

- Spurrier, M. H., & Reeck, G. R. (1976) *FEBS Lett.* 70, 81-84.
- Staynov, D. Z. (1976) *Nature (London)* 264, 522-525.
- Tatchell, K., & Van Holde, K. E. (1977) *Biochemistry* 16, 5295-5303.
- Thoma, F., & Koller, Th. (1977) *Cell* 12, 101-107.
- Weischet, W. D., Tatchell, K., Van Holde, K. E., & Klump, H. (1978) *Nucleic Acids Res.* 5, 139-160.
- Whitlock, J. P., Jr., & Simpson, R. T. (1976) *Nucleic Acids Res.* 3, 2255-2266.
- Whitlock, J. P., Jr., & Simpson, R. T. (1977) *J. Biol. Chem.* 252, 6516-6520.
- Wolf, B., Berman, S., & Hanlon, S. (1977) *Biochemistry* 16, 3655-3662.
- Wu, H.-M., Dattagupta, N., Hogan, M., & Crothers, D. M. (1979) *Biochemistry* 18, 3960-3965.

## Interaction of [<sup>14</sup>C]Dicyclohexylcarbodiimide with Complex V (Mitochondrial Adenosine Triphosphate Synthetase Complex)<sup>†</sup>

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**ABSTRACT:** The kinetics of interaction of [<sup>14</sup>C]dicyclohexylcarbodiimide ([<sup>14</sup>C]DCCD) with complex V of the mitochondrial oxidative phosphorylation system and inhibition of its ATPase and ATP-<sup>32</sup>P<sub>i</sub> exchange activities have been studied. DCCD inhibits ATP-P<sub>i</sub> exchange several times faster than ATP hydrolysis as catalyzed by complex V and is taken up by complex V in biphasic manner. The rapid, initial phase corresponds in time to the inhibition of ATP-P<sub>i</sub> exchange activity, while substantial inhibition of ATPase activity involves the second, slow phase of DCCD uptake as well. Dodecyl sulfate-acrylamide gels of [<sup>14</sup>C]DCCD-treated complex V show only a single, narrow protein band covalently labeled. The labeled polypeptide is extractable into acidified chloroform-methanol (2:1), and its capacity for labeling is 6 to 7 nmol of DCCD per mg of complex V protein. There is a linear correlation between [<sup>14</sup>C]DCCD labeling of this band and

inhibition of ATP-P<sub>i</sub> exchange and ATPase activities, at least up to 80-85% activity inhibition. Extrapolation to zero activity indicates that complete inhibition of ATP-P<sub>i</sub> exchange and ATPase activities corresponds, respectively, to labeling of about one-sixth and one-half of the total specific DCCD binding sites of complex V. Covalent interaction of [<sup>14</sup>C]DCCD with the DCCD-binding polypeptide of complex V is inhibited by prior treatment of the enzyme with rutamycin, venturicidin, carbonyl cyanide *m*-chlorophenylhydrazone, dibutyl(chloromethyl)tin chloride, diamide, and *N*-phenyl-*N'*-*n*-nonylthiourea (an uncoupler). Among these ATPase inhibitors and uncouplers (except diamide which inhibits neither the ATPase nor the ATP-P<sub>i</sub> exchange activities of complex V), venturicidin and dibutyl(chloromethyl)tin chloride were found at the concentrations used to inhibit covalent DCCD binding completely.

**B**eechey and co-workers (Beechey et al., 1966, 1967) discovered that dicyclohexylcarbodiimide is a specific inhibitor of ATP synthesis and hydrolysis in mammalian mitochondria. Subsequent studies showed that, similar to oligomycin, DCCD<sup>1</sup> reacts with the membrane sector (F<sub>0</sub>) of the mitochondrial ATPase complex (Hollaway et al., 1966), that the binding involves a low molecular weight (~8000) hydrophobic proteolipid extractable with chloroform-methanol (2:1) (Cattell et al., 1970, 1971), and that a similar DCCD-binding protein is present in chloroplasts (Nelson et al., 1977; Sigrist-Nelson et al., 1978; Sigrist-Nelson & Azzi, 1979), *Neurospora* and yeast mitochondria (Sebald et al., 1979), *Escherichia coli* plasma membrane (Fillingame, 1975, 1976; Altendorf, 1977), and the thermophilic bacterium PS3 (Sone et al., 1979). Experiments suggesting that oligomycin and DCCD bind to the same or interacting sites in mitochondria have been published (Enns & Criddle, 1977), and the isolated proteolipid from yeast has been shown to increase the proton conductance of phospholipid membranes in an oligomycin-sensitive manner (Criddle et al., 1977). The amino acid sequences of the DCCD-binding proteins isolated from *Neurospora crassa*,

*Saccharomyces cerevisiae*, and *E. coli* have been published (Sebald & Wachter, 1978). The DCCD-binding residue has been determined to be a glutamic acid at position 65 in the *Neurospora* and the same corresponding position in the yeast proteins and an aspartic acid at the same relative position in the *E. coli* protein (Sebald & Wachter, 1978). The DCCD-binding residue is located in the above three proteins in the center of a hydrophobic sequence of amino acids. In oligomycin-resistant mutants of *Neurospora* and yeast, single amino acid substitutions have been identified, which are four to six residues removed from the site of DCCD binding (Sebald & Wachter, 1978).

In spite of these important developments on the molecular and functional properties of DCCD-binding proteins (mainly from microorganisms) and the site of DCCD interaction in these molecules, very little has been done regarding the kinetics of DCCD binding to submitochondrial particles and ATPase complex preparations and regarding the relationship of binding to the kinetics of inhibition of ATP synthesis and hydrolysis. In most instances, binding studies involving radioactive DCCD have been performed after prolonged incubation of the enzyme

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<sup>1</sup> Abbreviations used: DCCD, dicyclohexylcarbodiimide; DNP, 2,4-dinitrophenol; pCMB, *p*-(chloromercuro)benzoate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; S-13, 5-chloro-3-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide; DBCT, dibutyl(chloromethyl)tin chloride; NPTU, *N*-phenyl-*N'*-*n*-nonylthiourea; NSPM, *N*-(*N*-*n*-nonyl-4-sulfamoylphenyl)maleimide; NaDodSO<sub>4</sub>, sodium dodecyl sulfate; di-amide, diazenedicarboxylic acid bis(dimethylamide).

with DCCD, e.g., 4 h (Stekhoven et al., 1972), which is far in excess of the incubation time needed for activity inhibition. The present manuscript examines the kinetics of DCCD binding and activity inhibition and presents data regarding the capacity of the enzyme system for DCCD binding in the presence and absence of various modifiers of mitochondrial energy-linked functions. The enzyme system used is complex V (Hatefi et al., 1974; Stiggall et al., 1978). This preparation has the advantage of being a much simpler structure than whole mitochondria or sonicated submitochondrial preparations. However, similar to submitochondrial particles, it catalyzes ATP (also GTP and ITP) hydrolysis and ATP- $P_i$  exchange (Stiggall et al., 1978). Both reactions are inhibited by oligomycin, DCCD, venturicidin, dibutyl(chloromethyl)tin chloride, and thiol reagents, and the ATP- $P_i$  exchange reaction is also inhibited by uncouplers and by valinomycin + nigericin (Stiggall et al., 1978).

#### Methods and Materials

Complex V was prepared from beef heart mitochondria or by phosphorylating submitochondrial particles (L6w & Vallin, 1963) as described (Stiggall et al., 1978). Protein was determined by the method of Lowry et al. (1951). ATP- $^{33}P_i$  exchange activity was measured at 30 °C according to published procedures (Stiggall et al., 1978). ATPase activity was measured spectrophotometrically at 37 °C according to the method of Pullman et al. (1960) using an assay medium containing 0.25 M sucrose, 50 mM Tris-acetate, pH 7.5, 60 mM KCl, 3 mM MgCl<sub>2</sub>, 0.27 mM NADH, 1.6 mM phosphoenolpyruvate, and 1.5 units/mL each of pyruvate kinase and lactate dehydrogenase. Dodecyl sulfate-acrylamide gel electrophoresis was performed according to Weber & Osborn (1976) at 12.5% acrylamide. Each sample was electrophoresed on duplicate gels. One gel was stained for protein with Coomassie blue in a solution containing 25% 2-propanol and 10% acetic acid (v/v), destained with 10% acetic acid, and then scanned in a Gilford gel scanner. These gels and the second unstained duplicate gels were then separately cut into 1-mm thick slices, using the Mickel gel slicer, and the individual slices were each dissolved in 0.5 mL of 28% H<sub>2</sub>O<sub>2</sub> by heating at 50 °C overnight. The radioactivity in each solution was measured in a Beckman LS-250 scintillation counter after addition of 5 mL of dioxane containing 0.5% 2,5-diphenyloxazole (PPO) and 10% naphthalene. The counting efficiency was 54–60%.

[<sup>14</sup>C]DCCD was synthesized from [<sup>14</sup>C]urea (ICN, 55.7 mCi/mmol) diluted with "cold" urea to 3.7 mCi/mmol). [<sup>14</sup>C]Urea (0.5 mmol) was heated at 120 °C for 24 h with 1 mL of cyclohexylamine. The reaction mixture was cooled, diluted with a small amount of water, and filtered. The white product, [<sup>14</sup>C]dicyclohexylurea (yield ~90%; mp 234 °C), was collected and dried in vacuo. The dry dicyclohexylurea (0.45 mmol) was placed in 2 mL of redistilled, dry pyridine, the mixture was heated to 50 °C, and 40  $\mu$ L of distilled POCl<sub>3</sub> was added. The temperature was raised to 90 °C and kept there for 2 h. The mixture was cooled, extracted with 8 mL of *n*-hexane, and the hexane solution was washed 4 times with 5 mL each of ice-cold water. The aqueous extracts were discarded and the hexane solution of [<sup>14</sup>C]DCCD was dried over anhydrous sodium sulfate and stored at -70 °C in small portions. The yield of [<sup>14</sup>C]DCCD was between 75 and 80%, and the specific radioactivity was 3300 cpm/nmol. The purity of [<sup>14</sup>C]DCCD was ascertained by the chromatographic procedure of Stekhoven et al. (1972) using unlabeled commercial DCCD as reference.

Experiments for comparison of [<sup>14</sup>C]DCCD binding to and ATPase activity inhibition of complex V were carried out as

follows. Complex V (0.55–0.65 mg/mL) was incubated at 0 °C in 0.25 M sucrose containing 50 mM Tris-acetate, pH 7.5, with 3–30 nmol of [<sup>14</sup>C]DCCD per mg of complex V as indicated. For this purpose, the 56 mM stock solution of [<sup>14</sup>C]DCCD was diluted in absolute ethanol to 4.3 mM immediately before addition to complex V. The complex V reaction mixture containing [<sup>14</sup>C]DCCD was divided in 1-mL portions into several tubes, and each tube was incubated at 0 °C for a certain period of time. ATPase activity was measured at the end of the incubation time, and the incubation with DCCD was stopped by addition of 20% potassium cholate to a final concentration of 2% followed by room temperature saturated ammonium sulfate to 0.42 saturation. After 10–15 min of standing on ice, the precipitated complex V was centrifuged at 40000 rpm for 30 min, and the pellet was dissolved in 0.1 mL of 0.25 M sucrose containing 50 mM Tris-acetate, pH 7.5. This solution was further treated with 0.1 mL of 0.1 M potassium phosphate, pH 7.5, containing 10% NaDodSO<sub>4</sub> and 10% 2-mercaptoethanol and diluted with water (0.1–0.16 mL) to give a protein concentration of ~1.8 mg/mL. Aliquots were taken for radioactivity measurement and for NaDodSO<sub>4</sub> gel electrophoresis. In the latter case, the mixture was heated for 1 min in a boiling water bath before application to the gels. Appropriate controls were run for the effects of the DCCD solvent on activity and for the procedure used to stop the interaction of DCCD with complex V.

For comparing the kinetics of DCCD labeling of complex V with the inhibition of ATP- $P_i$  exchange activity, duplicate experiments, one for [<sup>14</sup>C]DCCD binding and another for ATP- $^{33}P_i$  exchange activity measurement, had to be used, because  $^{33}P_i$  interfered with measurement of [<sup>14</sup>C]DCCD radioactivity bound to complex V. Thus, in duplicate tubes, complex V at 4 mg/mL was incubated at 0 °C in 0.25 M sucrose containing 50 mM Tris-acetate, pH 7.5, with 3–25 nmol of DCCD per mg of complex V. One tube received nonradioactive DCCD and was sampled as a function of time for measurement of ATP- $^{33}P_i$  exchange activity. The duration of the exchange assay was 5 min. The second tube received [<sup>14</sup>C]DCCD, aliquots were withdrawn from it at the indicated time intervals, and the reaction was stopped as indicated above. The effects of various ATPase inhibitors and uncouplers on [<sup>14</sup>C]DCCD binding to complex V were also studied as indicated above. The compounds were added to the complex V solution at 0 °C 5–10 min prior to the addition of [<sup>14</sup>C]DCCD, and the interaction of DCCD with complex V was stopped as above after the indicated periods of incubation.

Extraction of [<sup>14</sup>C]DCCD-labeled complex V with acidic and neutral chloroform-methanol was performed according to Tzagoloff & Akai (1972) after washing of the preparation with 90% methanol. Thin-layer chromatography of complex V on silica gel plates (Merck, 60 F-254) for analysis of the radioactive regions a and c (see text and Figure 3) was carried out according to Cuzner & Davidson (1967) or Skipski et al. (1964). The spots were visualized under short-wave UV light or with ninhydrin or iodine, and radioactivity was localized by autoradiography. Protein remained at the origin, and radioactivity of the a and c regions of NaDodSO<sub>4</sub> gels moved to two *R<sub>f</sub>* positions of about 0.5 and 0.8.

Cholic acid was obtained from Mann; nucleotides were from P-L Biochemicals; DCCD was from K and K Laboratories; hexokinase (type F-300), phosphoenolpyruvate, lactate dehydrogenase, glucose-6-phosphate dehydrogenase (type XV), *p*-(chloromercuri)benzoate, and 2,4-dinitrophenol were from Sigma; pyruvate kinase, diamide [diazenedicarboxylic acid bis(dimethylamide)], CCCP, and valinomycin were from

Table I: Occurrence of [ $^{14}\text{C}$ ]DCCD in  $M_r$  Regions a, b, and c of Complex V as a Function of DCCD Concentration<sup>a</sup>

[ $^{14}\text{C}$ ]DCCD added (nmol/mg of complex V)	incubn time (min at 0 °C)	ATPase inhibn (%)	[ $^{14}\text{C}$ ]DCCD bound (nmol/mg of complex V)		
			a	b	c
6	3	30	0.85	1.0	0.4
6	20	65	0.9	2.1	0.3
6	120	95	1.1	4.45	
12	3	45	1.2	1.6	1.1
12	20	79	1.3	2.7	1.0
12	120	97	1.8	6.3	0.5
24	120	100	7.2	6.0	1.0
50	10	100	8.5	6.0	0.8
12	aged		7.4	0.6	0.5

<sup>a</sup> a, region centered at apparent  $M_r \sim 22\,000$ ; b, band at apparent  $M_r \sim 13\,000$ ; c, region near the dye front. Termination of complex V interaction with DCCD, NaDodSO<sub>4</sub> gel electrophoresis, and radioactivity analyses were as described under Methods and Materials.

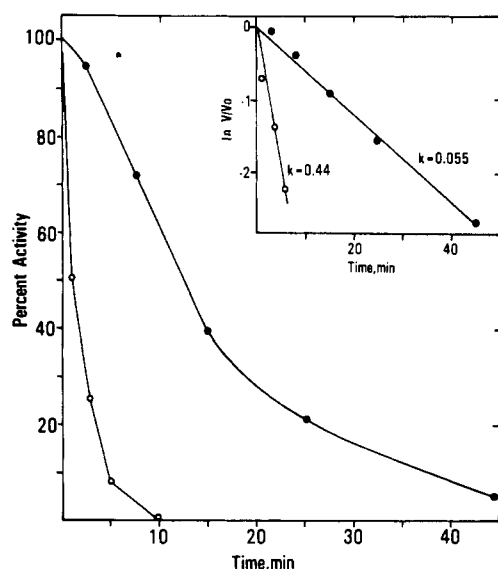


FIGURE 1: Kinetics of inhibition of complex V ATPase (●) and ATP- $^{33}\text{P}$  exchange (○) activities in the presence of 25 nmol of DCCD per mg of protein. Incubation temperature, 0 °C. ATPase and ATP- $\text{P}_i$  exchange activities at zero time were, respectively, 6  $\mu\text{mol}/(\text{min mg})$  at 37 °C and 100  $\text{nmol}/(\text{min mg})$  at 30 °C, both assayed in the absence of added phospholipids. The effect of [ $^{14}\text{C}$ ]DCCD on inhibition of ATPase activity was the same as that shown for unlabeled DCCD.

Calbiochem; [ $^{33}\text{P}$ ]phosphate was from New England Nuclear; [ $^{14}\text{C}$ ]urea and PPO were from ICN. Rutamycin and S-13 were gifts from Lilly and Monsanto Commercial Products Co., respectively; venturicidin and DBCT were gifts of Dr. D. E. Griffiths, University of Warwick, Coventry, England. NPTU<sup>2</sup> and NSPM<sup>3</sup> were synthesized, and the procedures will be reported elsewhere.

## Results

Figure 1 shows the kinetics of inhibition of ATP- $\text{P}_i$  exchange and ATPase activities of complex V treated with 25 nmol of DCCD per mg of protein. The inset shows that both inhibitions follow pseudo-first-order kinetics and that DCCD inhibits the ATP- $\text{P}_i$  exchange reaction several times faster than the ATPase activity of complex V.<sup>4</sup> Total [ $^{14}\text{C}$ ]DCCD binding by complex V is biphasic, as shown in Figure 2 [see also Norling et al. (1978)]. The rapid phase of [ $^{14}\text{C}$ ]DCCD

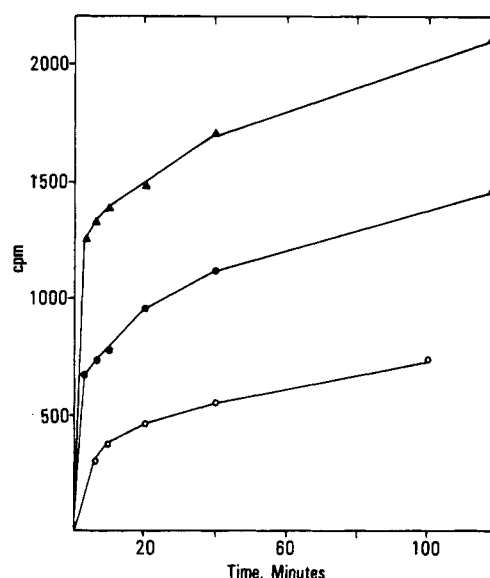


FIGURE 2: Kinetics of [ $^{14}\text{C}$ ]DCCD binding to complex V at 3 (○), 6 (●), and 12 (▲) nmol of [ $^{14}\text{C}$ ]DCCD per mg of complex V.

uptake by complex V corresponds in time to the kinetics of ATP- $\text{P}_i$  exchange inhibition (compare Figure 1 data with the rapid phase of the top curve of Figure 2), whereas, clearly, inhibition of ATPase activity does not correspond to this phase of [ $^{14}\text{C}$ ]DCCD uptake. Also, in Figure 2, the slow phases for the top two curves (i.e., uptake in the presence of 6 and 12 nmol of [ $^{14}\text{C}$ ]DCCD per mg of protein) are essentially superimposable, whereas the second phase of the bottom curve (3 nmol of [ $^{14}\text{C}$ ]DCCD per mg of protein) shows much slower DCCD uptake. Indeed, 3 nmol of DCCD per mg of protein is insufficient for complete labeling of the DCCD-binding protein of complex V. As shown in Figure 3, when [ $^{14}\text{C}$ ]DCCD-treated complex V is electrophoresed on 12.5% Weber-Osborn (Weber & Osborn, 1976) gels, three regions of radioactivity can be delineated. First is the diffuse region of low specific radioactivity (i.e., per unit weight of protein) centered at  $M_r \sim 22\,000$ , second is the region of high specific radioactivity at  $M_r \sim 13\,000$ , and third is the diffuse region of low radioactivity beyond the dye front. A somewhat similar labeling pattern has also been reported by Stekhoven et al. (1972). Table I shows the accumulation of radioactivity in these three regions, designated a, b, and c, respectively, as a function of added [ $^{14}\text{C}$ ]DCCD concentration and duration of incubation of complex V with the inhibitor at 0 °C. It is seen that region c takes up variable amounts of [ $^{14}\text{C}$ ]DCCD, but the maximum is  $\sim 1$  nmol/mg of protein. Region b takes up increasing amounts of [ $^{14}\text{C}$ ]DCCD up to about 6 to 7 nmol/mg of protein. Inhibitor uptake in this region is a

<sup>2</sup> R. Kiehl and Y. Hatefi, unpublished experiments.

<sup>3</sup> R. Kiehl and E. Bäuerlein, unpublished experiments.

<sup>4</sup> The rate constants ( $k$ ) of ATPase and ATP- $\text{P}_i$  exchange inhibition by a given amount of DCCD vary somewhat from one preparation of complex V to another, especially if the enzyme preparations have different phospholipid contents [see Stiggall et al. (1978)].

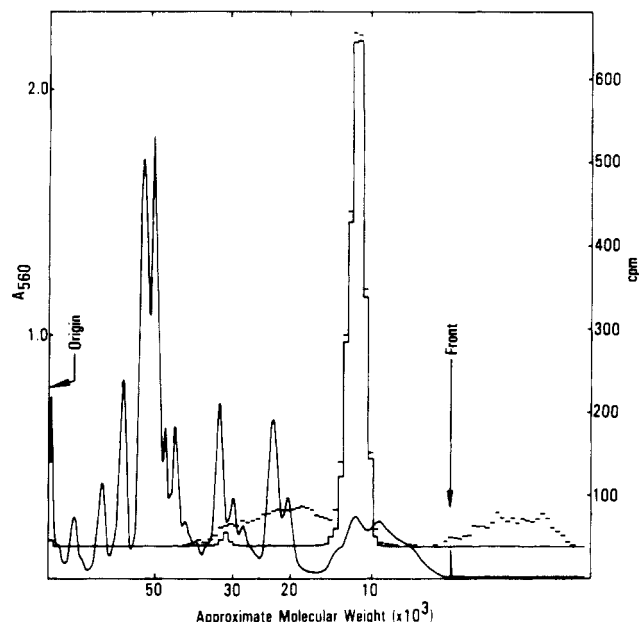


FIGURE 3: Distribution of radioactivity due to  $[^{14}\text{C}]\text{DCCD}$  in complex V electrophoresed on 12.5% Weber–Osborn  $\text{NaDodSO}_4$ -acrylamide gels. Complex V was incubated with 24 nmol of  $[^{14}\text{C}]\text{DCCD}$  per mg of protein for 4 h at  $0^\circ\text{C}$ . Continuous curve, protein bands of complex V (163  $\mu\text{g/gel}$ ) stained with Coomassie blue; continuous bars, radioactivity of gels stained with Coomassie blue and destained in 10% acetic acid; horizontal discontinuous bars, radioactivity before staining and destaining of the gels.

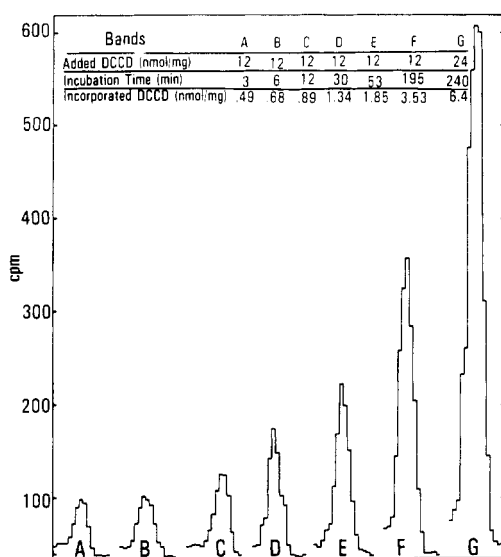


FIGURE 4: Incorporation of  $[^{14}\text{C}]\text{DCCD}$  into the DCCD-binding polypeptide of complex V as a function of time and DCCD concentration. Complex V treated with DCCD at  $0^\circ\text{C}$  was electrophoresed (197  $\mu\text{g/gel}$ ) on 12.5% Weber–Osborn  $\text{NaDodSO}_4$ -acrylamide gels.

function of both added DCCD concentration and duration of incubation (see also Figure 4); it also correlates with inhibition of ATPase activity. Similar to region c, the occurrence of label in region a is not correlated with activity inhibition and remains between 1 and 2 nmol/mg of protein until b is saturated. The capacity of a is  $\sim 10$ –12 nmol of DCCD per mg of protein. Also shown in Table I are data for a DCCD-treated complex V preparation which was aged for 2 weeks at room temperature. It is seen that there is a transfer of radioactivity from region b to a. However, as shown in Figure 3, when the gels were subjected to staining with Coomassie blue and destained as described, radioactivity in regions a and c was eluted from the gels while that in region

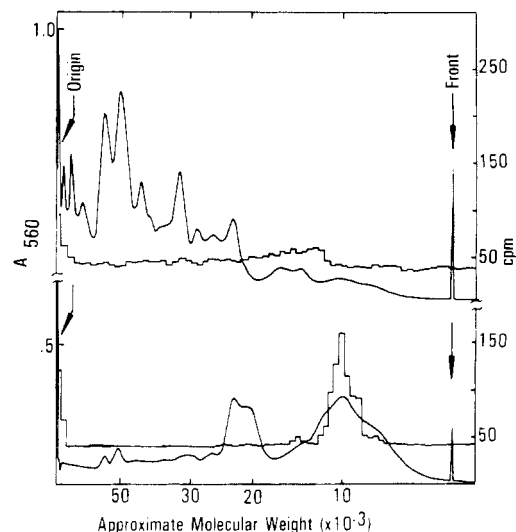


FIGURE 5: Distribution of radioactivity due to  $[^{14}\text{C}]\text{DCCD}$  in the acidified chloroform-methanol (2:1 plus 10 mM HCl) extract (bottom traces) and residue (top traces) of complex V treated for 2 h at  $0^\circ\text{C}$  with 25 nmol of  $[^{14}\text{C}]\text{DCCD}$  per mg of protein. In the top traces 65  $\mu\text{g}$  of protein and in the bottom traces 28  $\mu\text{g}$  of protein were electrophoresed on 12.5% Weber–Osborn  $\text{NaDodSO}_4$ -acrylamide gels.

b remained essentially unchanged. We have shown by chromatography on silica gel plates that radioactivity in extracts of regions a and c is not associated with protein. Therefore, it appears that the only band in  $\text{NaDodSO}_4$  gels of complex V, which is labeled with  $[^{14}\text{C}]\text{DCCD}$  and whose degree of labeling increases with inhibition of ATPase activity, is band b at the  $M_r$  region of 13 000. Since similar to DCCD-binding proteins from other sources the beef heart protein also appears to have a molecular weight of  $\sim 8000$  (Graf & Sebald, 1978), the banding of radioactivity on 12.5% Weber–Osborn gels at  $M_r \sim 13000$  (very close to the position of horse heart cytochrome c) appears anomalous [see also Sebald et al. (1979) for similar results]. However, this is in part due to difficulty in calibrating this region of Weber–Osborn gels. Indeed, on 12.5% Swank–Munkres (Swank & Munkres, 1971) gels, the calculated  $M_r$  value of the DCCD-binding polypeptide of complex V is  $\sim 6300$  (Galante et al., 1979). Therefore, in order to avoid confusion, we shall henceforth refer to this band simply as the DCCD-binding polypeptide or the specific DCCD binding site. Figure 5 shows that the labeled polypeptide can be extracted with chloroform-methanol (2:1) as in the case of the yeast DCCD-binding protein (Tzagoloff & Akai, 1972) and that the complex V residue after chloroform-methanol extraction is devoid of radioactivity. In agreement with these results and those of others, the water-soluble carbodiimide 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide did not inhibit the ATPase activity of complex V and did not interfere with  $[^{14}\text{C}]\text{DCCD}$  binding to the enzyme complex.

Figure 6 shows the relationships between inhibition of  $\text{ATP-P}_i$  exchange and ATPase activities of complex V and the extent of labeling of the DCCD-binding polypeptide by two concentrations of added  $[^{14}\text{C}]\text{DCCD}$ . Incorporation of DCCD into this polypeptide appears to be related to inhibition of both activities up to  $\sim 80\%$ . Thereafter, further inhibition of ATPase activity (maximum, 96% in the experiment shown) assumes a different relationship with labeling of the DCCD-binding polypeptide. Whether the same is true for inhibition of the remaining 20%  $\text{ATP-P}_i$  exchange activity was not experimentally feasible to determine. However, as seen in Figure 6, extrapolation of the steep lines to zero activity suggests

Table II: Effects of Modifiers of Mitochondrial Energy-Linked Functions on [ $^{14}\text{C}$ ]DCCD Binding to Complex V<sup>a</sup>

compd	concn (nmol/mg of complex V)	[ $^{14}\text{C}$ ] DCCD in complex V (% of control)	[ $^{14}\text{C}$ ] DCCD in the DCCD-binding polypeptide (% of control)	act. inhibn by the compd added (%)	
				ATP $\leftrightarrow$ P <sub>i</sub>	ATPase
rutamycin	38	78	45	100	100
venturicidin	94	69	0	100	68
CCCP	94	80-90	60	100	8
S-13	47	90-100		100	
DNP	540	90-100		100	-2
DBCT	94	159	0	100	
NSPM	94	100-120	80	100	25
pCMB	380	110		80	0
diamide	540	78-85	50	0	0
valinomycin	25 <sup>b</sup>	85-90		100	0
NPTU	80	85	51	100	40
NPTU	145	69	22	100	55

<sup>a</sup> The amount of [ $^{14}\text{C}$ ]DCCD added was 24 nmol/mg of complex V. Preincubation of complex V at 0 °C with each of the compounds listed in column 1 was for 5-10 min, and further incubation after addition of [ $^{14}\text{C}$ ]DCCD was for 3 min. Reactions were stopped, and complex V was precipitated and redissolved for NaDodSO<sub>4</sub> gel electrophoresis and radioactivity analysis as described under Methods and Materials.

<sup>b</sup> In the ATP-P<sub>i</sub> exchange assay, K<sup>+</sup> was also present, and inhibition by valinomycin + K<sup>+</sup> is due to the presence of NH<sub>4</sub><sup>+</sup> ions added together with the ammonium sulfate pelleted enzyme.

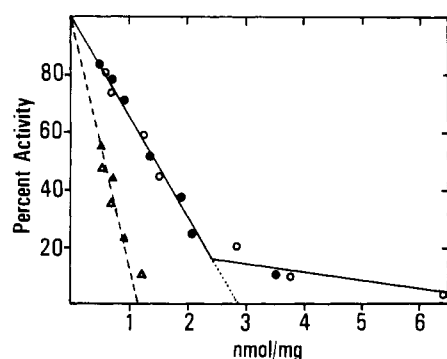


FIGURE 6: Correlation of [ $^{14}\text{C}$ ]DCCD incorporation into the DCCD-binding polypeptide of complex V with inhibition of ATP-P<sub>i</sub> exchange ( $\Delta$ ,  $\blacktriangle$ ) and ATPase ( $\circ$ ,  $\bullet$ ) activities. Complex V was incubated and assayed as in Figure 1 with 12 ( $\Delta$ ,  $\bullet$ ) and 24 ( $\Delta$ ,  $\circ$ ) nmol of [ $^{14}\text{C}$ ]DCCD per mg of protein. Complex V ATPase and ATP-P<sub>i</sub> exchange activities in the absence of DCCD were, respectively, 10  $\mu\text{mol}/(\text{min mg})$  at 37 °C and 106  $\text{nmol}/(\text{min mg})$  at 30 °C, both assayed in the absence of added phospholipids.

incorporation of about 1 and 3 nmol of [ $^{14}\text{C}$ ]DCCD per mg of complex V into the DCCD-binding polypeptide, respectively, for complete inhibition of ATP-P<sub>i</sub> exchange and ATPase activities. These titers vary somewhat depending on the purity of complex V and its content of phospholipids. However, on the assumption of a minimum molecular weight of 500 000 for complex V, complete inhibition of ATP-P<sub>i</sub> exchange activity appears to be correlated with substoichiometric amounts of DCCD covalently bound to the specific DCCD binding site, and complete inhibition of ATPase activity seems to be associated with greater than 1 mol of covalently bound DCCD per mol of complex V. Another relationship of possible interest is that in a number of experiments with preparations of complex V having somewhat different phospholipid contents (Stiggall et al., 1978), it seemed that the titer for complete inhibition of ATPase activity (i.e., the extrapolated abscissa intersect of the steep portion of the ATPase inhibition line to zero activity) was always  $\sim 50\%$  of the capacity of the specific DCCD binding site to take up [ $^{14}\text{C}$ ]DCCD. If we assume that the second portion of the ATPase curve, which has a shallow slope, is due to the presence of  $\sim 15\%$  complex V molecules having low sensitivity to DCCD, then the above results might mean that in the remaining "normal" molecules binding of DCCD to 50% of the available specific sites is sufficient for complete inhibition of ATPase activity. Indeed, if we carry

this reasoning over to the ATP-P<sub>i</sub> exchange data, it would seem that covalent labeling of only about one-sixth of the available specific sites with DCCD is sufficient for complete inhibition of ATP-P<sub>i</sub> exchange activity. However, this line of reasoning assumes that only DCCD covalently bound to the DCCD-binding protein results in inhibition. This may not be so, since noncovalently bound DCCD might also cause inhibition, and activation and covalent attachment of DCCD in a hydrophobic environment might be a relatively slow and secondary event.

It has been shown by Sebald et al. (1976) that oligomycin partially inhibits the binding of [ $^{14}\text{C}$ ]DCCD to submitochondrial particles from *Neurospora*, and Enns & Criddle (1977) and Criddle et al. (1977) have presented evidence regarding the interaction of oligomycin with the DCCD-binding protein of yeast mitochondria. Table II shows data regarding the effects of various ATPase inhibitors and uncouplers on [ $^{14}\text{C}$ ]DCCD binding to complex V and to the DCCD-binding polypeptide, as well as data on the effects of these compounds at the concentrations used on the ATP-P<sub>i</sub> exchange and the ATPase activities of the preparation. It is seen that rutamycin, venturicidin, CCCP, DBCT, diamide, and the substituted thiourea NPTU (an uncoupler; 50% uncoupling concentration with submitochondrial particles was  $\leq 10 \mu\text{M}$ ) effectively diminish the labeling of the DCCD-binding polypeptide of complex V with [ $^{14}\text{C}$ ]DCCD. That the effect of these compounds is not due simply to a uniform diminution of complex V labeling by [ $^{14}\text{C}$ ]DCCD is clear from a comparison of overall complex V labeling (Table II, column 3) and specific labeling of the DCCD-binding polypeptide (Table II, column 4). The results for CCCP, NPTU, DBCT, rutamycin, and venturicidin are also depicted in Figure 7, which shows the distribution of radioactivity due to [ $^{14}\text{C}$ ]DCCD among the components of complex V electrophoresed on 12.5% Weber-Osborn NaDodSO<sub>4</sub>-acrylamide gels. As seen in Table II and Figure 7, venturicidin, DBCT, and NPTU were highly effective in inhibiting DCCD binding to the specific DCCD binding site of complex V. In addition, DBCT appeared to have caused a transfer of label from the specific site to region a (Figure 7B), which as discussed above can be washed out of the gels, and its radioactivity is not associated with protein.

#### Discussion

As stated above, in spite of extensive work on the DCCD-binding protein from various sources, the present studies are

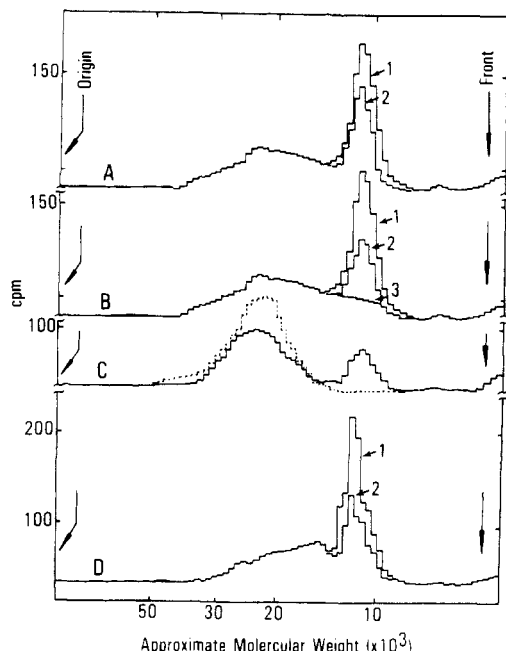


FIGURE 7: Effect of pretreatment of complex V with ATPase inhibitors and uncouplers on  $[^{14}\text{C}]$ DCCD binding. Complex V was treated for 5 min at  $0^\circ\text{C}$  with CCCP (94 nmol/mg), venturicidin (94 nmol/mg), rutamycin, (40 nmol/mg), dibutyl(chloromethyl)tin chloride (DBCT, 47 nmol/mg), or *N*-phenyl-*N'*-*n*-nonylthiourea (NPTU, 47 nmol/mg) and then with 24 nmol of  $[^{14}\text{C}]$ DCCD per mg of protein for 3 min at  $0^\circ\text{C}$  (in the rutamycin experiment, DCCD treatment was for 10 min). Gels (12.5% Weber-Osborn NaDodSO<sub>4</sub>-acrylamide) in A–C received 142  $\mu\text{g}$  of protein each; gels in D received 107  $\mu\text{g}$  of protein each. The gels sliced for determining radioactivity distribution were not stained with Coomassie blue. (A) 1, control; 2, plus CCCP; (B) 1, control; 2, plus NPTU; 3, plus venturicidin; (C) solid line, control; dashed line, plus DBCT; (D) 1, control; 2, plus rutamycin.

the first instance in which the labeling kinetics of the DCCD-binding polypeptide with radioactive DCCD has been investigated, and the binding data have been correlated with the inhibition kinetics of appropriate reactions. Regarding the latter, the results on complex V have shown that DCCD inhibits ATP- $\text{P}_i$  exchange several times faster than the ATPase activity. This difference in the sensitivity of the two reactions toward a common inhibitor is seen also in the case of several other compounds, including rutamycin, venturicidin, triethyltin sulfate, mercurials, butanedione, and phenylglyoxal (Stiggall et al., 1978; Frigeri et al., 1977). In the case of the arginyl-modifying reagents butanedione and phenylglyoxal, it has been shown that two essential arginyl residues are involved, one in  $\text{F}_1$ -ATPase [very likely the  $\beta$  subunit; see Esch & Allison (1978)] which is protectable by purine nucleotide diphosphates and another at or near the uncoupler binding site of complex V. However, in principle, a single inhibition site can be sufficient to render ATP- $\text{P}_i$  exchange more sensitive to inhibitors than ATP hydrolysis. If we assume that formally ATP- $\text{P}_i$  exchange involves ATP hydrolysis and resynthesis, then inhibition of the forward as well as the reverse reactions would result in the greater sensitivity of ATP- $\text{P}_i$  exchange than ATP hydrolysis toward various common inhibitors. Recently, Pougeois et al. (1979) have shown that DCCD also binds to the  $\beta$  subunit of  $\text{F}_1$ -ATPase and inhibits ATP hydrolysis when the enzyme is incubated at  $24^\circ\text{C}$  with several times higher levels of DCCD per milligram of protein than used in our studies. However, NaDodSO<sub>4</sub>-acrylamide gels of complex V treated with  $[^{14}\text{C}]$ DCCD at  $0^\circ\text{C}$  (Figure 3) show clearly that under our conditions no labeling of  $\text{F}_1$  subunits occurred. Indeed, the NaDodSO<sub>4</sub> gel results of Sebald et al. (1979) on

$[^{14}\text{C}]$ DCCD-treated mitochondria from yeast, *Neurospora*, and beef heart show appreciable labeling only in the region corresponding to the position of the DCCD-binding polypeptide. Therefore, we prefer to think of a single DCCD-modified site being responsible for inhibition of ATPase and ATP- $\text{P}_i$  exchange activities in mitochondria and complex V type preparations. While the observations of Pougeois et al. (1979) are interesting, it is possible that interaction of DCCD with  $\text{F}_1$ -ATPase is peculiar to the soluble form of the enzyme and does not occur under the same conditions with the membrane-bound form. On the other hand, because of their high reactivity (Khorana, 1953; Kurzner & Douraghi-Zadeh, 1967), carbodiimides would be expected to react under appropriate conditions with various proteins. [For example, see Carraway & Koshland (1968), Banks et al. (1969), Carraway & Triplett (1970), Beyer et al. (1972), and Casey et al. (1979), among which the latter two references are concerned with interaction of DCCD with the complex I region of the respiratory chain and with cytochrome oxidase preparations, respectively.]

As seen in Figure 2, the uptake of DCCD by complex V is biphasic. There is a rapid phase of uptake during the first several minutes of incubation, followed by a considerably slower phase. Analysis of bound DCCD in NaDodSO<sub>4</sub> gels of complex V (Table I and Figure 4) also shows this biphasicity in the labeling of the DCCD-binding polypeptide (region b in Table I) and suggests negative cooperativity in DCCD interaction with the specific DCCD binding site. Comparison of the inhibition kinetics of Figure 1 with the binding data of Figures 1 and 4 and Table I would suggest that nearly complete inhibition of ATP- $\text{P}_i$  exchange occurs during the rapid phase of DCCD uptake by complex V, while complete inhibition of ATP hydrolysis requires further DCCD binding in the second, slow phase. These conclusions are in agreement with the plots of Figure 6 which show the relationships between covalent inhibitor binding to the DCCD-binding polypeptide and the degree of inhibition of ATPase and ATP- $\text{P}_i$  exchange activities. The total capacity of the DCCD binding site of complex V is 6 to 7 (average of five experiments using five different complex V preparations,  $6.62 \pm 0.28$ ) nmol of DCCD per mg of protein. The amount of DCCD bound to the DCCD-binding polypeptide which correlates with 100% inhibition of ATPase activity, as shown by extrapolation of the steep portion of the Figure 3 curve to zero activity, was found to be  $3.23 \pm 0.26$  nmol of DCCD per mg of protein in the same set of five experiments, i.e.,  $\sim 50\%$  of the total capacity of the specific DCCD binding site of complex V. However, as seen in Figure 6, the extent of DCCD binding that correlates with complete inhibition of the ATP- $\text{P}_i$  exchange activity is between one-seventh and one-sixth of the total binding capacity of the DCCD binding site. These results recall the conclusions of Graf & Sebald (1978) and Sebald et al. (1979) that, as in *Neurospora* and yeast mitochondria, the beef heart system might also contain an oligomer of the DCCD-binding polypeptide composed of six to seven DCCD-reactive molecules. If so, then the above data would suggest that modification of only one site by DCCD is sufficient for complete inhibition of ATP- $\text{P}_i$  exchange activity. On the other hand, the data of Figure 6 show only the results of covalent DCCD binding as correlated with activity inhibition, and the possibility of noncovalent DCCD binding also causing inhibition should not be disregarded. Indeed, it should be kept in mind that the incubation times required for inhibition of ATP- $\text{P}_i$  exchange activity by DCCD are relatively short (see Figure 1) and that it has been shown by Bruni et al. (1971) that the inhibition brought about by short periods of incubation of submito-

chondrial particles with DCCD could be reversed to a considerable extent by the addition of phospholipids to the reaction mixture. Thus, it is possible that noncovalently bound DCCD might also cause inhibition and that the DCCD titer for complete inhibition of ATPase and ATP-P<sub>i</sub> exchange activities is greater than the extrapolated values of Figure 6. [<sup>14</sup>C]DCCD uptake data such as those shown in Figure 2 might have allowed a correlation between DCCD binding (i.e., both covalent and noncovalent) and activity inhibition. However, since nonspecific DCCD binding also occurs in complex V (e.g., in regions a and c of Table I and Figure 3), such a correlation is clearly not possible.

Another set of results deserving of consideration here is the data of Table II and Figure 7, which show inhibition of DCCD binding by rutamycin, venturicidin, CCCP, DBCT, diamide, NSPM (slight), and NPTU. Among these, the effect of rutamycin was expected and agrees with the previous results of others (Sebald et al., 1976; Enns & Criddle, 1977). Venturicidin is also considered to bind to a site at or near the oligomycin binding site (Lardy et al., 1975; Griffiths, 1976). Therefore, although a weaker ATPase inhibitor than rutamycin, it is seen in Table II and Figure 7 that at the concentration used venturicidin completely abolished DCCD binding to the DCCD-binding polypeptide. The available information suggests, therefore, that DCCD, oligomycin, and venturicidin bind to independent but interacting sites. Among the other reagents mentioned above, CCCP and NPTU are uncouplers of mitochondrial energy-linked functions, but their inhibition of DCCD binding does not seem to be related to their uncoupling property, because as seen in Table II the uncouplers S-13 and DNP have little or no effect on DCCD binding. On the other hand, CCCP, DBCT, diamide, and NSPM all react with thiols and dithiols. (DBCT is a potent dithiol inhibitor, and diamide oxidizes dithiols to disulfides.) However, among the DCCD-binding proteins with known amino acid sequences, only the yeast protein contains one cysteine six residues removed from the glutamic acid residue which reacts with DCCD (Sebald & Wachter, 1978). The beef heart protein also contains a single cysteine residue (Graf & Sebald, 1978), but its relation to the site of DCCD binding is not yet known. Lipophilicity and unspecific blockage of the DCCD binding site might be a common factor among the above compounds as well as NPTU, and the structural similarity of NPTU to the interaction product of DCCD with appropriate amino acid residues (e.g., O- and S-substituted ureas, isoureas, thioureas, and isothioureas) might also be responsible for its effect. Indeed, NPTU may displace DCCD from the protein after the latter has reacted to form the first covalent isourea adduct. With most of the above compounds, however, the more likely possibility is that they inhibit DCCD binding prior to its covalent interaction with carboxyl (or possibly cysteine) residues, since the covalently attached DCCD as its N-substituted urea (or thiourea) would be expected to be stable.

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#### References

- Altendorf, K. (1977) *FEBS Lett.* 73, 271-275.
- Banks, T. E., Blossey, B. K., & Shafer, J. A. (1969) *J. Biol. Chem.* 244, 6323-6333.
- Beechey, R. B., Holloway, C. T., Knight, I. G., & Robertson, A. M. (1966) *Biochem. Biophys. Res. Commun.* 23, 75-80.
- Beechey, R. B., Robertson, A. M., Holloway, C. T., & Knight, I. G. (1967) *Biochemistry* 6, 3867-3879.
- Beyer, R. E., Brink, T. W., Crankshaw, D. L., Kuner, J. M., & Pasternak, A. (1972) *Biochemistry* 11, 961-969.
- Bruni, A., Pitotti, A., Contessa, A. R., & Palatini, P. (1971) *Biochem. Biophys. Res. Commun.* 44, 268-274.
- Carraway, K. L., & Koshland, D. E., Jr. (1968) *Biochim. Biophys. Acta* 160, 272-274.
- Carraway, K. L., & Triplett, R. B. (1970) *Biochim. Biophys. Acta* 200, 564-566.
- Casey, R. P., Thelen, M., & Azzi, A. (1979) *Biochem. Biophys. Res. Commun.* 87, 1044-1051.
- Cattell, K. J., Knight, I. G., Lindop, C. R., & Beechey, R. B. (1970) *Biochem. J.* 117, 1011-1013.
- Cattell, K. J., Lindop, C. R., Knight, I. G., & Beechey, R. B. (1971) *Biochem. J.* 125, 169-177.
- Criddle, R. S., Packer, L., & Shieh, P. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 4306-4310.
- Cuzner, M. L., & Davidson, A. N. (1967) *J. Chromatogr.* 27, 388-397.
- Enns, R. K., & Criddle, R. S. (1977) *Arch. Biochem. Biophys.* 182, 587-600.
- Esch, F. S., & Allison, W. S. (1978) *J. Biol. Chem.* 253, 6100-6106.
- Fillingame, R. H. (1975) *J. Bacteriol.* 124, 870-883.
- Fillingame, R. H. (1976) *J. Biol. Chem.* 251, 6630-6637.
- Frigeri, L., Galante, Y. M., Hanstein, W. G., & Hatefi, Y. (1977) *J. Biol. Chem.* 252, 3147-3152.
- Galante, Y. M., Wong, S.-Y., & Hatefi, Y. (1979) *J. Biol. Chem.* 254, 12372-12378.
- Graf, T., & Sebald, W. (1978) *FEBS Lett.* 94, 218-222.
- Griffiths, D. E. (1976) in *The Structural Basis of Membrane Function* (Hatefi, Y., & Djavadi-Ohanian, L., Eds.) pp 205-214, Academic Press, New York.
- Hatefi, Y., Stiggall, D. L., Galante, Y., & Hanstein, W. G. (1974) *Biochem. Biophys. Res. Commun.* 61, 313-321.
- Holloway, C. T., Robertson, A. M., Knight, I. G., & Beechey, R. B. (1966) *Biochem. J.* 100, 79P.
- Khorana, H. G. (1953) *Chem. Rev.* 53, 145-166.
- Kurzner, F., & Douraghi-Zadeh, K. (1967) *Chem. Rev.* 67, 107-152.
- Lardy, H., Reed, P., & Lin, C.-H. C. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol.* 34, 1707-1710.
- Löw, H., & Vallin, L. (1963) *Biochim. Biophys. Acta* 69, 361-374.
- Lowry, D. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem.* 193, 265-275.
- Nelson, N., Eytan, E., Notsani, B.-E., Sigrist, H., Sigrist-Nelson, K., & Gitler, C. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 2375-2378.
- Norling, B., Glaser, E., & Ernster, L. (1978) in *Frontiers of Biological Energetics* (Dutton, P. L., Leigh, J. S., & Scarpa, A., Eds.) Vol. 1, pp 504-515, Academic Press, New York.
- Pougeois, R., Satre, M., & Vignais, P. (1979) *Biochemistry* 18, 1408-1413.
- Pullman, M. E., Penefsky, H. S., Datta, A., & Racker, E. (1960) *J. Biol. Chem.* 235, 3322-3329.
- Sebald, W., & Wachter, E. (1978) in *Energy Conservation in Biological Membranes* (Schäfer, G., & Klingenberg, M., Eds.) pp 228-236, Springer-Verlag, West Berlin.
- Sebald, W., Graf, T., & Wild, G. (1976) in *Genetics and Bioenergetics of Chloroplasts and Mitochondria* (Bucher, T., et al., Eds.) pp 167-174, Elsevier/North-Holland Biochemical Press, Amsterdam.



- Sebald, W., Graf, T., & Lukins, H. B. (1979) *Eur. J. Biochem.* 93, 587-599.
- Sigrist-Nelson, K., & Azzi, A. (1979) *Biochem. J.* 177, 687-692.
- Sigrist-Nelson, K., Sigrist, H., & Azzi, A. (1978) *Eur. J. Biochem.* 92, 9-14.
- Skipski, V. P., Peterson, R. F., & Barclay, M. (1964) *Biochem. J.* 90, 374-378.
- Sone, N., Yoshida, M., Hirata, H., & Kagawa, Y. (1979) *J. Biochem. (Tokyo)* 85, 503-509.
- Stekhoven, F. S., Waitkus, R. F., & van Moerkerk, H. T. B. (1972) *Biochemistry* 11, 1144-1150.
- Stiggall, D. L., Galante, Y. M., & Hatefi, Y. (1978) *J. Biol. Chem.* 253, 956-964.
- Swank, R. T., & Munkres, K. D. (1971) *Anal. Biochem.* 39, 462-477.
- Tzagoloff, A., & Akai, A. (1972) *J. Biol. Chem.* 247, 6517-6523.
- Weber, K., & Osborn, M. (1976) *Proteins, 3rd Ed.* 2, 179-223.

## Molecular Weight and Hydrodynamic Parameters of the Adenosine 5'-Diphosphate-Adenosine 5'-Triphosphate Carrier in Triton X-100<sup>†</sup>

Heinz Hackenberg\* and Martin Klingenberg

**ABSTRACT:** The Triton-solubilized ADP-ATP carrier from beef heart mitochondria was studied as the carboxyatractylate (CAT) complex by sedimentation in sucrose gradients, gel filtration, and analytical ultracentrifugation. The measurements of Triton binding and sedimentation velocity were not possible unless excess Triton and phospholipid were separated by an additional sucrose density gradient centrifugation. The absorption optical system of the analytical ultracentrifuge was used to analyze a multicomponent system consisting of protein, bound Triton, free Triton micelles, and Triton monomers. The isolated protein binds high amounts of Triton (1.5 g/g of protein) and phospholipid (0.25 g/g of protein) as would be expected for an intrinsic membrane protein. The sedimentation coefficient, measured on sucrose gradients and in the analytical ultracentrifuge, was  $s_{20,w} = 3.9 \times 10^{-13}$  s. The diffusion coefficient, calculated from the Stokes' radius ( $R_s = 65$  Å)

and determined in the analytical ultracentrifuge, was  $(3.4-3.5) \times 10^{-7}$  cm<sup>2</sup> s<sup>-1</sup> in both cases. The partial specific volume  $\bar{v}^*$  of the protein-Triton-phospholipid complex was evaluated from the fractions of the individual components and from sedimentation equilibrium measurements in solutions of different densities ( $\bar{v}^* = 0.845$  cm<sup>3</sup>/g). The molecular weight was calculated from the Svedberg equation ( $M^* 184\,000$ ) and from sedimentation equilibrium measurements ( $M^* 172\,000$ ). After subtraction of the bound Triton and phospholipid,  $M_r$  67 000 (63 000) was found for the protein moiety, indicating that the Triton-solubilized CAT-protein complex exists as a dimer composed of two peptide subunits. From geometrical considerations it is concluded that the protein is completely enveloped in an ellipsoid micelle of ~150 Triton and ~16 phospholipid molecules.

The ADP-ATP carrier from mitochondria has been isolated in the undenatured form as a carboxyatractylate-protein complex by using the nonionic detergent Triton X-100 (Klingenberg et al., 1974; Riccio et al., 1975b). It contains, according to NaDodSO<sub>4</sub><sup>1</sup>-polyacrylamide gel electrophoresis, a single polypeptide of  $M_r$  30 000. This polypeptide is the most abundant one in beef heart mitochondria, accounting for about 9% of total protein. From the amount of carboxyatractylate bound, a molecular weight of ~59 000 was calculated. Therefore, it was proposed that the isolated protein exists as a dimer with two subunits and one binding site.

With the isolated protein, studies in our laboratory were undertaken with the aim of elucidating the mechanism of this carrier, comprising functional and structural investigations (Klingenberg et al., 1975a,b, 1977, 1979; Aquila et al., 1978). For example, it was shown by reconstitution that the isolated carrier molecule is fully competent for transport (Krämer & Klingenberg, 1977).

The solubilization of the protein required high amounts of an efficient detergent and complete disruption of the phospholipid matrix. This was indicative for the strong hydrophobic nature of the protein in accordance with the expected deep embedment of this carrier in the membrane. Another particular feature of the solubilized CAT-protein was its nonadsorption to hydroxylapatite, in contrast to most other mitochondrial proteins; this is utilized as a main purification step. These results were explained by a large detergent coat shielding the protein (Klingenberg et al., 1978).

In the present study the molecular weight and the hydrophobic properties comprising the protein-detergent interaction were investigated by sucrose density gradients, gel filtration, and analytical ultracentrifugation. The CAT complex of the ADP-ATP carrier was used because of its particular stability. This analysis had to take into account the high amount of detergent and phospholipid bound to the protein and required

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<sup>1</sup> Abbreviations used: CAT, carboxyatractylate; Mops, 4-morpholinepropanesulfonic acid; EDTA, ethylenediaminetetraacetate;  $k$ , Boltzmann constant;  $R$ , gas constant;  $A_{280}$ , absorbancy at 280 nm;  $\eta$ , viscosity;  $\omega$ , angular velocity;  $T$ , absolute temperature;  $\rho_s$ , solvent density; NaDodSO<sub>4</sub>, sodium dodecyl sulfate.