Lineweaver, J., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.
Margoliash, E. (1954), *Biochem. J. 56*, 529, 535.
Minnaert, K. (1961), *Biochim. Biophys. Acta 54*, 26.
Slater, E. C. (1949), *Biochem. J. 44*, 305.
Smith, L. (1954), *Arch. Biochem. Biophys. 50*, 285.
Smith, L., and Conrad, H. (1956), *Arch. Biochem. Biophys. 63*, 403.
Tsou, C. L. (1952), *Biochem. J. 50*, 493.
Wharton, D. C., and Griffiths, D. E. (1962), *Arch. Biochem. Biophys. 96*, 103.
Yonetani, T. (1962), *J. Biol. Chem. 237*, 550.

The Reaction of Particle-bound Cytochrome c Oxidase with Endogenous and Exogenous Cytochrome c*

LUCILE SMITH† AND PAT W. CAMERINO‡

From the Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire

Received April 18, 1963

A systematic study has been made of the reaction of different kinds of particulate preparations from heart muscle with soluble ferrocytochrome c, *p*-phenylenediamine, and DPNH, and with combinations of these. The effect of treatment of the preparations with deoxycholate on these reactions was also studied.

The data show that the turnover rates of the cytochrome c oxidase of the different preparations in the reaction with soluble ferrocytochrome c are highly variable and always less than the maximal turnover rate possible, even when the rates are extrapolated to an infinitely large concentration of cytochrome c. The turnover rate of cytochrome a_3 in this reaction can always be increased by initiating electron transport from DPNH or by treatment with deoxycholate under specific conditions. Large consistent maximal turnover rates are observed in the deoxycholate-treated preparations at cytochrome c concentrations greater than 150 μM. This maximal rate is similar to the turnover of cytochrome a_3 in its reaction with oxygen. Conditions are described for a reproducible assay for heart muscle cytochrome c oxidase which appears to measure the maximal turnover rate possible.

The exogenous (soluble) cytochrome c has been found to react directly with the cytochrome a (or a_3) of the respiratory chain system of the heart muscle particles rather than with the endogenous cytochrome c.

The DPNH oxidase activity of Keilin-Hartree preparations is increased somewhat (as much as doubled) by addition of a low concentration of cytochrome c (2 μM); then further increases in the concentration of cytochrome c do not give further increases in the rate of oxygen uptake in the presence of DPNH.

Cytochrome c oxidase is the terminal part of the membrane-bound respiratory chain system where oxygen is reduced. The sequence of hydrogen and electron transport through this multienzyme system is usually represented as follows (Keilin and Hartree, 1938; Keilin and Slater, 1953; Chance, 1961):

DPNH → flavoprotein
↘
(ubiquinone) cytochrome b → cyto. c_1 → cyto. c → (cytochrome c oxidase) cyto. a → cyto. a_3 → O_2
↗
succinate → flavoprotein

The particle-bound oxidase can also oxidize purified soluble ferrocytochrome c. It is this reaction that is most often used as an assay for cytochrome c oxidase (Slater, 1949; Smith and Conrad, 1956). The kinetics of the oxidation of soluble ferrocytochrome c are unusual in that the rate constant decreases with increasing concentration of cytochrome c (oxidized plus reduced) in the reaction mixture (Smith and Conrad, 1956). This effect is observed with the oxidase on swollen mitochondria or heart muscle particles, as well as with purified preparations. Because of this decrease in rate constant with increasing concentration of cytochrome c a hyperbolic curve is observed on plotting the rate of the reaction against the total concentration of soluble cytochrome c added. This has led to the procedure of calculating the activity extrapolated to an infinitely large concentration of cytochrome c (Slater, 1949; Yonetani, 1962; Wharton and Griffiths, 1962), even though the rate constant for the reaction falls to quite low values at high concentrations of cytochrome c. The relationship of the cytochrome c oxidase activity of different preparations with a finite or at infinitely large concentration of added cytochrome c to the maximum activity attainable has not been investigated. It is also uncertain how the rate of oxidation of soluble cytochrome c reflects the reaction of the particle-bound oxidase with the endogenous cytochrome c. Turnover rates reported for the reaction of the oxidase with high concentrations of soluble cytochrome c (Yonetani, 1962) are low compared with some values reported for the enzyme in intact cells (Chance, 1952a, 1955).

A perusal of the literature on the subject reveals a great variability in the reactivity (usually expressed as Q_{O_2}) of different kinds of oxidase preparations with constant and with increasing concentrations of cytochrome c (Wharton and Griffiths, 1962; Smith and Conrad, 1961; Griffiths and Wharton, 1961; Yonetani, 1962). Although swollen mitochondria have a very active cytochrome c oxidase (Smith and Conrad, 1961), some particulate preparations derived from beef heart mitochondria were observed to react only slowly with soluble cytochrome c unless they were first treated with deoxycholate (Mackler and Green, 1956).

* This work was supported by a research grant (GM 06270) from the U. S. Public Health Service.

† Research Career Development Awardee of the U.S. Public Health Service (5-K3-GM-3865).

‡ Postdoctoral Trainee of the U. S. Public Health Service (5-T1-GM-174). Present address: Laboratory of Respiratory Enzymology, Oregon State University, Corvallis, Oregon.

We have systematically examined many different kinds of particulate preparations from heart muscle and found great variability in the rates of oxidation of soluble cytochrome c, both with increasing finite concentrations of the cytochrome c or extrapolated to an infinitely large cytochrome c concentration. The variability is seen whether the rates are expressed in terms of protein content or the content of cytochrome a_3. The maximum possible turnover rate of the oxidase was not obtained with any kind of preparation, even at infinite cytochrome c concentration, since the rates could always be increased by initiating additional electron transport through the respiratory chain or by pretreatment of the preparations with deoxycholate. Our data show that the usual methods of assaying for cytochrome c oxidase activity do not give results which can be related in a meaningful way to the maximal possible enzymatic activity.

When any of the heart muscle preparations was treated with deoxycholate under specific conditions, large consistent turnover rates of the oxidase were observed. These maximal rates are the same as the value obtained for the reaction of mammalian cytochrome a_3 with oxygen (Chance, 1955). This paper documents the variables involved in obtaining this maximal activity and suggests a method for consistently measuring the maximal turnover rate of cytochrome c oxidase of heart muscle.

Particulate preparations treated with deoxycholate to yield the maximal turnover rate of the reaction of the oxidase with soluble cytochrome c have lost the ability to oxidize DPNH or *p*-phenylenediamine. The oxidation of *p*-phenylenediamine is a measure of the content (or reactivity) of the endogenous cytochrome c. Thus these observations suggest that the soluble cytochrome c can react with the endogenous cytochromes ($a + a_3$), rather than with the endogenous cytochrome c. Further evidence that the soluble cytochrome c reacts with the particulate cytochromes ($a + a_3$) is furnished by observations on the oxidation of a combination of *p*-phenylenediamine and soluble cytochrome c.

Methods

Preparations.—The insoluble heart muscle particles bearing the respiratory chain system were prepared from beef heart by the Keilin-Hartree (1947) procedure[1] or by a modification of it employing the Waring Blendor for grinding the heart mince (Chance, 1952b). The minced heart was washed with (a) cold tap water, (b) a mixture of equal parts of tap water and distilled water, (c) a mixture of 1 part of tap water and 9 parts of boiled distilled water, or (d) boiled distilled water containing 1 mM $CaCl_2$. The insoluble particles were collected either by precipitation with acid (Keilin and Hartree, 1947) or by centrifugation at 30,000 rpm in the No. 30 rotor of the Spinco Model L centrifuge for 1 hour. These two types of preparations will be referred to as KHA and KHC, respectively.[2] Some preparations were made deficient in cytochrome c by washing the heart mince with strong phosphate buffer made from a mixture of Na_2HPO_4 and KH_2PO_4, following the directions of Tsou (1952).

Beef heart mitochondria were prepared by the method of Crane *et al.* (1956). Rat heart mitochondria were prepared in 0.32 M sucrose and 1 mM EDTA by the method of Cleland and Slater (1953).

Deoxycholate-treated preparations were made by adding 5% deoxycholate (neutralized to *p*H 7–8) to strong suspensions (13–20 mg protein/ml) in the ratio of 1 mg deoxycholate to 1 mg protein, then adding distilled water to make the volume at least twice that of the original suspension.

Cytochrome c was prepared from beef heart by the Keilin-Hartree (1945) and Margoliash (1954) procedures. The total concentration of cytochrome c was assessed from the optical density at 550 mμ of the compound reduced with $Na_2S_2O_4$ using the millimolar absorption coefficient reported by Margoliash (1954).

Polarographic Measurement of Cytochrome c Oxidase Activity.—The rate of oxygen uptake in the presence of ascorbate was measured with the Clark oxygen electrode according to the method given in the preceding paper (Smith and Camerino, 1963). Oxygen uptake rates in the presence of 33 mM *p*-phenylenediamine were also measured with the oxygen electrode in 0.05 M phosphate buffer at a final *p*H of 7.0. This reaction mixture was prepared by adding 0.5 M *p*-phenylenediamine dihydrochloride (Fisher Scientific Co.), neutralized to *p*H 6.7 in 0.01 M phosphate buffer to phosphate buffer of *p*H 7.4. The neutralized *p*-phenylenediamine solution was kept at 60° to prevent precipitation from solution. Higher concentrations of *p*-phenylenediamine in the reaction mixture gave no further increase in the rate of oxygen uptake.

Since cytochrome c, *p*-phenylenediamine, and deoxycholate showed a tendency to adhere to the membrane of the oxygen electrode, the electrode and cup were cleaned by washing with HCl (about 0.5 N) between each determination.

Assay of DPNH Oxidase.—DPNH oxidase was measured with the oxygen electrode in the same buffer (0.05 M phosphate, *p*H 7.0) used in the cytochrome c oxidase assay in the presence of 1 mM DPNH (Sigma or Pabst products gave identical rates of oxygen uptake). These rates were observed to be in agreement with rates determined from optical density changes at 340 mμ.

Assay of the Content of Cytochrome a_3.—The difference in absorption spectrum between an anaerobic suspension of heart muscle particles (in the presence of DPNH or succinate) and an aerobic suspension (no substrate added) was recorded with the Cary Model 14 spectrophotometer in the region of the α-absorption peak of cytochromes ($a + a_3$). The concentration of cytochrome a_3 was calculated from the ΔOD 605 minus 630 mμ, using the value $\Delta\epsilon = 23\ mM^{-1}\ cm^{-1}$ reported by Chance (1953). The same values for the ΔOD 605–630 mμ were obtained with the particles treated with deoxycholate and reduced with $Na_2S_2O_4$ and the mitochondria were assayed by this latter procedure. Turnover rates for cytochrome a_3 were calculated as follows:

$$\text{turnover number (TN) } sec^{-1} = \frac{O_2 \text{ uptake } (\mu M\ O_2\ sec^{-1}) \times 4}{\mu M \text{ cytochrome } a_3}$$

Protein Determination.—The protein content of the suspensions of heart muscle particles or mitochondria was measured with the biuret reaction (Gornall *et al.*, 1949) in the presence of 0.1% deoxycholate. An equivalent concentration of sucrose was added to the blank when measuring the protein content of mitochondria suspended in sucrose.

Results

Table I lists cytochrome c oxidase activities, meas-

[1] The washed heart muscle was ground in a mortar with sand for 1 hour.

[2] Abbreviations used in this paper: KHA, acid-precipitated heart mince; KHC, centrifuged heart mince; TN, turnover number.

TABLE I
CYTOCHROME C OXIDASE ACTIVITIES OF DIFFERENT HEART MUSCLE PREPARATIONS

Preparation	Wash Solution	Cytochrome c Oxidase Activity: μM O_2 Uptake sec^{-1} mg Protein in 3 ml (1) 25 μM Cyto. c	(2) Infinite Cyto. c	(2)/(1)	Turnover Numbers (sec^{-1}) 25 μM Cyto. c	Infinite Cyto. c
KHA-Blendor	Tap H_2O	0.7				
		0.68	2.17	3.2	26	81
		0.49				
		0.51	2.50	4.9		
		0.56			43	
		0.39	0.91	2.3		
		0.80	4.17	5.2		
KHA-Blendor	1 part tap + 1part distilled H_2O	1.24			39	
		0.93			36	
KHC-Blendor		1.53			40	
		1.06			33	
		1.22	2.76	2.3	40	90
KHC-Blendor	1 part tap + 9 parts distilled H_2O	0.93	2.53	2.7	32	86
KHC-sand ground		2.31	4.0	1.7	70	122
KHC-Blendor	1 mM $CaCl_2$	1.18	2.86	2.4	29	71
		1.20	3.77	3.2	29	90
Tsou-Blendor	tap H_2O	0.96			81	
		0.47				
		0.63	2.66	4.2	53	224
Beef heart mitochondria	—	1.44	3.3	2.3		
		1.83			84	
		1.27	4.08	3.2	39	124

ured polarographically with 33 mM ascorbate, for an assortment of heart muscle preparations in the presence of 25 μM cytochrome c and calculated at infinitely high cytochrome c concentration (Smith and Camerino, 1963). These rates of oxygen uptake vary considerably, whether expressed on the basis of protein content or on the basis of cytochrome a_3. The activities of the Keilin-Hartree type preparations vary with the methods of washing and grinding the mince. The turnover rate of the cytochrome a_3 of a Tsou-type preparation was about twice as great as the most active Keilin-Hartree preparation. The ratios of the rates of oxygen uptake at infinite cytochrome c concentration to those at 25 μM cytochrome c are also variable (from 2.3 to 5.9).

The rates of oxygen uptake at infinite cytochrome c in the cytochrome c oxidase assay do not represent the maximal possible turnover rates of the oxidase of Keilin-Hartree particles, since Figures 1 and 2 show that still greater rates can be obtained either by adding DPNH or by treating the preparations with deoxycholate. The data of Table II show the extent to which the turnover rate of the oxidase of several Keilin-Hartree preparations was increased by electron transport down the respiratory chain from DPNH or by pretreatment of the particles with deoxycholate.

The data of Figure 1 show that the DPNH oxidase activity of Keilin-Hartree preparations is increased somewhat on addition of a low concentration of cytochrome c (around 2 μM) and that further increases in the concentration of cytochrome c do not yield additional increases in the rate of oxygen uptake. This is in contrast to the effect of increasing the cytochrome c concentration in the cytochrome c oxidase assay. It should be pointed out that the conditions used for measurement of DPNH oxidase do not give maximal rates; the conditions are optimal for the reaction of cytochrome c oxidase. The DPNH oxidase activity is increased considerably in stronger phosphate buffer (0.15–0.2 M), particularly after a brief incubation in this stronger buffer, and is increased in the 0.05 M buffer by the addition of ascorbate. These effects will be described in detail in a subsequent publication.[3] The oxygen uptake rates in the presence of DPNH and with cytochrome c and ascorbate are additive at all concentrations of cytochrome tested, when the stimulatory effect of ascorbate on the DPNH oxidase is taken into consideration.

Keilin and Hartree (1938) and Slater (1949) showed that *p*-phenylenediamine can rapidly reduce the endogenous cytochrome c of the respiratory chain system, while ascorbate does this only very slowly. Table III lists the maximum rates of oxygen uptake obtained with our preparations in the presence of 33 mM *p*-phenylenediamine, with and without added soluble cytochrome c. Increasing the concentration of *p*-phenylenediamine above 33 mM in the absence of cytochrome c did not increase the rate. These rates with *p*-phenylenediamine were usually about the same as those obtained in the presence of DPNH. Addition of cytochrome c always increased the rate of oxygen uptake with *p*-phenylenediamine to about the extent expected from addition of the exogenous cytochrome c oxidase activity, measured with ascorbate.

The data of Table IV show that there is no constant increase in the turnover rates of the oxidase of the different preparations after pretreatment with deoxycholate. However, the deoxycholate treatment under

[3] The DPNH oxidase was completely inhibited by 1 mM CN^- or 1 μg/ml antimycin A under all conditions. The rate of O_2 uptake X 2 was always equal to the rate of disappearance of DPNH.

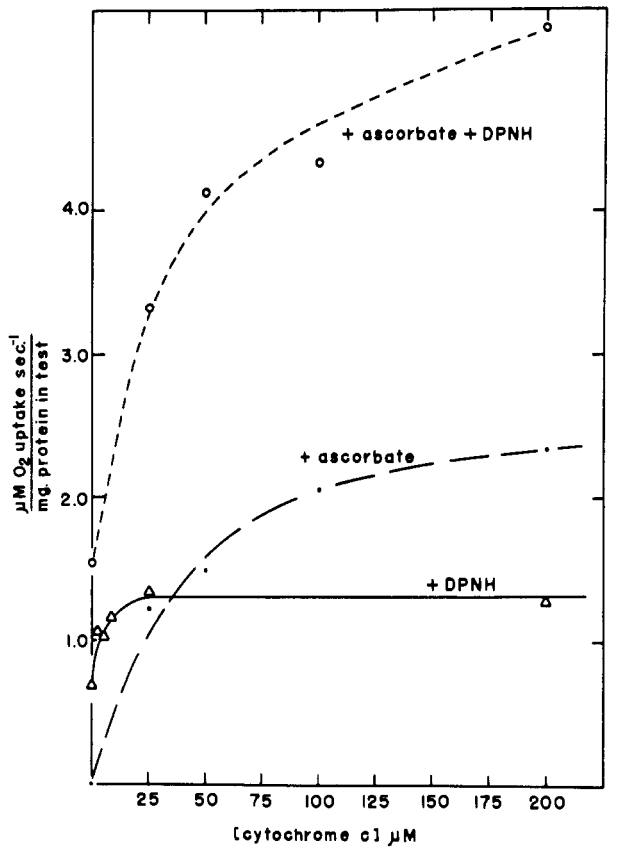

FIG. 1.—Oxygen uptake of a Keilin-Hartree preparation in the presence of DPNH (1 mM) or ascorbate (33 mM) or both with increasing concentrations of cytochrome c.

TABLE II

CYTOCHROME C OXIDASE ACTIVITIES OF KEILIN-HARTREE PARTICLES AND BEEF HEART MITOCHONDRIA WITH AND WITHOUT DPNH OXIDASE, AND BEFORE AND AFTER TREATMENT WITH DEOXYCHOLATE (DOC)

Preparation	Turnover Number of Cytochrome a_3 sec^{-1} at Infinite Cytochrome c Concentration		
	Cytochrome c Oxidase	Cytochrome c Oxidase plus DPNH Oxidase[a]	Cytochrome c Oxidase after DOC Treatment
KHC-Blendor washed with 1 part tap H_2O + 1 part distilled H_2O	90	200	
KHC-Blendor washed with 1 part tap H_2O + 9 parts distilled H_2O	86		359
KHC-Blendor washed with 1 mM $CaCl_2$	71 90	103 133	330 343
Beef heart mitochondria	123		357

[a] The maximal turnover number for DPNH oxidase plus cytochrome c oxidase was calculated in the following way. The turnover rate in the presence of DPNH and ascorbate was subtracted from the rates in the presence of DPNH, ascorbate, and cytochrome c. These rates were then used to obtain a turnover number at infinite cytochrome c concentration. The maximal turnover number was then calculated by adding the rate at an infinite cytochrome c concentration to the turnover rate in the presence of DPNH and ascorbate.

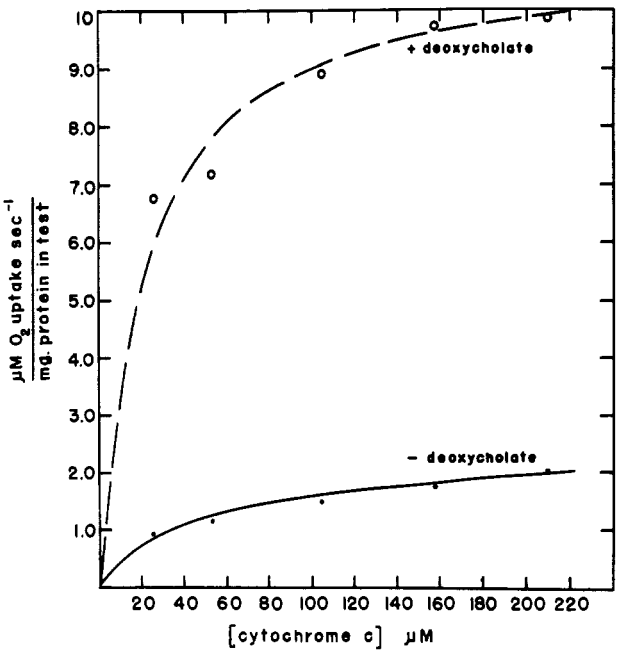

FIG. 2.—Oxygen uptake of a Keilin-Hartree preparation in the presence of 33 mM ascorbate and the cytochrome c concentration indicated. The preparation was assayed before and after pretreatment with deoxycholate (see Methods).

the conditions described in the table resulted in an increase in the turnover number of the oxidase of all preparations to a fairly constant value at infinite cytochrome c concentration (around 350 sec^{-1}). Also, after treatment with deoxycholate, the concentration of cytochrome c giving one-half the maximal rate changes to a lower value (Figs. 2 and 3). This may be 15 μM cytochrome c or lower; at concentrations above 150 μM cytochrome c, nearly maximal rates are obtained.

Table IV shows that the turnover rate of the oxidase of untreated rat heart mitochondria at infinite cytochrome c is higher than that of the oxidase in the other kinds of preparation (untreated with deoxycholate). Figure 3 is a plot of the reciprocals of the O_2 uptake against the reciprocals of the concentration of cytochrome c, which shows that the cytochrome c oxidase activity of the swollen rat heart mitochondria may

TABLE III

OXIDATION OF *p*-PHENYLENEDIAMINE BY HEART MUSCLE PREPARATIONS WITH AND WITHOUT CYTOCHROME C ADDED

Preparation	Turnover Numbers of Cytochrome a_3 (sec^{-1})			
	With 33 mM *p*-Phenylenediamine	33mM *p*-Phenylenediamine + 25 μM Cytochrome c	33 mM *p*-Phenylenediamine + Infinite cyto. c	With 1 mM DPNH
KHC-Blendor	30	57	90	22
	29	57		29
	28	66		28
	16	45		12
KHA-Blendor	31	63		23
	28	59		18
	34	48		25
Tsou-Blendor	10	75		13

TABLE IV
TURNOVER RATES OF CYTOCHROME C OXIDASE WITH AND WITHOUT PRETREATMENT OF STRONG PREPARATIONS WITH DEOXYCHOLATE[a]

Preparation	Turnover Number of Cytochrome c Oxidase (sec^{-1})			
	Without Deoxycholate		With Deoxycholate	
	22–27 μM Cyto. c	Infinite Cyto. c	22–27 μM Cyto. c	Infinite Cyto. c
K-H, washed with 1 part tap H_2O + 9 parts distilled H_2O	32	86	231	359
K-H, washed with 1 mM $CaCl_2$	29 29	71 90	153 180	330 343
Beef heart mitochondria	39	124	191	357
Rat heart sarcosomes	38	230	238	391

[a] K-H preparations are Keilin-Hartree-type particles prepared by disintegration of the heart mince with a Waring Blendor and the collection of the insoluble particles by high-speed centrifugation. The solutions used for washing the mince are indicated in the table. Strong suspensions of heart muscle particles or mitochondria (11–41 mg protein/ml) were treated with 5% deoxycholate (neutralized to *p*H 7–8) to give a final ratio of 1 mg deoxycholate/mg protein, then an aliquot was added to the assay mixture.

approach that of mitochondria pretreated with deoxycholate when both rates are calculated at infinite cytochrome c concentration, although at finite concentrations of cytochrome c the oxidase activities of the untreated mitochondria are considerably lower.

The stimulatory effects of deoxycholate on the cytochrome c oxidase shown in Table IV were obtained by treating strong suspensions (11–41 mg protein/ml) with 5% deoxycholate to give a ratio of 1 mg deoxycholate/mg protein. The data of Figure 4 show that with these strong suspensions higher ratios of deoxycholate to protein did not give further increase in the oxidase activity measured with 24 μM cytochrome c. However, the observed activity was also found to depend upon the protein concentration of the particle suspension treated with deoxycholate (Table V). Very little increase in activity was obtained when a suspension containing 0.38 mg protein/ml was treated with increasing concentrations of deoxycholate, while treatment of a more concentrated suspension to give the same ratios of deoxycholate to protein gave a pronounced increase in activity. It was established that the effect of the dilution of the suspension was not related to the concentration of inorganic phosphate present. When deoxycholate is added to dilute preparations to give the same final concentration of all components, variable effects are observed, often inhibition rather that stimulation. Similar observations of inhibition have been reported with cholate (Smith, 1955).

Under conditions where the cytochrome c oxidase activity, measured with ascorbate and cytochrome c, is most strongly stimulated by pretreatment with deoxycholate, the DPNH oxidase activity of all of the preparations was zero. The data of Figure 4 show that the ability to oxidize *p*-phenylenediamine in the absence of exogenous cytochrome c is also lost (see Ball and Cooper, 1957).

DISCUSSION

Our studies of the oxidation of soluble ferrocytochrome c by the oxidase of different kinds of prepara-

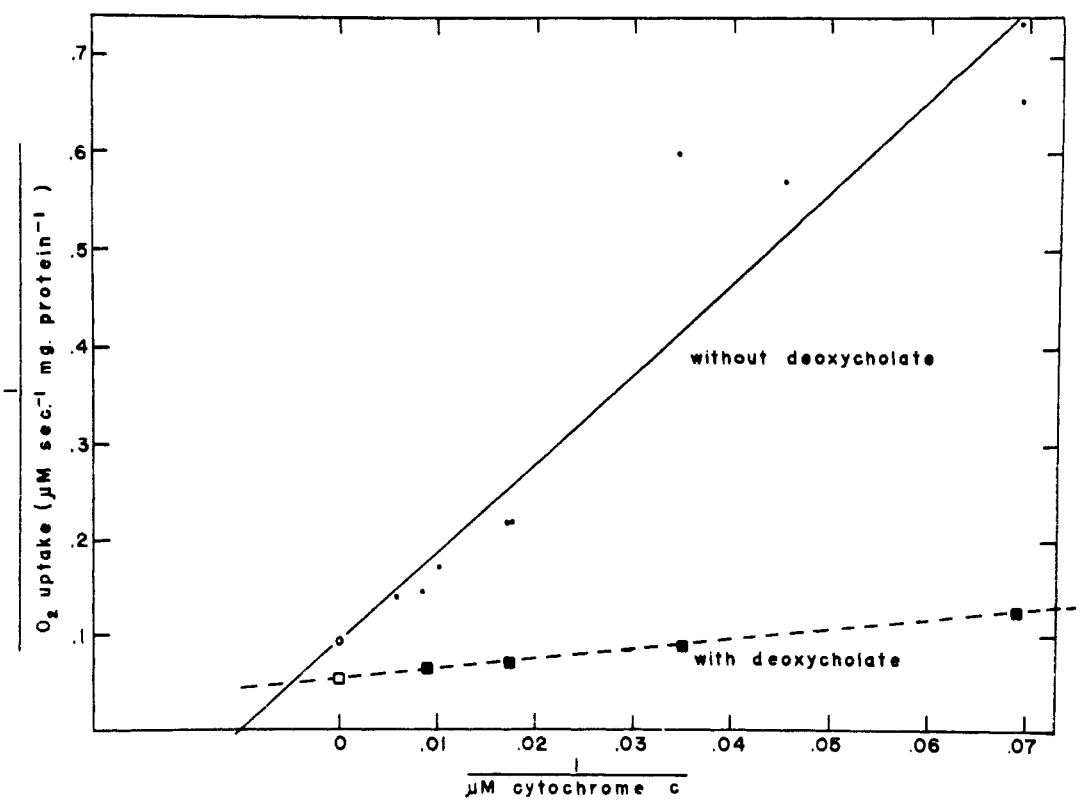

FIG. 3.—Plot of reciprocal of cytochrome c oxidase activity of rat heart sarcosomes against reciprocal of cytochrome c concentration. Pretreatment with deoxycholate is described under Methods.

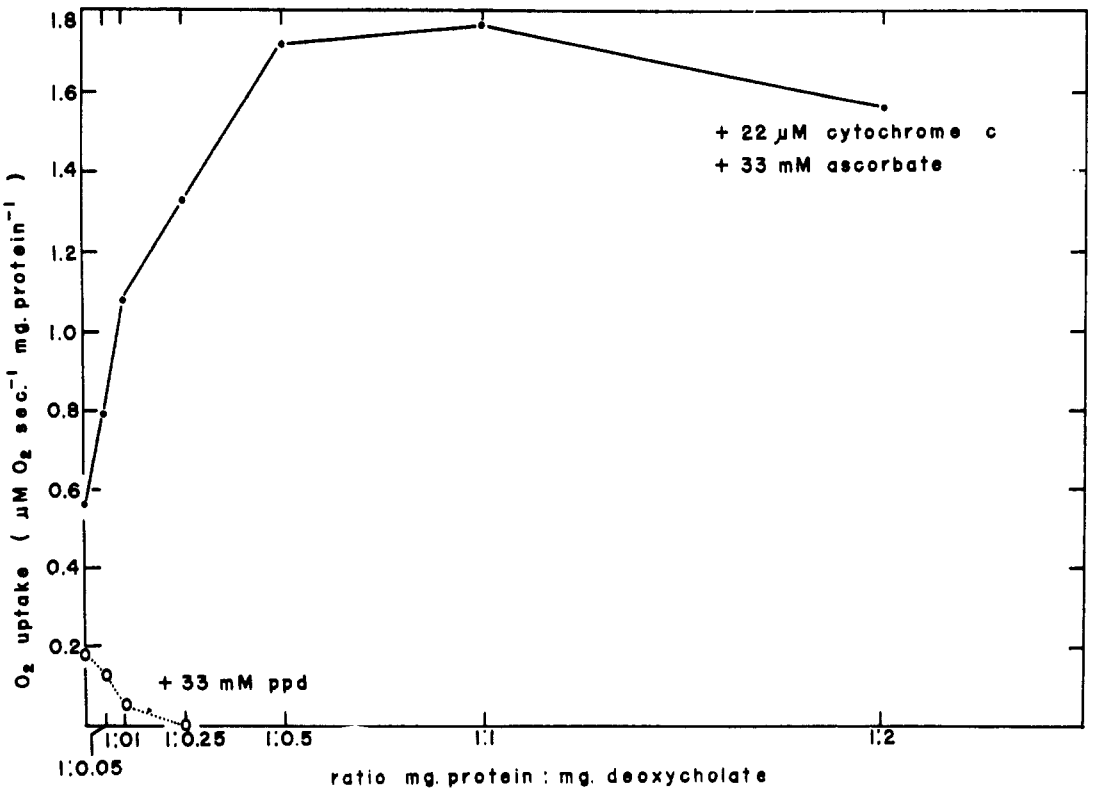

FIG. 4.—Effect of pretreatment of "strong" suspensions of Keilin-Hartree preparation with deoxycholate on cytochrome c oxidase activity and on oxidation of *p*-phenylenediamine. Aliquots of a "strong" suspension (24.6 mg protein/ml) were treated with 5% deoxycholate to give the ratios indicated.

tion which have not been subjected to special pretreatment show: (1) that the maximal turnover rate of the oxidase is never achieved, even at an infinitely large concentration of cytochrome c; and (2) that there is no obvious relationship between the rate of the reaction and the content of cytochrome c oxidase (cytochromes $a + a_3$) in the preparation.

The explanations for these observations appear to be twofold. First the cytochrome c oxidase may not be completely "exposed" for reaction with soluble cytochrome c. The evidence for this conclusion will be described in detail in a subsequent publication. Second, the rate constant for the oxidase reaction decreases as the concentration of soluble cytochrome c in the reaction is increased (Smith and Conrad, 1956); the rate constant is very low at high concentrations of cytochrome c. This effect of cytochrome c concentration on the rate constant can be explained either by the formation of some inactive compound between cytochrome c and the oxidase (Smith and Conrad, 1956; Slater, 1958) or by the existence of a rate-limiting step in the reaction of the soluble cytochrome c with the oxidase system (Minnaert, 1961). The *extent* to which increasing concentrations of cytochrome c decrease the rate constant is markedly variable among different types of preparations and, to a lesser extent, with preparations made in a consistent manner. This must mean that the structure of the oxidase system varies so that either the amount of formation of the inactive compound or the rate of reaction of soluble cytochrome c with the particulate system changes. In our hands, even the composition of the solution used to wash the heart mince and the method of disintegrating it affected these structural properties of the heart muscle particles.

The activity of the oxidase of swollen rat heart mitochondria in the reaction with soluble cytochrome c is greater (in terms of cytochrome a_3) than that of the small membrane fragments. The structure of the fragments differs from that of the intact mitochondrial membrane in the region of cytochrome c, since cytochrome c can be extracted from and reincorporated into swollen mitochondria or minced heart muscle by changing the salt concentration (Jacobs and Sanadi, 1960; Tsou, 1952), but cytochrome c cannot be extracted from heart muscle particles in this way. Our preparations of beef heart mitochondria (made in a Waring Blendor) resembled the heart muscle particles with re-

TABLE V

ADDITION OF DEOXYCHOLATE TO DIFFERENT CONCENTRATIONS OF HEART MUSCLE PARTICLE SUSPENSION[a]

mg Protein/ml Suspension Prior to Addition of Deoxycholate	mg Deoxycholate/ml Final Suspension					
	0	0.25	0.49	0.98	1.92	8.33	16.7
	Cytochrome c Oxidase Activity (μM O_2 sec^{-1}/mg protein)						
0.38	0.92	1.29	1.23	1.10	1.52	1.52	
0.84			1.43	1.42	2.07	2.10	
1.79			1.39	1.60	2.54	1.91	
7.82			1.28	1.40	1.60	2.89	
16.4							3.02

[a] Dilutions of the Keilin-Hartree preparation were made with cold distilled water. Then 5% deoxycholate (neutralized to *p*H 8.0) was added to each diluted suspension to give the final concentrations indicated, and the mixtures incubated for at least 5 minutes in an ice bath before assaying. All assays were run with 20 μM cytochrome c and 33 mM ascorbate.

spect to the extractability of cytochrome c and also the reactivity of the oxidase with soluble cytochrome c. The oxidase of the Tsou-type preparations was always more reactive (in terms of cytochrome a_3 content) than the enzyme on Keilin-Hartree particles, both at 25 μM and at infinite cytochrome c concentration. Again the change in structure resulting from the removal of the endogenous cytochrome c yields greater reactivity of the oxidase with soluble cytochrome c. The purified preparations of cytochrome c oxidase which have been described to date also show less than the maximal possible turnover rate in the reaction with soluble cytochrome c (see below).

The unfortunate result of the variability in the reactivity of the oxidase with soluble cytochrome c (expressed as turnover rate) is that the methods that have been used for assaying cytochrome c oxidase do not give values that are related in any meaningful way to the content of the enzyme in the preparation.

When all types of preparations from heart muscle are pretreated with deoxycholate under very specific conditions, the highly variable oxidase activities all increase to a consistent maximal turnover rate of about 350 sec^{-1}. This turnover rate is the same as that reported by Chance (1955) for the reaction of cytochrome a_3 of ascites tumor cells with 16 μM oxygen at 25°, which should be the maximal rate obtainable. (Chance did not measure the rate of reaction of the cytochrome a_3 of heart muscle particles with this concentration of oxygen.)

We have thus found a method by which the cytochrome c oxidase of preparations from heart muscle can be reproducibly assayed under conditions where the enzyme is turning over at its maximal rate. If further work shows that preparations from other tissues react similarly, the outlook for a generally applicable assay for cytochrome c oxidase is hopeful. After treatment with deoxycholate, nearly maximal turnover rates are obtained at finite concentrations of cytochrome c. It should not be necessary to extrapolate to infinite concentration of cytochrome c.

The effect of deoxycholate on the activity of the particulate preparations is puzzling. Mackler and Green (1956) described the increased reactivity with soluble cytochrome c after deoxycholate treatment as the "opening phenomenon," without speculating upon its nature. Under their experimental conditions, the "opening" was paralleled by a corresponding decrease in DPNH oxidase activity. Slater's (1958) method of treatment with cholate did not yield such a close parallel between the decrease in DPNH oxidase and the increased reactivity with soluble cytochrome c. The observation that *p*-phenylenediamine oxidation is lost after treatment with deoxycholate suggests a break in the chain of reactions between endogenous cytochromes c and a. This would be in agreement with the postulate (Keilin and Hartree, 1955; Slater, 1958) that bile salt treatment makes soluble cytochrome c more accessible to the two parts of the respiratory chain system. However, the result of the detergent treatment must be more complicated than a simple split in the respiratory chain system. The change in reactivity resulting from addition of deoxycholate depends upon the concentration of the suspension treated. As previously observed with cholate addition to heart muscle particles (Smith, 1955), treatment with deoxycholate may give varying degrees of stimulation or even inhibition, depending upon the concentration of the particle suspension to which the detergent is added. The significant observations are that the reaction between endogenous cytochromes c and a is inhibited and that the oxidase becomes more available for reaction with soluble cytochrome c. In addition, the depressing effect of cytochrome c on the rate constant for the oxidation of soluble cytochrome c becomes relatively smaller, so that the maximal turnover rate of the oxidase can be reached in the oxidation of concentrations of cytochrome c in excess of about 150 μM.

The observation that the effect of deoxycholate treatment depends upon the concentration of the suspension treated offers an explanation for the wide variation in activities of detergent-treated preparations reported from different laboratories. When the turnover rate of Yonetani's (1962) purified oxidase is recalculated in the same units as the data reported in this paper, a value of 126 sec^{-1} is obtained at infinite cytochrome c concentration. Calculated on the same basis, the preparation of Griffiths and Wharton (1961) gives a turnover number of about 200 sec^{-1} (applying the temperature correction factor of Minnaert [1961]). Thus these purified oxidase preparations show less than the maximal possible turnover rate in the reaction with soluble cytochrome c. The concentration of the particle suspension treated with detergent may determine the final turnover rate achieved with purified preparations.

The oxidation of DPNH by the Keilin-Hartree preparation is somewhat stimulated by the addition of soluble cytochrome c under the conditions of our experiments (0.05 M buffer), but, in contrast with the cytochrome c oxidase reaction, the DPNH oxidase is saturated at a relatively low concentration of cytochrome c. The small stimulatory effect of soluble cytochrome c on the DPNH oxidase results from a low rate of reduction of the exogenous cytochrome c by the "DPNH cytochrome c reductase" (Camerino and Smith, 1963). There is no evidence that additional endogenous cytochrome c is formed from the added soluble cytochrome c. There is also no evidence that the soluble cytochrome c affects the reaction of the endogenous cytochrome c with the oxidase. This is in agreement with the observation that polycations like salmine or poly-L-lysine inhibit the oxidation of soluble cytochrome, but not endogenous cytochrome c, by the oxidase of Keilin-Hartree particles (Minnaert and Smith, 1961). These observations will be elaborated in detail in another publication.

Several lines of evidence show that the soluble cytochrome c reacts directly with cytochrome a (or a_3) of the Keilin-Hartree heart muscle particles, rather than with the endogenous cytochrome c. The endogenous cytochrome c of the heart muscle particles is reduced by *p*-phenylenediamine. Thus the loss of ability to oxidize *p*-phenylenediamine after treatment with deoxycholate means that electron transport between endogenous cytochrome c and the oxidase is lost. Since the treated particles oxidize soluble ferrocytochrome c rapidly, the soluble cytochrome c can react directly with cytochromes a or a_3. In our Keilin-Hartree preparations the rate of oxygen uptake with saturating concentrations of *p*-phenylenediamine was usually the same as the rate with DPNH in the absence of added cytochrome c. Thus the rate with *p*-phenylenediamine is a measure of the content of endogenous cytochrome c (or the structural accessibility of endogenous cytochromes c and a + a_3). The data show that the addition of soluble cytochrome c does not form any further endogenous cytochrome c (see above), but that the addition of soluble cytochrome c to the particles increases the rate of oxygen uptake with saturating concentrations of *p*-phenylenediamine to the extent expected from the reaction of soluble cytochrome c with the oxidase (measured with ascorbate, which does not reduce the endogenous cytochrome c). The exogenous

cytochrome c must react directly with cytochrome a (or a_3).

ACKNOWLEDGMENT

The skillful assistance of Miss Marjorie Krause is appreciated.

REFERENCES

Ball, E. G., and Cooper, O. (1957), *J. Biol. Chem. 226*, 755.
Camerino, P. W., and Smith, L. (1963), *Fed. Proc. 22*, 654.
Chance, B. (1952a), *Nature 169*, 215.
Chance, B. (1952b), *J. Biol. Chem. 197*, 557.
Chance, B. (1953), *J. Biol. Chem. 202*, 407.
Chance, B. (1955), *Discussions Faraday Soc. 20*, 205.
Chance, B. (1961), *in* Hematin Enzymes, Falk, Lemberg, and Morton, eds., London, Pergamon, p. 597.
Cleland, K. W., and Slater, E. C. (1953), *Biochem. J. 53*, 547.
Crane, F. L., Glenn, J. L., and Green, D. E. (1956), *Biochim. Biophys. Acta 22*, 475.
Gornall, A., Bardawill, C., and David, M. (1949), *J. Biol. Chem. 177*, 751.
Griffiths, D. E., and Wharton, D. C. (1961), *J. Biol. Chem. 236*, 1850.
Jacobs, E. E., and Sanadi, D. R. (1960), *J. Biol. Chem. 235*, 531.
Keilin, D., and Hartree, E. F. (1938), *Proc. Roy. Soc. (London) Ser. B:125*, 171.
Keilin, D., and Hartree, E. F. (1945), *Biochem. J. 39*, 289.
Keilin, D., and Hartree, E. F. (1947), *Biochem. J. 41*, 500.
Keilin, D., and Hartree, E. F. (1955), *Nature 176*, 200.
Keilin, D., and Slater, E. C. (1953), *Brit. Med. Bull. 9*, 89.
Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.
Mackler, B., and Green, D. E. (1956), *Biochim. Biophys. Acta 21*, 1.
Margoliash, E. (1954), *Biochem. J. 56*, 529, 535.
Minnaert, K. (1961), *Biochim. Biophys. Acta 50*, 23.
Minnaert, K., and Smith, L. (1961), *Proc. Intern. Congr. Biochem., 5th, Moscow*, Abstracts, p. 467.
Slater, E. C. (1949), *Biochem. J. 44*, 305.
Slater, E. C. (1958), *Biochem. Soc. Symp. (Cambridge, Eng.) 15*, 76.
Smith, L. (1955), *J. Biol. Chem. 215*, 833.
Smith, L., and Camerino, P. W. (1963), *Biochemistry 2*, 1428 (preceding paper, this issue).
Smith, L., and Conrad, H. (1956), *Arch. Biochem. Biophys. 63*, 403.
Smith, L., and Conrad, H. (1961), *in* Hematin Enzymes, Falk, Lemberg, and Morton, eds., London, Pergamon, p. 260.
Tsou, C. L. (1952), *Biochem. J. 50*, 493.
Wharton, D. C., and Griffiths, D. E. (1962), *Arch. Biochem. Biophys. 96*, 103.
Yonetani, T. (1962), *J. Biol. Chem. 237*, 550.

Kinetic Studies of Bromelain Catalysis*

TADASHI INAGAMI† AND TAKASHI MURACHI

From the Department of Biochemistry, Nagoya City University School of Medicine, Nagoya, Japan

Received April 8, 1963

Bromelain has been found to hydrolyze esters of L-arginine derivatives much faster than esters of other amino acids. Kinetics of the hydrolyses of the ethyl ester and amide of benzoyl-L-arginine have been compared over *p*H 3.2–9.6 and 15–35°. The apparent first-order rate constant for the ester is about 140 times higher than that for the amide, a situation conspicuously different from other SH-proteinases. The apparent Michaelis-Menten constants for the two substrates are also different to the same extent, hence the ratios of the two constants for the ester and the amide are very close and behave similarly as *p*H and temperature are changed. The two constants are more or less unchanged over a wide neutral *p*H range with both substrates. With the ester both constants decrease parallel toward acidic and alkaline *p*H values, while with the amide the apparent Michaelis-Menten constant increases and the apparent first-order rate constant decreases. With the ester the apparent Michaelis-Menten constant is practically constant from 15° to 35°, while other constants for both substrates increase with temperature. By comparing these features with papain and ficin the apparent kinetic parameters have been characterized in terms of individual rate constants and it has been concluded that bromelain involves a unique mechanism of catalysis among the SH-proteinases.

Stem bromelain, a proteolytic enzyme from pineapple stem, has been shown to be a sulfhydryl enzyme by Murachi and Neurath (1960). It hydrolyzes proteins like casein and hemoglobin at high rates, while a synthetic substrate of smaller molecular size, like BAEE,[1] has also been shown to be hydrolyzed at a moderate rate (Murachi and Neurath, 1960). A preliminary experiment in this laboratory indicated that arginine derivatives like BAEE and BAA are the best substrates for the enzyme among various amino acid esters and amides, respectively. These facts seemed to indicate a close similarity between bromelain and other sulfhydryl enzymes from plants like papain and ficin. However, an important difference has been noted in that the rate of the BAEE hydrolysis is conspicuously higher than the rate for its amide analog, BAA, by a factor of more than 100, while both papain (Stockell and Smith, 1957; Smith and Parker, 1958) and ficin (Hammond and Gutfreund, 1959) have been shown to hydrolyze an ester (BAEE) and a corresponding amide (BAA) at almost identical rates. Since the similarity of the rates of the ester and amide hydrolyses has been considered as one of the important features of the enzymes from plant with a sulfhydryl group at the active center, as contrasted from other proteolytic

* This work was supported in part by grants from the Ministry of Education, Japan, and the National Institutes of Health, U. S. Public Health Service (GM 08714).

† Present address: Department of Chemistry, Yale University, New Haven, Connecticut.

[1] The abbreviations used are: BAEE, benzoyl-L-arginine ethyl ester; BAME, benzoyl-L-arginine methyl ester; TAME, *p*-toluene-sulfonyl-L-arginine methyl ester; BAA, benzoyl-L-argininamide.