Succinate Dehydrogenase. I. Purification, Molecular Properties, and Substructure*

K. A. Davis† and Y. Hatefi‡

ABSTRACT: Soluble succinate dehydrogenase has been purified from succinate–coenzyme Q reductase (complex II) particles by a simple procedure involving extraction in the presence of chaotropic agents followed by a two-step ammonium sulfate fractionation of the extract. The degree of extraction of succinate dehydrogenase from complex II is a function of the concentration and potency of the chaotropic agent used. The enzyme is homogeneous when examined by ultracentrifugation and moves as a single peak when chromatographed on Agarose.

Electrophoresis of succinate dehydrogenase treated with sodium dodecyl sulfate and 2-mercaptoethanol on polyacrylamide has shown that the preparation is essentially free of protein impurities. It is also free of any detectable lipids. Succinate dehydrogenase has a molecular weight of approximately 97,000 ± 5%, and contains 1 mole of covalently bound flavin, 7–8 g-atoms of iron, and 7–8 moles of acid-labile sulfide/97,000 g of protein. The enzyme is composed of two "subunits." One is a flavoprotein with a molecular weight of approximately 70,000 ± 7%. It contains iron and labile sulfide in an approximate ratio of 4 g-atoms of iron and 4 moles of labile sulfide per mole of flavin. The other subunit is an iron–sulfur protein with a molecular weight of approximately 27,000 ± 5%.

Succinate dehydrogenase from beef heart is a flavoprotein containing iron and labile sulfide, and is exclusively associated with the electron-transport system of the mitochondrial inner membrane. A soluble form of the enzyme was first prepared in 1954 by Singer and Kearney (Singer, 1966). This preparation was reported to have a molecular weight of approximately 200,000, and to contain 4.2–5.0 nmoles of flavin/mg of protein (Kearney, 1960) and 2–4 g-atoms of iron/mole of flavin. A number of preparations of succinate dehydrogenase have been reported since (for literature, see Singer, 1966). Among these the preparation extensively studied by King (1966) is of particular interest. This preparation, originally obtained by a modification of the isolation method of Wang *et al.* (1956), was shown by Keilin and King (1958) to differ from the Singer-type succinate dehydrogenase in one important respect. The Keilin–King preparation, but not the Singer-type preparation, was capable of electron transfer to the respiratory chain. Subsequent studies of King (1964) showed that the preparation contained 2.8–3.6 nmoles of flavin/mg of protein (average 3.04 ± 0.35) and approximately 8 g-atoms of iron and 8 moles of labile sulfide per mole of flavin.

A third preparation from mitochondria, which has high succinate dehydrogenase activity is complex II (Ziegler, 1961; Hatefi, 1966). This complex represents the segment of the respiratory chain from succinate to coenzyme Q (ubiquinone). It contains approximately 5 nmoles of flavin/mg of protein, and 7–8 g-atoms of nonheme iron and 7–8 moles of labile sulfide per mole of flavin. In addition to a highly active succinate dehydrogenase, preparations of complex II also contain 1 mole of cytochrome *b*/mole of flavin, small amounts of cytochrome c_1, other proteins, and lipid (Ziegler, 1961; Baginsky and Hatefi, 1969). In spite of the significant advances made in the laboratories of Singer (1966) and King (1966) on succinate dehydrogenase, a comparison of the above data pointed to the fact that much still remained to be learned about the molecular properties, composition, and catalytic activities of this enzyme.

Our studies on the resolution and reconstitution of complex II with the aid of chaotropic agents (Hatefi and Hanstein, 1969) have yielded a preparation of succinate dehydrogenase with molecular properties considerably different from the previous preparations of others. The present communication will show that succinate dehydrogenase from beef heart mitochondria has a molecular weight of approximately 97,000, and contains 1 mole of covalently bound flavin, 7–8 moles of acid-labile sulfide, and 7–8 g-atoms of iron per mole. The enzyme is composed of two unlike proteins: a flavoprotein containing iron and labile sulfide in an approximate ratio of 1 flavin:4 irons:4 labile sulfides, and an iron–sulfur protein with a molecular weight less than half that of the flavoprotein. The accompanying communication (Hanstein *et al.*, 1971) will describe the catalytic and reconstitution properties of succinate dehydrogenase as well as changes in the activities of complex II and purified succinate dehydrogenase upon resolution with chaotropic agents (for a preliminary report on this work, see Hatefi *et al.*, 1970).

Methods and Materials

Complex II was prepared from beef heart mitochondria as described earlier (Baginsky and Hatefi, 1969). Iron and flavin were estimated as before (Hatefi and Stempel, 1969). Acid-labile sulfide was estimated by the method of Fogo and Popowsky (1949) as modified by Davis *et al.*[1] Results were calculated on the basis of sodium sulfide standard curves, which gave an A_{667} value of 12.1 l./mmole. For absorption spectroscopy and ultracentrifugation, Beckman instruments DK-2A and E were used, respectively.

* From the Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California 92037. *Received October 7, 1970.* Supported by U. S. Public Health Service Grant AM-08126.

† Recipient of a San Diego County Heart Association advanced research fellowship.

‡ To whom to address correspondence.

[1] K. A. Davis, P. Tejada, and Y. Hatefi, in preparation.

Protein was estimated by the methods of Lowry *et al.* (1951), biuret (Gornall *et al.*, 1949), Kjeldahl nitrogen, and by dry weight. The first three methods were used for preparations which contained lipid; all four were used for estimating the protein of succinate dehydrogenase. The reason for the multiple protein determinations was that Bernath and Singer (1962) had reported a substantial overestimation of succinate dehydrogenase protein by the biuret method. In agreement with their report, we found that biuret overestimated the protein of several preparations of succinate dehydrogenase by 22% as compared to the other three methods, which were in excellent agreement with one another. Similarly, as compared to Lowry and Kjeldahl nitrogen (conversion factor of 0.625 was used), biuret overestimated the proteins of complex II and submitochondrial preparations by about 10%. In the case of complex II, this overestimation was over and above the cytochrome contribution to the biuret optical density at 540 nm, which gave rise to a further overestimation of approximately 14%. The biuret correction factor of 22% for succinate dehydrogenase was applied, without further correction, to the biuret values obtained for the proteins of the subunits of the enzyme. This concerns only the data of Table I and Figure 2, and the reason for not determining the correction factor for each subunit separately was because as prepared each fraction was cross-contaminated with small, but nevertheless appreciable, amounts of the other. Purification of the subunits of succinate dehydrogenase is in progress.

Cytochrome *c* (type III), urease, bovine serum albumin, and yeast alcohol dehydrogenase were obtained from Sigma Chemical Co.; ribonuclease, trypsin, glyceraldehyde 3-phosphate dehydrogenase, ovalbumin, and phosphorylase *a* from Worthington; and catalase from Boehringer. Agarose was Bio-Gel A-0.5 m, 200–400 mesh from Bio-Rad, and Merck thin-layer chromatographic sheets (silica gel F-254) on plastic were from Brinkman Instruments, Inc.

Results

1. Preparation. Succinate dehydrogenase was extracted from preparations of complex II in the presence of the chaotropic agent $NaClO_4$. The principle of the action of chaotropic agents has been discussed previously by Hatefi and Hanstein (1969) and Davis and Hatefi (1969). In brief, the stability of most membranes and enzyme complexes is largely due to the prevalence of hydrophobic interactions among the proteins and the lipids. The strength of hydrophobic interactions appears to be directly related to the ordered structure of the surrounding water and to the large negative entropies involved in transfer of the apolar components of membranes into the aqueous phase. Chaotropic agents disorder water. As a consequence hydrophobic bonds are weakened, the entropic barrier for the transfer of apolar components of membranes into the aqueous phase is lowered, and membrane destabilization takes place.

Preparations of complex II were suspended at a protein concentration of 10–12 mg/ml in 50 mM Tris-HCl (pH 8.0), containing 20 mM sodium succinate and 5 mM dithiothreitol. $NaClO_4$ was then added from an 8 M solution to a final concentration of 0.4 M and the mixture was allowed to stand in an ice bath for 20 min before centrifugation for 80 min at 49,000 rpm in rotor no. 50 of Spinco Model L ultracentrifuge. The supernatant obtained after centrifugation was fractionated with neutralized, saturated ammonium sulfate, and the fraction precipitating between 36 and 50% salt saturation was collected after 10-min centrifugation at 40,000 rpm and immediately frozen in liquid nitrogen. This preparation of succinate dehydrogenase shall be referred to as SD-A.

The pellet after the first centrifugation was taken up in the Tris-succinate–dithiothreitol buffer described above, and adjusted to 80% of the original volume. It was then reextracted as before in the presence of 0.75 M $NaClO_4$. The supernatant obtained after 80-min centrifugation at 49,000 rpm was fractionated with saturated ammonium sulfate, and the fraction precipitating between 34.5% and 50% salt saturation was collected and frozen in liquid nitrogen as before. This preparation of succinate dehydrogenase shall be referred to as SD-B. All the operations described above were conducted at 0–4°.

The fractions precipitating at 36 and 34.5% ammonium sulfate saturation also contain some soluble succinate dehydrogenase, which can be obtained after dissolving the two precipitates in a small volume of Tris-succinate–dithiothreitol buffer and centrifuging the suspension for 60 min at 40,000 rpm in order to remove some insoluble material which contains unwanted proteins of complex II. Although useful for certain enzymatic studies, this preparation of succinate dehydrogenase is impure and will not be discussed in this paper. The complex II pellet after the second extraction with 0.75 M $NaClO_4$ still contains some succinate dehydrogenase, which can be solubilized by further extraction with $NaClO_4$. However, the yield of succinate dehydrogenase at this point is very small as compared to SD-A and -B. In the procedure described above, the yield of purified succinate dehydrogenase (A plus B only) corresponds to 55–60% of the total flavin of complex II. The flavin content of complex II after the second perchlorate extraction is 1.3–1.5 nmoles/mg of protein, which indicates that about 82–85% of succinate dehydrogenase has been extracted.

2. Effects of Chaotrope Concentration and Complex II Concentration on the Yield of Succinate Dehydrogenase. As seen in Figure 1A, there is a linear relationship in the range shown (0.4–0.8 M $NaClO_4$) between the concentration of $NaClO_4$ and the amount of succinate dehydrogenase activity solubilized after a single extraction of complex II. This linear relationship holds between the concentration of $NaClO_4$ and the yield of purified succinate dehydrogenase even after the ammonium sulfate fractionation steps described above for isolation of SD-A. Figure 1A also shows that the specific activity of the solubilized enzyme in the PMS[2] reductase assay is the same regardless of the concentration of $NaClO_4$ (0.4–0.8 M) used for its extraction. The reason that in Figure 1A extrapolation to zero $NaClO_4$ concentration still shows some 10% resolution might be in part because the buffer employed leads to some resolution when the suspension of complex II is stored at −20° (Baginsky and Hatefi, 1969). Figure 1B shows a plot of the concentration of complex II in the extraction mixture at 0.4 M $NaClO_4$ *vs.* the succinate dehydrogenase activity and the amount of iron which appears in the extract after removal of insoluble complex II by centrifugation. Once again, it is seen that in the range tested (2.5–10 mg of complex II/ml) there is a nearly linear relationship between the concentration of complex II in the extraction mixture and the activity or the amount of succinate dehydrogenase rendered soluble. These results demonstrate the effectiveness and the degree of control with which chaotropic agents can be used for the resolution of an enzyme complex. The selectivity of the technique is demonstrated in Figure 6.

[2] Abbreviations used are: PMS, phenazine methosulfate; FP and IP, the flavoprotein and the iron–sulfur protein fractions of succinate dehydrogenase; SDS, sodium dodecyl sulfate.

TABLE I: Composition of Succinate Dehydrogenase, FP, and IP.

Preparation	Flavin (nmoles/mg of Protein)	Iron (ng-atoms/mg of Protein)	Acid-Labile Sulfide (nmoles/mg of Protein)
SD[a]	10.3 ± 4%[b]	70–80	70–80
FP[c]	12–13	45–55	45–55
IP	<0.5	95–110	90–100

[a] SD = succinate dehydrogenase. [b] Average of 12 preparations. [c] For analytical studies, FP was dissolved in 0.01 N NaOH. In the case of flavin analysis, tryptic digestion of FP was performed after neutralizing the solution with an equivalent amount of acid.

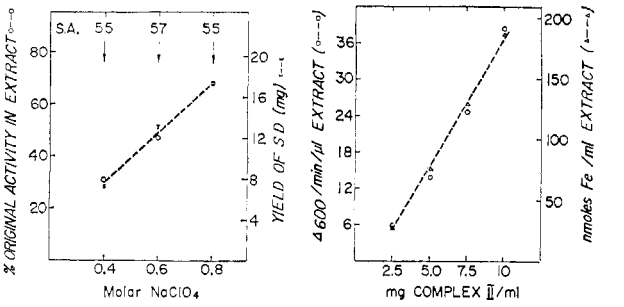

FIGURE 1: (A, left) Correlation between the concentration of $NaClO_4$ and the amount of succinate dehydrogenase extracted from complex II. Conditions were the same as described for preparation of SD-A at 12 mg of complex II protein/ml. Left-hand ordinate: per cent of complex II PMS reductase activity found in the 49,000-rpm supernatant after extraction with $NaClO_4$. Right-hand ordinate: yield of succinate dehydrogenase after ammonium sulfate fractionation. S.A.: specific activity of succinate dehydrogenase isolated at the $NaClO_4$ concentrations indicated. (B, right) Correlation between concentration of complex II and the amount of succinate dehydrogenase extracted from it with 0.4 M $NaClO_4$. Left-hand ordinate: PMS reductase activity of the extract. Right-hand ordinate: amount of iron in the extract, which is proportional to the amount of succinate dehydrogenase rendered soluble. Conditions were the same as described for the preparation of SD-A before ammonium sulfate fractionation.

As seen above, the resolution of complex II by chaotropic agents was achieved at ice-bath temperature. Indeed, preincubation of the complex II–perchlorate mixture for 20 min at 10, 20, 30, and 38°, instead of at 0°, prior to separation of succinate dehydrogenase by centrifugation had no effect upon the yield and activity of the enzyme found in the soluble fraction. This is quite unlike complex I whose resolution by chaotropic agents was shown earlier to have a high degree of temperature dependence and could not take place below 10° (Davis and Hatefi, 1969).

3. Composition. The composition of SD-B with respect to covalently bound flavin, iron, and acid-labile sulfide is given in Table I. As compared to complex II, purified succinate dehydrogenase contains twice as much flavin, iron, and labile sulfide per milligram of protein. Thus, in succinate dehydrogenase the ratio of iron : labile sulfide : flavin remains the same as in complex II. This ratio is approximately 7–8 : 7–8 : 1.

Preparations of succinate dehydrogenase appear to be free of lipids. Chloroform–methanol extracts of the enzyme were chromotographed on thin-layer chromatographic sheets, developed in a medium composed of chloroform–methanol–water in the ratio of 70:30:5, and placed in an iodine-saturated atmosphere for visualization of the lipids. Mitochondrial extracts were used as control, and by comparison extracts of succinate dehydrogenase showed no phospholipid spots. As will be seen in subsequent sections. SD-B is also free of any detectable cytochromes. Preparations of SD-A are not quite as pure as SD-B (see section 6). Therefore, values obtained for the flavin content of SD-A preparations were consistently about 6% lower than those for SD-B preparations.

4. Substructure. Succinate dehydrogenase has been resolved by chaotropic agents (or by sodium dodecyl sulfate, see section 6) into two unlike proteins. Preparations of the enzyme were dissolved at a protein concentration of 5–7 mg/ml in 50 mM sodium phosphate (pH 7.4–7.6), containing 5 mM dithiothreitol and 20 mM succinate. Sodium trichloroacetate, a potent chaotropic agent, was added from a 4 M solution to a final concentration of 0.6–0.7 M, the mixture was frozen in liquid nitrogen and thawed at room temperature three times. This treatment resulted in considerable protein precipitation. The mixture was then centrifuged for 2 min at 40,000 rpm, and the supernatant and the sedimented protein were thus separated. The former fraction, which is water soluble, was shown by its composition and spectral analysis (see section 5) to be an iron–sulfur protein, and the sedimented fraction to be a flavoprotein containing iron and labile sulfide. These two fractions will be referred to in subsequent sections and in the accompanying communication, respectively, as IP and FP. The compositions of FP and IP with regard to flavin, iron, and labile sulfide are given in Table I. It is seen that FP contains about 4 g-atoms of iron and 4 moles of labile sulfide per mole of flavin, whereas IP contains large amounts of iron and labile sulfide but negligible amounts of flavin. Since all the flavin in succinate dehydrogenase is covalently bound, the possibility of preferential adherence of flavin to the FP fraction is ruled out, and the presence of two different protein moieties in succinate dehydrogenase is clearly indicated from the data of Table I (see also section 6).

The resolution of succinate dehydrogenase into FP and IP can be achieved also by other chaotropic agents such as $NaClO_4$, NaSCN, or guanidine hydrochloride, and the effective concentration of each depends, as might be expected, on its chaotropic potency (see accompanying communication, Hanstein *et al.*, 1971). It is preferable, however, to avoid the use of NaSCN for this purpose because the SCN^- ion will combine with the iron moieties of FP and IP. The resolution of succinate dehydrogenase into FP and IP and the precipitation of FP are impeded at pH values above 7.8. Succinate and dithiothreitol are not necessary components of the resolution mixture; in fact, for estimation of the labile sulfide content of FP and IP, dithiothreitol should be omitted from the resolution mixture. However, the presence of dithiothreitol stabilizes both FP and IP, which under aerobic conditions tend to bleach rapidly and lose labile sulfide.

5. Absorption Spectra. The absorption spectra of succinate dehydrogenase, FP, and IP in the visible region are shown in Figure 2. Trace 1 of panel A is the absorption spectrum of oxidized succinate dehydrogenase. Treatment of this material with sodium mersalyl leads to the destruction of the iron–labile sulfide spectrum of the enzyme, thus leaving behind, as shown by trace 2 of panel A, the contribution of flavin to the oxidized spectrum of succinate dehydrogenase. Subtraction of the

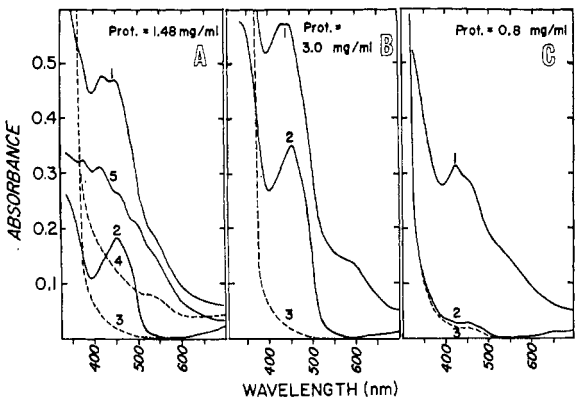

FIGURE 2: Absorption spectra of succinate dehydrogenase, FP, and IP. (A) Succinate dehydrogenase in 30 mM sodium phosphate and 1.6 M urea at pH 9.2. Trace 1, oxidized succinate dehydrogenase; trace 2, after addition of solid sodium mersalyl to oxidized succinate dehydrogenase of trace 1; trace 3, after addition of $Na_2S_2O_4$ to the mersalyl-treated succinate dehydrogenase of trace 2; trace 4, after addition of $Na_2S_2O_4$ to the succinate dehydrogenase of trace 1; trace 5, oxidized succinate dehydrogenase of trace 1 minus the mersalyl-treated succinate dehydrogenase of trace 2. Trace 5 was recorded by the spectrophotometer using material of trace 1 in the sample compartment and that of trace 2 in the reference compartment. (B) FP obtained from succinate dehydrogenase in 50 mM sodium phosphate (pH 7.4), containing 20 mM succinate and 5 mM dithiothreitol with the use of 0.7 M sodium trichloroacetate and three times freeze-thawing followed by 3-min centrifugation at 30,000 rpm. The precipitated FP so obtained was washed once with 50 mM Tris-HCl containing 5 mM dithiothreitol, then dissolved in 0.02 N NaOH and diluted with one volume of water. Trace 1, FP as prepared; trace 2, after addition of sodium mersalyl to the material of trace 1; trace 3, after addition of $Na_2S_2O_4$ to the material of trace 2. (C) IP obtained from succinate dehydrogenase in 50 mM sodium phosphate (pH 7.4), containing 20 mM succinate with the use of 0.7 M sodium trichloroacetate and three times freeze-thawing followed by 3-min centrifugation at 30,000 rpm. To 0.4 ml of the supernatant (IP) so obtained, 0.2 ml of a solution 2 M in urea and 0.025 N in NaOH was added before spectroscopy. Trace 1, IP as prepared; trace 2, IP of trace 1 after treatment with sodium mersalyl; trace 3, material of trace 2 after treatment with $Na_2S_2O_4$. The media used in these experiments were found to prevent development of turbidity upon addition of mersalyl. They did not alter the spectral characteristics of the samples.

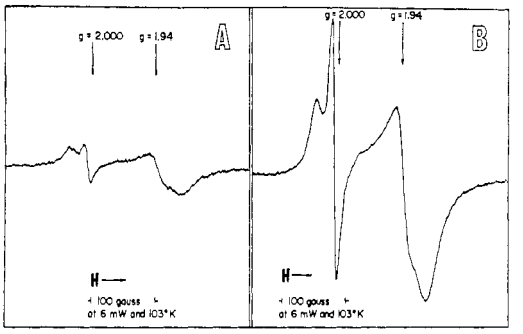

FIGURE 3: Electron paramagnetic resonance spectra of succinate dehydrogenase before (A) and after (B) treatment with succinate. Protein concentration = 8.8 mg/ml of 50 mM sodium phosphate (pH 7.4). The succinate dehydrogenase of part B was incubated with 20 mM succinate for 2.5 min at 38° before freezing in liquid nitrogen. Microwave frequency, 9.085 Gc; modulation width, 5 G.

spectrum of mersalyl-treated enzyme from untreated succinate dehydrogenase of the same concentration by differential spectrophotometry resulted in trace 5, which is equivalent to the contribution of the iron–labile sulfide system of succinate dehydrogenase to the spectrum of its oxidized form. Treatment of the sodium mersalyl treated enzyme with $Na_2S_2O_4$ resulted in reduction of its flavin and in trace 3 of panel A, thus indicating that flavin, iron, and labile sulfide are the only components of succinate dehydrogenase responsible for its absorption spectrum between 400 and 700 nm. Treatment of the oxidized succinate dehydrogenase of trace 1 with $Na_2S_2O_4$ resulted in trace 4, which is probably equivalent to the absorption spectrum of the reduced form of the iron–labile sulfide system of succinate dehydrogenase since $Na_2S_2O_4$ treatment bleaches the flavin completely.

Panel B of Figure 2 shows the spectra of oxidized FP (trace 1), oxidized FP treated with sodium mersalyl (trace 2), and sodium mersalyl treated FP after reduction by $Na_2S_2O_4$ (trace 3). It is seen that by comparison to succinate dehydrogenase, the contribution of iron–labile sulfide to the oxidized spectrum of FP is considerably diminished and the contribution of flavin enhanced. Qualitatively, the spectrum of FP is very similar to the spectrum of mitochondrial DPNH dehydrogenase in which the ratio of flavin:iron:labile sulfide is also 1:4:4 (Hatefi and Stempel, 1969).

Panel C of Figure 2 shows the spectrum of the IP fraction of succinate dehydrogenase. Trace 1 is the spectrum of oxidized IP, trace 2 is after treatment of same with sodium mersalyl, and trace 3 after addition of $Na_2S_2O_4$ to the sodium mersalyl treated IP. It is seen that in contrast to succinate dehydrogenase and FP, essentially all the absorption spectrum of IP is bleached upon treatment with sodium mersalyl, thus indicating the absence of much flavin. Gross contamination of IP by unresolved succinate dehydrogenase is easily detected by the difference between traces 2 and 3 when such spectral analyses are performed.

The extinction coefficients of the iron–labile sulfide systems of succinate dehydrogenase, FP and IP at 450 nm are approximately 2200, 1500, and 4000 per g-atom of iron (or per mole of labile sulfide) per l., respectively. These values are in excellent agreement with the distribution of succinate dehydrogenase protein into the FP (approximately 2.6 parts) and the IP (approximately 1 part) fractions. Moreover, the extinction coefficient of IP at 450 nm is in agreement with that calculated for plant-type ferredoxins isolated and purified by others.[3]

The electron paramagnetic resonance spectra of succinate dehydrogenase before and after treatment with sodium succinate are shown in Figure 3. The effect of succinate on the flavin radical at about $g = 2.003$ and the iron–labile sulfide signal at $g = 1.94$ is quite clear. In contrast to the preparations of others (Dervartanian *et al.*, 1969), the succinate dehydrogenase described here shows little or no extraneous iron signal at $g = 4.3$.

6. Gel Electrophoresis. Succinate dehydrogenase was subjected to SDS gel electrophoresis on polyacrylamide according to the method of Weber and Osborn (1969). Samples treated with SDS and 2-mercaptoethanol and containing 5–25 μg of succinate dehydrogenase protein were subjected to electrophoresis on gels containing 10% acrylamide and 0.2% methylenebisacrylamide, and the protein bands were visualized by the coomassie blue stain.

Figure 4 (right-half) shows the results obtained with B. It is seen that B is composed of two protein bands only. These bands represent the FP and the IP components of succinate dehydrogenase. The left-half of Figure 4 shows a photograph of the same gel at acid pH under ultraviolet light before treat-

[3] Dr. W. Orme-Johnson, private communication.

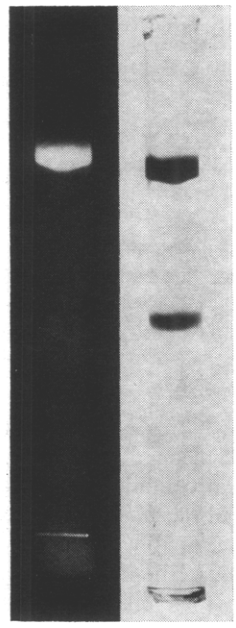

FIGURE 4: Components of succinate dehydrogenase on polyacrylamide gel (for conditions of electrophoresis see the text). Left-hand gel, the gel in acid under ultraviolet light showing the fluorescence of FP. Right-hand gel, same gel after treatment with coomassie blue showing the protein bands of FP and IP.

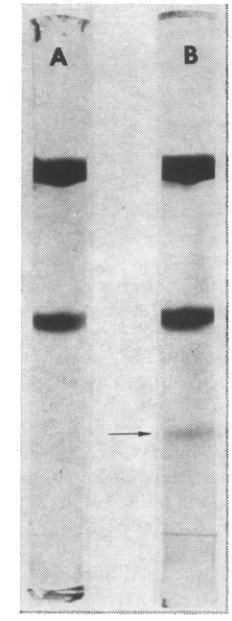

FIGURE 5: Electrophoresis of succinate dehydrogenase alone (A) and with added cytochrome *c* (2% by weight) as contaminant (B). Both samples were treated according to the method of Weber and Osborn (1969) as described in the text. Cytochrome *c* band is marked by an arrow.

ment of the gel with coomassie blue. The fluorescence of the upper band clearly marks the position of FP, whereas the lower band which corresponds to IP, does not fluoresce. We have also subjected the FP and IP fractions of succinate dehydrogenase (prepared as mentioned in section 4) to gel electrophoresis. While in each case the corresponding protein band showed considerable intensification as compared to the bands shown in Figure 4, it was found that both FP and IP as prepared still contained a small degree of cross-contamination.

In order to estimate the purity of our succinate dehydrogenase by the gel electrophoresis technique of Weber and Osborn, preparations of SD-B were admixed with 2% cytochrome *c* protein by weight. The mixture was then treated with sodium dodecyl sulfate plus 2-mercaptoethanol, and subjected to electrophoresis. As shown by the arrow in Figure 5, even 2% protein contamination applied on purpose to preparations of succinate dehydrogenase is clearly evident on the gel. Similar results were obtained with 1% cytochrome *c* contamination. Save for the possibilities that preparations of succinate dehydrogenase might contain proteins which would not stain with coomassie blue or would coelectrophorese with FP and IP, the experiment of Figure 5 gives a measure of the purity of our succinate dehydrogenase. That the possibility of appreciable protein contamination is indeed remote is supported by the facts that (a) succinate dehydrogenase sediments as a single, homogeneous peak in the ultracentrifuge (see section 7), and (b) it shows a single protein band when chromatographed on Agarose (see section 8). The controlled contamination experiment of Figure 5 with cytochrome *c* has an additional control value. It shows that stainable protein contaminants as small in size as cytochrome *c* would have been detected and could not have been lost during the prolonged dialysis of the samples.

Figure 6 shows on polyacrylamide gels materials from each step of the purification procedure of succinate dehydrogenase as described in section 1 above. Tube 1 in Figure 6 is the starting material, complex II. Tubes 2 and 3 are, respectively, the electrophoretic patterns of SD-A and -B, and tube 4 is the remainder of complex II after extraction of succinate dehydrogenase successively once with 0.4 M $NaClO_4$ and once with 0.75 M $NaClO_4$. The FP and the IP bands of succinate dehydrogenase are clearly apparent in tubes 2 and 3, and, as mentioned above, that SD-A of tube 2 shows additional, faint bands which are absent from SD-B (tube 3). A comparison of the protein bands of tube 4 with those of tube 1 also shows a

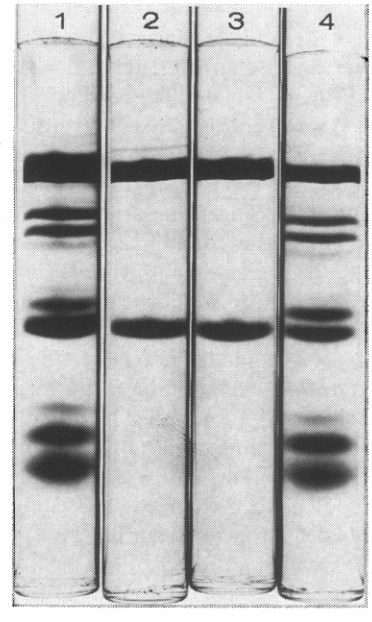

FIGURE 6: Profile of the purification procedure of succinate dehydrogenase depicted on polyacrylamide gels. 1, complex II; 2, SD-A; 3, SD-B; 4, particulate fraction of complex II remaining after removal of SD-A and -B. All protein samples were treated according to the method of Weber and Osborn as described in the text.

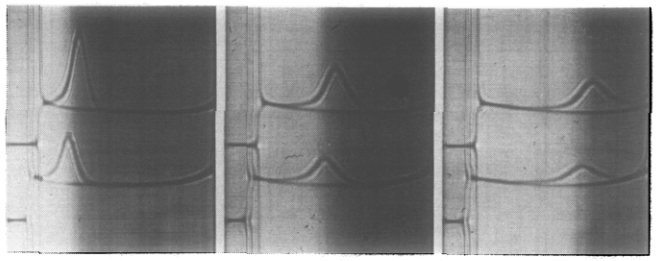

FIGURE 7: Sedimentation pattern of succinate dehydrogenase. SD-B was dissolved in 50 mM sodium phosphate (pH 7.5), containing 20 mM succinate and 5 mM dithiothreitol, and dialyzed at 0° against the same solution for 2 hr. It was placed in two double-sector cells of the ultracentrifuge. One double-sector cell contained 6.0 mg of succinate dehydrogenase protein/ml on one side and dialysate buffer on the other (upper pattern). The second cell contained 4.1 mg of succinate dehydrogenase protein/ml on one side and buffer on the other side (lower pattern). Centrifugation was conducted at 20° and 52,000 rpm, and the photographs shown (left to right) were taken at a bar angle of 60° after 24, 48, and 72 min, respectively. Because of the intense amber color of succinate dehydrogenase, an appropriate yellow filter was used during photography to minimize the contrast between the two portions of the cell. Nevertheless, it is clearly seen in the photographs that the color of succinate dehydrogenase moves with the protein peak.

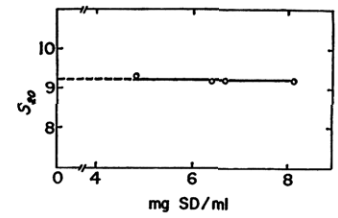

FIGURE 8: Effect of succinate dehydrogenase concentration on the sedimentation constant. Succinate dehydrogenase at the protein concentrations indicated was centrifuged at 20° under the conditions given in Figure 7.

diminution in thickness and intensity in tube 4 of the protein bands corresponding to FP and IP. By contrast, the other protein bands of tube 4 have approximately the same intensity as those of the starting complex II of tube 1, thus indicating selective extraction of succinate dehydrogenase by $NaClO_4$.

7. Sedimentation Properties. Figure 7 shows the sedimentation pattern of SD-B preparations at 6.0 mg (above) and 4.1 mg (below) of protein per ml. It is seen that the enzyme shows a single, symmetrical peak, which remains unchanged throughout the experiment. Figure 8 is a plot of s_{20} *vs.* the concentration of succinate dehydrogenase, and shows that sedimentation velocity of the enzyme is essentially independent of its concentration during ultracentrifugation. The value of s_{20} extrapolated to zero protein concentration is 9.22 S, which by comparison to the sedimentation constant of most globular proteins is large for a molecular weight of approximately 100,000. Whether this is characteristic of flavoproteins with a high content of iron and labile sulfide remains to be seen.[4]

Singer *et al.* (1956) reported a sedimentation constant of $s_{20} = 6.5$ S for their succinate dehydrogenase with a molecular weight of 200,000. Since our preparation has only half the molecular weight of the Singer preparation, we considered the possibility that under the conditions of ultracentrifugation, our enzyme might be present in a polymeric state. The following tests, however, failed to support this possibility. (a) Varying the concentration of succinate dehydrogenase in the range of 4–12 mg of protein/ml did not affect the sedimentation velocity of the enzyme. (b) Addition of powerful depolymerizing agents such as 0.4% sodium dodecyl sulfate (anionic), 0.4% cetyldimethylethylammonium bromide (cationic), 0.4% Triton X-100 (neutral), or 0.5 M sodium trichloroacetate (strong chaotrope) neither changed the sedimentation constant of succinate dehydrogenase, nor produced slower moving components. (c) Changing the pH of the medium from 7.0 to 8.0 to 9.0 was also ineffective even in the presence of depolymerizing agents. Furthermore, as will be seen in section 8, chromatographic behavior of succinate dehydrogenase on Agarose, under similar conditions of pH and buffer composition as used in the ultracentrifugation experiments, did not agree with the presence of polymeric forms of the enzyme.

8. Molecular Weights. The molecular weights of FP and IP were estimated from their relative mobilities during electrophoresis on polyacrylamide gels as compared to the monomeric polypeptides of phosphorylase *a*, urease, ovalbumin, glyceraldehyde 3-phosphate dehydrogenase, trypsin, ribonuclease, and cytochrome *c*. The results are shown in Figure 9, where the mean values (with standard deviations) of 12 separate experiments are shown as vertical bars for both FP and IP. Thus, the mobility of FP in these experiments corresponded to a molecular weight of 70,000 ± 7% and that of IP to a molecular weight of 27,000 ± 5%. Assuming 1 mole each of FP and IP per mole of succinate dehydrogenase, the molecular weight of succinate dehydrogenase would amount to 97,000 ± 6.6%. The molecular weight of succinate dehydrogenase as calculated from its flavin content of 10.3 ± 4% nmoles/mg of protein (Table I), is 97,000 ± 4%. The value obtained from the relative mobility of succinate dehydrogenase on Agarose columns (Figure 10) is 105,000 ± 7.6%, which is in good agreement with the other two determinations. As mentioned earlier, preparations of succinate dehydrogenase show only a single protein peak when chromatographed on Agarose. This peak is coincident with succinate dehydrogenase activity.

Discussion

It has been shown that soluble succinate dehydrogenase from beef heart has a molecular weight of approximately 97,000. The enzyme is homogeneous as examined by ultracentrifugation and chromatography on Agarose and contains 1 mole of covalently bound flavin, 7–8 g-atoms of iron, and 7–8 moles of acid-labile sulfide per 97,000 g of protein. That the enzyme is essentially pure and devoid of protein impurities has been demonstrated by the rigorous criterion of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and 2-mercaptoethanol.

In addition to the protein components of succinate dehydrogenase, preparations of complex II exhibit five major and several minor bands upon gel electrophoresis, essentially all of which are left behind during the selective extraction of succinate dehydrogenase by $NaClO_4$. The flavin content of complex II as compared to that of the purified succinate dehydrogenase indicates that approximately 50% of the protein of complex II is succinate dehydrogenase protein. Both cytochrome *b* (~5 nmoles/mg of protein) and cytochrome c_1 (maximum of 1.5 nmoles/mg of protein) are also present in

[4] The partial specific volume of succinate dehydrogenase was not determined because prolonged dialysis of the enzyme for complete removal of the salts used during its purification would lead to partial loss of iron and labile sulfide and, therefore, an uncertain value for $\bar{v}$.

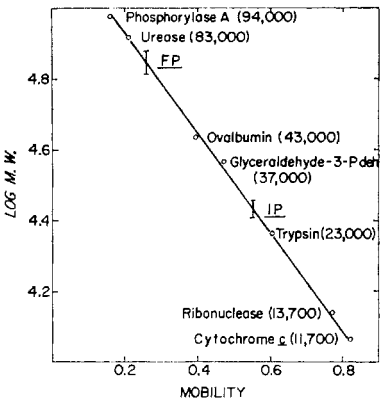

FIGURE 9: Relative mobilities of FP and IP during electrophoresis on polyacrylamide gels. All samples were treated according to the method of Weber and Osborn as described in the text.

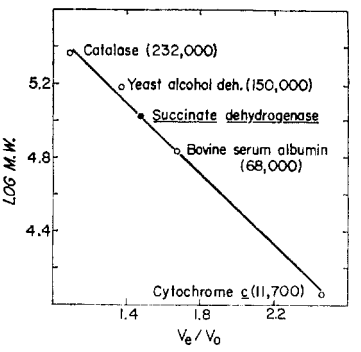

FIGURE 10: Estimation of the molecular weight of succinate dehydrogenase by chromatography on Agarose. All protein samples were dissolved in a degassed buffer containing 50 mM sodium phosphate (pH 7.0), 1 mM EDTA, 4 mM dithiothreitol, and 5 mM succinate. The column was prewashed and eluted with the same buffer. V_e, elution volume; V_0, void volume. The line drawn is the result of least-squares analysis of the data.

preparations of complex II. Based on the molecular weights published by Goldberger and Green (1963) for highly purified preparations of cytochrome *b* (mol wt 30,000) and cytochrome c_1 (mol wt 37,000), these hemoproteins at the amounts indicated would account for, at most, an additional 20% of the protein of complex II. Since the iron:labile sulfide:flavin ratio of purified succinate dehydrogenase is the same as that of complex II, the latter preparation cannot contain, as a major component, an iron–sulfur protein other than that which is present in succinate dehydrogenase itself.

The conditions used by others for extraction of succinate dehydrogenase from mitochondrial particles have led to the conclusions that succinate dehydrogenase is bound to the respiratory chain by covalent bonds (Singer, 1966), or by electrostatic attractions or hydrogen bonds (King, 1962). Wilson and King (1967) state that "A possible type of bond which would be consistent with the available data is a nonheme iron coordination complex. This may be visualized as a complex between a ligand group at the dehydrogenase binding site on the particle and a nonheme iron on the surface of the dehydrogenase protein." However, the fact that succinate dehydrogenase is extracted from particles by a neutral chaotropic salt such as $NaClO_4$ at pH values between 7 and 8, and the fact that the transition of succinate dehydrogenase from particle bound to the soluble state appears to be an equilibrium process aided by increasing concentrations of chaotropic agents (K. A. Davis and Y. Hatefi, in preparation) indicate that the attractive forces between succinate dehydrogenase and the respiratory chain are *mainly* hydrophobic. This conclusion is not in disagreement with the finding of King and coworkers that alkaline pH conditions lead to partial removal of succinate dehydrogenase from particles. Firstly, the material isolated by King and coworkers would correspond to only about 30% pure succinate dehydrogenase. Therefore, the link broken at alkaline pH could much more likely be associated with the 70% protein impurity of the preparation than with succinate dehydrogenase itself. Secondly, extremes of pH can impose coulombic attractions or repulsions which might overcome hydrophobic forces (Singer, 1962). That succinate dehydrogenase is bound to complex II mainly by hydrophobic bonds is also indicated by recent studies on the resolution of complex II in a D_2O medium (K. A. Davis, W. G. Hanstein, and Y. Hatefi in preparation). Since liquid D_2O is considered to be more structured than H_2O, it follows that the hydrophobic bonds of complex II suspended in D_2O should be stronger than in H_2O. Accordingly, it was found that the resolution of complex II with 0.4 M $NaClO_4$ in a D_2O medium yielded only 50% as much soluble succinate dehydrogenase as the control resolution in H_2O. This dramatic effect is not only strong support for the above contention, but it is also an important confirmation of the mechanism of action of chaotropic agents as formulated by Hatefi and Hanstein (1969).

The findings with regard to the substructure of succinate dehydrogenase recall our earlier studies on the composition of complex I (DPNH–coenzyme Q reductase) (Hatefi and Stempel, 1967, 1969; Davis and Hatefi, 1969). In these studies, two soluble proteins were isolated from complex I: a DPNH dehydrogenase flavoprotein containing iron and labile sulfide plus a separate iron–sulfur protein. The dehydrogenase had an approximate molecular weight of 70,000 (per mole of flavin), and a flavin:iron:labile sulfide ratio of 1:4:4. By comparison, the succinate dehydrogenase of complex II is composed of a flavoprotein fraction, with an approximate molecular weight of 70,000 and a flavin:iron:labile sulfide ratio of 1:4:4, plus a separate iron–sulfur protein. Although the similarities in size and composition between the mitochondrial DPNH dehydrogenase and the FP component of succinate dehydrogenase might be fortuitous, it should be added that in xanthine oxidase and aldehyde oxidase the flavin:iron:labile sulfide ratio is also 1:4:4.

The discovery of an iron–sulfur protein as a component of succinate dehydrogenase brings the number of the known iron–sulfur proteins of the mitochondrial electron-transport system to three, one each per complexes I, II, and III (Hatefi and Stempel, 1967; Davis and Hatefi, 1969; Rieske, 1965). While it is tempting to identify these iron–sulfur proteins by specific names, we prefer to refrain from doing so at this time because (a) the exact functions of these proteins are yet to be defined, and (b) the nomenclature and classification of iron–sulfur proteins have yet to be worked out satisfactorily.

Acknowledgments

The authors thank Dr. W. G. Hanstein for valuable discussions and for obtaining the electron paramagnetic resonance spectra of succinate dehydrogenase, Dr. A. J. Bearden for his help and advice in electron paramagnetic resonance spectroscopy, and Drs. W. B. Dandliker and V. De Saussure for the use of their analytical ultracentrifuge. They thank

Mr. P. Tejada and Mr. C. Muñoz for their most valuable technical assistance.

Added in Proof

Our discovery of the subunits of succinate dehydrogenase and their isolation, molecular weights, and composition was announced in September 1970, at the International Colloquium on Bioenergetics in Bari, Italy (Hanstein *et al.*, 1971). Subsequently, Righetti and Cerletti confirmed our findings and identified both subunits upon SDS–polyacrylamide gel electrophoresis of a succinate dehydrogenase preparation, which appears to be 70–80% pure as judged from its flavin content. In addition, they have reported that, after elution from the gel and removal of SDS by prolonged dialysis, both denatured polypeptides contained iron and labile sulfide. This is somewhat surprising, since in our experience SDS treatment destroys the iron–labile sulfide system of both subunits.

References

Baginsky, M. L., and Hatefi, Y. (1969), *J. Biol. Chem. 244*, 5313.

Bernath, P., and Singer, T. P. (1962), *Methods Enzymol. 5*, 597.

Davis, K. A., and Hatefi, Y. (1969), *Biochemistry 8*, 3355.

Dervartanian, D. V., Veeger, C., Orme-Johnson, W. H., and Beinert, H. (1969), *Biochim. Biophys. Acta 191*, 22.

Fogo, J. K., and Popowsky, M. (1949), *Anal. Chem. 21*, 732.

Goldberger, R., and Green, D. E. (1963), *Enzymes 8*, 81.

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.

Hanstein, W. G., Davis, K. A., Ghalambor, M. A., and Hatefi, Y. (1971), *Biochemistry 10*, 2517.

Hanstein, W. G., Davis, K. A., and Hatefii, Y. (1971), Energy Transduction in Respiration and Photosynthesis, Bari, Italy, Adriatica Editrice (in press).

Hatefi, Y. (1966), *Compr. Biochem. 14*, 199.

Hatefi, Y., Davis, K. A., Hanstein, W. G., and Ghalambor, M. A. (1970), *Arch. Biochem. Biophys. 137*, 286.

Hatefi, Y., and Hanstein, W. G. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 1129.

Hatefi, Y., and Stempel, K. E. (1967), *Biochem. Biophys. Res. Commun. 26*, 301.

Hatefi, Y., and Stempel, K. E. (1969), *J. Biol. Chem. 244*, 2350.

Kearney, E. B. (1960), *J. Biol. Chem. 235*, 865.

Keilin, D., and King, T. E. (1958), *Nature (London) 181*, 1520.

King, T. E. (1962), *Biochim. Biophys. Acta 58*, 375.

King, T. E. (1964), *Biochem. Biophys. Res. Commun. 16*, 511.

King, T. E. (1966), *Advan. Enzymol. 28*, 155.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Rieske, J. S. (1965), *in* Non-Heme Iron Proteins, San Pietro, A., Ed., Yellow Springs, Ohio, Antioch Press, p 461.

Righetti, O. O., and Cerletti, O. O. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 13, 181.

Singer, S. J. (1962), *Advan. Protein Chem. 17*, 1.

Singer, T. P. (1966), *Compr. Biochem. 14*, 127.

Singer, T. P., and Kearney, E. B. (1954), *Biochim. Biophys. Acta 15*, 151.

Singer, T. P., Kearney, E. B., and Bernath, P. (1956), *J. Biol. Chem. 223*, 599.

Wang, T. E., Tsou, C. L., and Wang, Y. L. (1956), *Sci. Sinica (Peking) 5*, 73.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Wilson, D. F., and King, T. E. (1967), *Biochim. Biophys. Acta 131*, 265.

Ziegler, D. M. (1961), *Biol. Struct. Funct. 2*, 253.