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PROTON-TRANSPORTING URINARY EPITHELIA

REACTIVITY WITH *N,N'*-DICYCLOHEXYLCARBODIIMIDE

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Dicyclohexylcarbodiimide (DCCD), a potent inhibitor of the F_0F_1-type H^+-translocating ATPase, was employed to determine the possible involvement of such an ATPase in urinary acidification. Two methods were used in this approach: (1) the reaction of [^{14}C]DCCD with tissues involved in urinary acidification and (2) the inhibition of ATPase activity by DCCD. Membrane components from epithelial cells of toad and turtle urinary bladder and brush borders of rabbit kidney were reacted with [^{14}C]DCCD and analyzed by polyacrylamide gel electrophoresis both before and after extraction with organic solvents. Although a DCCD-binding component was extracted from toad and turtle bladder membranes by chloroform/methanol (2:1, v/v), the binding was not saturable. Analysis of this DCCD-binding component by thin-layer chromatography indicated that there was no ninhydrin reactivity associated with the [^{14}C]DCCD binding. Moreover, all attempts to precipitate a DCCD-binding protein were unsuccessful. This and other evidence suggested that the observed DCCD binding was to phospholipid. In the second type of experiments, the ATPase activity present in brush borders from rabbit kidney was partially inhibited by DCCD, but at a concentration that is over two orders of magnitude greater than that required for typical DCCD-sensitive ATPase. We conclude from our failure to find positive evidence of a DCCD-reactive protein and from the relative insensitivity of the ATPase to DCCD that either urinary acidification is not accomplished by a typical F_0F_1-type translocating ATPase, or the F_0 has been modified so that the sensitivity to DCCD has been altered or lost.

Introduction

Two general types of H^+-translocating ATPases have been described. One type occurs in mitochondria, yeast, chloroplasts and bacteria (for reviews on the subject see Refs. 1–4). It is a multi-subunit F_0-F_1 complex consisting of a section intrinsic to the membrane (termed F_0) that functions as an H^+ channel and a multi-subunit complex extrinsic to the membrane (termed F_1) that is the catalytic (ATP-hydrolase) component. A second type of H^+-ATPase, described in *Neurospora, Saccharomyces* and gastric mucosa, contains a single protein subunit with a molecular weight of 100000–105000 that is the hydrolytic moiety of the H^+-translocating ATPase [5–10]. This H^+-ATPase bears analogies with two other membrane-bound ion-translocating ATPases, Ca^{2+}-ATPase [11] and $(Na^+ + K^+)$-ATPase [12].

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; TEMED, *N,N,N',N'*-tetramethylethylenediamine; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

In a previous study [19], we examined agents known to inhibit the F_0F_1-ATPase for effects on urinary acidification in the isolated urinary bladder of the toad and turtle. Some agents that inhibit intact F_0F_1-ATPase (oligomycin and EEDQ) failed to inhibit the H^+ pump responsible for urinary acidification under anaerobic conditions. On the other hand, other inhibitors of F_0F_1-ATPase such as DCCD and tyrosine-reactive chemicals did inhibit urinary acidification. The findings with respect to the individual subunits of F_0F_1-ATPase were also not conclusive. For example, oligomycin and DCCD both inhibit the F_0 H^+ channel in mitochondria, but only the latter inhibited anaerobic urinary acidification. However, the concentration of DCCD needed to inhibit urinary acidification was considerably different in that 10^{-4} M DCCD inhibited acidification only partially whereas 10^{-6} M DCCD inhibits F_0F_1-ATPase.

DCCD has been found to bind covalently at low concentrations with an F_0 protein in all systems in which it has been found with the exception of mutant systems [4,14–17]. With this in mind, we sought evidence for the presence of such a DCCD-reactive membrane protein in epithelia that conduct urinary acidification. Such an avenue of investigation seemed warranted in view of findings that DCCD inhibited both H^+ transport in urinary bladders of toad and turtle [18–20] and an ATPase in brush border preparations from rabbit kidney [21]. In this article we report our inability to provide evidence for a DCCD-binding protein in tissues involved in urinary acidification.

Materials and Methods

Preparation of membranes. Urinary bladders from toads (*Bufo marinus*) originating from Texas or Mexico, or turtles (*Pseudemys scripta*), were removed and the cells scraped from the mucosal side. The membrane fraction of the cells was prepared as described by Park and Edelman (Ref. 22, and Park, C.S., personal communication). The cells were disrupted with a Polytron homogenizer (Brinkmann Instruments) and spun at $700 \times g$ for 20 min. The supernatant was centrifuged at $25000 \times g$ for 45 min to obtain a crude membrane preparation. The apical membrane was further purified by density gradient centrifugation [22]. All studies reported were conducted on membranes carried through the gradient step.

Renal proximal tubule brush border membrane vesicles were prepared from 3-kg male New Zealand white rabbits according to the method of Malathi et al. [24]. The final preparation was suspended in 50 mM mannitol, 2 mM Tris-HCl, pH 7.0.

Binding of isethionyl[^{14}C]acetimidate to apical membrane. Toad hemibladders were tied on canula as bags, mucosa inside. The bladders were then filled and immersed in Ringer's solution of the following composition: 109.6 mM NaCl, 3.35 mM KCl, 4.8 mM $NaHCO_3$, 1.0 mM $MgCl_2$ and 2.7 mM $CaCl_2$, pH 8.7, and bubbled with air. The bladders were emptied after 30 min and refilled with Ringer's solution containing isethionyl-[^{14}C]acetimidate (10 μCi/ml) at a specific activity of 59 μCi/μmol. The bladders were allowed to react [23] for 2 h at 23°C. The reaction was terminated by draining the bladders and rinsing four times with Ringer's solution containing 20 mM Tris-HCl, pH 7.8. The amine buffer, Tris, was used to quench the reaction of isethionylacetimidate with tissue.

Binding of DCCD to mitochondrial hydrophobic protein. Beef heart mitochondria were suspended at a protein concentration of 30 mg/ml in 0.25 M sucrose and 10 mM Tris-HCl buffer at pH 7.6. Unlabeled DCCD was added as an ethanolic solution at 30 nmol/mg protein and incubated at 0°C for 24 h.

Enzymatic assays. Cytochrome *c* oxidase was measured according to the method of Smith [25]. The 5′-nucleotidase activity was determined by the method of Heppel and Hilmoe [26] with the modification that P_i was determined by the method of Chen et al. [27]. Alkaline phosphatase was measured according to the method reported by Mircheff and Wright [28]. The ATPase activity was determined following a 15 min incubation at 37°C in a medium containing 5 mM $MgCl_2$, 5 mM ATP and either 10 mM Tris-HCl, pH 7.8, or 10 mM Mes, pH 6.45. The reaction was initiated by the addition of ATP. All samples were preincubated for 15 min at 37°C with the following inhibitors, where indicated: 1 mM ouabain, 5 mM azide, oligomycin at 2 μg/mg protein and DCCD

at the various levels described in the text. DCCD and oligomycin were added as concentrated ethanolic solutions. The reaction was terminated by the addition of trichloroacetic acid at 4°C to a final concentration of 10%. P_i in the supernatant was determined by the method of Stantan [29].

[^{14}C]DCCD incorporation into membranes. Toad bladder membrane was suspended in 10 mM Tris-HCl, pH 7.4, whereas turtle bladder membrane was suspended in 10 mM Mes, pH 7.1, at 2–4 mg protein per ml. [^{14}C]DCCD was purified [14] and added as an ethanolic solution at 10 nmol/mg protein and incubated at 0°C for 24 h unless otherwise indicated.

Renal proximal tubule brush border membrane vesicles (25 mg protein/ml) were incubated with 0.5 nmol [^{14}C]DCCD at 0°C for 24 h in 50 mM mannitol, 2 mM Tris-HCl, pH 7.0.

Extraction of proteolipid. Proteolipids were extracted essentially by the method of Cattell et al. [31] after the membrane suspension was first adjusted to pH 6.8 with dilute HCl.

Proteolipid was precipitated by cooling the chloroform/methanol extract to −20°C followed by the addition of 5 vol diethyl ether at the same temperature. The resultant solution was covered and stored at −20°C for 24 h during which time a fluffy white precipitate developed. The precipitated protein, collected by centrifugation at $10000 \times g$ for 10 min, was dissolved in a small quantity of chloroform/methanol (2:1, v/v) and stored at −20°C.

Electrophoresis. The method developed by Swank and Munkres [32] for SDS-urea gel electrophoresis in highly cross-linked polyacrylamide gels was employed. This technique involved the use of 12.5% acrylamide and *N,N'*-methylenebisacrylamide (1:30) in a degassed solution containing 8 M urea, 0.1% Tris-phosphate, pH 6.8, and 0.06% ammonium persulfate. Polymerization was initiated by the addition of 0.01% TEMED. The electrophoresis buffer was 0.1% Tris-phosphate, pH 6.8, containing 0.1% SDS. Electrophoresis was conducted at 2.5 mA/tube and run until the tracking dye was approx. 2 cm from the end of the gel.

A modification of this method was used to examine higher molecular, weight components. A 3–15% acrylamide gradient was cast as a 1.5-mm thick slab gel (Bio-Rad Model 220, Richmond, CA). The TEMED concentrations were changed to 0.01 and 0.02% in the 3 and 15% acrylamide solutions and the ammonium persulfate concentrations were 0.06 and 0.085% respectively. All other components were unchanged. Gels were cast and maintained at 30–35°C to prevent crystallization of the urea.

Samples for electrophoresis were solubilized in 5% SDS, 1% β-mercaptoethanol and 8 M urea for 4 h at 60°C followed by heating to 100°C for 3 min. Following electrophoresis the gels were fixed in two changes of methanol/water/acetic acid (5:5:1, v/v) and stained for 4 h with 0.25% Coomassie blue in fixing solution. Gels were destained in water/methanol/acetic acid (625:250:75, v/v) until the background was clear. The gels were scanned at 590 nm on an RFT II scanning densitometer (Transidyne General Corp.). Quantitation of bound [^{14}C]DCCD was performed on 1.5-mm segments of the gel. The segments were digested by placing them in tightly capped scintillation vials containing 0.5 ml of 35% H_2O_2 for 12 h at 70°C. Following digestion, 10 ml of Betaphase scintillation fluid (Westchem Products) were added and the samples counted in a Searle Delta 300 liquid scintillation counter after chemiluminescence had decayed (approx. 8 h). The data were corrected for counter efficiency and quenching and the background subtracted except where indicated.

Procedures. Protein concentration was determined by the method of Lowry et al. [33]. In the quantitation of proteolipids the sample was first solubilized in 5% SDS at 60°C. TLC [31] was performed on activated silica gel G and developed with the solvent chloroform/methanol/acetic acid/water (25:15:4:2, v/v).

Materials. Toads (*B. marinus*) were purchased from Rand McNally, Summerset, WI. Turtles (*P. scripta*) were purchased from College Biological, Escondido, Ca. [^{14}C]DCCD (spec. act. 50 mCi/mmol) was obtained from Research Products International Corp., Elk Grove, IL. Isethionyl[^{14}C]-acetimidate was obtained from Amersham Corp., Arlington Heights, IL. ATP was purchased from Boehringer Mannheim, Indianapolis, IN. Aluminum oxide G (Merck 1090) was supplied by Merck, Darmstadt, F.R.G. DCCD and oligomycin

were purchased from Sigma, St. Louis, MO. All reagents used in SDS-polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories, Richmond, CA. All other reagents used were of analytical reagent grade.

Results

The crude membrane preparations were routinely analyzed using 5′-nucleotidase, a general marker enzyme for the plasma membrane. These preparations were enriched approx. 4-fold over the crude homogenate in this enzyme. Since it is not known whether 5′-Nucleotidase is a marker for apical plasma membrane of epithelia, a membrane-impermanent covalent label [23] was employed to follow the purification of apical membrane by density gradient centrifugation. A small number of toad bladders were incubated with isethionyl[^{14}C]acetimidate in the mucosal solution only. The crude membrane preparation obtained from these toad bladders was subjected to our usual density gradient centrifugation. Measurements of the specific activity of the density gradient revealed that the purified membrane had been enriched 6.8-fold in the ^{14}C label over the crude membrane which had been applied to the gradient.

Since mitochondria contain a hydrophobic protein which covalently binds DCCD at low levels [31], it was important to obtain preparations which were low in components of the mitochondrial inner membrane. As determined from the activity of cytochrome oxidase, the toad and turtle bladder membrane preparations routinely contained between 1 and 3% of the cytochrome oxidase activity (per mg protein) of that in toad bladder mitochondria. Enzymatic assay of freshly prepared brush border indicated that alkaline phosphatase, a marker enzyme for the brush border, increased 8-fold over the crude homogenate. The renal brush border preparation contained only 0.5% of the cytochrome oxidase activity (per mg protein) of the crude cellular homogenate.

Studies with the urinary bladder of the toad

Classical methods for isolation of the DCCD-binding protein from mitochondria, chloroplast and bacteria [4,14–17,34,35] have relied on extraction of proteolipids with organic solvents followed

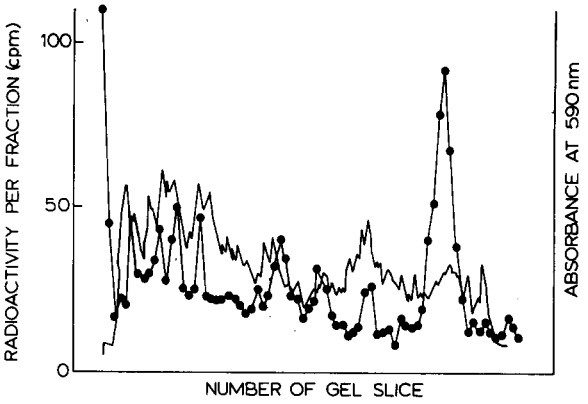

Fig. 1. SDS-polyacrylamide gel electrophoresis of ^{14}C-labeled membrane products from toad bladder. The membrane was incubated with [^{14}C]DCCD at a concentration of 10 nmol/mg protein for 24 h at 0°C. The membrane preparation was then solubilized in 5% SDS, 1% β-mercaptoethanol and 8 M urea. Electrophoresis was performd on a 12.5% acrylamide gel as described in Materials and Methods. (————) Protein absorbance; (●————●) ^{14}C radioactivity.

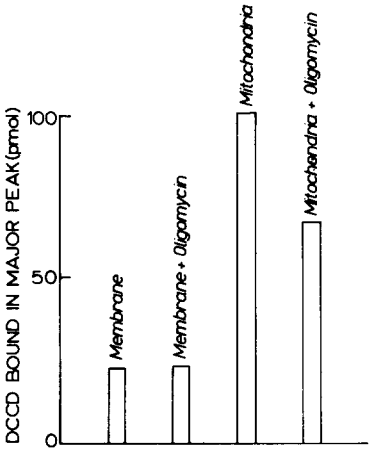

Fig. 2. Effect of oligomycin on [^{14}C]DCCD binding to the major component in toad bladder membrane and mitochondria. Oligomycin was added as a concentrated ethanolic solution to toad bladder mitochondria and membrane at 20 μg/mg protein; control preparations received only ethanol. Following brief mixing, [^{14}C]DCCD was added at 20 nmol/mg protein and the preparations were incubated for 4 h at 0°C. The reaction was terminated by the addition of 5% SDS, 1% β-mercaptoethanol and 8 M urea (final concentrations), followed by heating in a boiling-water bath for 5 min. The samples were applied to a 12.5% polyacrylamide gel. Electrophoresis and radioactivity were determined as described (see Materials and Methods).

by precipitation with ether to separate the DCCD-binding proteolipid from free lipid. Due to the small amount of membrane available from toad or turtle urinary bladder, an alternate approach was developed in which the entire membrane preparation was subjected to SDS-polyacrylamide gel electrophoresis after incubation with [^{14}C]DCCD. Since it had previously been demonstrated [31] that the staining and destaining process removed any unbound DCCD or DCCD hydrolysis products, radioactivity remaining in the gel would represent DCCD reacted with protein or lipid.

Initial binding experiments employed moderate levels of DCCD (10 nmol/mg protein) at 0°C for 24 h, since it has been shown that high concentrations of DCCD can label proteins other than the H^+ translocator [36–39]. The toad bladder membrane previously incubated with [^{14}C]DCCD was electrophoresed on both 6.25 and 12.5% acrylamide gels. The 6.25% gel showed no distinctive DCCD-binding peak; however, the 12.5% gel contained [^{14}C]DCCD-binding activity that migrated as a broad, low molecular weight band (Fig. 1) that was faintly stained by Coomassie blue. To eliminate the possibility that this band might be due to a small amount of mitochondrial contamination, samples of toad bladder membrane and mitochondria were incubated with [^{14}C]DCCD in the presence of oligomycin. Others have shown [13] that DCCD and oligomycin compete for the same binding site on the F_0 protein. Results indicate that while the DCCD binding in mitochondria was decreased 32% by oligomycin, the binding of DCCD in toad bladder membrane was unaffected (Fig. 2). Moreover, the molecular weight corresponding to the peak of DCCD binding was 8700 for mitochondria as compared to approx. 5000 for the toad bladder membrane.

The hallmark of the DCCD-binding protein has been its low molecular weight and solubility in chloroform/methanol (2:1, v/v). Consequently, toad bladder membrane previously incubated with [^{14}C]DCCD was subjected to extraction with chloroform/methanol (2:1, v/v). A fraction of the extract was subjected to electrophoresis on a 15% acrylamide slab gel. Analysis for ^{14}C revealed a single broad band that covered 19% of the length of the gel in the low molecular weight range. The remainder of the chloroform/methanol extract from 4.5 mg of membrane protein was subjected to ether precipitation. However, all attempts to precipitate proteolipid from the extract with ether were unsuccessful. Two possibilities for this were then considered. First, the [^{14}C]DCCD had reacted with a proteolipid, but the concentration of the protein was so low that a precipitate was not formed. Second, the [^{14}C]DCCD may have reacted with phospholipids [40] which would not be precipitated by ether. The first possibility was evaluated by adding to the chloroform/methanol extract of toad bladder membrane authentic DCCD-binding protein from mitochondria [31] to serve as a carrier protein in the precipitation procedure. In this experiment, mitochondrial 'carrier' hydrophobic protein previously reacted with a large excess of unlabeled DCCD was dissolved in a chloroform/methanol extract of toad bladder membrane which had previously been reacted with [^{14}C]DCCD. The combined extract was ether precipitated and separated from free lipids by centrifugation. The hydrophobic protein, precipitated in the presence of the extract of toad bladder membrane, failed to show any peak of bound [^{14}C]DCCD after polyacrylamide gel electrophoresis. This suggested that the broad peak of radioactivity previously seen in the chloroform/methanol extract of toad bladder membrane might not have been covalently bound to a proteolipid. Indeed, electrophoresis of the supernatant remaining after the ether precipitation step demonstrated that the broad, low molecular weight band of radioactivity that faintly stained with Coomassie blue remained in the supernatant (Fig. 3).

The material in the chloroform/methanol extract of toad bladder that had reacted with [^{14}C]DCCD was next analyzed by TLC, using a system in which an authentic F_0, DCCD-binding protein remains at the origin [31]. In contrast to F_0, the DCCD-reactive material in the toad bladder extract migrated with an R_f value of 0.89.

In another series of experiments, increasing concentrations of [^{14}C]DCCD were reacted with a preparation of toad bladder membrane. The membrane was extracted with chloroform/methanol and analyzed by gradient slab SDS-polyacrylamide gel electrophoresis. As shown in Fig. 4, there was no saturation in the labeling of the broad, low molecular weight peak, even at 1.05 mM DCCD.

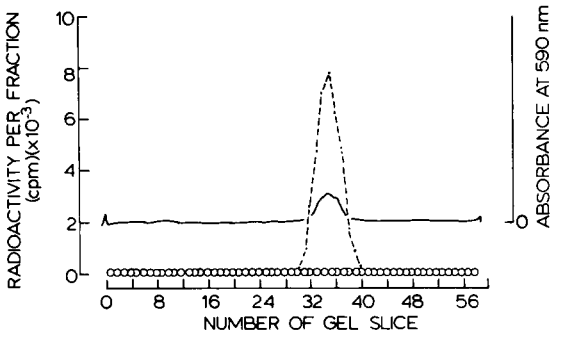

Fig. 3. SDS-polyacrylamide gel electrophoresis of the chloroform/methanol extract of toad bladder membrane following the precipitation of hydrophobic proteins. Toad bladder membrane was incubated with 10 nmol/mg protein of [^{14}C]DCCD for 24 h at 0°C. The reaction was terminated by the addition of 20 vol of chloroform/methanol (2:1, v/v). The extraction was continued for 18 h at room temperature. The filtrate was washed with water and the organic phase was concentrated under reduced pressure. Mitochondrial hydrophobic protein was dissolved in the extract followed by the addition of ether to precipitate the protein. The precipitated protein was removed by centrifugation. The supernatant was evaporated and subjected to electrophoresis on a 12.5% acrylamide gel and radioactivity determined (see Materials and Methods). (————) Protein absorbance, (------) radioactivity of 'chloroform/methanol extract' of membrane, (O------O) radioactivity of [^{14}C]DCCD run under identical conditions in the absence of membrane protein.

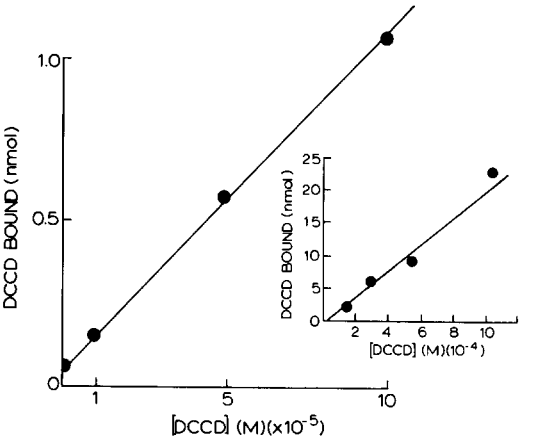

Fig. 4. Effect of DCCD concentration on [^{14}C]DCCD binding in a chloroform/methanol extract as analyzed by SDS-polyacrylamide gel electrophoresis. Aliquots of membrane were incubated with the various concentrations of DCCD for 24 h at 0°C. The reaction was terminated by the extraction of the membrane with chloroform/methanol (2:1, v/v). The individual samples of the extracts were analyzed on a 3–15% gradient slab gel and the ^{14}C radioactivity in the gel was determined (see Materials Methods). All samples contained only one peak of radioactivity which appeared in the low molecular weight section of the gel. The total amount of DCCD bound in this section of the gel was determined from the specific activity of the [^{14}C]DCCD. Inset shows higher concentrations of [^{14}C]DCCD.

Since all evidence was consistent with the [^{14}C]DCCD being bound by phospholipids, toad bladder membrane was reacted with [^{14}C]DCCD and the majority of the phospholipids were extracted with 90% acetone [41]. The acetone extract yielded the same broad low molecular weight peak of radioactivity that was present in the chloroform/methanol extract. Approx. 50% of the [^{14}C]DCCD binding in the toad bladder membrane was extracted by 90% acetone, as assayed by SDS-polyacrylamide gel electrophoresis. Moreover, the radioactivity remaining in the membrane after extraction with acetone migrated in the same manner on the gel as did that extracted by acetone (Fig. 5).

Studies with the urinary bladder of the turtle

The isolated turtle bladder membrane was reacted with [^{14}C]DCCD (10 nmol/mg protein) at pH 7.1 for 24 h at 0°C. The membrane was then extracted with chloroform/methanol (2:1, v/v). Examination of a fraction of this extract by SDS-polyacrylamide gel electrophoresis revealed a broad band of radioactivity in the low molecular weight portion of the gel. As was found with the toad bladder membrane, this material was only faintly stained by Coomassie blue.

The remaining chloroform/methanol extract from 200 μg of turtle bladder membrane protein was combined with 200 μg of hydrophobic carrier protein from beef heart mitochondria and ether precipitated. Both the pellet and supernatant were electrophoresed. As with toad bladder, analysis revealed no radioactivity in the precipitated protein. However, the lipid material in the ether supernatant did contain a low molecular weight component which bound DCCD (results not shown).

Studies with rabbit kidney brush border

Rabbit kidney brush border membranes were reacted with a low concentration of [^{14}C]DCCD

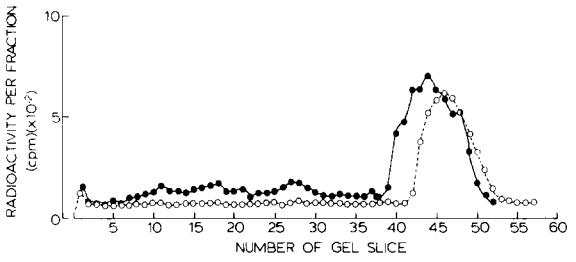

Fig. 5. Acetone extraction of DCCD bound to toad bladder membrane. Toad bladder membrane was incubated with 10 nmol/mg protein of [^{14}C]DCCD for 24 h at 0°C. The membrane preparation was made 90% in cold acetone. The suspension was mixed briefly and separated by centrifugation at 5000 × *g* for 5 min. The pellet was washed twice with cold 90% acetone. The combined acetone extracts were evaporated to dryness on a rotary evaporator. Samples of both the acetone extract and the acetone extracted membrane were prepared for analysis using a 3–15% gradient on SDS-polyacrylamide gel electrophoresis. (●———●) Acetone-extracted membrane containing 50 μg protein, (○———○) acetone-extractable lipid from an equal amount of membrane.

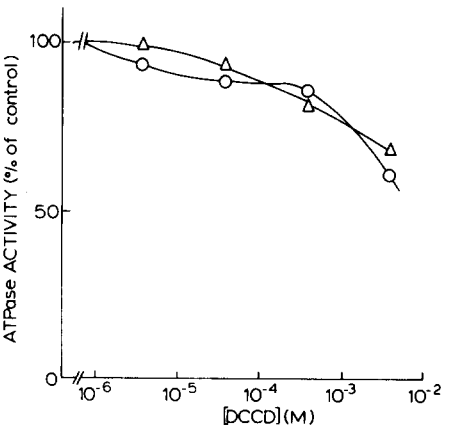

Fig. 6. Inhibition of ATPase activity in brush border by DCCD. The ATPase activity of freshly prepared rabbit kidney brush border was determined by a 15 min incubation at 37°C in a medium containing 5 mM $MgCl_2$, 5 mM ATP and 10 mM Tris-HCl, pH 7.8. All samples were preincubated for 15 min at 37°C with the following inhibitors: 1 mM ouabain, 5 mM sodium azide, oligomycin at 2 μg/mg protein and DCCD at the levels indicated. (○———○) ATPase activity of brush border prepared by the Ca^{2+}-precipitation method (see Materials and Methods); (△———△)ATPase activity of brush border prepared in an identical manner except for the substitution of Mg^{2+} for Ca^{2+}.

for 24 h at 0°C, conditions that label covalently and rather specifically a low molecular weight proteolipid in F_0 [31]. Following the incubation with [^{14}C]DCCD, a chloroform/methanol extract of the membranes was concentrated and ether precipitated three times to remove unbound DCCD. A final yield of 1.15 mg of proteolipid, precipitated from 219 mg of brush border protein, contained a low level of radioactivity. Calculations indicated that if this hydrophobic protein(s) had a molecular weight of 10000, (the approximate weight of most DCCD-binding proteins from F_0), then no more than one in 500 such molecules could have reacted with DCCD.

Two methods were used to determine if any of this low level of radioactivity in the precipitate represented protein covalently bound to the [^{14}C]DCCD. First, TLC under conditions where authentic DCCD-binding protein remains at the origin [31] demonstrated a ninhydrin-positive spot at R_f 0.45. However, the only radioactivity was found to coincide with a streak localized by iodine vapor with an R_f value of between 0.19 and 0.37. There was neither radioactivity nor ninhydrin reactivity at the origin.

In a final series of experiments we tested the ability of DCCD to inhibit ATPase activity of brush border membranes. The inhibitors azide, oligomycin and ouabain were used to eliminate any possible mitochondrial ATPase or ($Na^+ + K^+$)-ATPase activity. DCCD inhibition of ATPase at 0.4 mM was found to be less than 20% when

TABLE I

INHIBITION OF ATPase BY DCCD AT pH 6.45

Samples were preincubated with DCCD for 15 min at 37°C before addition of ATP. Percent inhibition in submitochondrial particles is expressed as a percent of the inhibition produced by oligomycin. — measurements not performed.

DCCD concentration (M)	Percent inhibition	
	Rat liver submitochondrial particles	Rabbit kidney brush border
$4 \cdot 10^{-8}$	80	–
$4 \cdot 10^{-7}$	94	–
$4 \cdot 10^{-6}$	–	21
$4 \cdot 10^{-5}$	100	38
$4 \cdot 10^{-4}$	–	58
$4 \cdot 10^{-3}$	–	71

assayed at pH 7.8 (Fig. 6). Although the inhibition of ATPase by DCCD in brush border membranes was greater at a lower pH, the concentration of DCCD needed to inactivate this enzyme was greatly different from that needed to inhibit the ATPase in rat liver mitochondria (Table I). The ATPase activity of the brush border membrane was not increased by levels of Triton X-100 ranging from 0.01 to 1%, indicating that the added ATP was not being excluded from latent ATPase in tightly sealed vesicles. We also verified the biological activity of the DCCD we used in these experiments by its ability to inhibit the ATPase of rat liver submitochondiral particles.

Discussion

The ubiquity of a DCCD-sensitive F_0F_1 H^+-translocating ATPase or H^+ pump in biology suggested to several groups of investigators interested in urinary acidification the possibility than an analogous DCCD-sensitive ATPase might be a component of the process required for urinary acidification. Indeed, at least three reports have appeared indicating that urinary acidification in amphibian or reptilian urinary epithelia can be inhibited by 10^{-4} M DCCD [18–20]. While this might be consistent with the presence of an F_0F_1-type ATPase, the concentration of DCCD (10^{-4} M) required to inhibit urinary acidification partially is about two orders of magnitude greater than that required to inhibit mitochondrial [42,43], yeast [44], bacterial [14,45] or chloroplast [34] F_0F_1-ATPase.

In the case of all known F_0F_1 H^+-translocating ATPases, with the exception of some mutants, an extremely hydrophobic protein in F_0 has been identified as the site of action of DCCD by virtue of the ability of low concentrations of [^{14}C]DCCD to react covalently with this protein while reacting to only a minor degree with other components.

The above considerations led us to conduct the studies reported here. The [^{14}C]DCCD we found in a broad, low molecular weight peak from the chloroform/methanol extract of membranes prepared from epithelial cells of the urinary bladder of the toad and turtle probably is accounted for by reaction of DCCD with lipids in the extract. The following findings are consistent with this interpretation: (1) the width of the radioactive band was greater than that expected for a single protein; (2) DCCD has been shown to react with lipid mixtures [40, 46]; (3) the bound radioactivity was not precipitated by ether, even in the presence of a carrier protein known to be an authentic F_0 hydrophobic protein; (4) the bound radioactivity was extractable by 90% acetone, which has been shown to solubilize lipids but not proteins [41]; (5) the bound [^{14}C]DCCD, when analyzed by TLC, exhibited a R_f value considerably less polar than that of previously reported DCCD-binding protein from F_0; (6) increasing the concentration of [^{14}C]DCCD to $4 \cdot 10^{-3}$ M failed to saturate the DCCD-reacting sites, suggesting no specific protein reactivity.

Similarly, in the case of rabbit kidney brush border, even though a hydrophobic, ether-precipitable protein was found, no [^{14}C]DCCD was bound to the protein after TLC or SDS-polyacrylamide gel electrophoresis.

Our failure to find a [^{14}C]DCCD-reactive protein in urinary bladder and kidney membranes cannot be due to faulty [^{14}C]DCCD. After our purification of the [^{14}C]DCCD, the compound inhibited respiration in liver mitochondria at 10^{-6} M; migrated with nonradioactive DCCD on TLC and was reactive with a 10000 dalton proteolipid from beef heart mitochondria under the conditions of Cattell et al. [31] (results not shown).

Our findings in aggregate fail to provide evidence for the existence of a DCCD-reactive F_0-like channel in urinary epithelia. Moreover, our failure to find a DCCD-reactive F_0-like protein is consistent with the relative insensitivity of DCCD to both the urinary acidification systems [2,18,20] and the ATPase in brush border (Ref. 21 and Fig. 6).

In summary, these results indicate that urinary acidification is not accomplished by the typical DCCD-sensitive H^+-translocating ATPase. We suggest two possible explanations. First, the ATPase concerned with urinary acidification might not be an F_0-type ATPase or, second, an evolutionary modification in the F_0 might have resulted in greatly decreased sensitivity to DCCD. It has been shown with mutant strains of *Escherichia coli* that the substitution of a single amino acid in the polypeptide chain of the F_0 can lead to the loss of

DCCD sensitivity [47]. In some cases the alteration can cause malfunction of the F_0; however, in other examples [14,48] the only apparent effect is the DCCD insensitivity of the membrane-bound ATPase. Our present data do not allow us to distinguish such an evolutionary modification from the possibility that the H^+-translocating ATPase involved in urinary acidification bears little or no resemblance to F_0-type ATPase.

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