AN ABSTRACT OF THE THESIS OF

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Title The Effect of Alkaline pH on the Succinate Oxidase System of Sub-mitochondrial Particles from Beef Heart Muscle

Abstract approved [redacted]
(Major professor)

The spectrophotometric method for the determination of the acid nonextractable flavin in the proteolytic digest of the trichloroacetic acid precipitate from the heart muscle preparation has been examined for the interference of the degraded hemeproteins. The proteolytic digest was chromatographically separated into the heme and flavin fractions. Spectrophotometric determinations made on the separated fractions have indicated that the heme interference is negligible for the heart muscle preparation. The validity of this method has thus been demonstrated.

In the course of the proteolysis, cytochrome $\underline{c}_1$ and $\underline{c}$ are evidently degraded to hemepeptides. The spectral behavior of the hemepeptides has been presented.

Based on the fluorescent characteristics of the acid nonextractable flavin, a fluorometric method has been devised. This method is approximately 100 times more sensitive than the spectrophotometric assay. Results from these two methods are in good agreement. It is suggested that the fluorometric method

may be more suitable for samples containing high concentrations of pigments adsorbing in the 450-530 mu region of the spectrum.

Alkaline inactivation of the succinate oxidase activity of the heart muscle preparation in the absence of substrate and presence of oxygen has been used by Keilin and King to prepare a particle which supplies all of the respiratory components except succinate dehydrogenase for reconstitution of succinate oxidase. The succinate dehydrogenase protein, as measured by its flavin coenzyme, was found to be dissociated from the particulate heart muscle preparation by alkaline treatment. The dissociation occurred at the same rate as the inactivation of the succinate oxidase. It was therefore concluded that the original site of binding of the dehydrogenase to the particle is available to bind active succinate dehydrogenase during reconstitution of the system.

The inactivation of succinate oxidase under the experimental conditions used was found to follow zero order kinetics. The apparent zero order rate constant varied as the first power of the initial enzyme concentration and the second power of the hydroxyl ion concentration. The temperature dependence of the apparent zero order rate constant was complex. A mechanism is presented which is consistant with the observed kinetics.

The equilibrium dissociation of the succinate oxidase system by alkaline treatment in the presence of succinate and absence of oxygen was studied using the acid nonextractable flavin content as a measure of the succinate dehydrogenase concentration. The percent of the dehydrogenase which was soluble was a linear

function of the hydroxyl ion concentration and the concentration of the soluble dehydrogenase was proportional to the total concentration bound and soluble, confirming the report of King. The equilibrium constant for the dissociation decreased with increasing temperature and buffer concentration.

THE EFFECT OF ALKALINE pH ON THE SUCCINATE OXIDASE SYSTEM OF SUB-MITOCHONDRIAL PARTICLES FROM BEEF HEART MUSCLE

by

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THE EFFECT OF ALKALINE pH ON THE SUCCINATE OXIDASE SYSTEM OF SUB-MITOCHONDRIAL PARTICLES FROM BEEF HEART MUSCLE

GENERAL INTRODUCTION

The existence of a reaction in which succinate is oxidized to fumarate by molecular oxygen was discovered by Thunberg (61) in 1909 and this reaction has been the subject of a very large volume of literature since that time. Among the important developments in the study of this enzyme system are, in chronological order, (a) the discovery by Keilin (25) of the cytochromes and his recognition of their function in intracellular oxidations, (b) the isolation by Keilin (26, 27) of a cell free particulate preparation containing a much purified intact succinate oxidase system, (c) the application by Chance (see Chance and Williams (8)) of sensitive spectrophotometric methods to the study of the kinetics and steady state of the oxidation and reduction of the cytochromes during the oxidation of succinate by oxygen, (d) the solubilization and purification by Wang (68, 69) and Singer (23, 55) and coworkers of a protein catalysing the oxidation of succinate by artificial electron acceptors, (e) the solubilization and partial purification by Keilin and King (29, 30) of a protein able not only to catalyze the oxidation of succinate by artificial dyes but also active in the reconstitution of succinate oxidase.

The developments in the field up to and including the isolation and purification of the soluble succinate - phenazine methosulfate

(PMS)[1] reductase[2] (d) have been reviewed (8, 16, 44, 50, 54, 56, 57, 66, 71). The literature related to the reconstitution of succinate oxidase (e) is, however, presently growing very rapidly and no up-to-date reviews are available. A review by Veeger (66) covers the literature through 1962. An attempt will be made to review the available literature in order to give a perspective of the data presented in this thesis.

The reconstitution of succinate oxidase by Keilin and King (29, 30) has been followed by a considerable number of papers on the properties and nature of the reconstitution. This is not surprising because the separation and then reconstitution of the components of the particulate enzyme complex is the most powerful tool available in the continuing effort to understand the structure and function of the intact complex. This experimental approach allows the properties of the intact system to be compared with the properties of the isolated components. In addition, separation and reconstitution of the components can be studied by conventional kinetics and equilibrium measurements to gather information about the interaction among the individual components. In this respect the

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[1] The following abbreviations are used in this thesis:
FAD - Flavin adenine dinucleotide
FMN - riboflavin 5' phosphate
EDTA - Ethylenediamine tetraacetate
NADH - Reduced nicotinamide dinucleotide
PMS - phenazine methosulfate

[2] In this thesis the enzyme which catalyses the oxidation of succinate by artificial dyes such as phenazine methosulfate and ferricyanide but which is reconstitutively inactive is referred to as succinate-PMS oxidoreductase, or simply succinate-PMS reductase. This is in accordance with the recommendation submitted by the Enzyme Committee of the International Union of Biochemistry. The reconstitutively active enzyme is referred to as succinate dehydrogenase.

reconstitution of the succinate oxidase is particularly useful because the succinate dehydrogenase is a soluble protein. Although cytochrome c was the first member of the particulate complex to be solubilized and then reincorporated under special conditions (64), the reconstitution of the succinate dehydrogenase has been more amenable to experimental evaluation.

In 1940 Keilin and Hartree (27) observed that when the Keilin-Hartree heart muscle particles were incubated at pH 9.0 and room temperature, the succinate dehydrogenase (as measured by its methylene blue activity) was much more rapidly inactivated than the cytochrome oxidase activity. These observations were extended by Keilin and King (30) using phenazine methosulfate as an electron acceptor. The succinate methylene blue reductase was inactivated at the same rate as the succinate oxidase but more rapidly than the succinate -PMS reductase. A particle was prepared by alkaline treatment (pH 9.3 at 0^{o} and then incubation at 38^{o}) that was totally inactive in the succinate-methylene blue and succinate-oxygen assays but which retained 60-70 percent of its original cytochrome oxidase activity (30). When this alkali-treated particle was combined with soluble succinate dehydrogenase prepared by a modification of the method of Wang et al. (68) an active succinate oxidase appeared (29, 30).

This process was interpreted to be a reconstitution on the basis of the following observations on the reactivated system: 1) the unstable soluble dehydrogenase reacquired the stability of the particulate form, 2) the oxidation of succinate by molecular oxygen

was mediated by the cytochrome system and was antimycin sensitive, 3) methylene blue and 2, 6-dichlorophenolindophenol could again be used as electron acceptors as they had been for the untreated preparation, 4) there was a stoichiometric relationship between the quantities of alkali-treated particles and dehydrogenase required for reconstitution, 5) after the alkali-treated particle and the soluble dehydrogenase were mixed, the dehydrogenase was bound to the particle and the two could not be separated by high-speed centrifugation or repeated washing.

These important findings have been confirmed by several laboratories (14, 32, 66) although some confusion is introduced in subsequent papers by Singer and coworkers. These workers first reported a failure to obtain reconstitution (51) then a non-linear relationship between the incorporation of the succinate dehydrogenase and the increase in succinate oxidase activity and a low and variable degree of reconstitution (52). In their most recent report (32), however, they state "the experimental findings of Keilin and King were fully confirmed" with respect to points 1-5 listed above.

In a series of papers on the reconstitution of succinate oxidase King has reported that 1) there is no species specificity for the reconstitution and the soluble dehydrogenase and alkali-treated particles from beef, pig, horse and human heart can be cross reconstituted (35, 39), 2) the inactivation of the soluble succinate dehydrogenase occurs with a non-parallel loss of reconstitutive and artificial dye reductase activities (34, 38, 39), 3) the presence of a reducing agent (succinate, NADH, dithionite or ascorbate) is

required in order to isolate an active succinate dehydrogenase (37, 39), 4) when the alkaline treatment is carried out at 0^{o} in the presence of succinate and absence of oxygen, the dehydrogenase is reversibly dissociated from the particle and an equilibrium constant can be calculated for the dissociation (36, 39).

With regard to 2), King (34, 38, 39) observed that the soluble dehydrogenase is very labile and on standing in air it is changed to a reconstitutively inactive succinate-PMS reductase that in turn loses this activity. Kimura and Hauber (31) fractionated a succinate dehydrogenase preparation on Sephadex G-100 in the presence of air and found at least three and possible four forms of the protein. Only one of these forms was reconstitutively active. This is consistent with the data of King. One of the other forms found by chromatography on Sephadex was a succinate-PMS reductase that was capable of binding to the alkali treated particle but was incapable of reconstituting succinate oxidase activity. Although this would seem to suggest that the initial site of inactivation of the dehydrogenase does not involve the binding site, the active form was preferentially bound and some modification of the binding site must have occurred.

The lability of the soluble dehydrogenase may be the reason succinate dehydrogenase can not be isolated in the absence of reducing agents. In the absence of reducing agents the isolated enzyme is always a succinate-PMS reductase which has no reconstitutive activity (37, 39). It appears likely that the function of the reducing agent is to stabilize the soluble dehydrogenase either by keeping it in the reduced form, by removing the oxygen, or by a combination of the

two effects.

The reversible dissociation of the succinate dehydrogenase from the particle by alkaline treatment, in the presence of succinate and absence of oxygen, of a heart muscle preparation which was pre-incubated with succinate as reported by King (36, 39) is of singular importance. The dissociated soluble dehydrogenase is functionally identical to that prepared by the butanol method of Keilin and King (30) and the particle formed when the dehydrogenase is dissociated is functionally identical (toward reconstitution) to that prepared by alkaline inactivation of the succinate oxidase in the absence of succinate and presence of oxygen. The report of the reversible dissociation not only supported the contention of King (31, 33) that the reconstitution is a physiological phenomenon but also defined the experimental conditions that could be used to study the thermodynamics of the interaction between the soluble dehydrogenase and the particle.

Singer and coworkers (32, 52) have questioned the existence of the reversible dissociation as reported by King. There is, however, no conflict in the data presented, because the former authors used experimental conditions very different from those of the latter. King reported that when the alkaline treatment is carried out in the presence of succinate and absence of oxygen the succinate dehydrogenase, as measured by its reconstitutive activity, is dissociated from the particle and becomes soluble. Under these conditions the dissociation is reversible and if the suspension is neutralized before centrifugation the dehydrogenase is reincorporated into the particle.

Thus the amount of dehydrogenase in the supernatant liquid after high speed centrifugation is dependent on the pH of the medium during centrifugation. Singer and coworkers (32, 52) have reported that succinate dehydrogenase, as measured by its peptide bound flavin content, is not solubilized during the alkaline treatment of the heart muscle preparation (absence of succinate and presence of oxygen). Under these conditions the succinate dehydrogenase is irreversibly inactivated (27, 30). In view of the great difference in the effect of the two sets of conditions on the succinate dehydrogenase (100 percent recovery of the activity vs. greater than 95 percent inactivation) it would seem that this latter data is not "contrary to King's (1962) report" as stated (52). The experimental conditions used by Singer and coworkers are comparable to those in Parts 1, 2 and 3 of this thesis.

Although the soluble succinate dehydrogenase was isolated in 1958 and purified to approximately 40 percent purity at that time, it has resisted further purification due to the extreme lability of its reconstitutive activity. The time required to inactivate 50 percent of the enzyme in air has been reported to be approximately 80 minutes at 0-4^{o} (34, 35) and approximately 15 minutes at 22^{o} (31). When the enzyme is stored in vacuum at 0-4^{o} the half life is increased to approximately 12 hours (35). Veeger (66) has reported the preparation in their laboratory of an enzyme of 50-75 percent purity but no data is given.

Electron spin resonance studies of the partially purified dehydrogenase have been made in two laboratories (40, 66) and

compared with studies on the purified succinate-PMS reductase (2, 18, 66) and the particulate system (1, 2, 9, 20). These data are discussed in detail by Veeger (66).

It is the purpose of this thesis to present a study of the interaction of the succinate dehydrogenase and the particulate cytochrome system. The study consists of four related phases or parts. The first part (1) is the development and characterization of a sensitive assay method for the determination of the succinate dehydrogenase protein that is independent of the enzymatic activity. The second part (2) is the application of this method to the problem of the fate of the succinate dehydrogenase protein during alkaline inactivation of the succinate oxidase activity. The third part (3) is a kinetic study of the inactivation of succinate oxidase by alkali and the fourth part (4) is the application of the method developed in part 1 to a study of the equilibrium dissociation of the dehydrogenase during alkaline treatment in the presence of succinate and absence of oxygen. Each of these parts will be treated separately in order to avoid confusion as to the experimental methods used. The short introduction to each part is included to give a background for that particular section of the thesis.

GENERAL EXPERIMENTAL PROCEDURES

Materials

Crystalline chymotrypsin, recrystallized trypsin (type III), FMN, and FAD were obtained from the Sigma Chemical Company. The purity of the FMN and FAD was determined spectrophotometrically. "1 — 300 trypsin" was purchased from the Nutritional Biochemicals Corporation.

The Keilin-Hartree preparation was prepared from beef heart as previously reported (41). The cytochrome b-c_1 particle, which was completely free of cytochrome c, was prepared according to the method of Takemori and King (60).

Methods

Protein determination - the total fat-free dry solid determined by the Slater method (58) was used as the protein content of the heart muscle preparation.

pH determination - a Beckman Zeromatic pH meter with manual temperature compensation was used for all operations for which the pH is given to the nearest 0.1 pH unit and for the data in Fig. 8. A Beckman model 76 expanded scale pH meter was used for all pH measurements given to the nearest 0.01 pH unit. In all cases time was allowed for thermal equilibration of the electrodes. Beckman buffers were used for calibration. When the expanded scale pH meter was used all solutions were maintained within 0.5^{o} of the desired temperature by using a thermostatted beaker.

Enzymatic activity determinations - the succinate-PMS reductase activity was measured manometrically at 37° in a system composed of 60 mM succinate, 100 mM phosphate pH 7.4, 1 mM potassium cyanide and an appropriate amount of enzyme; after temperature equilibration sufficient phenazine methosulfate was added from the side arm to give a final concentration of 0.5 mg ml^{-1}.

The succinate oxidase activity was measured by either manometry or polarography, the latter using a Gilson Medical Electronics Oxygraph. The assay medium contained 40 mM sodium succinate, 100 mM phosphate pH 7.4, 28 uM cytochrome c and 50 uM EDTA.

The cytochrome oxidase activity was determined by using either manometry or polarography to measure the oxygen uptake in a system containing 28 uM cytochrome c, 70 mM phosphate pH 7.4, 16 mM ascorbate and 0.8 mM EDTA. Boiled enzyme controls were used to measure the nonenzymatic oxygen uptake.

All enzymatic activities were determined for at least three different enzyme concentrations. The reported value is the slope of the straight line plot of activity against enzyme concentration.

1. THE DETERMINATION OF THE ACID NONEXTRACTABLE FLAVIN IN MITOCHONDRIAL PREPARATIONS FROM HEART MUSCLE

INTRODUCTION

Although the existence of bound forms of flavins in natural materials has been recognized (5, 17, 45, 46) the methods usually employed to assay for this component have not been well characterized. Boukine (5) has demonstrated, in certain plants, the presence of a form of flavins that is not extractable by acid but can be rendered soluble by proteolytic digestion. Independently, Green et al. (17) have shown this type of a flavin in a particulate enzyme preparation known as "succinate dehydrogenase complex". Kearney (22, 24), Wang (68, 69), and their coworkers have more specifically reported that the prosthetic group of their enzyme preparations, which catalyze the oxidation of succinate by ferricyanide or phenazine methosulfate, is a flavin adenine dinucleotide covalently linked to the protein. This form of flavin is now generally known as the acid nonextractable flavin[1] and is assumed to be exclusively derived from succinate dehydrogenase, at least in mammalian heart. There are indications, however, that other tissues may contain similarly bound flavin coenzymes that are not associated with succinate dehydrogenase (7, 11).

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[1] Actually the acid nonextractable flavin even after proteolysis was still in the form of flavin peptide(s). For simplicity in description, sometimes the term "flavin" is used. Acid hydrolysis under the conditions described decomposes the FAD peptide to FMN peptide.

Methods have been devised to determine the acid nonextractable flavin by first treating the sample with trichloroacetic acid to remove the acid soluble flavins. The precipitate, which contains the acid nonextractable flavin, is subjected to proteolytic digestion. The flavin thus solubilized is assayed spectrophotometrically (41) by assuming that its molar absorbancy index (oxidized-reduced) at 450 mu is equal to that of FAD. During proteolysis, however, some hemepeptides are also solubilized. These hemepeptides are also reducible by dithionite. Because the spectral properties of the hemepeptides are unknown, they may contribute a possible source of error in the flavin determination by this method. Consequently, a study was undertaken to ascertain how extensive an error may be involved. A separation of the hemepeptides from the flavin was accomplished and the absorbancy determinations were made on the individual fractions. This paper presents evidence to show the error is actually negligible. Thus the spectrophotometric method as described previously (41) is useful and reliable.

Mainly through the work of Kearney (22) and Wang et al. (68, 69), the acid nonextractable flavin has been shown to have fluorescent characteristics different from those of free FAD and FMN. The molar fluorescent intensity of the acid nonextractable flavin in the mononucleotide form at pH 3.2-3.5 is identical to that of FMN, but in neutral media it exhibits a negligible fluorescence. On the other hand, the heme fraction does not in practice contribute fluorescence under the conditions tested. The results obtained from an assay method based on the fluorescence are in agreement with

those from the spectrophotometric method.

EXPERIMENTAL PROCEDURES

All manipulations during flavin analysis were carried out under dim light in low actinic glassware unless otherwise indicated.

Spectrophotometric Assay - The spectrophotometric method for the determination of the acid nonextractable flavin was detailed previously (40). A correction[1] for contamination by acid extractable flavins was made by fluorometric measurement at pH 7.0. The acid nonextractable flavin concentration was calculated by equation 1.

(1) Acid nonextractable flavin = (Flavin determined spectrophotometrically) - (Flavin determined fluorometrically at pH 7.0 equivalent to FMN + FAD)

The second term of equation 1, the spectrophotometrically determined flavin, was calculated from the molar absorbancy index at 450 mu (oxidized minus reduced) of $10.3 \times 10^6 cm^2 \times mole^{-1}$. The change in absorption at 450 mu on reduction of the flavin was computed from the base at 530 mu as follows:

(2) $(A^{ox}_{450mu} - A^{red}_{450mu}) - (A^{ox}_{530mu} - A^{red}_{530mu})$

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[1] This correction for the contamination of acid extractable flavins is always made in the determination of the acid nonextractable flavin from this laboratory by either the spectrophotometric or the fluorometric method but this was not made clear in the procedure of a previous article (4). Our negligence in emphasizing this correction may have caused the misunderstanding by Cerletti et al. (6).

The last term of equation 1, the flavin determined fluorometrically at pH 7.0 equivalent to FMN + FAD, was determined after hydrolysis by using a FMN standard according to a previous report (41) under "fluorometric method."

Fluorometric Assay - The sample containing not less than 1.5 mumoles of acid nonextractable flavin was mixed with 50 % trichloroacetic acid to give a final concentration of 10%. The mixture was allowed to stand at 0° for 30 minutes, and then centrifuged. The residue was washed twice with 1% trichloroacetic acid and finally suspended in 50 mM phosphate buffer. The pH of the suspension was adjusted to 7.5-8.0 with 2.5 N NaOH and made up to a final volume of 6 ml with 50 mM phosphate buffer. The proteolytic digestion was carried out as described previously (41). At the end of the digestion 50% trichloroacetic acid was added to the mixture to give a final concentration of between 8 and 10%. The sample was then incubated overnight at 38° to hydrolyze the flavin to its mononucleotide form. Alternate methods of hydrolysis employed incubations at 100° for 10 to 15 minutes or 110° in an autoclave for 6 minutes. After hydrolysis the samples were diluted to a known volume and the precipitate was removed by centrifugation.

A Farrand spectrofluorometer with a Hanovia D.C. Xenon arc lamp and RCA IP21 photomultiplier tube was used for fluorescent measurements. The excitation wave length was 450 mu and the fluorescent (emission) wave length was 520 mu. The slit widths of the excitation monochrometer were 5 mu while those of the fluorescence monochrometer were 10 mu. The fluorescence of each

flavin sample was measured before and after reduction by dithionite at two or more flavin levels between 2×10^{-2} uM and 6×10^{-2} uM in 30 mM citrate buffer, pH 3.2-3.3 and between 6×10^{-2} uM and 1.5×10^{-1} uM in 30 mM phosphate buffer at pH 7.0-7.4. The standards were subjected to exactly the same measurements and consisted of at least four levels of either "the acid nonextractable flavin standard" (see below) after hydrolysis or free FMN.[1] The net fluorescent intensity (oxidized minus dithionite reduced) of both the standards and the samples varied linearly with their concentrations (see Fig. 1 of ref. 39 for standard curves). The correction of the fluorescence at pH 7.0 was small but variable. This fluorescence was evidently due to contamination by the acid extractable flavins. The acid nonextractable flavin was calculated by equation 3.

(3) Concentration of acid nonextractable flavin = Equivalent concentration of FMN at pH 3.2 − Equivalent concentration of FMN at pH 7.4

Chromatography - Sephadex column chromatography was carried out using Sephadex G-25 in a column of 1.2 cm x 65 cm. The flavin sample in a 3.4 ml volume was applied onto the column and distilled water was used for elution. Florisil chromatography was performed according to Huennekens (21). Paper chromatography with a solvent system of 5% Na_2HPO_4 was made by the ascending technique using Whatman filter paper No. 1 or No. 52. After the chromatographs were sprayed with 10% acetic acid, the flavin bands were detected by their fluorescence under ultraviolet light. In some

[1] In routine assay, FMN was used as the standard.

instances authentic FMN and FAD were mixed with the sample as reference compounds.

Preparation of "the Acid Nonextractable Flavin Standard" - Several samples of the proteolytic digest from a number of batches of the heart muscle preparation (cf. 41) were separately chromatographed on Sephadex G-25 columns. The flavin fractions thus obtained were pooled and further purified by adsorption chromatography on florisil. The eluate containing the flavin from the florisil column was concentrated by lyophillization and finally purified by paper chromatography. The intense yellow band with an R_f value of 0.7 on the paper chromatograph was cut out and eluted with water. This purified flavin peptide was in the dinucleotide form. It was used as the acid nonextractable flavin standard and is sometimes called the "purified flavin peptide" in this paper.

Alkaline Treatment I - The alkaline treatment was made essentially according to a previous report (29). The heart muscle preparation, containing approximately 10 mg protein per ml of 0.05 M phosphate-borate buffer, pH 7.8, was cooled to 0° and then adjusted to pH 9.4-9.5 with 2.5 N NaOH. The sample was rapidly warmed to 37° (approximately 3 minutes) and incubated for the specified length of time. The treatment was stopped by rapidly cooling the sample in an ice bath followed by addition of a predetermined volume of 3 N HCl to give a pH of 7.4-7.6. The mixture was centrifuged for 90 minutes at 30,000 RPM in a No. 30 rotor of a Spinco ultracentrifuge, model L. The supernatant liquid and the residue were then separately assayed for acid nonextractable flavin.

RESULTS

Possible Error in the Spectrophotometric Assay for the Acid Nonextractable Flavin Made Directly in the Proteolytic Digest

The proteolytic digest (i.e. the material solubilized by trypsin-chymotrypsin digestion of the trichloroacetic acid precipitate of the heart muscle preparation) was found to contain, in addition to the acid nonextractable flavin, hemepeptide(s). The absorption spectrum of the proteolytic digest is shown in Fig. 1. This digest exhibited absorbancy maxima at 408-410 mu in the oxidized form and at 415 mu in the reduced form. The flavin absorption peak at 450 mu was evident only in the different spectrum.

When the proteolytic digest was subjected to chromatography on a Sephadex G-25 column, it was separated into two components that exhibit absorbancy at 450 mu. The elution pattern is depicted in Fig. 2. The absorbancy at 450 mu was used because the oxidized flavin absorbs maximally at this wave length. The heme contaminant also contributed absorption in this region as will be seen later and was responsible for Peak I of Fig. 2.

The fractions under Peak I, i.e. from No. 20 through 28 of Fig. 2, were combined and concentrated by lyophilization to approximately one-fifth of the original volume. In the same manner, the fractions under Peak II, i.e. from No. 29 through 39, were concentrated. The spectra of Peak II were characteristic of a flavin as witnessed by Fig. 3C and 3D, although a minute amount of heme was still present. The flavin contamination in Peak I was very

Fig. 1. The absorption spectra of the proteolytic digest of the precipitate from trichloroacetic acid treatment of the heart muscle preparation as used for the spectrophotometric flavin assay. From 1.2 g (protein) of the heart muscle preparation, 20 ml of the solution was obtained. The insert is the difference spectrum (the oxidized minus the dithionite reduced).

Fig. 2. The elution pattern of the proteolytic digest (as used in Fig. 1) from a Sephadex G-25 column. The solution in a volume of 4 ml derived from 1.0 g (protein) of the heart muscle preparation was placed on a 1.2 x 65 cm Sephadex G-25 column. The eluant was distilled water. Each fraction was approximately 2 ml.

Figure 1

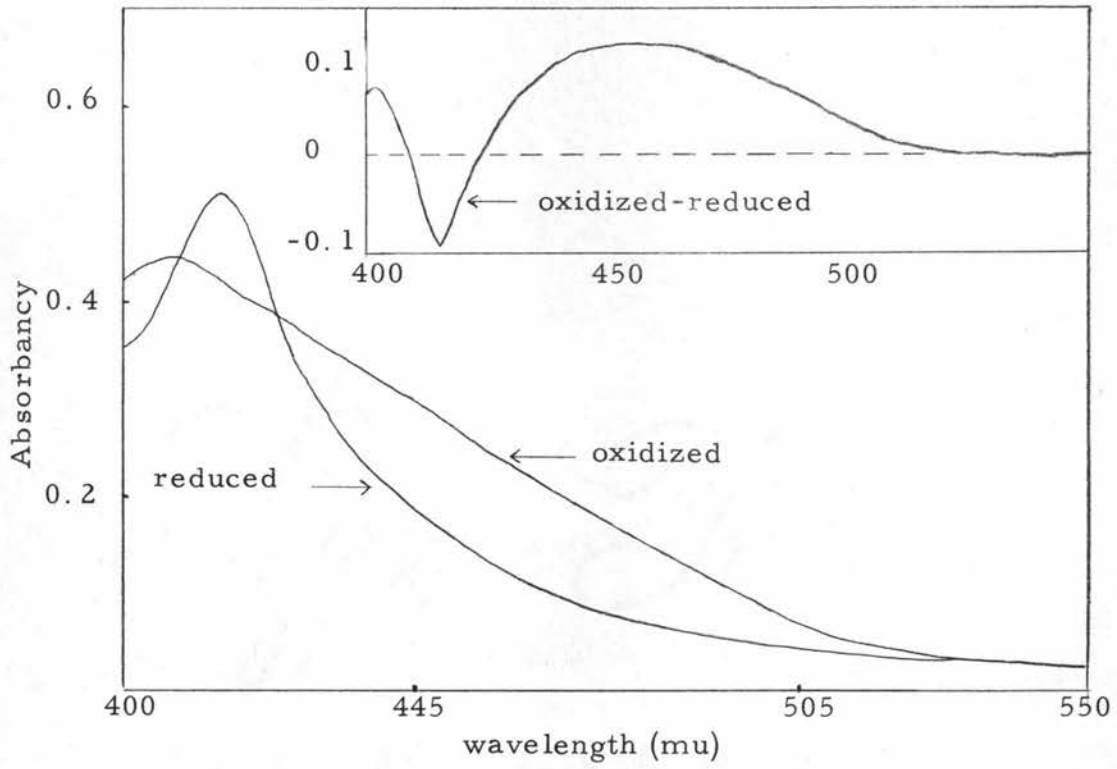


Figure 2

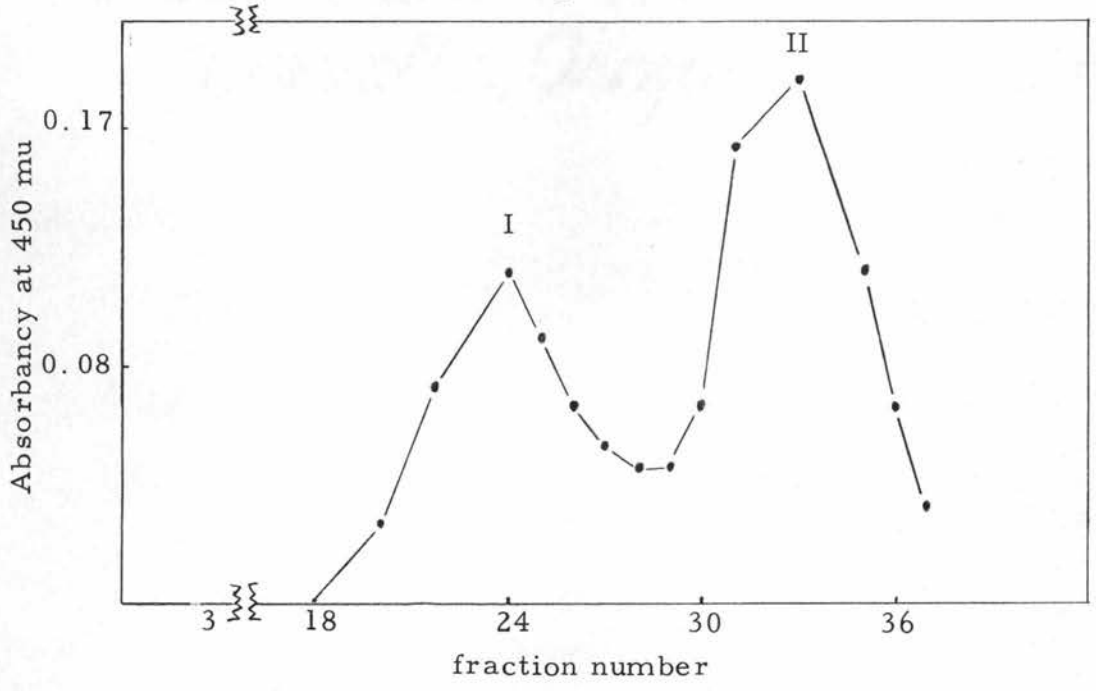


Fig. 3. The absorption spectra of Peak I and Peak II from the Sephadex column.

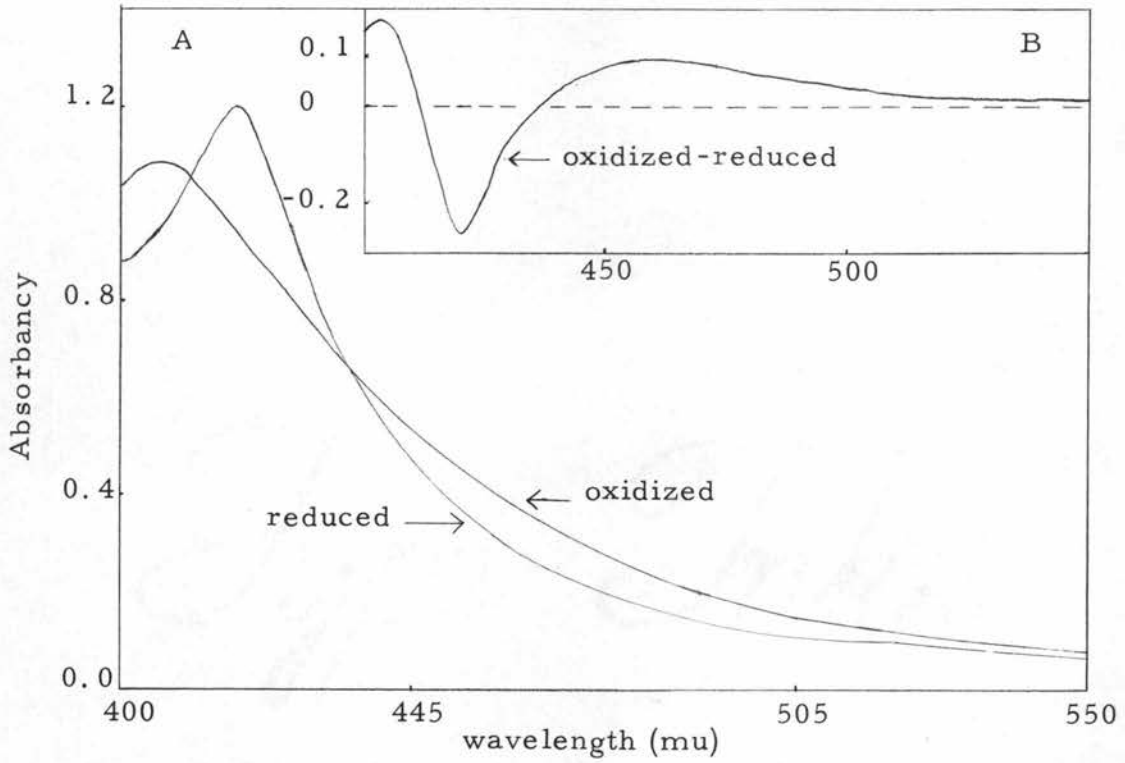
A. The absolute spectra of Peak I. Fractions 20 through 28 were combined and the volume was reduced to 4 ml by lyophilization.

B. The difference spectrum of Peak I, the oxidized minus the dithionite reduced sample.

C. The absolute spectra of Peak II. Fractions 29 through 39 were combined and the volume was reduced to 16 ml by lyophilization.

D. The difference spectrum of Peak II, the oxidized minus the dithionite reduced.

Figure 3


A
B
0.1
0
-0.2
1.2
0.8
0.4
0.0
Absorbancy
oxidized-reduced
450
500
oxidized
reduced
400
445
505
550
wavelength (mu)

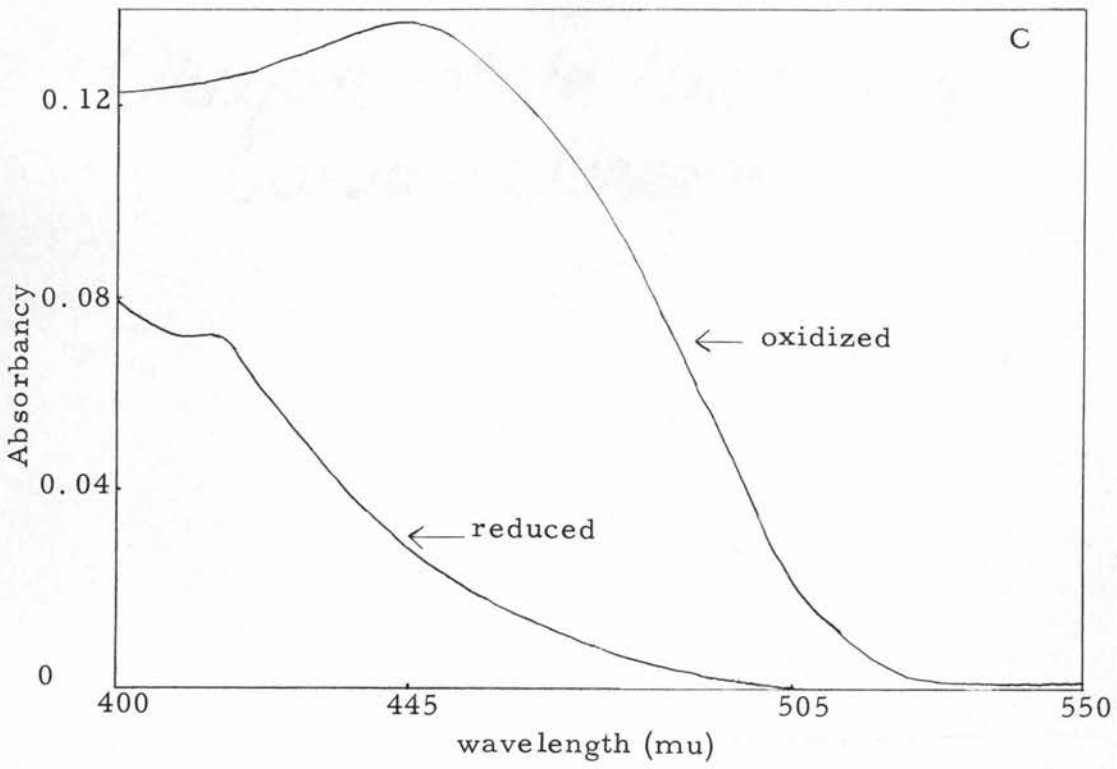
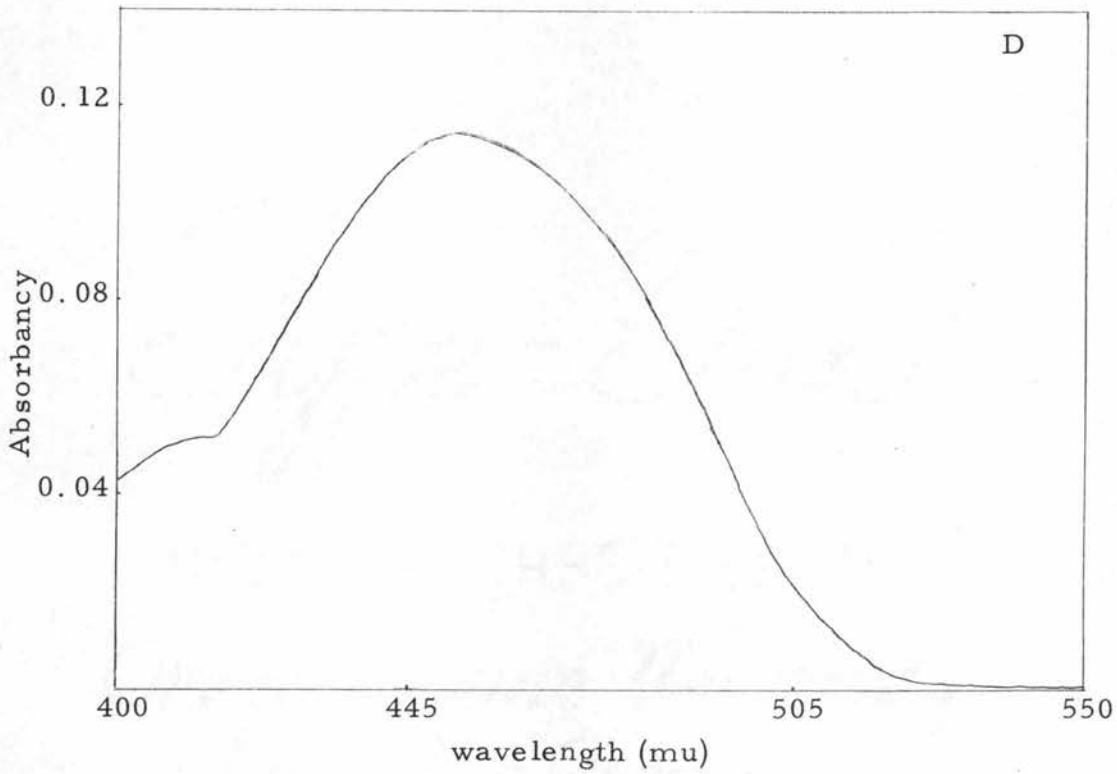

C
0.12
0.08
0.04
0
Absorbancy
oxidized
reduced
400
445
505
550
wavelength (mu)

Figure 3 (Continued)



small as may be seen in Fig. 3A and 3B. Peak I was most probably hemepeptide(s) derived from proteolytically degraded cytochrome c_1 and/or c rather than from cytochrome oxidase or cytochrome b. This separation of the two components allowed the estimation of the extent of the contribution of non-flavin to the flavin determination using the spectrophotometric assay (41), in the proteolytic digest directly without chromatographic separation, as employed by this (41) and other (4) laboratories.

The flavin content of a number of batches of heart muscle preparation was determined before and after chromatographic separation of the flavin and heme. The results are summarized in Table 1. The flavin content in Peak II was found to be an average of 82% of that determined directly in the proteolytic digest. This was true also for those samples that were different from the heart muscle preparation such as the supernatant and the residue fractions after alkali treatment. The mechanical loss due to manipulation accounted for approximately 10% of the total. This figure was obtained by carrying a known amount of FAD through the complete procedure and by rechromatography of Peak II. It should be considered that Peak I might also have contained a small amount of the flavin. Thus the error in the spectrophotometric method made directly in the proteolytic digest due to the contribution of absorbance at 450 mu by the heme was not more than 8%. It must be emphasized that 8% is the estimated maximal error. As may be seen from Table 3 the true error may be negligible. However, if the sample had a high cytochrome content, the error would be accordingly increased.

TABLE 1

The apparent recovery of the acid nonextractable flavin of heart muscle preparations after Sephadex chromatography

The flavin content is reported as umoles per gram of protein in the original heart muscle preparation.

Sample	Time of pH 9.5 treatment (minutes)	Acid nonextractable flavin (umole per g protein) Determined directly in the proteolytic digest	Determined in Peak II from Sephadex column	Recovered in Peak II (percent)
A. Heart muscle preparation				
219	0	0.136	0.111	82
224	0	0.159	0.137	86
230	0	0.153	0.124	81
234	0	0.148	0.115	78
247	0	0.167	0.142	85
Mean		0.153	0.125	82
B. The pellet of the pH 9.5 treated heart muscle preparation *				
224A (P)	45	0.107	0.084	78
230A (P)	30	0.086	0.071	83
234A (P)	15	0.096	0.074	77
247A (P)	60	0.108	0.096	89
247AA (P)	90	0.108	0.100	93
Mean		---	---	84
C. The supernatant fraction of the pH 9.5 treated heart muscle preparation *				
224A (S)	45	0.042	.030	71
230A (S)	30	0.074	.059	80
234A (S)	15	0.048	.041	85
247A (S)	60	0.051	.049	96
247AA (S)	90	0.042	.036	85
Mean		---	---	83

* The flavin data given were based on the protein content of the heart muscle preparation originally employed. The retention of supernatant fluid in the residue was not corrected. Thus, the true value for the supernatant fraction should be somewhat higher and that in the residue should be accordingly lower.

Characteristics of the Heme Components

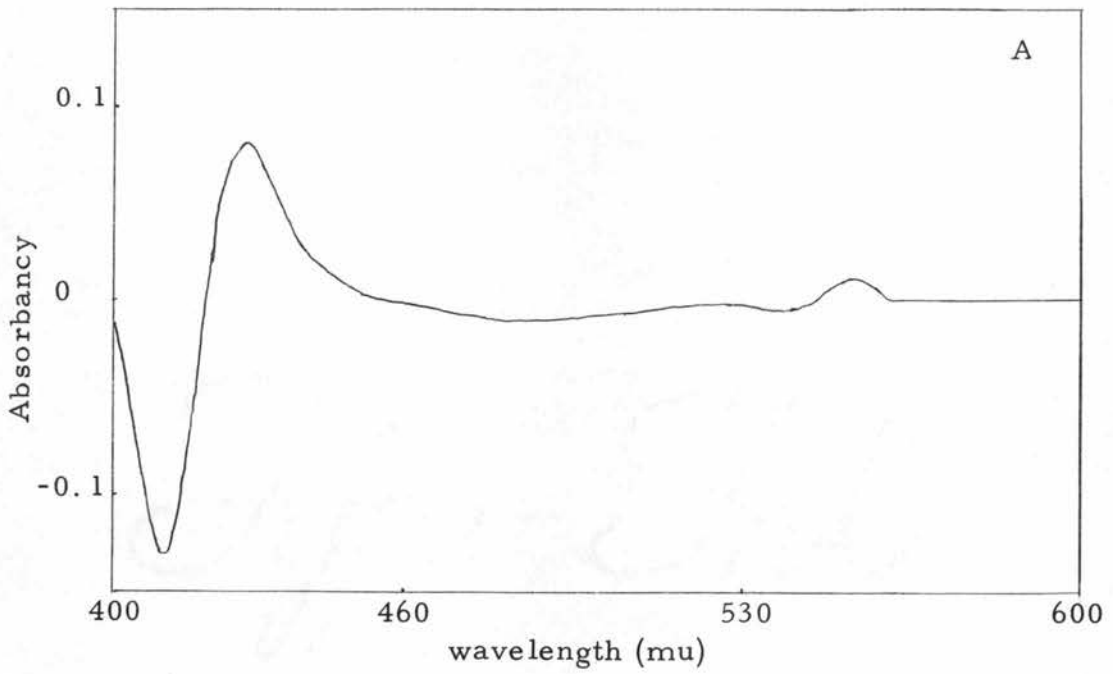
The heme component separated from the Sephadex column (Peak I) was reducible by dithionite. The reduced heme could be re-oxidized in air. However, the re-oxidized heme showed an absorption spectrum different from the original; its absorbancy at 408 mu was much lower. In the reduced, but not the oxidized state, the heme reacted with carbon monoxide and the Soret peak shifted from 417 to 413 mu. The difference spectrum of the CO treated sample is shown in Fig. 4A.

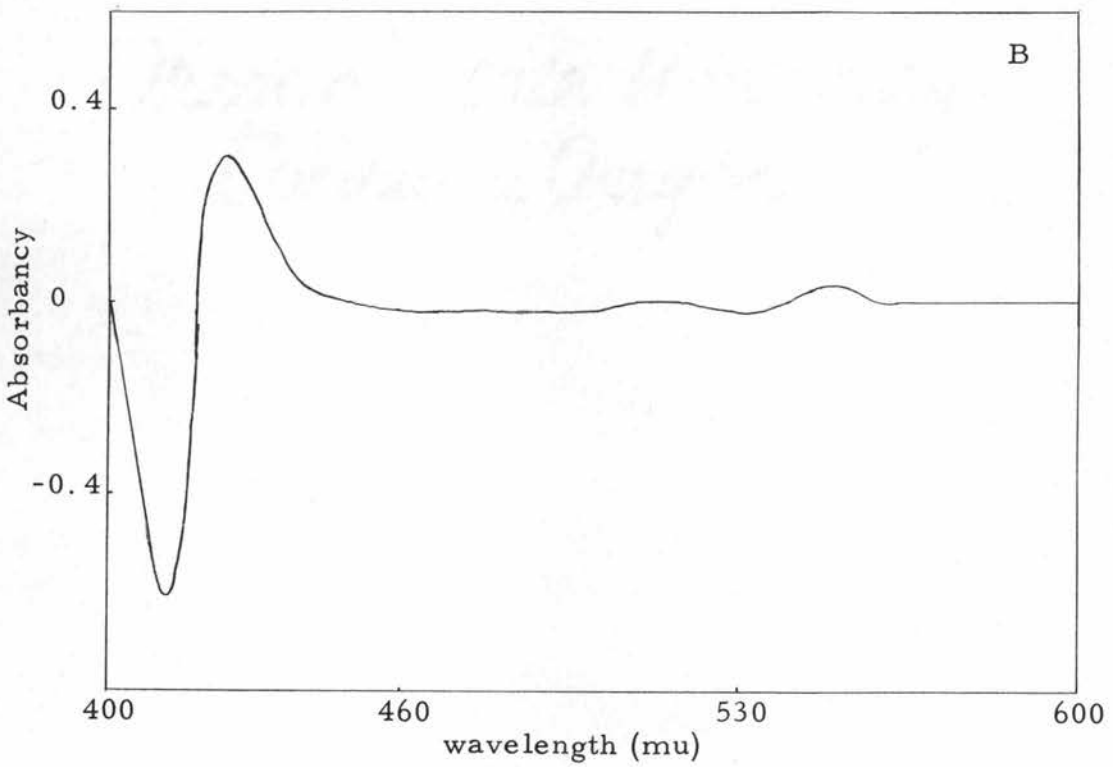
Several lines of evidence indicate that the heme component was the product of proteolytic degradation of cytochromes c and c_1. The heme component could be largely eliminated by washing the first trichloroacetic acid precipitate (prior to proteolysis) with water. Cytochrome c was isolated from the water wash by reprecipitation with 10% trichloroacetic acid. Proteolytic digestion of cytochrome c (Sigma type III) resulted in the formation of heme-peptide(s) that was not precipitated by 10% trichloroacetic acid or by boiling. The hemepeptide was auto-oxidizable and reactive with carbon monoxide (Fig. 4B). This observation is in agreement with the report of Tsou (63).

In addition to cytochrome c, the heme in Peak I also contained a degradation product from cytochrome c_1. Samples of the cytochrome b-c_1 particle (60), which was completely free of cytochrome c, were subjected to the same treatment as the heart muscle preparation. The proteolytic digest thus prepared showed the heme spectra similar to those depicted in Fig. 3A and 3B.

Fig. 4. Difference spectra of the reduced minus the CO-reduced heme fraction. (A) the heme from Peak II; (B) the heme from proteolytic digestion of Sigma type III cytochrome c. The concentration of the latter was equivalent to approximately 6 uM cytochrome c.

Figure 4


A
0.1
0
-0.1
Absorbancy
400
460
530
600
wavelength (mu)


B
0.4
0
-0.4
Absorbancy
400
460
530
600
wavelength (mu)

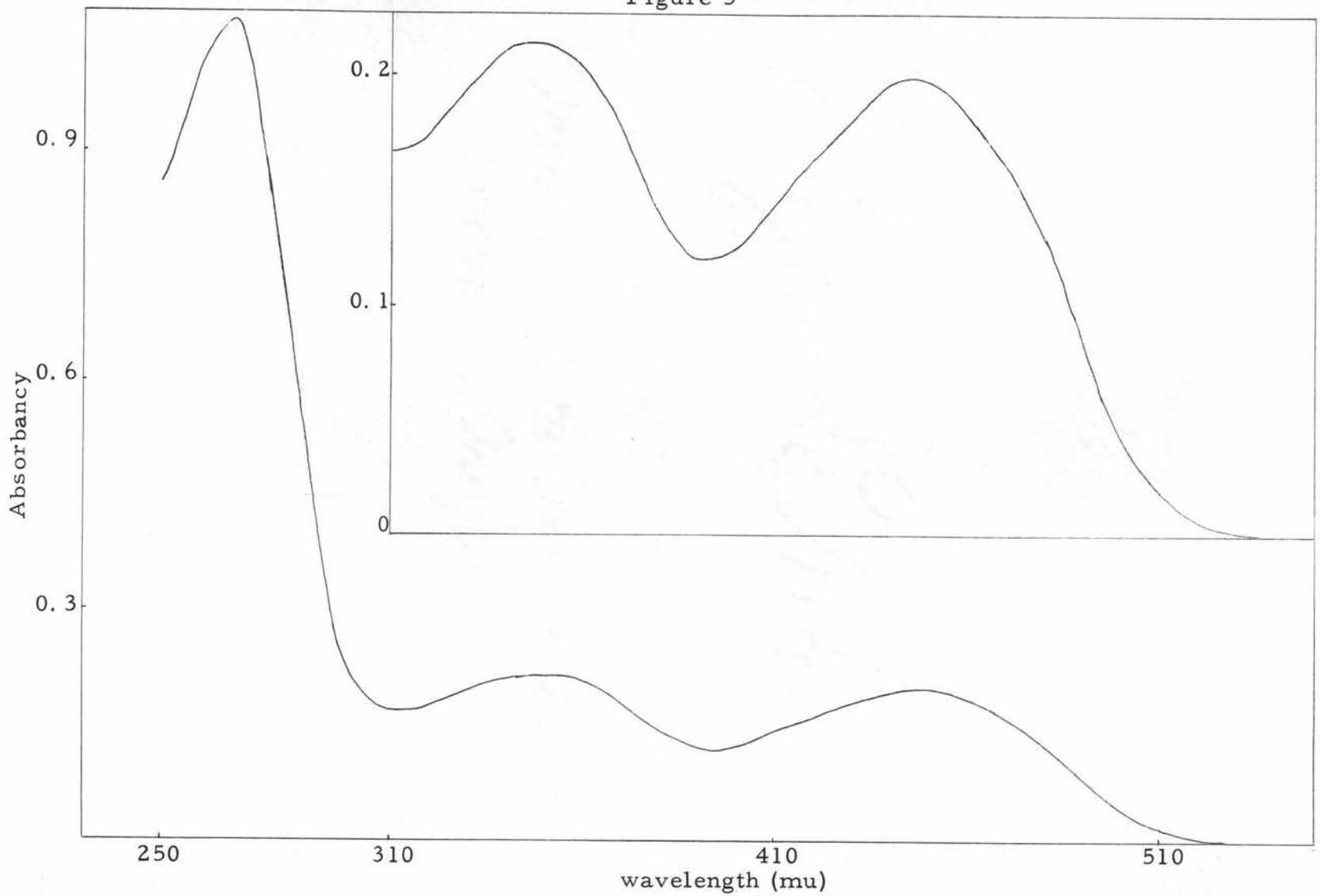
In the determination of acid nonextractable flavin, Singer et al. (53) recommended washing the trichloroacetic acid precipitate with acid-acetone prior to proteolysis to "remove the type of cytochrome apt to interfere with the spectrophotometric determination." This wash would dissociate and then extract the heme groups of cytochrome oxidase and cytochrome b (cf. 47, 48) but would not affect those heme groups covalently bound to proteins such as cytochromes c and c_1. Indeed, it was found that acid-acetone wash did not cause any significant change in the spectra of the proteolytic digest. This observation again indicates that the heme contamination was from cytochromes c and c_1 but not from cytochrome b or cytochrome oxidase.

Characteristics of the Flavin

The flavin peptide purified by Sephadex, florisil, and paper chromatography exhibited spectral properties similar to those reported by Wang et al. (68, 69) and Kearney (22). Its absorption maxima were found to be at 447, 347, and 267 mu and minima at 392 and 310 mu (cf. Fig. 5). The absorbancy ratios for 260 mu/450 mu and 350 mu/450 mu were 5.1 and 1.06 respectively. It should be pointed out that the purification of the flavin peptide could not be achieved by paper chromatography without prior purification with Sephadex and florisil chromatography. In the 5% Na_2HPO_4 solvent system for paper chromatography, the flavin peptide moved with an R_f value of 0.7; and traces of fluorescence were sometimes observed for components with R_f values of 0.46 and 0.36.

Fig. 5. The absorption spectrum (oxidized) of the flavin standard in water. The flavin concentration was 17.5 uM. The insert is an enlarged scale.

Figure 5



Authentic FMN and FAD samples added to the sample exhibited R_f values of 0.46 and 0.36 respectively. The flavin peptide of succinate-PMS reductase, FAD, and FMN have been reported (22) to have R_f values of 0.81, 0.50 and 0.39 respectively in this solvent system.

The fluorescence of the hydrolyzed flavin peptide was dependent on the pH of the medium as shown in Fig. 6. Its maximal fluorescent intensity was at pH 3.2 to 3.5, and the molar fluorescent intensity in this pH range was identical with that of FMN. The fluorescence of the hydrolyzed flavin peptide at pH 7.4 was less than 4% of the maximum.

The effect of fluorescent wave length on the fluorescent intensity of the hydrolyzed flavin peptide at a constant excitation energy was compared with that of FMN as shown in Fig. 7. The wave length showing the maximal fluorescence was 520 mu for both FMN and the hydrolyzed flavin peptide. The excitation maxima of the hydrolyzed flavin peptide were likewise compared with those of FMN. The results are summarized in Table 2. The data for FMN reported by Duggan *et al.* (10), which are corrected for the variation of the energy output of the xenon light source at different wave lengths, are also included in the table. It can be seen that the hydrolyzed flavin peptide differs slightly with free FMN.

Neither the isolated heme compound from Sephadex column (Peak I) nor the proteolytic digest of cytochrome *c* showed significant fluorescence at pH 3.2-3.5, with an excitation wave length of 450 mu and fluorescent wave length at 520 mu. Under these conditions, it was found that the heme component of Peak I contributed

Fig. 6. The effect of pH on the fluorescence of the hydrolyzed flavin peptide. The pH values were taken immediately after the fluorescent intensity was measured. The flavin concentration was approximately 5×10^{-2} uM in 30 mM citrate buffer.

● : Hydrolyzed flavin standard

▫ : Hydrolyzed proteolytic digest

○ : FMN

△ : Hydrolyzed acid extractable flavins

Fig. 7. The fluorescence spectra of FMN and the hydrolyzed flavin standard. The ordinate is the fluorescent intensity in arbitrary units. Corning filter No. 3-73 was used as a secondary filter. The excitation wave lengths used were 355 mu and 375 mu, respectively without filter, for the flavin peptide (7.4×10^{-2} uM) and FMN (6.0×10^{-2} uM) in 30 mM citrate buffer, pH 3.25. No corrections were made for variation of the photomultiplier tube sensitivity with wave length. The intensity readings for these two samples cannot be quantitatively compared because of different instrument settings.

Figure 6

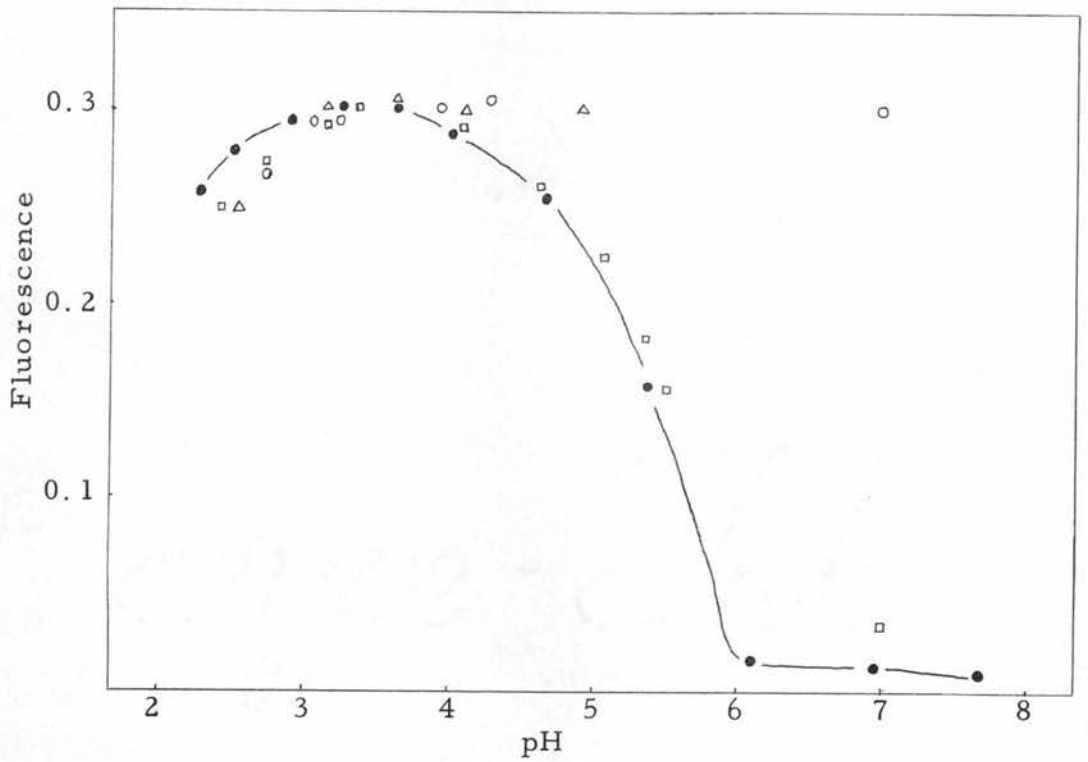


Figure 7

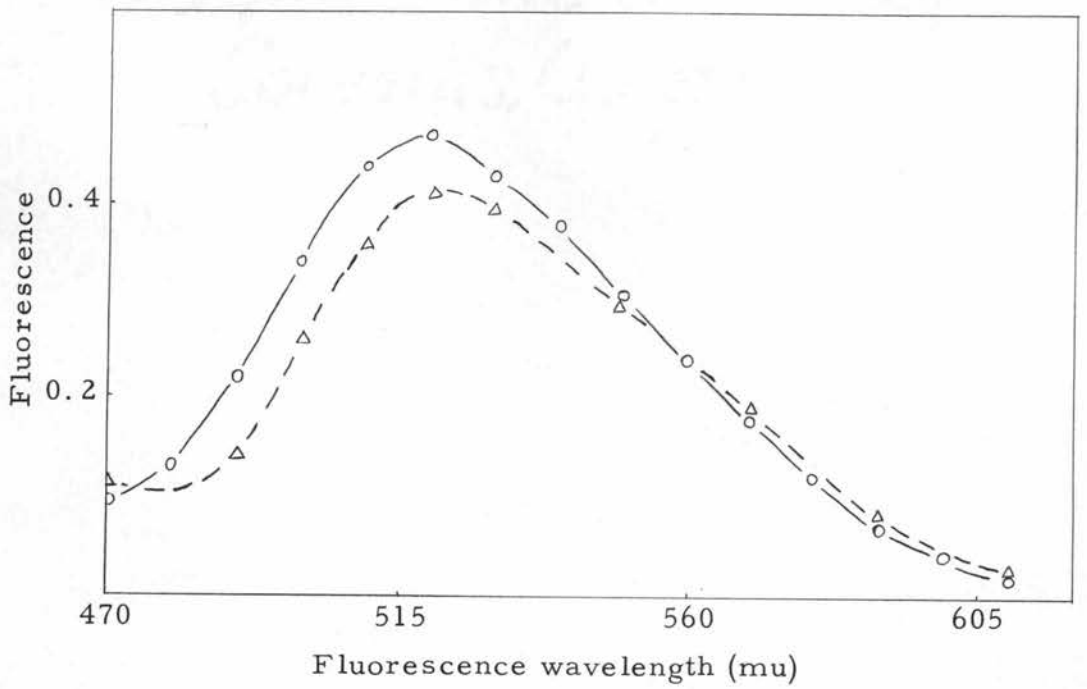


TABLE 2

The excitation maxima of the hydrolyzed flavin peptide and FMN.

The fluorescence was measured at 520 mu with a Farrand spectrophotometer equipped with an IP21 photomultiplier. Both the flavin standard and FMN were 0.3 uM in 30 mM citrate buffer, pH 3.24. A Corning filter No. 7-54 was used for excitation wave lengths near 350 mu. The fluorescence light was filtered through Corning filter No. 3-72 or 3-73. No corrections were made for variations of the energy output of the xenon light source with wave length. The values of Duggan et al. (10) are for FMN in water and are corrected for the energy difference of the light source.

Hydrolyzed acid nonextractable flavin	FMN	FMN (Duggan et al.)
mu	mu	mu
447-452	450-460	445
355-360	370-375	370

between 2-4% to the flavin concentration of the proteolytic digest prior to the chromatographic separation. This contribution was evidently due to incomplete separation of the heme and the flavin peptides because the fluorescence of the heme fraction (a) was also maximal at pH 3.2-3.5, (b) disappeared on the addition of dithionite, and (c) showed a fluorescent maximum at 520 mu and an excitation maximum at 450 mu. The fluorescence of the hemepeptide of cytochrome c under these conditions could be detected only at concentrations equivalent to 10 uM, or higher, of cytochrome c. In the actual assay system for the heart muscle preparation, the equivalent concentrations for cytochromes c plus c_1 was less than 0.2 uM.

A Fluorometric Method for the Determination of Acid Nonextractable Flavin in the Heart Muscle Preparation

With the cognizance of the properties of the flavin peptide, a method based on its fluorescent behavior was devised and described in the Experimental Procedures. This method was also used for direct comparison with the spectrophotometric method.

A number of batches of the heart muscle preparation were analyzed for the acid nonextractable flavin by both the fluorometric and spectrophotometric methods. The results are summarized in Table 3. No difference was observed in the acid nonextractable flavin content of the heart muscle preparations as determined by these two methods. It is unlikely therefore that the heme component interferes significantly with the spectrophotometric method.

TABLE 3

A comparison of the acid nonextractable flavin content of the heart muscle preparation determined by the spectrophotometric and fluorometric methods.

The determination was made directly in the proteolytic digest. Each sample was assayed in duplicate.

Heart muscle preparation number	Spectrophotometric method	Fluorometric method	Spectrophotometric / Fluorometric
	umoles per g protein		
332	0.180	0.170	1.06
333	0.183	0.176	1.04
336	0.166	0.179	.95
338	0.154	0.163	.95
Mean	0.171	0.172	1.00

The acid nonextractable flavin in the heart muscle preparation was found to average 0.15 (Table 1), and 0.17 (Table 3) umole per g protein in two different sets of experiments at different times of the year. Previously a mean value of 0.12 umoles per g of protein was reported (41). The reason for the variation is not known. However, preliminary experiments showed that those batches of the heart muscle preparation with higher acid nonextractable flavin content exhibited proportionately higher Q_{O_2} values for succinate oxidation. The turnover number, computed from per mole of acid nonextractable flavin, was found to be always approximately $7.5\text{-}8.1 \times 10^3$ moles succinate oxidized per minute at 37° in the presence of 20 uM of cytochrome c.

DISCUSSION

The close agreement between the spectrophotometric and the fluorometric assay methods coupled with the evidence for the non-interference of the heme contaminants in these systems indicates the validity for both methods, at least in the assay of the material used. The absolute value is, however, dependent on the reliability of the molar absorbancy indices applied to the acid nonextractable flavin. The values used here are assumed to be identical with the indices for FMN and FAD and may be in error. However, this error is not likely to be large in view of the similarity of both the absorption and fluorescence spectra of the acid nonextractable flavin on the one hand and FAD and FMN on the other.

A priori considerations make the fluorometric method seem more useful than the spectrophotometric method for routine assays. The sensitivity of the fluorometric assay is more than a hundredfold greater than the spectrophotometric method. The fluorometric method should be more specific for those samples with very high contents of hemeproteins, since the flavins have excitation and fluorescence maxima at longer wave length and have a greater fluorescence efficiency than most other biological compounds (cf. also 10).

Although the characteristics of the acid nonextractable flavin as reported here are in accord with those reported by Kearney (22), the data of Strom and Cerletti (59) disagree on two important points: (a) they report that the succinate dehydrogenase flavin in the mononucleotide form is only 85% as fluorescent as riboflavin under their assay conditions (0.03 M phosphate, 0.1 M KCl) and (b) the maximum fluorescence of the hydrolyzed flavin peptide was exhibited only at pH 2.6-2.8 in 0.02 M phosphate plus 0.1 M KCl. These differences may be the result of the different assay conditions. However, it cannot be neglected that they have used a Klett fluorometer with glass filters instead of a monochromatic light source. These differences indicate a need for control of all details in the use of the fluorometric assay.

Singer et al. (53) have concluded on the basis of circumstantial evidence that in the mammalian heart the succinate dehydrogenase content may be calculated from bound (acid nonextractable) flavin analysis. This is consistent with our tentative identification of the

acid nonextractable flavin fraction of the heart mitochondria and the heart muscle preparation (41) and with the data of Cerletti and co-workers (6) for heart tissue. However, many old methods (e.g. (18)) may not measure the true flavin content of natural materials that contain peptide bound flavin.

SUMMARY

The spectrophotometric method for the determination of the acid nonextractable flavin in the proteolytic digest of the trichloroacetic acid precipitate from the heart muscle preparation has been examined for the interference of the degraded hemeproteins. The proteolytic digest was chromatographically separated into the heme and flavin fractions. Spectrophotometric determinations made on the separated fractions have indicated that the heme interference is negligible for the heart muscle preparation. The validity of this method has thus been demonstrated.

In the course of the proteolysis, cytochrome c_1 and c are evidently degraded to hemepeptides. The spectral behavior of the hemepeptides has been presented.

Based on the fluorescent characteristics of the acid nonextractable flavin, a fluorometric method has been devised. This method is approximately 100 times more sensitive than the spectrophotometric assay. Results from these two methods are in good agreement. It is suggested that the fluorometric method may be more suitable for samples containing high concentrations of pigments absorbing in the 450-530 mu region of the spectrum.

2. THE DISSOCIATION OF SUCCINATE DEHYDROGENASE FROM THE HEART MUSCLE PREPARATION BY ALKALINE TREATMENT

INTRODUCTION

The inactivation of the succinate oxidase activity of the Keilin-Hartree heart muscle preparation by pH 9.0 at room temperature was first observed by Keilin and Hartree (27) in 1940. When the pH of this preparation was adjusted to 7.4 after one hour of alkaline treatment, the particles were almost devoid of succinate dehydrogenase activity as measured by methylene blue but still retained 70% of the cytochrome oxidase activity of the original untreated preparation.

The mechanism of this inactivation became of great interest when Keilin and King (29, 30) succeeded in reconstituting an active succinate oxidase system from an alkali-treated preparation devoid of succinate oxidase activity and a soluble succinate dehydrogenase preparation. During reconstitution, the particulate preparation binds the unstable soluble dehydrogenase to form a stable particulate system. The reconstituted system has all the properties of the succinate oxidase of the original heart muscle preparation with regard to solubility, stability, and inhibitor specificity (29, 30, 39). An understanding of the nature of the two components necessarily depends on a knowledge of the fate of the succinate dehydrogenase which was inactivated by the alkaline treatment.

This part of the thesis presents the results of an investigation of the fate of the succinate dehydrogenase during alkaline inactivation

of the heart muscle preparation. The succinate dehydrogenase, as measured by its flavin coenzyme, is dissociated from its binding site on the particle. The rate of dissociation is equal to the rate of inactivation of the succinate oxidase activity.

EXPERIMENTAL PROCEDURE

Flavin determination - the acid nonextractable flavin was determined by the fluorometric method as given in Part 1.

Alkaline treatment I - See Methods Part 1.

Alkaline treatment II - a single batch of heart muscle preparation containing approximately 10 mg of protein per ml of 0.05 M phosphate-borate buffer was used for 4 to 5 alkaline treatments. At suitable time intervals 30 ml aliquots were transferred to a thermostatted beaker and the pH adjusted to $\pm$ .02 pH unit of the desired value with 2.5 M NaOH. The alkali was added from a microburet with the delivery tip immersed in the stirred sample in order to minimize localized pH and temperature variations. The temperature of the preparation was within 0.5^{o} of the incubation temperature while the pH was being adjusted.

Two No. 50 Spinco centrifuge tubes were filled with the alkaline suspension, a 5 ml portion was placed in a test tube, and the incubation of both portions continued. When each of the 30 ml aliquots had been similarly treated, the centrifuge tubes were placed in a centrifuge rotor which was at the incubation temperature and centrifuged for 30 minutes at 50,000 RPM in a Spinco Ultracentrifuge Model L. Six to seven minutes after the centrifuge was started the

portions in the test tubes were neutralized and 0.2 ml of 0.6 M sodium succinate was added to each. These uncentrifuged portions were then allowed to stand at room temperature for at least 10 minutes to "fully activate" and the succinate oxidase activities were measured. Aliquots of the clear supernatant liquid obtained from the centrifuged portions were assayed for acid nonextractable flavin. The time of alkaline treatment was the interval between the time the final pH was attained and 6-7 minutes after the centrifuge was started.

RESULTS

The solubilization of a significant fraction of the acid non-extractable flavin of the heart muscle preparation by alkaline treatment I has been reported in Part 1. It was noticed at that time that the fraction of the flavin solubilized was quite variable among treatments (24-45%) but was apparently independent of the time of incubation at 37° (Table 1). The time invariance of the amount of flavin solubilized was partly explicable because the shortest measured incubation time at 37° was 15 minutes and the succinate oxidase activity was already more than 90% inactivated (Table 4). The reason for the variability of the amount of flavin solubilized was not as obvious. In these experiments neutralization was used only to stabilize the succinate oxidase activity and the final pH was not accurately measured. It was suspected, therefore, that the amount of flavin in the supernatant liquid was partly dependent on the pH during centrifugation.

TABLE 4

The effect of the time of treatment of alkaline treatment I on the enzymatic activity of the heart muscle preparation.

The enzymatic activities were measured by Warburg manometry at 37° and the activities of the untreated preparation in terms of umoles substrate oxidized/min/mg protein were: succinate oxidase, 0.72; succinate-PMS reductase, 1.35; cytochrome c oxidase, 4.0.

time of treatment	succinate oxidase	succinate PMS reductase	cytochrome oxidase
	percent of initial activity remaining		
2 minutes	40	45	60
15 minutes	4	27	67
40 minutes	0	7	56
70 minutes	0	3	61

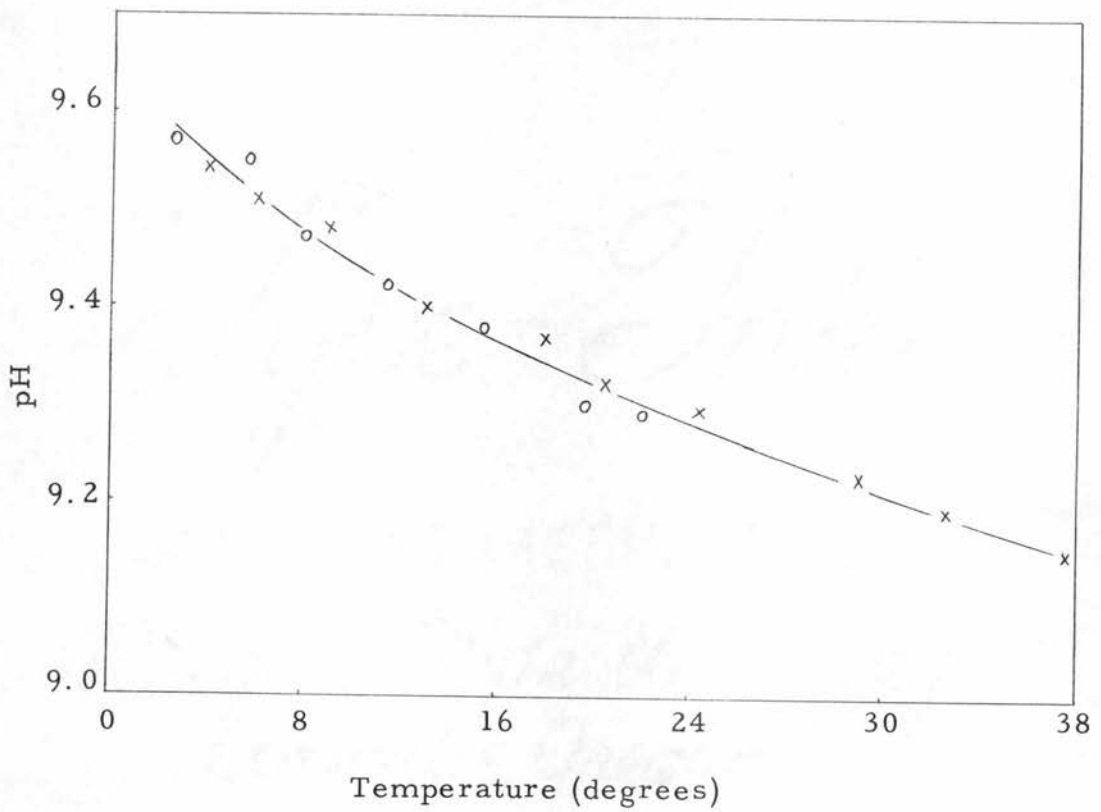
In preliminary experiments for which the medium from alkaline treatment I was not neutralized prior to centrifugation, it was found that more than one-half of the acid nonextractable flavin remained in the supernatant fraction. This observations suggested that if the alkaline treatment were carried out under conditions where the rate of solubilization were measurable, it would be possible to compare the rates of solubilization of the flavin with the rate of inactivation of the succinate oxidase.

The Temperature Dependence of the pH of a Heart Muscle Preparation under Alkaline Conditions. Under the conditions for alkaline treatment I, the incubation temperature (37^{o}) is much higher than the temperature at which the pH is measured ($0\text{-}4^{o}$). The pH of the alkaline heart muscle preparation varied with temperature as seen in Fig. 8. This behavior is approximately that which is expected for a borate buffer.

A Comparison of the Rate of Inactivation of the Succinate Oxidase Activity and the Rate of Dissociation of the Acid Non-extractable Flavin. The alkaline treatment was modified to allow a more precise control of the temperature and pH (alkaline treatment II of Methods). Aliquots of a single batch of heart muscle preparation were used for each of four or five alkaline treatments with a final pH of $\pm$ .02 pH unit of the same value and the same incubation temperature, but with different incubation times. Each of the treated aliquots of heart muscle preparation was divided into two portions. One portion was centrifuged at the incubation temperature and the other portion was neutralized 6 to 7 minutes after the

Fig. 8. The variation of pH with temperature of the heart muscle preparation under alkaline conditions. A heart muscle preparation diluted with an equal volume of water was adjusted to pH 9.29 at 23°C with 2.5 N NaOH, and the pH measured during cooling and rewarming. The abscissa is the temperature at the time the pH was determined. The pH was determined using a Beckman zeromatic pH meter with manual temperature compensation.

Figure 8



centrifuge was started. The time interval before neutralization was assumed to equal the time required for the particles to be sufficiently well sedimented in the centrifuge to end the transfer of the dissociated dehydrogenase from the residue to the supernatant liquid. The supernatant liquid from the centrifugation was used to determine the dissociated flavin and the neutralized portion was used to determine the remaining succinate oxidase activity.

The data from two experiments are given in Table 5, and Table 6 summarizes the data from five experiments. Both the rate of dissociation of the succinate dehydrogenase protein and the rate of inactivation of the succinate oxidase activity were zero order. The rates are therefore expressed as the time in minutes required to inactivate 20% of the initial succinate oxidase activity or to solubilize 20% of the total acid nonextractable flavin respectively. As may be seen from Table 6, the rates for the two processes are equal within experimental error.

Only in the case of heart muscle preparation batch number 324 did the data for the processes coincide. In the other three batches an initial decrease in the succinate oxidase activity occurred which was greater than could be accounted for by the dissociation of the succinate dehydrogenase (c.f. Table 5). This initial decrease in the succinate oxidase activity was 12%, 20% and 10% for batches number 321, 331 and 344 respectively.

As mentioned in Methods under alkaline treatment II, all of the samples assayed for succinate oxidase activity by oxygraph were preincubated for at least 10 minutes at room temperature with

TABLE 5

The effect of the time of incubation during alkaline treatment II on the succinate oxidase activity and the acid nonextractable flavin.

Heart muscle preparation batch number 321 was incubated at 16° and batch number 324 at 24°. Both the enzymatic activity and the acid nonextractable flavin are based on the protein content of the original heart muscle preparations.

Batch number	pH	Minutes of treatment	Succinate oxidase activity ul O_2 per hr per mg protein	Acid nonextractable flavin in the supernatant liquid umoles per g protein
321	---	---	540	.188*
321 a)	9.35	50	323	.053
321 b)	9.35	38	329	.045
321 c)	9.34	27	362	.043
321 d)	9.35	17	370	.038
324	---	---	490	.136*
324 a)	9.20	47	187	.087
324 b)	9.21	35	212	.074
324 c)	9.20	25	246	.071
324 d)	9.20	15	278	.050

* The acid nonextractable flavin content of the untreated samples are the total acid nonextractable flavin content of the heart muscle preparation. Prior to alkaline treatment less than 2% of the flavin is in the supernatant liquid.

TABLE 6

A comparison of the rates of succinate oxidase inactivation and the acid nonextractable flavin solubilization during alkali treatment II.

Batch number	pH	Incubation temperature	Rate* of inactivation of the succinate oxidase activity	Rate* of flavin solubilization
321	9.35	15.8°	82 min	88 min
324	9.45	16.0	62 "	62 "
324	9.20	23.8	27 "	27 "
331	9.15	24.0	54 "	60 "
344	9.11	23.8	59 "	63 "

* The rates are expressed as the time required for 20% inactivation or solubilization of the control value for succinate oxidase inactivation and acid nonextractable flavin solubilization respectively.

20 mM succinate in order to "fully activate" the enzyme. It was observed that under these conditions both untreated heart muscle preparation and heart muscle preparation which had been partially inactivated by alkaline treatment increased in specific activity. In addition, the lag in the succinate oxidase activity which was observed for the partially inactivated preparation was abolished. This activation by succinate was not investigated except to determine the conditions necessary for maximal activity.

DISCUSSION

The original observations of Keilin and King on the reconstitution of succinic oxidase have been verified in several laboratories (14, 32, 66). Although Singer and co-workers experienced some difficulty in getting reconstitution (51) and then in getting a good correlation between the amount of succinate dehydrogenase incorporated into the particle and the increase in succinate oxidase activity (52), in a more recent publication they have been able to resolve these difficulties (32). They have, however, maintained that it (the reconstitution) is a process of reactivation of the original dehydrogenase (32, 52). This conclusion was based on the observation that the succinate dehydrogenase flavin was in the residue fraction after centrifugation of the alkaline treated preparation.

King (36, 39) found that if the alkaline treatment is carried out at 0^{o} in the presence of succinate and the absence of air, the succinate dehydrogenase, as measured by its ability to reconstitute succinate oxidase activity, is actually dissociated from the particle

and becomes soluble. This solubilization is reversible and an equilibrium constant for the dissociation may be calculated (39).

Doubts that these observations are pertinent to the question of the fate of the succinate dehydrogenase during "normal" alkaline treatment (absence of succinate and presence of air) have been voiced by Massey (43) in view of the greatly differing conditions of the treatment. Singer et al. (32, 52, 54) reported that under the conditions of "normal" alkaline treatment, no succinate dehydrogenase, as measured by bound (acid nonextractable) flavin analysis, was dissociated from the particle "unless the inactivation is carried out at a much more alkaline pH than is required for complete inactivation of the dehydrogenase and oxidase in a relatively short time." In addition they found "it makes no difference in our experiments whether the alkali-treated particles are centrifuged before or after neutralization." This is not consistant with the data presented in this thesis and no explanation of the difference may be made.

The observation that the succinate dehydrogenase which is inactivated during alkaline treatment is dissociated from the particle shows conclusively that the original site on the particle is available for binding of the succinate dehydrogenase during reconstitution. In addition, the mechanism of inactivation of the succinate oxidase by alkali is intimately related to the dissociation of the dehydrogenase protein from the particle. Although the dissociation of the dehydrogenase protein is not necessarily the rate limiting step, it occurs at the same rate as the inactivation under the conditions employed. If the solubilization of the dehydrogenase is not rate limiting during

succinate oxidase inactivation by alkali it must follow and be very rapid compared to the actual rate limiting reaction.

SUMMARY

The succinate dehydrogenase protein, as measured by its flavin coenzyme, was found to be dissociated from the particulate heart muscle preparation by alkaline treatment. The dissociation occurred at the same rate as the inactivation of the succinate oxidase. It was therefore concluded that the original site of binding of the dehydrogenase to the particle is available to bind active succinate dehydrogenase during reconstitution of the system.

3. THE KINETICS OF THE ALKALINE INACTIVATION OF SUCCINATE OXIDASE

INTRODUCTION

The inactivation of succinate oxidase by alkaline treatment has been used to prepare a particle devoid of succinate oxidase activity that is capable of binding soluble succinate dehydrogenase to form an active succinate oxidase system (30). In Part 2 of this thesis it was shown that the alkaline inactivation proceeds at the same rate as the dissociation of the original succinate dehydrogenase from the particle in an inactive form. A kinetic study of the inactivation was therefore undertaken as a means of gaining information about the nature of the interaction between the dehydrogenase and the rest of the respiratory chain.

EXPERIMENTAL PROCEDURES

Alkaline treatment III. This treatment is similar to alkaline treatment II (Part 2). The heart muscle preparation at about 10 mg of protein per ml of 0.05 M phosphate-borate buffer and in the absence of substrate was brought to the incubation temperature and placed in a thermostatted beaker. The gently stirred preparation was then titrated to $\pm$ .02 pH unit of the desired value by adding 2.5 M NaOH. The NaOH was added from a microburet with the delivery tip immersed in the stirred sample in order to minimize localized pH and temperature variations. The incubation was carried out in a constant temperature water bath ($\pm$ .05°). At time intervals during

the incubation, 5 ml aliquots were rapidly transferred to chilled test tubes containing 0.2 ml of 0.6 M sodium succinate and sufficient H_3PO_4 to give a final pH between 7 and 8. They were then allowed to stand for at least ten minutes at room temperature. Any deviation in the method for a particular experiment is given in the legend of the appropriate table or figure.

RESULTS

When the alkaline treatment of the heart muscle preparation was carried out as given for alkaline treatment III and the succinate oxidase activity of the aliquots measured, the inactivation of the succinate oxidase proceeded as shown in Fig. 9. The activity of an aliquot removed before the addition of NaOH was used as 100%, and the time of treatment is the time interval for which the enzyme was at the alkaline pH. The reaction was zero order until the succinate oxidase was about 70% inactivated. The zero order kinetics were observed over certain pH and temperature ranges.

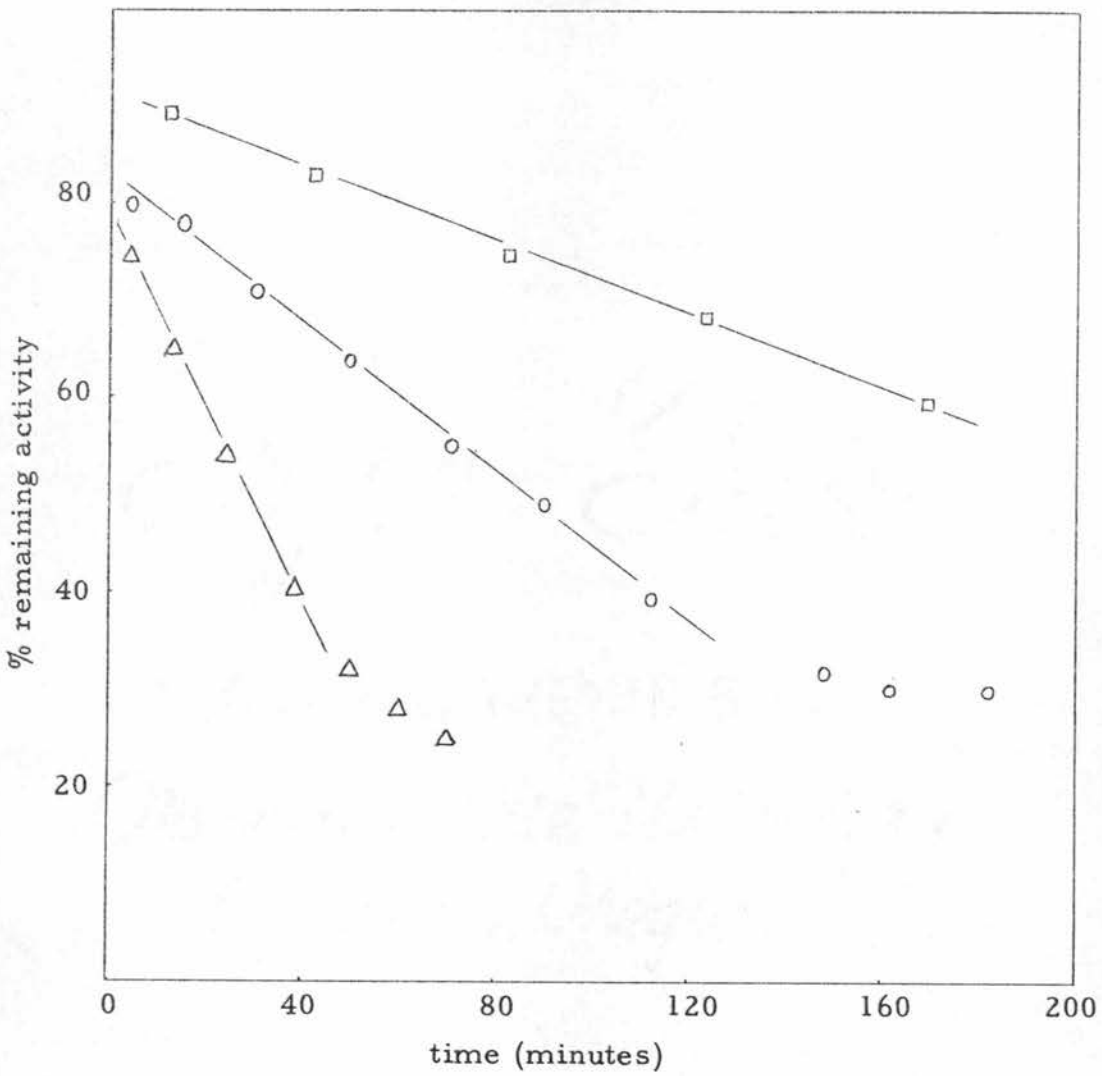
For convenience the rate of the zero order inactivation will be expressed as $t_{20\%}$, i.e. the time in minutes required for inactivation of 20% of the control succinate oxidase activity. This is a useful term for several reasons. It is an experimentally determined value and is independent of the initial succinate oxidase activity. It is also proportional to the reciprocal of the zero order rate constant for a single enzyme concentration.

Fig. 9. The time course of the alkaline inactivation of succinate oxidase during alkaline treatment III. The heart muscle preparation concentration was 10 mg protein per ml in 0.05 M phosphate-borate buffer. The control (100%) activity is the activity of an aliquot removed before the NaOH was added, and the zero time is the time at which the final pH was reached. The succinate oxidase activities of the aliquots were measured at room temperature with a GME oxygraph.

□-pH 9.02 and 22.0° ○-pH 9.17 and 22.0°

△-pH 9.40 and 22.0°

Figure 9


% remaining activity
80
60
40
20
0
40
80
120
160
200
time (minutes)

The Effect of Protein Concentration on the Rate of Inactivation. When the buffer concentration, pH and temperature were held constant for a series of alkaline treatments and the heart muscle preparation concentration varied from 4.3 to 17.5 mg per ml, no change was observed in the $t_{20\%}$ for the inactivation of the succinate oxidase activity as may be seen in Fig. 10. Since the initial succinate oxidase activity was proportional to the heart muscle preparation concentration, the amount of enzyme inactivated per unit time was proportional to the heart muscle preparation concentration. The rate of inactivation was therefore first order with respect to the initial succinate oxidase activity.

The Effect of Buffer Concentration on the Rate of Inactivation. A series of alkaline treatments were carried out for which the protein concentration, pH, and temperature were held constant and the molarity of the phosphate-borate buffer varied from 0.025 to 0.17. The $t_{20\%}$ for the inactivation of the succinate oxidase activity did not change (Fig. 11), showing that the rate of inactivation was independent of the buffer concentration over the range tested.

The Effect of pH on the Rate of Inactivation. When the temperature, protein concentration and buffer strength were held constant and the pH varied, the negative logarithm of the $t_{20\%}$ of the inactivation was a linear function of the pH as shown in Fig. 12. The slope of the straight line is about 2, indicating that the rate of inactivation was proportional to the square of either the hydroxyl ion concentration or the reciprocal of the hydrogen ion concentration. In a series of five experiments the slope of the curves varied from

Fig. 10. The effect of protein concentration on the rate of inactivation of succinate oxidase. The temperature was 23.5°, the buffer 0.05 M phosphate-borate and the pH 9.16 ± .02. The concentration of the heart muscle preparation is plotted on the abscissa as mg protein per ml of suspension and the $t_{20\%}$ for the inactivation is plotted on the ordinate. O and △ represent two different heart muscle preparations.

Fig. 11. The effect of buffer concentration on the rate of inactivation of succinate oxidase. During each treatment the protein concentration was 9.5 mg ml^{-1}, the pH was 9.16 ± .02 and the temperature was 23.5°. The molarity of the phosphate-borate buffer is plotted on the abscissa and the $t_{20\%}$ for the inactivation plotted on the ordinate. O and △ represent two different batches of heart muscle preparation.

Figure 10

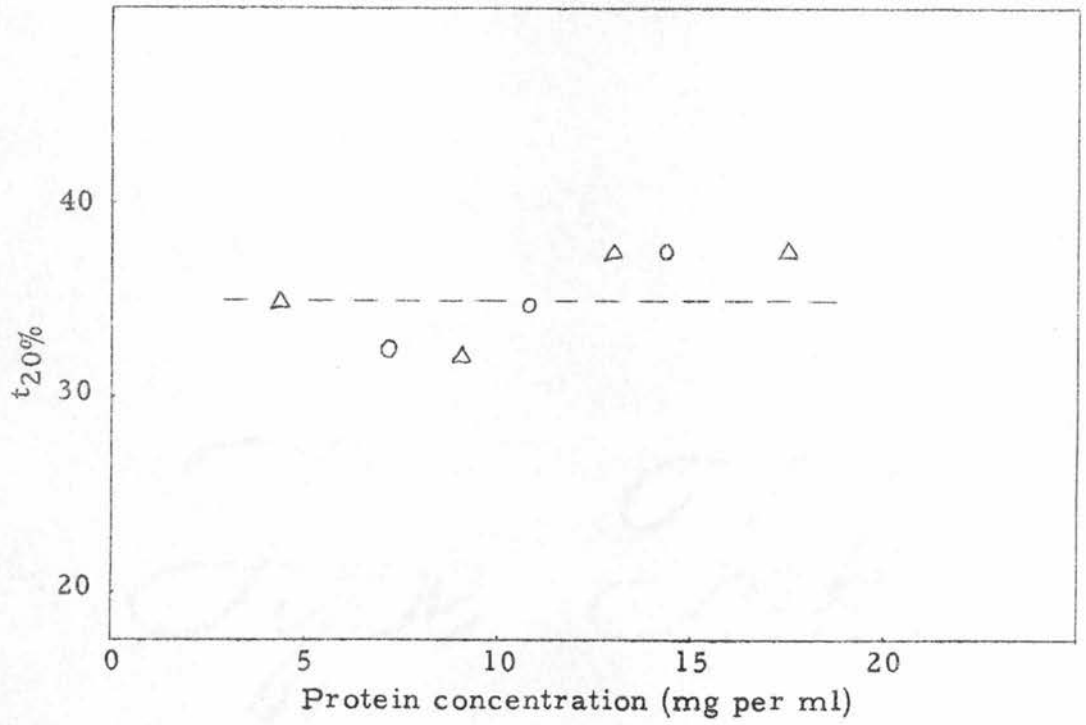


Figure 11

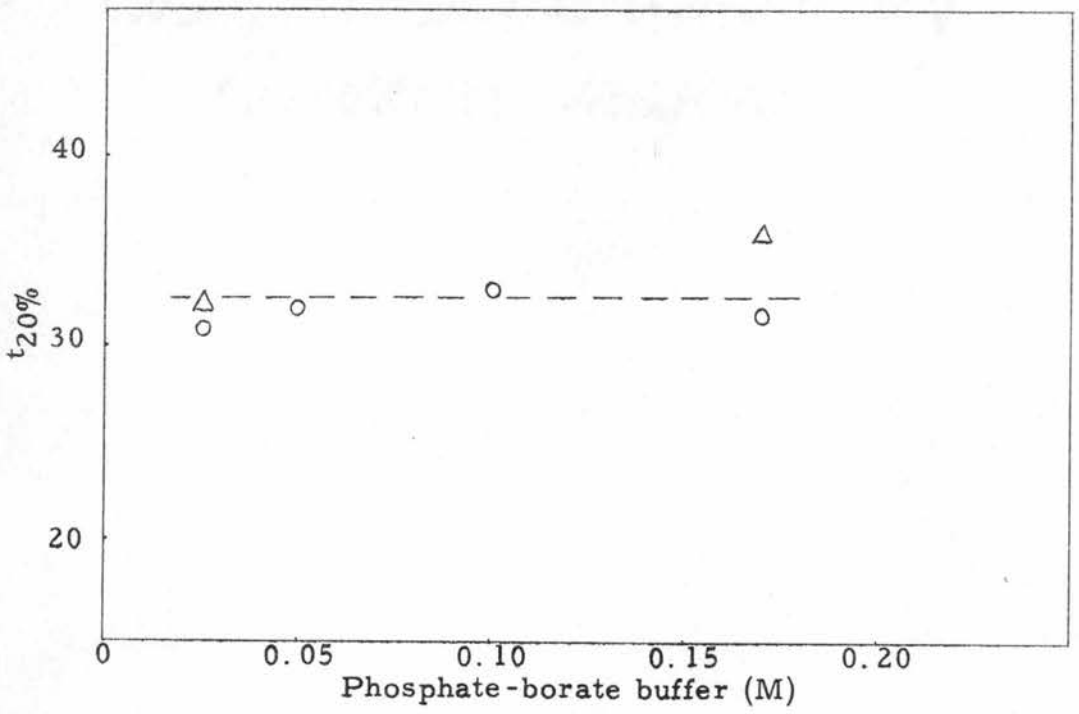
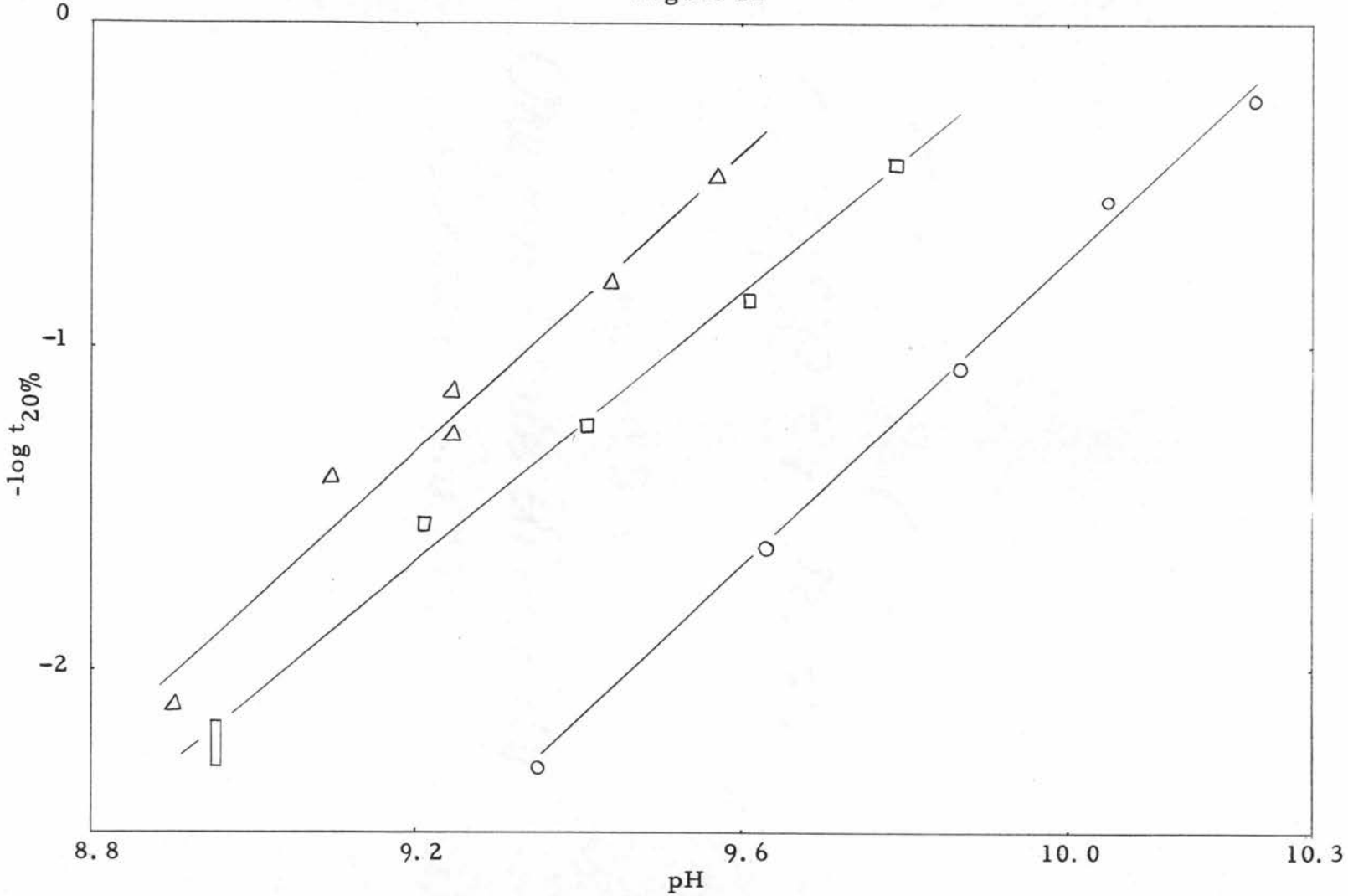


Fig. 12. The effect of pH on the rate of inactivation of succinate oxidase. The heart muscle preparations were 9.5 mg per ml in 0.05 M phosphate-borate buffer. The measured pH of the alkaline treatment is plotted on the abscissa and the negative logarithm of the $t_{20\%}$ for the inactivation is plotted on the ordinate. O -8.7° □ - 21.8° Δ - 25.0°.

Figure 12



1.9 to 2.4 with an average of 2.2. The $t_{20\%}$ for the inactivation was somewhat variable among batches of heart muscle preparation. A single batch was therefore used for each experiment.

The Temperature Dependence of the Rate of Inactivation at Constant pH. When the rate of inactivation was measured at constant pH and the temperature varied over a narrow range (10^{o}) the Arrhenius plots were linear within experimental error and apparent Arrhenius activation energies were determined. When larger temperature ranges were used the plots showed positive curvature, indicating that the apparent activation energy increases with temperature. Quantitative evaluation of the curvature was not achieved. Table 7 summarizes the observed activation energies, the pH for which they were determined, and the mean of the temperature range used for the Arrhenius plot.

The Temperature Dependence of the Rate of Inactivation at Constant pOH. When the pH is held constant and the temperature is varied, the hydroxyl ion concentration is a temperature dependent variable. The apparent activation energies at constant pH may be related to those at constant pOH by the temperature dependence of the ion product of water. If the rate of inactivation is second order with respect to the OH^{-} ion concentration, then the apparent activation energies at constant pH will be 26 k cal greater than those at constant pOH.

This decrease in the apparent activation energy allowed a much wider temperature range to be used for the Arrhenius plots at constant pOH than could be used at constant pH (Fig. 13). The

TABLE 7

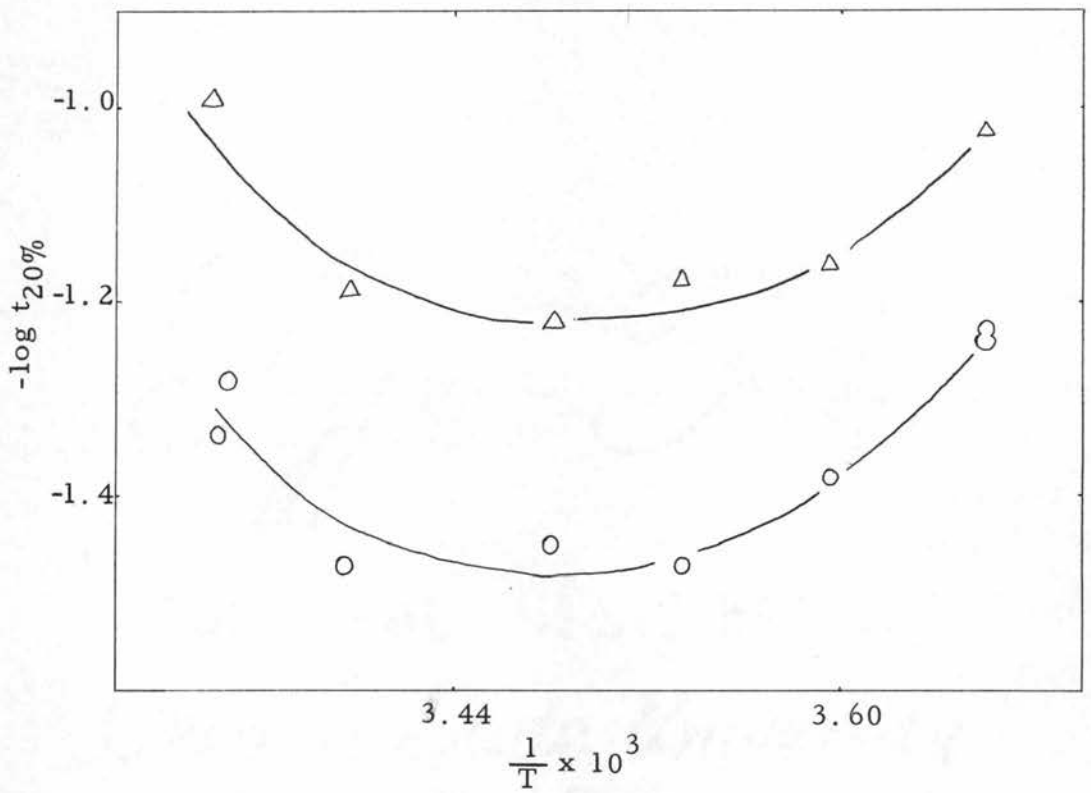
The effect of pH on the apparent Arrhenius activation energy for the alkaline inactivation of succinate oxidase.

The protein concentration was 10 mg per ml in 0.05 M phosphate-borate buffer. The apparent activation energies were determined for a series of inactivations at constant pH. For each plot the temperature range was 8-10°. Listed are the pH during the inactivation, the apparent Arrhenius activation energy in K cal per mole and the mean of the temperature range of the Arrhenius plot.

pH	Apparent Arrhenius Energy in Kcal $mole^{-1}$	Mean temp.	pH	Apparent Arrhenius Energy in Kcal $mole^{-1}$	Mean temp.
8.79	76	30°	9.29	39	20°
8.89	60	29°	9.40	33	18°
8.99	48-54	28°	9.56	26	15°
9.09	45	26°	9.7	21	8°
9.19	41	23°	9.8	20	8°
9.20	38	24°	9.91	20	8°

Fig. 13. The temperature dependence of the rate of alkaline inactivation of succinate oxidase at constant pOH. The heart muscle preparation concentration was 9.5 mg protein per ml of 0.05 M phosphate-borate buffer. For each temperature the dissociation constant of water was used to calculate the required pH and the heart muscle preparation titrated to within $\pm$ .02 pH units of that value. O - pOH = 4.74 Δ - pOH = 4.90

Figure 13



rate of inactivation at constant pOH had a minimum at 12°-13° and the apparent activation energy was strongly temperature dependent, indicating that the reaction was complex.

The Effect of Preincubation at 0° and Alkaline pH on the Rate of Inactivation. A batch of heart muscle preparation was cooled to 0° and the pH of the stirred sample brought to the desired value by addition of 2.5 N NaOH. After standing for five minutes at 0° the sample was warmed rapidly to 24° and incubated at that temperature. All further treatment was as described in Methods. The time of treatment was measured from the time that the sample temperature reached the incubation temperature until the aliquot was neutralized. An aliquot removed before the NaOH was added was used to determine the control succinate oxidase activity.

As may be seen from Fig 14, the incubation at 0° and high pH changed the inactivation in two ways. First, the rate of inactivation was greatly increased and second, the order was changed from pseudo zero order to a complex order (i.e. the inactivation no longer had a unique order for the measured time interval).

The inactivation shown in Fig. 14 is that which would be expected for two simultaneously occurring reactions, one first order and the other zero order (Fig. 15). The half life for the first order reaction is 2.3 minutes at pH 9.51, 5.7 minutes at pH 9.37 and 15 minutes at pH 9.14.

Fig. 14. The inactivation of succinate oxidase after preincubation of the heart muscle preparation at 0° and alkaline pH. Samples of heart muscle preparation at 10 mg protein per ml of 0.05 M phosphate-borate buffer were made alkaline at 0°C and allowed to stand at that temperature for 5 minutes. The temperature was then raised to 24° and the pH again measured. The inactivation at 24° was followed by determining the succinate oxidase activity of aliquots removed and neutralized at the time intervals indicated. The time of treatment is the time interval for which the aliquot was alkaline and at 24°. The succinate oxidase activity of an aliquot removed before the NaOH was added was used as 100%.

O - pH 9.84 at 0° and pH 9.51 at 24°;

Δ - pH 9.72 at 0° and pH 9.37 at 24°;

$\square$ - pH 9.49 at 0° and pH 9.15 at 24°.

Figure 14

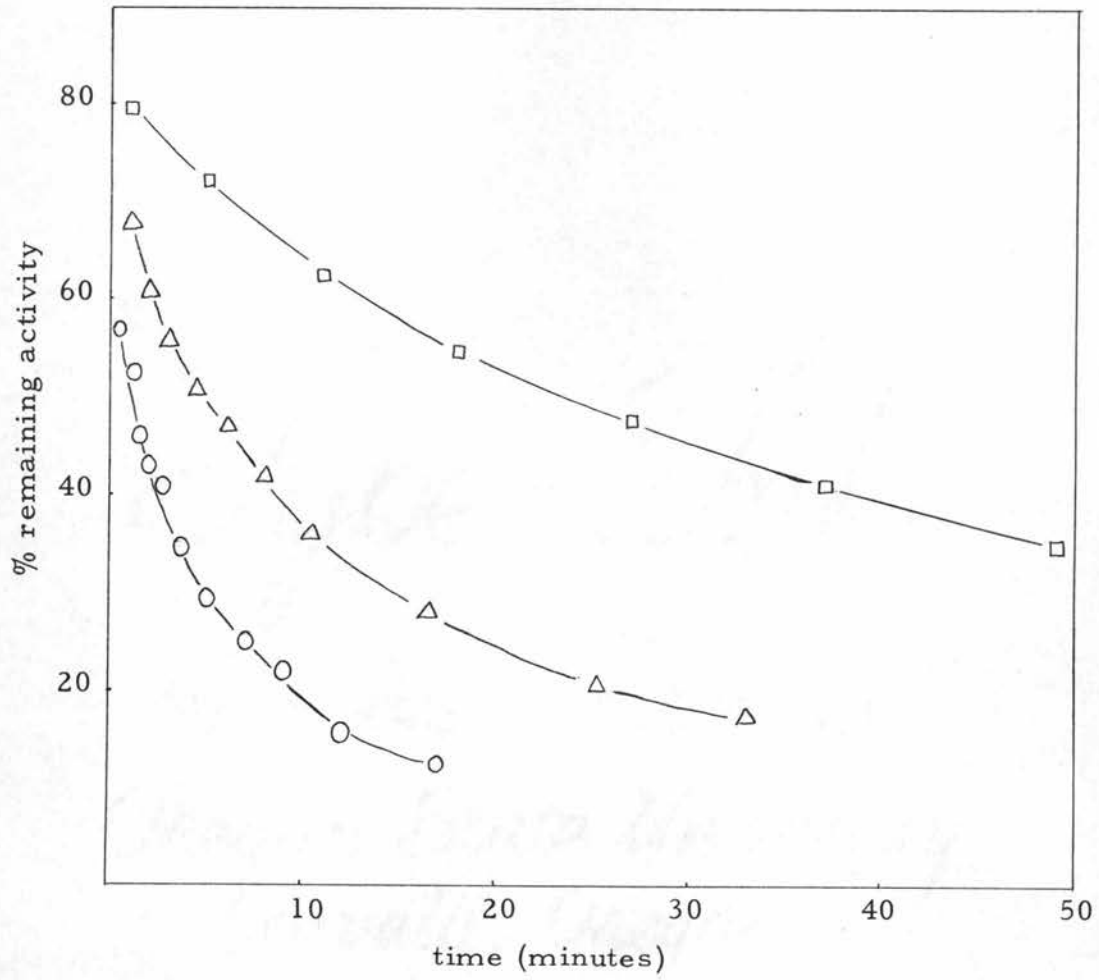


Fig. 15. The resolution of the rate of inactivation. The data is for the inactivation of heart muscle preparation at pH 9.51 and 24° after preincubation for 5 minutes at pH 9.84 and 0°.

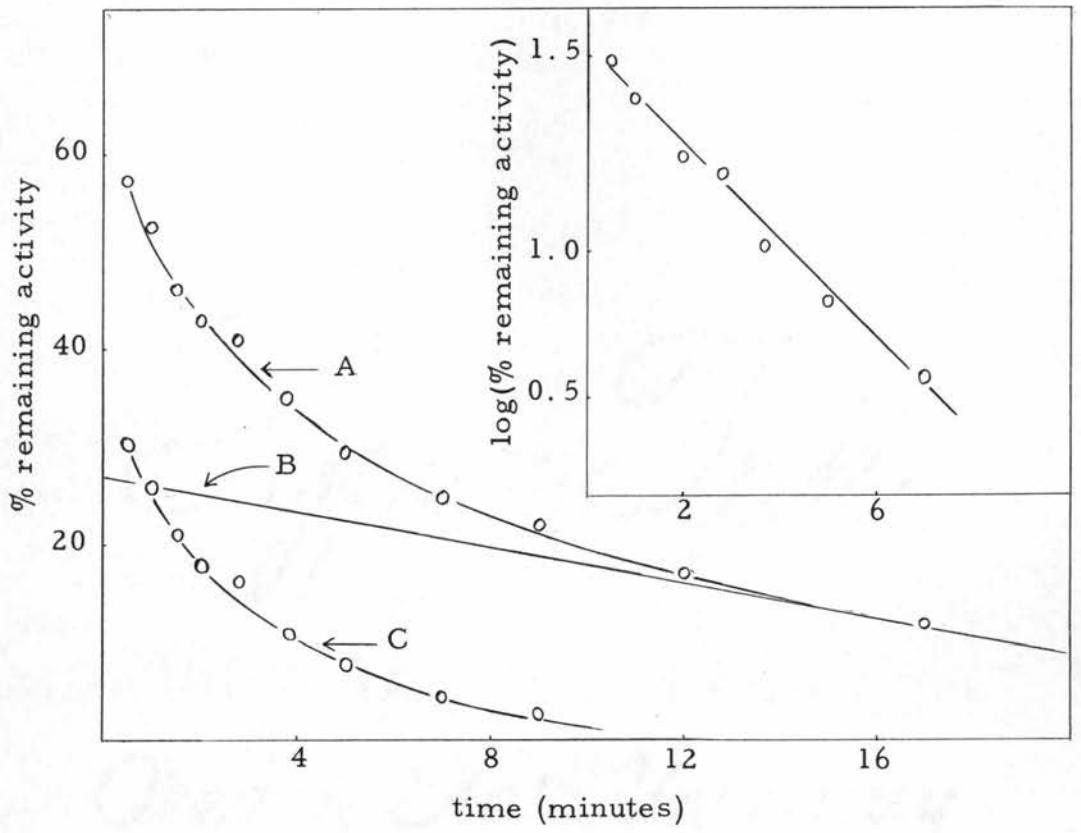
Curve A, the experimentally determined values;

Curve B, the estimated zero order reaction;

Curve C, the difference between the experimentally determined values and the estimated zero order reaction;

The insert is Curve C replotted as the log of the percent remaining activity against time. Curve B was estimated by a series of approximations designed to make Curve C most nearly first order.

Figure 15


% remaining activity
60
40
20
A
B
C
4
8
12
16
time (minutes)
log(% remaining activity)
1.5
1.0
0.5
2
6

AN INQUIRY INTO THE MECHANISM OF THE INACTIVATION

The rate of inactivation of the succinate oxidase may be expressed as

$$1. \quad \frac{-d\,[SO]}{dt} = C\,[SO]_i\,X^2$$

where C is a constant, $[SO]_i$ is the initial succinate oxidase activity and X^2 is either the square of the hydroxyl ion concentration, the square of the reciprocal of the hydrogen ion concentration, or the ratio of the two concentrations. The rate of inactivation of the succinate oxidase activity is equal to the rate of dissociation of the succinate dehydrogenase protein (Part 2), therefore

$$2. \quad V_d = C\,[SD]_i\,X^2$$

is the expression for the rate of dissociation of the dehydrogenase from the particle. $[SD]_i$ is the initial concentration of bound succinate dehydrogenase. Equation 2 is assumed to be true throughout the pH and temperature ranges for which the rate of inactivation was measured. The rate of dissociation was actually measured and compared to the rate of inactivation over a relatively narrow range (Part 2). In view of the consistant pH dependency of the rate of inactivation at constant temperature (Fig. 12) and the observation of King (30) and Kimura et al. (32) that relatively wide pH (9.0-9.7) and temperature (0-38°) ranges can be used to prepare alkali inactivated particles for reconstitution, this extrapolation of the dissociation data is not unreasonable.

The curvature of the Arrhenius plots at constant pH and constant pOH can be explained only if the constant (C) consists of two or

more rate constants. The form of equation 1 and the observed pseudo zero order rate of inactivation strongly suggest that the reaction is a "steady state" reaction. In addition this steady state reaction consists of at least two distinguishable parts. The first part is the reaction of the heart muscle preparation with hydroxyl ion and the second part is the actual inactivation reaction. A comparison of Figs. 9 and 14 suggests that the activation energy of the first step is less than that of the second since preincubation at alkaline pH and 0^{o} increased the rate of inactivation and changed the order of the reaction.

The following mechanism for the inactivation was designed by considering both the kinetic data and the data from the equilibrium dissociation of the succinate dehydrogenase presented in Part 4.

Reaction 1 $$S_b^2 + OH^- \underset{k\text{-}1}{\overset{k_1}{\rightleftharpoons}} S_b^1$$

" 2 $$S_b^1 + OH^- \underset{k\text{-}2}{\overset{k_2}{\rightleftharpoons}} S_b^o + OH^-$$

" 3 $$S_b^o \underset{k\text{-}3}{\overset{k_3}{\rightleftharpoons}} S_o^o + SD$$

" 4 $$S_b^1 + OH^- \underset{k\text{-}4}{\overset{k_4}{\rightleftharpoons}} S_o^o + OH^- + SD \text{ (inactive)}$$

" 5 $$SD \xrightarrow{k_5} SD \text{ (inactive)}$$

The heart muscle preparation with the succinate dehydrogenase bound to a specific site on the particle is represented by S_b^2. The succinate dehydrogenase is dissociated from the particle under

alkaline conditions by the reaction of a hydroxyl ion with the binding site either on the particle or the dehydrogenase. Only one hydroxyl ion is consumed in the dissociation but the reaction is catalysed by hydroxyl ion. For convenience the forms of the binding site are designated by superscript numbers and the subscripts "b" and "o" are used to indicate whether a succinate dehydrogenase molecule is bound to the site (S_b) or whether the site is open (S_o). Soluble succinate dehydrogenase is represented by SD.

The assumptions used during the evaluation of the mechanism are as follows:

A) Steady state conditions exist during the inactivation.

B) The rate constant k_5 is very large in the absence of succinate and presence of oxygen so that reaction 3 is effectively irreversible.

C) Reaction 2 is much slower than reaction 1 so that reaction 1 is near equilibrium.

D) The equilibrium for reaction 1 lies far to the left and under the experimental conditions $K_1 [OH^-] \ll 1$.

E) $k_{-2} [OH^-] \ll k_3$ under the experimental conditions.

The following equations may now be written:

3. $$\frac{-d [SO]}{dt} = k_3 [S_b^o] + k_4 [S_b^1] [OH^-]$$

4. $$K_1 = \frac{[S_b^1]}{[S_b^2] [OH^-]}$$

5. $$\frac{-d [S_b^o]}{dt} = 0 = k\text{-}2 [S_b^o] [OH^-] + k_3 [S_b^o] - k_2 [S_b^1] [OH^-]$$

From equation 4 and assumption C and D

$$6.\quad \left[S_b^1\right] = K_1 \left[OH^-\right] \left[S_b^2\right]_i$$

Where $\left[S_b^2\right]_i$ is equal to the initial concentration of S_b^2 or the total concentration of succinate dehydrogenase.

From equation 5, equation 7 is obtained

$$7.\quad \left[S_b^o\right] = \frac{k_2 \left[S_b^1\right]\left[OH^-\right]}{k\text{-}2 \left[OH^-\right] + k_3}$$

The rate of inactivation of the succinate oxidase may be expressed as a function of $\left[S_b^2\right]_i$ and $\left[OH^-\right]$ by substitution of $\left[S_b^1\right]$ from equation 6 into equation 7

$$8.\quad \left[S_b^o\right] = \frac{k_2 K_1 \left[S_b^2\right]_i \left[OH^-\right]^2}{k\text{-}2 \left[OH^-\right] + k_3},$$

and then the values of $\left[S_b^o\right]$ and $\left[S_b^1\right]$ from equations 6 and 8 into equation 4

$$9.\quad -\frac{d\left[SO\right]}{dt} = \frac{k_2 k_3 K_1 \left[S_b^2\right]_i \left[OH^-\right]^2}{k\text{-}2 \left[OH^-\right] + k_3} + k_7 K_1 \left[S_b^2\right]_i \left[OH^-\right]^2$$

This expression simplifies to (assumption E)

$$10.\quad -\frac{d\left[SO\right]}{dt} = \left\{k_2 + k_7\right\} K_1 \left[S_b^2\right] \left[OH^-\right]^2$$

This rate expression (eq 10) is of the same form as the experimentally determined rate expression (eq's 1 and 2). The mechanism is also consistant with the available data in the following points:

a) The dissociation of the succinate dehydrogenase protein occurs at the same rate as the inactivation of the succinate oxidase (Part 2).

b) The particle remaining after the dissociation of the dehydrogenase is reconstitutively active (29, 30).

c) Alkaline conditions are required for the dissociation of the dehydrogenase protein (36).

d) The apparent zero order rate constant contains the sum of two rate constants and makes possible the increase of the apparent activation energy with increasing temperature (Table 7 and Fig. 13) if k_4 has a much larger activation energy than k_2, but the rates are comparable. A brief discussion of this phenomenon may be found in (67). In addition, reaction 4 may be complex because it would be expected to be affected by structural changes in the dehydrogenase protein.

SUMMARY

The inactivation of succinate oxidase during the alkaline treatment III in the absence of succinate or any reducing agent was found to follow zero order kinetics. The apparent zero order rate constant varied as the first power of the initial enzyme concentration and the second power of the hydroxyl ion concentration. The temperature dependence of the apparent zero order rate constant was complex. A mechanism is presented which is consistant with the observed kinetics and thermodynamics.

4. THE REVERSIBLE DISSOCIATION OF SUCCINATE OXIDASE

INTRODUCTION

The electron transport system of heart mitochondria has been the subject of intensive investigation since 1925 (26). A great deal is now known about its composition and function, but very little is known about how the individual components are bound together to form the insoluble enzyme complex.

A powerful tool for the study of this important question became available when Keilin and King (30) reconstituted the succinate oxidase system from a soluble succinate dehydrogenase and a particle containing all of the components except an active dehydrogenase. King (36, 39) has more recently demonstrated the equilibrium dissociation of succinate dehydrogenase from the Keilin-Hartree heart muscle preparation under well defined conditions.

When the heart muscle preparation is treated with alkali, the succinate dehydrogenase protein is dissociated from the particle either reversibly (36, 39) or irreversibly (Part 2) depending on the conditions of the treatment. It is therefore of the utmost importance that the experimental conditions be considered carefully in the evaluation of the experimental data (cf. 52 vs. 36). In the present paper the experiments are carried out in the presence of succinate and the absence of oxygen. King (36, 39) has shown that under these conditions the dissociated succinate dehydrogenase is reconstitutively active and the dissociation is reversible.

It is the purpose of Part 4 to present a study of the equilibrium dissociation of the dehydrogenase.

EXPERIMENTAL PROCEDURE

Acid Nonextractable Flavin Assay. The acid nonextractable flavin content was determined by fluorometry.

Alkaline Treatment IV. Forty ml of the heart muscle preparation at a final concentration of 10 gm of protein per ml in 35 mM succinate and 50 mM phosphate-borate buffer was incubated for 10 minutes at the temperature for which the dissociation was to be measured. The pH of the suspension was then adjusted to the desired value by addition of 2.5 N NaOH from a microburet with the delivery tip immersed in the gently stirred sample. The sample was in a thermostatted beaker during the addition of the NaOH to keep the temperature of the sample within 0.5° of the incubation temperature. Care was taken at all times to minimize the diffusion of oxygen into the sample.

Immediately after the final pH was reached, two tubes for the No. 50 rotor of the Spinco Model L preparative ultracentrifuge were filled and sealed. For incubation the filled tubes were placed in a No. 50 Spinco rotor which was immersed in the constant temperature water bath.

During the usual experiment, three or four samples were titrated to different pH values during a 35 minute or less time interval. When the last of the filled tubes had been incubated for five minutes, they were centrifuged for 30 minutes at 50,000 RPM. At the end of the centrifugation the rotor temperature was within 2^{o} of the incubation temperature.

The volume of the clear, yellow-orange supernatant liquid was measured and the acid nonextractable flavin content assayed. The volume of the protein in the original heart muscle preparation was neglected and the acid nonextractable flavin content of the supernatant fraction was compared directly with the content of an equal volume of heart muscle preparation taken immediately after the pH was adjusted. The time of treatment was the interval between the time the final pH was achieved and six minutes after the centrifuge was started.

The succinate dehydrogenase in the supernatant liquid is referred to as dissociated or soluble.

RESULTS

The Effect of the Time of Incubation on the Fraction of the Acid Nonextractable Flavin in the Supernatant Liquid. A single batch of heart muscle preparation was used to measure the solubilized succinate dehydrogenase at a constant temperature and pH but with a variable time of incubation at the alkaline pH (Table 8). The fraction of the succinate dehydrogenase in the supernatant liquid was constant for the measured time interval (15-40 minutes). This

TABLE 8

The effect of the time of incubation during alkaline treatment IV on the fraction of the acid nonextractable flavin which was soluble.

The heart muscle preparations were two different batches of 10 mg of protein per ml in 50 mM phosphate-borate buffer and 35 mM sodium succinate.

Temperature	pH	Minutes of treatment	Percent of the acid nonextractable flavin in the supernatant liquid
16.4°	10.14	16	42
16.4°	10.18	24	44
16.4°	10.14	43	44
14.3°	10.01	17	33
14.3°	10.02	23	33
14.3°	10.01	39	31

is in agreement with the data of King (39) and is strong evidence for the existance of a true equilibrium which was attained in less than 15 minutes.

The experimental design was such that the test of reversibility could not be applied. The succinate oxidase activity of the preparation was used to maintain oxygen free conditions. This activity decreased very rapidly with increasing pH for pH values above 9. It was therefore essential that immediately after the final pH was attained the samples were transferred to centrifuge tubes and the tubes immediately sealed to exclude air. The entire operation from the start of the NaOH addition until the tubes were sealed required not more than 2.5 minutes. No way was found to exclude air and yet allow the incubation (15 min) and neutralization which were required to test reversibility without excessively modifying the procedure. It may be pointed out that the process of oxygen diffusion from air to the liquid phase is rather slow and was significant only over the longer time interval. For the purposes of this communication the system was assumed to be in equilibrium as long as the amount of solubilized flavin was independent of the time of incubation and the data was consistant with that reported by King (39) for the equilibrium at 0^{o}.

The Hydroxyl Ion Dependence of the Fraction of the Acid Nonextractable Flavin in the Supernatant Liquid. It has been previously reported (39) that at 0^{o} (the only temperature used) the fraction of the succinate dehydrogenase which was solubilized was a linear function of the hydroxyl ion concentration. In the present

study this was confirmed (Fig. 16). In addition it was found to be true for all temperatures from 0^{o} to 23.5^{o} for which the dissociation was measured.

The pH to which the plots remained linear depended on the batch of heart muscle preparation. Batches made from heart muscle which contained unusually high amounts of fatty tissue could not be used as the resulting plots were nonlinear and the data erratic. After the fat was removed by washing the preparation twice with phosphate-borate buffer, the plots were linear. Batches with high specific activities for succinate oxidation ($Q_{O_2} > 300$ at 24^{o}) gave the most consistent data.

The Temperature Dependence of the Equilibrium Constant for the Dissociation of the Acid Nonextractable Flavin. The ratio of the fraction of the flavin which was dissociated to the hydroxyl ion concentration was a constant (K_e) for any given temperature. This constant decreased with increasing temperature (Fig. 16). In Fig. 17 the logarithm of this constant (K_e) is plotted as a function of the reciprocal of the absolute temperature. The plot has a positive curvature but the points are somewhat scattered. The scatter may represent experimental error or variation among the batches of heart muscle preparation or both.

The equilibrium constant (K_e) reported by King (39) for 0^{o} is $3 \times 10^{4} M^{-1}$ and is two fold larger than the $1.5 \times 10^{4} M^{-1}$ reported here (Fig. 17). This difference is such that the fraction of the flavin solubilized at a given pH during the studies of King is equivalent to the fraction solubilized in the present study at

Fig. 16. The variation of the percent of acid nonextractable flavin solubilized with hydroxyl ion concentration and temperature. The heart muscle preparation was 11.5 mg protein per ml in 50 uM phosphate-borate buffer and 38 mM sodium succinate. Each mg of heart muscle preparation protein contained 0.17 mu moles of acid nonextractable flavin. Curve A, 0^{o}; Curve B, 5^{o}; Curve C, 10^{o}; Curve D, 23.5^{o}.

Fig. 17. The temperature dependence of the logarithm of the equilibrium constant (K_e) for the dissociation of the acid non-extractable flavin from the particle. The heart muscle preparations were 8-11 mg of protein per ml in 50 mM phosphate-borate buffer and 35 mM sodium succinate.

Figure 16

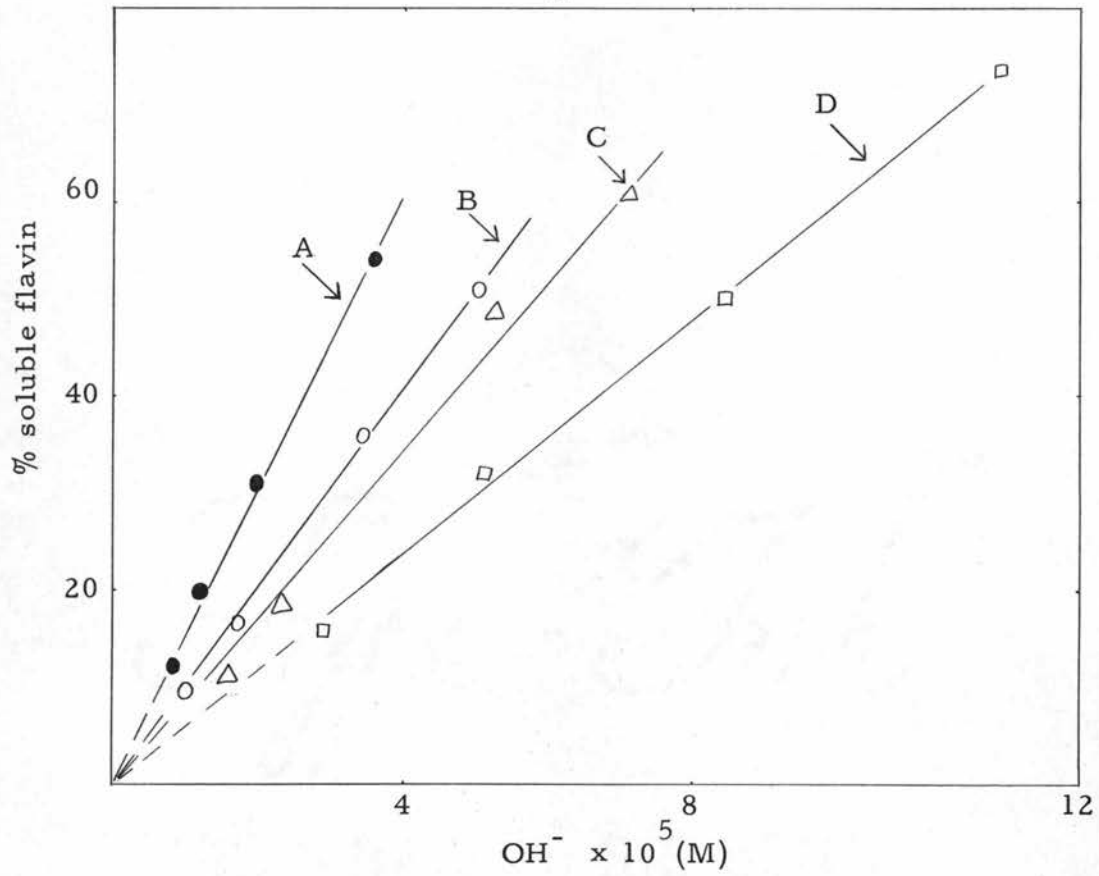
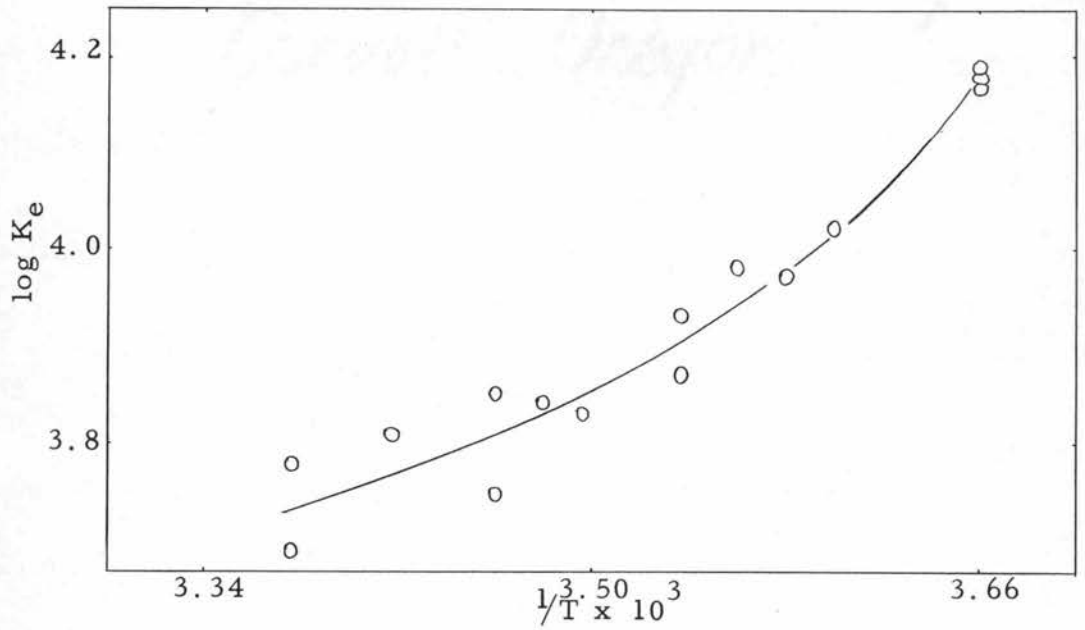


Figure 17



approximately 0.3 pH unit higher (pH 10.0 versus pH 10.3 etc.). This disparity, although comparately small, judging from the complicated operation, may be due to the difference in measurements; the present study used a pH meter with a higher precision.

The Effect of the Concentration of Heart Muscle Preparation on the Dissociation of the Acid Nonextractable Flavin. As shown in Fig. 18, the fraction of the succinate dehydrogenase which was soluble at a given hydroxyl ion concentration and temperature was independent of the concentration of the heart muscle preparation. Thus the concentration of the soluble succinate dehydrogenase was proportional to the total concentration of succinate dehydrogenase, bound and soluble. King (39) reported a very slight deviation from proportionality when a greater range of heart muscle preparation concentrations was used.

The Effect of the Buffer Concentration on the Equilibrium Constant for the Dissociation of the Acid Nonextractable Flavin. The dissociation of the flavin as a function of the hydroxyl ion concentration was measured at different concentrations of phosphate-borate buffer (Fig. 19). An increase in the concentration of phosphate-borate buffer from 29 mM to 160 mM decreased the fraction of the succinate dehydrogenase dissociated at a given pH and temperature by more than 20%.

Fig. 18. The effect of protein concentration on the equilibrium constant for the dissociation of the acid nonextractable flavin. The heart muscle preparation was suspended in 50 mM phosphate-borate buffer and 40 mM sodium succinate. O - 7 mg per ml; x - 10.5 mg per ml; △ - 14 mg per ml.

Fig. 19. The effect of the concentration of phosphate-borate buffer on the equilibrium constant for the dissociation of the acid nonextractable flavin. The heart muscle preparation was 10 mg protein per ml in 30 mM sodium succinate. The temperature was 14.3°. Curve A, 29 mM phosphate-borate; Curve B, 50 mM phosphate-borate; Curve C, 160 mM phosphate-borate.

Figure 18

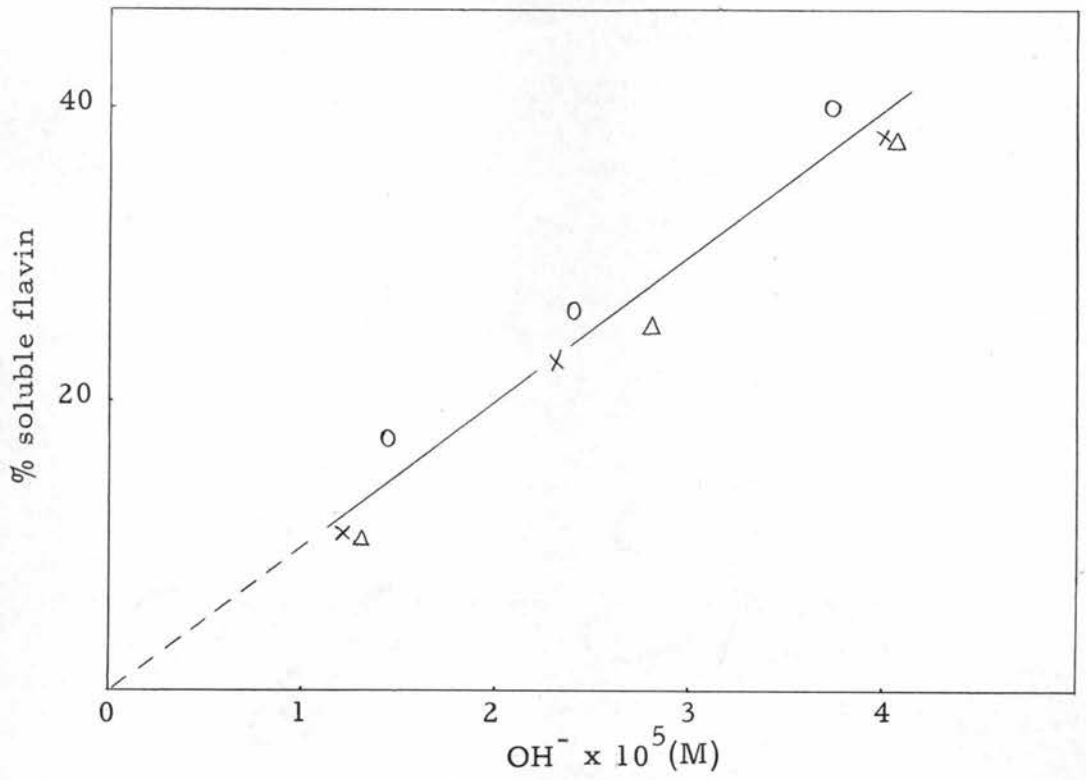
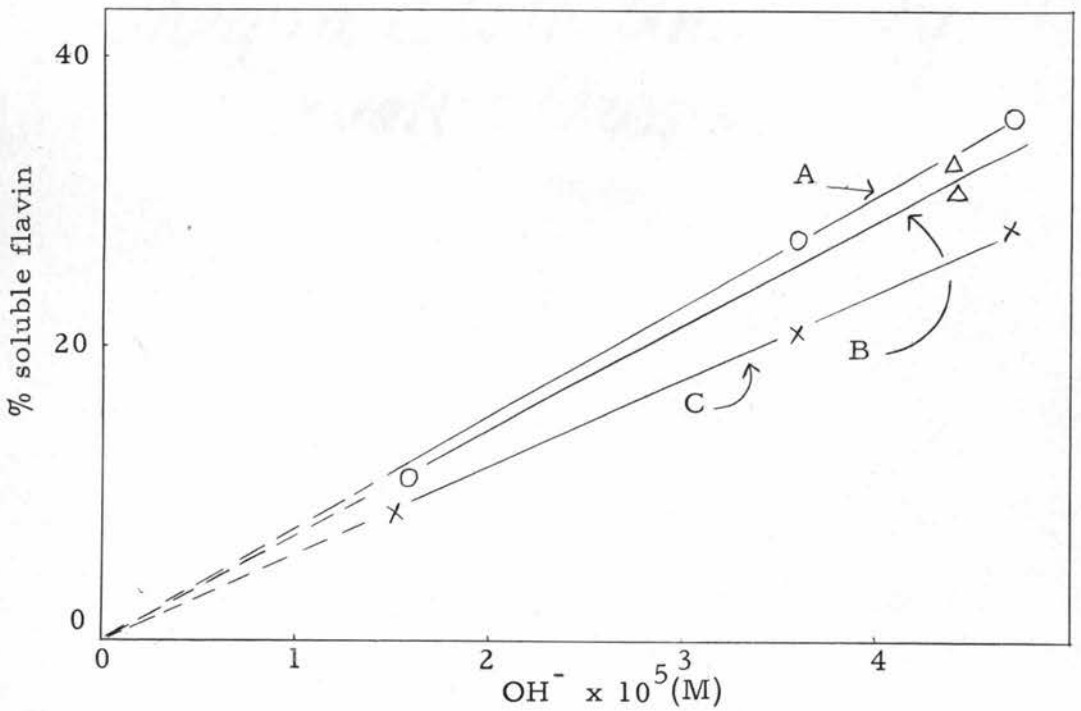


Figure 19



DISCUSSION

The equilibrium constant for the dissociation of the succinate dehydrogenase appears to have an unusual form. King (39) has previously expressed this equilibrium constant as the ratio of the fraction of the succinate dehydrogenase solubilized to the hydroxyl ion concentrations as shown in equation (1).

$$(1) \qquad K_e = \frac{[SD]}{[SD_t]} \cdot \frac{1}{[OH^-]}$$

where K_e represents the equilibrium constant and $[SD]$ and $[SD_t]$ represent the concentrations of the dissociated dehydrogenase and the total dehydrogenase respectively. This form is supported by the data presented in this paper. It is evident that the dissociation is not a simple process.

The alkaline treatment of the heart muscle preparation has been carried out under two different sets of conditions. When the treatment was in the absence of succinate and presence of oxygen, the succinate dehydrogenase was dissociated and irreversibly inactivated (27, 30, Part 2) but the particle remained reconstitutively active. This irreversible system was used to determine the kinetics of the inactivation of the succinate oxidase. In the presence of succinate and the absence of oxygen, however, the succinate dehydrogenase was reversibly dissociated from the particle (36, 39) and both the soluble dehydrogenase and the particle were reconstitutively active.

Since both processes result in the dissociation of the dehydrogenase protein from its binding site on the particle, the difference may be attributed to the increase lability of the dissociated succinate dehydrogenase in the absence of succinate and presence of oxygen as compared to that in the presence of succinate and absence of oxygen. It has been reported by King (37, 39) that reconstitutively active succinate dehydrogenase can be prepared only in the presence of succinate or other suitable reducing agents. In the absence of the reducing agents a succinate-PMS reductase is solubilized which has no reconstitutive activity and appears to be a degradation product of the dehydrogenase (31, 34, 39).

The data which are available on the reversible dissociation of the dehydrogenase are, unfortunately, not adequate to allow a mechanism to be proposed for the dissociation, even when they are considered in conjunction with the kinetic data.

SUMMARY

The equilibrium dissociation of the succinate oxidase system was studied using the acid nonextractable flavin content as a measure of the succinate dehydrogenase concentration. The percent of the dehydrogenase which was soluble was a linear function of the hydroxyl ion concentration and the concentration of the soluble dehydrogenase was proportional to the total concentration bound and soluble, confirming the report of King (39). The equilibrium constant for the dissociation decreases with increasing temperature and buffer concentration.

GENERAL DISCUSSION

The available data are not consistent with the possibility that either hydrophobic or electrostatic interactions alone form the link between the dehydrogenase and the particle. Hydrophobic interaction would not be expected to show stoichiometry with hydroxyl ion concentration either kinetically or thermodynamically. It would appear that the role of butanol in the isolation of succinate dehydrogenase (30, 68) is to make the dissociation irreversible by destroying the binding site on the dehydrogenase deficient particle which is formed or by inhibiting the recombination in some other way. This is quite possible, since cytochromes are sensitive to the alcohol and it has been reported that trace amounts of butanol interfer with reconstitution (32). The dehydrogenase can thus be dissociated in good yield at pH values (8.9-9.0) much less than are required to dissociate significant amounts of the enzyme under reversible conditions (pH 9.5-10.5).

Electrostatic interaction could show stoichiometry if the hydroxyl ion were neutralizing the positively charged groups. The rate constant for such a neutralization reaction is about $10^{10} M^{-1}$ sec^{-1} (3). This large rate constant assures a complete equilibration of the charge neutralization reaction during the inactivation of the succinate oxidase and the reaction with hydroxyl ion could not be one of the slow reactions of the steady state. Figs. 9 and 14 are not compatible with this because a preincubation of the heart muscle preparation at alkaline pH and 0^{o} increases the rate and

changes the order of the reaction. Since the rate of the first order portion of the inactivation (Figs. 14 and 15) is also pH dependent the rate of reaction with the hydroxyl ion is probably slow.

Electrostatic interaction is also inconsistent with the observed decrease in the equilibrium dissociation constant with increasing buffer concentration (Fig. 19). It is very much possible, however, that hydrophobic or electrostatic interactions have secondary binding effects which contribute to the binding of the dehydrogenase to the particle.

One possible type of bond which would be consistent with the available data and the proposed mechanism for the inactivation is a nonheme iron coordination complex. This may be visualized as a coordination complex between a ligand group on the particle binding site and a bound iron on the surface of the succinate dehydrogenase protein. At alkaline pH the hydroxyl ion would dissociate the dehydrogenase from the particle by replacing the ligand from the particle in a reaction catalized by hydroxyl ion.

After the soluble succinate dehydrogenase is separated from the particle and the medium neutralized, the decrease in the hydroxyl ion would lead to an equilibration between the hydroxyl ion and water as the iron ligand. The aquo form would be favorable for reconstitution.

Cyanide ion has been shown to irreversibly inactivate the succinate oxidase activity of the heart muscle preparation (15, 28, 62). The cyanide inactivation is very similar to the inactivation by alkaline treatment. The succinate oxidase, succinate-cytochrome

c reductase and succinate-methylene blue reductase are inactivated at the same rate but the succinate-PMS reductase is not affected (28). This last point is not certain (15). This is quantitatively the same effect as is observed for the inactivation by hydroxyl ion in the absence of succinate and presence of oxygen except that the succinate-PMS reductase is less stable at alkaline pH. In addition the particle which remains after either cyanide or hydroxyl ion inactivation is reconstitutively active.

It has also been observed that the inactivation by cyanide is prevented by succinate and dithionite (15, 62), is first order with respect to the cyanide concentration (62), and that the rate increases with increasing pH (15). This behavior would be expected if the cyanide ion reacted in place of the hydroxyl ion in reaction 2 of the mechanism for the inactivation. In the presence of oxygen and absence of reducing agent the solubilized dehydrogenase would be irreversibly inactivated and form a succinate-PMS reductase. In the presence of succinate or dithionite the succinate dehydrogenase would be stable and the dissociation reversible. Thus when the cyanide was removed by dialysis a complete succinate oxidase system would be reconstituted.

One apparent difference is that the inactivation by cyanide gives first order kinetics with respect to the enzyme activity (15, 62) while the inactivation by hydroxyl ion is apparent zero order (Part 3). The lowest concentration of cyanide ion used by Tsou (62) was 5 mM while the highest hydroxyl ion concentration used (Part 3) was 3×10^{-2} mM, more than one hundred fold less. This

concentration difference is adequate to change the kinetics from steady state to those observed by Tsou (62). Metal chelating reagents such as thenoyltrifluoroacetone have been found to strongly inhibit succinate oxidation in mitochondria (72). Whittaker and Redfearn (70) have investigated the inhibition by thenoyltrifluoroacetone with several acceptors and found that at 1 mM the chelator inhibits 83% of the succinate-cytochrome c reductase; 68% of the succinate-methylene blue reductase; 67% of the succinate-methylene blue reductase mediated by ubiqninone and 23% of the succinate-phenazine methosulfate reductase. They have interpreted these results to mean that the chelator has at least two sites of action, one before the site of action of phenazine methosulfate and another between the sites of action for phenazine methosulfate and methylene blue. The second proposed site could be the nonheme iron which is suggested here as the reaction site for the cyanide and hydroxyl ions.

No data has been presented which is pertinent to the question of the nature of the ligand for the bound form of the enzyme. Electron flow must occur between the succinate dehydrogenase and the rest of the electron chain. Although both the inactivation by cyanide and by hydroxyl ion can be interpreted as a physical separation of the two parts of the system, the possibility of nonheme iron at the point of contact suggests that this nonheme iron may be involved in electron transport. This would be possible if the ligand on the particle were sulfhydryl or imidazole.

Both of these groups form coordination complexes with iron and both have been suggested as active in oxidation and reduction in conjunction with nonheme iron. Fridovich and Handler (12, 13) have postulated a role for the nonheme-iron sulfhydryl complex in xanthine oxidase. Urry and Eyring (65) have proposed an imidazole pump model for electron transport.

Experiments are being carried out to investigate further the bond between succinate dehydrogenase and the particulate cytochrome system.

GENERAL SUMMARY

The spectrophotometric method for the determination of the acid nonextractable flavin in the proteolytic digest of the trichloroacetic acid precipitate from the heart muscle preparation has been examined for the interference of the degraded hemeproteins. The proteolytic digest was chromatographically separated into the heme and flavin fractions. Spectrophotometric determinations made on the separated fractions have indicated that the heme interference is negligible for the heart muscle preparation. The validity of this method has thus been demonstrated.

In the course of the proteolysis, cytochrome $\underline{c}_1$ and $\underline{c}$ are evidently degraded to hemepeptides. The spectral behavior of the hemepeptides has been presented.

Based on the fluorescent characteristics of the acid nonextractable flavin, a fluorometric method has been devised. This method is approximately 100 times more sensitive than the spectrophotometric assay. Results from these two methods are in good agreement. It is suggested that the fluorometric method may be more suitable for samples containing high concentrations of pigments adsorbing in the 450-530 mu region of the spectrum.

Alkaline inactivation of the succinate oxidase activity of the heart muscle preparation in the absence of substrate and presence of oxygen has been used by Keilin and King to prepare a particle which supplies all of the respiratory components except succinate dehydrogenase for reconstitution of succinate oxidase.

The succinate dehydrogenase protein, as measured by its flavin coenzyme, was found to be dissociated from the particulate heart muscle preparation by alkaline treatment. The dissociation occurred at the same rate as the inactivation of the succinate oxidase. It was therefore concluded that the original site of binding of the dehydrogenase to the particle is available to bind active succinate dehydrogenase during reconstitution of the system.

The inactivation of succinate oxidase under the experimental conditions used was found to follow zero order kinetics. The apparent zero order rate constant varied as the first power of the initial enzyme concentration and the second power of the hydroxyl ion concentration. The temperature dependence of the apparent zero order rate constant was complex. A mechanism is presented which is consistant with the observed kinetics.

The equilibrium dissociation of the succinate oxidase system by alkaline treatment in the presence of succinate and absence of oxygen was studied using the acid nonextractable flavin content as a measure of the succinate dehydrogenase concentration. The percent of the dehydrogenase which was soluble was a linear function of the hydroxyl ion concentration and the concentration of the soluble dehydrogenase was proportional to the total concentration bound and soluble, confirming the report of King. The equilibrium constant for the dissociation decreased with increasing temperature and buffer concentration.

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