Inactivation of *Escherichia coli* BF_1-ATPase by Dicyclohexylcarbodiimide. Chemical Modification of the β Subunit†

Michel Satre, Joël Lunardi, Richard Pougeois, and Pierre V. Vignais*

ABSTRACT: Addition of dicyclohexylcarbodiimide (DCCD) to BF_1-ATPase isolated from *Escherichia coli* resulted in irreversible loss of enzymic activity. The kinetics of inactivation were consistent with 1 mol of DCCD reacting with one active site of BF_1-ATPase to give an inactive complex. Inactivation was more rapid at acid pH than at alkaline pH, the half-maximum effect being obtained at about pH 7. The half-time of inactivation was increased 4 times by 10 mM $MgCl_2$ and twice by 10 mM ADP or ATP. Inactivation of BF_1-ATPase was accompanied by the covalent binding of DCCD to the β subunit of BF_1-ATPase as shown by the use of (^{14}C)DCCD. The loss of enzymic activity was proportional to the incorporation of (^{14}C)DCCD. Linear extrapolation to zero activity corresponded to the binding of 1 mol of (^{14}C)-DCCD to 1 mol of BF_1-ATPase. Glycine ethyl ester, a nucleophilic reagent, which reacts with the DCCD-activated carboxyl group to form an amide bond, did not relieve the inactivation brought about by DCCD but prevented the binding of (^{14}C)DCCD, indicating that DCCD binds to a carboxyl group. Further evidence for a modification of a carboxyl group by DCCD was provided by isoelectrofocusing assays which suggested the disappearance of a negative charge in DCCD-modified BF_1-ATPase. The formation of the fluorescent aurovertin–BF_1-ATPase complex was not prevented by DCCD, neither was the enhancement of fluorescence caused by ADP. In contrast, the quenching of aurovertin fluorescence due to ATP was prevented by DCCD. DCCD had no apparent effect on the binding affinity of BF_1-ATPase for (^{14}C)ADP. The presence of a carboxyl group at the active site of BF_1-ATPase is supported by the nature of the data presented in this paper.

Dicyclohexylcarbodiimide (DCCD)[1] is a potent inhibitor of membrane-bound ATPase in mitochondria, chloroplasts, and bacteria (Beechey et al., 1967; Cattell et al., 1970; Roberton et al., 1968; Hare, 1975; Nelson et al., 1977). The increasing interest in the inhibitory effect of DCCD on the bacterial ATPase complex stems from the potential use of DCCD-resistant mutants as tools to identify the DCCD-binding protein. In fact, DCCD-resistant *Escherichia coli* mutants have been isolated, and the mutation has been located in one of the peptides of the hydrophobic sector of the ATPase complex (Fillingame, 1975, 1976; Friedl et al., 1977). The effect of DCCD on the hydrophilic sector of the bacterial ATPase complex is less documented, and it has been repeatedly reported that the isolated BF_1-ATPase is not very sensitive or not sensitive at all to DCCD as opposed to the BF_1–F_0 complex (Roisin & Kepes, 1973; Abrams & Smith, 1974; Fillingame, 1975; Hare, 1975; Patel & Kaback, 1976; Feinstein & Fisher, 1977).

The present paper describes experiments carried out with BF_1-ATPase isolated from *E. coli*. The results show that, under appropriate conditions of incubation, DCCD binds to a carboxyl group of the β subunit of BF_1-ATPase and that the binding of 1 mol of DCCD to 1 mol of BF_1-ATPase results in full inactivation of the enzyme.

Materials and Methods

Materials. Aurovertin D was purified from cultures of *Calcarisporium arbuscula* (NRRL 3705) (Osselton et al., 1974) and stored at −20 °C as an ethanolic solution protected from light. The molar extinction coefficient of our aurovertin preparation was 35 100 cm^{-1} at 368 nm. Aurovertin fluorescence was measured at 25 °C with a Perkin-Elmer MPF2A fluorometer. The medium consisted of 0.25 M sucrose, 10 mM Tris-HCl, and 0.5 mM EDTA, pH 7.4. The excitation wavelength was set at 365 nm and the emission wavelength was set at 470 nm.

(^{14}C)DCCD (54.5 Ci/mol) was obtained from the Commissariat à l'Energie Atomique (CEA, Saclay). It was more than 97% radiochemically pure as judged by thin-layer chromatography (Cattell et al., 1971), followed by autoradiography. It showed concentration vs. inhibition curves identical with unlabeled DCCD curves. Unlabeled DCCD was freed of any dicyclohexylurea according to the procedure described by Fieser & Fieser (1967). DCCD was used as a methanolic solution, and appropriate controls were run with methanol alone, the final methanol concentration being always less than 1%.

Organism and Growth Conditions. The *E. coli* strain used in this study was strain AN180 (*arg*E3, *thi*-1) (Butlin et al., 1971). Bacteria were grown at 37 °C on peptone–yeast extract medium (Miller, 1972) and stored at −80 °C.

BF_1-ATPase Purification. Membranes were prepared by disruption of the cells in a Sorvall-Ribi press (Roisin & Kepes, 1972). BF_1 was released from the membranes by chloroform treatment (Beechey et al., 1975). It was purified by ion-

† From the Laboratoire de Biochimie, Département de Recherche Fondamentale, Centre d'Etudes Nucléaires, 38041 Grenoble-cedex, and the Laboratoire de Biochimie de la Faculté de Médecine de Grenoble, France. *Received February 12, 1979.* This investigation was supported by research grants from the Centre National de la Recherche Scientifique (E.R.A. no. 36), the Fondation pour la Recherche Médicale, and the Délégation Générale à la Recherche Scientifique et Technique.

* Address correspondence to this author at the Laboratoire de Biochimie, Département de Recherche Fondamentale, Centre d'Etudes Nucléaires, 38041 Grenoble-cedex, France.

[1] Abbreviations used: DCCD, *N,N'*-dicyclohexylcarbodiimide; DIPC, *N,N'*-diisopropylcarbodiimide; EDAC, 1-ethyl-3-[(dimethylamino)-propyl]carbodiimide; CMCD, 1-cyclohexyl-3-(2-morpholino-4-ethyl)-carbodiimide *p*-methyltoluenesulfonate; Woodward's reagent K, *N*-ethyl-5-phenylisoxazolium 3'-sulfonate; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; IIDQ, *N*-[(isopropyloxy)carbonyl]-2-(isopropyloxy)-1,2-dihydroquinoline; Mops, 3-(*N*-morpholino)propanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; NBD, 4-chloro-7-nitrobenzofurazan; F_1, beef heart mitochondrial coupling factor; BF_1, *E. coli* coupling factor.

0006-2960/79/0418-3134$01.00/0

exchange chromatography (Vogel & Steinhart, 1976) on a DEAE-cellulose column (DE-52; Whatman) at 0–4 °C using a Tris-HCl gradient up to 0.75 M in 50 mM Tris-HCl, 2.5 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM ATP, pH 7.4, and 20% methanol (v/v). Fractions containing the ATPase activity were eluted at approximately 0.2 M Tris-HCl. They were concentrated on Amicon XM-100 membranes and further purified by gel filtration on a Sepharose 6B column equilibrated in the above buffer. Purified BF_1-ATPase was stored at 0–4 °C at a protein concentration of about 10 mg/mL. Its specific activity varied between 25 and 35 (μmol/min)/mg of protein under the described assay conditions. For calculation of binding data, a molecular weight of 360 000 (Hanson & Kennedy, 1973) was assumed.

(^{14}C)DCCD-Binding Experiments. After incubation of BF_1-ATPase with (^{14}C)DCCD, the (^{14}C)DCCD-bound ATPase was freed from unreacted (^{14}C)DCCD by the elution–centrifugation method described by Penefsky (1977). A 1-mL plastic syringe equipped with a porous polyethylene disk was filled with 1 mL of Sephadex G-50 fine equilibrated with the same buffer (50 mM Mops), pH 6.5, as BF_1-ATPase. The packed syringe was placed in a centrifuge tube and spun at low speed to remove the excess of the buffer. A sample of (^{14}C)DCCD-modified BF_1-ATPase was applied to the top of the column. The eluate after centrifugation contained 90–95% of the (^{14}C)DCCD-bound ATPase. Radioactivity counting was corrected for background by omission of the enzyme in the reaction mixture.

Gel Electrophoresis. Electrophoresis in 7.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate was carried out as described by Weber & Osborn (1969). After migration, the gels were stained for 4–6 h with a Coomassie blue solution made of 0.05% Coomassie blue R-250, 25% isopropyl alcohol, and 10% acetic acid and destained according to Fairbanks et al. (1971). The densitometric traces were recorded with a Joyce-Loebl Scan 400. To determine the distribution of radioactivity in the gels, the gels previously fixed, stained, and destained were frozen in solid CO_2 and sliced in 1-mm slices with a Joyce-Loebl gel slicer. Slices were digested by overnight incubation in 1 mL of 15% H_2O_2 at 55–60 °C and counted in 10 mL of a scintillation fluid (Patterson & Greene, 1965).

Isoelectric focusing in polyacrylamide gels was performed in glass tubing (120 × 3 mm inside diameter). The gels contained 2% Ampholines (LKB, pH range 3–10) and were prepared according to the procedure of O'Farrell (1975) except that Nonidet P-40 was replaced by Triton X-100. BF_1-ATPase samples (50–80 μg of protein) were lyophilized and then dissolved in 20 μL of 9.5 M urea, 2% Ampholines (pH range 3–10), 2% Triton X-100, and 5% 2-mercaptoethanol. Focusing was run as described by O'Farrell (1975).

At the end of the run, the gels were cut into 1-cm sections which were placed in capped tubes containing H_2O. The tubes were shaken for 30 min and then the pH was measured. In this way, the pH gradient was accurately determined. The other gels were stained in 0.1% Coomassie blue R-250, 10% trichloroacetic acid, 3% sulfosalicylic acid, and 25% methanol for 1 h and destained in a mixture of 30% ethanol and 10% acetic acid.

ATPase Assay. ATPase activity was assayed at 37 °C as described by Butlin et al. (1973). The reaction mixture contained 20 mM ATP, 10 mM $MgSO_4$, and 100 mM Tris–sulfate buffer, pH 8.5, in a final volume of 0.5 mL. After incubation for 10 min, the reaction was terminated by 0.1 mL of 2.5 N perchloric acid, and the amount of phosphate released was measured colorimetrically (Fiske & SubbaRow, 1925).

Table I: Inactivation of BF_1-ATPase by Carboxyl-Group Reagents[a]

reagent	concn (mM)	ATPase % inactivation	cross-linking
DCCD	0.01	55	no
	0.05	98	no
DIPC	0.05	2	
EDAC	0.25	31	yes ($\alpha\beta$, β_2, $\beta\epsilon$)
CMCD	0.5	73	yes (β_2, $\beta\epsilon$)
Woodward's reagent K	2.5	11	
EEDQ	0.020	47	yes ($\alpha\beta$, $\alpha\gamma$)
	0.100	88	yes ($\alpha\beta$, $\alpha\gamma$)
IIDQ	0.02	77	very slight

[a] BF_1 (0.5 mg/mL) was preincubated at 37 °C for 15 min with the carboxyl-group reagents in 25 mM Mops buffer, pH 6.5. After preincubation, aliquot samples were diluted 25-fold in 50 mM Tris–sulfate buffer, pH 8.5, and immediately assayed for ATPase activity (see Materials and Methods). Another sample was depolymerized in 2% $NaDodSO_4$, 2% 2-mercaptoethanol, and 10% glycerol and submitted to polyacrylamide gel electrophoresis.

Protein Assay. The protein concentration was determined by the dye-binding method using Coomassie blue G-250 as described by Bradford (1976). Bovine serum albumin was used as a standard.

Results

Inactivation of BF_1-ATPase from E. coli by Carboxyl-Group Reagents. Table I shows the effect on BF_1-ATPase of several carboxyl-group reagents, capable of reacting with proteins to give stable derivatives or to promote cross-linkages between carboxyl and amino groups in these proteins (Kurzer & Douraghi-Zadeh, 1967; Belleau & Malek, 1968; Belleau et al., 1968; Timkovich, 1977a,b; Pougeois et al., 1978). Among the reagents tested, DCCD, EEDQ, and IIDQ were the most effective inhibitors. EDAC and CMCD were less effective. DIPC and Woodward's reagent K were virtually inefficient. Following reaction of BF_1-ATPase with EDAC, CMCD, and EEDQ, intramolecular cross-linking occurred. The cross-linked subunits were tentatively identified on the basis of the molecular weight of the cross-linked products, as measured by $NaDodSO_4$ gel electrophoresis (Table I). No cross-linking was observed after inactivation of BF_1-ATPase by IIDQ and particularly with DCCD. The following experiments are concerned with the inactivation of BF_1-ATPase by DCCD.

Kinetics of Inactivation of BF_1-ATPase by DCCD. Incubation of BF_1 with DCCD resulted in a time- and concentration-dependent decrease of ATPase activity. This progressive effect of DCCD was not reversed by subsequent dilution or by removal of the inhibitor by gel filtration. The time course of inactivation followed pseudo-first-order kinetics until at least 80% of inactivation was reached. A plot of the log of the half-time of inactivation ($T_{1/2}$) against log [DCCD], according to Levy et al. (1963), gave a straight line (Figure 1A) with a slope close to 1, indicating that 1 mol of DCCD reacts with one catalytic site of BF_1-ATPase to form an inactive complex. A plot of $T_{1/2}$ against the reciprocal of the DCCD concentration also gave a straight line (Figure 1B) starting from the origin, thus excluding the formation of a reversible BF_1–DCCD complex prior to the inactivation step (Petra, 1971; Piszkiewicz & Smith, 1971). Inactivation of BF_1-ATPase depended on pH. As shown in Figure 2, the profile of inactivation between pH 5.5 and 9.5 was sigmoidal, the inactivation being more marked at acidic pH. The half-maximal effect was between pH 7.0 and 7.5.

The protective effect of ATP, ADP, and divalent cations against DCCD inactivation is shown in Table II. The

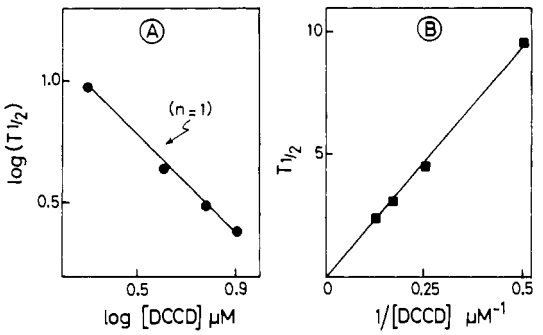

FIGURE 1: Kinetics of inactivation of BF_1-ATPase by DCCD. (A) Plot of the half-times of inactivation ($T_{1/2}$) against the log of the DCCD concentration. (B) Plot of half-times of inactivation ($T_{1/2}$) vs. the reciprocal of the DCCD concentration. BF_1 (0.14 mg/mL) was preincubated at 30 °C in 50 mM Mops buffer, pH 6.3, in the presence of 2, 4, 6, and 8 μM DCCD. At various times, aliquot samples were diluted 25-fold in 50 mM Tris–sulfate buffer, pH 8.5, and immediately assayed for ATPase activity (see Materials and Methods). $T_{1/2}$ (min) was calculated from semilogarithmic plots of ATPase activity at different DCCD concentrations.

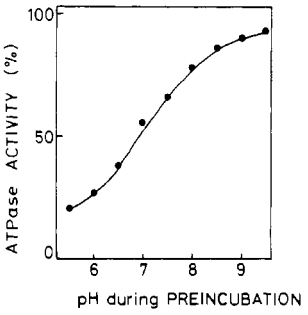

FIGURE 2: Effect of pH on inactivation of BF_1-ATPase by DCCD. BF_1-ATPase (0.15 mg/mL) was incubated at 37 °C with 10 μM DCCD for 15 min in 25 mM Mops and 25 mM Tris buffers at the indicated pH. Aliquots were removed, diluted 25-fold in 50 mM Tris–sulfate buffer, pH 8.5, and immediately assayed for ATPase activity.

Table II: Effect of Adenine Nucleotides and Divalent Cations on Inactivation of *E. coli* BF_1-ATPase by DCCD[a]

additions	half-time of inactivation (min)
none	6
ATP	12
ADP	11
$MgCl_2$	23
$MnCl_2$	25
$CaCl_2$	10

[a] BF_1-ATPase (0.04 mg/mL) was preincubated for 5 min at 37 °C in 25 mM Mops buffer with 10 mM nucleotides or cations as indicated in the table, and the pH was adjusted to 6.5. The time course of inactivation was followed after addition of 25 μM DCCD. The half-time of inactivation was deduced from the semilogarithmic plot of ATPase activity as a function of time (see Figure 1). For each condition, a control without DCCD was run.

half-time of inactivation was increased by a factor of 2 in the presence of 10 mM ATP, 10 mM ADP, or 10 mM $CaCl_2$ and by a factor of 4 by 10 mM $MgCl_2$ or 10 mM $MnCl_2$. The following nucleophilic compounds, 2-mercaptoethanol, dithiothreitol, hydroxylamine, and hydrazine, had no effect on inactivation of BF_1 by DCCD, even when added prior to DCCD.

Binding of (^{14}C)DCCD to BF_1-ATPase. Stoichiometry of the Inactivation Process and Identification of the DCCD-Binding Subunit. (^{14}C)DCCD bound covalently to BF_1. The amount of reagent bound during incubation with (^{14}C)DCCD was linearly related to the decrease in enzyme activity until

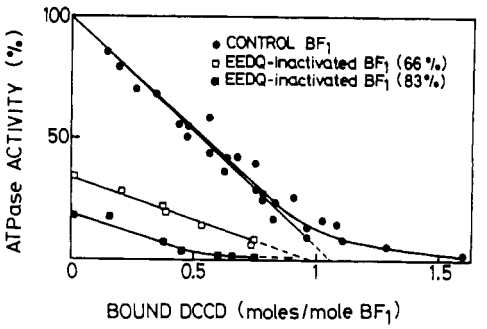

FIGURE 3: Correlation of inactivation of BF_1-ATPase activity and incorporation of (^{14}C)DCCD. Effect of EEDQ on the binding of (^{14}C)DCCD. BF_1-ATPase (0.7 mg/mL) in 50 mM Mops buffer, pH 6.5, was inactivated by incubation with increasing concentrations of (^{14}C)DCCD up to 100 μM for 15 min at 37 °C. ATPase activity and (^{14}C)DCCD binding (see Materials and Methods) were measured on samples removed at intervals (●; control). Two other fractions of BF_1-ATPase in 50 mM Mops, pH 6.5, were incubated for 10 and 20 min at 37 °C with 40 μM EEDQ to obtain respectively 66 and 83% inactivation. The action of EEDQ was stopped by centrifugation–filtration, according to Penefsky (1977) (see Materials and Methods), and the eluates were further incubated with (^{14}C)DCCD. ATPase activities and (^{14}C)DCCD binding were measured [BF_1-ATPase was inactivated by EEDQ to 66 (□) and 83% (■)].

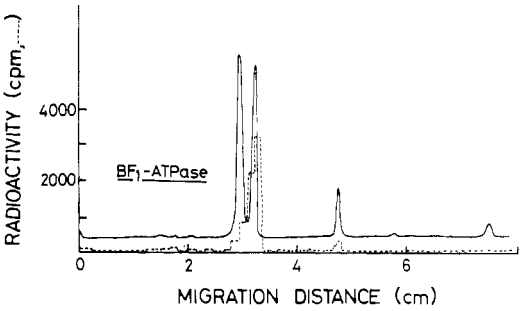

FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis of DCCD-modified BF_1-ATPase. BF_1-ATPase was inactivated by incubation with 8 μM (^{14}C)DCCD up to 90% inactivation. After removal of unbound (^{14}C)DCCD by centrifugation–filtration (see Materials and Methods), the enzyme (20 μg of protein) was subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. (—) Scan at 600 nm; (- - -) ^{14}C radioactivity.

about 85% inactivation was reached. By extrapolating binding data to zero activity, it was found that incorporation of 1 mol of (^{14}C)DCCD per mol of enzyme resulted in complete inactivation of BF_1 (Figure 3). Longer incubation periods with excess DCCD resulted in the incorporation of more than 1 mol of DCCD per mol of BF_1-ATPase. This extra binding of (^{14}C)DCCD was probably not specific.

To identify which subunit of BF_1 reacts with DCCD, $NaDodSO_4$–polyacrylamide gel electrophoresis was performed with samples of enzyme previously inactivated to some 90% with (^{14}C)DCCD. After recording the densitometer traces, we sliced the gels and determined the radioactivity. More than 75% of the total radioactivity was bound to the β subunit (Figure 4). One can calculate from Figure 4 that 1.1–1.2 mol of (^{14}C)DCCD is bound per mol of BF_1, which is fully consistent with the 1:1 stoichiometry deduced from the gel filtration data in Figure 3. As previously mentioned, the densitometer trace of the protein, after staining, showed no evidence for subunit cross-linking.

Effect of Glycine Ethyl Ester on the Binding of (^{14}C)DCCD to BF_1-ATPase. As DCCD may react with residues other than carboxyl groups in proteins (Kurzer & Douraghi-Zadeh, 1967), glycine ethyl ester is often used to control whether the group modified by DCCD is indeed a carboxyl group. In fact, DCCD-activated carboxyl groups react with glycine ethyl ester

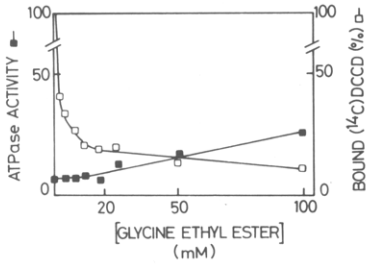

FIGURE 5: Effect of glycine ethyl ester on inactivation of BF_1-ATPase by DCCD and (^{14}C)DCCD binding. BF_1-ATPase was preincubated for 15 min at 37 °C with 20 μM (^{14}C)DCCD in 50 mM Mops, pH 6.5, and increasing concentrations of glycine ethyl ester. Then, ATPase activity and (^{14}C)DCCD incorporation were measured as described in the legend of Figure 3.

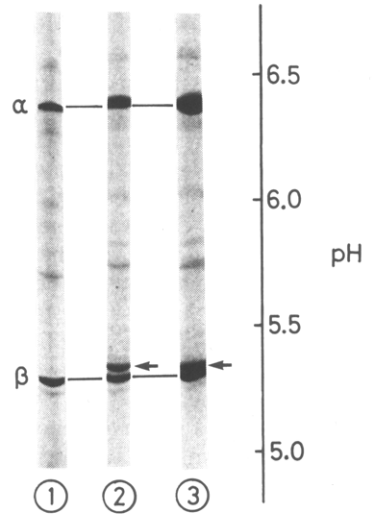

FIGURE 6: Isoelectric focusing of control BF_1-ATPase and DCCD-inactivated BF_1-ATPase. BF_1-ATPase was inactivated to about 70% by incubation with 4 μM (^{14}C)DCCD. After removal of free (^{14}C)DCCD by centrifugation–filtration (see Materials and Methods), the DCCD-modified enzyme (80 μg) was subjected to isoelectric focusing (gel 2) as described under Materials and Methods. The arrows point to the new protein band revealed in the DCCD-modified BF_1-ATPase. Control BF_1-ATPase (80 μg of protein; gel 1) and a mixture of control and DCCD-treated BF_1-ATPase (80 μg of protein each; gel 3) were also focused.

to form an amide bond (Hoare & Koshland, 1967; Carraway & Koshland, 1972). Inactivation of BF_1-ATPase by (^{14}C)-DCCD was measured in the presence of increasing amounts of glycine ethyl ester (Figure 5). The rates of inactivation were the same in the absence and in the presence of glycine ethyl ester up to 20 mM. However, the amount of (^{14}C)DCCD bound to BF_1-ATPase steadily decreased when increasing amounts of glycine ethyl ester were included in the medium, 80% decrease being reached with 20 mM glycine ethyl ester. This result strongly suggests that the DCCD-reactive residue in BF_1-ATPase is a carboxyl group.

Isoelectric Focusing of DCCD-Modified BF_1. Subunits α and β were clearly separated by isoelectrofocusing in urea–polyacrylamide gel (Figure 6). Their respective isoelectric points were pH 6.4 and 5.3. After DCCD inactivation, the β subunit migrated with a satellite band, identified by autoradiography as a (^{14}C)DCCD-bound β subunit, quite distinct from that corresponding to the unmodified β subunit (gel 2). The DCCD-modified β subunit was more basic than the unreacted β subunit, which is consistent with the binding of DCCD to a carboxyl group.

As pointed out by O'Farrell (1975), artifactual charge heterogeneity in a single protein is frequently detected, even

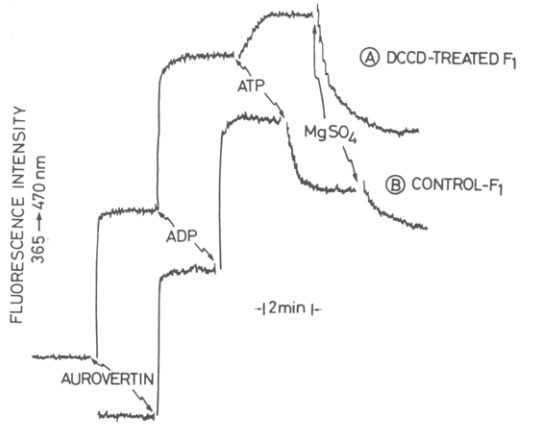

FIGURE 7: Effect of adenine nucleotides and $MgSO_4$ on the fluorescence intensity of the aurovertin–BF_1-ATPase complex and aurovertin–DCCD-inactivated BF_1-ATPase. BF_1 was inactivated to 70% by incubation with 50 μM DCCD for 10 min at 37 °C. Inactivation was stopped by removal of unbound DCCD (see Materials and Methods). The fluorescence assays were made at 25 °C with control BF_1-ATPase or DCCD-inactivated BF_1-ATPase (85 μg of protein) incubated with 0.4 μM aurovertin in 2 mL of 0.25 M sucrose, 10 mM Tris-HCl, and 0.5 mM EDTA, pH 7.4. ADP, ATP, and $MgSO_4$ were added sequentially to obtain the following final concentrations: 0.2 mM ADP, 2 mM ATP, and 2.5 mM $MgSO_4$.

under mild conditions such as storage. Charge heterogeneity is revealed in electrofocusing by consecutive spots on the gel, the spacing of which is consistent with single charge differences. The isoelectric point of the charge-modified protein is usually more acidic than that of the native protein. In keeping with this proposal, the first artifactual band found below the β subunit (see Figure 6) would correspond to a β chain with a single charge change, resulting in a more acidic isoelectric point. This artifactual band is hardly detectable in native F_1-ATPase; it increases with aging, which rules out the possibility that it could correspond to an impurity present in the ATPase preparation. The distances between the β subunit and that more acidic band on one hand and the β subunit and the more basic DCCD-modified β subunit on the other (gel 2) are virtually the same. This observation suggests that 1 mol of DCCD binds to one carboxyl group per β subunit.

Effect of EEDQ on the Binding of (^{14}C)DCCD to BF_1-ATPase. As shown in Table I, EEDQ is, in addition to DCCD, one of the most potent carboxyl reagents to inhibit *E. coli* BF_1-ATPase. The following assay was conducted to investigate whether EEDQ bound to the same site in BF_1-ATPase as did (^{14}C)DCCD. First, the enzyme was some 65% inactivated with EEDQ. Unreacted EEDQ was eliminated by Sephadex G-50 gel chromatography (see Materials and Methods). Next, (^{14}C)DCCD was added and the incorporated radioactivity was monitored together with the ATPase activity. As shown in Figure 3, (^{14}C)DCCD was incorporated to the same maximal extent (about 1 mol/mol of enzyme) in control BF_1 and in EEDQ-treated BF_1, suggesting that DCCD binds to a different carboxyl group than does EEDQ. However, it is not excluded that EEDQ binding unmasks another carboxyl group which then becomes accessible to DCCD. A more complex pattern was apparent when BF_1 was inactivated by more than 80% by EEDQ; a partial interference with (^{14}C)DCCD binding was then observed (Figure 3). This could be due to secondary cross-linking effects of EEDQ (Table I).

Aurovertin Binding to DCCD-Inactivated BF_1-ATPase. Effect of ADP and ATP. It has been recently shown that aurovertin binds to *E. coli* BF_1-ATPase to form a fluorescent complex (Satre et al., 1978). The basal fluorescence of the

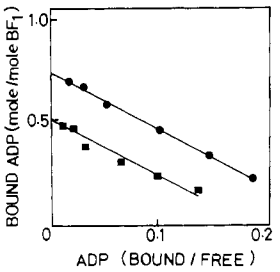

FIGURE 8: Binding of (^{14}C)ADP to BF_1-ATPase. BF_1-ATPase (2.5 mg/mL) in 25 mM Mops buffer, pH 6.5, was 80% inactivated by incubation with 100 μM DCCD for 10 min at 37 °C (see Materials and Methods). Control BF_1-ATPase and DCCD-inactivated BF_1 (both 0.5 mg/mL) were incubated for 15 min at 37 °C in 50 mM Tris-HCl and 0.5 mM EDTA, pH 7.4, containing 3–100 μM (^{14}C)ADP. Bound (^{14}C)ADP was measured as described by Penefsky (1977).

aurovertin–BF_1 complex is strongly enhanced by addition of ADP as well as by small concentrations of ATP (below 0.2 mM). The ADP enhanced aurovertin fluorescence is quenched by high concentrations of ATP (above 0.2 mM). In the experiment presented in Figure 7, BF_1-ATPase was partially inactivated with DCCD. This did not prevent the increase of fluorescence caused by the binding of aurovertin to BF_1-ATPase, indicating that binding of DCCD to BF_1-ATPase does not interfere with aurovertin binding. DCCD binding did not affect the enhancement of the fluorescence caused by addition of ADP nor its quenching by Mg^{2+} ions; a subsequent addition of ATP had either no effect or resulted in a further enhancement of fluorescence (Figure 7), indicating that DCCD binding either interferes directly with the binding of ATP or prevents the ATP binding indirectly by inducing a conformational change of the β subunit.

Effect of DCCD on the Binding Affinity of BF_1-ATPase for (^{14}C)ADP. In agreement with Bragg & Hou (1977), BF_1-ATPase was found to rapidly bind (^{14}C)ADP. The Scatchard plot in Figure 8 reveals essentially one type of ADP binding site for ADP concentrations ranging between 3 and 100 μM. The maximal amount of bound ADP was 0.74 mol/mol of BF_1-ATPase with a K_d of 6 μM, which probably corresponds to a binding stoichiometry of 1:1 (Figure 8). An 80% inactivation of BF_1-ATPase by DCCD was accompanied by a relatively small loss (30%) of the (^{14}C)ADP binding capacity of the enzyme; further, the binding affinity was not modified. These data corroborate those reported above on aurovertin fluorescence, showing that DCCD had no significant effect on ADP binding.

Discussion

The results presented in this paper demonstrate that BF_1-ATPase isolated from *E. coli* is irreversibly inactivated by the covalent binding of DCCD. The amount of (^{14}C)DCCD incorporated linearly follows the loss of enzyme activity, full inactivation occurring when 1 mol of (^{14}C)DCCD is bound to 1 mol of BF_1-ATPase. Kinetic data also indicate that the reaction of 1 mol of DCCD per mol of catalytic sites results in full inactivation of the enzyme. The binding of DCCD to a carboxylic group is strongly suggested by isoelectric focusing experiments demonstrating the disappearance of a negative charge in the DCCD-modified BF_1-ATPase. The modification of a carboxyl residue by DCCD is also indicated by the effect of glycine ethyl ester on the binding of (^{14}C)DCCD; although (^{14}C)DCCD binding was extensively prevented by this reagent, the extent of inactivation remained the same. The persistency of the loss of ATPase activity under these conditions could be due to the disappearance of a strategic carboxyl residue to give

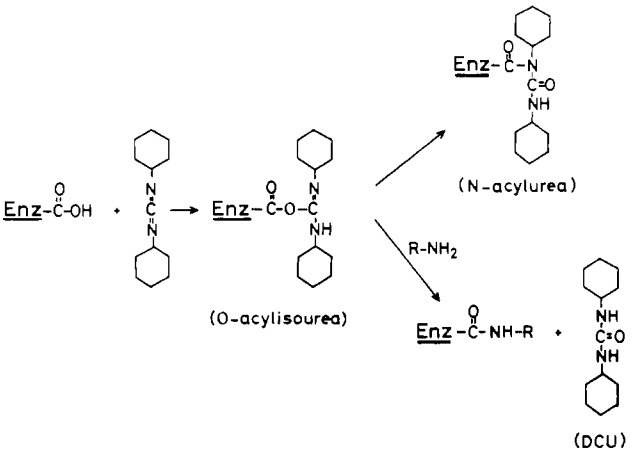

FIGURE 9: Reaction scheme of DCCD with carboxyl groups.

a neutral amide (Figure 9). Extensive formation of amide bonds to explain the BF_1-ATPase inactivation is ruled out because there are no significant quantities of cross-linked species produced during the inactivation process, in contrast to that which occurs with other carboxylic reagents, such as EEDQ, EDAC, and CMCD. Concerning the DCCD-modified carboxylic group, it could be argued that the pK of this group is about 7.5, as indicated by the study of the pH dependence of the enzyme inactivation. This is not astonishing as inactivation of beef heart F_1-ATPase by EEDQ, a selective carboxyl-group reagent, brings also into play a carboxyl group with a pK as high as 7.5 (Pougeois et al., 1978). It is known that carboxyl groups located in a hydrophobic environment exhibit unusually high pK values (Tanford, 1962; Basch & Timasheff, 1967; Ba Pho et al., 1977; Braun et al., 1977). The DCCD-modified carboxyl group of the BF_1-ATPase is located in the β subunit. This is consistent with other observations indicating that the catalytic center for ATP hydrolysis is also located on subunit β.

It has been recently reported that DCCD inactivates F_1-ATPase (Satre et al., 1978; Pougeois et al., 1979). In both cases, F_1-ATPase and BF_1-ATPase, the DCCD-binding site is located on the β subunit. However, the binding stoichiometry is different. Whereas BF_1-ATPase is fully inactivated by the binding of 1 mol of (^{14}C)DCCD to 1 mol of BF_1-ATPase, complete inactivation of F_1-ATPase requires the binding of 2 mol of (^{14}C)DCCD to 1 mol of F_1-ATPase. No explanation can yet be offered for this difference.

Because of its oligomeric structure (either $\alpha_2\beta_2$ or $\alpha_3\beta_3$ type), BF_1-ATPase possesses at least two copies of the β subunit and thus two available catalytic sites, by assuming a symmetrical arrangement of the β subunits, yet only one DCCD-binding site can be detected. A similar situation has been described for three other group-specific reagents in the case of beef heart F_1-ATPase; modification of one tyrosine residue by NBD (Ferguson et al., 1975) or one arginine residue by butanedione or phenylglyoxal (Marcus et al., 1976; Kohlbrenner & Cross, 1978) completely inhibits its hydrolytic activity. This behavior is characteristic of enzymes exhibiting the half-of-the-sites reactivity (Levitzki et al., 1971; Seydoux et al., 1974). We shall now briefly discuss the DCCD-binding data in light of two of the alternatives put forward by Levitzki et al. (1971) to explain the enzymic mechanism of half-of-the-sites reactivity, and we shall assume that BF_1-ATPase possesses two β subunits. One possible explanation of half-of-the-sites reactivity of BF_1-ATPase with respect to DCCD binding is that the binding of DCCD to one given β subunit induces, by means of cooperative interactions, a conformational change

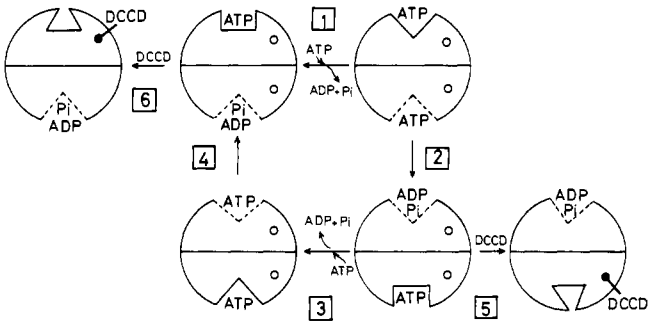

FIGURE 10: A model of alternating-site sequence compatible with DCCD-binding data. ATP binding to one β subunit (step 1) promotes the release of the previously bound ADP (and P_i) at the same subunit. Besides, it induces in the other β subunit the alternate conformation of the ATP site which permits hydrolysis of the bound ATP (step 2). A symmetrical sequence of events occurs in steps 3 and 4. DCCD binds to only one of the two β subunits in spite of the existence of a potential DCCD-reactive carboxyl group in each β subunit (see Discussion).

in the second β subunit, which becomes unable to bind DCCD. The binding of only one DCCD to one β subunit fully inactivates the enzyme. In the second alternative, it is supposed that the conformation of the catalytic sites located on each of the β subunits alternates between two states, one of which is able to bind DCCD (Figure 10). This is reminiscent of the alternating-site mechanism proposed by Moudrianakis & Adolfsen (1975) and by Kayalar et al. (1977) for beef heart F_1-ATPase, in which two identical catalytic sites function cooperatively in such a way that binding of ADP and P_i at one site promotes ATP release at the other and vice-versa. In the scheme of Figure 10, it is supposed that the conformation of the nucleotide site in each β subunit alternates between two states, one of which is able to bind ADP and the other of which is able to bind ATP. At any time the two nucleotide sites are filled with either ATP or ADP. Each of the two β subunits possesses a potential DCCD-reactive carboxyl group, but only in one β subunit at a given time is the carboxyl group accessible to DCCD. To ensure the formation of a stable adduct with DCCD, the peptide chain in the DCCD-binding site would have to be folded so as to provide a proper environment. One of the two conformational states would provide that environment but not the other one. A possible reason for a nonappropriate environment for DCCD binding may be the exposure of the potential DCCD-binding carboxyl group to water; water would act as a nucleophile to regenerate the carboxyl group, whereas DCCD would be released as free dicyclohexylurea (Figure 9).

References

Abrams, A., & Smith, J. B. (1974) *Enzymes, 3rd Ed. 10*, 395–429.

Ba Pho, D., Roustan, C., Nguyen Ti Tot, A., & Pradel, L. A. (1977) *Biochemistry 16*, 4533–4537.

Basch, J. G., & Timasheff, S. N. (1967) *Arch. Biochem. Biophys. 118*, 37–47.

Beechey, R. B., Roberton, A. M., Holloway, C. T., & Knight, I. G. (1967) *Biochemistry 6*, 3867–3879.

Beechey, R. B., Hubbard, S. A., Linnett, P. E., Mitchell, A. D., & Munn, E. A. (1975) *Biochem. J. 148*, 533–537.

Belleau, B., & Malek, G. (1968) *J. Am. Chem. Soc. 90*, 1651–1652.

Belleau, B., Martel, R., Lacasse, G., Menard, M., Weinberg, N. L., & Perron, Y. G. (1968) *J. Am. Chem. Soc. 90*, 823–824.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Bragg, P. D., & Hou, C. (1977) *Arch. Biochem. Biophys. 178*, 486–494.

Braun, H., Cogoli, A., & Semenza, G. (1977) *Eur. J. Biochem. 73*, 437–442.

Butlin, J. D., Cox, G. B., & Gibson, F. (1971) *Biochem. J. 124*, 75–81.

Butlin, J. D., Cox, G. B., & Gibson, J. (1973) *Biochim. Biophys. Acta 292*, 366–373.

Carraway, K. L., & Koshland, D. E., Jr. (1972) *Methods Enzymol. 25*, 616–623.

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.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Feinstein, D. L., & Fisher, R. J. (1977) *Biochem. J. 167*, 497–499.

Ferguson, S. J., Lloyd, W. J., Lyons, M. H., & Radda, G. K. (1975) *Eur. J. Biochem. 54*, 117–126.

Fieser, L. F., & Fieser, H. (1967) in *Reagents for Organic Synthesis*, Vol. 1, pp 231–236, Wiley, New York.

Fillingame, R. H. (1975) *J. Bacteriol. 124*, 870–883.

Fillingame, R. H. (1976) *J. Biol. Chem. 251*, 6630–6637.

Fiske, C. A., & SubbaRow, Y. (1925) *J. Biol. Chem. 66*, 375–400.

Friedl, P., Schmid, B. I., & Schairer, H. U. (1977) *Eur. J. Biochem. 73*, 461–468.

Hanson, R. L., & Kennedy, E. P. (1973) *J. Bacteriol. 114*, 772–781.

Hare, J. F. (1975) *Biochem. Biophys. Res. Commun. 66*, 1329–1337.

Hoare, D. G., & Koshland, D. E. (1967) *J. Biol. Chem. 242*, 2447–2453.

Kayalar, C., Rosing, J., & Boyer, P. D. (1977) *J. Biol. Chem. 252*, 2486–2491.

Kohlbrenner, W. E., & Cross, R. L. (1978) *J. Biol. Chem. 253*, 7609–7611.

Kurzer, F., & Douraghi-Zadeh, K. (1967) *Chem. Rev. 67*, 107–152.

Levitzki, A., Stallcup, W. B., & Koshland, D. E., Jr. (1971) *Biochemistry 10*, 3371–3378.

Levy, H. M., Leber, P. D., & Ryan, E. M. (1963) *J. Biol. Chem. 238*, 3654–3659.

Marcus, F., Schuster, S. M., & Lardy, H. A. (1976) *J. Biol. Chem. 251*, 1775–1780.

Miller, J. H. (1972) in *Experiments in Molecular Genetics*, pp 201-205, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Moudrianakis, E. N., & Adolfsen, R. (1975) in *Electron Transfer Chains and Oxidative Phosphorylation* (Quagliariello, E., Papa, S., Palmieri, F., Slater, E. C., & Siliprandi, N., Eds.) pp 373–378, North-Holland/Elsevier, Amsterdam, Oxford, and New York.

Nelson, N., Eytan, E., Notsani, B., Sigrist, H., & Sigrist-Nelson, K. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2375–2378.

O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.

Osselton, M. D., Baum, H., & Beechey, R. B. (1974) *Biochem. Soc. Trans. 2*, 200–202.

Patel, L., & Kaback, H. R. (1976) *Biochemistry 15*, 2741–2746.

Patterson, M. S., & Greene, R. C. (1965) *Anal. Chem. 37*, 854–857.

Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.

Petra, P. H. (1971) *Biochemistry 10*, 3163–3177.
Piszkiewicz, D., & Smith, E. L. (1971) *Biochemistry 10*, 4544–4552.
Pougeois, R., Satre, M., & Vignais, P. V. (1978) *Biochemistry 17*, 3018–3023.
Pougeois, R., Satre, M., & Vignais, P. V. (1979) *Biochemistry 18*, 1408–1413.
Roberton, A. M., Holloway, C. T., Knight, I. G., & Beechey, R. B. (1968) *Biochem. J. 108*, 445–456.
Roisin, M. P., & Kepes, A. (1972) *Biochim. Biophys. Acta 275*, 333–346.
Roisin, M. P., & Kepes, A. (1973) *Biochim. Biophys. Acta 305*, 249–259.
Satre, M., Pougeois, R., Lunardi, J. Dianoux, A. C., Klein, G., Bof, M., & Vignais, P. V. (1978) in *Frontiers in Bioenergetics* (Dutton, P. L., Leigh, J., & Scarpa, A., Eds.) Vol. I, pp 467–476, Academic Press, New York.
Seydoux, F., Malhotra, O. P., & Bernhard, S. (1974) *CRC Crit. Rev. Biochem. 2*, 227–257.
Tanford, C. (1962) *Adv. Protein Chem. 17*, 70–165.
Timkovich, R. (1977a) *Anal. Biochem. 79*, 135–143.
Timkovich, R. (1977b) *Biochem. Biophys. Res. Commun. 74*, 1463–1468.
Vogel, G., & Steinhart, R. (1976) *Biochemistry 15*, 208–216.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.

Studies of the Kinetics of Oxidation of Cytochrome *c* by Cytochrome *c* Oxidase: Comparison of Spectrophotometric and Polarographic Assays†

Lucile Smith,* Helen C. Davies, and Maria Elena Nava

ABSTRACT: The kinetics of oxidation of cytochrome *c* by cytochrome *c* oxidase were studied by spectrophotometric assays of the oxidation of soluble ferrocytochrome *c* and by polarographic measurements of O_2 uptake in the presence of *N,N,N′,N′*-tetramethylphenylenediamine plus ascorbate. Kinetic measurements by the two methods were compared by using different kinds of cytochrome *c* oxidase preparations and varying experimental conditions with cytochrome *c* concentrations between 0.05 and 5 μM. With both the spectrophotometric and the polarographic methods the most rapid rates per cytochrome *c* concentration were found at concentrations of cytochrome *c* around 0.1 to 0.25 μM. However, the two methods showed very different responses to changes of experimental conditions. Under some conditions the rates of O_2 uptake measured by the polarographic method were the same as the rates calculated from the spectrophotometric measurements of cytochrome *c* oxidation; under other conditions the measured rates of O_2 uptake were as much as 30-fold greater than the calculated rates. "Apparent K_M" values derived from plots of v/S against v are different when derived from data from the two methods under some experimental conditions. The rates of O_2 uptake in the presence of *N,N,N′,N′*-tetramethylphenylenediamine, ascorbate, and increasing concentrations of cytochrome *c* correlate with the concentrations of cytochrome *c*, which remain oxidized in the aerobic state under these conditions. The data suggest that some conditions promote the formation of an especially reactive cytochrome *c*–cytochrome oxidase complex.

Two different kinds of methodology have been utilized in recent studies of the kinetics of cytochrome *c* oxidase (EC 1.9.3.1) using different species or derivatives of cytochrome *c*, with the aim of gaining insight into its mechanism of action (Staudenmayer et al., 1976; Smith et al., 1976, 1977; Errede & Kamen, 1978; Ferguson-Miller et al., 1976, 1978a).

(1) First is the oxidation of pure soluble cytochrome *c* by the oxidase, followed spectrophotometrically. The reaction is first order in ferrocytochrome *c* with concentrations of cytochrome *c* between 0.04 and 100 μM in buffers such as 0.05 M Tris[1]–acetate, Tris–maleate, or phosphate or 0.1 M Mes or phosphate at pH values between 6 and 7.4 (Smith & Conrad, 1956; Smith et al., 1973; Errede & Kamen, 1978). However, the first-order rate constant decreases with increasing concentration of cytochrome *c* in the reaction mixture, but not in a linear fashion. The observed kinetics have been interpreted as reflecting rates of association and dissociation of cytochrome *c* and the oxidase (Minnaert, 1961; Yonetani & Ray, 1965; Errede & Kamen, 1978). Most recently Errede & Kamen (1978) obtained data over a wide range of concentrations of cytochrome *c* and could explain the observed kinetics by assuming the binding of two molecules of cytochrome *c* with different affinities for the oxidase.

(2) Second is the polarographic measurement of O_2 uptake in the presence of a reductant of cytochrome *c*. Ascorbate has often been utilized as the reductant, where the rate of O_2 uptake divided by four was found to equal the rate of oxidation of cytochrome *c* followed spectrophotometrically when the cytochrome *c* was kept nearly completely reduced during the reaction; rather high concentrations of ascorbate are required (Smith & Camerino, 1963a). Ferguson-Miller et al. (1976) employed a combination of TMPD plus a lower concentration of ascorbate and reported that the cytochrome *c* remained

† From the Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03735, and the Department of Microbiology, University of Pennsylvania Medical School, Philadelphia, Pennsylvania 19104. *Received February 5, 1979.* This research was supported by Grant GM-06270 from the National Institutes of Health and by General Research Support Grant RR-05392 to L.S. and by a grant from the Delaware Heart Association.

* Address correspondence to this author at Dartmouth Medical School.

[1] Abbreviations used: SMP, submitochondrial particles; TMPD, *N,N,N′,N′*-tetramethylphenylenediamine dihydrochloride; DOC, sodium deoxycholate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris-(hydroxymethyl)aminomethane.

0006-2960/79/0418-3140$01.00/0