cm in length),

equilibrated with 0.01 M phosphate buffer (pH 7.4) containing 0.1% Emasol 1130. It was subsequently developed with the same buffer. R_f of the complex band was 0.58. Three-tenths ml of the fraction was collected.

(B) One and four-tenths μ moles of oxidase and 2.4 μ moles cytochrome c were mixed and homogenized thoroughly. The mixture was fractionated as before.

(C) One and four-tenths μ moles of oxidase was dissolved in five ml of 0.025 M phosphate buffer containing 0.25% Emasol at 0° C. The solution was sonicated for 20 minutes at 4° C in the Raytheon sonic oscillator. A one-minute interlude followed each two minutes of sonic exposure. One ml of 2.4 mM crystalline cytochrome c solution was added. The mixture was sonicated another 10 minutes. Five-tenths ml of the mixture was applied to the column and fractionated as before. Six-tenths ml of fraction was collected with the fraction collector.

(D) and (E) Seven-tenths μ mole and 1.4 μ moles of oxidase, 1.2 μ moles and 2.5 μ moles of cytochrome c were used respectively.

(F) Two and eight-tenths μ moles of oxidase and 5.6 μ moles of cytochrome c were mixed in 10 ml of 0.025 M phosphate buffer (pH 7.4). The mixture was sonically irradiated for 45 minutes. (Five-tenths ml of the sonicated mixture was applied to the same column as described above.) Six-tenths ml of fraction was collected in every five-minute interval. The sonic treatment was the same as above except for the time period. Nos. *21, 22, and 23 fractions were extracted for infrared spectroscopy. R_f value of the complex band was about 0.6 and of the cytochrome c band, about 0.1.

(G) Two and eight-tenths μ moles of oxidase and 3.2 μ moles were used. Total volume of sonicated mixture was six ml, which was irradiated for 45 minutes.

(H) One and nine-tenths μ moles of cytochrome oxidase and 3.2 μ moles of cytochrome c were used. They were dissolved in seven ml of 0.025 M phosphate buffer containing 0.25% Emasol and sonically irradiated for 60 minutes.

(I) One and four-tenths μ moles of oxidase and 5.5 μ moles of cytochrome c were used. The mixture was dissolved in 10 ml of phosphate buffer containing Emasol, and irradiated for 45 minutes before fractionation.

Fig. 3A. Absorption spectra of cytochrome c-cytochrome oxidase complex.

For absolute spectrum of the oxidized form of the cytochrome c-cytochrome oxidase complex, the complex solution consisting of 3.3 μM each component was used without any further treatment. The solvent of the complex was a 0.05 M phosphate buffer (pH 7.4) containing 0.25% Emasol 1130. The reference solution consisted of the same buffer solution.

For absolute spectrum of the reduced form of the complex, the oxidized form of the complex was reduced with a few grains of sodium dithionite in the presence of 10^{-3} M sodium azide. After addition of the reducing reagent, the mixture was allowed to stand for five minutes in an ice bath. For absolute CO spectrum, the reduced form of the complex was flushed with a fine stream of pure CO for one minute in the ice bath. For the reference of the above two, the same buffer solution containing the same amount of dithionite and azide was used. These spectra were scanned with a Cary spectrophotometer, model 11, at 25° C.

Fig. 3B. The absolute absorption spectra of the complex (1:1) in the ultraviolet region.

Concentration of the complex was 1.87 μM (oxidized form).

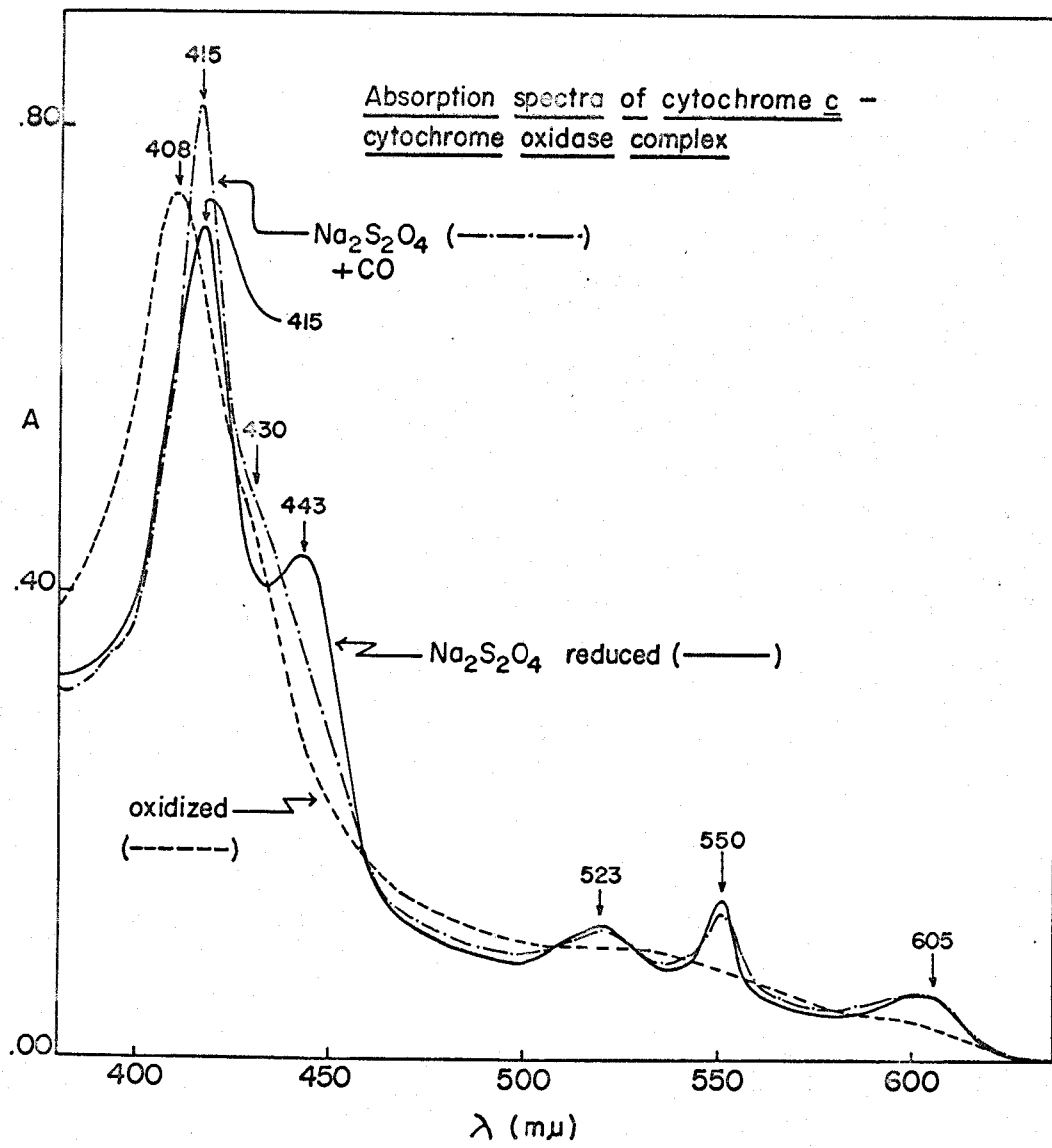


Fig. 3A

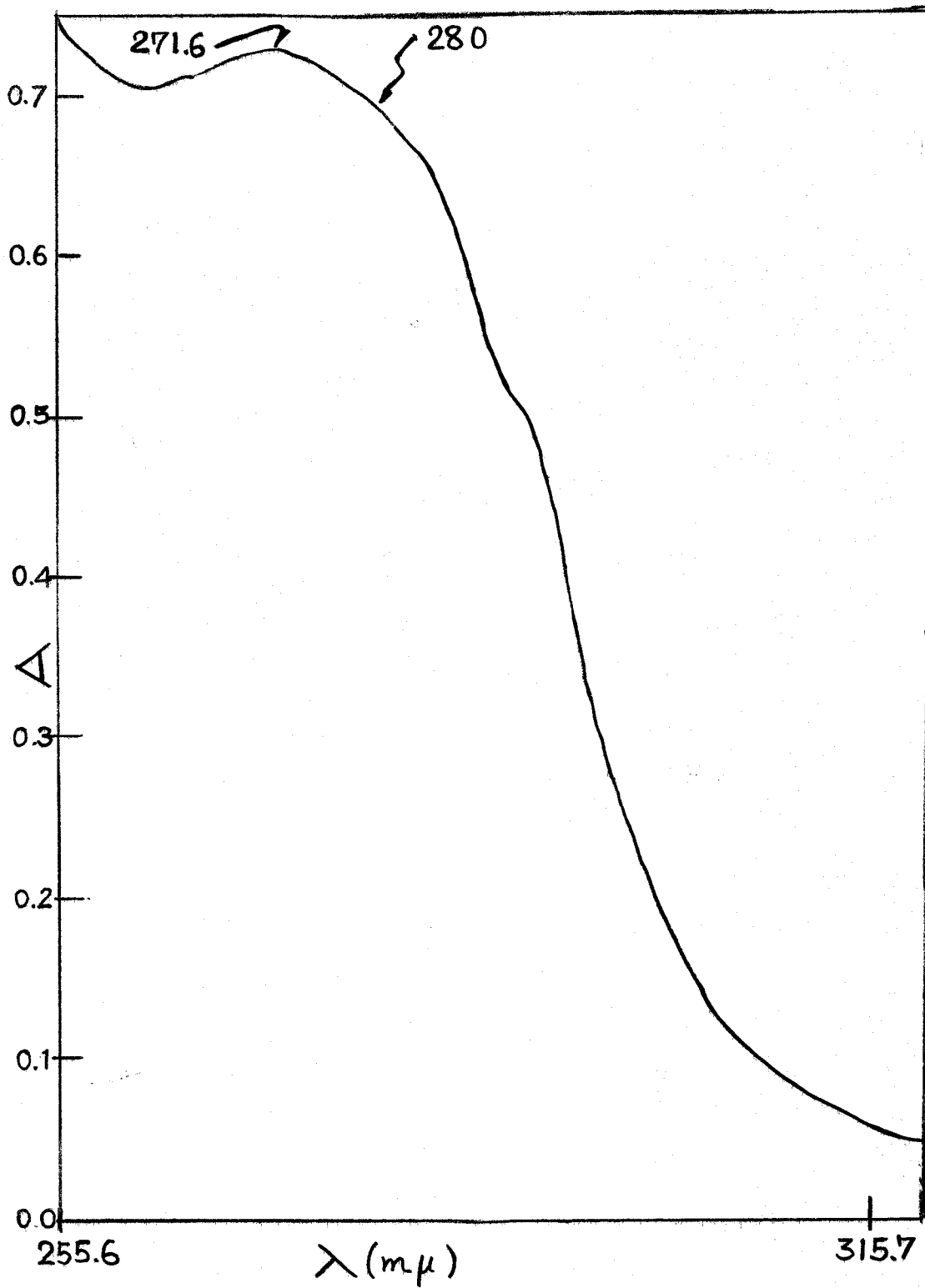


Fig. 3B

Fig. 4. Difference spectra of the cytochrome c-cytochrome oxidase complex.

The cytochrome c-cytochrome oxidase complex consisted of five μ M of each component. A few grains of sodium dithionite were used for reduction of the complex with 10^{-3} M sodium azide. For the oxidized form, the isolated complex solution was used without any further treatment but with addition of 10^{-3} M azide. The solvent used for the complex solution was 0.05 M phosphate buffer (pH 7.4) containing 0.25% Emasol. The spectra were scanned with a Cary spectrophotometer, model 11, at 25° C.

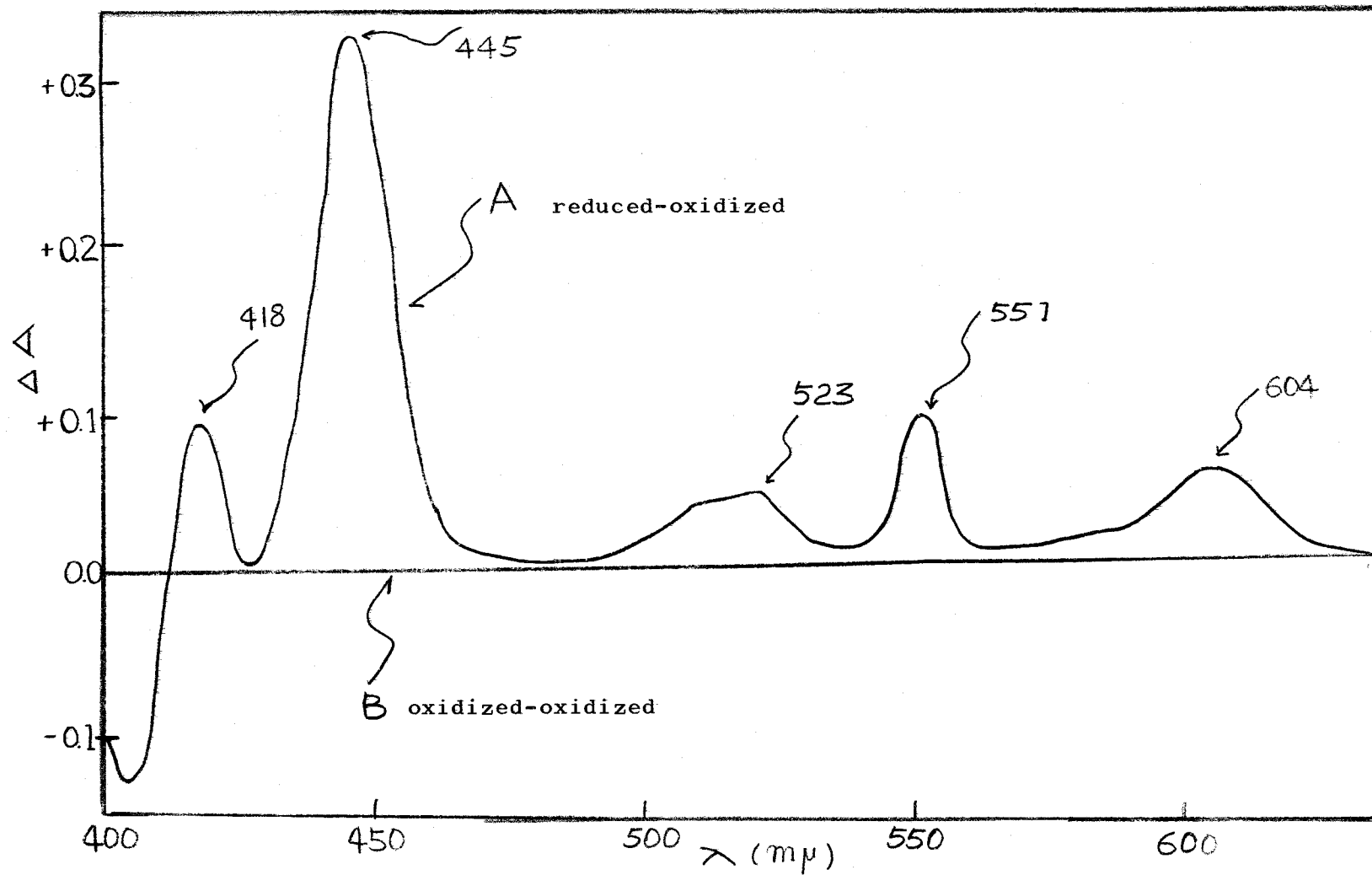


Fig. 4

These maxima are essentially in agreement with the literature values (5, 68, 97, 99).

The effect of sonic irradiation on the molar ratio of the two components was also examined and the results are shown in Table II. It is more likely that prolonged treatment of the mixture produced a complex of higher cytochrome c to cytochrome oxidase ratio. At zero time the ratio was 0.2; 30 minutes after sonic treatment the ratio was 0.5. However, the ratio was never greater than unity even after 60 or 90 minute sonic irradiation.

Infrared Spectra of the Complex and Individual Components

The samples of the enzymes for infrared spectroscopy were prepared from a dry powdered purified preparation by the KBr disc method. The disc was made under the pressure of 22,000 Psi and the thickness of the disc was an average of 0.7 mm. Only transparent, good pellets were employed for the spectrum and the enzyme preparations were kept at 0° C until spectroscopic operation. The cytochrome c-cytochrome oxidase complex was taken from the Sephadex column and lyophilized after confirming the ratio of component content as shown in Table II. Infrared spectra of cytochrome c-cytochrome oxidase and their complex (1:1) are shown in Figs. 5, 6 and 7.

From the protein nature of all samples, strong bands are observed at around 3300, 1650 and 1525 cm^{-1} due to N-H

Fig. 5. Infrared spectrum of cytochrome c-cytochrome oxidase complex.

The cytochrome c-cytochrome oxidase complex was obtained, as previously described, from Sephadex chromatography of the sonically irradiated mixture of cytochrome c crystalline preparation and cytochrome oxidase. After confirming the ratio of cytochrome c to cytochrome oxidase as one to one, the sample was lyophilized. The preparation was kept in a desiccator at cold temperature. The KBr disc for the complex was prepared under a pressure of 22,000 Psi; the thickness of the disc was 0.700 mm. The sample disc was kept at 0° C until spectroscopic operation.

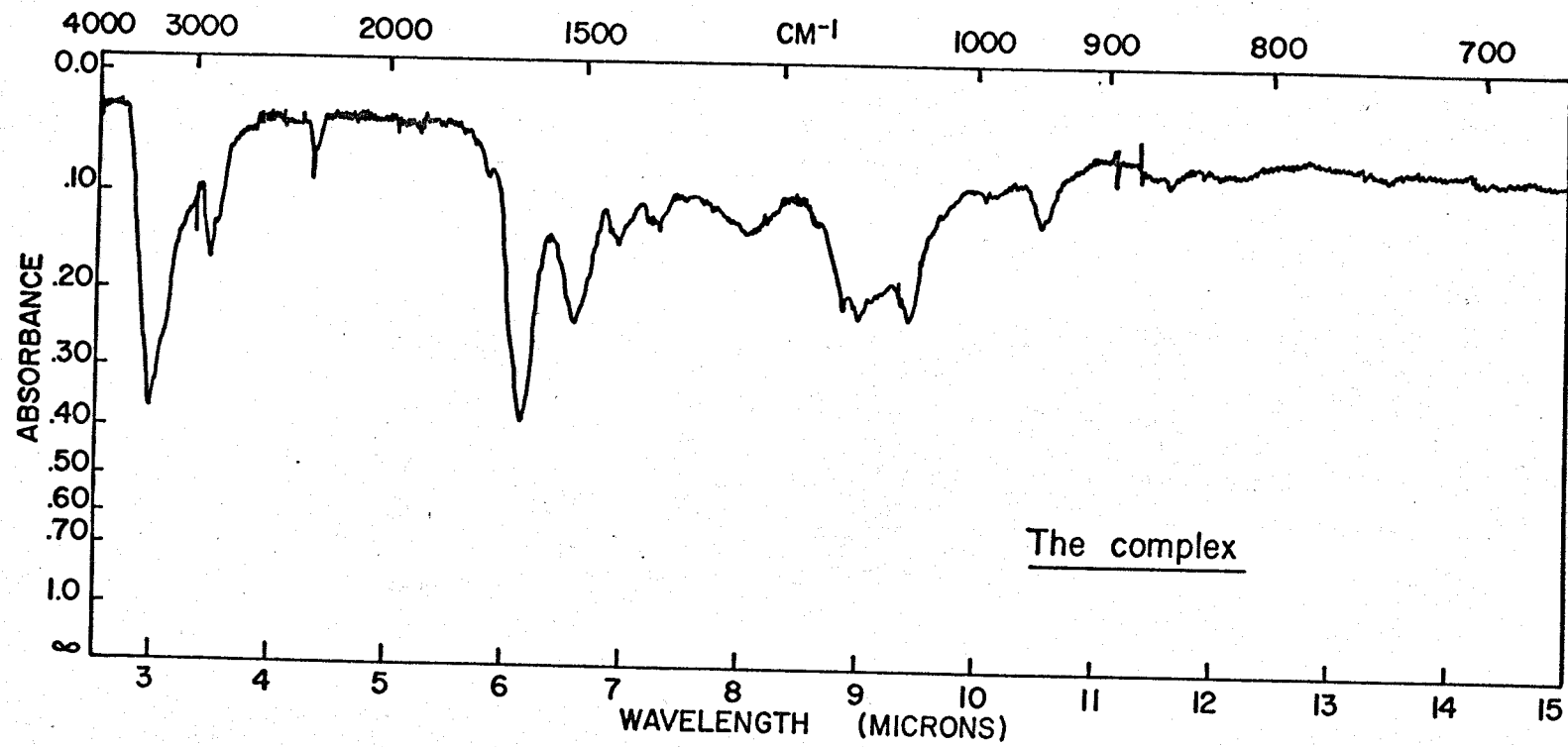


Fig. 5

Fig. 6. Infrared spectrum of cytochrome c.

The crystalline cytochrome c preparation used was made from beef heart muscle by the method of Margoliash. The iron content of the material was estimated at 0.45%. The KBr disc was prepared under a pressure of 22,000 Psi and the thickness of the pellet was 0.680 mm. The sample was kept at 0° C until spectroscopic operation.

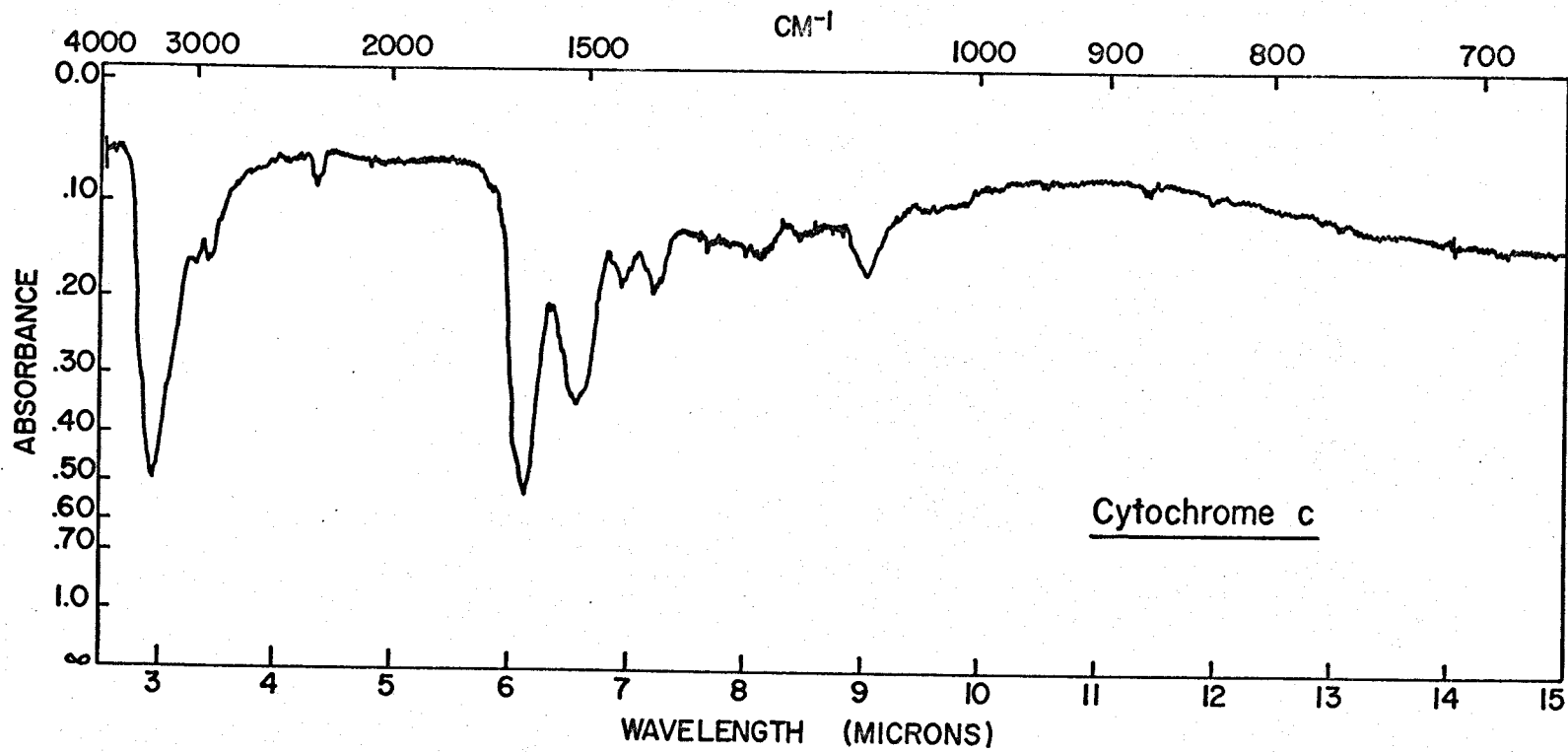


Fig. 6

Fig. 7. Infrared spectrum of cytochrome oxidase.

The cytochrome oxidase prepared from beef heart muscle by means of Okunuki's method (67) was dialyzed extensively to remove any detergent at the final stage of the preparation; 12 hours against 0.01 M phosphate buffer (pH 7.4), and another 12 hours against glass-distilled water, both at 4° C. The dialyzed material was lyophilized and kept in a desiccator at -8° C. The infrared sample was made by KBr disc method; thickness, 0.695; pressure used for making the pellet, 22,000 Psi. The sample was kept at 0° C until spectroscopic operation.

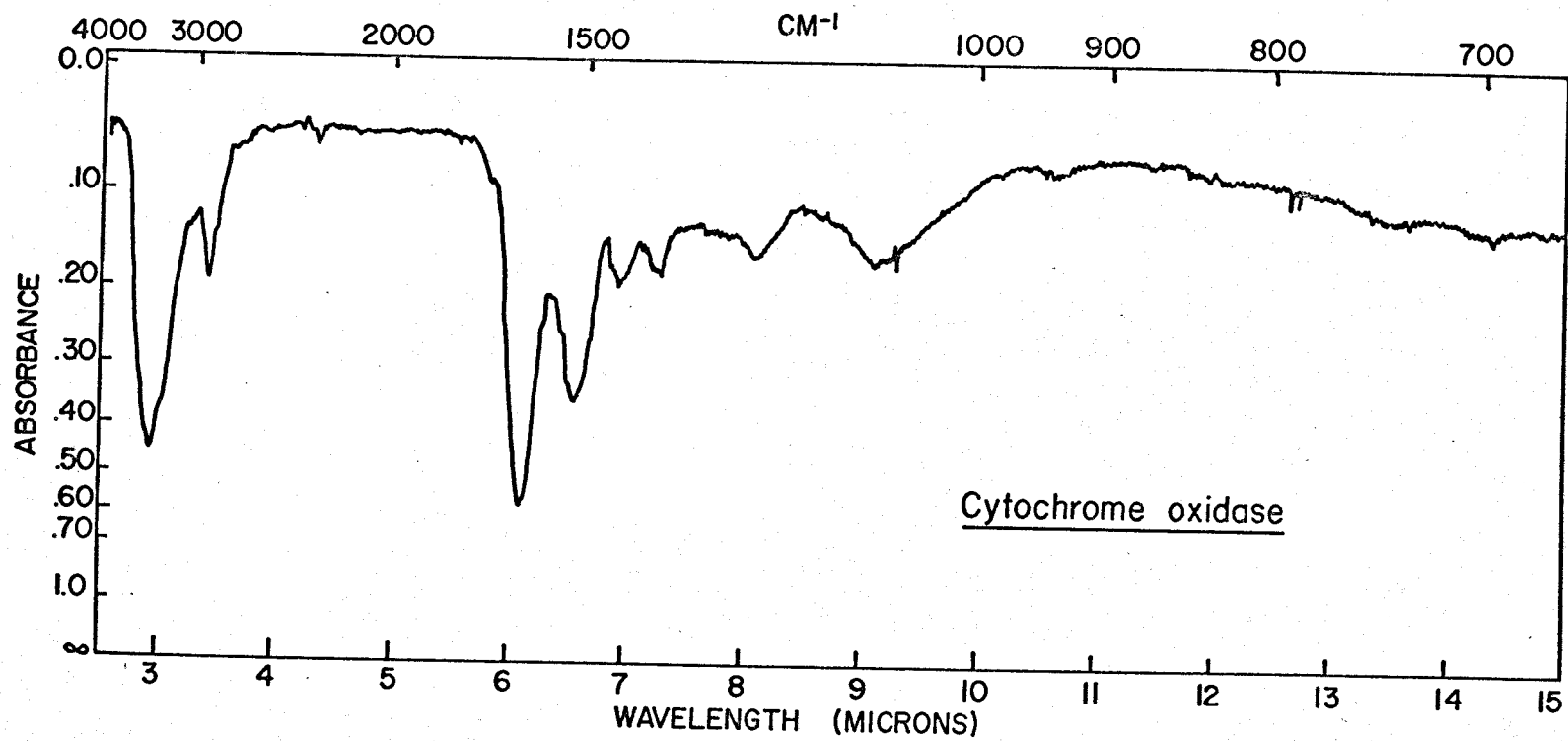


Fig. 7

stretching mode, C=O, and N-H bending mode of typical amide linkage respectively (4, p. 203). The spectrum of the complex differs mainly in the appearance of the new bands near 950 and 1050 cm^{-1} . The complex absorption is not likely a superposition of cytochrome c and cytochrome oxidase as would be expected from mechanical mixture. The fading peak at 1110 cm^{-1} and the appearance of a peak at 1050 cm^{-1} can be interpreted as some interaction between OH and anionic groups (47). The marked shift in the absorption peak of NH bands in the complex is noticed compared with those in other authentic samples. They can also be interpreted as the interaction among the NH_2 residues of cytochrome c, known to have 18 lysine residues (58), and other functional groups, probably anionic, in cytochrome oxidase.

Integrity of the Cytochrome c-Cytochrome Oxidase

Complex; Dissociation of the Complex

One-half ml of complex solution (cytochrome c : cytochrome oxidase ratio, 1:1; heme concentration, 36 μM each; both in oxidized form), after being concentrated about three times with the aid of polyvinylpyrrolidone, was rechromatographed under the same conditions with the same column of the Sephadex G-200 as was used for the isolation. One single homogeneous band descended through the 12 cm column with an

R_f value of about 0.6. The eluted fraction contained an average of 11.2 μM cytochrome c and 10.9 μM cytochrome oxidase. Two ml of the complex solution was recovered. There was no diffusion or broadening of the band in the column for several hours on stopping the flow of the developing solvent.

One ml of isolated cytochrome c-cytochrome oxidase complex (ratio 1:1; and about 35 μM each) was mixed with 6.7 ml of 0.025 M Na-phosphate buffer (pH 7.4) at 4° C and the mixture was centrifuged at 114,000 x g for 120 minutes using a Spinco preparative centrifuge, model L, No. 20.7 rotor at 4° C. The concentration of the components in the resultant precipitate was determined spectrophotometrically after one washing with the same buffer. The ratio between component concentrations was maintained unity after centrifugation under the above conditions.

Examination was made on the effect of ionic strength on the dissociation of the complex. The basal medium used consisted of 0.01 M phosphate buffer (pH 7.4), then a series of media of different ionic strengths (that is, 0.03, 0.11, 0.18, and 0.41) were prepared by adding 0.6 M NaCl to this buffer. A 0.8 ml aliquot of the complex solution (ratio, 1:1; 33.6 μM) was mixed thoroughly with the prepared media. They were centrifuged with a Spinco preparative centrifuge, model L, No. 50 rotor, at 151,000 x g for 300 minutes at 4° C. Supernatant and precipitate were analyzed to determine the

concentrations of both components spectrophotometrically. The percentage of dissociation was plotted against the ionic strength, as shown in Fig. 8.

The complex was subjected to Amberlite IRC-50 column chromatography which consists of a weak cation exchange resin. A small column with dimensions of 0.5 cm in diameter and 3 cm in length of resin bed was made to handle a small amount of sample. The resin was equilibrated with 0.15 M ammonium phosphate (in terms of NH_4^+) containing 0.25% Emasol 1130. After application of 1.0 ml of the sample solution to the upper surface of the resin bed with a dropping-pipette, the sample was developed with the same solvent. The cytochrome oxidase fraction, easily identified by its yellowish-green color, passed through the column, while reddish cytochrome c was trapped in a thin layer near the surface of the column. Three ml of the developing solvent was added to completely elute cytochrome oxidase. Then the cytochrome c fraction was eluted with 0.25 M ammonium phosphate. After standardizing the collected volume of each fraction combined with washings, the concentration of each component was determined spectrophotometrically with each eluting solvent as reference. Not only was the molar ratio of each component maintained at unity after fractionation, but also the absolute molar value of each component was in good accord with that in the complex solution.

Fig. 8. Dissociation of cytochrome c-cytochrome oxidase complex at different ionic strengths.

The basal medium used was 0.01 M phosphate buffer (pH 7.4) and a series of solutions of different ionic strengths (0.03, 0.11, 0.18, and 0.41) was made by adding 0.6 M NaCl aqueous solution to this medium. Eight-tenths ml of cytochrome c-cytochrome oxidase complex consisting of 36.6 μ M of each component was made up to 10.2 ml with the media and mixed thoroughly at 0° C. The duplicate samples were then centrifuged at 151,000 x g for 300 minutes at 4° C. The centrifugal rotor was stopped without braking. The supernatant was carefully sucked out with a dropping-pipette. The precipitate was suspended in the same medium and homogenized. The concentrations of cytochrome c and cytochrome oxidase were determined spectrophotometrically. Cytochrome c was found in the supernatant with a negligible amount of cytochrome oxidase. Dissociation percentage was calculated from the concentration of cytochrome c in the supernatant to that in the precipitate.

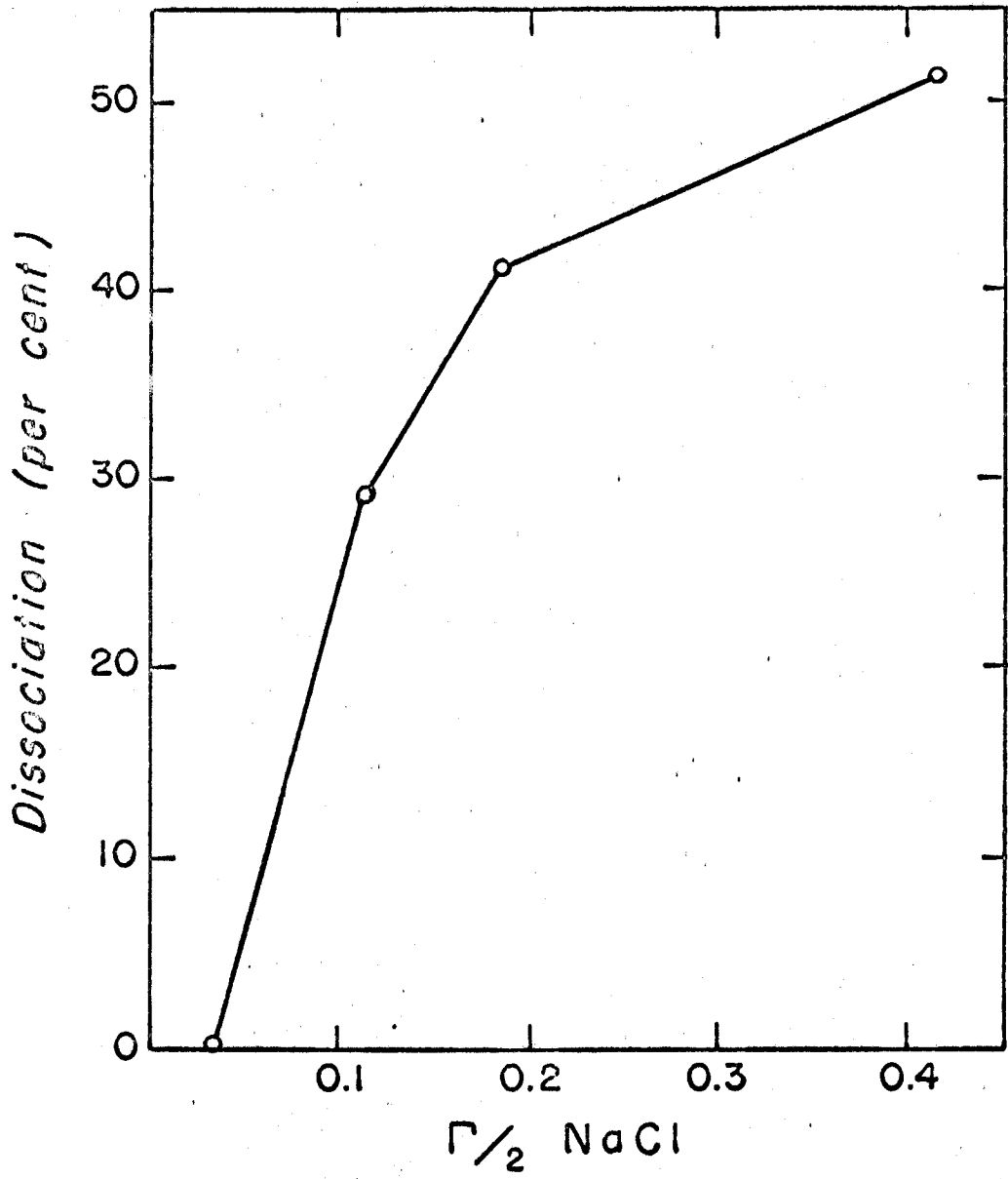


Fig. 8

The separated complex component was used for CO treatment, which will be described later.

Complex Formation of Chemically Modified Cytochrome c
with Cytochrome Oxidase

The chemical modification of cytochrome c has been reported to result in the loss of the activity or activation in its interaction with cytochrome oxidase (80). It may constitute a critical test for the hypothesis of complex formation of cytochrome c and cytochrome oxidase--due to their electrostatic nature--to examine the parallelism between activity and complex formation of modified cytochrome c, which is believed to be sealed in its lysine residue with imported chemical groups.

The acetylated, succinylated and guanidinated cytochrome c was prepared from a crystalline preparation of cytochrome c. The modified cytochrome c was mixed with a 20-minute sonically irradiated sample of cytochrome oxidase in 0.025 M phosphate buffer (pH 7.4) containing 0.25% Emasol 1130 and sonically treated for another 30 minutes. Subsequent fractionation with Sephadex G-200 was conducted as previously.

As shown in Table III, while the guanidinated cytochrome c formed the complex with cytochrome oxidase in 1:1 ratio as well, both acetylated and succinylated cytochrome c failed to

TABLE III

Formation and activity of the cytochrome c-cytochrome oxidase complex from chemically modified cytochrome c

Cytochrome <u>c</u>	Formation	Activity of complex	Activity of "free" components in mixture
"Native" (not modified)	+	++	+
Acetylated	-	-	-
Succinylated	-	-	-
Guanidinated	+	++	+

form a complex of any kind. The guanidinated cytochrome c and cytochrome oxidase complex also gave activity comparable with that of intact cytochrome c, in both ascorbate oxidation and succinate oxidation in reconstitutive activity, measured as described later. Both acetylated and succinylated cytochrome c, 20 μ M each in concentration, were entirely inactive to 1 μ M cytochrome oxidase when ascorbate or the reconstituted succinate cytochrome c reductase served as an electron donor.

CO-compound and CN-compound of the Complex

It was observed that the cytochrome c-cytochrome oxidase complex gave a distinctive peak at 415 m μ in the CO difference

spectrum (Fig 9).

On the other hand, sonically irradiated cytochrome c and cytochrome oxidase did not show any anomaly in their CO spectra (Fig. 10).

The complex containing cytochrome c and cytochrome oxidase in 1:1 ratio was separated into individual components by IRC-50 resin chromatography. The separated components were treated with CO. No 415 m μ peak, as seen in the complex, was found with either fraction (Fig. 11).

Furthermore, the complex solution was pretreated with 2% cholate at 30° C for varying durations of time (0, 10, and 20 minutes) before CO treatment. The 415 m μ peak in the CO difference spectrum diminished with the increase of incubation time to complete disappearance, while other peaks remained in good accord with reported data (Fig. 9) (5, 68, 97).

A peak similar to that at 420 m μ in the heart muscle preparation CO spectrum was found in the reconstituted succinate oxidase, as described later (cf. page 66) (Fig. 12).

The complex in the oxidized state was treated with 0.02 M KCN for 20 minutes at 25° C (86). The 408 m μ peak shifted to the red region but less than 5 m μ . According to Tsou (86) exogenous cytochrome c gives more than 5 m μ shift on CN incubation.

Fig. 9. CO difference spectrum of cytochrome c-cytochrome oxidase complex and effect of cholate.

The cytochrome c-cytochrome oxidase complex consisted of 5.6 μ M of each component in 0.025 M phosphate buffer (pH 7.4) containing 0.1% Emasol 1130. The solution was incubated with 2% sodium cholate in a 30° C water bath for 0, 10, and 20 minutes. After incubation, each solution was cooled in the ice bath, a few grains of dithionite and 10^{-3} M sodium azide were added to each, then each solution was treated with pure CO as described previously. The scanning was made 15 minutes after CO treatment--during which the reaction mixture was kept in the ice bath--against the dithionite reduced form of the complex. The base line was scanned with reduced form of the complex against the same reference.

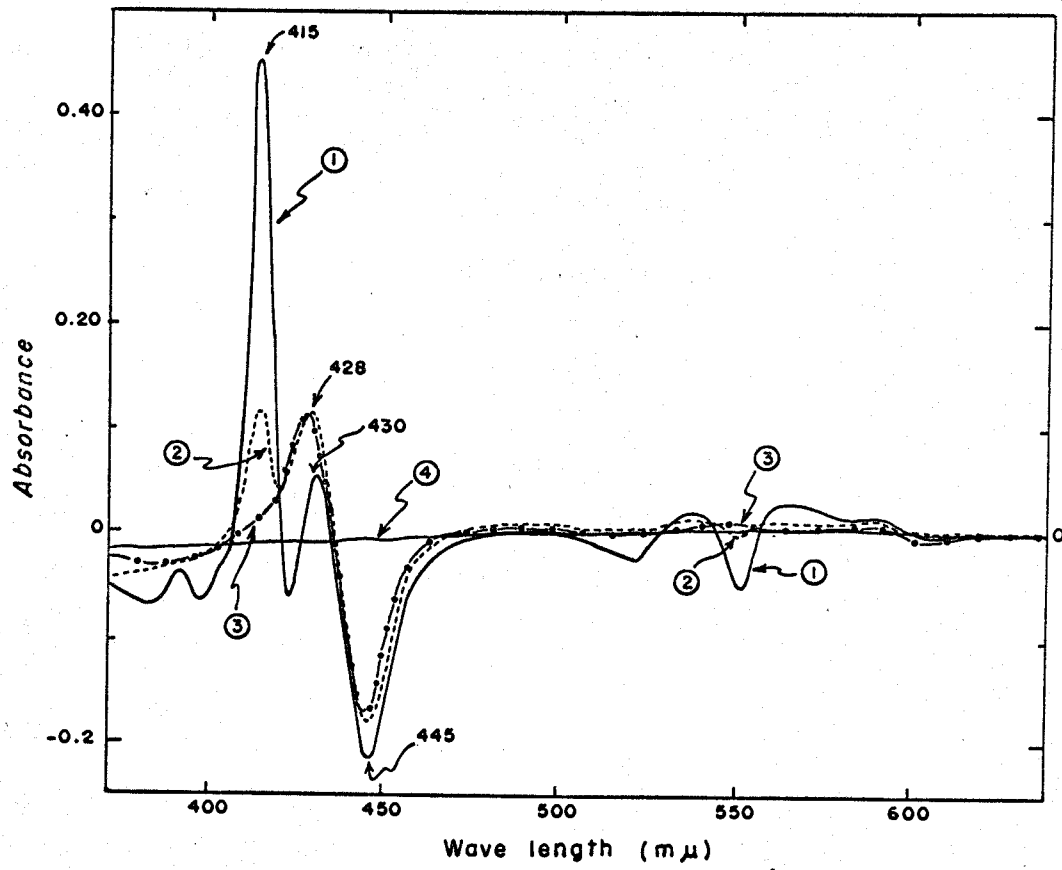


Fig. 9

Fig. 10. Absorption spectra of cytochrome oxidase after 50 minutes of sonic irradiation.

Four μ moles of cytochrome oxidase preparation were dissolved in 10 ml of 0.25 M phosphate buffer containing 0.25% Emasol 1130. The solution was subjected to sonic irradiation at 4° C for 50 minutes with a Raytheon sonic oscillator at full output. The sample for spectrum was made by diluting the sonically irradiated enzyme solution 50 times with the same phosphate buffer solution. The enzyme was reduced with a few grains of dithionite and also treated with pure CO for one minute after reduction.

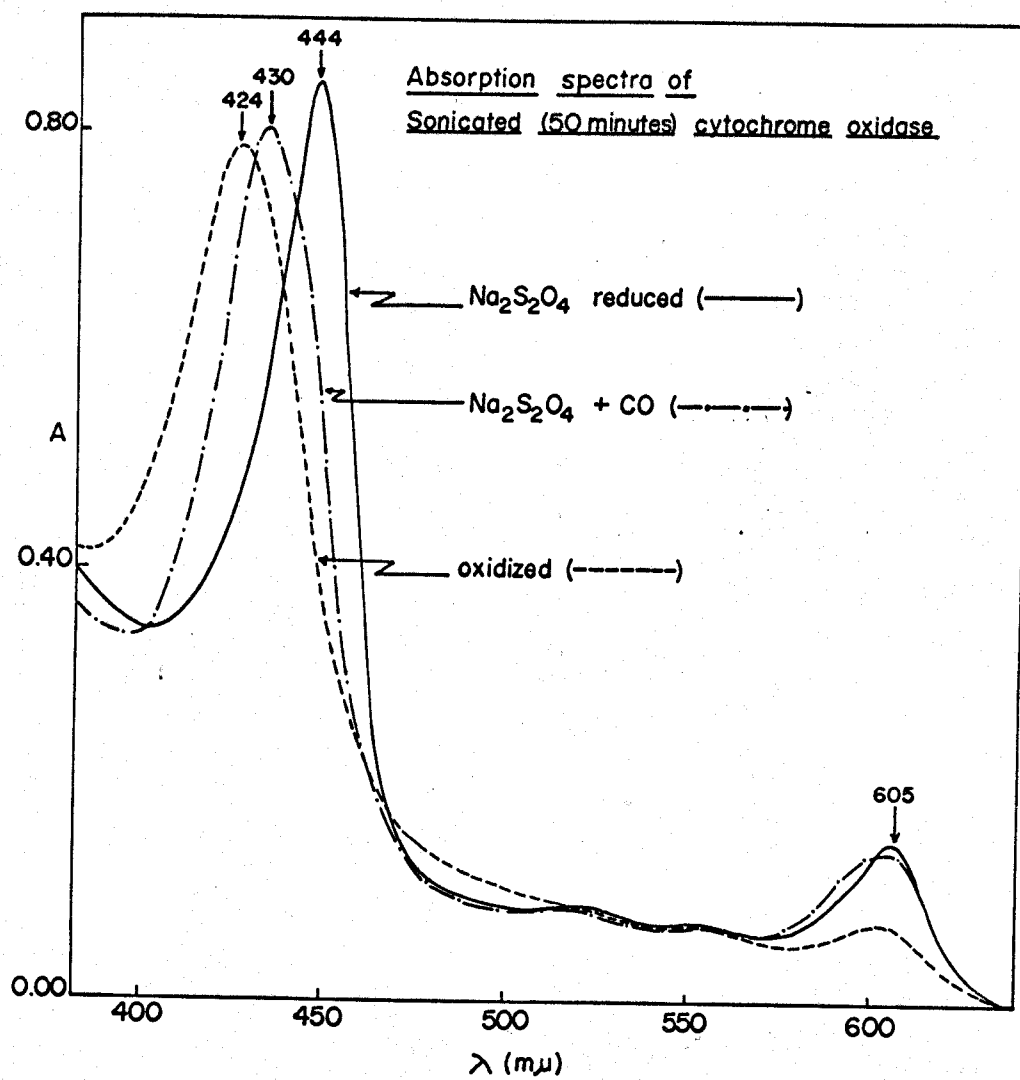


Fig. 10

Fig. 11. Absorption spectra of cytochrome c separated from the cytochrome c-cytochrome oxidase complex.

The cytochrome c fraction of the cytochrome c-cytochrome oxidase complex was obtained by means of the procedure described before. About 3.5 μM of cytochrome c fraction was treated with pure CO for one minute after reduction with a few grains of dithionite, as described previously. The absolute oxidized, reduced, and CO spectra were made with a Cary spectrophotometer, model 11, with the same salt solution (0.25 M ammonium phosphate, pH 7.4), containing the same amount of dithionite as that used for the reduction of the component, as a reference at 25° C.

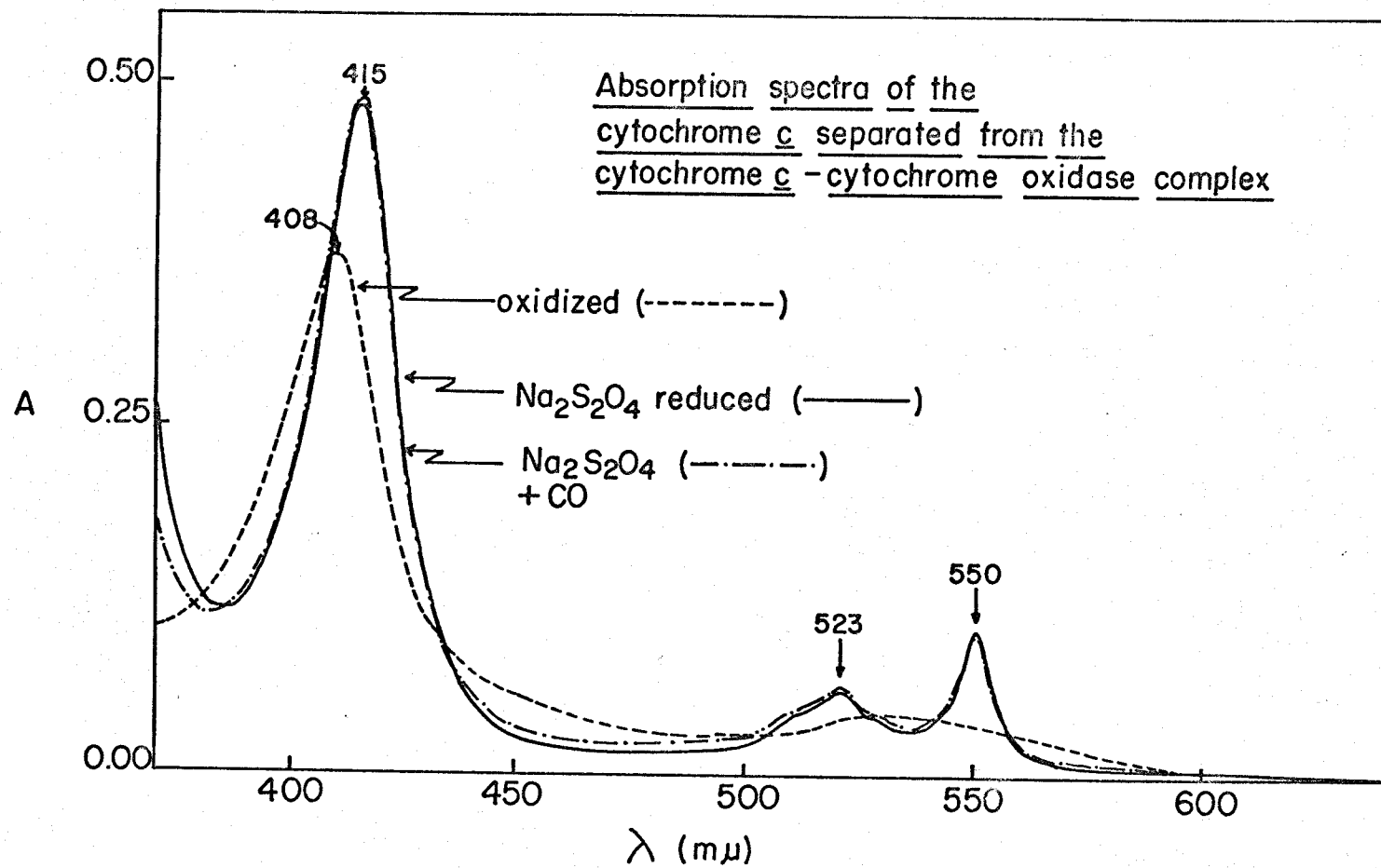


Fig. 11

Fig. 12. CO spectra:

- (A) The cytochrome c-cytochrome oxidase complex.
- (B) Reconstituted succinate oxidase.
- (C) The Keilin-Hartree heart muscle preparation.

The cytochrome c-cytochrome oxidase solution of 0.025 M phosphate buffer (pH 7.4) in 0.1% Emasol 1130 contained each component in a ratio of 1:1 and 10 μ g protein in one ml. The reconstituted succinate oxidase was prepared as described in Part II, and suspended in the same buffer at a concentration of 1.5 mg per ml. The Keilin-Hartree heart muscle preparation was made as previously and diluted to 2.0 mg per ml with the same buffer. Each enzyme solution was treated by bubbling with CO for one minute after reduction with a few grains of sodium dithionite. The scanning in the Soret region was made by a Cary spectrophotometer, model 11, with the corresponding reduced-form solution as a reference, at 25° C.

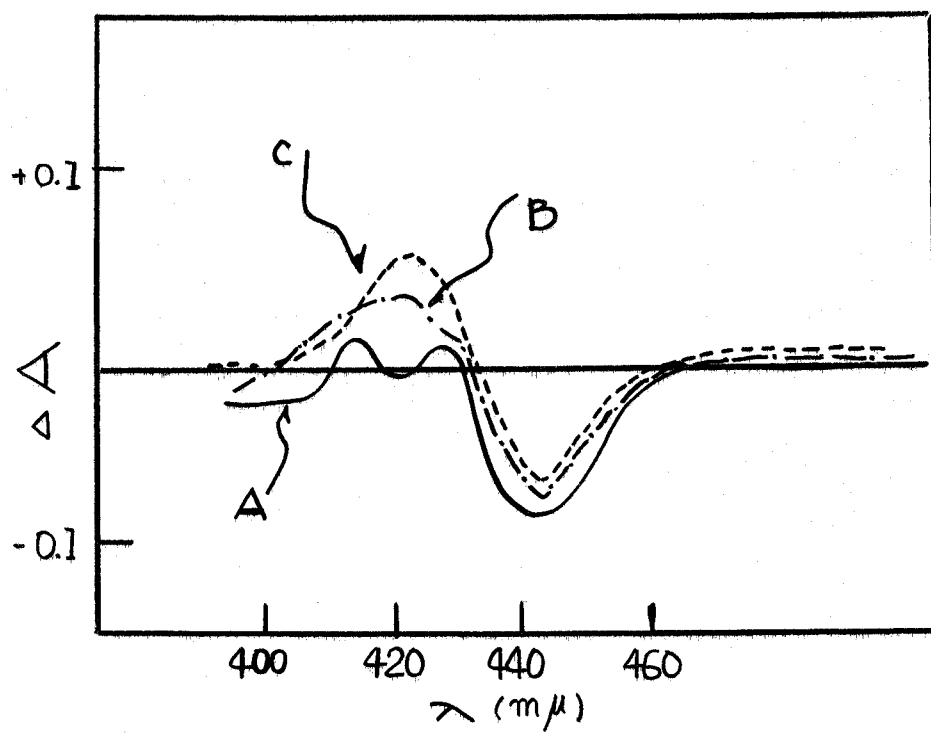


Fig. 12

Fig. 13. CN compound of the cytochrome c-cytochrome oxidase complex.

KCN, to a final concentration of 0.02 M, was added to the oxidized form of cytochrome c-cytochrome oxidase complex consisting of 4.2 μ M of each component in 0.025 M phosphate buffer (pH 7.4) containing 0.25% Emasol 1130. The mixture was allowed to stand at 25° C for 20 minutes. The average shift of the peak at the Soret band to longer wave lengths was about 4.3 μ .

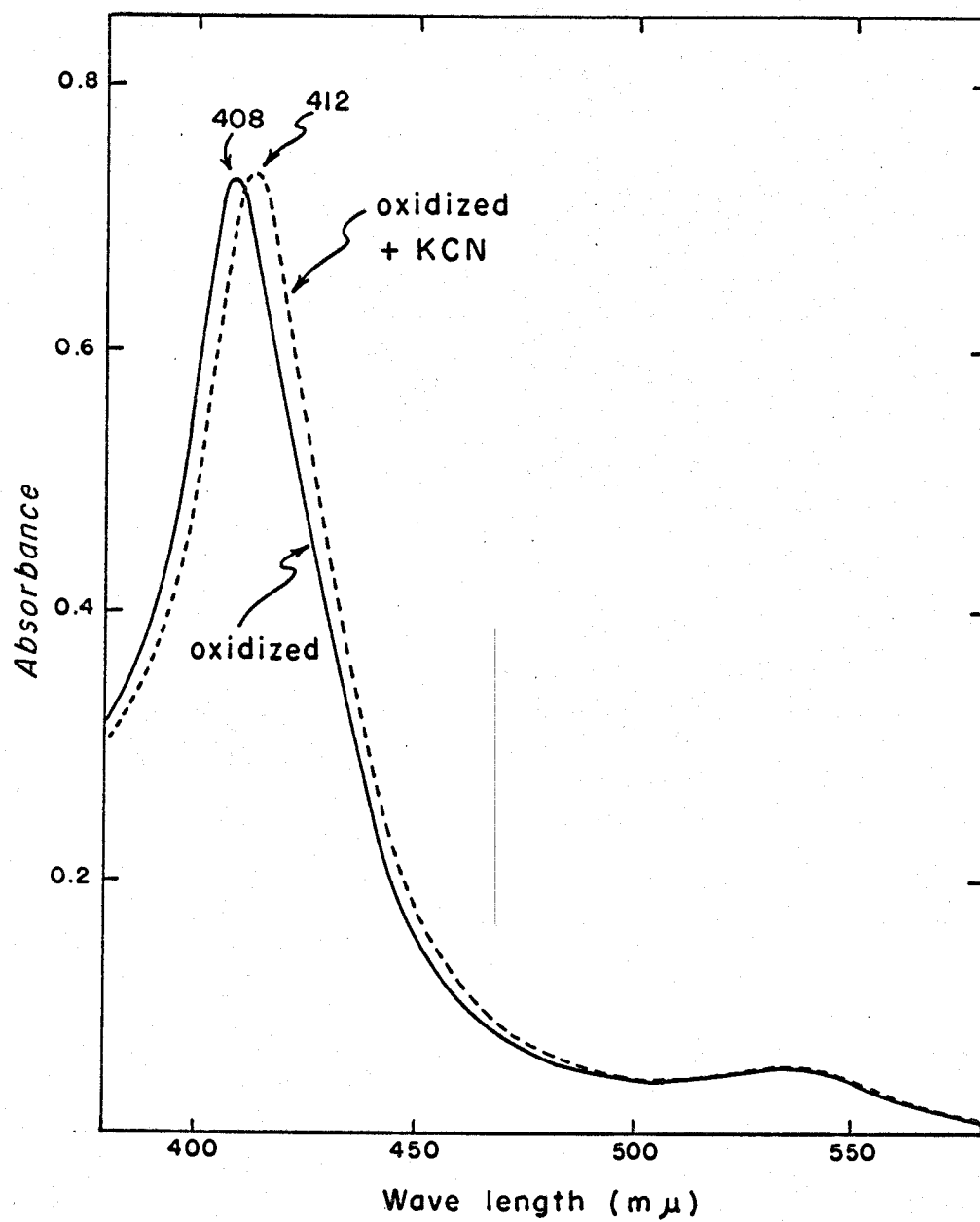


Fig. 13

Ascorbate Oxidation with Cytochrome c-Cytochrome

Oxidase Complex and TMPD Stimulation

The behavior of the complex in the oxidation of ascorbate was also examined. The reaction mixture contained 25 mM sodium phosphate buffer, 25 mM ascorbate and 2.5 mM EDTA, cytochrome c-cytochrome oxidase complex consisting of 1.7 μ M of each component; pH 7.4; total volume, 2.5 ml; temperature, 25° C. The oxygen uptake was as low as 1.5 μ M O₂ per μ M oxidase per minute. To this reaction mixture 0.2 mM TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine) (23) freshly prepared, was added. The remarkable increase in oxygen uptake was observed as high as thirty times that of the system without TMPD. The oxygen absorption of the system was completely inhibited by 1×10^{-4} M potassium cyanide (Fig. 14).

The Keilin-Hartree heart muscle preparation has been known to be rather inactive in ascorbate oxidation, as carefully examined by Slater (73). Three-tenths of mg of the heart muscle preparation per ml was added to the same reaction mixture above mentioned minus the cytochrome c-cytochrome oxidase complex. The comparable extent of stimulation in oxygen uptake was also observed on the addition of an equal amount of TMPD (Fig. 14). The behavior of the ascorbate-TMPD-heart muscle preparation system toward cyanide was also identical with that of the cytochrome c-cytochrome oxidase complex.

Fig. 14. Ascorbate oxidation with the cytochrome c-cytochrome oxidase complex and with the Keilin-Hartree heart muscle preparation.

The reaction mixture contained 25 mM sodium phosphate buffer, 25 mM ascorbate and 2.5 mM EDTA, cytochrome c-cytochrome oxidase complex consisting of 1.7 μ M of each component or 0.3 mg per ml of the heart muscle preparation; pH 7.4; total volume 2.5 ml; temperature 25° C. The O₂ uptake was measured with a G.M.E. oxygraph, model K. The reaction was initiated by addition of the enzyme to the reaction mixture. The time course is shown in decrease of O₂ concentration in μ M. About three minutes after starting reaction, freshly prepared TMPD was tipped into the reaction mixture to a final concentration of 0.2 mM. Finally, 1×10^{-4} M KCN was added to the reaction mixture.

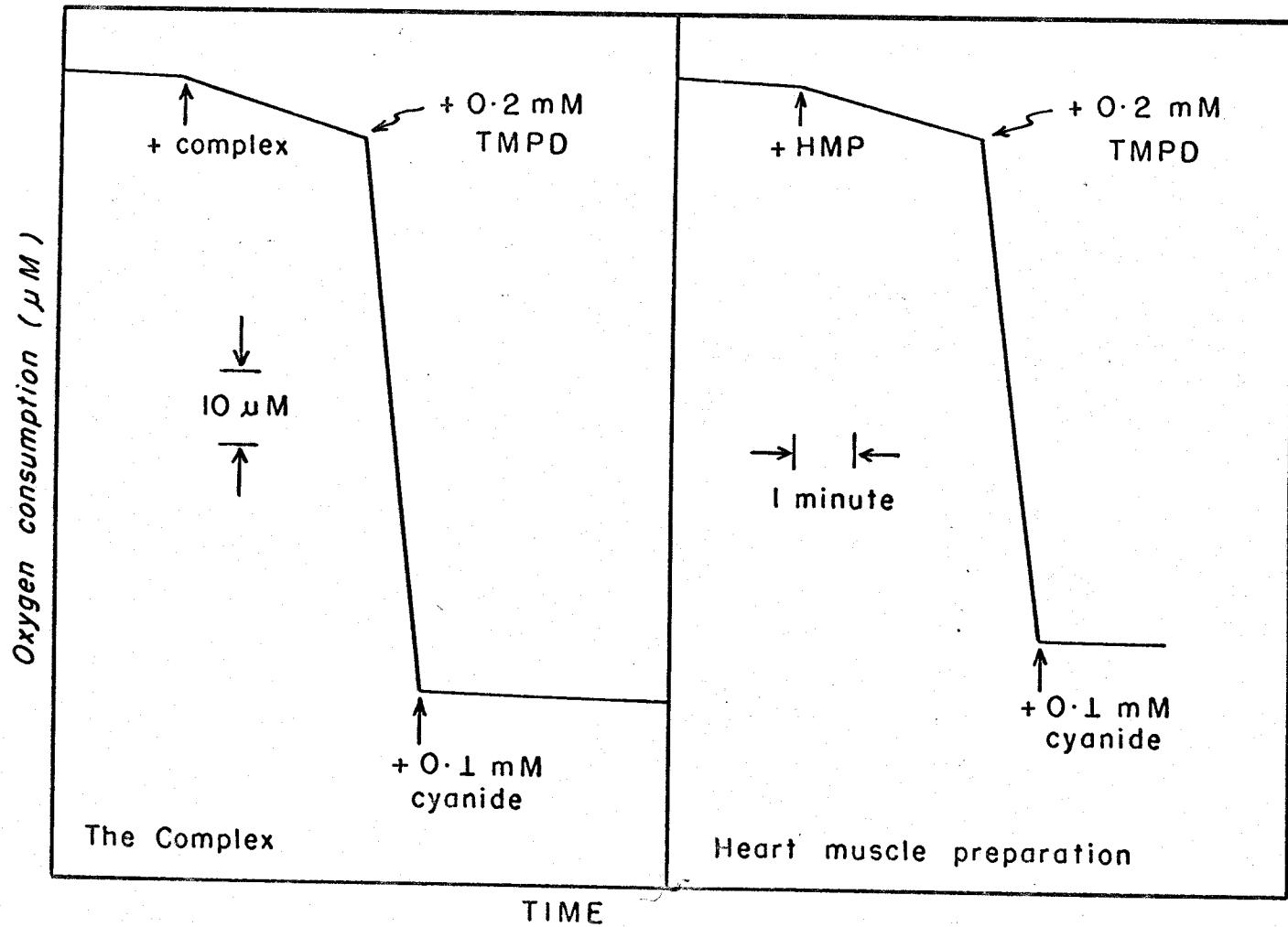


Fig. 14

DISCUSSION

The purified cytochrome oxidase has been reported to be polymerized (79). The molar ratio of cytochrome c to cytochrome oxidase (heme a) in the complex depends upon the time of the treatment by sonic irradiation; the longer the treatment, the higher the ratio of the components of the product. However, no complex of a ratio higher than one was obtained even after prolonged sonication of the mixture. The state of cytochrome oxidase in mitochondrial preparations is not known; a priori considerations indicate that it may not be in a polymeric form.

The classic experiment of Tsou (86) has clearly demonstrated a parallel between the restoration of activity and structural composition of cytochrome c in a deficient preparation. The formation of the complex is not due to non-specific adsorption of cytochrome c to the cytochrome oxidase, since a definite stoichiometric relation is followed. Also the complex showed behaviors similar to those of more complicated mitochondrial preparations towards a number of agents or treatments such as: inseparability by differential centrifugation, dissociation in concentrated salt solutions, extraction of cytochrome c by bile salt. The formation of the complex differs from that of the lipid-soluble cytochrome c, although the cytochrome oxidase used contained about 20%

lipid. The lipid-soluble cytochrome c may be formed in a much higher but varying concentration with respect to cytochrome c. Moreover, cytochrome c may be "dissolved" in or adsorbed on other particulate preparations such as the cytochrome b-c₁ particle and succinate cytochrome c reductase (40, 81), almost in unlimited proportions rather than in a definite stoichiometric relationship as in the cytochrome c-cytochrome oxidase complex. Richardson and Fowler (71) have reported the binding of cytochrome c by their DPNH oxidase preparation. Depending on the cytochrome c concentration used, molar ratio (cytochrome c : heme a) from 0.5 to 2.8 have been found.

Ball, Strittmatter and Cooper (2) have observed maxima at 415 m μ and 430 m μ in the carbon monoxide difference spectrum of their "electron transmitter system" (1) when ascorbate is used as the reductant. Using their data, the corresponding carbon monoxide difference spectrum was re-plotted as shown in Fig. 15.

It can be seen that these maxima are at the same positions for those observed in the cytochrome c-cytochrome oxidase complex, although the absorbance ratio of 415 to 430 m μ of these preparations differs. An absorption maxima similar to this has been reported in some bacterial preparations of electron transport enzymes (64, 94). When dithionite

Fig. 15. Replotted CO difference spectrum of Ball's
"electron transmitter system" (1, 2).

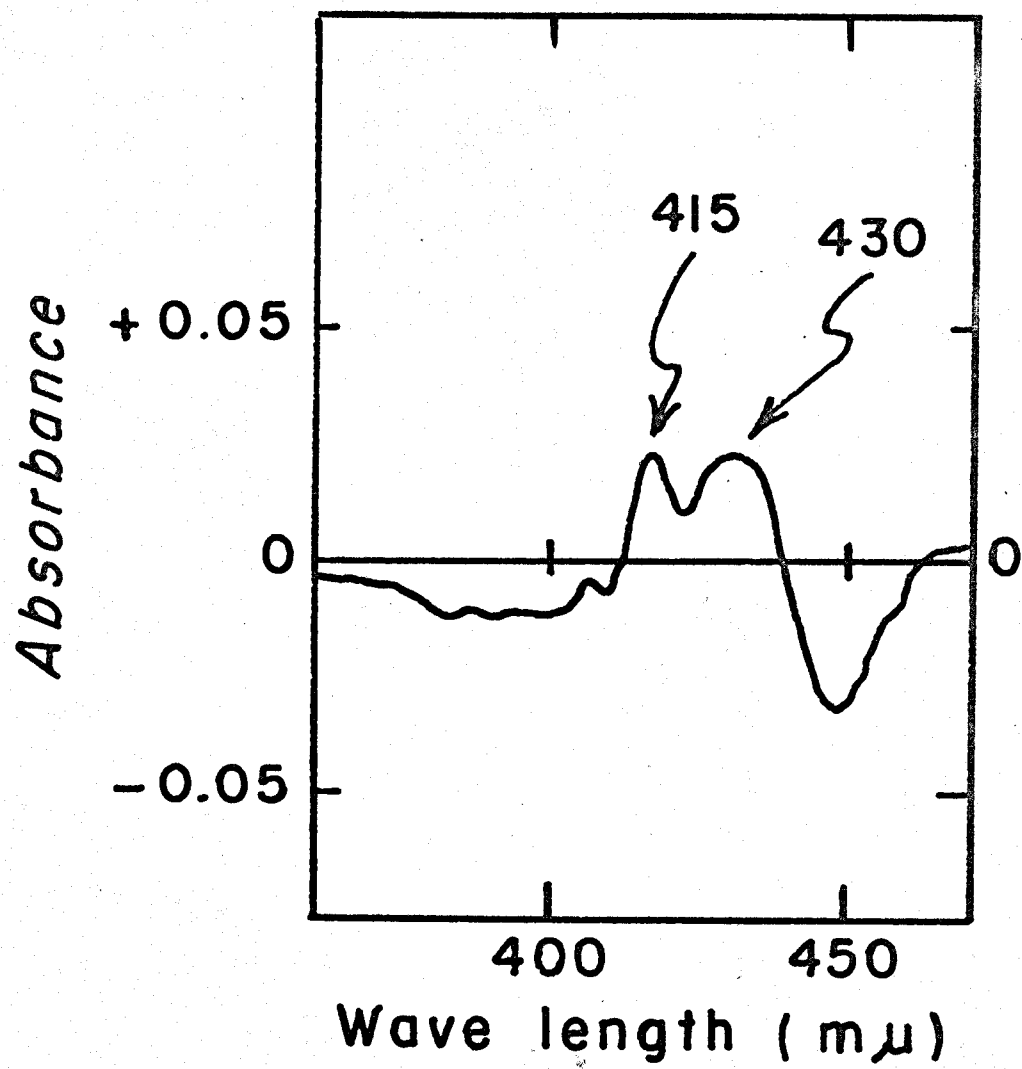


Fig. 15

is used as the reducing agent, the 415 and 430 m μ maxima are replaced by a maximum at 423 m μ (2).

Chance (6, 7, 8), in his study of carbon monoxide reaction with cytochrome oxidase, has reported that preparations with appreciable hemoglobin content gave a peak in the CO-difference spectrum at 424 m μ , and Ball's 423 m μ peak might be due to this pigment. The higher the K_2 ($K_2 = \frac{\Delta D_{445}}{\Delta D_{433}}$), the lower the hemoglobin contamination. Thus from those preparations in which K_2 is approximately equal to six, Chance (6) observed a maximum at 430 m μ only. Slater, Colpa-Boonstra and Minnaert (11, 75) have implied that the observation of 423 m μ in the carbon monoxide difference spectrum of the heart muscle preparation may be due to myoglobin. The carbon monoxide difference spectrum of myoglobin shows a maximum at 422 m μ (75). Judging from the history of the preparation of the complex, it is not likely to have hemoglobin or myoglobin as a contaminant. The K_2 value of the complex is very high and approaches infinity. That the 415 m μ peak in the difference spectrum in the cytochrome c-cytochrome oxidase complex is not due to contamination or to an alteration of the components during the preparation, comes from various line of evidence: (1) The components, either prior to the preparation of the complex, or subsequently dissociated from the complex, do not show the 415 m μ maximum. (2) The 415 m μ

maximum disappears when the complex is incubated with cholate (i.e. when cholate has dissociated the complex into its components). (3) When the complex is reconstituted with cytochrome c reductase, no 415 m μ peak is observed; instead a 423 m μ peak appears. (4) A "free mixture" of both components exhibits no absorption peak at the same wave length in the presence of carbon monoxide.

It is speculative whether the 415 m μ maximum is due to a conformational change of cytochrome c and/or cytochrome oxidase in the complex. In this connection, it may be mentioned that the trichloroacetic acid modified cytochrome c or polymer of cytochrome c forms a carbon monoxide compound which gives a 415 m μ maximum (57, 59).

Butts and Keilin re-examined the carbon monoxide compound of cytochrome c at extreme pH (below three and above twelve) and found an absorbancy decrease in the α region and an increase at 415 m μ at these pH (5). One can say that a definite conformational change in the protein moiety of cytochrome c takes place at extreme pH. The heme group which is situated in a crevice of the protein in the physiological state, according to Theorell and Åkesson (83), might be exposed to reaction with carbon monoxide by such a conformational change. Thus, it may be concluded that the cytochrome responsible for the 415 m μ peak in the carbon monoxide spectrum is most probably cytochrome c and such an anomaly can be caused by

conformational change as a result of interaction with cytochrome oxidase resulting in complex formation. Close observation of the spectrum reveals a trough at about the 550 $m\mu$ region, occurring simultaneously with the 415 $m\mu$ peak. Pre-treatment of the complex with cholate diminishes both the 415 $m\mu$ peak and the 550 trough in the carbon monoxide spectrum.

These conformational changes can be also substantiated from the infrared spectra of the complex. The definite changes in radical groups are reflected in shifts at various regions and in the occurrence of new peaks.

Here it is well to recall the question on the carbon monoxide spectrum raised by Ball et al. (2). Due to the fact that the ascorbate reduced CO difference spectrum of the complex also shows 415 $m\mu$ and 430 $m\mu$ maxima, and that cytochrome b is rather insensitive to ascorbate reduction, a 423 $m\mu$ peak in the dithionite reduced CO spectrum could be attributed to the superposition of the absorptions of cytochrome oxidase, cytochrome c and cytochrome b in part, without any contamination by myoglobin. In this connection, Yonetani's interpretation of the disparity between his and Chance's observations on the 430 $m\mu$ peak of the CO difference spectrum of cytochrome oxidase is noteworthy (96). This can be substantiated by the fact that reconstituted succinate oxidase exhibits a 423 $m\mu$ maximum in a CO spectrum which

preparation may be completely free from myoglobin. Based on Chance's criterion, a cholate-treated Keilin-Hartree heart muscle preparation, in which the K_2 value is much higher than six, also shows a 423 $m\mu$ maximum.

Then why does Chance (6) observe a maximum at 430 $m\mu$ but not at 415 $m\mu$ in the heart muscle preparation? This can be explained from a consideration of "solubility" of the preparation and accessibility to carbon monoxide of the other cytochromes, excepting cytochrome a₃. Chance did not treat the preparation with detergent. The "solubility" of his preparation might not be comparable to Ball's electron transmitter system (1), or the the cytochrome c-cytochrome oxidase complex where detergent was extensively applied.

Final judgement of the validity of the hypothesis that the CO 415 $m\mu$ peak is due to conformational change on the part of cytochrome c resulting from complex formation with cytochrome oxidase (which differs from polymerized cytochrome c or alkaline- and acid-treated cytochrome c) may come from examination of the physiological activity of the cytochrome c-cytochrome oxidase complex. This will be described in the following part.

2. RECONSTITUTION OF SUCCINATE OXIDASE WITH CYTOCHROME c-
CYTOCHROME OXIDASE COMPLEX (50)

INTRODUCTION

While the reconstitution of succinate oxidase and succinate cytochrome c reductase identical to the intact oxidase and the intact reductase has been successful (35, 36, 40), reconstitutive study of the terminal part of the electron transferring chain is proposed, especially with the cytochrome c-cytochrome oxidase complex described previously.

This also constitutes a physiological test of the complex per se. The attempt was made to reconstitute the succinate-cytochrome oxidase system from soluble succinate dehydrogenase and the cytochrome b-c₁ particle with the cytochrome c-cytochrome oxidase complex.

This part of the thesis describes the successful reconstitution of the succinate oxidation system and its kinetic examination. A comparison was also made between the reconstituted succinate oxidase and the intact preparation, such as the Keilin-Hartree heart muscle preparation.

EXPERIMENTAL PROCEDURES

Materials

The soluble succinate dehydrogenase was prepared from the Keilin-Hartree heart muscle preparation up to the stage of the gel eluate by method III (36). The cytochrome b-c₁ was made from the heart muscle preparation (81).

Antimycin A was obtained from Wisconsin Alumni Research Foundation; thenoyltrifluoroacetone from Aldrich Chemical Company.

RESULTS

Reconstitution of Succinate Oxidase with Cytochrome c- Cytochrome Oxidase Complex, Soluble Succinate Dehydrogenase and Cytochrome b-c₁ Particle; Turnover Number

The reconstituted succinate cytochrome c reductase was made from both freshly made soluble succinate dehydrogenase (0.4 mg/ml) and cytochrome b-c₁ particle (6 mg/ml) at a volume ratio of 4 to 1. The cytochrome c-cytochrome oxidase complex was prepared as previously (1:1, oxidized form, 30 μ M each).

The soluble succinate dehydrogenase (80 μ g/ml) plus cytochrome b-c₁ particle (1.2 mg/ml) or reconstituted succinate cytochrome c reductase consisting of the same amount of succinate dehydrogenase and cytochrome b-c₁ particle was inactive, even in the presence of an additional 20 μ M cytochrome c, in the catalytic oxidase of succinate. The cytochrome c-cytochrome oxidase complex alone was also inactive in catalyzing the oxidation of succinate.

The reconstitution of the succinate oxidation system was conducted as follows: the reaction mixture contained the final concentrations of 25 mM phosphate buffer, 40 mM succinate, either soluble succinate dehydrogenase plus cytochrome b-c₁ or the reconstituted succinate cytochrome c reductase, the cytochrome c-cytochrome oxidase complex of 1:1

ratio; total volume 2.5 ml; pH 7.4; temperature, 25° C. The concentrations of succinate dehydrogenase and cytochrome b-c₁ were 80 µg/ml and 1.2 mg/ml, respectively. The reductase consisted of 80 µg of soluble succinate dehydrogenase and 1.2 mg of cytochrome b-c₁ particle per ml. The cytochrome c and cytochrome oxidase contents of the complex were 1.2 µM each. The components were added to the buffer solution in the following order: succinate, succinate dehydrogenase and cytochrome b-c₁ particle or succinate cytochrome c reductase, and the cytochrome c-cytochrome oxidase complex. The Q_{O₂} value of this system amounted to approximately 55,000 µl of oxygen per hour per µmole of cytochrome oxidase content of the complex. This system did not require any additional free cytochrome c (Fig. 16).

Titration of Reconstituted Succinate Cytochrome c Reductase
with Cytochrome c-Cytochrome Oxidase Complex; Turnover
Number of the Reconstituted Succinate Oxidase

The catalytic activity of the system was proportional to the concentration of the cytochrome c-cytochrome oxidase complex. When the same amount of succinate cytochrome c reductase was titrated with the complex, the activity measured as oxygen consumption increased linearly at first and gradually fell off as shown in Fig. 17. The calculated molar ratio of cytochromes b : c₁ : c : cytochrome oxidase at the

Fig. 16. Reconstitution of succinate oxidase with succinate cytochrome c reductase and cytochrome c-cytochrome oxidase complex.

The reaction mixture contained the final concentration of 25 mM phosphate buffer, 40 mM succinate, succinate cytochrome c reductase and the cytochrome c-cytochrome oxidase complex; total volume, 2.5 ml; pH 7.4; temperature 25° C. The reductase consisted of 80 µg of soluble succinate dehydrogenase at the gel eluate stage and 1.2 mg of cytochrome b-c₁ particle per ml. The cytochrome c and the cytochrome oxidase contents of the complex were 1.2 µM each. Additions: (1) succinate; (2) succinate cytochrome c reductase; and (3) the cytochrome c-cytochrome oxidase complex.

Reconstitution of Succinate
oxidase from SDH, $b-c_1$, and
 c -oxidase

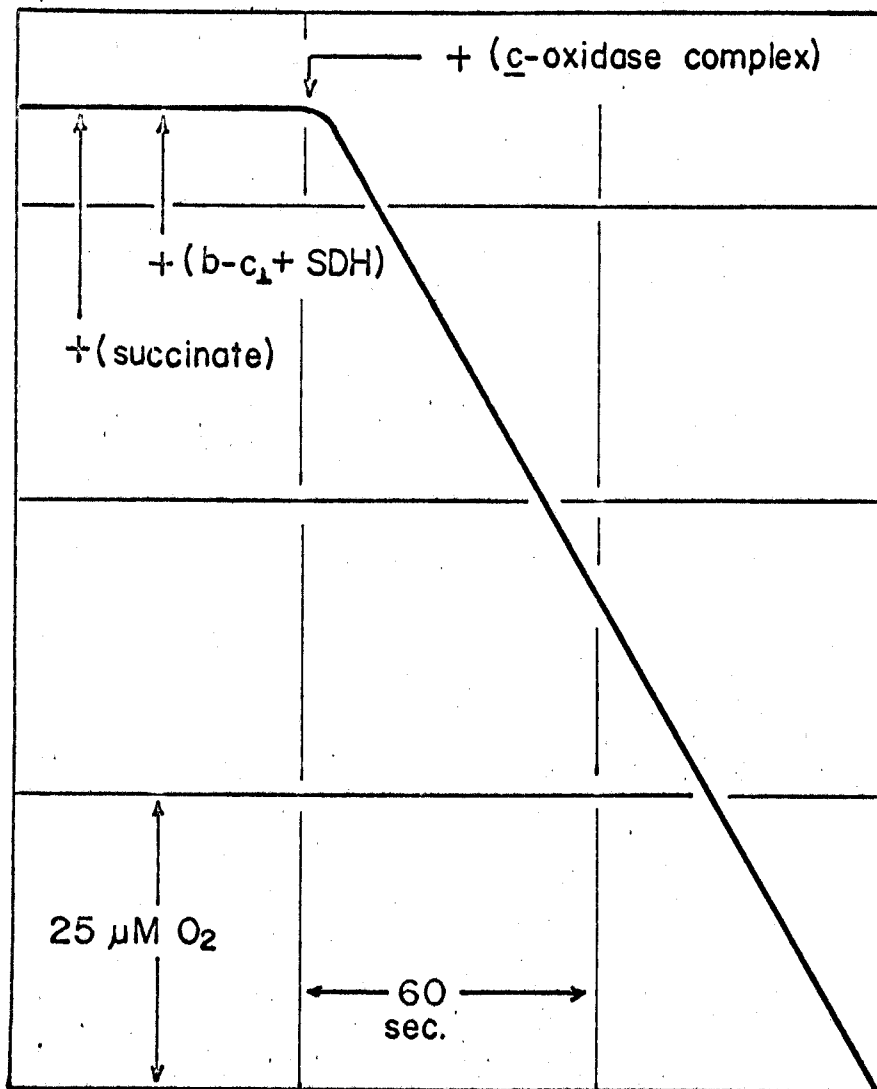


Fig. 16

Fig. 17. Effect of the concentration of the cytochrome c-cytochrome oxidase complex on the activity of the reconstituted succinate oxidase.

The reaction mixture contained 25 mM phosphate buffer, 40 mM succinate, succinate cytochrome c reductase (80 μ g of soluble succinate dehydrogenase plus 1.2 mg of the cytochrome b-c₁ particle in one ml) and the indicated amounts of the cytochrome c-cytochrome oxidase complex; total volume, 2.5 ml; pH 7.4. The ordinate is the activity in μ l of oxygen consumed at 25° C per minute of the complex of one to one molar ratio (cytochrome c to cytochrome oxidase). The abscissa is the concentration of the complex in μ M cytochrome oxidase present.

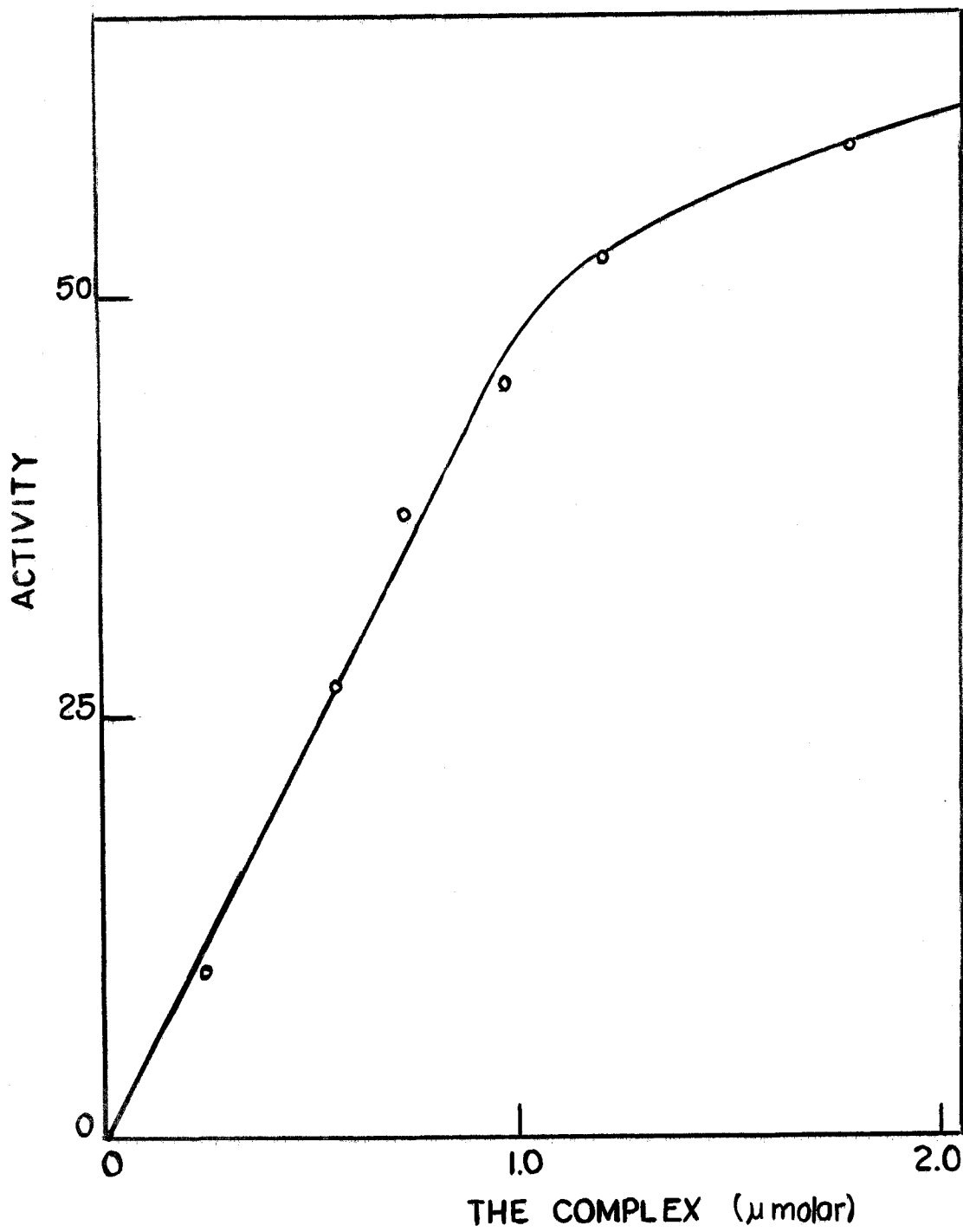


Fig. 17

highest specific activity was about 2 : 1 : 1 : 1. King reported turnover number of the succinate oxidase to be 7,500-10,000 in the heart muscle preparation and the reconstituted system, which consists of succinate dehydrogenase and alkaline-treated heart muscle preparation (42). The turnover numbers are expressed in moles of succinate oxidized per minute per mole of succinate dehydrogenase flavin added or the cytochrome oxidase present. The turnover number of the present reconstituted succinate oxidase is shown in Table IV, with King's data for comparative purposes.

These three preparations appear to be comparable with respect to both succinate dehydrogenase and cytochrome oxidase.

Inhibition of the Reconstituted Succinate Oxidase;

CO Inhibition and the Effect of Light

The activity of the system was almost completely inhibited by the usual respiratory poisons, such as Antimycin A, cyanide, and azide, but not by Amytal. It was also inhibited by thenoyltrifluoroacetone and competitively by malonate (Table V). The heart muscle preparation was inhibited in its oxygen uptake in succinate oxidation by carbon monoxide treatment. It was observed that this carbon monoxide inhibition was noticeably relieved with illumination by white light on the reaction cell of the oxygraph, in the presence of a few percent of dissolved oxygen. Likewise the reconstituted

TABLE IV

Turnover number of the reconstituted succinate oxidase

Heart muscle preparation (42)	Succinate dehydrogenase	7.5×10^3	
	Cytochrome oxidase	7.6×10^2	
Reconstituted system	King (42)	Succinate dehydrogenase	10.0×10^3
		Cytochrome oxidase	5.4×10^2
	The present	Succinate dehydrogenase	2.1×10^3
		Cytochrome oxidase	8.0×10^2

The turnover numbers are expressed in moles of succinate oxidized per minute per mole of succinate dehydrogenase flavin added or cytochrome oxidase content. For the present system, the flavin content of the succinate dehydrogenase was calculated on the basis of 1.2 μ moles flavin per mg protein at the stage of gel eluate.

TABLE V

Inhibition of reconstituted succinate oxidase

Inhibitor	Concentration	Inhibition rate
	M	%
KCN	1×10^{-4}	94
	4×10^{-5}	78
NaN_3	3×10^{-4}	60
Antimycin A	3 $\mu\text{g/ml}$	78
Malonate	8×10^{-3}	70
Thenoyltrifluoro- acetone	3×10^{-5}	83
Amytal	3×10^{-3}	0

succinate oxidation system was relieved with light after treatment with carbon monoxide (Fig. 18). These results agree with the previous reports (8, 9; 90, p. 144).

Fig. 18. Photo-irradiation effect on the carbon monoxide inhibited reconstituted succinate oxidase and the Keilin-Hartree heart muscle preparation.

The reaction mixture of 2.5 ml contained 40 mM succinate, 25 mM sodium phosphate buffer (pH 7.4) and the enzyme system (1.3 mg of the Keilin-Hartree heart muscle preparation in one ml, or the reconstituted succinate-cytochrome oxidase consisting of cytochrome c-cytochrome oxidase complex --1.2 μ M of each component--1.2 mg cytochrome b-c₁, and 80 μ g succinate dehydrogenase in one ml). The reaction mixture was bubbled with pure CO in the oxygraph cell at 25° to reduce the oxygen level to 3.3 μ M. Then the oxygen level was enlarged to a full scale of 30 cm span by manipulation of the sensitivity adjustment. The scanning was operated at the maximum speed to determine oxygen uptake. The illumination on the water-jacketed reaction cell was made with "Viewlex" slide projector (500 W) at a distance of 60 cm during the scanning. The light beam was focused to the cell by placing a magnifying lens (7 cm in diameter) in the light path at 40 cm from the projector.

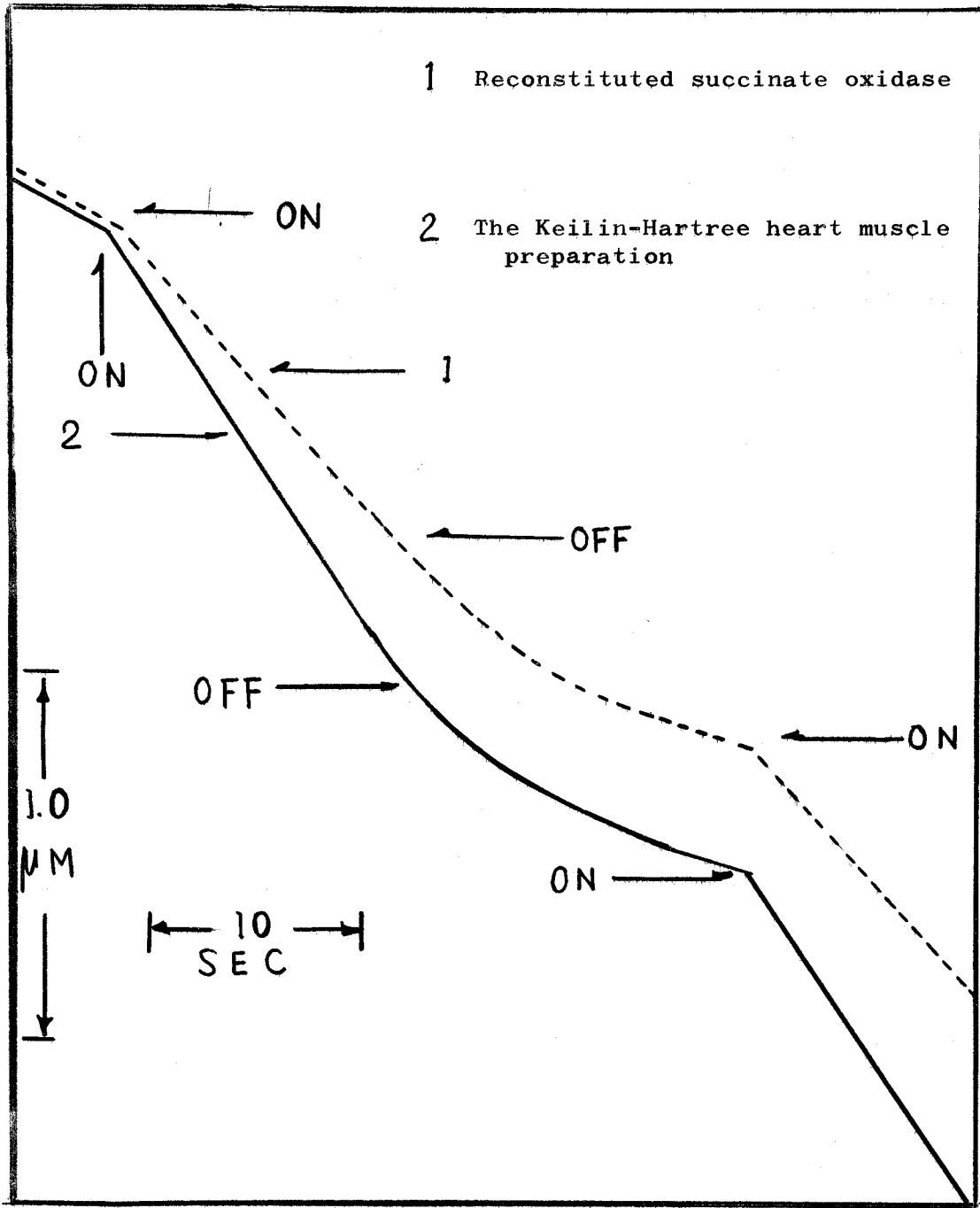


Fig. 18

Activity of Complexes of Different Ratios
of Cytochrome c to Cytochrome Oxidase

As previously described, other forms of the cytochrome oxidase complex with 0.5 and 0.2 ratios of cytochrome c to cytochrome oxidase have been isolated. A comparison was made of the activities of these complexes as well as the mixture of the free components in the reconstitution of succinate oxidase.

The reaction mixture contained 25 mM sodium phosphate buffer, 40 mM succinate, succinate cytochrome c reductase (70 μ g of succinate dehydrogenase plus 1.3 mg of the cytochrome b-c₁ particle) and the complex or "free components" at 1.0 μ M cytochrome oxidase; total volume, 2.5 ml; pH 7.4; temperature 25° C. "Free components" (cytochrome c and cytochrome oxidase) were added in the same amount as that in the complex. Before addition, the cytochrome oxidase was subjected to sonic irradiation for about 45 minutes. It can be seen that the activity increases with the ratio. Furthermore, in the reconstitution of the succinate oxidase, the specific activity of the complex of 1:1 ratio of cytochrome c to cytochrome oxidase was nearly twice as much as that of its components when the latter were added in the free form. On the other hand, those forms of the complex with the lower ratios of cytochrome c to cytochrome oxidase exhibited almost the same activity as if the components were in the uncombined

state (Table VI).

TABLE VI

Comparison of the activity of the cytochrome c-cytochrome oxidase complex with the free components in the reconstitution of succinate oxidase

Molar ratio of the complex (<u>cytochrome c</u>) (<u>cyt. oxidase</u>)	Specific activity (μ l O ₂ consumed/minute/ μ mole cytochrome oxidase)		Activity ratio (<u>complex</u>) (<u>free components</u>)
	Complex	Free components	
1.0	760	420	1.8
.5	340	280	1.2
.2	190	170	1.1

The reaction mixture contained 25 mM phosphate buffer, 40 mM succinate, succinate cytochrome c reductase (70 μ g of succinate dehydrogenase plus 1.3 mg of the cytochrome b-c₁ particle) and the complex or "free components" at 1.0 μ M of cytochrome oxidase; total volume, 2.5 ml; pH 7.4; temperature 25° C. "Free components" (cytochrome c and cytochrome oxidase) were added in the same ratio as that in the complex. Before addition, the cytochrome oxidase was subjected to sonic irradiation for about 45 minutes.

Effect of Buffer Concentration, Detergent and pH on the Succinate Oxidase Activity of the Reconstituted System

The reaction mixture of 2.5 ml contained 40 mM of succinate, 25 mM sodium phosphate buffer, unless otherwise specified, and an enzyme system, which was either 1.3 mg of the Keilin-Hartree heart muscle preparation in one ml or the reconstituted succinate cytochrome oxidase consisting of cytochrome c-cytochrome oxidase complex (1.2 μ M of each), 1.2 mg of cytochrome b-c₁ particle and 80 μ g of succinate dehydrogenase per ml.

Employing the sodium phosphate buffer (pH 7.4), the effect of phosphate buffer concentration on the succinate oxidase activity was determined in the range of concentration from 0.01 to 0.1 M. The succinate oxidase activity of the reconstituted system was inhibited 30% at 50 mM and 50% at 0.1 M.

The Emasol 1130, non-ionic detergent, inhibited 40% at a concentration of 5% (v/v). Sodium cholate, anionic detergent, at 0.5% inhibited 50% of the succinate oxidase activity.

The optimum pH was also examined with the reconstituted succinate oxidase using both 25 mM of sodium phosphate buffer and 25 mM sodium-citrate-phosphate buffer. The optimal pH of the reconstituted system coincided with that of the heart muscle preparation at 7.4-7.6 in both buffers (Figs. 19 and 20).

Fig. 19. Optimal pH for succinate oxidase activity of the reconstituted succinate oxidase.

The pH of the 25 mM sodium-phosphate buffer ranged from 5.6 to 7.8; 25 mM sodium-citrate-phosphate buffer 5.8 to 8.0. The reaction mixture of 2.5 ml contained 40 mM succinate, 25 mM buffer and the reconstituted succinate oxidase (1.2 mg of cytochrome b-c₁ and 80 µg succinate dehydrogenase in one ml; 1.2 µM cytochrome c-cytochrome oxidase complex, one to one ratio).

- (1) Sodium-citrate-phosphate buffer
- (2) Sodium-phosphate buffer

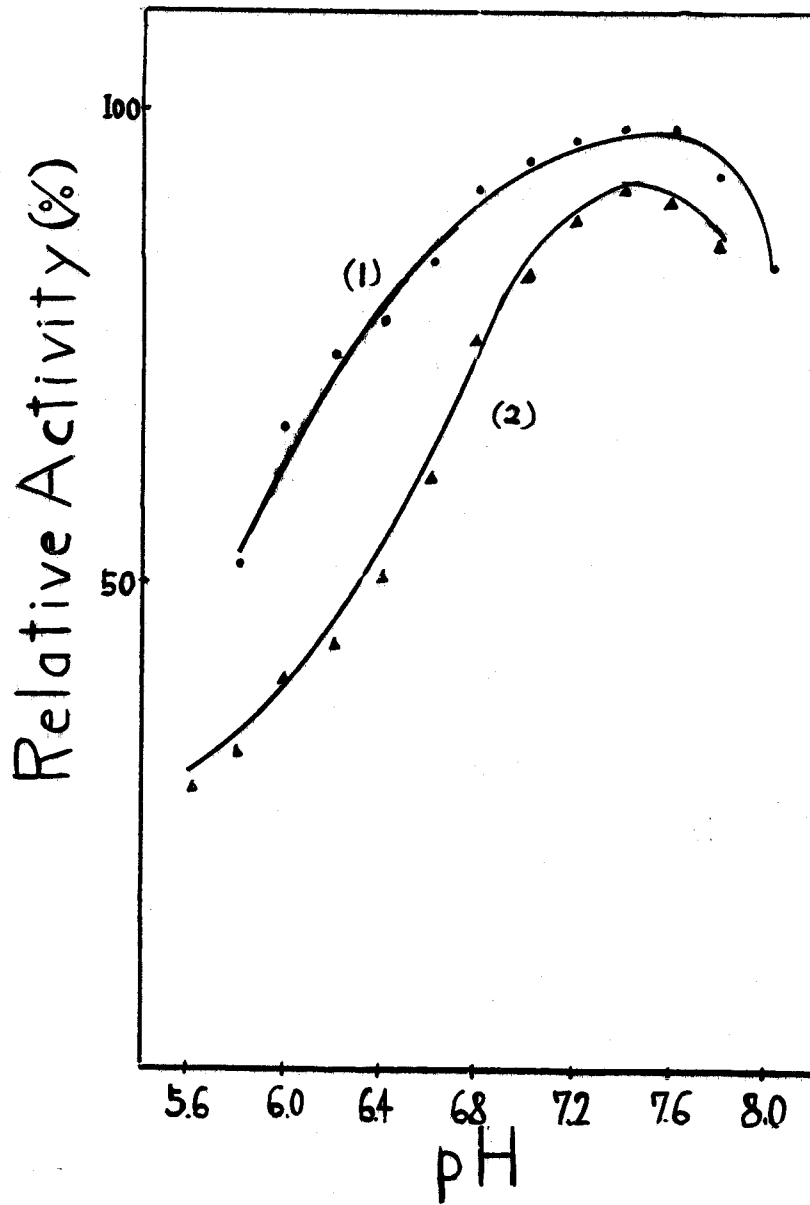


Fig. 19

Fig. 20. Optimal pH for succinate oxidase activity of the Keilin-Hartree heart muscle preparation.

The pH of the 25 mM sodium-phosphate buffer ranged from 5.6 to 7.8; 25 mM sodium-citrate-phosphate buffer 5.8 to 8.0. The reaction mixture of 2.5 ml contained 40 mM succinate, 25 mM buffer and 1.3 mg of the Keilin-Hartree heart muscle preparation in one ml.

(1) Sodium-citrate-phosphate buffer

(2) Sodium-phosphate buffer

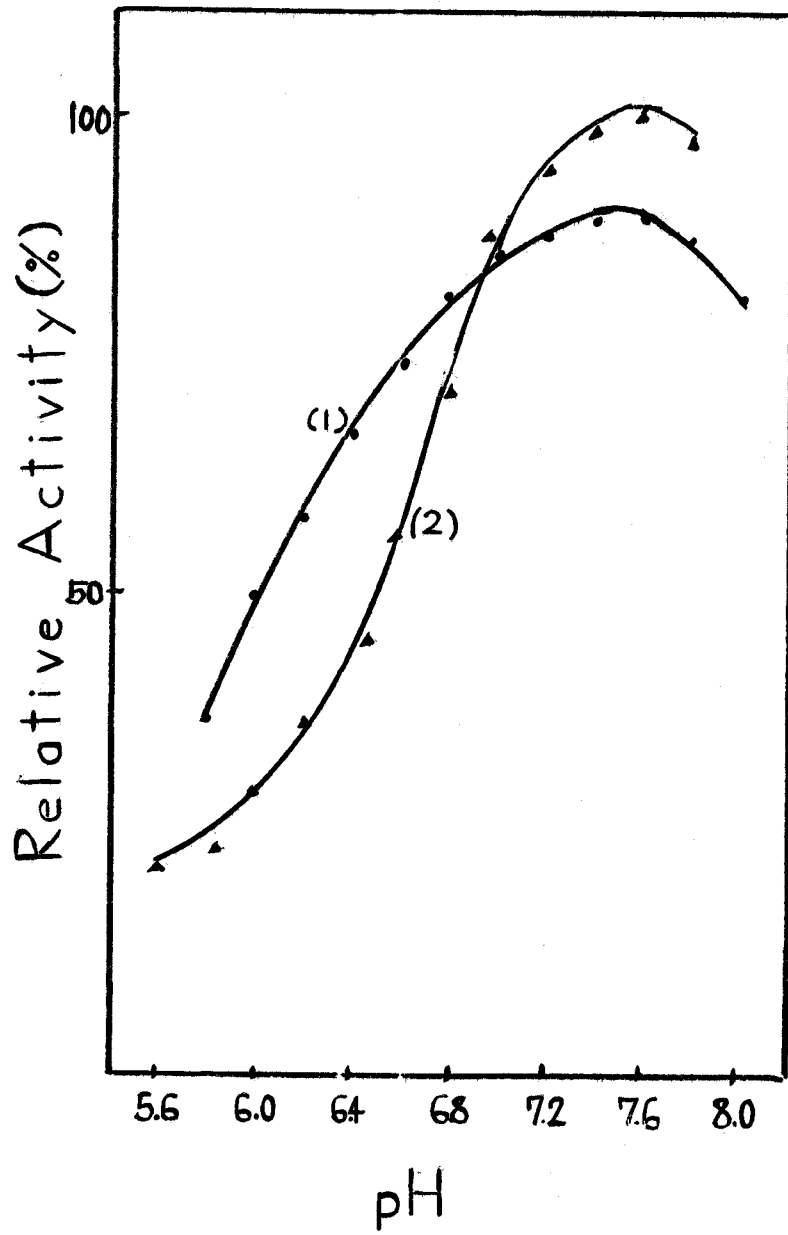


Fig. 20

DISCUSSION

In reference to the anomaly of a carbon monoxide compound of the cytochrome c-cytochrome oxidase complex, the polymerized form of cytochrome c was mentioned. This polymerized form of cytochrome c was inactive toward cytochrome oxidase; the active sites of cytochrome c could be sterically hindered or block each other (57, 59). The cytochrome c in the cytochrome c-cytochrome oxidase complex does not belong to this category of polymerized cytochrome c because the complex on the basis of its molar composition is more active than the free cytochrome c in the oxidation of succinate. Butt and Keilin (5) have viewed the formation of a carbon monoxide compound by cytochrome c as an indication of its autoxidizability. They state (5):

The increment in the ability to combine with CO was accompanied by an increase in the ability to catalyze the oxidation of ascorbate and a decrease in the ability to react with the succinic dehydrogenase-cytochrome system of the heart muscle preparation.

It is clear that the cytochrome c in the complex does not conform to this behavior because of the fact that it is much less active in the oxidation of ascorbate than in that of succinate (56, 84, 85). It has been observed that an exact parallel exists between the decrease of catalytic activity of the complex and the dissociation of the complex by the addition of concentrated salt solution to the reaction medium.

"Endogenous" cytochrome c has been shown to be incorporated into the respiratory chain (34, 86) and more active than the exogenous component (14, 73, 86); the catalytic activity of the complex is twice as high as that of the free components. Moreover, the behavior of the complex toward the N,N,N',N'-tetramethyl-p-phenylenediamine-ascorbate system is exactly the same as that of mitochondrial preparation, such as the Keilin-Hartree heart muscle preparation. The maxima of CO spectrum similar to those of the complex were also observable in a clarified intact succinate oxidase, such as Ball's "electron transmitter system," when ascorbate was employed as a reductant.

The CO spectrum, optimal pH and behaviors toward available respiratory poisons of the reconstituted succinate oxidase with the complex were found to highly resemble the intact preparation.

In comparing the turnover numbers, it was found that the present reconstituted succinate oxidase accorded a lower value with respect to succinate dehydrogenase than either the heart muscle preparation or King's reconstituted succinate oxidase. This discrepancy is explainable by the fact that the succinate dehydrogenase is extremely labile under the conditions of the reconstitution experiment and the amount of enzyme actually incorporated is lower than that of the total amount employed. On the other hand, the turnover number with cytochrome oxidase

is still comparable.

In view of these considerations, one can think the cytochrome c-cytochrome oxidase may exist as such in the respiratory chain. The isolated components show properties different from those in the chain but once the components are re-incorporated, the original properties are resumed. In the case of the complex, the behavior resembles that of the segment in the chain containing cytochrome c and cytochrome oxidase.

3. "RECONSTITUTION" OF CYTOCHROME OXIDASE FROM
CYTOCHROME a AND HEMATIN a (52)

INTRODUCTION

The dispute on the identity or non-identity, functional as well as structural, of cytochrome a and a₃ has already been outlined. Recent isolation of cytochrome a free from cytochrome a₃ by Horie and Morrison (21) leads toward a convincing direction for resolving the question. The criterion used by Horie and Morrison is, however, based only on the spectral properties.

Enzymatic activity of the cytochrome a preparation has been examined recently by King and Kuboyama (46) and it has been found to be active as an electron acceptor.

The isolated cytochrome a is essentially insensitive to carbon monoxide, does not react with oxygen and exhibits absorption spectra different from cytochrome oxidase.

It may be proposed that during the isolation of cytochrome a, the heme a attributable to cytochrome a₃ might be modified or destroyed so that the resulting heme a could no longer function as a prosthetic group of cytochrome a₃. If this is the case, cytochrome oxidase activity should be restored by introduction of intact heme a to the cytochrome a preparation under proper conditions.

This part of the thesis presents restoration of cytochrome

oxidase activity of cytochrome a preparation with incorporated heme a, and spectral properties of the reconstituted cytochrome oxidase.

EXPERIMENTAL PROCEDURES

Materials

Cytochrome a was isolated from cytochrome oxidase by a modification of Horie and Morrison (21). Initial dithionite reduction was conducted for five minutes, followed by 15 mM cyanide incubation for 30 minutes at 0-4° C. The final preparation was dialyzed against 50 mM sodium phosphate buffer, pH 7.4, containing 0.5% Emasol 1130 at 0-4° C for 18 hours. Hemin a was freshly prepared from cytochrome oxidase lyophilized preparation by means of a procedure similar to those by Morrison and Horie (65) and Takemori and King (82). Hemin a was also made from beef heart mince by means of metalation of purified porphyrin a (63). Palladium-asbestos for reduction of cytochrome c was obtained from Fisher Scientific Company. Crystalline catalase was procured from Worthington Biochemical Corporation, Freehold, New Jersey.

Methods

Hematin a Solution -- Hemin a was dissolved into a minimal amount of 0.05 M sodium hydroxide and, after it was completely dissolved, was diluted 10 to 20 times its volume with 0.1 M phosphate buffer, pH 7.4. If any particulate material was present, the solution was clarified by filtration. The final concentration of hematin a solution was determined

by a pyridine hemochrome method (82). The pyridine was kept over KOH and purified by distillation.

Determination of the Oxygen Uptake -- Oxygen uptake by the enzyme system was determined by a G.M.E. oxygraph at 25° C as previously described. The rate of oxidation of the reduced cytochrome c was measured spectrophotometrically with a Cary, model 11 (98).

Cytochrome a Concentration -- Cytochrome a concentration was determined for the reduced form, using an extinction coefficient of $22.8 \text{ mM}^{-1} \times \text{cm}^{-1}$ at 600 m μ (21).

RESULTS

Absorption Spectra of Cytochrome a Preparation

The absorption spectra of the reduced form and the carbon monoxide compound of the cytochrome a preparation are shown in Fig. 21. The maxima in the absolute spectrum of the dithionite reduced form are at 602 m μ , 515 m μ , and 440 m μ for the α -, β -, and Soret bands, respectively. The maxima in the carbon monoxide absolute spectrum are at 602 m μ and 439 m μ for the α - and Soret bands, respectively. The present cytochrome a preparation was practically insensitive to carbon monoxide and very similar to that of Horie and Morrison (21).

"Reconstitution" of Cytochrome Oxidase from Cytochrome a and Hematin a

The prepared cytochrome a showed no cytochrome oxidase activity, namely it could not consume the dissolved oxygen with the ascorbate-cytochrome c electron donor system and failed to oxidize the reduced cytochrome c with molecular oxygen.

Incubation mixtures were made from cytochrome a, ascorbate-EDTA, and cytochrome c (oxidized and reduced), as tabulated in Table VII. These systems were allowed to stand at room temperature. The oxygen uptake was measured by successive sampling of aliquots from the incubation mixture.

Fig. 21. Absorption spectra of cytochrome a preparation.

Cytochrome a was dissolved in 0.1 M sodium phosphate buffer (pH 7.4) containing 1% Emasol 1130 after 18 hours dialysis. The concentration was 7 μ M, or 1.26 mg protein per ml.

_____ Reduced form
----- Treated with CO after reduced

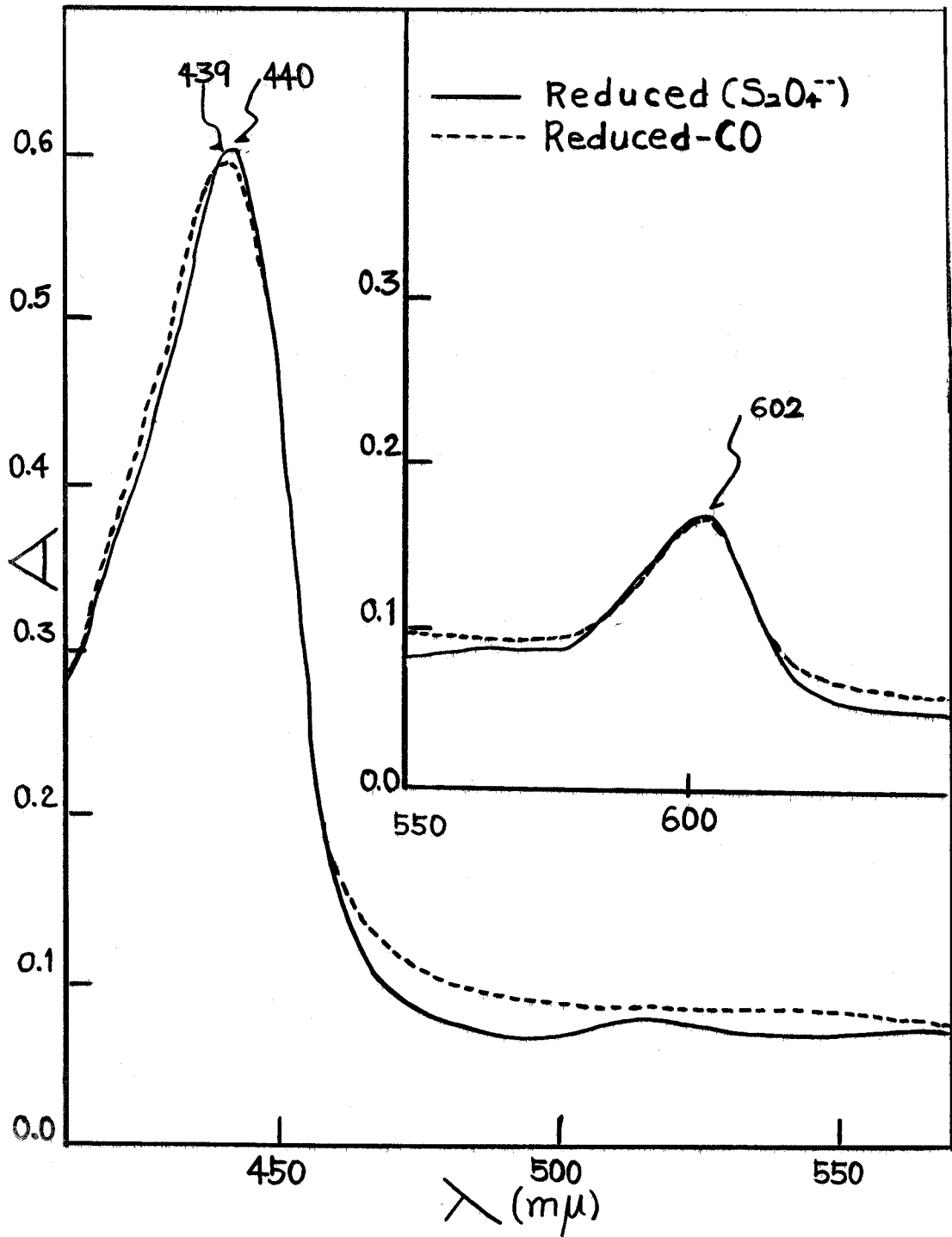


Fig. 21

TABLE VII

Systems used for "reconstitution" of cytochrome oxidase
from cytochrome a and hematin a*

System	Cyt. <u>a</u> μM	Cyt. <u>c</u> (oxidized) μM	Cyt. <u>c</u> (reduced) μM	Hematin <u>a</u> μM	Ascorbate mM	EDTA mM
1	30	30	--	30	--	--
2	30	--	30	30	--	--
3	30	30	--	30	20	1
4	30	--	--	30	20	1
5	--	--	--	30	20	1
6	30	--	--	--	20	1

* Basal medium: phosphate buffer, 0.1 M, pH 7.4

The reaction mixture for polarographic assay consisted of: 0.1 ml of enzyme, 20 μM cytochrome c, 30 mM ascorbate, 1.5 mM EDTA, and 50 mM phosphate buffer; total volume, 2 ml.

As shown in Fig. 22, the "reconstitution" of cytochrome oxidase proceeded gradually and reached a plateau in 45 minutes at room temperature by incubation of cytochrome a and hematin a in the presence of an electron donor system, namely ascorbate and cytochrome c in a neutral pH. The reconstitution took place in the system with a sufficient amount of reductants. Even in the system (System 2 of Table VII) containing equimolar concentrations of cytochrome a, hematin a and reduced

Fig. 22. Reconstitution of cytochrome oxidase from cytochrome a and hematin a.

The numbers labeled on the curves refer to the Systems used as shown in Table VII. At time intervals indicated, aliquots of the incubation mixture were withdrawn for determination of the cytochrome oxidase activity. The activity as expressed on the ordinate was determined polarographically at 25° C in a mixture containing 50 mM phosphate buffer, pH 7.4; ascorbate, 30 mM; EDTA, 1.5 mM; cytochrome c, 20 μ M; and an amount of enzyme equivalent to 0.75 μ M cytochrome a plus 0.75 μ M hematin a for Systems 1, 2, 3, 4, and 6, or equivalent to 1.5 μ M hematin a for System 5; total volume, 2.0 ml.

"Reconstitution" of Cytochrome Oxidase from
Cytochrome a and hematicin a

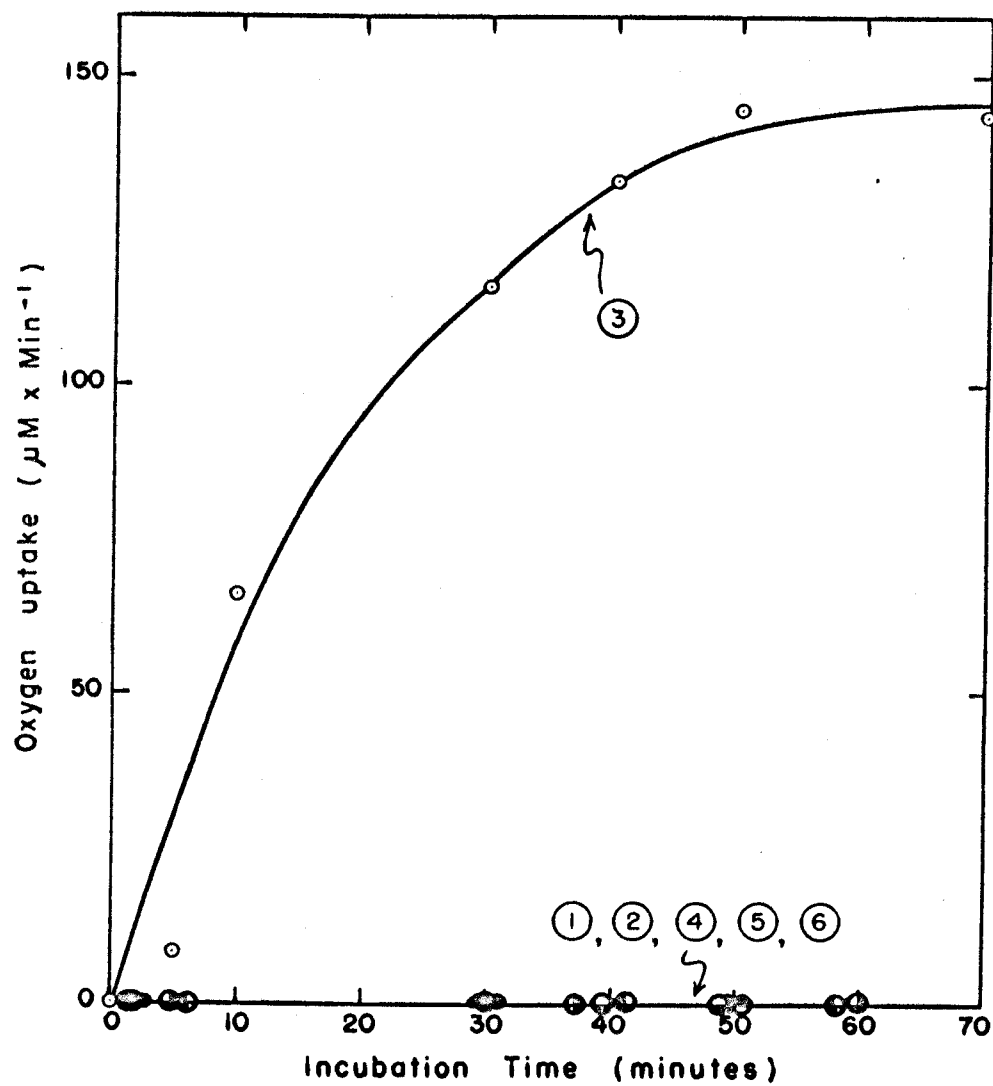


Fig. 22

cytochrome c, no reconstitution was observed. Calculations from four experiments similar to the one depicted in Fig. 23 gave an average value of 250 $\mu\text{moles O}_2$ reduced per minute per μmole of cytochrome a at 25° C, determined both polarographically and spectrophotometrically with reduced cytochrome c at 50 μM .

The recovery of cytochrome oxidase activity of the reconstituted system registered 60% of the cytochrome oxidase used as a control. The oxygen uptake was not affected by addition of catalase up to as high as 1 mg of crystalline catalase per ml, but was inhibited to about 90% by 0.1 mM cyanide or 1 mM azide.

Spectral Properties of the "Reconstituted" Cytochrome Oxidase

The spectral study of the "reconstituted" cytochrome oxidase was conducted with samples purified by chromatography on Sephadex G-75 column, using 0.1 M sodium phosphate buffer containing 1% Emasol 1130. On development of the applied sample, three distinct bands were observed: the first band was of a greenish color and retained full cytochrome oxidase activity; the second band contained only cytochrome c; and the third one consisted of hematin a, as detected spectrally. Fig. 23 shows the spectra of the oxidized and reduced forms of the reconstituted cytochrome oxidase and its carbon monoxide compound. It may be noted that all absorption

Fig. 23. Absolute spectra of reconstituted cytochrome oxidase.

Curve 1, the dithionite-reduced; Curve 2, the dithionite-reduced and subsequently treated with carbon monoxide; and Curve 3, the oxidized.

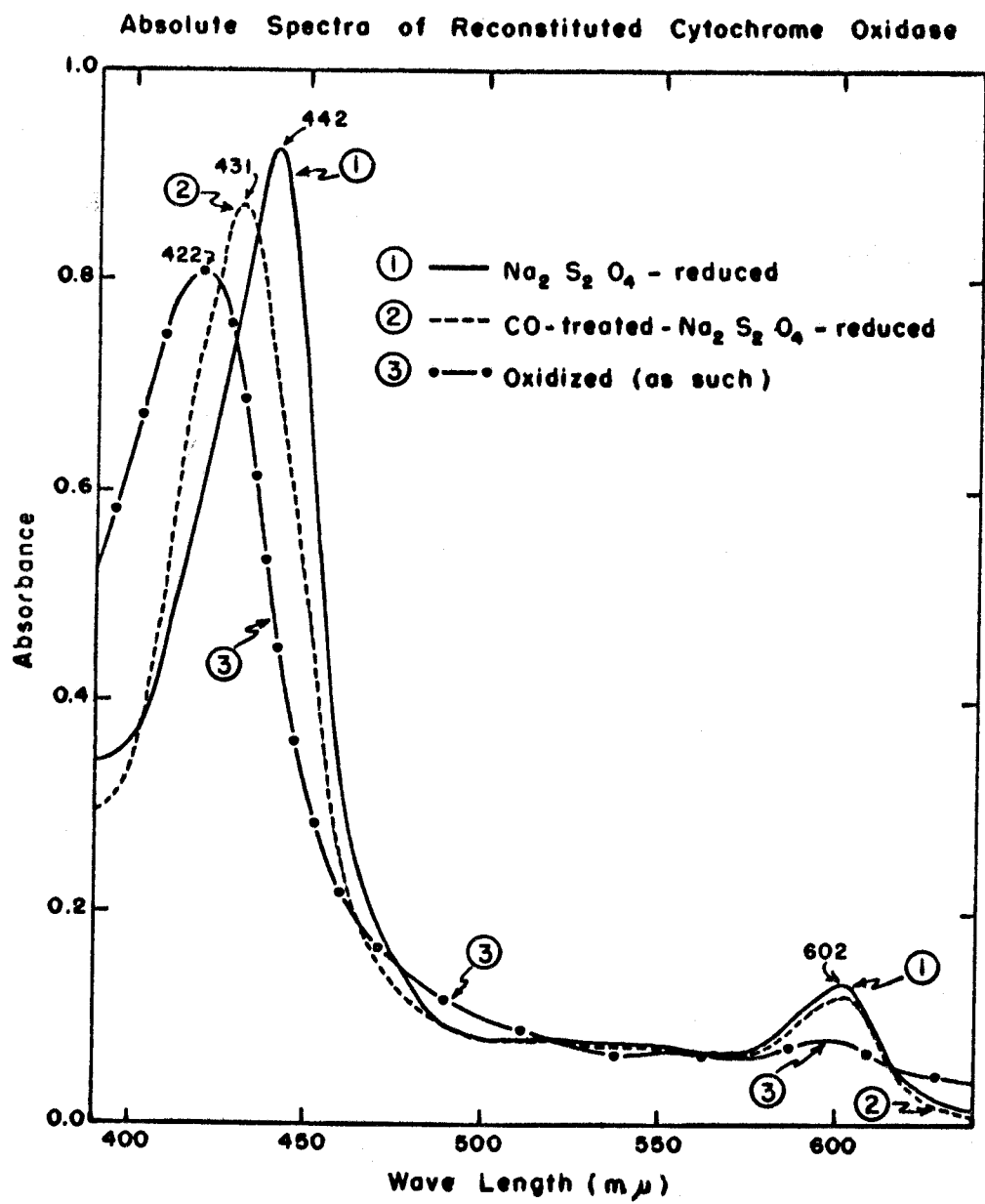


Fig. 23

maxima occurred at the same positions as in the intact cytochrome oxidase. Likewise, the Soret peak of the carbon monoxide compound was 431 m μ . The difference (reduced-oxidized) spectrum and the spectrum of the cyanide compound of the reconstituted cytochrome oxidase (Curve 1, Fig. 24) were also essentially the same as those of the intact preparation. Moreover, treatment of the cyanide complex with carbon monoxide shifted the Soret peak from 441-3 to 430-32 m μ (Curve 2, Fig. 24); in other words, carbon monoxide displaced cyanide attached to the reconstituted oxidase as it does in intact cytochrome oxidase.

Fig. 24. Effect of cyanide and carbon monoxide on absolute spectrum of reconstituted cytochrome oxidase.

Curve 1, the cyanide compound of the dithionite-reduced oxidase; Curve 2, the above system was then saturated with carbon monoxide.

Effect of CN and CO on Absolute Spectra of Reconstituted Cytochrome Oxidase

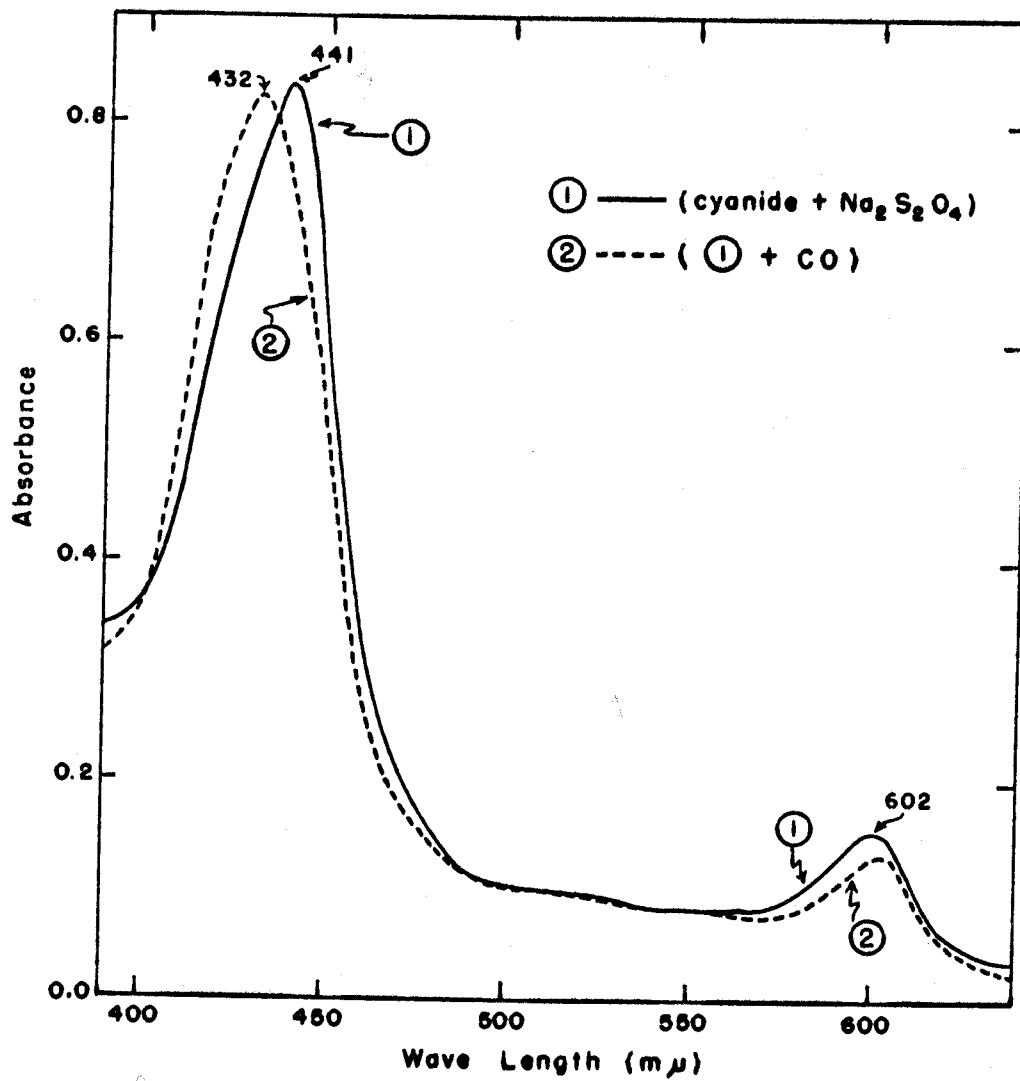


Fig. 24

DISCUSSION

Recently Horie (22) commented on the absorption maxima of his cytochrome a preparation, assuming a similarity between ferricytochrome a and ferricytochrome a₃-cyanide complex. However, any shift in absorption maxima occurring in the cytochrome a preparation is mainly attributable to the extent of destruction or modification of the cytochrome oxidase by the preparative reagents. The observed maxima of the reduced form and the carbon monoxide compound of the present cytochrome a preparation ranged from 600 to 602 m μ and 440 to 439 m μ in the α - and Soret bands, respectively. According to Horie, α - and Soret maxima of cytochrome a are more likely at 602 m μ and 441 to 440 m μ , respectively.

Although it may be noted that all absorption maxima occurred at the same positions as in the intact cytochrome oxidase, the shift at the red region for the carbon monoxide compound of reconstituted cytochrome oxidase differed slightly from intact cytochrome oxidase. The α -bands of practically all spectra of the reconstituted cytochrome oxidase were slightly less symmetrical than those of intact cytochrome oxidase. These disparities might possibly be due to: (1) the reconstituted cytochrome oxidase was not saturated (or was oversaturated) by added hematin a and/or (2) the linkages between added hematin a and protein differed from those in

the original.

Similar attempts have been made by Kiese (37) and Paul (70) with horse-radish peroxidase and by Yamanaka and Okunuki (90) with pseudomonas cytochrome oxidase. It seems reasonable that the total picture of a cytochrome oxidase molecule consists of protein (which is composed of one or more than one peptide chain) and heme a of a single chemical species which coordinates with ligand groups located at different sites in the tertiary structure of the protein moiety (44). Such a structural difference in the periphery of heme a might cause a dramatically functional difference between cytochrome a and cytochrome a₃. The heme a group attributable to cytochrome a₃ can be easily subject to carbon monoxide and cyanide attachment and also to borohydride modification after cyanide treatment in the reduced state, while the heme a group attributable to cytochrome a remains hindered for these treatments. A similar view was also extended by Horie (22).

Several possibilities can be considered for the mechanism of "reconstitution" of cytochrome a by introduction of heme a. The removal of heme a from cytochrome oxidase may furnish the opportunity for the intact heme a to re-coordinate to the intact ligand groups. However, the possibility of modified heme a remaining at the original site on the protein cannot be ruled out. In this case, reconstitution can take place by means of a simple exchange reaction between modified inactive

heme a and intact heme a.

In analogy with the activation of induced tryptophan pyrrolase by hematin (17), original oxidase may not be saturated with heme a groups, with unoccupied potential coordination sites occurring on the protein moiety. The third possibility of reactivation is that it may be due to the supplementation of intact heme a to those potential coordination sites.

The "reactivation" of cytochrome a could happen only under certain conditions; that is, by the incubation at room temperature of the mixture of cytochrome a, hematin a, and cytochrome c with a sufficient amount of ascorbate at neutral pH. It was noted that the oxidized form of cytochrome c with ascorbate could not be replaced by the same concentration of reduced cytochrome c.

A priori consideration of the process of biosynthesis of hemoprotein and the asymmetric form of heme a-1:3:5 trimethyl-2 α -hydroxyalkyl-4 β -alkylvinyl-8-formyl-6:7-di (β -carboxyethyl) iron porphyrin--may rationalize the requirement for the reconstitution of specific conditions such that physiological processes are operative.

GENERAL DISCUSSION

The reconstitution study offers not only confirmation of sequential arrangement of the electron carriers, but also an opportunity to examine the interaction mechanism between the adjacent ones.

From the spectral anomaly of the carbon monoxide compound of the cytochrome c-cytochrome oxidase complex, it has been proposed that a conformational change in the protein moiety could be taking place when both components are brought into complex formation, analogous to the behavior of cytochrome c in solutions of extreme pH.

From the experiment with modified cytochrome c and cytochrome oxidase, it has also been concluded that the interaction between cytochrome c and cytochrome oxidase involves electrostatic forces.

The isolated complex was in the oxidized form; in the reduced state of the complex, cytochrome c was found to be susceptible to carbon monoxide. The recent studies on cytochrome c disclosed the difference between the reduced and oxidized states with respect to the physico-chemical properties of this hemoprotein, that is: monomolecular layer (24), optical rotary dispersion (12), crystalline forms (18), digestion by proteolytic enzymes (93), chromatography (56), dissociation of the ligand complexes (72), and proton

magnetic resonance (48). These differences due to the oxidation state of the hemoprotein can be best explained only on the basis of a change in the protein conformation (59).

These observations may reveal the close relationship between the charge distribution in the whole molecule of hemoprotein and the protein conformation (54).

This view can be further extended in the respiratory chain enzyme system. The electron flow through the chain may cause change in the state of the electron carriers and that can be associated with reversible structural changes in the sequential arrangement in order to facilitate a close contact between adjacent components. A conformational change in the conjugated protein molecules, a closer contact at active sites between discrete components and a change in the reduction-oxidation state in electron carriers may represent a "Trinitarian" manifestation of an electron transport mechanism in the respiratory chain.

The original proposal of a physical approach to the electron transport enzyme system might be, to some extent, demonstrated in the present thesis. The same approach to other subjects in the biochemical field has been developed in a profusion of ways, enhanced by the development of borderline fields. This may represent a trend in modern biochemistry.

However, biochemistry was originally established as a discipline by the abiogenesis dispute between Liebig and

Pasteur in the middle 1800's over the mechanism of fermentation. This can serve as a clue to the formulation of a way of thinking intrinsic to biochemistry, a "biological way of thinking." Rather than mystifying biological phenomena, this may suggest that the physico-chemical approach is not the ultimate methodology in biochemistry as illustrated by a great deal of examples as landmarks in the history of biochemistry.

SUMMARY

1. Preparation of a cytochrome c-cytochrome oxidase complex was achieved by means of molecular sieve chromatography.
2. Spectral (visible, U.V., and infrared) properties of the complex were examined.
3. Based on the observation of the spectral anomaly of carbon monoxide compound of the complex, the possibility of a conformational change in hemoproteins on complex formation was hypothesized.
4. Chemically modified cytochrome c preparations were employed to elucidate the interaction mechanism of cytochrome c and cytochrome oxidase in the complex.
5. The physiological significance of the complex was reflected by demonstration of the reconstitution of succinate oxidase with soluble succinate dehydrogenase and the cytochrome b-c₁ particle.
6. Reconstitution of cytochrome oxidase from cytochrome a and hematin a was achieved.

BIBLIOGRAPHY

1. Ball, Eric G. and Octavia Cooper. The activity of succinate oxidase in relation to phosphate and phosphorus compound. *Journal of Biological Chemistry* 180:113-124. 1949.
2. Ball, Eric G., Cornelius F. Strittmatter and Octavia Cooper. Reaction of cytochrome oxidase with carbon monoxide. *Journal of Biological Chemistry* 193:635-647. 1951.
3. Ball, Eric G. and Octavia Cooper. Observation on the function of cytochromes c and c₁. *Journal of Biological Chemistry* 226:755-763. 1957.
4. Bellamy, L. J. The infra-red spectra of complex molecules. 2d ed. London, Methuen, 1957. 425 p.
5. Butt, W. D. and D. Keilin. Absorption spectra and some properties of cytochrome c and of its compounds with ligands. *Proceedings of the Royal Society, London* B156:429-458. 1962.
6. Chance, Britton. The carbon monoxide compounds of the cytochrome oxidases. I. Difference spectra. *Journal of Biological Chemistry* 202:383-396. 1953.
7. _____ The carbon monoxide compounds of the cytochrome oxidases. II. Photodissociation spectra. *Journal of Biological Chemistry* 202:397-406. 1953.
8. _____ The carbon monoxide compounds of the cytochrome oxidases. III. Molecular extinction coefficients. *Journal of Biological Chemistry* 202:407-416. 1953.
9. Chance, Britton, Lucile Smith and Laroy Castor. New methods for the study of the carbon monoxide of respiratory enzymes. *Biochimica et Biophysica Acta* 12:289-298. 1953.
10. Chance, Britton and G. R. Williams. The respiratory chain and oxidative phosphorylation. *Advances in Enzymology* 17:65-134. 1956.
11. Colpa-Boonstra, J. P. and K. Minnaert. Myoglobin in heart-muscle preparations and the effect of nitrite

- treatment. *Biochimica et Biophysica Acta* 33:527-534. 1959.
12. Eichhorn, G. L. and J. F. Cairns. Rotary dispersion of cytochrome c. *Nature* 181:994. 1958.
 13. Ernster, Lars and Chuan-pu Lee. Biological oxidations. In: *Annual Review of Biochemistry*, vol. 33. Palo Alto, Annual Review Inc., 1964. p. 729-788.
 14. Estabrook, R. W. Factors influencing the activity of exogenous cytochrome c. *Federation Proceedings* 8:223. 1959.
 15. Gibson, Q. H. et al. Reactions of cytochrome oxidase with cytochrome c. *Federation Proceedings* 23:322. 1964.
 16. Green, David E. Electron transport and oxidative phosphorylation. *Advances in Enzymology* 21:73-129. 1959.
 17. Greengard, Olga and Philip Feigelson. The purification and properties of liver tryptophan pyrrolase. *Journal of Biological Chemistry* 237:1903-1907. 1962.
 18. Hagihara, B. et al. Crystalline animal cytochrome c. In: *Biochemical Preparations*, vol. 6. New York, Wiley, 1959. p. 1-7.
 19. Hjertén, Stellan and Rolf Mosbach. "Molecular-sieve" chromatography of proteins on columns of cross-linked polyacrylamide. *Analytical Biochemistry* 3:109-118. 1962.
 20. Hollocher, Thomas C. Kinetic consideration of the cytochrome c-cytochrome oxidase reaction. *Archives of Biochemistry and Biophysics* 98:12-16. 1962.
 21. Horie, Shigeo and Martin Morrison. Cytochrome c oxidase components. V. A cytochrome a preparation free of cytochrome a₃. *Journal of Biological Chemistry* 239:1438-1441. 1964.
 22. Horie, Shigeo. On the absorption spectrum of cytochrome a₃. *Journal of Biochemistry* 56:57-66. 1964.
 23. Jacobs, Earl E. Phosphorylation coupled to electron transport initiated by substituted phenylenediamine. *Biochemical and Biophysical Research Communications* 3:536-539. 1960.

24. Jonxis, J. H. P. On the spreading of different haemoglobins, muscle haemoglobins and cytochrome c. *Biochemical Journal* 33:1743-1751. 1939.
25. Keilin, D. On cytochrome, a respiratory pigment, common to animals, yeast and higher plants. *Proceedings of the Royal Society, London* B98:312-339. 1925.
26. _____ A comparative study of turacin and haematin and its bearing on cytochrome. *Proceedings of the Royal Society, London* B100:129-151. 1926.
27. _____ Cytochrome and respiratory enzyme. *Proceedings of the Royal Society, London* B104:206-252. 1929.
28. _____ Cytochrome and intercellular oxidase. *Proceedings of the Royal Society, London* B106:418-444. 1930.
29. _____ Supposed direct spectroscopic observation of the "oxygen-transporting ferment." *Nature* 132:783. 1933.
30. _____ Cytochrome and the supposed direct spectroscopic observation of oxidase. *Nature* 133:290-291. 1934.
31. Keilin, D. and E. F. Hartree. Cytochrome oxidase. *Proceedings of the Royal Society, London* B125:171-186. 1938.
32. _____ Cytochrome and cytochrome oxidase. *Proceedings of the Royal Society, London* B127:167-191. 1939.
33. _____ Succinic dehydrogenase-cytochrome system of cells, intercellular respiratory system catalysing aerobic oxidation of succinic acid. *Proceedings of the Royal Society, London* B129:277-306. 1940.
34. _____ Relationship between certain components of the cytochrome system. *Nature* 176:200-206. 1955.
35. Keilin, D. and Tsao E. King. Reconstitution of the succinic oxidase system from soluble succinic dehydrogenase and a particulate cytochrome system preparation. *Nature* 181:1520-1522. 1958.
36. _____ Effect of inhibitors on the activity of soluble succinic dehydrogenase and on the reconstitution of the succinic dehydrogenase-cytochrome system from its

- components. Proceedings of the Royal Society, London B152:163-187. 1960.
37. Kiese, Manfred and Hermann Kurz. Verbindung des Ferment hämins mit Peroxydase Protein. Biochemische Zeitschrift 332:11-17. 1959.
 38. King, T. E. Energy transfer with special reference to biological systems. (Discussion) Discussions of the Faraday Society 27:262-263, 269. 1959.
 39. _____ Reconstitution of respiratory chain enzyme systems. IV. "Cross-reconstitution" of sacrosomal succinic oxidase from soluble succinic dehydrogenase of different species. Journal of Biological Chemistry 236: 2342-2346. 1961.
 40. King, T. E. and S. Takemori. Reconstitution of respiratory enzyme systems. VIII. Reconstitution of a succinate-cytochrome c reductase system. Biochimica et Biophysica Acta 64:194-196. 1962.
 41. King, Tsao E. Is the reconstitution of respiratory succinate oxidase really a process of reactivation? Nature 198:366-368. 1963.
 42. _____ Reconstitution of respiratory chain enzyme systems. XII. Some observations on the reconstitution of the succinate oxidase from heart muscle. Journal of Biological Chemistry 238:4037-4051. 1963.
 43. King, T. E., Karen Sahlstrom Nickel and Donald R. Jensen. Iron, copper, cytochrome and lipid contents of heart muscle preparation and heart mitochondria. Journal of Biological Chemistry 239:1989-1994. 1964.
 44. King, Tsao E., M. Kuboyama and S. Takemori. On cardiac cytochrome oxidase. Presented at An International Symposium on Oxidases and the Related Oxidation-Reduction Systems, Amherst, Mass. July 15-19, 1964. New York, Wiley. (In press)
 45. King, T. E., J. E. Hucko, M. Kuboyama and S. Takemori. Sequential fragmentation and systematic reconstitution of succinate oxidase. (Abstract) In: Sixth International Congress of Biochemistry, New York City, July 26-Aug. 1, 1964.

46. King, T. E. and M. Kuboyama. Enzymic activity of cytochrome a free of cytochrome a₃ from cardiac muscle. Biochemical and Biophysical Research Communications (In press) 1964.
47. Klots, Irving M., Patricia Griswold and Dieter M. Gruen. Infrared spectra of some proteins and related substances. Journal of American Chemical Society 71:1615-1620. 1949.
48. Kowlsky, A. Nuclear magnetic resonance studies of proteins. Journal of Biological Chemistry 237:1807-1819. 1962.
49. Kuboyama, M., S. Takemori and T. E. King. Reconstitution of respiratory chain enzyme systems. IX. Cytochrome c-cytochrome oxidase complex of heart muscle. Biochemical and Biophysical Research Communications 9:534-539. 1962.
50. _____ Reconstitution of respiratory chain enzyme systems. X. Reconstitution of succinate oxidase with cytochrome c-cytochrome oxidase of heart muscle. Biochemical and Biophysical Research Communications 9: 540-544. 1962.
51. _____ Some properties of the cytochrome c-cytochrome oxidase complex. Federation Proceedings 22: 466. 1963.
52. Kuboyama, M. and T. E. King. Reconstitution of cytochrome oxidase from cytochrome a and hematin a. Biochimica et Biophysica Acta (In press) 1964.
53. Lemberg, Rudolf. Cytochromes of group a and their prosthetic groups. Advances in Enzymology 23:265-321. 1962.
54. Lumry, Rufus. Some aspects of the thermodynamics and mechanism of enzyme catalysis. In: The enzymes, ed. by Paul D. Boyer, Henry Lardy and Karl Myrback. vol. 1. New York, Academic Press, 1959. p. 157-231.
55. Margoliash, E. Chromatographic behavior of cytochrome c on cation exchangers. Biochemical Journal 56:535-543. 1953.
56. Margoliash, E., Nehaman Frohwirt and Edith Wiener. A study of the cytochrome c haemochromogen. Biochemical Journal 71:559-570. 1959.

57. Margoliash, E. and J. Lustgarten. Interconversion of horse heart cytochrome c monomer and polymers. *Journal of Biological Chemistry* 237:3397-3405. 1962.
58. Margoliash, E. et al. Amino acid composition of horse heart cytochrome c. *Journal of Biological Chemistry* 237:2148-2150. 1962.
59. Margoliash, E. Some structural aspects of cytochrome c function. In: *Brookhaven Symposia in Biology No. 15, Enzymic models and enzyme structure.* New York, Brookhaven National Laboratory, 1962. p. 266-281.
60. Margoliash, E. Cytochrome c preparation (crystalline). Personal communication. 1963.
61. Minnaert, K. The kinetics of cytochrome c oxidase. I. The system: Cytochrome c-cytochrome oxidase-oxygen. *Biochimica et Biophysica Acta* 50:23-34. 1961.
62. _____ The kinetics of cytochrome c oxidase. II. The system: Reducing agent-cytochrome c-cytochrome oxidase-oxygen. *Biochimica et Biophysica Acta* 54:26-41. 1961.
63. Morell, D. B., J. Barrett and P. S. Clezy. The prosthetic group of cytochrome oxidase. I. Purification as porphyrin a and conversion into haemin a. *Biochemical Journal* 78: 793-797. 1961.
64. Morita, S. and S. F. Conti. Localization and nature of the cytochromes of *rhodospirillum rubrum*. *Archives of Biochemistry and Biophysics* 100:302-307. 1963.
65. Morrison, Martin and Shigeo Horie. Cytochrome c oxidase components. IV. Chemical distinction between cytochrome a and a₃. *Journal of Biological Chemistry* 239:1432-1437. 1964.
66. Nicholls, Peter. The cytochrome c-cytochrome oxidase complex. (Abstract) In: *Fifth International Congress of Biochemistry, Moscow, 1962.* p. 468.
67. Okunuki, et al. Studies on cytochrome a. 1. Extraction, purification and some properties of cytochrome a. *Journal of Biochemistry* 45:847-854. 1958.
68. Okunuki, Kazuo. Cytochrome oxidase. In: *Oxygenases*, ed. by Osamu Hayaishi. New York, Academic Press, 1962. p. 409.

69. Oorii, Y., I. Sekuzu and K. Okunuki. Studies on cytochrome c₁. II. Oxidation mechanism of cytochrome c₁ in the presence of cytochromes a and c. *Journal of Biochemistry* 51:204-215. 1962.
70. Paul, K. G. Die Darstellung von reinen Hämine. *Acta Chemica Scandinavica* 12:1611-1621. 1958.
71. Richardson, S. H. and L. R. Fowler. Studies on the electron transfer system. LII. Binding of cytochrome c by reconstituted DPNH oxidase. *Archives of Biochemistry and Biophysics* 100:547-553. 1963.
72. Schejter, A. and P. George. Formation and dissociation of ferrihaemochrome c complex with cyanide. *Federation Proceedings* 21:46. 1962.
73. Slater, E. C. The measurement of the cytochrome oxidase activity of enzyme preparations. *Biochemical Journal* 44:305-318. 1949.
74. _____ The constitution of the respiratory chain in animal tissues. *Advances in Enzymology* 20: 147-199. 1958.
75. Slater, E. C. and J. P. Colpa-Boonstra. The role of cytochrome b in the respiratory chain. In: *Haematin enzymes*, pt. 2. ed. by J. E. Falk, R. Lemberg and R. K. Morton. Oxford, Pergamon, 1961. p. 575-596.
76. Slater, E. C., B. F. van Gelder and the late K. Minnaert. Cytochrome c oxidase. Presented at An International Symposium on Oxidases and the Related Oxidation-Reduction Systems, Amherst, Mass. July 15-19, 1964. New York, Wiley. (In press)
77. Smith, Lucile and Helen Conrad. A study of the kinetics of the oxidation of cytochrome c by cytochrome c oxidase. *Archives of Biochemistry and Biophysics* 63:403-413. 1956.
78. Stotz, E., A. M. Altschul and T. R. Hogness. The cytochrome c-cytochrome oxidase complex. *Journal of Biological Chemistry* 124:745-754. 1938.
79. Takemori, S., I. Sekuzu and K. Okunuki. Studies on cytochrome a. VII. Physico-chemical properties of purified cytochrome a. *Biochimica et Biophysica Acta* 51:464-472. 1961.

80. Takemori, Shigeaki, et al. Reaction of cytochrome a with chemically modified cytochrome c and basic proteins. *Journal of Biochemistry* 52:28-37. 1962.
81. Takemori, S. and T. E. King. Reconstitution of respiratory enzyme systems. VII. Preparation of the cytochrome b-c₁ complex from heart muscle. *Biochimica et Biophysica Acta* 64:192-194. 1962.
82. Takemori, S. and T. E. King. Effect of alkali and borohydride on cardiac cytochrome oxidase: formation of Schiff base. *Journal of Biological Chemistry* (In press) 1964.
83. Theorell, H. and Å. Åkesson. Studies on cytochrome c. *Journal of American Chemical Society* 63:1804-1827. 1949.
84. Tsou, C. L. Cytochrome c modified by digestion with proteolytic enzyme. I. Digestion. *Biochemical Journal* 49:362-367. 1951.
85. _____ Cytochrome c modified by digestion with proteolytic enzyme. II. Properties of pepsin modified cytochrome c. *Biochemical Journal* 49:367-374. 1951.
86. _____ Exogenous and endogenous cytochrome c. *Biochemical Journal* 150:493-499. 1952.
87. Warburg, Otto. Über Eisen, den sauerstoffübertragenden Bestandteil des Atmungsferments. *Biochemische Zeitschrift* 152:479-494. 1924.
88. _____ Über die Wirkung des Kohlenoxyds auf den Stoffwechsel der Hefe. *Biochemische Zeitschrift* 177:471-486. 1926.
89. _____ Das sauerstoffübertragende Ferment der Atmung. *Angewandte Chemie* 45:1-6. 1932.
90. _____ Heavy metal prosthetic groups and enzyme action. tr. by A. Lawson. London, Oxford University Press, 1949. 230 p.
91. Wainio, W. W. Reduction of cytochrome oxidase with ferrocyclochrome c. *Journal of Biological Chemistry* 216: 593-599. 1955.

92. Wainio, W. W. and S. J. Cooperstein. Some controversial aspects of the mammalian cytochromes. *Advances in Enzymology* 17:329-392. 1956.
93. Yamanaka, T. et al. Studies on cytochrome c. III. Determination of "native" mammalian heart muscle cytochrome c and its physiological properties. *Journal of Biochemistry* 46:121-132. 1959.
94. Yamanaka, T. and K. Okunuki. Crystalline pseudomonas cytochrome oxidase. II. Spectral properties of the enzyme. *Biochimica et Biophysica Acta* 67:394-406. 1959.
95. _____ Reconstitution of pseudomonas cytochrome oxidase from haem a₂ and its protein moiety. *Biochemische Zeitschrift* 338:62-72. 1963.
96. Yonetani, T. Studies on cytochrome a. III. Effect of synthetic detergents upon the activity of cytochrome a. *Journal of Biochemistry* 46:917-924. 1959.
97. _____ Studies on cytochrome oxidase. I. Absolute and difference absorption spectra. *Journal of Biological Chemistry* 235:845-852. 1960.
98. _____ Studies on cytochrome oxidase. III. Improved preparation and some properties. *Journal of Biological Chemistry* 236:1680-1688. 1961.
99. _____ The a-type cytochromes. In: *The enzymes*, ed. by Paul D. Boyer, Henry Lardy and Karl Myrback. vol. 8. New York, Academic Press, 1963. p. 41-79.