The present communication is devoted to the description of the isolation, properties, and composition of a particulate unit which we shall refer to as the succinic dehydrogenase complex (SDC). The working hypothesis underlying this and subsequent communications in this series is that SDC is a unit of enzymatic action in the same sense as any well-defined soluble enzyme.

Since the discovery of succinic dehydrogenase (1), a vast literature on this enzyme has accumulated, much of which has been amply reviewed in recent years (2-4). The important investigations which are pertinent to or form the basis of the observations reported in this paper may be classified as follows: relationship of succinic dehydrogenase to cytochrome c and the cytochromes generally (5-9), 2,3-dimercapto-1-propanol (BAL) sensitivity of succinic dehydrogenase (10-14), rôle of cytochrome b in succinic dehydrogenation (15-17), bacterial succinic dehydrogenase (18), development of new techniques for the separation of particulate enzymes (19-27), purification of succinic dehydrogenase by ammonium sulfate-cholate mixtures (28-30), and preparation of a soluble succinic dehydrogenase (31).

Most recently Neufeld et al. (29) have described the isolation of two purified (particulate?) preparations of succinic dehydrogenase from heart muscle extracts. The first (Preparation A), obtained by fractionation with ammonium sulfate in the presence of cholate and by selective heat denaturation, is about 20 times more active than the whole homogenate of heart muscle. It is free of cytochrome c and cytochrome oxidase. The $Q_{O_2}$ at $30^\circ$ is 46. The reaction of Preparation A with cytochrome c is relatively slow compared to the reaction with indophenol. The second (Preparation B), obtained by extensions of the procedures applied in the making of Preparation A, is 30 times more active than the starting material. It reacts with indophenol but not with cytochrome c. The

* Postdoctoral Trainee of the National Heart Institute.
final preparation which is amber-yellow in color contains both "a small amount of a hemoprotein which could not be removed" and flavin. Clark et al. (28) have isolated a third component (SC) from heart muscle (30-fold purified) which they report functions as an antimycin-sensitive link between their succinic dehydrogenase Preparation B and cytochrome c, although the SC preparation already contains a very active succinic dehydrogenase. The spectrophotometric analysis of the SC preparation (30) discloses the presence of two types of hemes, one of which has been characterized as cytochrome b (peak at 563 m\(\mu\)) and the other as cytochrome c (peak at 554 m\(\mu\)). Both hemes are partially reducible by succinate.

**Methods**

**Assay of Enzymatic Activity**—The activity of the enzyme has been quantitatively determined with ferricyanide, 2,6-dichlorophenolindophenol, cytochrome c, and phenazine methosulfate as alternative electron acceptors.

**Reduction of Ferricyanide**—The enzymatic oxidation of succinate by ferricyanide has been followed manometrically by measurement of carbon dioxide evolution (32). The main compartment of each manometer flask contained 0.1 mg. of enzyme, 50 \(\mu\)moles of potassium bicarbonate, 50 \(\mu\)moles of succinate, and 15 mg. of albumin (Armour’s crystalline bovine plasma albumin) in a final volume of 1.3 ml. The side arm contained 50 \(\mu\)moles of ferricyanide in 0.2 ml. The gas space contained a mixture of 95 per cent nitrogen and 5 per cent carbon dioxide. The specific rate of \(\text{CO}_2\) evolution expressed as micromoles of \(\text{CO}_2\) per mg. of protein per 5 minutes at 38° has been the basis on which purity has been evaluated.

**Reduction of Indophenol**—The enzymatic oxidation of succinate by indophenol has been followed spectrophotometrically at 600 m\(\mu\). A cuvette of 1 ml. capacity was filled with 0.98 ml. containing 10 \(\mu\)moles of phosphate (pH 7.4), 0.5 mg. of human serum albumin, \(^1\) 2 \(\mu\)moles of potassium cyanide, 10 \(\mu\)moles of succinate, and 20 \(\gamma\) of 2,6-dichlorophenolindophenol. Then 5 to 15 \(\gamma\) of enzyme in 0.02 ml. were added and readings were taken at 30 second intervals against a blank containing all components except succinate. Specific activity is defined as micromoles of indophenol reduced per mg. of protein per minute at 38°. The optical density change at 600 m\(\mu\) divided by 19.1 is the value for micromoles of indophenol reduced (33).

\(^1\) Bovine plasma albumin (Armour) is not as satisfactory in this assay as human serum albumin. We are grateful to Dr. D. M. Surgenor of Harvard University for the gift of samples of human serum albumin.
Reduction of Cytochrome c—This assay is based on spectrophotometric measurement of change in absorbance at 550 μm. A mixture of 10 μmoles of phosphate (pH 7.4), 10 mg. of bovine plasma albumin, and 5 to 10 γ of enzyme in 0.22 ml. was incubated at 30° for 5 minutes. At the end of this period 1 μmole of KCN and 5 μmoles of succinate were added and the volume was made up to 0.9 ml. The mixture was brought to 38° and at time zero the reaction was started by the addition of 1 mg. of cytochrome c (Sigma Chemical Company) in 0.1 ml. Readings were taken at 30 second intervals against a blank containing all components except succinate. The molar extinction coefficients for oxidized and reduced cytochrome c at 550 μm were taken to be, respectively, $2.87 \times 10^3$ and $0.9 \times 10^3$ sq. cm. per mole, the difference, $1.97 \times 10^3$ sq. cm. per mole (34), representing the change in absorbance at 550 μm which accompanies reduction of cytochrome c. Specific activity is defined as micromoles of cytochrome c reduced per minute per mg. of enzyme at 38°.

Reduction of Phenazine Methosulfate—A manometric assay procedure with phenazine methosulfate (35) as electron acceptor has already been described by Singer and Kearney (31). A more linear reaction obtains for the particulate enzyme when histidine buffer (50 μmoles per ml.) of pH 7.5 is used instead of phosphate buffer and when the level of cyanide is raised from 0.001 to 0.002 M. Specific activity is defined as micro-atoms of oxygen taken up per 5 minutes per mg. of enzyme at 38°.

Analytical Procedures—Iron was estimated with o-phenanthroline after wet ashing with concentrated nitric acid (36) and copper was estimated with dithizone after wet ashing with concentrated sulfuric acid and hydrogen peroxide (36). The enzyme preparations were dialyzed for 48 hours against 0.02 M phosphate buffer of pH 7.8 for copper analysis, and against 0.02 M tris(hydroxymethyl)aminomethane acetate of pH 7.5 for iron analysis, with several changes of buffer. Protein was determined colorimetrically by the biuret method, crystalline bovine plasma albumin serving as the primary standard (37). Flavin was determined by measurement of the change in absorbancy at 450 μm of a 0.6 per cent perchloric acid filtrate (neutralized) on addition of dithionite. The release of flavin from the protein by acid treatment is not quantitative unless the preparation has been digested with crude trypsin (Nutritional Biochemicals, 1:300) in the ratio of 0.5 mg. of trypsin to 1 mg. of protein. The digestion is carried out for 1 hour at 38° in the presence of sodium cholate (1 mg. per mg. of SDC). The molar extinction coefficient of flavin was taken to be $11.3 \times 10^6$ sq. cm. per mole (38).

Total heme was determined as follows: Enzyme equivalent to about 2 mg. of protein was mixed with 10 μmoles of phosphate (pH 7.4) and 30 mg. of deoxycholate in a final volume of 1 ml. of water. The mixture was
incubated at 38° for 2 minutes. The optical density at the peak of the Soret band and the α-band was determined after reduction of the heme with hydrosulfite. The extinction coefficient for the Soret band of the reduced enzyme heme was assumed to be $1.4 \times 10^8$ sq. cm. per mole, which is the molar extinction coefficient of reduced cytochrome $c$ at 415 mμ, while that of the reduced α-band was assumed to be $0.287 \times 10^8$ sq. cm. per mole. The heme content was estimated on the basis both of the Soret and α-bands. If turbid, the solution should be clarified by centrifugation.

On several occasions heme was determined by an independent method, i.e. that of the pyridine hemochromogen (39). The two methods were in substantial agreement.

**Preparation of Enzyme Complex**

*Step 1*—Beef heart mitochondrial suspension prepared as described previously (40) was mixed with 4 volumes of 0.9 per cent KCl and allowed to stand for 12 hours at 5°. Even when not explicitly stated, all manipulations were carried out within the range of 0–5°. The suspension was then frozen slowly at −10°. The frozen suspensions can be stored indefinitely at that temperature.

*Step 2*—The frozen suspension was thawed and centrifuged for 1 hour at 3000 × g. The mitochondrial sediment was mixed with an equal volume of a mixture which was 0.14, 0.044, and 0.005 M with respect to sucrose, potassium chloride, and succinate. The mixture was thoroughly homogenized in a Waring blendor run at half maximal speed and then frozen.

*Step 3*—The thawed suspension was homogenized by stirring in a Potter-Elvehjem type of homogenizer (Teflon pestle), mixed with 0.11 volume of tert-amyl alcohol (cooled to 0°), and allowed to stand for 10 minutes. Then 0.04 volume of 0.5 M phosphate buffer of pH 7.6 was added, and the mixture was again mechanically homogenized. The mixture was centrifuged for 5 minutes at 30,000 r.p.m. in the No. 30 head of the Spinco preparative ultracentrifuge. The actual centrifugation time from starting the motor to switching off was 15 minutes in all. The precipitate should contain two layers, a loose top layer and a well packed bottom one. The supernatant fluid is poured off together with the loose top layer. The critical requirement of this step is the complete separation of the two sedimented layers. The supernatant suspension was diluted with 4 volumes of 8.5 per cent sucrose, homogenized, and centrifuged at 30,000 r.p.m. for 15 minutes under the conditions described above. The sediment was resuspended in 4 volumes of 0.25 M sucrose (0.005 M with respect to succinate) and the mixture was frozen.
Step 4—The frozen suspension was thawed and centrifuged at 40,000 r.p.m. for 5 minutes. The pellet was suspended in 2 volumes of 0.12 M potassium chloride and the mixture was thoroughly homogenized before addition of 0.11 volume of isobutyl alcohol (23, 27) at 0°. After 10 minutes, 0.04 volume of 0.5 M phosphate buffer, pH 7.2, was added. Again the suspension was homogenized thoroughly before centrifugation for 5 minutes at 17,000 r.p.m. in the No. 40 head of the Spinco preparative ultracentrifuge. The tightly packed sediment was discarded, while the supernatant suspension was thoroughly homogenized and then centrifuged at 40,000 r.p.m. for 5 minutes. The pellet was mixed with 3 to 4 volumes of 0.25 M sucrose solution and the suspension, after homogenization, was again centrifuged at 40,000 r.p.m. for 5 minutes. Finally the pellet was suspended in the minimal volume of 0.25 M sucrose and the suspension, after homogenization, was stored in the frozen state at −10°.

When, during the isobutyl alcohol fractionation, sedimentation of the particles of SDC has progressed almost to the tip of the centrifuge, the viscous red layer thus formed is perfectly clear. However, an immediate turbidity develops when this layer is disturbed. The optical effect appears to be analogous to that observed in concentrated solutions of tobacco mosaic virus (41) and is probably an expression of spontaneous orientation of asymmetric particles (42). This orientation is also manifest when shearing forces are applied to concentrated suspensions of SDC in dilute solutions of isobutyl alcohol.

Table I contains a summary of the degree of purification and the recovery of protein and activity during a typical isolation procedure. A convenient amount of starting material would be about 750 ml. of mitochondrial suspension diluted as in Step 2. The yield of SDC at the final stage is about 600 mg.

The final frozen preparation is stable when stored at −10° for several weeks.

Results

Enzymatic Activities of Purified Preparation—The succinic dehydrogenating complex prepared as described above catalyzes the oxidation of succinate by ferricyanide, indophenol, phenazine methosulfate, and cytochrome c, but not significantly by molecular oxygen. The preparation at the highest purity level contains traces, if any, of pyruvic, α-ketoglutaric, malic, isocitric, α-glycerophosphoric, choline, and reduced triphosphopyridine nucleotide (TPNH) dehydrogenases, cytochrome oxidase, and fumarase. Also none of the enzymes of the glycolytic or fatty acid oxidation cycle was present in measurable amount. Furthermore, nucleic acid
of either type is not present to any significant degree, as judged by the level of bound phosphate which is accounted for almost quantitatively by the amount of flavin dinucleotide in the preparation. At the stage of Step 3 in the purification, the main residual activities are diphosphopyridine nucleotide (DPNH) cytochrome \( c \) reductase and reduced cytochrome \( c \) oxidase. After the fractionation with isobutyl alcohol the preparation contains only traces of the latter activity. Particulate enzyme preparations appear to become inactivated by prolonged manipulation and this disability imposes a limit on the number of purification procedures which can be applied successfully.

In subsequent sections chemical and spectroscopic evidence bearing on the functional purity of SDC will be presented.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Purification of SDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step No.</td>
<td>Total protein</td>
</tr>
<tr>
<td>1. Original mitochondrial suspension</td>
<td>103.9</td>
</tr>
<tr>
<td>2. After suspension in sucrose</td>
<td>59.9</td>
</tr>
<tr>
<td>3. &quot; amyl alcohol fractionation</td>
<td>12.2</td>
</tr>
<tr>
<td>4. &quot; isobutyl alcohol fractionation</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* Micromoles of CO\(_2\) per 5 minutes per mg. in ferricyanide assay.
† Protein (mg.) \( \times \) specific activity.

Rates of Dehydrogenation with Various Electron Acceptors—Under the assay conditions described above the maximal rates of oxidation of succinate per minute per mg. of protein in the best preparations of SDC are with the different electron acceptors, respectively, ferricyanide (7\( \mu \)moles), phenazine methosulfate\(^2\) (5 \( \mu \)moles), cytochrome \( c \) (4.5 \( \mu \)moles), and indophenol (0.62 \( \mu \)mole).

The dependence of the velocity of oxidation of succinate on the concentrations of cytochrome \( c \), ferricyanide, phenazine methosulfate, indophenol, and succinate has been determined under the particular conditions specified in the description of the assay systems. The calculated \( K_m \) values obtained by a Lineweaver-Burk plot of the data are \( 2.8 \times 10^{-5} \) M for cytochrome \( c \), \( 7.8 \times 10^{-4} \) M for ferricyanide, \( 1.9 \times 10^{-4} \) M for phenazine methosulfate, and \( 2.5 \times 10^{-5} \) M for 2,6-dichlorophenolindophenol. The concentrations of electron acceptor at which saturation of the enzyme obtains are, respectively, \( 1.25 \times 10^{-4} \) M for cytochrome \( c \), \( 2.6 \times 10^{-2} \) M

\(^2\) It is assumed in the calculation that the oxidation of reduced phenazine methosulfate requires 2 atoms of oxygen per molecule of reductant.
for ferricyanide, $1 \times 10^{-3} \text{M}$ for phenazine methosulfate, and $8 \times 10^{-5} \text{M}$ for 2,6-dichlorophenolindophenol. The $K_m$ figure for succinate ($1.8 \times 10^{-4}$) is in fair agreement with the values of $2.7 \times 10^{-4}$ and 2.5 to $5.3 \times 10^{-4}$ reported, respectively, by Franke and Siewardt (43) and Thorn (44) in studies on crude particle suspensions.

**Fig. 1.** Absorption spectrum of SDC. The test solution contained 1.7 mg. of protein, 17 mg. of deoxycholate, and 118 μmoles of phosphate (pH 7) in 1 ml. The measurements were made in the automatic Beckman DK model.

**Fig. 2.** The spectrum of the pyridine hemochromogen of the heme of SDC. The test solution contained the extract from 1.64 mg. of SDC protein. The pyridine hemochromogen was prepared as described in the text. Dithionite was used to reduce the hemochromogen to the ferrous form.

**Absorption Spectrum**—The spectrum of the preparation of SDC, after treatment with deoxycholate in the oxidized and reduced state, is shown in Fig. 1 (cf. (30)). To convert the enzyme entirely to the oxidized state ferricyanide was added at 3 times the concentration level of the enzyme-bound heme. The enzyme was then washed free of any excess reagent by sedimentation and resuspension in fresh sucrose solution. The totally oxidized enzyme was reducible either by borohydride or dithionite. The peak of the Soret band is shifted from 412 μm for the oxidized to 428 μm for the reduced enzyme. In addition, there are two other bands in
the visible spectrum of the reduced enzyme, viz. at 525 and 560 m\(\mu\). The band maxima are the same whether a given preparation is examined directly or is first made soluble by deoxycholate.

The pyridine hemochromogen of SDC was prepared as follows: The enzyme suspension (2.33 mg. per ml.) was made 0.1 N with respect to KOH and then mixed with an equal volume of pyridine. The insoluble residue was removed by centrifugation. Fig. 2 shows the comparison of the spectrum for the pyridine hemochromogen of the hemes derived from SDC and that for the pyridine hemochromogen of authentic protohemin (45). The positions of the maxima are identical in the two cases, but there are some discrepancies particularly in the 590 and 520 m\(\mu\) regions. The spectrum suggests that protoheme is the main, if not the only, constituent heme of SDC, and also that some other non-heme substance or substances extracted into alkaline pyridine contribute to the absorption at 525 and 590 m\(\mu\). The prosthetic heme will have to be characterized by direct isolation and analysis before the identification with protoheme can be considered to be established.

Components of SDC—Table II summarizes the results of the analyses of preparations of SDC at the highest purity level. Flavin was determined after digestion of the preparation with crude trypsin, and the estimate was based on the change in absorbancy at 450 m\(\mu\) following reduction with dithionite. Only 10 to 30 per cent of the total extractable flavin was estimated as flavin dinucleotide in the \(\beta\)-amino acid oxidase test.

The estimate of heme based on the absorbancy of the \(\alpha\)-band was uniformly only 86 per cent of the estimate based on the Soret band. There are two uncertainties inherent in both these estimates: (a) the possibility of non-heme substances absorbing either in the Soret region or at 560 m\(\mu\) and (b) the possibility that the extinction coefficients for cytochrome \(c\), which are used as the basis of the calculation of heme content, are significantly different from those of SDC heme. We have decided that the average of the two separate estimates of heme might be a closer approximation to the actual heme content than either of the two estimates singly. When the iron equivalent to the averaged heme content is subtracted from the total iron, the difference is taken to represent the non-heme iron content of SDC. Copper was not detected in the preparations used in these analyses.

The ratio, moles of flavin to moles of heme to atoms of non-heme iron, is, in round numbers, 1:4:16. According to the data for titration of the enzyme with antimycin (see a subsequent section for details), \(1 \times 10^6\) gm. of SDC protein combine with 1 mole of antimycin. According to the flavin determination, \(1.16 \times 10^6\) gm. of protein contain 1 mole of flavin.
When the preparation of SDC was dialyzed against 0.01 M phosphate for 48 hours and then lyophilized, 60 per cent of the total dry weight was extractable into absolute alcohol heated to 60°. The alcohol-extracted material was soluble in organic solvents but not in water. No heme or protein was extracted by alcohol. Cold alcohol does not extract the enzyme-bound lipide. Ball and Cooper (46) have reported that 37 per cent of the dry weight of their particulate preparations of beef heart succinic dehydrogenase could be accounted for as lipide.

Whenever an activity or concentration is expressed per mg. of enzyme in the text of the present communication, this weight of enzyme is always on a lipide-free basis, since the weight is a calculation based on the biuret method of protein determination. When the contribution of lipide is included in the dry weight, a specific statement to that effect will be made.

**TABLE II**

*Composition of SDC Based on Averaged Determinations*

<table>
<thead>
<tr>
<th>Constituent</th>
<th>No. of determinations</th>
<th>μmoles × 10⁻³ X mg⁻¹</th>
<th>Microatoms × 10⁻³ X mg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavin</td>
<td>5</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>Heme (Soret)</td>
<td>6</td>
<td>3.53</td>
<td></td>
</tr>
<tr>
<td>&quot; (α-band)</td>
<td>6</td>
<td>3.07</td>
<td></td>
</tr>
<tr>
<td>&quot; (average of two estimates)</td>
<td>3</td>
<td>3.28</td>
<td></td>
</tr>
<tr>
<td>Total iron</td>
<td>3</td>
<td></td>
<td>17.4</td>
</tr>
<tr>
<td>Non-heme iron</td>
<td>3</td>
<td></td>
<td>14.1</td>
</tr>
<tr>
<td>Antimycin titer</td>
<td>12</td>
<td>1.01</td>
<td></td>
</tr>
</tbody>
</table>

**Non-Heme Iron**—Non-heme iron is not released from the enzyme under the following conditions: cold 5 per cent trichloroacetic acid, pH 1 for 20 minutes at 0°, 50 per cent alcohol for 20 minutes at 0°, pH 9 for 1 hour at 100°. It is, however, released in whole or part under the following conditions: 1 hour at 100° at neutral pH (about 5 per cent yield), 12 hours in N hydrochloric acid at room temperature (about 25 per cent yield), 1 hour at 100° in N HCl (100 per cent yield).

The enzymatic activity of SDC is not inhibited by relatively high concentrations of chelating agents for iron and other metals such as Versene, 8-hydroxyquinoline, o-phenanthroline, and dithizone. This insensitivity of SDC is in line with the tenacity with which the metallic components of SDC (heme and non-heme iron) are bound to the protein.

**Reversible Reduction and Oxidation**—When a purified suspension of SDC is reduced with dithionite or borohydride, and the absorbancy of the reduced form is measured against that of the oxidized form as a blank, a difference spectrum such as shown in Fig. 3 is obtained. The reduction
SUCCINIC DEHYDROGENASE

and oxidation of the hemes can thus be followed by changes either in the Soret region or at 560 m\(\mu\) (cf. also (30)). When chemical reducing agents are replaced by succinate, a similar difference spectrum is obtained, but the magnitude of the change is less. If we can assume that the value for \(+\Delta D_{430} m\(\mu\) - \(\Delta D_{410} m\(\mu\)\) of the dithionite-reduced enzyme compared to the oxidized control represents 100 per cent reduction, then it would follow that about 80 per cent of the total heme in the preparation is reducible by succinate within 1 minute. With inactivation of the enzyme this per-

Fig. 3. Difference spectrum. An aliquot of a suspension of SDC washed free of succinate and converted to the oxidized state was used in the control cuvette, while another aliquot of the same suspension was used in the experimental cuvette to which either succinate (1 \(\mu\)mole per ml.) or \(\text{Na}_2\text{S}_2\text{O}_4\) has been added. The cuvettes contained 3.3 mg. of enzyme protein, 5 \(\mu\)moles of phosphate, 0.75 \(\mu\)mole of cyanide, and 50 \(\mu\)moles of tris(hydroxymethyl)aminomethane (pH 8.5) per ml.

centage declines and reaches zero. In some preparations\(^3\) complete reduction of the heme is attained after some minutes but not immediately. Ferricyanide instantaneously reoxidizes the reduced heme in the preparation and thus is a useful reagent for converting reduced enzyme to the oxidized state. The ferricyanide-treated particles are then washed by centrifugation and suspension in fresh sucrose before a test is made. The ferrohemes of SDC are rapidly oxidized by cytochrome c (cf. Fig. 4), phenazine methosulfate, benzoquinone, and indophenol, while the ferrihemes are reducible by succinate but not by DPNH.

\(^3\)These preparations contained cytochrome c in bound state. The binding of cytochrome c by preparations of SDC is described in a later section.
When SDC is heated at 55° for various lengths of time, cytochrome c reductase activity declines in a regular fashion which is exactly paralleled by the decline in the percentage of endogenous heme reducible by succinate. For example, after exposures of 4, 6, and 8 minutes cytochrome c reductase activity was, respectively, 62, 22, and 11 per cent of the original activity, while the extent of hemin reduction was 68, 20, and 15 per cent of that of the untreated enzyme (taken as 100 per cent).

**Inhibition by Antimycin**—SDC is completely inhibited by antimycin A (47) at unusually low levels. This property has been taken advantage of by Reif and Potter (48) for direct titration of crude preparations of succinic dehydrogenase. The inhibition by low concentrations of antimycin applies only to the reduction of cytochrome c. No inhibition is observed with the other electron acceptors. The reaction of the enzyme

![Figure 4](http://www.jbc.org/)

**Fig. 4.** Spectrophotometric evidence of the oxidation of the hemes of SDC by cytochrome c. A succinate-free suspension of SDC was used to obtain the difference spectrum between the dithionite-reduced enzyme and the oxidized enzyme by the procedure described in the legend for Fig. 3. Then 0.0166 μmole of cytochrome c, equivalent to 1.3 times the heme content of the enzyme, was added to both the experimental (reduced) and control (oxidized) cuvettes. The difference spectrum was measured again. Each cuvette contained 3 mg. of enzyme protein, 62.5 μmoles of phosphate (pH 7.4), 1.25 μmoles of cyanide, 22.5 mg. of deoxycholate, and 0.05 mg. of benzyl viologen. The viologen was used as an internal indicator for detection of traces of residual dithionite which had not been removed by aeration.
SUCCINIC DEHYDROGENASE

with antimycin is not instantaneous. Thus, when the enzyme is titrated with antimycin (a sample prepared according to Dunshee et al. (49) and supplied by Dr. F. Strong) at both 30° and 0°, some 100 times more antimycin is required for complete inhibition at 0° than at 30° (cf. Table III). In practice we have incubated the enzyme with antimycin at 30° for 5 minutes before conducting the assay at 0°. Once the antimycin-enzyme complex is formed at 30°, then there is an advantage in conducting the assay at 0°, since the stability of the complex is apparently greater at the lower temperature.

The average minimal molar concentration of antimycin required for complete inhibition is \(1.0 \times 10^{-3}\) μmole per mg. (cf. Table II), which is

$$\text{Table III}$$

Antimycin Titer As Function of Time of Incubation, Temperature of Incubation, and Temperature of Assay

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Time of incubation</th>
<th>Temperature of incubation °C.</th>
<th>Temperature of assay °C.</th>
<th>Antimycin titer μmoles (\times 10^{-3}) per mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>75</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>10</td>
<td>10</td>
<td>39</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>10</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>30</td>
<td>30</td>
<td>4.0</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>10</td>
<td>10</td>
<td>3.6</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>30</td>
<td>10</td>
<td>1.7</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>30</td>
<td>10</td>
<td>1.9</td>
</tr>
<tr>
<td>8</td>
<td>15</td>
<td>30</td>
<td>10</td>
<td>1.6</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>30</td>
<td>0</td>
<td>0.71</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>30</td>
<td>0</td>
<td>1.14</td>
</tr>
</tbody>
</table>

approximately one-fourth the heme content and nearly equivalent to the flavin content. The value for complete inhibition is calculated by extrapolation from the antimycin concentrations which produce partial inhibitions in the range of 20 to 40 per cent (cf. Fig. 5). The rapid reduction of SDC-bound heme by succinate is inhibited completely at the same minimal concentration at which reduction of cytochrome c is suppressed (cf. Fig. 6). Exposure of SDC to BAL under the conditions outlined by Slater (10-14) does not lead to any inhibition of the capacity to react with cytochrome c.

Incorporation of Cytochrome c—When SDC suspended in 8.5 per cent sucrose is incubated with excess cytochrome c (0.25 μmole per 5 mg. of enzyme protein) for 30 minutes at 0°, a considerable amount of cytochrome c is taken up by the enzyme (cf. Tsou (50) for similar results). A maximum of 5 moles of cytochrome c per mole of endogenous heme is taken
up. The near equivalence of the cytochrome taken up \((17.7 \times 10^{-3}\ \mu\text{mole per mg.})\) and the non-heme iron present in the preparation \((14.1 \times 10^{-3}\ \text{microatom per mg.})\) are quite striking. The particulate enzyme can be washed indefinitely with sucrose by repeated centrifugation and suspension without release of this bound cytochrome \(c\). When, however, the enzyme is exposed to the conditions which obtain in Steps 3 and 4 of the method of preparation, all the cytochrome \(c\) which had been bound is released. Exposure to 10 per cent isobutyl alcohol or tert-amyl alcohol is, in fact, the condition which leads to release.

Antimycin does not inhibit this binding of cytochrome \(c\) nor does it release cytochrome \(c\) already taken up by the enzyme.

The cytochrome \(c\) taken up by the enzyme is rapidly reduced by succinate and behaves like an internal indicator of the state of oxidation-reduction of the enzyme. Also the reduction is partly antimycin-sensitive, in contrast to the complete antimycin sensitivity of the endogenous heme of SDC.

Modification of Absorption Bands—The \(\alpha\)- and \(\beta\)-bands of the hemes
of SDC are decidedly different from the corresponding bands of pyridine hemochromogens or of cytochrome c and, in fact, from any synthetic or protein-free iron porphyrin compounds. These differences are two-fold: (a) the bands, particularly the $\beta$, are not sharp and show a tendency to flatten out, and (b) the difference between the absorbancy of oxidized and reduced enzyme in the region of the $\alpha$- and $\beta$-bands is much smaller than is normally found. It was of interest to determine whether cytochrome c after becoming bound to the enzyme would show these anomalies of spectrum. Since 5 times as much cytochrome c is taken up as the amount of endogenous heme present in the preparation, the extent of interference of endogenous heme with the spectrum of bound cytochrome c is not excessive. Examination of the spectrum shows that cytochrome c undergoes a change of spectrum as a consequence of attachment to the enzyme. Thus, clearly, the spectral anomalies noted above are related to the manner in which heme is bound to the protein. According to Shibata, Benson, and Calvin (51), orientation of hemes in layers with interaction between the central iron atoms such as obtain in the solid state leads to precisely the modification in spectrum exhibited by the hemes of SDC.

Reaction with Cytochrome c—Since inhibitory metals are present occasionally in samples of cytochrome c (Sigma), the addition of albumin to the assay system is essential. Versene ($0.001 M$) is about 80 per cent as effective as albumin. When suspensions of SDC are washed exhaustively with sucrose solutions (8.5 per cent), the cytochrome c reductase activity usually declines. Partial restoration of activity can be accomplished by heating the washed preparation for several minutes at 45-50°.

**SUMMARY**

1. A particulate form of succinic dehydrogenase (SDC) has been isolated from beef heart mitochondria 10 to 15 times more active than the starting mitochondrial suspension. The isolation procedure which is based on fractionation with aqueous solutions of tert-amyl alcohol and isobutyl alcohol is described in detail.

2. The final preparations of SDC are essentially free from all other dehydrogenases of the citric acid cycle, the enzymes of the fatty acid oxidation cycle, the glycolytic enzymes, fumarase, TPNH, $\alpha$-glycerolphosphoric and choline dehydrogenases, and cytochrome oxidase. They contain no nucleic acid.

3. 1 mg. of SDC at the stage of highest purity catalyzes the oxidation of 7.5, 5.0, 4.5, and 0.62 $\mu$moles of succinate per minute at 38° in the presence of ferricyanide, phenazine methosulfate, cytochrome c, and 2,6-dichlorophenolindophenol, respectively.
4. Preparations of SDC contain only one type of heme component which shows a single band in the oxidized state at 412 m\(\mu\) and three bands in the reduced state, viz. at 428, 525, and 560 m\(\mu\). The pyridine hemo-chromogen of the heme component of SDC resembles that of protoporphyrin.

5. Flavin, heme, and non-heme iron, respectively, are present in SDC at the following average levels: \(0.86 \times 10^{-3}\) \(\mu\)mole per mg., \(3.28 \times 10^{-3}\) \(\mu\)mole per mg., and \(14.1 \times 10^{-3}\) microatom per mg. Thus for each mole of flavin there are in round numbers 4 moles of heme and 16 atoms of non-heme iron. 10 to 30 per cent of the flavin can be accounted for in the form of flavin adenine dinucleotide.

6. 60 per cent of the dry weight of SDC is extractable by absolute alcohol or ether after heat denaturation of the protein.

7. The non-heme iron of SDC is not released except after prolonged hydrolysis in strong acid.

8. The heme of SDC is reduced by succinate to the extent of 80 per cent. The reduced heme is oxidized by ferricyanide, phenazine methosulfate, quinone, or cytochrome \(c\). The proportion of reducible heme decreases as the activity of the enzyme declines and is reduced to zero with complete inactivation of the enzyme.

9. Antimycin \(A\) at low levels inhibits the reduction both of cytochrome \(c\) and of SDC-bound heme but has no effect on the oxidation of succinate by any other electron acceptors.

10. Antimycin \(A\) reacts with SDC in what appears to be a stoichiometric fashion. A unit of SDC containing 1 mole of flavin combines roughly with 1 mole of antimycin.

11. SDC binds cytochrome \(c\) to the extent of 5 moles of cytochrome per mole of endogenous heme.

This investigation was supported by a research grant, No. H-458(C5), from the National Heart Institute of the National Institutes of Health, Public Health Service, and also by a grant from the Nutrition Foundation, Inc. We are indebted to Oscar Mayer and Company for generously supplying us with fresh slaughter-house material. It is a pleasure to record our thanks to Dr. H. Reinert, Dr. D. R. Sanadi, and Dr. H. R. Mahler for their invaluable advice and many suggestions and to Dr. Robert Basford for carrying out determinations of heme and flavin.

Addendum—Since this paper was submitted for publication, the presence of two heme components with \(\alpha\)-bands at 555 and 562 m\(\mu\), respectively, has been demonstrated in SDC. These hemes can be extracted from denatured SDC and separated quantitatively from one another. The lack of symmetry and sharpness of the bands of SDC can thus be referred to the presence of two heme components with overlapping spectra.
SUCCINIC DEHYDROGENASE

BIBLIOGRAPHY

STUDIES ON THE TERMINAL ELECTRON TRANSPORT SYSTEM: I. SUCCINIC DEHYDROGENASE
D. E. Green, S. Mii, P. M. Kohout and With the technical assistance of Howard Tisdale


Access the most updated version of this article at http://www.jbc.org/content/217/2/551.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/217/2/551.citation.full.html#ref-list-1