Studies on the Straub Diaphorase. I. Isolation of Multiple Forms*

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ABSTRACT: The Straub diaphorase has been resolved on columns of DEAE-cellulose to yield three components, of which two are obtained in highly purified form. A similar pattern of fractionation is obtained with the flavoprotein derived from the urea-resolved complexed fraction of the enzyme. All fractions thus obtained yield the same spectrum of enzymatic activities in assays with a variety of electron acceptors. Electrophoresis of the three DEAE-cellulose fractions of the enzyme yields different protein patterns, with a total number of components in excess of thirteen. Some aspects of the reactions catalyzed by this enzyme are considered.

Reports from various laboratories have dealt with the properties and modification of the purification of a loosely bound fluorescent flavoprotein of pig heart mitochondria. Significantly pure preparations were first obtained by Straub (1939), who followed purification of the protein by assay of the heat-labilized FAD.[1] This preparation was shown by Corran *et al.* (1939) to catalyze the specific oxidation of DPNH by methylene blue. The early literature on the enzyme and the origin of the term *diaphorase* have been cited by Savage (1957). The literature to 1961 has been reviewed by Massey (1963). Since the early work on the pig heart enzyme, several other diaphorases have been purified, i.e., preparations catalyzing the oxidation of reduced pyridine nucleotides by nonbiological electron acceptors but with diverse properties, differing from the Straub diaphorase in several respects. The term "Straub diaphorase," now synonymous with lipoyl dehydrogenase, has come into use to describe the flavoprotein first isolated by Straub and will be retained in the present paper.

This flavoprotein has been found to utilize a broad range of electron acceptors in the oxidation of DPNH. Edelhoch *et al.* (1952) reported the use of DCIP in the assay of diaphorase activity; this redox dye has been used extensively because of its relatively low rate of autoxidation in the reduced form. Mahler *et al.* (1955) reported the activity of a variety of quinones in this system. Weber and Kaplan (1956, 1957) reported that the Straub diaphorase catalyzed with marked activity a symmetrical transhydrogenase reaction specific to DPN, i.e., the oxidation of DPNH by the acetylpyridine analog of DPN, and suggested on the basis of deuterium-exchange studies (Weber *et al.*, 1957) that a reduced enzyme intermediate was formed in the reaction of flavoprotein with DPNH. Spectrophotometric evidence for this intermediate was obtained by Savage (1957). In 1958 Massey reported that Straub diaphorase preparations possessed dihydrolipoic acid dehydrogenase activity, and that flavoproteins indistinguishable from the Straub diaphorase were isolatable from α-ketoglutaric acid dehydrogenase preparations (Massey, 1960b; see also: Koike *et al.*, 1960; Searls and Sanadi, 1960). More recently, Lusty and Singer (1964) have estimated the distribution of lipoyl dehydrogenase activity in α-ketoglutarate and pyruvate dehydrogenases and in the free forms in heart and liver; Hayakawa *et al.* (1964) have purified both dehydrogenases from pig heart.

In his original preparation, Straub effected the solubilization of the enzyme by heating the ground heart muscle in dilute ethanol at slightly acidic *p*H. Further purification was obtained by adsorption and elution from alumina C_γ, additional heat treatment, and repeated ammonium sulfate fractionation. Mahler (1955) modified the procedure by extracting the enzyme from mitochondria of pig heart isolated by precipitation at *p*H 4.6 and addition of dilute ammonium sulfate to the extraction medium. Further purification in low yield was obtained by Savage (1957), by application of boundary electrophoresis, to obtain a preparation, homogeneous by sedimentation, with a ratio of 6.0 for the absorbances at 280 and 450 mμ. She was also able to demonstrate the extraction of the enzyme by heating in dilute ammonium sulfate in the absence of ethanol. In 1958, Massey reported extraction of the enzyme in cold 1% ammonium sulfate and purification by repeated ammonium sulfate fractionation and chromatography on columns of calcium phosphate gel suspended on cellulose. Subsequently, to improve yield (Massey *et al.*, 1960), this purification was modified to include extraction and additional

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[1] Abbreviations used in this report are: FAD, flavin adenine dinucleotide; DPN, diphosphopyridine nucleotide; DPNH, reduced DPN, CoQ_0, coenzyme Q_0; APDPN and TNDPN, respectively, the acetylpyridine and thionicotinamide analogs of DPN; DCIP, 2,6-dichlorophenolindophenol.

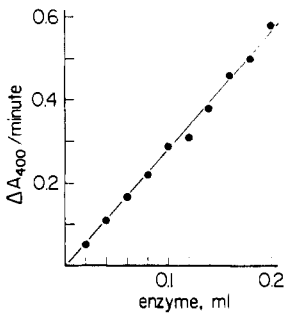

FIGURE 1: Dependence of the rate of the DPNH-TNDPN transhydrogenase reaction on enzyme concentration. Peak III, 8.7 μg in 0.1 M potassium phosphate buffer, *p*H 7.7, and 1 mg bovine serum albumin per ml, was added as indicated. The initial rates of reaction, measured on the Gilford Model 2000, are plotted.

heating steps similar to those described by Mahler (1955); a value of 5.35 was reported for the ratio of absorbances at 280 and 455 mμ (Massey, 1963). Atkinson *et al.* (1962) have reported the electrophoretic resolution of the Straub diaphorase on starch-gel supporting medium into four major and two minor components stated to vary in lipoyl dehydrogenase and diaphorase activity. Lusty (1963) reported the purification of a homologous beef liver enzyme, fractionation of the enzyme on columns of DEAE-cellulose and DEAE-Sephadex, as well as subfractionation of the column components by starch-gel electrophoresis. A total of six electrophoretic components were reported, in agreement with Atkinson *et al.* (1962).

In the present study a modification of the preparation of the enzyme is reported, the enzymatic properties of the fractions obtained by chromatography are examined, and a preliminary characterization of their electrophoretic behavior is presented.

Experimental

Materials. DPN, DPNH, CoQ_6, and menadione were obtained from Sigma Chemical Co.; DL-lipoamide from Farmaco Cutolo-Calosi; duroquinone, aurantioglliocladin, and CoQ_0 were gifts from Dr. Britton Chance; 2,3-dimethyl-5,8-quinoxalinedione and 2,3,6-trimethyl-5,8-quinoxalinedione were gifts from Dr. Madeleine Joulliè. APDPN and TNDPN were prepared by published procedures (Kaplan and Ciotti, 1956; Stein *et al.*, 1963, respectively). Crystalline bovine serum albumin was obtained from the Pentex Corp., nitro blue tetrazolium chloride from the Nutritional Biochemicals Co. DEAE-cellulose was obtained from the Bio-Rad Corp. and further purified as follows: DEAE-cellulose (0.45 kg) was stirred in 2–3 liters of 1 M NaOH in 0.001 M EDTA for 1 hour, filtered, and washed with water; the treatment was repeated. The filter cake was then stirred for 1 hour in 0.2 M K_2HPO_4 in 0.001 M EDTA and filtered, and the treatment was repeated. The filter cake was then washed with large volumes of 0.01 M potassium phosphate buffer, *p*H 7.7, in EDTA as before. DEAE-Sephadex was obtained from Pharmacia, Uppsala. Calcium phosphate gel was prepared by the method of Keilin as described by Colowick (1955). Hydroxylapatite was prepared by the method of Tiselius *et al.* (1956). In the early part of this study ammonium sulfate, analytical reagent, was recrystallized from 0.001 M EDTA and ammonia at *p*H 9; subsequently the product of Mann Research Laboratories labeled "Enzyme Grade" was used.

Procedures. Protein chromatography on columns of DEAE-cellulose and hydroxylapatite was carried out as described by Sober *et al.* (1956) and Tiselius *et al.* (1956), respectively. The fluorescence of the eluate was monitored at room temperature in an Aminco-Keirs spectrophosphorimeter with excitation at 460 mμ and emission at 520 mμ, and was found to be proportional to protein concentration in the range 1–400 μg/ml. Electrophoresis on 5% acrylamide gel (Raymond and Weintraub, 1959) was carried out in the vertical electrophoresis equipment described by Raymond (1962). A discontinuous buffer system was used: the gel slabs were preequilibrated for at least 30 minutes with 0.074 M Tris-citrate, *p*H 8.7. The buffer was then changed to 0.1 M sodium borate, *p*H 9.0, and the samples, containing 0.25 M sucrose, were introduced in the sample wells. Electrophoresis was carried out for about 4 hours at 400–500 v with ice water circulating through the plates. The gel slabs were photographed under an ultraviolet lamp and either stained with amido black or allowed to react for about 2 hours in 50 ml of reaction mixture containing 30 mg DPNH and 15 mg nitro blue tetrazolium chloride in 0.10 M potassium phosphate buffer, *p*H 7.7, then washed in 1 M acetic acid.

Enzyme Assays. A 3-ml reaction mixture was used in all the spectrophotometric assays described below. The transhydrogenase reaction was measured essentially as described previously (Stein *et al.*, 1959) in 0.1 M potassium phosphate buffer, *p*H 7.7, and 10^{-5} M *o*-phenanthroline or 10^{-5} M EDTA containing 0.5 mg DPNH and 0.6 mg APDPN or TNDPN. No effect of addition of bovine serum albumin to this system could be detected. Lipoyl dehydrogenase activity was measured according to Massey *et al.* (1960), with 0.4 mg DPNH, 0.2 mg DPN, 2 mg bovine serum albumin in 10^{-5} M EDTA, and 0.1 M potassium phosphate buffer, *p*H 5.8. DPNH-ferricyanide diaphorase activity was measured as described by Massey (1960a); a nonenzymatic blank of ΔA_{420} = 0.004/minute was applied to all readings. Diaphorase activity with DCIP was measured in 0.1 M potassium phosphate buffer, *p*H 7.7, with 0.4 mg DPNH and sufficient DCIP to yield A_{600} = 2.0. Menadione and other quinone reductase activities were followed in the same phosphate buffer used in the lipoyl dehydrogenase reaction, with 0.4 mg DPNH, 0.2 mg of menadione, 0.6 mg CoQ_0, and 0.4 mg. of the other quinones prepared in ethanolic solution. The quinoxalinediones were prepared in acetone. Reactions were followed in a Zeiss PMQ-II spectro-

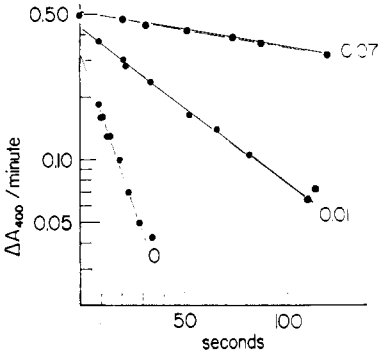

FIGURE 2: Enzyme inactivation by DPNH. A dilution of peak I in 0.1 M Tris-acetate, *p*H 7.5, and 1 mg bovine serum albumin per ml was added at zero time to a reaction mixture, 0.1 M Tris-acetate *p*H 7.5, 10^{-5} M *o*-phenanthroline, and 0.5 mg DPNH. At variable time, TNDPN and phosphate buffer were added to the reaction mixture and the initial rate was measured. The numbers represent the final molar concentration of phosphate in the three experiments.

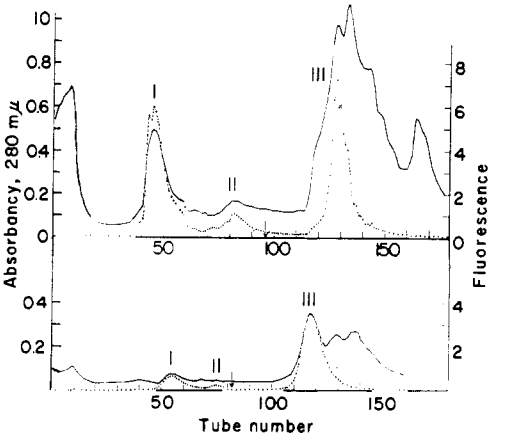

FIGURE 3: Chromatography of the Straub diaphorase on DEAE-cellulose. Above, separation of the 0.45–0.70 saturated ammonium sulfate fraction. Column, 2.3 × 20 cm; mixer volume, 1000 ml; fraction volume, 5 ml. At arrow, the reservoir concentration was increased to 1 M in phosphate. Below: separation of 0–0.45 saturated ammonium sulfate fraction, resolved in urea. Column, 1.2 × 21 cm; other details as above. The solid line is absorbance at 280 mμ; the broken line is fluorescence.

photometer or a Gilford Model 2000 recording spectrophotometric system. A unit is defined conventionally as 1 μmole of substrate oxidized or reduced per minute. Millimolar absorbancies are taken as A_{340} DPNH = 6.22 (Horecker and Kornberg, 1948), A_{400} TNDPNH = 11.9 (Stein *et al.*, 1963), A_{600} DCIP = 19 (Savage, 1957), A_{420} ferricyanide = 1.0 (Minakami *et al.*, 1962). Protein was estimated by the biuret method described by Gornall *et al.* (1949). Enzyme FAD was estimated by the absorbance of the samples at 455 mμ, using a millimolar absorbancy of 11.3 (Beinert, 1960).

Results

Transhydrogenase Assay. The activity of the enzyme in the transhydrogenase reaction is proportional to enzyme concentration over as wide a range of reaction rates as has been found practicable using an automatic recording spectrophotometer (Figure 1). A true initial linear rate is not observed; the reaction is quantitated by estimating the rate as close to zero time as possible. In general, when using hand-operated spectrophotometers, the quantitation of rates faster than 0.15 absorbance unit per minute is not attempted. The observed K_m values for DPNH and TNDPN are 5×10^{-5} M and 2.8×10^{-5} M, respectively, estimated at single concentrations of TNDPN and DPNH, 2.4×10^{-4} M and 2.8×10^{-4} M, respectively. In the absence of a chelating agent in the transhydrogenase reaction mixture, the rate of reaction decays very rapidly (Stein *et al.*, 1960); 8-hydroxyquinoline, cyanide, citrate, EDTA, 2,2-dipyridyl, and *o*-phenanthroline have been found variously effective in retarding the decay of rate of reaction. The effect of the chelating agent is variable and appears to depend in part on the preparation of enzyme used, the sample of DPNH, and the purity of the buffer salts used in the reaction mixture; with varying conditions, the relative effectiveness of these agents may vary. The chelating agents appear to react by protecting the reduced enzyme in the reaction mixture from inactivation by trace metals. Figure 2 shows the rate of decay of enzyme activity in reaction mixtures, containing 10^{-5} M *o*-phenanthroline, with varying phosphate concentrations. Inactivation appears to be first order with respect to concentration of active enzyme. Similar results are obtained with EDTA in Tris buffer. Thus, in 10^{-7}, 2×10^{-7}, 3×10^{-7}, and 10^{-6} M EDTA the half-life of a highly purified sample of enzyme was found to be 2 (est.), 20, 36, and 235 seconds. These data are compatible with the occurrence of a trace ($\sim 10^{-7}$ M) of heavy metal in the reaction mixture combining with the reduced enzyme ($\sim 4 \times 10^{-9}$ M). Basu and Burma (1960) have noted a stimulatory effect of phosphate on the lipoyl dehydrogenase reaction catalyzed by a spinach leaf preparation, which similarly may have been an effect on trace metal contaminants.

It is of interest to note that under the conditions of short-term incubation with DPNH, such as are presented in Figure 2, the diaphorase activity with DCIP is not affected, although the transhydrogenase reaction may be virtually abolished.

Enzyme Purification. All steps are carried out in a cold room maintained at 2–5°. Acid-precipitated pig heart mitochondria are prepared after Keilin and Hartree (1945). Fresh, chilled pig hearts are trimmed and the ventricles are passed twice through an electric meat grinder. The mince is washed four to six times with large volumes of iced tap water and allowed to drain overnight in a parachute cloth sack. The mince is

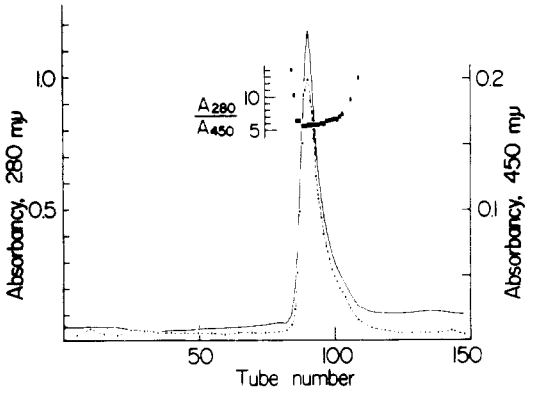

FIGURE 4: Rechromatography of peak III on DEAE-Sephadex A-60. Column, 1.2 × 42 cm; mixer, 250 ml 0.01 M potassium phosphate buffer, *p*H 7.4, and 10^{-6} M *o*-phenanthroline. Reservoir, 0.6 M potassium phosphate buffer, *p*H 7.4, and 10^{-6} M *o*-phenanthroline. Fraction volume, 1.2 ml per tube. The solid line is absorbance at 280 mμ; the broken line is absorbance at 450 mμ.

homogenized in 3.2 volumes of 0.02 M Na_2HPO_4 for 30 seconds at the low speed of a 1-gallon Waring Blendor. Debris and nuclei are removed in a 3-liter conical centrifuge rotor (Lourdes, 3RA) by accelerating the rotor to 5000 rpm and applying the brake. The supernatant is collected by decantation through cheesecloth and titrated to *p*H 5.4 with 1 N acetic acid. The precipitate is collected in a refrigerated air-driven Sharples Supercentrifuge at a bowl speed of 50,000 rpm and a flow rate of 400 ml/minute. Transhydrogenase activity is solubilized by the cold leaching procedure described by Massey (1958). The tightly packed sediment is homogenized in 1% ammonium sulfate, 1 liter per kg of starting mince, and is allowed to stand overnight. The bulk of the insoluble material is removed by centrifugation in the Lourdes 3-liter rotor at about 7000 rpm for 20–30 minutes. The slightly turbid supernatant is collected and titrated to *p*H 7.5 with 1 M K_2HPO_4, and 5-ml aliquots are titrated with calcium phosphate gel to determine the amount required to adsorb 95–97% of the transhydrogenase activity. The gel is removed by passing the suspension through the Sharples Supercentrifuge; inpurities are eluted with 0.1 M potassium phosphate buffer, *p*H 7.7, 100 ml/kg of starting mince, followed by elution of the enzyme by the same buffer in 5% (w/v) ammonium sulfate in two portions, 50 and 20 ml, respectively, per kg of starting mince. Ammonium sulfate is added to 0.45 saturation and the precipitate is removed and reserved for further treatment. To the supernatant, ammonium sulfate is added to 0.7 saturation; the precipitate is dissolved in 0.01 M potassium phosphate, *p*H 7.7, in 10^{-6} M EDTA and dialyzed for 2–3 days against several 6-liter changes of the same buffer, 10^{-3} M in phosphate, 10^{-6} M in EDTA. The dialysate is applied to a column of DEAE-cellulose preequilibrated with 10^{-2} M potassium phosphate in 10^{-6} M EDTA. The proteins are eluted with the usual concave downward gradient employing a 1-liter mixer containing the preequilibrating buffer. The reservoir contains initially 0.15 M potassium phosphate, *p*H 7.7, in 10^{-6} M EDTA; after peak II emerges, the phosphate concentration of the reservoir is increased to 1 M. A typical chromatogram is shown in Figure 3; the pattern obtained is quite reproducible. Routinely, peak locations may be identified with an ultraviolet lamp in the darkened cold room; the protein fractions are concentrated with ammonium sulfate and dialyzed as before. Peaks I and III are applied to 1.2- × 15-cm columns of hydroxylapatite; in the preparation shown in Table I, peak II was applied to a 0.6- × 12-cm column. The columns are irrigated with 50–100 ml of 0.1 M potassium phosphate, *p*H 6.8, followed by 50–75 ml of the same buffer in 2% (w/v) ammonium sulfate. The bands are eluted with 0.1 M potassium phosphate, *p*H 6.8, in 5% (w/v) ammonium sulfate, concentrated with ammonium sulfate, and dialyzed as before. Peak I appears to be eluted as a pure component, whereas examination of the chromatogram of peak III on hydroxylapatite shows that impurities are eluted at the point of emergence of the enzyme. As is shown in Table I, rechromatography of peak III on DEAE-Sephadex leads to some further purification. The resulting chromatogram and experimental conditions are shown in Figure 4. The contents of the tubes with low A_{280}/A_{450} ratios are pooled, concentrated, and dialyzed as before.

The 0–0.45 saturated ammonium sulfate fraction previously reserved is washed with 0.45 saturated ammonium sulfate in 0.1 M potassium phosphate buffer, *p*H 7.7, dissolved in the same buffer, and reprecipitated at 0.45 saturated ammonium sulfate. In the course of this treatment a considerable amount of activity is lost into the phase soluble in 0.45 saturated ammonium sulfate. In the experiment shown in Table I, the loss is about 3200 units. In later preparations the 0.45 saturated ammonium sulfate pellet was extracted with 0.45 ammonium sulfate and the extract was combined with the first supernatant for further purification. The pellet is dissolved in 0.001 M potassium phosphate buffer, *p*H 7.7, in 10^{-6} M *o*-phenanthroline and treated with 5–6 M urea for 1.5 hours following Massey's (1960b) demonstration of the resolution of complexed flavoprotein with this reagent. The urea solution is then fractionated between 0.45 and 0.70 saturated ammonium sulfate. The resolved fraction is dialyzed and chromatographed on DEAE-cellulose in a gradient elution system scaled down from that indicated above. A sample chromatogram is shown in Figure 3. In general, chromatograms of the resolved fraction have proved to be less uniform than those obtained from the 0.45–0.70 saturated ammonium sulfate fraction; the results given in Table I are more typical.

In agreement with Massey (1960a), the initial extract in cold 1% ammonium sulfate contains little or no cytochrome *c* reductase activity. The constancy of the ratios of the activities of transhydrogenase, lipoyl dehydrogenase, and ferricyanide diaphorase through-

TABLE I: Summary of Purification Procedure.

Fraction	Volume (ml)	Protein (mg/ml)	DPNH + TNDPN (units/ml)	DPNH + TNDPN (total units)	DPNH + TNDPN (units/mg)	Activity Ratio $K_3Fe(CN)$/TNDPN	Activity Ratio Lipoamide/TNDPN	Spectral Ratio 280/450	Spectral Ratio 450/405	(DPNH + TNDPN)/A_{450}
Mitochon dria extract[a]	8900	4.00	4.30	38,300	1.07	2.82	2.43			
Combined gel eluates	628	5.86	40.3	25,300	6.87	2.34	2.43			
Ammonium sulfate, 0.45–0.70 satd	74	6.49	148	10,900	22.8	2.65	2.32			
DEAE-cellulose: peak I	13.2	2.83	272	3,570	96.2	2.54	2.46	5.61	2.40	460
peak II	6.0	1.23	53.8	323	43.7	2.73	2.45	7.12	1.15	224
peak III	17.0	3.33	171	2,920	51.5	2.29	2.16	7.45	1.42	359
Hydroxylapatite: peak I	4.5	6.56	692	3,110	105	2.38	2.32	5.33	2.55	523
peak II	1.2	1.90	104	125	55	2.64	2.56	8.03	2.16	390
peak III	4.7	4.74	401	1,880	84.90	2.49	2.53	6.00	2.22	436
DEAE-Sephadex, peak III[b]	2.5	2.62	232	580	88.50	2.41	2.16	5.60	2.49	442
Ammonium sulfate, 0–0.45 satd	27.5	51.7	215	5,920	4.16	2.92	2.39			
do, resolved with urea	10.1	8.50	218	2,200	25.7	2.29	2.28	16.9	2.18	497
DEAE-cellulose: peak I	2.3	1.90	109	240	57.0	2.29	2.36	6.58	2.40	469
peak II	1.7	3.60	141	240	39.0	2.50	2.55	9.81	2.24	435
peak III	2.6	4.30	151	393	35.0	2.40	2.38	10.5	2.28	502

[a] Mitochondria derived from 10 kg of pig heart mince. [b] Derived from 3 ml of hydroxylapatite, peak III fraction.

TABLE II: Properties of the Purified Peaks.

Peak	s_0 [a]	A_{280}/A_{455}	A_{280}/A_{260}	A_{455}/A_{395}	A_{455}/A_{360}	Protein (g/mole FAD)	Trans-hydrogenase (units/mg)
I[b]	5.50	5.24		3.28	1.28	52,700	105
III[c]	5.43	5.41		3.17	1.28	54,300	88.5
I[d]		5.28	1.23	3.44	1.31	61,000	92.6
II[d]		5.73	1.25	2.21	1.13	70,700	39.3
IIIa[d]		5.14	1.22	3.62	1.42	59,500	97.4

[a] Determined at 0.46 mg biuret protein per ml, in 0.08 M potassium phosphate buffer, *p*H 7.7. [b] Sample described in Table I, 0.45–0.70 saturated ammonium sulfate, hydroxylapatite fraction. [c] Sample described in Table I, 0.45–0.70 saturated ammonium sulfate, DEAE-Sephadex fraction. [d] Samples fractionated from two preparations, combined at the 0.45–0.70 saturated ammonium sulfate step and chromatographed on a column of DEAE-cellulose, 2.3 × 50 cm. A portion of peak III, judged to be free of contaminating protein, was isolated. Fractions were purified further on columns of hydroxylapatite, as described in text.

TABLE III: Enzymatic Activities of the DEAE-Cellulose Fractions.[a]

	Ammonium Sulfate Fraction					
	0.45–0.70 Satd			0–0.45 Satd		
Column Fraction	I	II	III	I	II	III
DPNH + TNDPN	0.448	0.423	0.415	0.462	0.394	0.446
DPNH + APDPN	0.124	0.102	0.118	0.112	0.099	0.124
DPNH + DCIP	0.012	0.016	0.061	0.015	0.013	0.016
			0.017[b]			
	0.014[c]		0.019[c]			
DPNH + menadione	0.079	0.085	0.089	0.058	0.075	0.077
DPNH + CoQo	0.543	0.579	0.621	0.566	0.603	0.651

[a] Relative to lipoyl dehydrogenase activity of the various fractions. Preparation described in Table I. The column fractions derived from the 0.45–0.70 saturated ammonium sulfate fraction were carried through the hydroxylapatite purification. [b] At DEAE-cellulose step. [c] Separate preparation at DEAE-cellulose step.

out the purification procedure suggests that essentially all of the easily extracted transhydrogenase from pig heart mitochondria is associated with lipoyl dehydrogenase. Similar results have been obtained in the purification of a homologous beef heart mitochondrial enzyme. Examination of the transhydrogenase activity of the residue of exhaustively extracted beef heart mitochondria yields a ratio of 0.33 with TNDPN and APDPN as acceptors, a number in close agreement with the data of Minakami *et al.* (1963).[2] The large difference in reactivity of the DPNH dehydrogenase and the Straub diaphorase with pyridine nucleotide analogs might be used to evaluate the contribution of the two flavoproteins to the transhydrogenase activity of the whole mitochondrion.

Properties of the Components. The chromatogram in Figure 3 shows that peak III is eluted from DEAE-cellulose along with several contaminating proteins; further purification on hydroxylapatite yields a preparation of substantially lower specific activity and FAD content. To obtain more significant data on this component, in one experiment chromatography was carried out on a 50-cm column of DEAE-cellulose at a slow flow rate, and a reasonably homogenous section of peak III was isolated and worked up separately. Some values derived from this preparation and the one presented in Table I are shown in Table II. Identical sedimentation values are obtained for peaks I and III and the numbers are in agreement with those published by Savage (1957) and Massey *et al.* (1962); sedimentation of peaks I and III at higher protein concentration yields symmetrical schlieren patterns. The spectral ratios obtained compare with the values given by Massey (1963); the values for the equivalent weight of the various peaks, fortuitously the upper and lower

[2] A. M. Stein and N. O. Kaplan, unpublished data.

limits of all the values obtained, compare to a value of 57,000 reported by Massey *et al.* (1962). The data in Table II for peak II, the purest preparation of this component obtained thus far, suggest that the specific activity of this fraction is indeed lower than that of peaks I and III. Further preparations will be required to establish this point.

The question of nonequivalence of the electrophoretic forms raised by Atkinson *et al.* (1962) prompted an examination of the enzymatic activities of the six column fractions using a variety of acceptors. The data obtained from a single preparation are shown in Table III. All rates are related to lipoyl dehydrogenase activity. Within experimental error, the profile of activities is the same for all fractions. In addition, no differences between fractions were found in the lipoyl dehydrogenase reaction carried out in acid and alkaline reaction medium, with and without added DPN. Typical data are shown in Figure 5; in agreement with the data of Basu and Burma (1960), Koike *et al.* (1960), and Searls and Sanadi (1961) for the spinach, *E. coli*, and heart enzyme, respectively, no requirement for DPN is evident at higher *p*H.

Quinone Reduction. The efficacy of CoQ_0 as an acceptor in the Straub diaphorase reaction prompted an examination of some other quinones. Some data

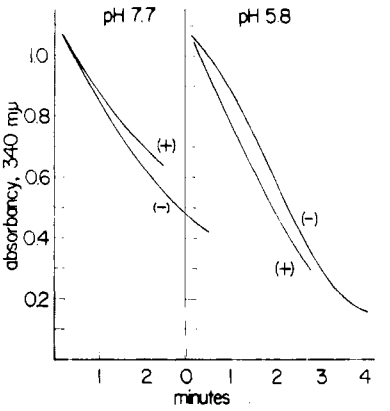

FIGURE 5: Effect of DPN on the lipoyl dehydrogenase reaction. The reaction was carried out as indicated in the experimental section and with replacement of the *p*H 5.8 phosphate buffer with 0.1 M potassium phosphate buffer, *p*H 7.7. The addition of 0.2 mg DPN per 3 ml reaction mixture is indicated by the + and − signs.

TABLE IV: Quinone Reductase Activity of the Straub Diaphorase.

	Relative Activity[a] ($-A_{340}$/min)		
	−Enzyme	+Enzyme (net)	K_m[b] (M)
DL-Lipoamide	0	0.390	7.4×10^{-4}
p-Quinone	0.185	0.175	
Duroquinone	0	0.007	
Aurantiogliocladin	0	0.034	
CoQ_0	0	0.198	1.7×10^{-4}
CoQ_6[c]	0.002	0.003	
Menadione	0	0.023	1.0×10^{-4}
2,3-Dimethyl-5,8-quinoxalinedione	0.180	0.061	
2,3,6-Trimethyl-5,8-quinoxalinedione	0.018	0.092	5.1×10^{-4}

[a] At arbitrary concentrations of substrates (concentration of DPNH in all cuvets, 0.13 mg/ml).
[b] Determined at fixed DPNH concentration, 0.13 mg/ml.
[c] Suspension.

are shown in Table IV. Menadione appears to be a relatively poor acceptor as compared to the benzoquinones. *p*-Quinone appears to be reduced enzymatically at about the same rate as is CoQ_0. Of interest is the observation that nonenzymatic reduction by DPNH requires at least two unsubstituted positions on the quinone ring, whereas enzymatic reduction requires at least one unsubstituted position. In view of the activating effect of DPN on the lipoyl dehydrogenase reaction at acid *p*H, it was of interest to investigate this effect in the quinone reductase reaction. In fact, at *p*H 5.8 DPN proved to be markedly inhibitory, with a K_i of 7×10^{-5} M. Further, as is the case in the transhydrogenase reaction, chelating agents retard the decay of reaction rate.

Electrophoresis. Figure 6 shows the acrylamide-gel electropherograms of the peaks from a single preparation (see note (*d*), Table II). The gel slab is seen as viewed under an ultraviolet lamp and after staining with amido black. Typically, at least five components can be seen resolved in each of peaks I and III. Three components may be detected in the electropherogram of peak II. It will be noted that the distribution of protein differs in each of the three peaks. This is shown semiquantitatively in Figure 7, a scan of the amido-black patterns of peaks I and III with a Joyce-Lobell microdensitometer. Figure 8 shows the zymogram obtained with peaks I and III in the DPNH–nitro blue tetrazolium chloride reaction mixture. Seven to eight components can be distinguished easily in the patterns.

Discussion

In order to minimize the possibility of artifactual modification of the protein in isolation, the mildest procedure was chosen at the expense of yield. Treatment at high temperatures on freezing of the samples either during purification or storage was avoided. The purity of the samples prepared for electrophoresis as

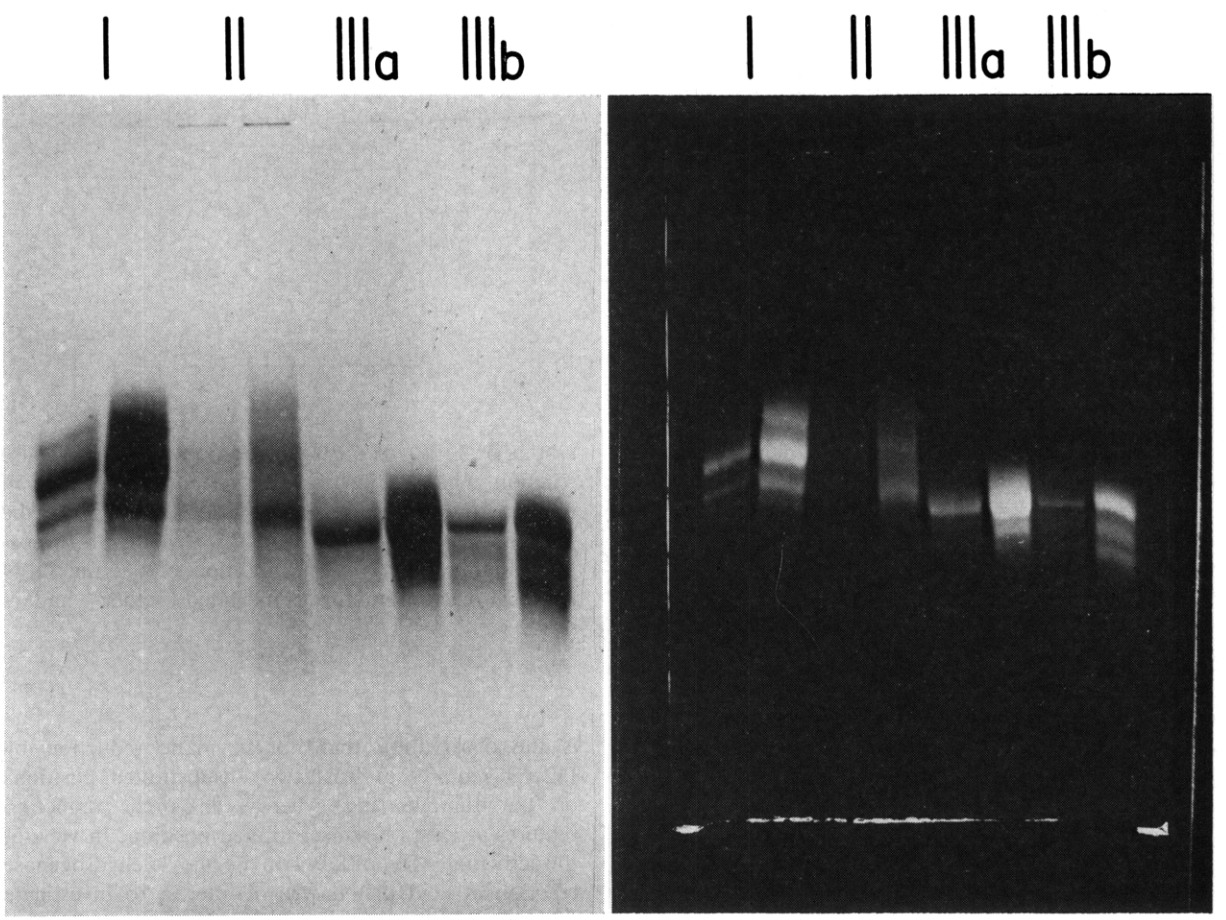

FIGURE 6: Acrylamide-gel electrophoresis of the Straub diaphorase components derived from the 0.45–0.70 saturated ammonium sulfate fraction. Approximately 0.1 and 0.2 mg of biuret protein of each component was applied to replicate slots. Samples were migrated at 550 v for 3.5 hours. Photograph at right taken under ultraviolet lamp; at left after staining with amido black. See text for conditions of electrophoresis and sample preparation.

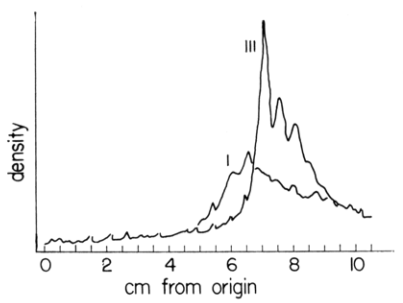

FIGURE 7: Densitometer scan of electropherograms of peaks I and III. Samples were migrated on a single slab 2.5 hours at 450 v, then stained with amido black.

well as the close correspondence of the electrophoretic patterns of protein staining, fluorescence, and deposition of formazan precipitates permit the identification of the bands observed as components of the Straub diaphorase. Three *major* components are detected in the electropherograms of both peaks I and III; thus it appears that the bulk of the uncomplexed enzyme isolated by these procedures is accounted for by six electrophoretic forms. In addition, a number of minor components occur, associated with both peaks I and III. Some variability in these has been encountered in various electropherograms; the factors involved are not known. For a discussion of variability of lactic acid zymograms on starch gel, see Vesell and Brody (1964). Less information is available on the electrophoretic behavior of peak II. However, the elution patterns of some of the DEAE-cellulose chromatograms do suggest the presence of three components of this peak. Additional evidence for the preexistence of the electrophoretic forms of the enzyme is afforded by the difference in the protein distribution of samples IIIa and IIIb in Figure 6. Fraction IIIa represents the portion of peak III first eluted from the column; the electropherogram of IIIa is correspondingly richer than fraction IIIb in the slower-moving electrophoretic components. The

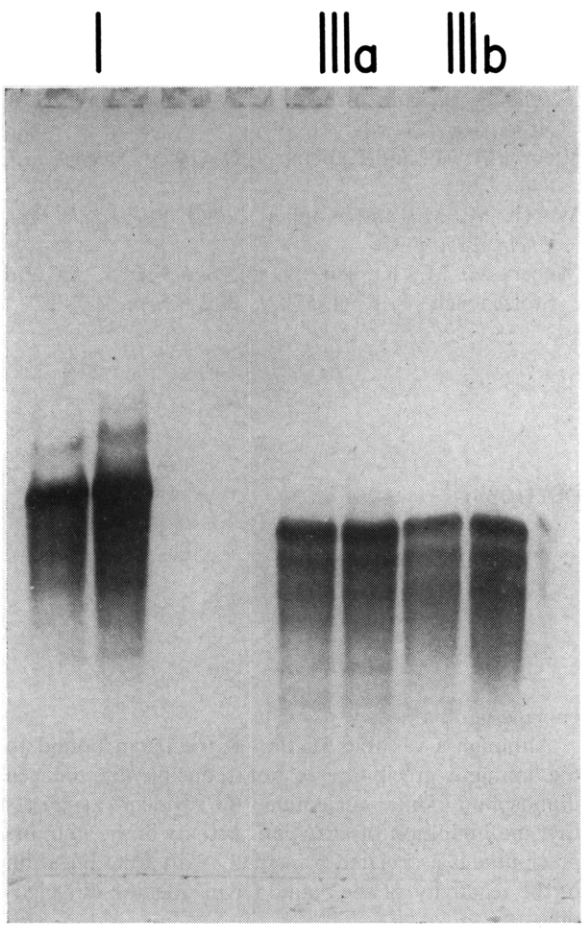

FIGURE 8: Zymograms of peaks I and III developed in the DPNH–nitro blue tetrazolium reaction mixture. Approximately 0.05 and 0.1 mg biuret protein of each component were applied to replicate slots. Samples migrated 4 hours at 450 v. See text for details of sample preparation.

converse is true for the faster-moving components.

Atkinson *et al.* (1962) reported in a preliminary communication the resolution of four major and two minor components of the pig heart enzyme on starch-gel electrophoresis. It should be pointed out that in the present investigation additional resolution was obtained by prior separation of components of the enzyme on DEAE-cellulose. Further, as is the case for the esterases (Hunter *et al.*, 1964), it is possible that acrylamide gel may offer intrinsically more resolving capacity than the starch-gel system.

As yet, no evidence for the molecular basis of the multiple forms of the Straub diaphorase is available. The DEAE-cellulose fractions possess the same spectrum of enzyme activities and they appear to be identical by immunochemical criteria (Stein *et al.*, 1965). Further study of the electrophoretic components is in progress.

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References

Atkinson, M. R., Dixon, M., and Thornber, J. M. (1962), *Biochem. J. 82*, 29P.

Basu, D. K., and Burma, D. P. (1960), *J. Biol. Chem. 235*, 509.

Beinert, H. (1960), *Enzymes 2*, 339.

Colowick, S. P. (1955), *Methods Enzymol. 1*, 90.

Corran, H. S., Green, D. E., and Straub, F. B. (1939), *Biochem. J. 33*, 793.

Edelhoch, H., Hayaishi, O., and Teply, L. J. (1952), *J. Biol. Chem. 197*, 97.

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

Hayakawa, T., Muta, H., Hirashima, M., Ide, S., Okabe, K., and Koike, M. (1964), *Biochem. Biophys. Res. Commun. 17*, 51.

Horecker, B. L., and Kornberg, A. (1948), *J. Biol. Chem. 175*, 385.

Hunter, R. L., Rocha, J. T., Pfrender, A. R., and DeJong, D. C. (1964), *Ann. N.Y. Acad. Sci. 121*, 532.

Kaplan, N. O., and Ciotti, M. M. (1956), *J. Biol. Chem. 221*, 823.

Keilin, D., and Hartree, E. F. (1945), *Biochem. J. 39*, 289.

Koike, M., Shah, P. C., and Reed, L. J. (1960), *J. Biol. Chem. 235*, 1939.

Lusty, C. J. (1963), *J. Biol. Chem. 238*, 3443.

Lusty, C. J., and Singer, T. P. (1964), *J. Biol. Chem. 239*, 3733.

Mahler, H. R. (1955), *Methods Enzymol. 2*, 707.

Mahler, H. R., Fairhurst, A. S., and Mackler, B. (1955), *J. Am. Chem. Soc. 77*, 1514.

Massey, V. (1958), *Biochim. Biophys. Acta 30*, 205.

Massey, V. (1960a), *Biochim. Biophys. Acta 37*, 310.

Massey, V. (1960b), *Biochim. Biophys. Acta 38*, 447.

Massey, V. (1963), *Enzymes 7*, 275.

Massey, V., Gibson, Q. H., and Veeger, C. (1960), *Biochem J. 77*, 341.

Massey, V., Hoffman, T., and Palmer, G. (1962), *J. Biol. Chem. 237*, 3820.

Minakami, S., Cremona, T., Ringler, R. L., and Singer, T. P. (1963), *J. Biol. Chem. 238*, 1529.

Minakami, S., Ringler, R. L., and Singer, T. P. (1962), *J. Biol. Chem. 237*, 569.

Raymond, S. (1962), *Clin. Chem. 8*, 455.

Raymond, S., and Weintraub, L. (1959), *Science 130*, 711.

Savage, N. (1957), *Biochem. J. 67*, 146.

Searls, R. L., and Sanadi, D. R. (1960), *J. Biol. Chem. 235*, 2485.

Searls, R. L., and Sanadi, D. R. (1961), *J. Biol. Chem.*

236, 580.
Sober, H. A., Gutter, F. J., Wyckoff, M. M., and Peterson, E. A. (1956), *J. Am. Chem. Soc. 78*, 756.
Stein, A. M., Kaplan, N. O., and Ciotti, M. M. (1959), *J. Biol. Chem. 234*, 979.
Stein, A. M., Kaufman, B. T., and Kaplan, N. O. (1960), *Biochem. Biophys. Res. Commun. 2*, 354.
Stein, A. M., Lee, J. K., Anderson, C. D., and Anderson, B. M. (1963), *Biochemistry 2*, 1015.
Stein, A. M., Wolf, B., and Stein, J. H. (1965), *Biochemistry 4*, 1500 (this issue; following paper).
Straub, F. B. (1939), *Biochem. J. 33*, 787.
Tiselius, A., Hjerten, S., and Levin, Ö. (1956), *Arch. Biochem. Biophys. 65*, 132.
Vesell, E. S., and Brody, I. A. (1964), *Ann. N.Y. Acad. Sci. 121*, 544.
Weber, M. M., and Kaplan, N. O. (1956), *Science 123*, 844.
Weber, M. M., and Kaplan, N. O. (1957), *J. Biol. Chem. 225*, 909.
Weber, M. M., Kaplan, N. O., San Pietro, A., and Stolzenbach, F. E. (1957), *J. Biol. Chem. 227*, 27.

Studies on the Straub Diaphorase. II. Properties of an Antibody to the Straub Diaphorase*

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ABSTRACT: Rabbit antibody has been prepared against a single fraction of the Straub diaphorase obtained by chromatography on DEAE-cellulose. The antibody reacts in the Ouchterlony double-diffusion test to give a line of identity with all chromatographic forms of the enzyme. In the immune precipitate the flavin fluorescence is only slightly affected; markedly variable inhibition of the several reactions catalyzed by this enzyme is obtained.

Although a variable fraction of the flavin bound to the immune precipitate is not reducible by reduced diphosphopyridine nucleotide, the evidence suggests that the inhibition of enzymatic activity in the immune precipitate is associated principally with a modification of the reactivity of the bound flavin–adenine dinucleotide.

The Straub diaphorase, identical with lipoyl dehydrogenase (Massey, 1958, 1960b; Searls and Sanadi, 1959, 1961), has been separated into several electrophoretic components (Atkinson *et al.*, 1962) with variable enzymatic activities. A homologous liver enzyme has been separated into several fractions on DEAE-cellulose and DEAE-Sephadex by Lusty (1963), who also carried out starch-gel electrophoretic subfractionation of the column fractions. The first paper (Stein and Stein, 1965) in this series has reported the separation on DEAE-cellulose of the pig heart enzyme into three separate fractions which react identically in several enzymatic tests.

This study was undertaken to explore the immunochemical relationships between the three peaks; in the tests applied thus far, these appear to be identical. Some of the properties of the immune reaction and of the immune precipitate are described here.

Experimental

Enzyme Preparations. The procedures used to prepare and separate the Straub diaphorase into several chromatographic fractions were described in the first publication in this series. The DEAE-cellulose fractions resulting from the chromatography both of the free form of the enzyme and from the urea-resolved complex were used in this study.

Preparation of Immune Globulins. Two separate samples of peak I, with spectral ratios of 280/455 mμ of about 5.25, were used as antigens. Doses (10 ml) of 10–15 mg protein, emulsified in an equal volume of incomplete Freund's adjuvant, were injected in two separate sites, both gastrocnemius muscles, of two large New Zealand rabbits. The first dose was followed by two doses at intervals of 1 month. Samples of serum were obtained immediately before and a month following the third antigenic dose. The sera were fractionated between 0.3 and 0.5 saturated ammonium sulfate; the globulins were reprecipitated with ammonium sulfate and the fractionations were repeated. In the final preparation the globulin solutions represented approximately a 2-fold increase in concentration over

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