BBA 45513

RECONSTITUTION OF RESPIRATORY-CHAIN ENZYME SYSTEMS

XVI. THE EFFECT OF ALKALI ON THE SUCCINATE OXIDASE SYSTEM

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(Received March 18th, 1966)
(Revised manuscript received August 29th, 1966)

SUMMARY

1. The succinate dehydrogenase protein, as measured by the acid non-extractable flavin, was found to be dissociated from the particulate succinate oxidase (the heart-muscle preparation) by alkali treatment in the absence of succinate and presence of oxygen. The dissociation occurred at the same rate as the inactivation of the succinate oxidase. From the kinetic and the equilibrium results reported in this paper, it was concluded that the original site for binding of the dehydrogenase to the particle was available to link active succinate dehydrogenase during reconstitution of the succinate oxidase system.

2. The inactivation of succinate oxidase during the alkali treatment, in the absence of succinate or any reducing agent, was found to be linear with time. The rate of inactivation varied as the first power of the initial enzyme concentration and the second power of the hydroxyl ion concentration. The temperature dependence of the rate constant, however, was complex.

3. The equilibrium dissociation of the particulate succinate oxidase by alkali in the presence of succinate and absence of oxygen was examined using the acid non-extractable flavin as a measure of succinate dehydrogenase. The per cent of the dehydrogenase dissociated is a linear function of the hydroxyl ion concentration. The equilibrium constant for the dissociation is decreased by increasing the ionic strength and the Van 't Hoff isochore is not linear but exhibits positive curvature. The equilibrium constant for the dissociation determined by the acid non-extractable flavin is practically the same as that estimated by reconstitutive activity.

INTRODUCTION

The electron-transport system of heart mitochondria has been the subject of intensive investigation since 1925. Considerable information is available on its composition and function, but little is known about how the individual components are bound together to form the insoluble, organized complex in the mitochondrion. The

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dearth of knowledge on the intricate problem of the linkages between the respiratory components is due to the lack of suitable methodology. One of the methods is the study of the reversible dissociation of respiratory components. However, only two components—cytochrome *c* (ref. 1) and succinate dehydrogenase[2,3]—have been isolated from the respiratory chain into true soluble form and, in turn, can be reconstituted to form the original.

Of these two components, succinate dehydrogenase is particularly suitable for study because this enzyme is extremely unstable in the soluble form in contrast to the great stability of its bound form in the respiratory chain. Furthermore, the dehydrogenase (either in the form of reconstitutively active or inactive) can be structurally equated with the acid non-extractable (peptide-bound) flavin for the samples derived from cardiac mitochondria[4–7]. Succinate dehydrogenase was first isolated[2,3] to a reconstitutively active form in 1958. When this active enzyme reacts with a particle which contains all respiratory components except the dehydrogenase, a stable succinate oxidase system is reformed. The particle is prepared by an alkali treatment of succinate oxidase; the treated particle is completely devoid of succinate oxidase or succinate dehydrogenase activity[2,3,8]. More recently the equilibrium dissociation of succinate dehydrogenase from the Keilin–Hartree heart-muscle preparation under well-defined conditions has been demonstrated[8,9]; this achievement has further increased the usefulness of the reconstitution technique.

When the heart-muscle preparation is treated with alkali, the succinate oxidase activity may be either irreversibly lost[9] or reversibly dissociated into soluble succinate dehydrogenase and a dehydrogenase-free particle[8,9] depending on the conditions of the treatment. It is, therefore, of utmost importance that the experimental conditions be considered carefully in the evaluation of the experimental data (for example, ref. 10 *vs.* 9).

In this investigation we have examined the fate of the succinate dehydrogenase protein during both irreversible inhibition (in the presence of oxygen and absence of succinate) and reversible dissociation (in the presence of succinate and absence of oxygen). It has been found that the dehydrogenase protein is dissociated from the particle during irreversible inhibition by alkali and that the dissociation occurs at the same rate as the inactivation. Both the irreversible dissociation and the reversible dissociation of the succinate oxidase system have been examined as a function of several parameters.

EXPERIMENTAL PROCEDURE

Flavin determination

The acid non-extractable flavin was determined by the fluorimetric method described previously[4]. This partition of flavin was equated to succinate dehydrogenase, either active or inactivated. This equation is perhaps justified at least for the mitochondrial preparations from heart (*cf.* refs. 4–7).

Alkali treatment I

The alkali treatment was made according to the method previously described[2,8].

Alkali Treatment II

Alkali treatment II was adapted from alkali treatment I in order to allow the

specific design of the experiment. Aliquots from a single batch of the heart-muscle preparation containing approx. 10 mg of protein per ml of 0.05 M phosphate buffer were carefully adjusted to a desirable pH (± 0.02 unit). Each aliquot was then divided into two portions: one was placed in a centrifuge tube and the other in a test tube. These tubes were incubated at a desired temperature. After a suitable interval the portion in the centrifuge tube was centrifuged for 30 min at 50000 rev./min. 6 min after the start of the centrifugation*, the portion in the test tube was neutralized and 0.2 ml of 0.6 M succinate was added to each 5 ml. The mixture was allowed to stand for 10 min and the succinate oxidase was determined[4]. The clear supernatant liquid obtained from the centrifuged sample was then neutralized and used for the determination of the acid non-extractable flavin.

Alkali treatment III

The heart-muscle preparation containing about 10 mg of protein per ml of 0.05 M phosphate–borate buffer (in the absence of succinate) was titrated to ±0.02 unit of a desired pH value by adding 2.5 M NaOH. The mixture was then incubated at a specified temperature (±0.05°). At specified time intervals aliquots were rapidly transferred to chilled test tubes containing 0.2 ml of 0.6 M succinate and sufficient H_3PO_4 to give a final pH of between 7 and 8. They were then allowed to stand for approx. 10 min at room temperature and the succinate oxidase activity was determined[4].

The adjustment to alkaline pH was made by adding 2.5 M NaOH from a microburet with the delivery tip immersed in the gently stirred sample which was placed in a thermostatted beaker. A Beckman expansion scale of pH meter with a precision of 0.005 unit was used. Volume changes from additions of reagents and the retention of the supernatant fraction in the residue were always corrected. The time of alkali treatment was computed from the interval between the attaining of the final pH of the sample and 6 min after the centrifugation was started. The temperature at all steps of manipulation, unless otherwise specified, was within 0.5° of the temperature in the incubation; the latter had a precision of 0.05°.

Alkali treatment IV

The heart-muscle preparation containing approx. 10 mg protein per ml of 50 mM phosphate–borate in the presence of 35–40 mM succinate was used. The pH of the preparation was adjusted to a desired value by addition of 2.5 M NaOH from a microburet as detailed in *Alkali treatment III*. Care was taken at all times to minimize the diffusion of oxygen into the sample. Immediately after the pH was attained, aliquots were transferred to centrifuge tubes, sealed, and placed in a Rotor No. 50 of a Spinco preparative centrifuge. The rotor was immersed in a water bath at a specified temperature (±0.05°) for a desired length of time. The mixture was then centrifuged for 30 min at 50000 rev./min.

The volume of the clear, amber supernatant liquid was measured and the acid non-extractable flavin was determined. Volume changes from addition of reagents and the retention of the supernatant fraction in the residue were corrected. The time

* This 6-min delay for neutralization was used to compensate for the time required for the particle to be sufficiently sedimented in the centrifuged portion to end transfer of the solubilized dehydrogenase protein from the particulate (pellet) fraction to the supernatant.

of alkali treatment was computed from the interval between the attaining of the final pH of the sample and 6 min after the centrifugation was started. The temperature at all steps of manipulation unless otherwise specified, was within 0.5° of the temperature during incubation.

Deviations in the method of testing any particular parameter are given in the text.

Other methods and materials

These are described in previous papers[4,8,11].

RESULTS

Presence of oxygen and absence of succinate

A significant solubilization of the acid non-extractable flavin of the heart-muscle preparation by alkali treatment I has been reported earlier[4,11,12]. The observations suggested that if the alkali treatment was carried out while the rate of solubilization was slow enough for measurement, it would be possible to compare directly the rates of solubilization of the flavin with the rate of inactivation of the succinate oxidase activity. Consequently, the experiment described in the following section was designed.

Parallelism of the rate of inactivation of succinate oxidase and the rate of dissociation of the acid non-extractable flavin. Protocols of the rate of alkaline inactivation of the succinate oxidase activity and that of dissociation of the acid non-extractable flavin are given in Table I; both processes were linear with time. The rates might,

TABLE I

THE EFFECT OF THE TIME OF ALKALINE TREATMENT ON THE SUCCINATE OXIDASE ACTIVITY AND THE ACID NON-EXTRACTABLE FLAVIN

Heart-muscle preparation (approx. 10 mg protein per ml) batch number 321 was incubated at 16.0° and batch number 324 at 24.0° under the condition of alkali treatment II. Both the enzymatic activity and the acid non-extractable flavin are based on the protein content of the original heart-muscle preparations.

Batch number	*pH*	*Time of treatment (min)*	*Succinate oxidase activity (μl O_2/h per mg protein)*	*Acid non-extractable flavin in the supernatant liquid ($m\mu moles$/mg protein)*
321	7.8	0	540	0.188*
321a	9.35	50	323	0.053
321b	9.35	38	329	0.045
321c	9.34	27	362	0.043
321d	9.35	17	370	0.038
324	7.8	0	490	0.136*
324a	9.20	47	187	0.087
324b	9.21	35	212	0.074
324c	9.20	25	246	0.071
324d	9.20	15	278	0.050

* The acid non-extractable flavin content of the untreated samples was the total acid non-extractable flavin content of the heart-muscle preparation.

therefore, be expressed as the time in min required to inactivate 20 % ($t_{20\%}$)* of the initial succinate oxidase activity (prior to the addition of NaOH) or to solubilize 20 % of the total acid non-extractable flavin respectively. Table II depicts the rates of these two reactions. Allowing for experimental error, they were practically identical.

Kinetics of alkali inactivation of succinate oxidase. The course of the inactivation of succinate oxidase is shown in Fig. 1. For this experiment, alkali treatment III was used. The activity of an aliquot removed before the addition of NaOH was used as 100 %, and the zero time was taken as the time at which the sample reached its final alkaline pH. The reaction was linear with respect to time until the succinate oxidase was about 70 % inactivated, even when as many as 12 samples were taken during this interval. The apparent zero-order kinetics was observed over wide ranges of

TABLE II

A COMPARISON OF THE RATES OF SUCCINATE OXIDASE INACTIVATION AND THE SOLUBILIZATION OF THE ACID NON-EXTRACTABLE FLAVIN DURING ALKALINE TREATMENT

$t_{20\%}$ is the time required for 20 % (based on the values before the addition of alkali) inactivation of succinate oxidase or solubilization of the acid non-extractable flavin.

Batch number	*pH*	*Incubation temperature* (°)	*Rate of inactivation of the succinate oxidase activity,* $t_{20\%}$ *(min)*	*Rate of flavin solubilization,* $t_{20\%}$ *(min)*
321	9.35	15.8	82	88
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

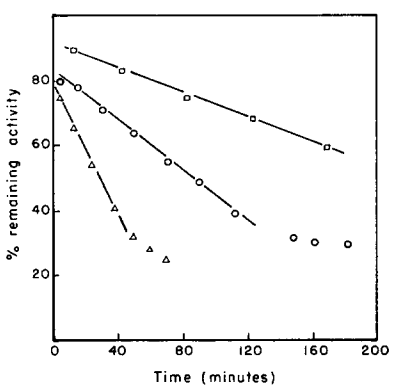

Fig. 1. The time course of the alkaline inactivation of succinate oxidase by alkaline treatment III. The heart-muscle preparation was at 10 mg protein per ml of 0.05 M phosphate–borate buffer. The control (100 %) activity was the activity of an aliquot removed before the addition of NaOH, and the zero time was the time at which the final pH was reached. The succinate oxidase activities of the aliquots were measured polarographically at room temperature. □, pH 9.02, 22.0°; ○, pH 9.17, 22.0°; △, pH 9.40, 22.0°.

* The symbol, $t_{20\%}$, used in this paper is in the dimension of time and has the similar meaning as $t_{50\%}$ (half-time) which is widely used in chemical kinetics. The difference is, of course, in the present case, 20 % of the reactant reacted either in the solubilization of the acid non-extractable flavin or inactivation of the oxidase activity as properly specified.

pH values and temperatures from numerous experiments employing many batches of the heart-muscle preparation. The apparent zero-order behavior was not unexpected because in this heterogenous system, the particle concentration should not be considered equivalent to the homogenous solution. On the other hand, the deviation from the zero-order kinetics after 70 % inactivation would indicate that the particle could not be exactly independent as in a simple solid–gas or solid–liquid system*. The plots, however, failed to extrapolate to 100 % at zero time, partly because the graphic zero time was not the true zero time (see METHODS). Moreover, it was inevitable that localized high pH would occur during the addition of NaOH and that time would be required (approx. 2 min) to reach the desirable pH.

For convenience, the rate of the inactivation is expressed as $t_{20\%}$ because it was an experimentally determined value and independent of the initial succinate oxidase activity. The latter was demonstrated at least up to 70 % inactivation.

The effect of protein concentration on the rate of inactivation. The alkaline inactivation of succinate oxidase is not affected by the protein concentration of the heart-muscle preparation tested between 4.3 and 17.5 mg/ml at constant buffer concentration, pH and temperature as shown in Fig. 2. Because the initial succinate oxidase activity is a linear function of the concentration of the heart-muscle preparation, therefore the amount of the enzyme inactivated per unit time is proportional to the concentration of the heart-muscle preparation. This fact makes the rate of inactivation to be first order with respect to the initial succinate oxidase activity.

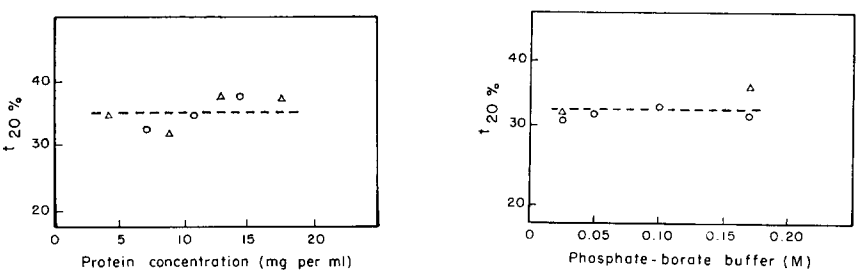

Fig. 2. The effect of protein concentration of the heart-muscle preparation on the rate of inactivation of succinate oxidase. The temperature was 23.5°, the buffer 0.05 M phosphate–borate and the pH 9.16 ± 0.02. The abscissa is the concentration of the heart-muscle preparation in mg protein per ml and the ordinate is $t_{20\%}$ for the inactivation. ○ and △ represent two different batches of the heart-muscle preparation.

Fig. 3. The effect of buffer concentration on the rate of inactivation of succinate oxidase. During each treatment the protein concentration of the heart-muscle preparation was 9.5 mg/ml, the pH was 9.16 ± 0.02 and the temperature was 23.5°. The abscissa is in molarity of the phosphate–borate buffer and the ordinate is $t_{20\%}$ for the inactivation. ○ and △ represent two different batches of the heart-muscle preparation.

The effect of buffer concentration on the rate of inactivation. When 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. The independence of buffer concentration at several constant pH values was demonstrated. Fig. 3 illustrates the findings at pH 9.16 for two batches of the heart-muscle preparation.

* See ref. 31 (p. 188) for more detailed discussion on the weight of particle concentration in computing equilibrium and kinetics of the heart-muscle preparation system.

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. 4. The slope of the straight line was approx. 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 experiments, using five different batches of the heart-muscle preparation, the slope of the curves was found in the range between 1.9 and 2.4 with a mean of 2.2. The $t_{20\%}$ for the inactivation was slightly variable among batches of the heart-muscle preparation. However, for a single batch, the $t_{20\%}$ values were constant and therefore, a single batch was used for each series of experiments.

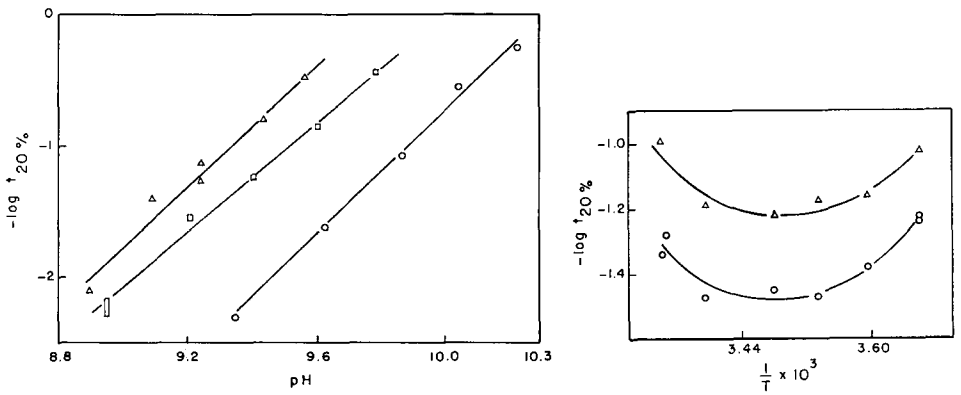

Fig. 4. The effect of pH on the rate of inactivation of succinate oxidase. The heart-muscle preparation was at 9.5 mg protein per ml of 0.05 M phosphate–borate buffer. The abscissa is the measured pH value of the alkaline treatment and the ordinate is the negative logarithm of the $t_{20\%}$ for the inactivation. ○, 8.7°; □, 21.8°; △, 25.0°.

Fig. 5. The temperature dependence of the rate of alkaline inactivation of succinate oxidase at constant pOH. The heart-muscle preparation was 9.5 mg protein per ml of 0.05 M phosphate–borate buffer. For each temperature the dissociation constant of water (from International Critical Tables) was used to calculate the required pH and the heart-muscle preparation titrated to within ± 0.02 pH units of that value. ○, pOH = 4.74; △, pOH = 4.90.

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°), the Arrhenius plots approximated straight lines, and the apparent Arrhenius activation energy could be calculated. When wider temperature ranges were used, the plots showed positive curvature (Fig. 5); the apparent activation energy increased with temperature. Quantitative evaluation of the curvature was achieved. Table III summarizes the observed activation energies at different pH values with the mean temperature range used for the Arrhenius plot.

The temperature dependence of the rate of inactivation at constant pOH. When the pH was held constant and the temperature was varied, the hydroxyl ion concentration was a temperature-dependent variable. The apparent activation energies (for the alkaline inactivation of succinate oxidase) at constant pH might be related to those at constant pOH by the temperature dependence of the ion product of water. If the rate of inactivation was second order with respect to the OH^- concentration,

TABLE III

THE EFFECT OF pH ON THE APPARENT ARRHENIUS ACTIVATION ENERGY FOR THE ALKALINE INACTIVATION OF SUCCINATE OXIDASE

The heart-muscle preparation was adjusted to 10 mg protein per ml of 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 span was approx. 10°. Listed are the pH during the inactivation, the apparent Arrhenius activation energy in kcal/mole and the mean of the temperature range of the Arrhenius plot.

pH	*Mean temperature* (°)	*Apparent Arrhenius energy in* $kcal \cdot mole^{-1}$	*pH*	*Mean temperature* (°)	*Apparent Arrhenius energy in* $kcal \cdot mole^{-1}$
8.79	30	76	9.29	20	39
8.89	29	60	9.40	18	33
8.99	28	51	9.56	15	26
9.09	26	45	9.7	8	21
9.19	23	41	9.8	8	20
9.20	24	38	9.91	8	20

then the apparent activation energies at constant pH would be 26 kcal greater than those at constant pOH. A much wider temperature range could therefore be used for the Arrhenius plots at constant pOH than at constant pH. The Arrhenius plot at constant pOH (Fig. 5) had a minimum at 12–13°, and the apparent activation energy was strongly temperature dependent. This fact suggests the complex nature of the reaction (*vide infra*).

The effect of prior incubation at 0° and alkaline pH on the rate of inactivation. The heart-muscle preparation was cooled to 0°, and the pH of the stirred sample brought to the desired alkaline value. After standing for 5 min at 0°, the sample was warmed rapidly to 24° and incubated at that temperature.

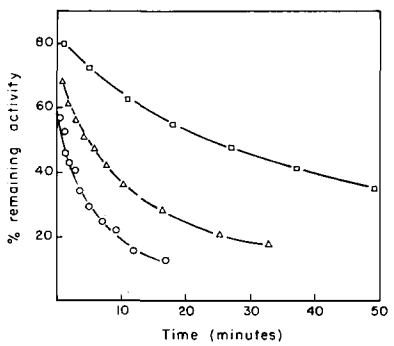

Fig. 6. The inactivation of succinate oxidase after prior incubation of the heart-muscle preparation at 0° and alkaline pH. Samples of the heart-muscle preparation at 10 mg protein per ml of 0.05 M phosphate–borate buffer were made alkaline at 0° and allowed to stand at that temperature for 5 min. The 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 was the time interval for which the aliquot was alkaline; temperature, 24°. The succinate oxidase activity of an aliquot removed before the addition of NaOH was used at 100%. ○, pH 9.84 at 0° and pH 9.51 at 24°; △, pH 9.72 at 0° and pH 9.37 at 24°; □, pH 9.49 at 0° and pH 9.15 at 24°.

As may be seen from Fig. 6, the incubation at 0° and high pH values changed the inactivation in two ways: the rate of inactivation was greatly increased, and the order was changed from zero to complex. In other words, the inactivation no longer had a unique order for the measured time interval. The change in the time course of the inactivation by preincubation at alkaline pH is consistant with the interpretation that the observed apparent zero-order kinetics result from a pH-dependent steady-state concentration of reaction intermediate.

Presence of succinate and absence of oxygen

Great care must be exercised in comparison of the results described in this section with those in the previous part. In the presence of succinate and absence of oxygen, the dissociation of the succinate dehydrogenase from the heart-muscle preparation is an equilibrium process and is freely reversible; *i.e.* upon neutralization, the dissociated succinate dehydrogenase is immediately (within the operation time) recombined with the particle (*cf.* refs. 8,9). On the other hand, irreversible dissociation occurs when oxygen is present and succinate is absent.

TABLE IV

THE EFFECT OF THE TIME OF ALKALI TREATMENT ON THE DISSOCIATION OF SUCCINATE DEHYDROGENASE MEASURED AS THE ACID NON-EXTRACTABLE FLAVIN IN THE SOLUBLE FRACTION

Two different batches of the heart-muscle preparation were used, 10 mg of protein per ml of 50 mM phosphate–borate buffer containing 35 mM succinate for alkali treatment IV.

Temperature (°)	*pH*	*Treatment* (*min*)	*Per cent of the acid non-extractable 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

Effect of time on the dissociation of succinate dehydrogenase measured as acid non-extractable flavin. Table IV depicts the relationship between the time of incubation of the heart-muscle preparation in alkaline media (alkaline treatment IV) and the dissociation of succinate dehydrogenase measured as acid non-extractable flavin at constant temperature and pH. As shown, the dissociation or solubilization of succinate dehydrogenase is not a function of time within the limits of the operation time, but the dissociation is dependent upon pH. This is in agreement with the earlier data[8] which are obtained mostly from activity determination. The present observation is consistent with the claim[8] of a true equilibrium that is attained within the time of operation.

Advantage was taken of the high succinate oxidase activity ($Q_{O_2} > 300$ at 24°) of the preparation to maintain oxygen-free conditions as witnessed by the reduced state of the cytochromes. The latter was always ascertained by parallel examination

of the samples under a microspectroscope. However, the oxidase activity decreased rapidly with the increase of pH at values more alkaline than 9. It was therefore essential that immediately after the final desired pH was reached the sample be transferred to a centrifuge tube and the tube sealed without delay.

In this paper the system was taken to be at equilibrium as long as the amount of the solubilized acid non-extractable flavin was independent of the time of incubation (*i.e.* completed within the time of manipulation). The results were consistent with those previously reported[8] at 0–4° for which complete reversibility was clearly established mainly by activity determination.

Dependence of hydroxyl ion concentration on the dissociation of succinate dehydrogenase. A previous report[8] shows that the fraction of the succinate dehydrogenase solubilized is directly proportional to the hydroxyl ion concentration at 0–4°, the only temperature studied. When this observation was confirmed and extended to several temperatures, it was found that the degree of the dissociation of succinate dehydrogenase from succinate oxidase was dependent not only upon hydroxyl ion concentration but also upon temperature (*cf.* Fig. 7).

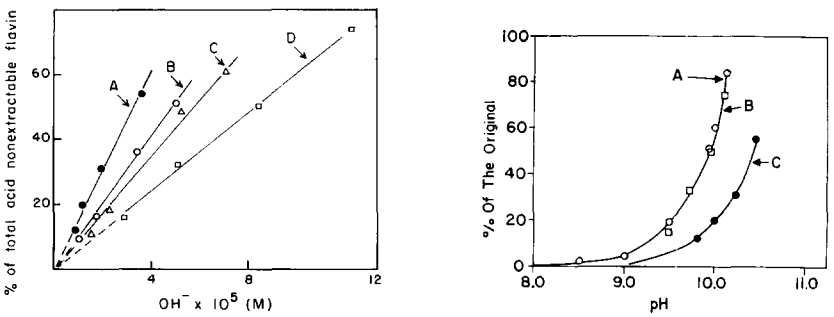

Fig. 7. Effects of temperature and hydroxyl ion concentration on the dissociation of succinate dehydrogenase measured as the acid non-extractable flavin solubilized. The heart-muscle preparation contained 11.5 mg protein per ml of 50 mM phosphate–borate buffer in the presence of 35 mM succinate. The untreated heart-muscle preparation containing 0.17 mμmole of acid non-extractable flavin per mg of protein was used as the reference, *i.e.* 100%. Curve A, 0°; Curve B, 5°; Curve C, 10°; and Curve D, 23.5°.

Fig. 8. Comparison of the functional and the structural criteria for the alkaline dissociation of succinate dehydrogenase from the heart-muscle preparation. Curve A (○) is constructed from activity determination of the solubilized dehydrogenase from the data obtained at 0–4° (ref. 8). Curves B (□) and C (●) are plotted from the data based on the acid non-extractable flavin solubilized at 23° and 0° respectively; see Table IV for other details.

The acid non-extractable flavin liberated to the soluble fraction was used as a measure for the dissociation of succinate dehydrogenase. The comparison of this measure with the criterion, which uses the succinate dehydrogenase activity determined by reconstitution[8], is depicted in Fig. 8. It can be seen that Curve A, constructed from the activity determination, is the same as the curve (Curve B) obtained from structural analysis—*i.e.* from acid non-extractable flavin determination. However, as elaborated below, a displacement of 0.3 pH unit (the average from three experiments) exists, *i.e.* Curves A and B *versus* C of Fig. 8.

Effect of temperature on the dissociation of succinate dehydrogenase. The ratio of the fraction of succinate dehydrogenase dissociated, measured as the acid non-

extractable flavin, to the hydroxyl ion concentration was found to be a constant (K_e) at constant temperature. This constant decreased with the increase of temperature (*cf.* Fig. 9). When log K_e was plotted as a function of the reciprocal of the absolute temperature, it was not a straight line but possessed a positive curvature (Fig. 9). Thus the thermodynamic quantities could not be easily calculated.

K_e reported previously from the activity determination[8] at 0–4° is $3 \cdot 10^4$ M^{-1} and is two-fold larger than the $1.5 \cdot 10^4$ M^{-1} reported here at 0° (Fig. 9). This difference is equivalent to a displacement of approx. 0.3 pH unit (*i.e.* pH 10.3 in this report *versus* pH 10.0 in the previous one, *etc.*). This disparity, though small when considered as a detail of a very complex system, may also be due to the difference of precision in measurements; the present investigation used a pH meter with a higher accuracy and the temperature control was more rigorous than the previous study.

The effect of the concentration of the heart-muscle preparation on the dissociation of succinate dehydrogenase. As shown in Fig. 10, the fraction of the succinate dehydrogenase dissociated, measured as the acid non-extractable flavin at a given hydroxyl ion concentration and temperature, was independent of the concentration of the

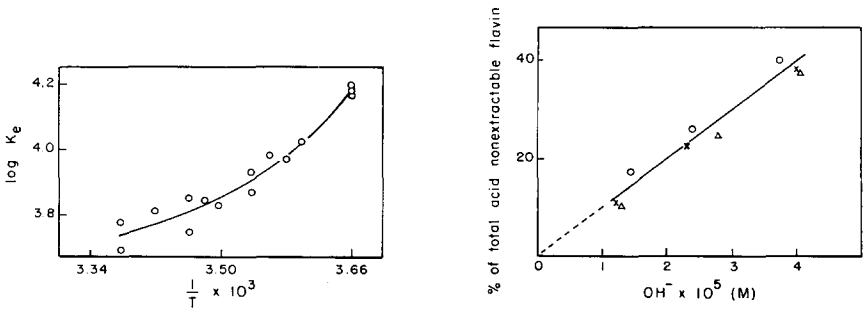

Fig. 9. The temperature dependence of the equilibrium constant (K_e) for the dissociation of succinate dehydrogenase measured as the acid non-extractable flavin. The heart-muscle preparation contained 8–11 mg of protein per ml of 50 mM phosphate–borate buffer in the presence of 35 mM succinate.

Fig. 10. The effect of protein concentration on the dissociation of succinate dehydrogenase at different pH measured as the acid non-extractable flavin. The heart-muscle preparation was suspended in 50 mM phosphate–borate buffer in the presence of 40 mM succinate. ○, 7 mg protein per ml; ×, 10.5 mg/ml; △, 14 mg/ml.

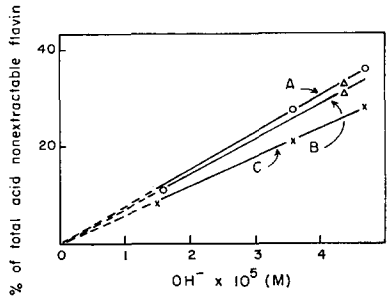

Fig. 11. The effect of the concentration of phosphate–borate buffer on the dissociation of the acid non-extractable flavin. The heart-muscle preparation contained 10 mg protein per ml of 30 mM succinate and the concentration of buffer were: Curve A, 29 mM phosphate–borate; Curve B, 50 mM phosphate–borate; and Curve C, 160 mM phosphate–borate. Temperature, 14.3°.

heart-muscle preparation. Thus, the concentration of the soluble succinate dehydrogenase was proportional to the total (bound and soluble) concentration of succinate dehydrogenase. From activity measurement[8], a slight deviation from proportionality was observed when a greater range of concentrations of the heart-muscle preparation was used.

The effect of buffer concentration on the equilibrium constant for the dissociation of succinate dehydrogenase. Fig. 11 summarizes the effect of buffer concentration on the dissociation of succinate dehydrogenase measured as the acid non-extractable flavin. An increase of the concentration of phosphate–borate buffer from 0.020 M to 0.16 M decreased the dissociation less than 20 % in the pH range tested.

DISCUSSION

The original observations[2,3] on the reconstitution of succinate oxidase have been verified and extended by many laboratories (*e.g.* refs. 13, 14–17). SINGER and co-workers[10,13,18] have reported however, that under the conditions of alkali treatment, in the presence of oxygen and absence of succinate, no succinate dehydrogenase as measured by bound (acid non-extractable) flavin analysis was dissociated from the particle "unless the inactivation was carried out at a much more alkaline pH than was required for complete inactivation of the dehydrogenase and oxidase in a relatively short time". In addition, they have found "it makes no difference in our experiment whether the alkali-treated particles are centrifuged before or after neutralization". These observations conflict directly with the experimental data reported in this paper and no explanation is yet available.

The data presented in this paper clearly show that during the alkali treatment of succinate oxidase by the method used for the preparation of the dehydrogenase-free particle for reconstitution experiments[2,3,8,12], the succinate dehydrogenase protein is dissociated from the particle at a rate equal to the rate of the inactivation of succinate oxidase. The dissociation is irreversible as evidenced by the reconstitutive inactivity of succinate dehydrogenase solubilized in the absence of succinate and presence of oxygen[8,12]. The original site for binding succinate dehydrogenase on the particle is thus available to bind the soluble enzyme during the reconstitution.

The alkali treatment of the heart-muscle preparation has been carried out under two different sets of conditions. When the treatment was conducted in the absence of succinate and in the presence of oxygen, the succinate dehydrogenase was dissociated (Table I) and irreversibly inactivated[3,3,19]. The particle obtained from the treatment may serve as a source of the respiratory components other than the dehydrogenase[3]. In the presence of succinate and in the absence of oxygen, the succinate dehydrogenase was reversibly dissociated from the particle[8,9] and both the solubilized dehydrogenase and the particle were reconstitutively active.

Because both processes result in the dissociation of the dehydrogenase protein from its binding site on the particle, the difference may be attributed to the increased lability of the dissociated dehydrogenase in the absence of succinate and presence of oxygen as compared to that in the presence of succinate and absence of oxygen (see also refs. 3, 8). This interpretation requires that the irreversible inactivation be a kinetic expression of the dissociation of the soluble dehydrogenase from the particle under reversible conditions. The irreversible inactivation is proportional to the square

of the hydroxyl ion concentration (Fig. 4) while the equilibrium constant for the dissociation has been expressed[8] as shown in Eqn. 1.

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

[SD] and [SD_t] represent the concentrations of the dissociated dehydrogenase and the total dehydrogenase respectively. From these experimental results, it is concluded that one of the two kinetically active hydroxyl ions must therefore act only to catalize the dissociation reaction since only one hydroxyl ion appears in the equilibrium constant.

The available data cannot be explained by the possibility that either hydrophobic or electrostatic interactions alone form the linkage between the dehydrogenase and the particle. Hydrophobic interactions would not be expected to show stoichiometry with hydroxyl ion concentration either kinetically or thermodynamically but would be consistant with the observation that increasing the ionic strength decreases the reversible dissociation of the succinate oxidase (Fig. 11). It would appear that the role of butanol in the isolation of succinate dehydrogenase[3,7] is to make the dissociation "irreversible" by destroying the binding site on the dehydrogenase-free particle or by inhibiting the reconstitution. This explanation is in accord with the observation that the cytochromes are sensitive to ethanol and that traces of butanol interfere with reconstitution[13]. Therefore, in the presence of butanol, the dehydrogenase can be dissociated in good yield at pH values (8.9–9.0) much lower than are required to dissociate any significant amount of the enzyme under reversible conditions.

Electrostatic interactions could show stoichiometry if the hydroxyl ion were neutralizing the positively charged groups. The rate constant for such a neutralization reaction in about 10^{10} $M^{-1} \cdot sec^{-1}$ (*e.g.*, ref. 20). Figs. 1 and 6 are not compatible with this explanation. Moreover, both the reversible and irreversible dissociations are temperature dependent whereas the activation energy of neutralization is essentially zero. It is likely, however, that electrostatic and hydrophobic interactions have secondary binding effects which reinforce the linkage (see next paragraph) between the dehydrogenase and the rest of the respiratory chain.

A possible type of bond which would be consistent with the available data is a non-heme iron coordination complex. This may be visualized as a complex between a ligand group at the dehydrogenase-binding site on the particle and a non-heme iron on the surface of the dehydrogenase protein. At alkaline pH the hydroxyl ion would displace the dehydrogenase from the particle by replacing the ligand in a reaction catalyzed by hydroxyl ion. After the succinate dehydrogenase is separated from the particle and the medium neutralized, the decrease in the hydroxyl ion would lead to an equilibrium between the hydroxyl ion and water as an iron ligand. The aquo form would favor reconstitution.

On the other hand, electron flow must occur between the succinate dehydrogenase and the rest of the respiratory chain. Although the inactivation by hydroxyl ion can be interpreted as a physical separation of the two parts of the system, the possibility of non-heme iron at the point of contact suggests that this non-heme iron may also be involved in electron transport[21–25]. A recent study[26] from this laboratory has shown that one difference between the reconstitutively active succinate dehydro-

genase and the reconstitutively inactive succinate phenazine reductase (such as those preparations by the SINGER group[27,23]) is in the ratio of flavin to non-heme iron; in the former the ratio is 1:8 whereas in the latter it is 1:4 or 1:2. The reconstitutively active dehydrogenase also contains labile sulfide[26] with a molar content equal to that of non-heme iron. This observation is again in line with the postulation that non-heme iron may be a structural link with the respiratory chain. These observations together with the results reported by DERVARTANIAN[29] may suggest that soluble succinate dehydrogenase contains two kinds of non-heme iron. One type is essential for reconstitution and responsible for the decay of spectral absorbance[29]. This interpretation explains well the reconstitutive inability of those SINGER preparations[27,28] which contain only 2 or 4 non-heme iron per flavin. Independently WANG AND WANG[30] have inferred a similar explanation for the role of the non-heme iron in succinate dehydrogenase obtained from their reconstruction experiment using our technique. Finally, it may be pointed out that the non-heme iron hypothesis is reached only by the elimination of other explanations which are not compatible to the results described and it should not be construed as a conclusion with direct experimental evidence.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Science Foundation, the U.S. Public Health Service, the American Heart Association, the Office of the Naval Research and the Life Insurance Medical Research Fund. One of the authors (D.F.W.) was Predoctoral Fellow from the National Institutes of Health, 1962–1964.

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