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The Effects of Carbodiimides on Functions Associated with the Energy-Conservation Mechanism in Beef Heart Sub-Mitochondrial Particles

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Abstract

N,N'-di-*n*-propyl-, *N,N'*di-*n*-butyl-, *N,N'*-di-*n*-pentyl-, *N,N'*di-*n*-hexyl-, *N,N'*di-*n*-octoyl-, *N,N'*-dibenzhydryl-, and *N,N'*-dibenzhydrylcarbodiimides were synthesized. They were all effective inhibitors (2 nmoles carbodiimide per milligram protein) of the ATP-driven reduction of NAD by succinate and the ATP-driven transhydrogenase activities catalyzed by beef heart submitochondrial particles (SMP). They had no effect on the nonenergy-linked transhydrogenase and stimulated the succinate-driven aerobic transhydrogenase activity of beef heart SMP. It was concluded that they exert their effects by reacting with the *N,N'*-dicyclohexylcarbodiimide-binding protein. Water-soluble carbodiimides were not effective inhibitors.

Introduction

N,N'-dicyclohexylcarbodiimide (DCCD, structure I, R = cyclohexyl) is a potent and specific inhibitor of the ATP-synthetase system in energy-conserving membranes [1, 2].

$$R-N=C=N-R$$

Structure I

It has been shown that DCCD interacts with a specific proteolipid in (a) the membranes of beef heart mitochondria [3–5], (b) *Escherichia*

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coli vesicles [6–8], (c) lettuce chloroplast membranes [9], and (d) fungal mitochondrial membranes of *Neurospora crassa* [10] and *Saccharomyces cereviseia* [11].

It inhibits the ATPase/ATP-synthetase activity of a wide range of bacteria, chloroplasts, and mitochondria [12]. DCCD also inhibits the proton permeability of spinach chloroplast membranes [13, 14], *E. coli* cells and vesicles [15–17], and *Streptococcus faecalis cells* [18, 19].

The experiments reported here were designed to investigate the optimization of the inhibitory activity of carbodiimides on functions associated with energy-conservation mechanisms and to give some information about the site at which carbodiimides act. The results show that symmetrical *N,N'-n*-alkylcarbodiimides (structure I, R = *n*-propyl, *n*-butyl, *n*-pentyl, *n*-hexyl, and *n*-octyl) affect to different extents the aerobic and ATP-driven transhydrogenases and the ATP-driven reduction of NAD by succinate catalyzed by BH-SMP. *N,N'*-dibenzyl- and *N,N'*-dibenzhydrylcarbodiimides are also effective modulators, but *N,N'*-ditritylcarbodiimide has no inhibitory action on these energy-linked reactions.

Methods

Preparation of Beef-Heart Mitochondria, Submitochondrial Particles, and EDTA-Particles

The methods used to prepare these biological materials were those used by Beechey et al. [1].

Assay of Protein

Protein was estimated colorimetrically by the Biuret method [20], after solubilization of the protein with 1.5% (w/v) potassium cholate.

Assays of Partial Reactions of Oxidative Phosphorylation

The nonenergy-linked transhydrogenase activity, the aerobic energy-linked transhydrogenase driven by succinate, the ATP-driven energy-linked transhydrogenase, and the ATP-driven reduction of NAD by succinate activities of BH-SMP and EDTA-particles were assayed using the methods described by Beechey et al. [1].

Synthesis of N,N'-*di*-n-*alkylcarbodiimides*

The appropriate *N,N'*-di-*n*-alkyl thioureas were prepared from the *n*-alkylamine and carbon disulfide. The corresponding carbodiimides were

prepared by refluxing in diethyl ether the thiourea with freshly prepared yellow mercuric oxide. The course of the reaction was followed by measuring the intensity of the IR absorption band at 4.76 μm. The ethereal layer was separated and dried over $MgSO_4$. The diethyl ether was removed and the crude products were redistilled *in vacuo*. The precise experimental details are given in [21].

Solutions of these carbodiimides (10 mM) were prepared in ethanol and stored at −20°C. These proved to be stable over a period of months.

Syntheses of N,N′*-dibenzyl- and* N,N′*-dibenzhydrylcarbodiimides*

The thioureas were prepared by the method of Fry and Farquhar [22]. *N,N′*-dibenzylcarbodiimide was prepared from the thiourea essentially as described for the preparation of the *n*-alkylcarbodiimides save that the solvent for the reaction was acetone. The reaction product was purified by distillation *in vacuo*. *N,N′*-dibenzhydrylcarbodiimide was prepared by the method of Zetzche and Fredrich [23]. It was purified by recrystallization from petroleum spirit (bp 40–60):ethanol, 3:1; mp 100–101°C.

(*Safety note:* The vapor from these liquid carbodiimides is extremely irritating to the eyes. It is essential that all these manipulations be done in a well-ventilated fume hood and that the exhaust from the vacuum pump be directed into the fume hood.)

Results and Discussion

The Effects of N,N′*-di-*n*-alkylcarbodiimides on the ATP-Driven Reduction of NAD by Succinate*

Aliquots of ethanolic solutions of *N,N′*-di-*n*-alkylcarbodiimides were added to suspensions of BH-SMP to give a final carbodiimide titer of 2 nmoles mg^{-1} protein. The SMP were incubated at 0°C and samples were removed after different incubation times. The abilities of the control and carbodiimide-treated SMP to catalyze the ATP-driven reduction of NAD by succinate were then measured. The results are presented in Fig. 1. It can be seen that all the carbodiimides tested inhibited this reaction, but the rate of onset of inhibition and, to a lesser extent, the degree of the inhibition varied with the chain lengths of the alkyl substituents.

The Effects of N,N′*-di-*n*-alkylcarbodiimides on the ATP-Driven Transhydrogenase Reaction*

An experiment similar to that described in the legend to Fig. 1 was performed save that the ability of the *N,N*-di-*n*-alkylcarbodiimides to inhibit

the ATP-driven transhydrogenase reaction catalyzed by BH-SMP was measured. The results obtained were essentially similar to those illustrated in Fig. 1. The rate of the ATP-driven transhydrogenase in the control BH-SMP to which ethanol had been added was 220 nmoles NADPH produced per minute per milligram protein, after correction for a nonenergy-linked transhydrogenase rate of 15 nmoles min^{-1} mg^{-1} protein.

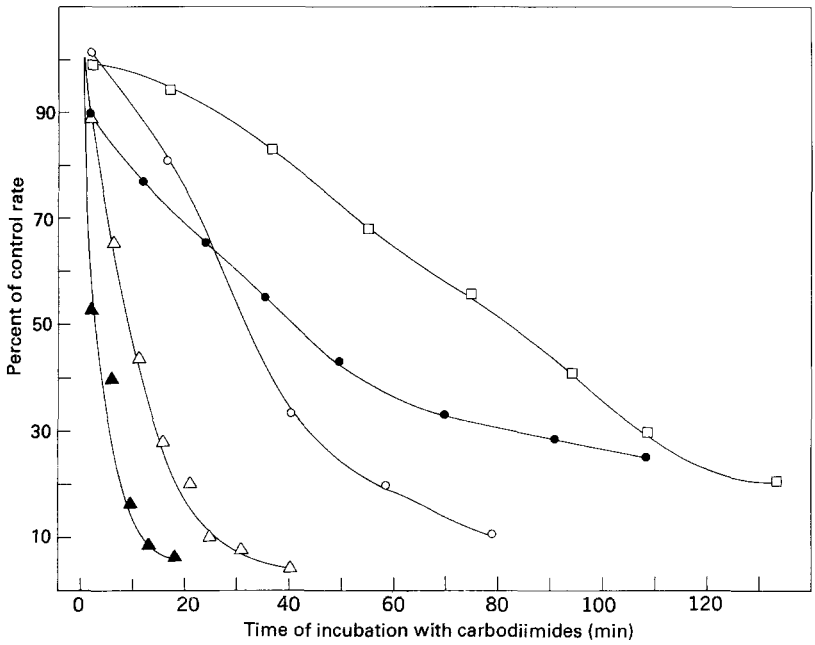

Figure 1. The inhibition of the ATP-driven reduction of NAD by succinate activity of BH-SMP by *N,N'*-di-*n*-alkylcarbodiimides. Aliquots (3 μl) of 10 mM ethanolic solutions of *N,N'*-di-*n*-alkylcarbodiimides were added to 1 ml samples of a suspension of BH-SMP (15 mg of protein) in a solution that contained 250 mM sucrose, 10 mM Tris-SO_4, 4 mM $MgSO_4$, 1 mM succinate, and 1 mM ATP, pH 7.7 at 4°C. Alcohol was added to the control. Samples were taken at the indicated times, and the ability of the BH-SMP to catalyze the ATP-driven reduction of NAD was assayed as described in the Methods section. Because of the number of assays involved, the actual duration of the experiment was 3 h. During this time the control rate was 127.7 ± 3.9 (6) nmoles min^{-1} mg^{-1} protein ± standard deviation. ○, *N,N'*-di-*n*-propylcarbodiimide; △, *N,N,'*-di-*n*-butylcarbodiimide; ▲, *N,N'*di-*n*-pentylcarbodiimide; ●, *N,N'*-di-*n*-hexylcarbodiimide; □, *N,N'*-di-*n*-octylcarbodiimide.

The speed of inhibition by these *N,N'*-di-*n*-alkylcarbodiimides is optimal with *N,N'*-di-*n*-pentylcarbodiimide. Thus the speed of inhibition cannot be entirely due to the hydrophobicity of the carbodiimide molecules since this would increase with the increasing carbon chain lengths of the alkyl substituent. Neither can variations in the reactivity of the carbodiimide moiety totally explain this pattern of speed of inhibition, since the reactivity would vary directly with the chain length of the alkyl substituents. One parameter that could be important is the actual size of the molecule (see Table I). Presumably the carbodiimide molecules penetrate into the hydrophobic region of the membrane and assume an orientation which is parallel to that of the phospholipid alkyl side chains, i.e., more or less perpendicular to the plane of the membrane. Thus the $-N=C=N-$ moiety of these molecules will assume different average depths of penetration into the membrane. In the mitochondrial membrane the carbodiimide-binding protein is associated with a complex of proteins thought to be related to the ATP-synthetase complex [5]. The proximity of these protein molecules could limit the space available for carbodiimides to approach the carbodiimide-binding protein. It is thought that the *N,N'*-di-*n*-pentylcarbodiimide molecule fits into the available space close to the carbodiimide-binding protein such that the $-N=C=N-$ moiety of the molecule and the functional group that reacts with the carbodiimide are very close. The *N,N'*-di-*n*-propyl- and *N,N'*-di-*n*-butylcarbodiimides are less rapid in their inhibitory action because they can oscillate in the plane perpendicular to the membrane plane, thus decreasing the chances of interaction. The longer alkyl-chain carbodiimides are less effective, possibly because the appropriate approach of the carbodiimide moiety to the reactive functional group on the carbodiimide-binding protein requires the twisting of the longer alkyl chains.

TABLE I. Lengths of the *N,N'*-di-*n*-alkylcarbodiimides and *N,N'*-dicyclohexylcarbodiimide

Molecule	Length (Å)[a]
N,N'-di-*n*-propylcarbodiimide	12.6
N,N'-di-*n*-butylcarbodiimide	15.2
N,N'-di-*n*-pentylcarbodiimide	17.9
N,N'-di-*n*-hexylcarbodiimide	20.6
N,N'-di-*n*-octylcarbodiimide	25.9
N,N'-dicyclohexylcarbodiimide	15.2

[a]Calculated from bond lengths given in refs. 32 and 33.

The Effects of N,N′-*di*-n-*pentylcarbodiimide on the Partial Reactions of Oxidative Phosphorylation*

BH-SMP were incubated with 2 nmoles of *N,N′*-di-*n*-pentylcarbodiimide per milligram protein at 0°C and assays were made after different incubation times for the following activities: (a) the ATP-driven reduction of NAD by succinate, (b) the ATP-driven transhydrogenase, and (c) the succinate-driven aerobic transhydrogenase. The results obtained are illustrated in Fig. 2. Both the ATP-driven functions were completely and rapidly inhibited, the transhydrogenase being inhibited at a slower rate.

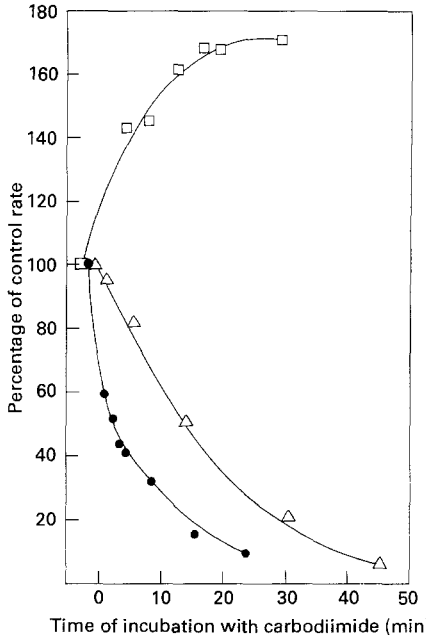

Figure 2. The effects of *N,N′*-di-*n*-pentylcarbodiimide on the partial reactions of oxidative phosphorylation. Aliquots (4 μl) of a 10 mM ethanolic solution of *N,N′*-di-*n*-pentylcarbodiimide were added to 1-ml samples (20.1 mg. of protein) of BH-SMP suspended in the medium described in the legend to Fig. 1. Alcohol was added to the controls. Samples were taken at the indicated times, and the ability of the BH-SMP to catalyze the following reactions was assayed: □, succinate-driven aerobic transhydrogenase, control rate 125.9 ± 5.8 (6) nmoles min^{-1} mg^{-1} protein; △, ATP-driven transhydrogenase, control rate 218.7 ± 3.5 (3) nmoles min^{-1} mg^{-1} protein; ●, ATP-driven reduction of NAD by succinate, control rate 126 nmoles min^{-1} mg^{-1} protein.

However, the aerobic transhydrogenase activity was greatly stimulated. Identical experiments were performed with *N,N'*-di-*n*-propyl-, *N,N'*-di-*n*-butyl-, *N,N'*-di-*n*-hexyl-, and *N,N'*-di-*n*-octylcarbodiimides, with very similar results. The rates of onset of inhibition of the ATP-driven reactions were very similar to those illustrated in Fig. 1. The ATP-driven transhydrogenase was always inhibited at a slower rate and the aerobic transhydrogenase was always stimulated. However, none of these carbodiimides gave a stimulation greater than 20% over the control rates.

The Effects of Different Titers of N,N'-*di*-n-*pentylcarbodiimide on the Partial Reactions of Oxidative Phosphorylation*

Samples of BH-SMP were incubated with titers of *N,N'*-di-*n*-pentylcarbodiimide from 0–2 nmoles mg^{-1} protein at 0°C for 60 min, and the activities of the ATP-driven reduction of NAD by succinate, the ATP-driven transhydrogenase, and the succinate-driven aerobic transhydrogenase were assayed. The results are presented in Fig. 3. It can be seen that as the titer of the *N,N'*-di-*n*-pentylcarbodiimide increased, there was a gradual increase in the inhibitions of the ATP-driven functions and a stimulation of the aerobic transhydrogenase. Maximum inhibition is achieved at 2 nmoles mg^{-1} protein. Assuming an ATPase content of 8.5% of the total membrane protein [24] and that all of the carbodiimide reacts at a single site, this result suggests that there are at least eight carbodiimide-reacting protein molecules per molecule of ATPase on the beef heart mitochondrial membrane. This contrasts with the results of Roberton et al. [25] where complete inhibition was obtained with approximately 1 nmole of DCCD per milligram BH-SMP protein, indicating four DCCD-binding sites per ATPase molecule. However, in the case of *N,N'*-di-*n*-pentylcarbodiimide there is no evidence that all of the compound reacts at a specific site in the membrane, while there is good evidence for DCCD reacting at a single site in the beef heart mitochondrial membrane [3, 4]. The highly reactive *n*-alkylcarbodiimides could be interacting with sites that do not relate to the functions assayed in these experiments. Also, they could be reacting with water to form the *N,N'*-di-*n*-alkylureas and thus are removed from the test system.

The Stimulation by Oligomycin-A and N,N'-*di*-n-*pentylcarbodiimide of the ATP-Driven Reduction of NAD by Succinate in EDTA Particles*

The incubation of EDTA particles with varying titers of *N,N'*-di-*n*-pentylcarbodiimide for 1 h and oligomycin-A for 3 min resulted in a stim-

ulation of the ATP-driven reduction of NAD by succinate at low titers and inhibition at high titers (see Fig. 4). Compared to oligomycin-A, *N,N'*-di-*n*-pentylcarbodiimide is both less potent and less effective as a stimulator of this reaction. EDTA particles have a high proton permeability which is inhibited by low titers of oligomycin-A, thus stimulating the

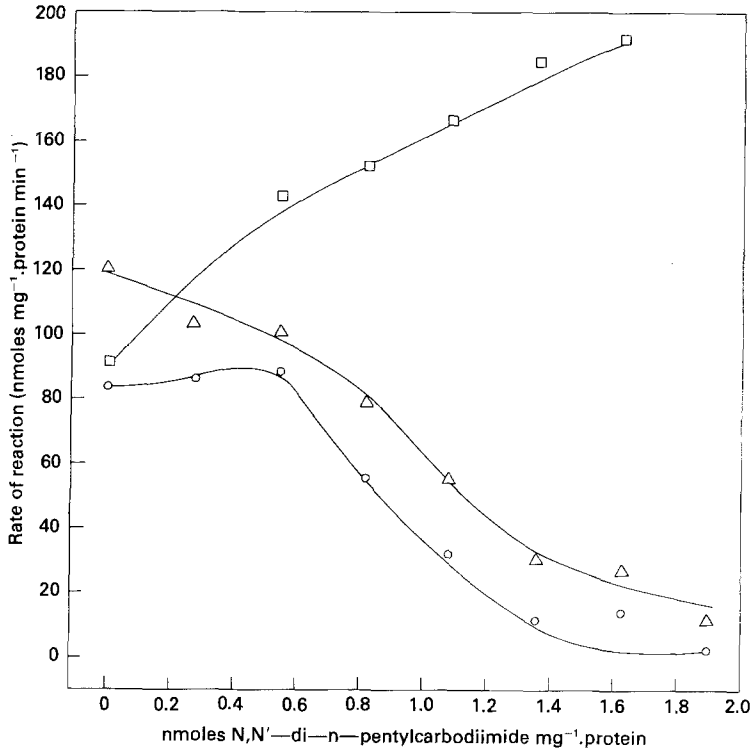

Figure 3. The effects of different titers of *N,N'*-di-*n*-pentylcarbodiimide on the partial reactions of oxidative phosphorylation. Aliquots (0.5 ml) of a suspension of BH-SMP (7.3 mg of protein) were mixed with aliquots of either 1 mM or 10 mM *N,N'*-di-*n*-pentylcarbodiimide dissolved in ethanol and ethanol to give a final volume of 10 µl ethanol. They were incubated for 1 h at 0°C, and the ability to catalyze the following reactions was assayed: □, succinate-driven aerobic transhydrogenase, control rate 92.4 ± 13.4 (4) nmoles min^{-1} mg^{-1} protein; △, ATP-driven transhydrogenase, control rate 122.5 ± 14.8 (4) nmoles min^{-1} mg^{-1} protein; ○, ATP-driven reduction of NAD by succinate, control rate 84.7 ± 6.2 (4) nmoles min^{-1} mg^{-1} protein.

ATP-driven reaction [26]. It would appear that oligomycin-A has a higher affinity for the exposed proton translocating sites than for those associated with the competent ATP-synthetase complexes. The relatively low potency and low effectiveness of *N,N'*-di-*n*-pentylcarbodiimide in stimulating this ATP-driven reaction suggests that the affinities of this compound for the exposed proton pores and those associated with the ATP-synthetase complex are not very different. This situation is similar to that found for DCCD [25].

The Effects of Other Carbodiimides on Partial Reactions of Oxidative Phosphorylation

N,N'-diisopropylcarbodiimide gave 86% inhibition of the ATP-driven transhydrogenase activity at titers of 1 nmole mg^{-1} BH-SMP protein when incubated at 30°C for 30 min.

The effects of *N,N'*-dibenzyl-, *N,N'*-dibenzhydryl-, and *N,N'*-ditrityl-carbodiimides on the ATP-driven reduction of NAD by succinate and the ATP-driven transhydrogenase activities of BH-SMP were tested. The pro-

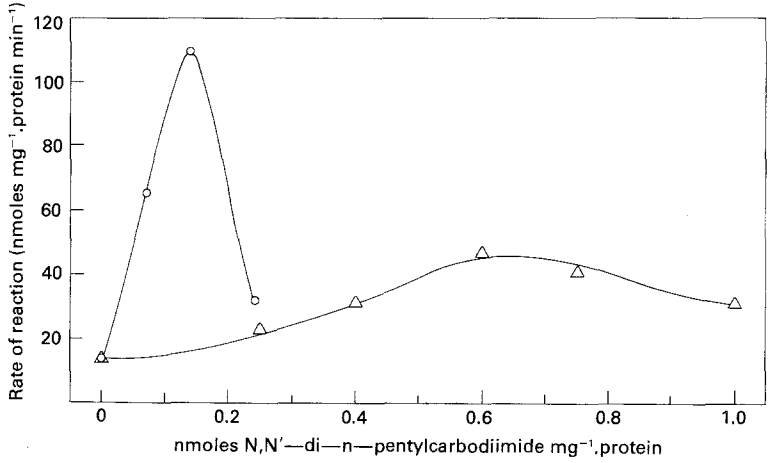

Figure 4. The stimulation by oligomycin-A and *N,N'*-di-*n*-pentylcarbodiimide of the ATP-driven reduction of NAD by succinate catalyzed by EDTA particles. EDTA particles were incubated with the indicated titers of either *N,N'*-di-*n*-pentylcarbodiimide for 1 h at 0°C or oligomycin-A for 3 min at 30°C in the assay medium. The ability of the EDTA particles to catalyze the ATP-driven reduction of NAD by succinate was then measured. ○, oligomycin-A; △, *N,N'*-di-*n*-pentylcarbodiimide.

cedure used was that described in the legend to Fig. 1 save that the incubation temperature was 30°C. Under these conditions *N,N'*-dibenzyl- and *N,N'*-dibenzhydrylcarbodiimides rapidly inhibited both these functions, 90% inhibition of the ATP-driven transhydrogenase being achieved by 2 nmoles mg^{-1} protein after 18 min incubation with *N,N'*-dibenzylcarbodiimide and after 80 min incubation with *N,N'*-dibenzhydrylcarbodiimide. The ATP-driven reduction of NAD by succinate was inhibited more rapidly. No inhibition of either reaction was noted with *N,N'*-ditritylcarbodiimide after 24 h incubation at 0°C.

Examination of a space-filling molecular model of *N,N'*-ditritylcarbodiimide suggests that approach to the $-N=C=N-$ moiety of the molecule is difficult because of the bulk of the trityl groups. However, care must be taken in ascribing the lack of inhibition by this carbodiimide to steric hindrance, since the reactivity of substituted alkylcarbodiimides decreases with the degree of branching in the alkyl chain [27].

The inhibitory effects of two charged water-soluble carbodiimides, *N,N'*-di-*p*-(*N*-dimethylamino)phenylcarbodiimide and *N*-cyclohexyl-*N'*-2-morpholinyl-(4)ethylcarbodiimide metho-*p*-toluenesulfonate were insignificant, i.e., less than 10% inhibition of the ATP-driven transhydrogenase and the ATP-driven reduction of NAD by succinate, after 24 h incubation at 0°C with 10 nmoles of carbodiimide per milligram BH-SMP protein. This lack of activity contrasts with the high biological activity shown by all the uncharged, hydrophobic carbodiimides, with the exception of *N,N'*-ditritylcarbodiimide. Presumably the charged carbodiimides cannot penetrate the mitochondrial membrane and react with the carbodiimide-sensitive site located in the hydrophobic region of the membrane.

Abrams and Baron [28, 29] also found that the membrane-bound ATPase isolated from *Streptococcus faecalis* was inhibited by hydrophobic carbodiimides and was not affected by water-soluble carbodiimides. However, Patel and Kabak [30] observed that the ATPase activity associated with vesicles isolated from *E. coli* AN180 was sensitive both to the hydrophobic *N,N'*-dicyclohexyl- and *N,N'*-diisopropylcarbodiimides and the hydrophilic *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide and *N*-cyclohexyl-*N'*-2-morpholinyl-(4)ethylcarbodiimide metho-*p*-toluenesulfonate. However, the titers of these different carbodiimides required to give approximately 80% (maximal) inhibition of the ATPase were 20, 500, 5000, and 5000 nmoles mg^{-1} protein respectively. Neither we nor Abrams and his colleagues have used such high concentrations of carbodiimides.

The results presented here show that all of the alkylcarbodiimides and *N,N'*-dibenzyl- and *N,N'*-dibenzhydrylcarbodiimides are potent inhibitors

of the ATP-driven transhydrogenase activity of BH-SMP. It is unlikely that they are inhibiting directly the transhydrogenase molecule since these compounds do not inhibit either the nonenergy-linked or the succinate-driven transhydrogenase activities associated with the BH-SMP. Thus they have a site of action that lies in the ATP-synthetase region. This conclusion was confirmed by the observations that (a) treatment of BH-SMP with 2 nmoles of either *N,N'*-di-*n*-butyl- or *N,N'*-di-*n*-octyl- or *N,N'*-dibenzylcarbodiimides per milligram of protein led to the complete inhibition of the ATPase activity and (b) the addition of any one of these carbodiimides to BH-mitochondria (100 nmoles mg^{-1} protein) gave immediate inhibition of ADP-stimulated respiration, as does DCCD [31].

All of the inhibitory carbodiimides described in this paper are effective at titers comparable with those used for DCCD [1]. Also they exert their inhibitory effects more rapidly. Thus Beechey et al. [1] reported that the ATP-driven reduction of NAD by succinate was inhibited after 2 h incubation of BH-SMP with 2 nmoles of DCCD per milligram protein compared with 20 min for *N,N'*-di-*n*-pentylcarbodiimide (Fig. 1). However, DCCD is probably the inhibitor of choice for beef heart mitochondrial preparation since the longer times required for the onset of inhibition facilitates better control of the reaction. Also DCCD is commercially available and may be purchased in a radiolabeled form, and finally DCCD is much easier to handle than the liquid carbodiimides, the vapors of which have potentially dangerous toxic effects.

Because of the low titers of carbodiimides required to inhibit the ATP-dependent reactions, we conclude that it is probable that these inhibitory carbodiimides are acting at the DCCD-binding protein [4]. The requirement for hydrophobicity in the carbodiimide molecules for inhibition supports this conclusion since the DCCD-binding protein is also very hydrophobic.

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