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F_0 "Proton Channel" of Rat Liver Mitochondria

RAPID PURIFICATION OF A FUNCTIONAL COMPLEX AND A STUDY OF ITS INTERACTION WITH THE UNIQUE PROBE DIETHYLSTILBESTROL*

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The F_0 portion of the rat liver mitochondrial ATP synthase (F_0F_1-ATPase) has been purified by a rapid, high yield procedure. F_0 is selectively extracted from inner membrane vesicles with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) after prior treatment of the vesicles with guanidine HCl to remove F_1. The resultant F_0 is functional in proton translocation assays and separates in sodium dodecyl sulfate-polyacrylamide gel electrophoresis into four major and three minor Coomassie-stainable bands, all with apparent molecular masses below 30 kDa. This CHAPS-purified F_0 preparation was characterized in detail for its capacity to interact with the unique probe diethylstilbestrol (DES) which, depending on conditions, has been shown to interact with rat liver F_0F_1 to either inhibit or promote ATP hydrolysis (McEnery, M. W., and Pedersen, P. L. (1986) *J. Biol. Chem.* 261, 1745-1752).

DES-inhibitory sensitivity could be conferred on F_1-ATPase activity with the same concentration dependence on F_0 as conferral of oligomycin sensitivity. DES was shown also to inhibit the magnitude of valinomycin induced proton influx, while initiating proton efflux in asolectin vesicles reconstituted with F_0 and loaded with K^+. The potency of DES in producing the latter effects was shown to be highly dependent on hydroxyl groups in "para" positions of the two benzene rings within the DES molecule. Finally, in the absence of F_0, DES was shown to act as a catalyst of proton influx in K^+-loaded asolectin vesicles upon addition of valinomycin. A model based on the structure of DES is presented to account for both the inhibitory and uncoupling properties of this compound.

In eukaryotic cells, diethylstilbestrol (DES)[1] is the only agent known to inhibit all three classes of proton ATPases, *i.e.* the *P* or "E_1E_2" type (1), the *V* or "vacuolar" type (2), and the *F* or "F_0F_1" type (3). Consequently, there is considerable interest in the mechanism by which DES affects proton ATPases localized in eukaryotic membranes. Although high concentrations of DES can exert damage to membrane bilayers and produce other nonspecific effects (4-6), it is significant to note that concentrations of DES that inhibit eukaryotic proton ATPases are quite low. Thus, I_{50} values for DES of 10, 15, and 27 μM have been reported, respectively, for proton ATPases isolated from mitochondria (3), chromaffin granules (2), and the yeast plasma membrane (1). Both the chromaffin granular and mitochondrial ATPases also undergo an "uncoupling effect" when the same low concentration of DES is added before or after addition of DCCD (2, 3). In this case, ATPase activity inhibited by either agent alone is restored in the presence of both agents (2, 3).

In a previous study from this laboratory on the effect of DES on the purified F_0F_1-ATPase of rat liver mitochondria, we suggested that the primary site of action of DES may reside at the level of F_0 (3). This suggestion was based on the finding that the ATPase activity of the purified F_1 moiety remained completely active at a concentration of DES (10 μM) that provided half-maximal inhibition of purified F_0F_1-ATPase activity (3). Unfortunately, that study could not be extended further because a functional rat liver F_0 preparation was unavailable.

Here we report a novel, rapid procedure for obtaining a functional rat liver F_0 preparation in high yield and purity and demonstrate directly that DES can affect the properties of this complex. We also provide data which show that the effects of DES on F_0 and F_0F_1 preparations from rat liver are strongly dependent on the structure of DES, in particular the two hydroxyl groups located at *para* positions on the two benzene ring systems.

EXPERIMENTAL PROCEDURES

Materials

Adult, male CB albino rats were obtained from the Charles River Breeding Laboratories, Wilmington, MA, and were fed *ad libitum* with Charles River RHM-1000 rat chow pellets. CHAPS was purchased from Calbiochem. Asolectin was obtained from Associated Concentrates, Woodside, NY. DES, analogs of DES, oligomycin, pyruvate kinase, lactate dehydrogenase, guanidine HCl, bovine serum albumin, valinomycin, and CCCP were purchased from Sigma. Acrylamide, methylene bisacrylamide, Coomassie Blue R-250, and Bio-Rad protein assay dye reagent were purchased from Bio-Rad. Venturicidin was purchased from Gallard/Schlesinger. The bath sonicator used in these experiments was the cylindrical ultrasonic tank and generator type from Laboratory Supply Company, Inc., Hicksville, NY. 9-Amino-6-chloro-2-methoxyacridine was generously donated by Drs. P. Friedl, H. Schairer, and W. Sebald, Gesellschaft fur Biotechnologische Forschung GmbH, Braunschweig-Stockheim, Germany

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[1] The abbreviations used are: DES, diethylstilbestrol; DCCD, dicyclohexylcarbodiimide; DNP, 2,4-dinitrophenol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS, sodium dodecyl sulfate; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

(original source, Dr. P. Overath, Max-Planck-Institut for Biology). FCCP was generously donated by Dr. P. G. Heytler of Du Pont.

Methods

Preparation of Rat Liver Mitochondria—The procedure of Schnaitman and Greenawalt (7) was used. The isolation medium contained 220 mM D-mannitol, 70 mM sucrose, 2 mM Hepes, pH 7.4, and 0.5 mg/ml defatted bovine albumin. Bovine albumin was omitted when mitochondria were to be used to test directly the effect of DES on ATPase activity.

Preparation of Inner Membrane Vesicles—The procedure of Wehrle *et al.* (8) was used. Protein-depleted, high ATPase activity membranes (3× membranes) were prepared according to the procedure of McEnery *et al.* (9) which involves washing inner membrane vesicles three times in PA buffer (0.15 M K_2HPO_4, 1 mM ATP, 25 mM EDTA, 0.5 mM dithiothreitol, 5% ethylene glycol, pH 7.9). The membrane pellets (3× membranes) were suspended to 15 mg/ml in PA buffer and stored at −70 °C until use. The 3× membranes have a specific activity in the range of 2.5–5.0 μmol of ATP hydrolyzed per min/mg protein.

Preparation of Guanidine-treated Membranes (3×G Membranes)—3× membranes (3 mg in 0.2 ml) were added to 1.8 ml of GPA buffer (315 g of guanidine HCl added to 500 ml 2 × PA buffer and brought to a final volume of 1.0 liter with water). The solution was incubated for 5 min on ice, after which time it was diluted with 5.5 ml of PA buffer and centrifuged for 30 min at 48,000 rpm (148,000 × *g*) in the Sorvall T-865.1 rotor at 2 °C. The resulting pellet was then resuspended in 7 ml of PA buffer and spun as before. The pellet (3×G membranes) was suspended in TA buffer (50 mM Tricine, 1 mM ATP, 25 mM EDTA, 0.5 mM dithiothreitol, and 5% ethylene glycol, pH 7.9) at a protein concentration of 5–10 mg/ml and stored at −70 °C until use. The 3×G membranes have no detectable ATPase activity when assayed by the spectrophotometric procedure described below.

Isolation of F_0 from 3× Membranes—3×G membranes were incubated at a protein concentration of 3.5 mg/ml in TA buffer plus 1.0% CHAPS for 15 min on ice. After this time, the samples were centrifuged for 1 h at 48,000 rpm in the Sorvall T-865.1 rotor at 2 °C. The soluble fraction (F_0) was diluted to 0.6% CHAPS by the addition of fresh TA buffer and stored at −70 °C until use.

Purification of the F_0F_1-ATPase—The procedure for the purification of the rat liver mitochondrial F_0F_1-ATPase was according to the method of McEnery *et al.* (9) with one exception. A 25% sucrose solution was used in the final step of the purification (10). The average specific activity of F_0F_1 preparations used in this study was 8–11 μmol of ATP hydrolyzed per min/mg protein. Samples could be stored for greater than 6 months at −70 °C without loss of activity.

Preparation of F_1-ATPase—The F_1-ATPase of rat liver mitochondria was isolated according to the procedure of Catterall and Pedersen (11) and stored as a lyophilized preparation at −20 °C. The average specific activity was 20–25 μmol of ATP hydrolyzed per min/mg protein. The stored preparation was reconstituted with TA buffer plus 0.6% CHAPS prior to use.

Assay for Reconstitution "Activity" of F_0—For comparative purposes, the assay for F_0 activity is based on the conditions employed by Alfonzo *et al.* (12). The conditions are as follows: 3×G membranes (0–100 μg) and F_0 (0–50 μg) were incubated with 2 μg of purified F_1-ATPase in TA buffer plus 0.6% CHAPS (final volume of 0.2 ml) at room temperature for 1 h. After this time, 25-μl samples were removed and assayed for total ATPase activity and inhibition of ATPase activity. Reconstitution assays were also routinely carried out at an F_1-ATPase concentration of 12 μg/0.2 ml. In this case, the amount of F_0 protein was in the range of 12–60 μg.

Reconstitution of F_0 Preparations for Determination of Passive Proton Flux (Dilution Method)—This procedure is based upon the method of Huang *et al.* (13). F_0 (protein concentration 100–200 μg/ml) was incubated with asolectin sonicated in TA buffer plus 0.6% CHAPS at a ratio of 15:1 asolectin:protein (mg/mg) in a final volume of 0.5 ml for 5 min on ice. After this time, 7.7 ml of 210 mM KCl, 10 mM Tricine, pH 7.5, was added. The samples were centrifuged for 2 h at 2 °C at 48,000 rpm in the Sorvall T-865.1 rotor. The vesicles were resuspended to 1 mg/ml with 0.25 M sucrose, 10 mM Tricine, pH 7.5. The vesicles were stable on ice for 1.5 h. The vesicle suspension (25 μl) was added to 2 ml of 200 mM NaCl, 10 mM Tricine, pH 7.5, 25 nM 9-amino-6-chloro-2-methoxyacridine dye. After initiating the reaction by addition of 50 ng of valinomycin to elicit K^+ efflux, proton influx was assayed fluorimetrically by monitoring the quenching of the 9-amino-6-chloro-2-methoxyacridine dye as described previously (14, 15).

In control studies, K^+-loaded asolectin vesicles were prepared by omitting the step involving incubation with F_0.

Assay for ATPase Activity—ATPase activity was measured spectrophotometrically at 340 nm by coupling the production of ADP to the oxidation of NADH via the pyruvate kinase and lactate dehydrogenase reactions (16). The reaction mixture contained (final volume of 1 ml at pH 7.5 and at room temperature), 4.0 mM ATP, 4.8 mM $MgCl_2$, 2.5 mM KCl, 0.4 mM NADH, 0.6 mM phosphoenolpyruvate, 5 mM KCN, 1 unit of lactate dehydrogenase, 1 unit of pyruvate kinase, and 0.25–25 μg of protein (depending upon the form of the enzyme assayed).

Where indicated, ATPase activity was assayed also by monitoring the release of inorganic phosphate. In this case, the assay medium contained in a final volume of 0.6 ml, 25 mM Hepes, 10 mM ATP, 8.3 mM $MgCl_2$, and amounts of protein as indicated. The assay was carried out for 5 min at room temperature and then stopped by addition of 0.1 ml, 2.5 N $HClO_4$. After addition of 0.1 ml of 2.5 M KOH, the resultant $KClO_4$ precipitate was removed by centrifugation. Aliquots of 0.4 ml were then removed for P_i determination (17).

Gel Electrophoresis in SDS—Gel electrophoresis was carried out according to the procedures of Laemmli (18) using a polyacrylamide gradient gel system (12–20%) as described by Kaplan *et al.* (19). The samples were precipitated with 10% trichloroacetic acid for 15 min on ice and then centrifuged for 15 min in a microcentrifuge. The pellets were resuspended with 2 × sample buffer which contained 8 M urea. The samples were heated for 15 min at 65 °C. The molecular mass standards were supplied by Bio-Rad and were phosphorylase *b* (92.5 kDa), bovine serum albumin (67.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and lysozyme (14.3 kDa). The apparent molecular weights in SDS-polyacrylamide gel electrophoresis for the subunits of the F_1-ATPase, α 62,000, β 57,000, γ 36,000, δ 12,500, and ϵ 7,500 (20), were used as internal molecular weight standards. The gels were stained for 2 h in 50% methanol, 10% acetic acid, and 0.05% Coomassie Blue and destained in 10% ethanol, 10% acetic acid overnight.

Determination of Protein—Membrane protein was estimated by the biuret method in the presence of 0.25% sodium cholate (21). Soluble protein was measured by the method of Bradford (22). Bovine serum albumin was used as a standard in both cases. All samples were normalized with respect to detergent and buffer concentration.

RESULTS

Purification and Subunit Composition of Rat Liver F_0 Prepared Using the Zwitterionic Detergent CHAPS—In order to characterize further the interaction of diethylstilbestrol with the ATP synthase complex of rat liver, it was essential to have at hand a functionally active F_0 preparation. To obtain such a preparation from rat liver mitochondria we took advantage of our previous experience with the zwitterionic detergent CHAPS which extracts rather selectively the intact ATP synthase complex (F_0F_1) from washed submitochondrial particles. We rationalized that if F_1 could be removed first from such particles, F_0 may be rather selectively extracted also by using CHAPS. This, in fact, proved to be the case leading to the purification of rat liver F_0 in a novel, two-step procedure involving guanidine HCl to first remove F_1 from washed inner membrane vesicles (3× membranes) followed by treatment with CHAPS.

Data presented in Table I show that 1% CHAPS solubilizes 44% of the total F_0 activity of 3×G membranes while solubilizing only 13.6% of the total 3×G membrane protein. This corresponds to 4.3% of the starting inner membrane vesicle protein and illustrates the selectivity of CHAPS in extracting rat liver F_0. Subsequent additions of CHAPS did not result in the extraction of additional F_0 activity. (It should be noted that F_0 activity is defined, as recommended previously (12), on the basis of its capacity to confer oligomycin inhibitory sensitivity on the ATPase activity of F_1.)

Fig. 1 compares the SDS-gel electrophoretic patterns of purified rat liver F_0F_1 (9) and the CHAPS-purified F_0 in 12–20% polyacrylamide gels. Fig. 1*A* presents photographs of representative Coomassie-stained gels in which the protein

load was 20 μg for both F_0F_1 and F_0. The scans of such gels presented in Fig. 1, *B–E*, reveal more than can be visualized by eye. Rat liver F_0F_1, as described previously (9), contains in addition to the five F_1 subunits (α, β, γ, δ, and ε) five different polypeptide species designated 1, 2, 3, 4, and 5. There can be detected also in most preparations three minor components which we designate here as 1′, 4′, and 5′. These eight different polypeptide species all exhibit a molecular mass of less than 30 kDa. Polypeptides 1′, 1, 2, and 3 fall into what we refer to here as the large peptide cluster (*LPC*) whereas polypeptides 4, 4′, 5, and 5′ lie in a small peptide cluster (*SPC*).

CHAPS-purified F_0, which is completely functional in proton translocation (see below), contains seven of the eight "non-F_1" polypeptides observed in F_0F_1. Polypeptide 3, present in F_0F_1, is absent in F_0, and polypeptide 1, present in F_0F_1 at very low levels, is greatly enriched in F_0. All seven polypeptides were observed in three different CHAPS-solubilized F_0 preparations (Fig. 1, *C–E*). Moreover, all seven polypeptides remain with F_0 even after sedimentation into a 25% sucrose solution containing 0.2% CHAPS (data not presented).

Fig. 1*F* summarizes SDS-gel electrophoretic patterns of both F_1-ATPase (50 μg) and purified F_0 run at a very high protein load (125 μg) and for a longer time period than that in Fig. 1*A*. Although high molecular weight contaminants are observed at these high protein loads, F_0 resolves into the seven different species. With the possible exception of small amounts of ε, F_0 is devoid of F_1 subunits. Apparent molecular masses for these seven species are: polypeptide 1′, 28 kDa; polypeptide 1, 23 kDa; polypeptide 2, 20 kDa; polypeptide 4, 13.5 kDa; polypeptide 4′, 11 kDa; polypeptide 5, 8–10 kDa; and polypeptide 5′, 7 kDa. A polypeptide running just below *1′* in Fig. 1*F*, which appears as a shoulder in Fig. 1, *C–E*, must be considered as a possible eighth species. Reaction of F_0F_1 with [^{14}C]DCCD results in labeling of a single species which comigrates with the 8–10-kDa component (data not presented).

TABLE I

Scheme summarizing the purification of F_0 from rat liver inner membrane vesicles

Membranes and CHAPS-solubilized F_0 were prepared exactly as described under "Methods." F_0F_1-ATPase activity was reconstituted in a 0.20-ml system containing 50 mM Tricine, pH 7.9, 1.0 mM ATP, 25 mM EDTA, 0.50 mM dithiothreitol, 5% ethylene glycol, 0.6% CHAPS, 2.0 μg of purified F_1-ATPase, and 0–50 μg of 3×G membranes or F_0. After incubation at room temperature for 1 h, 25-μl aliquots were removed and assayed for ATPase activity in the presence of 0.5% methanol (control) or 5 μg/ml oligomycin (prepared as a methanolic solution). The percent inhibition was determined from these two values. One F_0 unit is defined as the amount of protein needed to give 50% inhibition of ATPase activity under these conditions. Values indicate below represent averages of four separate preparations of 3×G membranes and eight separate preparations of F_0.

Fraction	Total protein		Specific F_0 activity[a]	Total F_0 activity[a]	
	mg	*%*	*units/mg protein*	*units*	*%*
Inner membrane vesicles	256	100			
3× membranes	176	69			
3×G membranes	80.6	31.5	70	5642	100
F_0	11.0	4.3	228	2508	44

[a] Because the units of F_0 are defined on the basis of its capacity to confer oligomycin inhibitory sensitivity on the ATPase activity of purified F_1, F_0 "activity" values cannot be calculated for inner membrane vesicles and 3× membranes which contain F_1.

Capacity of CHAPS-purified Rat Liver F_0 to Confer DES

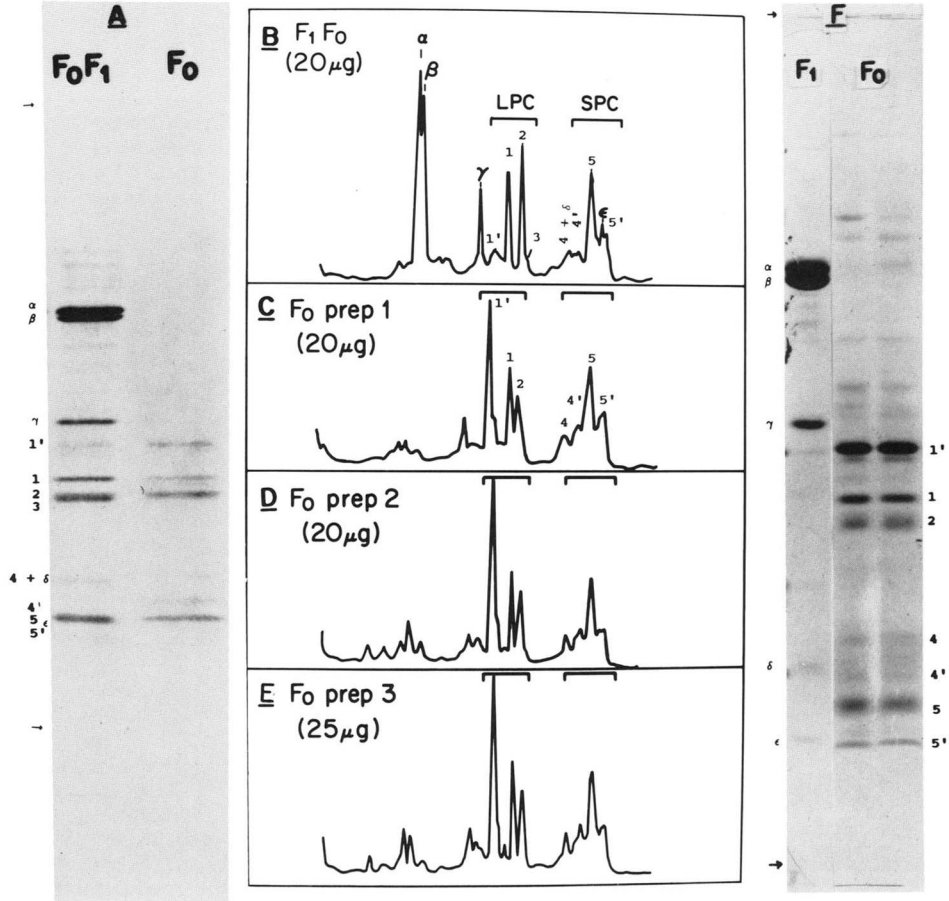

FIG. 1. **SDS-PAGE profiles of control rat liver F_0F_1 and different preparations of CHAPS-purified rat liver F_0.** $F_1F_0F_1$, and F_0 were purified and processed for SDS-PAGE as described under "Methods." *A*, representative gels of F_0F_1 and F_0 run at a protein load of 20 μg of protein and stained with Coomassie dye. *Arrows* mark the top of the gels and the dye front. *B–E*, gels scanned with a Zeineh soft laser scanning densitometer model SL-504-XL. Peaks corresponding to apparent F_0 subunits in F_0F_1 in *B* can be conveniently subdivided into a large polypeptide cluster (*LPC*) and a small polypeptide cluster (*SPC*). It will be noted that in the three different F_0 preparations (*C–E*), all peaks designated as possible F_0 subunits in the intact F_0F_1, with the exception of polypeptide 3, are present. These include protein peaks designated *1′, 1, 2, 4, 4′, 5, 5′*. *F*, gels of F_1-ATPase (50 μg) and F_0 run at a very high protein load (125 μg) and for a longer period of time than in *A*. *Arrows* mark the top of the gel and the dye front. It will be noted that the seven apparent F_0 subunits are resolved and that they are distinct from F_1 subunits.

TABLE II

CHAPS-purified F_0 recombines with F_1 to form an F_0F_1 complex inhibited by DES and known F_0 inhibitors

In A, CHAPS-purified F_0 (60 μg) was reconstituted with 12 μg of F_1-ATPase for 1 h in a final volume of 200 μl following the procedure stated in the legend to Table I. Aliquots (25 μl) were removed and assayed without prior incubation for ATPase activity in the absence and presence of inhibitor. The intact F_0F_1-ATPase, also purified using CHAPS as described under "Methods," was assayed at a concentration of 0.5 μg/ml. Values represent an average of three independent determinations. One hundred percent corresponds to rates of 240 nmol of ATP hydrolyzed per min/ml and 4.5 nmol of ATP hydrolyzed per min/ml for the reconstituted and control purified F_0F_1. The latter had a specific ATPase activity of 9 μmol of ATP hydrolyzed per min/mg. In B, the effect of DES on F_1-ATPase activity was examined after incubating F_1 under conditions identical to those described at the top without F_0. The F_1 preparation used had a specific ATPase activity of 20 μmol of ATP hydrolyzed per min/mg and was present at a final concentration of 1.5 μg/ml in the ATPase assay medium.

Compound	Remaining ATPase activity	
	Reconstituted F_0F_1-ATPase (I)	Purified F_0F_1-ATPase (II)
	%	
A.		
None	100	100
DES (25 μM)	15	17
Oligomycin (5 μg/ml)	5	4
Venturicidin (1 μg/ml)	33	30
Tricyclohexyltin (25 μM)	15	5
DCCD (25 μM)	9	10
DCCD (25 μM) + DES (25 μM)	100	100

Compound	Remaining F_1-ATPase activity
	%
B.	
None	100
DES (25 μM)	85

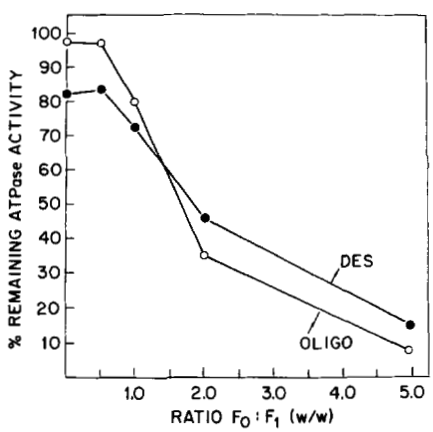

FIG. 2. **Relative capacities of DES and oligomycin to confer inhibitory sensitivity on rat liver F_1 at different F_0/F_1 ratios.** Different amounts of CHAPS-purified rat liver F_0 were added to a constant amounts (12 μg) of rat liver F_1 in a total volume of 200 μl as described in Table I. After 1 h at room temperature, a 25-μl aliquot of each sample was removed and tested for total ATPase activity in the presence of 0.5% methanol (control), 0.5 μg/ml oligomycin (○), or 25 μM DES (●).

Sensitivity on F_1—Results summarized in Table IIA show that when the CHAPS-purified F_0 preparation is recombined with purified rat liver F_1, the ATPase activity of the reconstituted F_0F_1 complex is inhibited 85% by 25 μM DES (Column I). This corresponds well with the capacity of DES to inhibit the ATPase activity of purified rat liver F_0F_1 (Column II). Under identical conditions of assay, it will be noted that 25 μM DES inhibits purified F_1 ATPase activity by only 15% (Table IIB). These results demonstrate that CHAPS-purified rat liver F_0 has recombined with F_1 in such a way as to confer DES sensitivity on the ATPase activity of F_1 and, therefore, implicate F_0 as the primary site of action of DES within the F_0F_1 complex. This suggestion gains support from additional data presented in Table IIA, which show that CHAPS purified F_0 also confers on the ATPase activity of F_1 sensitivity to known F_0-directed inhibitors (*i.e.* oligomycin, venturicidin, tricyclohexyltin, and DCCD).

In a previous study (3) we reported that, in addition to inhibiting F_0F_1-ATPase activity when added alone, DES is able to restore ATPase activity to F_0F_1 inhibited by DCCD. As shown in Table II, this "uncoupling" property of DES is retained also in F_0F_1 reconstituted from CHAPS-purified F_0 and F_1. Thus, when 25 μM DES is added to the reconstituted complex inhibited by 25 μM DCCD, the original ATPase activity is restored completely.

Comparison of the Capacity of DES Relative to That of Oligomycin to Confer Inhibitory Sensitivity on F_1 at Different

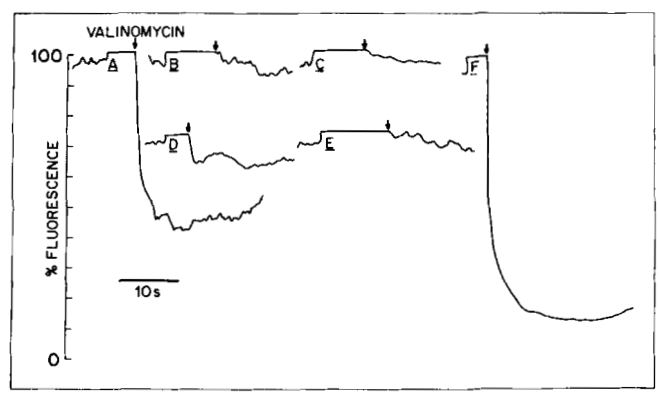

FIG. 3. **The capacity of CHAPS-purified rat liver F_0 to catalyze passive proton permeability and the sensitivity of this activity to F_0-directed inhibitors.** F_0 was incorporated into K^+-loaded asolectin vesicles by the dilution procedure described under "Methods" and assayed immediately for passive proton translocation also as described under "Methods": *A*, control F_0 (0.5% methanol present); *B*, F_0 + oligomycin (5 μg/ml); *C*, F_0 + DCCD (25 μM); *D*, F_0 + venturidicin (5 μg/ml); *E*, F_0 + trialkyltin (12.5 μM); and *F*, F_0 + CCCP (0.4 μM). The *arrow* refers to the point of addition of valinomycin.

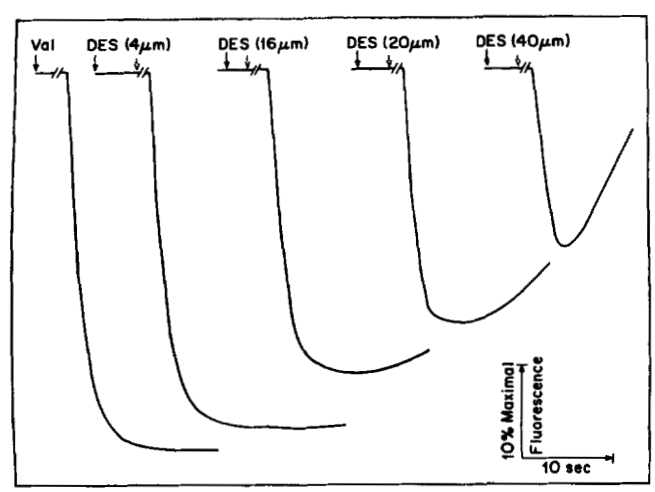

FIG. 4. **The capacity of DES to inhibit passive proton permeability catalyzed by asolectin vesicles reconstituted with F_0.** All conditions were identical to those noted in the legend to Fig. 3. Concentrations of DES used are indicated in the figure. The second *arrow* refers to the point of addition of valinomycin (*Val*).

F_0/F_1 Ratios—In order to better evaluate the primary site of action of DES, its capacity to confer DES inhibitory sensitivity on F_1 was determined by titrating a fixed concentration of F_1 with F_0. The logic behind this experiment was to establish whether the conferral of DES inhibitory sensitivity on F_1 by addition of increasing amounts of F_0 followed the same titration pattern as that of a known F_0 inhibitor such as oligomycin. As shown in Fig. 2, the capacity of both DES and oligomycin to confer 50% inhibitory sensitivity on F_1-ATPase activity occurs at almost the same F_0/F_1 weight/weight ratio. Data presented in Fig. 2 show also that both DES and oligomycin are capable of inhibiting the ATPase activity of F_1 as much as 80–90% when the F_0/F_1 ratio is further increased. These data complement those presented in Table I and further implicate F_0 as the primary site of action of DES within the F_0F_1 complex.

Capacity of DES to Inhibit Proton Permeability Catalyzed by F_0 Reconstituted with Asolectin Liposomes—Results presented in Fig. 3*A* demonstrate that CHAPS-purified rat liver F_0, after incorporation into asolectin vesicles loaded with K^+, readily catalyzes proton permeability when valinomycin is added. Further evidence for the functional intactness of this F_0 preparation is the finding that the known F_0-directed inhibitors oligomycin, DCCD, venturicidin, and trialkyltin inhibit proton translocation through F_0 (Fig. 3, *B–E*). As expected, the uncoupler CCCP bypasses F_0 allowing for proton entry at an enhanced rate (Fig. 3*F*).

The effects of DES on proton permeability catalyzed by F_0-reconstituted liposomes is presented in Fig. 4. Here, it is seen that as the concentration of DES if increased from 4 to 40 μM proton influx catalyzed by F_0 is increasingly inhibited. However, at the higher DES concentrations there is a significant reestablishment of the fluorescent intensity, suggestive of proton efflux. This raised the possibility that DES may exhibit a dual effect in this system, one to inhibit the normal proton pathway through F_0, and the other to initiate an efflux pathway, either within the lipid bilayer or at the F_0-lipid boundary.

Comparison of the Effects of DES on Proton Permeability in Asolectin Vesicles in the Absence and Presence of F_0—Results presented in Fig. 5 show that, in the absence of F_0, DES catalyzes proton influx into K^+-loaded vesicles upon addition of valinomycin, inducing a fluorescence quenching signal similar to the protonophoric uncouplers FCCP and DNP. DES concentrations which give maximal and half-maximal responses are, respectively, 40 and 12.5 μM, whereas a concentration of 80 μM DES appears to damage the vesicles preventing any further response (Fig. 5*A*, *inset*). When added to K^+-loaded vesicles containing F_0, DES no longer mimics the action of simple protonophoric uncouplers. Thus, DES, unlike the uncouplers FCCP (Fig. 5*B*, *2*) and DNP (data not shown), is unable to bypass the DCCD block in F_0 and catalyze proton influx (Fig. 5*B*, *3*).

In confirmation of results presented in Fig. 4, DES is seen to act on F_0 to induce an inhibition of the magnitude of proton influx while initiating proton efflux (Fig. 5*B*, *4*). At 40 μM DES, inhibition of influx is near 50% (Fig. 5*B*, *inset*). Results presented in Fig. 5*B*, *5*, show that proton efflux alone can be initiated by adding DES after proton influx has taken place.

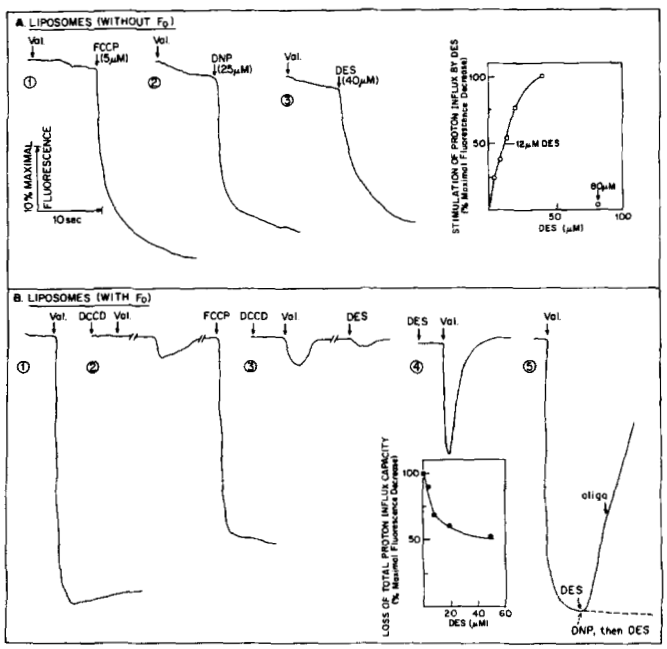

FIG. 5. **Effect of DES on passive proton permeability catalyzed by K^+-loaded asolectin vesicles in the absence and presence of CHAPS purified F_0.** *A*, K^+-loaded asolectin vesicles were prepared exactly as described under "Methods" and assayed immediately for passive proton translocation as described under "Methods." Additions are indicated on the graph. The *inset* summarizes the effect of DES concentration on proton permeability in the same system. *B*, F_0 was incorporated into K^+-loaded asolectin vesicles by the dilution procedure described under "Methods" and also assayed immediately for passive proton translocation. Where indicated, the following compounds were added: DCCD (25 μM), FCCP (5 μM), DES (40 μM), and DNP (40 μM). The *inset* summarizes the effect of DES concentration on the magnitude of proton influx. *Val*, valinomycin.

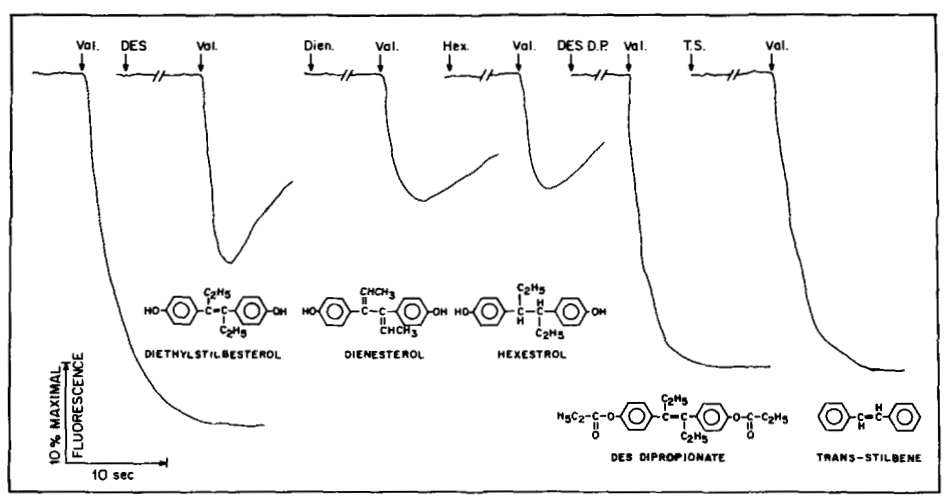

FIG. 6. **Comparison of the capacity of structural analogs of DES to replace this synthetic estrogen as an inhibitor of proton translocation through F_0.** Methods for the incorporation of F_0 into K^+-loaded vesicles and its assay for passive proton permeability were identical to those employed in experiments summarized in Figs. 3 and 4. DES and all analogs thereof were tested at a final concentration of 20 μM. *Val.*, valinomycin; *Dien.*, dienestrol; *Hex.*, hexestrol; *D.P.*, dipropionate; *T.S.*, trans-stilbene.

Proton efflux initiated by DES is not inhibited by addition of the F_0 directed inhibitor oligomycin, but can be prevented completely when the uncoupler DNP is added prior to DES (Fig. 5*B*, *5*, see *arrow*). In experiments not presented here, DNP was shown also to initiate proton influx after the fluorescence signal had recovered completely, demonstrating that the efflux pathway is not due simply to a slow destruction of the vesicles by DES.

Taken together, these results indicate that, when added to asolectin vesicles containing F_0, DES preferentially targets F_0 and in so doing dramatically alters its proton translocating function.

Specificity of DES in Altering Proton Permeability in F_0 Containing Asolectin Vesicles and in Inhibiting the ATPase Activity of Purified F_0F_1—Experiments summarized in Fig. 6 show that when tested at a concentration of 20 μM, the DES analogs dienestrol and hexestrol are actually more effective than DES in inhibiting the magnitude of proton influx and somewhat less effective in stimulating proton efflux. In contrast, neither DES-diproprionate nor trans-stilbene affected either proton influx or efflux. These findings are of particular significance since the effective compounds are those with hydroxyl groups at *para* positions on the two benzene rings.

Experiments summarized in Table III show that, in analogy to DES, dienestrol and hexestrol are also equally effective inhibitors of the ATPase activity catalyzed by the purified F_0F_1-ATPase of rat liver. In contrast, trans-stilbene is without effect while DES dipropionate inhibits ATPase activity only 13%.

These experiments show that the hydrophobic properties of the DES molecule alone are impotent in producing effects reported here on either F_0 or F_0F_1. Clearly, the hydroxyl groups of the DES molecule are critical for its mechanism of action.

Effect of DES on the ATPase Activity Catalyzed by F_0F_1 in Intact Mitochondria—Results of experiments presented in Fig. 7 show that the ATPase activity of F_0F_1, catalyzed by freshly isolated rat liver mitochondria, is stimulated by low concentrations of DES (<20 μM) and inhibited by higher concentrations (Fig. 7*A*). The stimulatory effect is similar to that observed in intact mitochondria by uncouplers like FCCP and DNP (23). In fact, if DNP is first added to maximally stimulate F_0F_1 ATPase activity, only the inhibitory phase induced by DES is observed (Fig. 7*B*). Under the assay conditions described here, F_1 ATPase activity undergoes no inhibition by DES.

These results show that in intact mitochondria, DES, depending on its concentration, can act to promote or inhibit the ATP hydrolytic activity of F_0F_1. As F_1-ATPase activity is not suppressed under these conditions, it seems likely that DES is targeting the F_0 moiety of the ATP synthase complex.

TABLE III

Comparison of the capacity of structural analogs of DES to inhibit the ATPase activity of rat liver F_0F_1

F_0F_1 was prepared and assayed for ATPase activity exactly as described under "Methods." The compounds tested were added directly to the assay cuvette to give a final concentration of 37 μM. F_0F_1 was present at a concentration of 0.75 μg/ml. Assays were conducted without prior incubation of inhibitor with F_0F_1. Values represent an average of three independent determinations.

Inhibitor present	Specific ATPase activity	Remaining ATPase activity
	μmol ATP hydrolyzed/min/mg	%
None	8.50	100
DES	1.39	16.4
Hexesterol	0.96	11.3
Dienesterol	1.20	14.2
DES dipropionate	7.40	87.0
Trans-stilbene	8.50	100

DISCUSSION

The major objective of this study was to gain greater insight into the mode of action of diethylstilbestrol on the mitochondrial ATP synthase complex (F_0F_1). As an earlier study from this laboratory had implicated F_0 rather than F_1 as the primary site of action of DES, the initial part of this study focused on obtaining a highly purified, functionally intact F_0 preparation. To this end, a rapid procedure was developed which employed guanidine HCl to first remove F_1 from inner membrane vesicles followed by the use of the zwitterionic detergent CHAPS to selectively extract F_0 in high yield.

The rat liver F_0 preparation described here is essentially free of F_1 subunits and molecular weight components above 28 kDa (Fig. 1). It contains four major Coomassie-stainable components together with three to four minor components. The latter are reproducibly obtained in all preparations of F_0 and F_0F_1 examined. As reports on bovine heart (12, 24–27) have placed the number of possible F_0 subunit types in the range of 6 to ≥8, rat liver F_0 may have a similar degree of complexity. This is not surprising as several lines of evidence (27, 28–31) indicate that higher eukaryotes contain several F_0 polypeptides (OSCP, F_6, F_B, A6L, and subunit d) which are not present as distinct species in *Escherichia coli* where only three polypeptide types comprise F_0 (32). Nevertheless, further experimentation will be necessary to establish which of the seven to eight apparent F_0 subunits described here are functional or structural components of rat liver F_0 and which might be contaminants. Significantly, the "triplet" represented by polypeptides 1′, 1, and 2 corresponds to a similar triplet in bovine heart F_0F_1 where the three polypeptides have been identified respectively as subunit b, OSCP, and subunit d (27). In addition, the DCCD binding site in rat liver appears to comigrate with polypeptide 5.

Four different experimental approaches described here support our earlier suggestion (3) that the F_0 moiety of rat liver ATP synthase is the preferential site of action of DES within

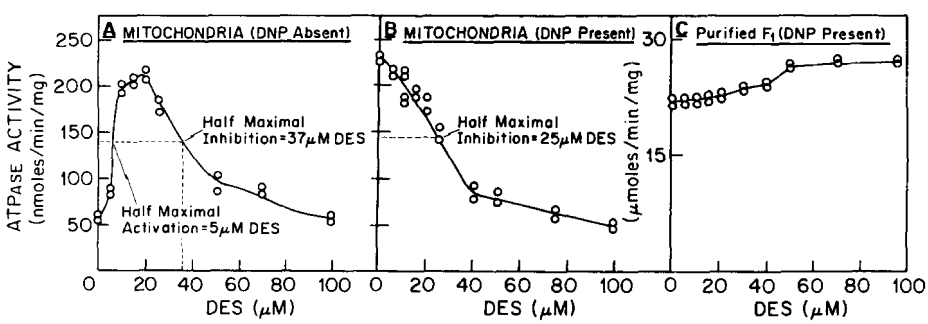

FIG. 7. **Capacity of DES to affect F_0F_1-ATPase activity catalyzed by intact rat liver mitochondria.** ATPase activity was assayed by monitoring P_i release exactly as described under "Methods." Where indicated, 0.5 mg of mitochondria or 10 μg of F_1 was present. DNP was present at 1.6×10^{-4} M. Concentrations of DES are as indicated.

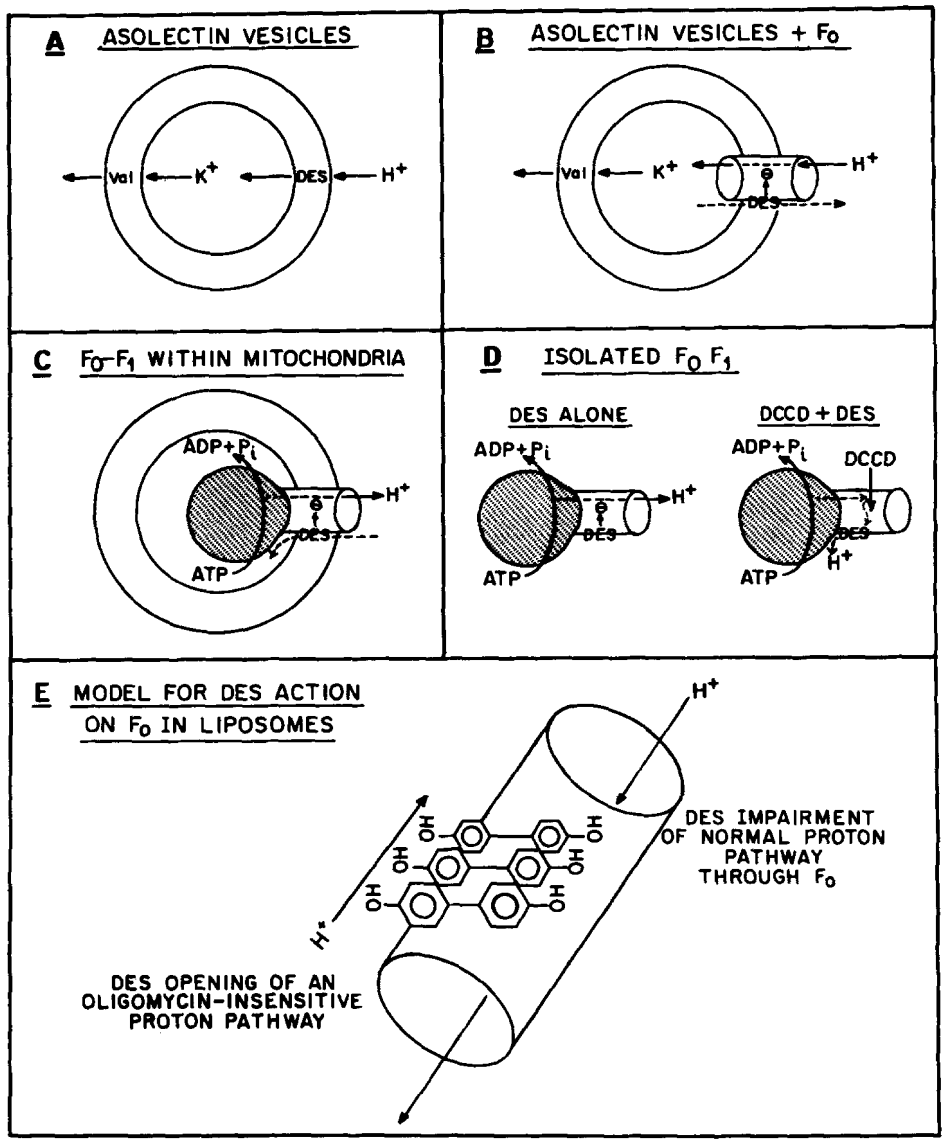

FIG. 8. **Summary diagrams illustrating the effect of DES on the four different systems (*A–D*) examined in this study (see "Discussion").** *E*, model depicting how DES may interact with F_0 contained within liposomal vesicles (see "Discussion"). The model depicts "stacking" of DES molecules along the F_0-lipid boundary with partial insertion into F_0. One of the polar hydroxyl groups of DES may inhibit the normal proton pathway through F_0 while its symmetrical partner closer to the F_0-lipid interface, may promote proton efflux through an oligomycin-insensitive pathway. As the hydroxyl groups of DES do not have readily dissociable protons, it seems likely that the proton efflux pathway induced by this probe operates quite differently from proton translocation pathways induced by uncouplers like DNP, FCCP, and CCCP. *Val*, valinomycin.

this complex. First, experiments carried out under two different assay conditions showed little or no effect of DES on the ATPase activity of isolated F_1 (Table IIB; Fig. 7*C*). Second, similar to other well known F_0-directed inhibitors, oligomycin, venturicidin, trialkyltin, and DCCD, the capacity of DES to inhibit F_1-ATPase is restored upon recombining with F_1 (Table II), as is the capacity of DES to restore ATPase activity to F_0F_1 inhibited by DCCD (Table II). Third, the DES inhibition pattern obtained upon titrating F_1 with F_0 is very similar to that obtained with oligomycin (Fig. 2). Finally, DES and closely related analogs have the capacity to directly modulate proton translocation catalyzed by rat liver F_0 reconstituted with asolectin vesicles (Figs. 4–6). It should be emphasized that all of these studies were conducted using low concentrations of DES, rarely exceeding 50 μM.

The illustrations presented in Fig. 8 represent an attempt to rationalize the effects of DES which, depending on conditions, may exhibit either an inhibitory or apparent uncoupling effect on the mitochondrial ATP synthase complex (Table II; Ref. 3). Fig. 8, *A* and *B*, depicts how DES may mimic the action of protonophoric uncouplers when added to liposomes alone (Fig. 5*A*), but target F_0 in F_0 containing liposomes (Fig. 5*B*). In this latter system, DES produces an impairment of proton influx while initiating proton efflux (Fig. 5*B*). In Fig. 8*B*, the site of action of DES is depicted as involving direct interaction with F_0 and with the boundary lipid associated with F_0. In Fig. 8*C*, it is assumed that the biphasic ATPase pattern observed when DES is added to mitochondria (Fig. 7*A*) is related to the effect of DES on F_0. Thus, the stimulation of ATPase activity may arise from proton influx into the organelle at the F_0-lipid interface at low concentrations of DES followed by inhibition of ATPase activity as higher DES concentrations impair the normal proton pathway through F_0. Consistent with the above interpretations, and as illustrated in Fig. 8*D*, DES would be predicted to inhibit and not stimulate the ATPase activity of isolated F_0F_1. This is because isolated F_0F_1 is not in a lipid bilayer, leaving only the normal proton pathway through F_0 to be affected by DES. Significantly, DES does have only an inhibitory effect on isolated F_0F_1 (Table II; Ref. 3). Finally, as illustrated also in Fig. 8*D*, the capacity of DES to restore ATPase activity to F_0F_1 (Table II; Ref. 3), inhibited by the F_0-directed compound DCCD, may reflect the capacity of DES to catalyze proton translocation. Thus, protons may be "sidetracked" to the medium by DES when the normal proton pathway is blocked by DCCD.

As emphasized by experiments summarized in Fig. 6 and Table III, the hydroxyl groups at *para* positions of the two benzene rings of the DES molecule are essential for both its capacity to modulate F_0 and to inhibit the ATPase activity of F_0F_1. This was suggested also in early reports (33, 34) indicating that DES is an apparent uncoupler of mitochondrial oxidative phosphorylation and in a more recent report on the effects of DES on the H^+ ATPase of chromaffin granules (2). On the basis of these observations and those described above, it is suggested by the illustration depicted in Fig. 8*E* that the hydroxyl groups of DES may promote impairment of the normal proton pathway through F_0 while promoting a reverse, oligomycin-insensitive pathway at the F_0-lipid interface. Hydrogen bonding involving the essential OH groups of DES may be involved in both events.

It is important to reemphasize that the reverse proton pathway stimulated by low concentrations of DES is not due to a slow destruction of the F_0 containing liposomes. Thus, uncouplers like DNP completely prevent DES from inducing proton efflux (Fig. 5*B, 5*), or if efflux is allowed to proceed to completion (*i.e.* to reestablish maximal fluorescence intensity) DNP induces influx (fluorescence quenching). An obvious question that arises from these observations concerns the driving force for proton efflux. The negative inside potential of the liposomes, generated by efflux of K^+ upon addition of valinomycin, is the normal driving force for proton influx and maintenance of the protons within the liposomes. Therefore, in inducing proton efflux it seems likely that DES allows Na^+ to enter the liposomes. (The external liposomal medium normally contains 200 mM NaCl (see "Methods").)

It is interesting to note that Strid *et al.* (6, 35) have examined recently the effects of DES on several activities catalyzed by the phototropic bacterium *Rhodospirillum rubrum* and on proton permeabilities of asolectin vesicles. In confirmation of our earlier study (3), they too find that DES inhibits F_0F_1 ATPase activity and that its action can be reversed by bovine albumin. However, for reasons which are not clear, these workers chose to use conditions which would be expected to produce interactions of DES with soluble oligomeric proteins like F_1 as well. Preincubation plus incubation times as long as 40 min were used, a time in which the hydrophobic DES molecule would be expected to penetrate oligomeric proteins in general and disrupt hydrophobic forces holding subunits together. In studies not reported here, we also observed inhibition of rat liver F_1 by incubating for long periods of time with DES, a process that could be facilitated when DES was presented to F_1 in a detergent like CHAPS. Although we agree with the conclusion of Strid *et al.* (35) that one step in the mode of action of DES may be to enter protein-lipid interfaces, we do not agree that the effect of DES on membrane proteins is based solely on its hydrophobicity. Clearly, this is not the case as supported by experiments reported here in Fig. 6 and Table III which demonstrate the importance of hydroxyl groups at both ends of the DES molecule. Along these lines, it is not clear why Strid *et al.* (35) failed to observe the apparent uncoupling property of DES in asolectin vesicles at low concentrations (Fig. 5*A*), and only observed disruption of the vesicles when DES was used at very high concentrations.

One of the most interesting properties of DES, as pointed out earlier in this report, is its capacity to inhibit all three classes of eukaryotic proton ATPases (P, V, and F) at low concentrations, and its capacity with the F and V type enzymes to restore ATPase activity after prior incubation with DCCD. From the study reported here, we believe that DES, when used at low concentration and with little or no incubation, may prove to be a useful probe for proteolipid centers involved in proton translocation. Thus, the hydrophobicity of DES appears to first target it to protein-lipid boundaries where its insertion into proteolipid complexes permits the hydroxyl groups to either inhibit or promote proton translocation pathways (Fig. 8*E*). Significantly, our earlier work on DES (3) has proved useful already in demonstrating similarities between vacuolar and mitochondrial proton ATPases in eukaryotic cells (2).

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