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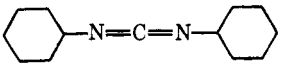
Effect of *N,N'*-Dicyclohexylcarbodiimide and Other Carbodiimides on Electron Transfer Catalyzed by Submitochondrial Particles†

Robert E. Beyer,* Thomas W. Brink,‡ Daune L. Crankshaw,§ Jerry M. Kuner,¶ and Alice Pasternak

ABSTRACT: Compound I inhibited NADH oxidation in beef heart sonic submitochondrial particles (ETPH) but was not inhibitory with succinate as substrate or with electron donors which transfer electrons on the oxygen side of coenzyme Q. The oxidative phosphorylation uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) released the I-induced inhibition of NADH oxidation and thereby mimicked respiratory control observed in intact mitochondria. Inhibitor studies with a series of hydrophobic and water-soluble carbodiimides revealed that only nonpolar carbodiimides had significant inhibitory activity at reasonably low concentrations, suggesting that the inhibitory site within the mitochondrial membrane is also hydrophobic. *N,N'*-Dicyclohexylurea (hydrophobic) and 1-cyclohexyl-3-(2-morpholinoethyl)urea metho-*p*-toluenesulfonate (water soluble) were without inhibitory effect. Treatment of ETPH with I in the presence of glycine methyl ester increased the inhibitory action of I and prevented release by FCCP, suggesting the involvement of available carboxyl groups at the I-sensitive site. Gel filtration studies suggested that I and GlyOMe, reacted in the presence of I, are firmly bound to ETPH. Electron spin resonance (epr) spectrometry of I-inhibited ETPH revealed that nonheme iron of NADH dehydrogenase was reducible by NADH while other nonheme iron compounds of the electron-transfer chain giving signals at $g = 1.94$, and copper at $g = 2.00$, remained oxidized under such conditions. In addition, coenzyme Q was not reduced by NADH in I-inhibited preparations under conditions where succinate did reduce the quinone in the inhibited particle and both NADH and succinate reduced coenzyme Q in uninhibited particles. The epr and coenzyme Q data suggest that the site sensitive to hydrophobic carbodiimides lies on the oxygen side of NADH dehydrogenase nonheme iron and on the substrate (NADH) side of coenzyme Q. Experiments with submitochondrial particles from Mung bean etiolated seedlings, which are resistant to inhibition by rotenone, barbiturates, and piericidin A, as well as to I, suggested that such inhibitors all react with a common, sensitive component. A mechanism involving conformational alteration of the inhibitory site is suggested.

In addition to being a valuable reagent in the study of phosphate esters of biological importance (Khorana, 1961), compound I has received attention recently as a tool in studies of oxidative phosphorylation and respiratory metabolism. Beechey *et al.* (1966, 1967) have reported that I acts at low concentrations as an inhibitor of oxidative phosphorylation



I

intact mitochondria and submitochondrialp articles. Racker in and Horstman (1967) have shown that I mimics the effects of rutamycin in that it stimulates the synthesis of ATP[1] by

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[1] Abbreviations used are: ATP and ADP, adenosine tri- and diphosphates; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HBHM, heavy-layer beef heart mitochondria; ETPH, electron-transfer particles prepared from HBHM by sonic vibrations (20 kc) in the presence of salts which are indicated in parentheses; epr, electron spin resonance; MB-ETP, electron-transfer particles prepared from Mung bean seedling mitochondria; UQ, coenzyme Q or ubiquinone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; PMS, phenazine methosulfate; $FeNH_{N,S,\text{ or }R}$, nonheme iron proteins of the NADH dehydrogenase, succinic dehydrogenase, and cytochrome b–c_1 complex (Reiske protein), respectively.

submitochondrial particles at low concentrations and inhibits at high concentrations. McCarty and Racker (1967) have observed similar effects of I on photophosphorylation catalyzed by chloroplasts. Compound I also appears to mimic the effects of oligomycin as an inhibitor of coupled respiratory metabolism in intact yeast cells (Kováč *et al.*, 1968). I has been employed to study the ability of inorganic ions to interact with inhibited respiratory states in submitochondrial particles (Beyer *et al.*, 1969).

In addition to the relatively intact biochemical systems noted above, I has also been used to study reactions catalyzed by partially resolved submitochondrial systems. Penefsky (1967) has noted inhibition of ATPase activity after reacting purified coupling factor 1 with I. Holloway *et al.* (1966) have reported that I binds to the CF_0 complex described by Kagawa and Racker (1966), while more recent studies (Knight *et al.*, 1968) indicate that I forms covalent bonds with mitochondrial proteolipids.

The experiments to be described herein were designed to provide further information on the location and characteristics of the I-inhibited site. Some of these data have been presented in preliminary form (Beyer *et al.*, 1967a,b, 1968a,b).

Methods

Preparation of Mitochondria and Submitochondrial Particles. HBHM were prepared from fresh bovine cardiac ventricles as described by Löw and Vallin (1963) with modifications as described by Fritz and Beyer (1969). The Willem's Polytron, Model BEW, used to homogenize cardiac ventricles revolved at a speed of 14,500 rpm while unloaded. ETPH (Mg, Mn) and ETPH (EDTA-1) were prepared as described by Beyer (1967). Submitochondrial particles from Mung bean (*Phaseolus aureus*) etiolated seedlings were prepared as described previously (Beyer *et al.*, 1968c).

Analytical Methods. Protein was estimated by a biuret procedure (Gornall *et al.*, 1949) in which protein was solubilized by the use of approximately 1 mg of deoxycholate/mg of mitochondrial protein. Oxygen consumption was determined with a Clark electrode (Yellow Springs Instrument Co.) in 1.5- to 1.7-ml water-jacketed glass cells (Norman Erway Glass Blowing, Oregon, Wis.) or a 3-ml water-jacketed Plexiglass cell (Estabrook, 1967) maintained at 25°. Signals from the Clark electrode were monitored as described by Estabrook (1967) using a Heath EUW-20A servorecorder. Dissolved oxygen was assumed to be 240 μM at 25°. All assays of oxygen consumption were determined in a medium (medium A) consisting of 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, and 5 mM Tris-phosphate (pH 7.5), in addition to those components indicated in the tables and figures. Carbodiimides were added to assays in small volumes; those not soluble in water were added as ethanol solutions, the ethanol concentrations of which had negligible effects on rates of substrate oxidations. X-band (9 GHz) epr spectra were obtained with a Varian V-4500 spectrometer equipped with a microwave bridge and a circulator (Palmer, 1967). A low-temperature accessory resembling that described by Hansen *et al.* (1967) was employed. Samples of normal or carbodiimide-inhibited ETPH (approximately 100 mg of protein/ml) were prepared for observation of epr spectra exactly as described previously (Beyer and MacDonald, 1970). Epr instrumental settings were: Microwaver power, 25 W; modulation amplitude, 12 G; temperature, −190°; V = 9.23. Steady-state oxidation–reduction levels of UQ were assayed under the following conditions. Portions (0.6 ml) of a suspension of freshly prepared ETPH (Mg, Mn) in 0.25 M sucrose–0.01 M Tris-HCl (pH 7.5) were pipetted into screw-topped test tubes containing 0.4 ml of a solution of medium A and a small Teflon-covered magnetic bar added for aeration. Three particle types were prepared: control; inhibited with excess I (preincubated); and particles incubated for 8 min in the presence of 10 mM I (inhibited). All suspensions were incubated at 30° for 2 min to establish temperature equilibrium. The reaction was initiated by the rapid addition of excess NADH or succinate. The reaction was terminated after 10 sec by the rapid addition of 4 ml of methanol containing 1 mg/ml of pyrogallol at −20°. Total and steady-state oxidation–reduction levels of UQ were determined by the method of Redfearn (1967) using a Beckman DU-2 spectrophotometer or a Unicam SP-800 recording spectrophotometer for photometric analysis.

Materials

All reagents were of analytical reagent grade or equivalent whenever possible and were used without further purification. Filtered, deionized, glass-distilled water, or redistilled ethanol, was used for all solutions. FCCP was a generous gift of Dr. Peter Heytler. Compound I was purchased from Aldrich Chemical Co. and Schwarz BioResearch Inc. No difference in the effects of the two I samples was observed. All other carbodiimides and urea derivatives were purchased from Aldrich Chemical Co. NADH was a product of P-L Biochemicals. Using the rate of NADH oxidation catalyzed by ETPH with increasing NADH concentrations, samples from this source appeared to contain considerably less of the inhibitor of NADH oxidase reported by Fawcett *et al.* (1961) than those of several other sources of NADH.

Results

Inhibition of NADH Oxidation by I. Increasing concentrations of I progressively inhibit the oxidation of NADH catalyzed by ETPH (Mg,Mn) (Figure 1). Half-maximal inhibition (K_i) was observed at approximately 43 μM I. This inhibition constant may also be reported as half-maximal inhibition at approximately 172 nmoles of I/mg of enzyme protein. Further additions of NADH to inhibited enzyme did not result in increases in rates of oxidation, indicating that inhibition was not of the competitive type. However, this point is not absolutely clear since the study of such inhibition kinetics is rendered difficult of interpretation due to an inhibitor of NADH oxidation present in commercial NADH preparations (Fawcett *et al.*, 1961). The concentration of NADH which we have employed, 8 mM, gave maximal rates of oxidation. Higher concentrations of NADH as substrate resulted in progressive inhibition. Since the curve in Figure 1 is not hyperbolic, the K_i values mentioned above should be regarded as approximations.

Release of Carbodiimide Inhibition by FCCP. As reported above, and previously (Beyer *et al.*, 1967a), uncouplers of oxidative phosphorylation appear to release the inhibition of NADH oxidation imposed by I and, in that regard, simulate respiratory control in intact mitochondrial systems imposed by adenine nucleotides. The question arose as to whether FCCP was merely stimulating those electron-transfer assemblies not combined with carbodiimides, similar to the stimulation of ATPase catalyzed by submitochondrial particles in the presence of I (Beyer *et al.*, 1967a). In order to answer the question a series of experiments were performed in which ETPH (Mg, Mn) and ETPH (EDTA-1) were assayed for the

TABLE I: Effect of I on Segments of the Electron-Transfer Chain.[a]

Substrate	Electron Acceptor	I (μM)	Mean Rate[b]
Succinate	Oxygen	0	86
		2–133	119
Ascorbate, PMS, antimycin A	Oxygen	0	768
		2–133	720
Succinate, TMPD, antimycin A	Oxygen	0	216
		2–100	223

[a] Assay conditions were as detailed in the Methods section using the oxygen electrode at 25°. Antimycin A was present at 4 μg/mg of protein during the ascorbate–PMS assay of the terminal portion of the electron-transfer chain. Ascorbate = 4.16 mM; PMS = 0.83 μM; TMPD = 0.3 mM. All assays contained 0.75 mg of ETPH (Mg, Mn) protein. [b] natoms of acceptor reduced/min per mg of protein.

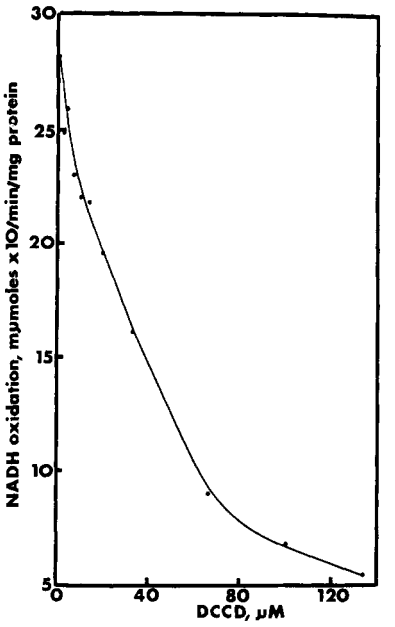


FIGURE 1: Inhibition of NADH oxidation by increasing concentrations of I. Each assay cuvet contained 2.9 ml of medium A and 0.75 mg of ETPH (Mg, Mn) protein, and the reaction was initiated by the addition to the oxygen electrode cuvet of 2.5 μmoles of NADH. Oxidation was allowed to proceed until enough trace enabling a rate to be measured was obtained, when I was added. The points in the figure reflect the final rate after I addition. Note that ADP was not present.

oxidation of NADH before (A) and after (B) the addition of uncoupling concentrations of FCCP, and the ratio B:A noted. The particles were then assayed with respect to NADH oxidation in the presence of various concentrations of I (C) and after the addition of FCCP (D) and the ratio D:C noted. If FCCP was capable merely of stimulating NADH oxidation in electron-transfer assemblies not bound by I then the ratio D:C would not be expected to exceed the ratio B:A at any concentration of I. If, on the other hand, the ratio D:C was observed to exceed the ratio B:A, this would lend support to the contention that inhibition by I may be released by the uncoupler.

At 89 and 167 nmoles of I per mg of protein addition of the uncoupler, FCCP, resulted in D:C ratios which exceeded B:A ratios. These data suggest that at least a portion of the electron-transfer assemblies inhibited by I were stimulated by FCCP with regard to the oxidation of NADH.

I and Portions of the Electron-Transfer Chain. Segments of the electron-transfer chain may be functionally isolated with the use of inhibitors and various artificial electron donors or acceptors (Lee *et al.*, 1965). In an attempt to localize the inhibitory site of I, its inhibitory capacity has been noted using several functionally isolated portions of the electron-transfer chain. Compound I, even at extremely high concentrations, did not inhibit the oxidation of succinate (Table I). Since electrons from succinic dehydrogenase flavin enter the electron-transfer chain at the level of coenzyme Q, *i.e.*, on the oxygen side of the NADH dehydrogenase complex, the lack of inhibition of succinate oxidation by I indicates that the site sensitive to I resides within the NADH dehydrogenase complex. This conclusion was supported by the lack of inhibition by I of assays which measure electron transfer over the cytochrome *c* to oxygen segment of the electron-transfer chain (ascorbate–PMS–antimycin A) and the cytochrome *b* to cytochrome *c* portion of the chain (succinate–TMPD–antimycin A (Table I). Since the rates of oxidation were similar for any given substrate in the presence of 0–133 mM I, mean values for rates are given in Table I.

EPR Analysis of I Inhibition. Two pieces of information, namely the lack of inhibition of the submitochondrial succinic oxidase system by I and the appearance of a crossover point between NADH dehydrogenase flavin and cytochrome *b* in I-inhibited particles as observed in the Aminco-Chance dual-wavelength spectrophotometer (Beyer *et al.*, 1967a), suggested that the site within the electron-transfer chain susceptible to I resided on the substrate side of cytochrome *b*, *i.e.*, within the NADH dehydrogenase complex. In order to further delineate the I-sensitive site, epr spectra were obtained using I-inhibited and control submitochondrial particles (Figure 2). Instead of the highly phosphorylating submitochondrial particle ETPH (Mg, Mn) (Beyer, 1967), particles prepared in the absence of metals, ETPH (EDTA-1), were employed to avoid the strong Mn^{2+} epr signal which obscures paramagnetic nonheme iron and copper signals. Both control and I-inhibited ETPH (EDTA-1), in the oxidized state, exhibited normal epr spectra in that they both contained a copper signal at g = 2.00 and no evidence of reduced nonheme iron signals at g = 1.94 (Figure 2). Control particles incubated with NADH for 30 min. at 25° displayed loss of the cytochrome oxidase copper signal at g = 2.00 and appearance of g = 1.94 signals characteristic of $FeNH_N$, $FeNH_S$, and $FeNH_R$ (Palmer, 1967) (Figure 2A). Conversely, the epr spectrum of I-inhibited particles incubated with NADH did not show a loss of the copper signal at g = 2.00 and of the three nonheme iron signals seen in the control particle, only that characteristic of $FeNH_N$ was observed (Figure 2A). The data in Figure 2A indicate that I blocked the flow of electrons from NADH to $FeNH_S$, $FeNH_R$, and copper of cytochrome oxidase but did not interfere with the reduction of $FeNH_N$ by NADH. These findings suggest that the I-sensitive site in the electron-transfer chain resides on the oxygen side of $FeNH_N$ and on the substrate (NADH) side of $FeNH_R$.

The addition of succinate to the epr tubes after obtaining the spectra shown in Figure 2A resulted in a sizeable reduction of the strength of the copper signal in the control tube

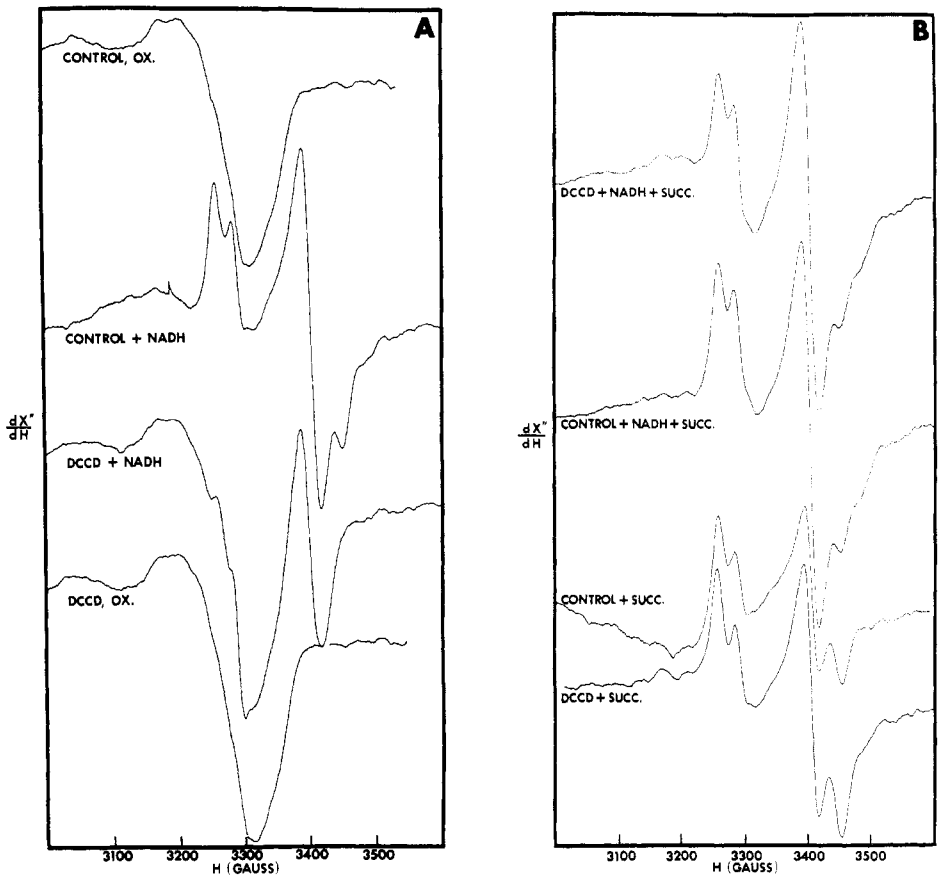


FIGURE 2: The effect of I on the epr spectra of ETPH (EDTA-1). Inhibited submitochondrial particles were prepared by the slow addition of 60 μl of 1 M I in ethanol to centrifuge tubes containing 150 mg of particle protein and incubating at 30°. Control particles were treated similarly except that 60 μl of ethanol was added slowly with stirring. Portions of the incubating enzyme were removed periodically during incubation and assayed for NADH oxidase activity. After 45-min incubation the rate of NADH oxidase activity was 6.9 natoms of oxygen/min per mg of protein in the I-treated preparation while the rate catalyzed by control particles was 907 natoms of oxygen/min per mg of protein. The submitochondrial particles were diluted with 0.25 M sucrose–0.01 M Tris-HCl (pH 7.5) and centrifuged at 155,000*g* for 30 min. The pellets were drained of excess fluid, rinsed with 0.25 M sucrose–0.01 M Tris-HCl (pH 7.5), and used for epr spectroscopy as reported previously (Beyer and MacDonald, 1970).

while a significant portion of the copper signal was maintained in the I-treated (no NADH) tube (Figure 2B). These data indicated that at the very high concentrations of I used to inhibit the ETPH (EDTA-1) for purposes of epr spectroscopy some small interference with electron flow from succinate to cytochrome oxidase copper occurred. These two preparations also exhibited normal, pure $FeNH_S$ signals. The addition of succinate to the preparations previously incubated with NADH (upper two traces, Figure 2B) did not result in a significant alteration of the spectrum for the control enzyme preparation. On the other hand, the I-treated enzyme, after incubation with succinate, exhibited normal $g = 1.94$ signals for $FeNH_S$ and $FeNH_R$, as well as a reduction in the intensity of the $g = 2.00$ cytochrome oxidase copper signal, indicating essentially normal functional capacity for such components in the succinic oxidase pathway of electron transfer. An additional 12% reduction of components occurred upon the addition of dithionite, indicating good continuity between substrate dehydrogenases and the rest of the electron-transfer chain.

Steady-State Oxidation–Reduction Levels of Coenzyme Q. Because UQ is generally considered to occupy a position in the electron-transfer chain linking the succinic and NADH dehydrogenases to the cytochrome portion of the chain, the steady-state oxidation–reduction levels of UQ were measured during the oxidation of either succinate or NADH by control and two types of ETPH (Mg, Mn) treated with I. Since seventy distinct preparations were assayed for their UQ contents, it is noteworthy to report the values obtained. The mean UQ content was 5.24 nmoles/mg of protein with a standard deviation of the mean of 1.22. The values ranged from 3.2 to 8.2 and fitted a normal distribution around the mean. These values are in agreement with those for similar submitochondrial particles from beef heart mitochondria reported by Green and Wharton (1963) and for submitochondrial particles prepared from *Phaseolus aureus* etiolated seedlings reported by Beyer *et al.* (1968c).

Submitochondrial particles, inhibited by I, and with NADH as electron donor, contained primarily oxidized UQ, in contrast to control particles in which approximately two-thirds of the UQ was found to be in the reduced state (Table II). It should also be noted that the two preparations treated with I had a limited capacity to oxidize NADH (Table II). ETPH (Mg, Mn) catalyzed succinate oxidation at nearly normal rates and, as a consequence, were capable of maintaining approximately half of the UQ in the reduced state (Table II). These data indicate that the site which is susceptible to I resides prior to UQ within the NADH dehydrogenase complex, in agreement with data derived from oxygen electrode studies reported previously (Beyer *et al.*, 1967a).

Effect of I on Mung Bean Submitochondrial Particles. In conjunction with studies on the properties of submitochondrial particles from etiolated seedlings of *Phaseolus aureus* (Ikuma and Beyer, 1966; Beyer *et al.*, 1968c) it was observed that, in contrast to submitochondrial particles isolated from beef heart, MB-ETP were not inhibited by rotenone and other NADH dehydrogenase inhibitors. These data supplement the observations of Ikuma and Bonner (1967) that extremely high concentrations of rotenone are required to inhibit malate oxidation in intact Mung bean mitochondria and that, even at very high rotenone concentrations, 50% of the malate oxidation is completely insensitive to rotenone in the presence of ADP (*i.e.*, state 3) while malate-supported respiration in the absence of ADP (state 4) is completely insensitive to rotenone. The observations of Ikuma and Bonner (1967) and our own data clearly indicate that a portion of the electron-transfer chain in the NADH dehydrogenase complex in intact Mung bean mitochondria, and all of such complexes in MB-ETP, either are devoid of the rotenone-sensitive component or contain it in such a modified form that it is not capable of interaction with rotenone. It was of interest, therefore, to compare the effect of I on both types of submitochondrial particle preparations.

The addition of I at 0.13 mM to ETPH (EDTA-1) resulted in a reduction in the rate of NADH oxidation of 64% while the same treatment reduced NADH oxidation in the Mung bean preparation 43%. The addition of the uncoupler FCCP (Heytler, 1963; Heytler and Prichard, 1962) caused a further depression of NADH oxidation in MB-ETP while the beef heart system was stimulated by 35%. In this respect I appeared to be capable of inducing respiratory control in the mammalian system (Beyer *et al.*, 1967a, 1969), but not in the plant submitochondrial system. That the Mung bean system reacts differently than the mammalian system to I is evident from data on the effect of I on succinate oxidation by submitochondrial particles. With increasing concentrations of I succinate oxidation was first stimulated and then depressed in ETPH (EDTA-1) while only inhibition was seen with the Mung bean preparation.

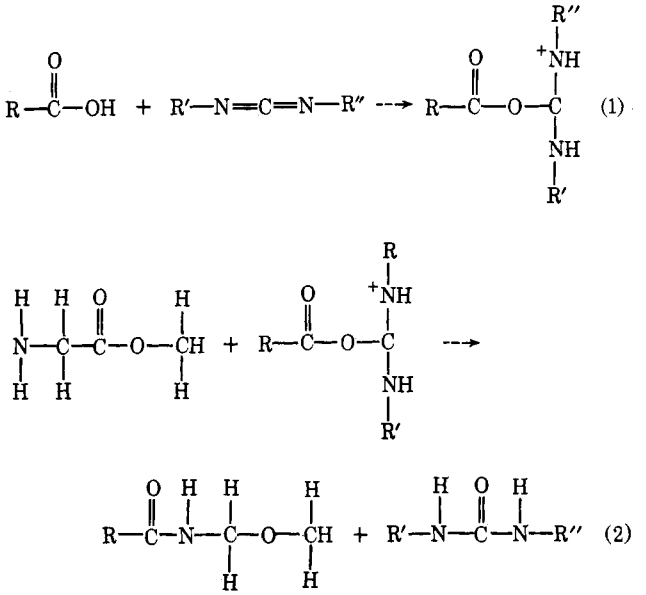
A comparison of the effects of I on the rate of substrate oxidations in the two types of submitochondrial particles is seen in Figure 3 where the rate of oxidation is plotted against the amount of I per milligram of submitochondrial protein. Compound I inhibited NADH oxidation by both types of preparations at all concentrations employed, although the depression of NADH oxidation was considerably more severe in the beef heart preparations (Figure 3). With the beef heart preparation I caused an initial increase in succinate oxidation followed by inhibition to oxidation values slightly below control (no I) in the presence of extremely high concentrations of the carbodiimide. No stimulation was observed with the Mung bean preparation (Figure 3).

I Inhibition and Carboxyl Groups. The observation that uncouplers of oxidative phosphorylation, possessing nucleophylic properties, partially released the inhibition imposed on the NADH dehydrogenase portion of the electron transfer chain suggested the possibility that I was binding to the enzyme *via* carboxyl groups. Such binding could lead to inhibition of electron transfer as a result of a conformational alteration of an electron-transfer component. Hoare and Koshland (1966, 1967) have published a method of determining the involvement of carboxyl groups in water-soluble proteins by condensation with GlyOMe in the presence of water-soluble carbodiimide according to reactions 1 and 2 in which it would be expected that the stable "amidification" of the carboxyl group by GlyOMe would result in strong inhibition not reversible by an oxidative phosphorylation uncoupler nucleophile such as FCCP.

TABLE II: I Inhibition and Oxidoreduction Levels of UQ.[a]

Particle Type	Substrate	UQ Oxidized (%)	O_2 Consumption, % Inhibited
Control	NADH	36.8	
Inhibited	NADH	95.0	84.8
Preinhibited	NADH	100	94.3
Control	Succinate	49.7	
Inhibited	Succinate	44.7	16.5
Preinhibited	Succinate	57.1	8.1

[a] Experimental procedures as described in the Methods section. Prior to incubation an aliquot of each ETPH (Mg, Mn) preparation was assayed with the Clark oxygen electrode for rate of substrate oxidation.



Initial experiments involved the effect of GlyOMe on the rate of NADH oxidation catalyzed by ETPH (Mg, Mn) since the carboxyl group technique of Hoare and Koshland (1966, 1967) requires the presence of high concentrations of nucleophile. GlyOMe, at concentrations up to 180 mM, increased the rate of NADH oxidation by approximately 400% while higher concentrations resulted in inhibition. Since the concentration of GlyOMe recommended by Hoare and Koshland (1966, 1967) is as high as 1 M, and since the inclusion of 0.1 M GlyOMe stimulated NADH oxidation by about 300%, we employed GlyOMe at 0.1 M in subsequent experiments. Since we felt it desirable to conform as closely as possible to the conditions of Hoare and Koshland (1966, 1967) for the determination of "active" carboxyl groups possibly involved in the inhibition of electron transfer imposed by I, the water-soluble carbodiimide, 1-cyclohexyl-3(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate, was used in preliminary experiments. However, inhibition of NADH oxidation was obtained only at extremely high concentrations of this carbodi-

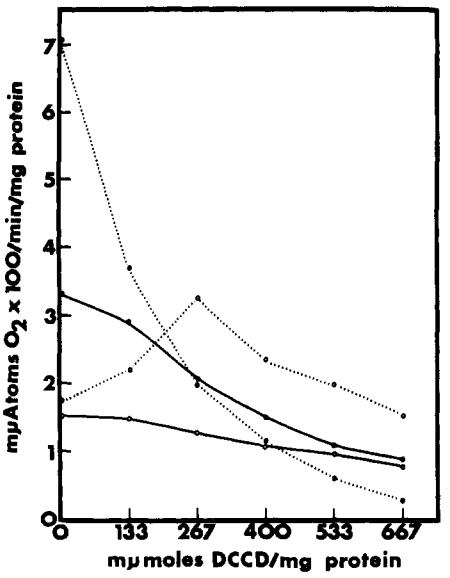


FIGURE 3: The effect of I on NADH and succinate oxidations catalyzed by Mung bean and beef heart submitochondrial particles. Assay conditions were as described in Table IV. (●- - - - -●) ETPH (EDTA-1), NADH as substrate; (○- - - -○) ETPH (EDTA-1), succinate as substrate; (●——●) MB-ETP, NADH as substrate; (○——○) MB-ETP, succinate as substrate.

imide and, in addition, the slight inhibition observed was not released by uncouplers. Consequently, the most effective carbodiimide, I, was used.

In the presence of GlyOMe, ETPH (Mg, Mn) catalyzed the oxidation of NADH at twice the rate of controls containing low salt or 0.1 M KCl (Table III). The addition of I inhibited the KCl and low-salt controls 61 and 53%, respectively, while the enzyme in the presence of GlyOMe was inhibited 87% by I. The uncoupler of oxidative phosphorylation, FCCP, which released the inhibited electron-transfer activity in the KCl and low-salt controls (+71 and +68%, respectively) was relatively ineffective in stimulating the enzyme in the presence of GlyOMe (Table III).

In order to ensure that the submitochondrial particle system was capable of significant stimulation of NADH oxidation by uncoupler in the presence of GlyOMe, the second experiment in Table III was performed. In the presence of both GlyOMe or KCl, FCCP was capable of increasing electron-transfer activity by 53 and 54%, respectively, although the low-salt control was stimulated by 85%.

In order to determine whether the interaction between I, GlyOMe, and the inhibited site in the NADH dehydrogenase complex of the electron-transfer chain was a stable, covalent complex as predicted from reactions 1 and 2 above, a series of experiments, a typical result of which appears in Table IV, were performed. The submitochondrial preparation, ETPH (Mg, Mn), was treated with I in the absence and presence of GlyOMe and passed through a column of Sephadex G-25 coarse. The resulting particles were then assayed for their ability to oxidize NADH, the effect of FCCP on NADH oxidation was noted, and the rates thus obtained were compared to rates of oxidation prior to treatment with Sephadex (Table IV). In each of the three types of treatments, the rate at which NADH was oxidized by ETPH (Mg, Mn) was slightly reduced following passage through Sephadex and subsequent washing. The addition of FCCP to control particles doubled the rate of NADH oxidation both before and after Sephadex treatment, while FCCP increased the inhibited rate of NADH oxidation by I-treated particles 2.8 times before Sephadex treatment and 2.4 times after Sephadex treatment (Table IV). In contrast, those particles treated with I in the presence of 0.1 M GlyOMe oxidized NADH at a rate considerably lower than control preparations and those treated with I alone. Such was

TABLE III: Effect of GlyOMe and I on Oxidations Catalyzed by ETPH (Mg, Mn).[a]

	Rate (natoms of O_2/min per mg of Protein)					
Additions	Control	%	+GlyOMe	%	+KCl	%
NADH	403		911		406	
NADH, I	191	−53	121	−87	159	−61
NADH, I FCCP	320	+68	145	+20	272	+71
NADH	398		1183		503	
NADH, FCCP	734	+85	1736	+53	774	+54

[a] Rates of oxygen consumption were assayed with the Clark oxygen electrode. The electron chamber contained 1.6 ml of medium A, 0.6 mM I, 0.9 μM FCCP, 0.3 mg of ETPH (Mg, Mn) protein, 0.1 M GlyOMe or 0.1 M KCl, and 2.5 μmoles of NADH. Reaction was initiated by the addition of substrate. Control assays were performed in the basic medium A described in the Methods section with KCl omitted. GlyOMe and KCl were added prior to enzyme which was allowed to incubate for 60 sec before the addition of substrate.

TABLE IV: Binding of I and GlyOMe to ETPH (Mg, Mn).[a]

	NADH Oxidation (natoms of O_2/min per mg of Protein)					
	Control		I		I + GlyOMe	
Additions	Pre-Seph	Post-Seph	Pre-Seph	Post-Seph	Pre-Seph	Post-Seph
NADH	651	508	328	296	216	209
NADH, FCCP	1414	1068	929	709	259	244

[a] 1.5 ml of freshly prepared ETPH (Mg, Mn) at 30 mg of protein/ml was incubated at 25° and 5-μl aliquots of 30 mM I added until NADH oxidation was reduced to 50% of the control activity. Another 1.5-ml sample was similarly treated but in the presence of 0.1 M GlyOMe. A control sample was treated identically except that 5-μl portions of ethanol were added instead of I and no GlyOMe was present. One milliliter of each of the three samples was passed through 1 × 30 cm columns of Sephadex G-25 coarse equilibrated with 0.25 M sucrose–0.01 M Tris-HCl (pH 7.5), maintained at 0–2°. The submitochondrial particle preparations eluted from the column were centrifuged at 144,000*g* for 40 min, suspended in 0.25 M sucrose–0.01 M Tris-HCl (pH 7.5), centrifuged again, and suspended in sucrose–Tris to a protein concentration of 30 mg/ml. Each preparation was then assayed for its ability to catalyze the oxidation of NADH and the effect of the uncoupler FCCP. Conditions of assay as under Table III.

the case both before and after Sephadex treatment of the particles (Table IV). The addition of FCCP to the I + GlyOMe-treated particles resulted in a low degree of stimulation of electron transfer from NADH. These data support the supposition that (1) I was firmly bound to the inhibited site and (2) although highly water soluble, GlyOMe also appeared to be firmly bound since its effect in the presence of I was observed after Sephadex treatment (Table IV). In separate experiments the stimulatory effect of GlyOMe, on NADH oxidation (in the absence of I) was not observed following Sephadex treatment. Further, the addition of higher concentrations of NADH to particles inhibited by I did not increase the rate of NADH oxidation.

Comparison of Various Carbodiimides as Inhibitors of NADH Catalyzed by ETPH (Mg, Mn). In an attempt to gain insight into the molecular arrangements required of carbodiimides as inhibitors of electron transfer in submitochondrial particles, as well as to possibly gather information on the degree of hydrophobicity of the carbodiimide-combining site within the membrane catalyzing the I-sensitive portion of the electron-transfer chain, inhibitor constants (K_i) were determined for a series of commercially obtained carbodiimides. The most effective carbodiimides with respect to inhibition of the rate of NADH oxidation were the highly water-insoluble I: di-*p*-tolylcarbodiimide, di-*o*-tolylcarbodiimide, and *N*-cyclohexyl-*N'*-β-morpholinoethylcarbodiimide. However, the only compounds listed in Table V the inhibitory character of which could be significantly reversed by the uncoupler FCCP were I, di-*p*-tolylcarbodiimide, and di-*o*-tolylcarbodiimide. None of the water-soluble carbodiimides showed any release of inhibition by FCCP (Table V).

Since carbodiimides may be transformed to urea derivatives in aqueous solvent by the addition of water, two such urea derivatives were also tested in the submitochondrial system. Neither of the urea derivatives significantly affected the rate of NADH oxidation by the submitochondrial preparation (Table V, part II). It is also of interest to note that the last two carbodiimides listed in Table VA stimulated NADH oxidation and were not inhibitory even at very high concentrations.

TABLE V: Inhibitor Constants (K_i) for Various Carbodiimides as Inhibitors of NADH Oxidation.[a]

Compound	K_i (μmoles/mg of Protein)
Part A	
I	0.107
Di-*p*-tolylcarbodiimide	0.127
Di-*o*-tolylcarbodiimide	0.218
N-Cyclohexyl-*N'*-β-morpholinoethyl-carbodiimide	0.300
1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate[b]	41
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride[b]	64
N-Ethyl-*N'*-β-dimethylaminoethylcarbodiimide hydrochloride[b]	82
N,N'-Diisopropylcarbodiimide	0.880
N,N'-Bis(triphenylmethyl)carbodiimide[c]	Stimulates
N,N'-Bis(2,6-diethylphenyl)carbodiimide[c]	Stimulates
Part B	
1-Cyclohexyl-3-(2-morpholinoethyl)urea metho-*p*-toluenesulfonate[d]	453
N,N'-Dicyclohexylurea[d]	No effect

[a] All assays were performed as detailed in Table III. Reactions were initiated by the addition of 1.5 μmoles of NADH exactly 10 min following the addition of carbodiimide to the reaction chamber maintained at 25°. K_i is hereby defined as that concentration of carbodiimide which results in half-maximal inhibition of NADH oxidation under the above conditions and is expressed in terms of micromoles of carbodiimide per milligram of submitochondrial protein. [b] Water soluble. [c] Insoluble in acceptable solvent. Compound was prepared for incubation in the assay system by sonication with soy bean asolectin to produce an inclusion micellar suspension. [d] Substituted urea.

Discussion

Since the report by Beechey and his colleagues (1966) that I mimics the effect of oligomycin and aurovertin in intact mitochondria, *i.e.*, inhibits mitochondrial ATPase and electron transfer in well-coupled systems, the locus of interaction between I and the mitochondrial inner membrane has been the subject of research in several laboratories. Holloway *et al.* (1966) and Roberton *et al.* (1966) reported that I binds irreversibly to the F_0 fraction isolated by Kagawa and Racker (1966). Knight *et al.* (1968) provided evidence that I combines with at least two proteolipid fractions in mitochondria and that the binding is covalent in nature. Compound I has also been reported to inhibit mitochondrial coupling factor 1 (ATPase) (Penefsky, 1967). Bruni *et al.* (1971) have recently implicated phosphatidylserine and phosphatidylcholine in the I-induced inhibition of mitochondrial ATPase. At somewhat higher concentrations, I also appears to inhibit the electron-transfer chain in submitochondrial particles (Beyer *et al.*, 1967a, 1969).

Four distinct types of evidence allow for the assignment of the site inhibited by carbodiimides within the NADH dehydrogenase complex of the electron-transfer chain. First, I inhibits the oxidation of NADH and not the oxidation of succinate or other electron donors which enter the chain on the oxygen side of UQ. Second, in I-inhibited submitochondrial particles oxidizing NADH, UQ is found to be predominately in the oxidized form in contrast to uninhibited particles where UQ, under the same conditions of incubation, is approximately 50% reduced. Third, in submitochondrial particles inhibited by I only $FeNH_N$ of the nonheme iron compounds is reduced by NADH. Fourth, I is relatively inactive in inhibiting NADH oxidation in submitochondrial particles from Mung beans. Since the MB-ETP system oxidizing NADH is not inhibited by rotenone or other inhibitors active in this region of the electron-transfer chain in mammalian systems (R. E. Beyer and C. Woermann, unpublished results), it would appear reasonable to assume that the component which normally binds inhibitors in the NADH dehydrogenase complex is either altered or missing in the Mung bean submitochondrial system. The observation that I was without significant inhibitory activity in this preparation is consistent with an identical inhibitory site for I and other inhibitors active in this region. These data, in addition to the spectral data published previously (Beyer *et al.*, 1967a), provide strong evidence that the site within the electron-transfer chain

sensitive to I lies on the oxygen side of $FeNH_N$ and on the substrate side of UQ. This site, although as yet unidentified with respect to the component involved, also appears to be sensitive to such other inhibitors as barbiturates, rotenone, and piericidin A (*cf.* Garland *et al.*, 1969). The most likely candidate for this site would appear to be the ferroprotein fraction B isolated by Hatefi and Stempel (1967). For a pertinent discussion of this possibility, see Garland *et al.* (1969).

The increased efficacy of I as an inhibitor in the presence of GlyOMe, the inability of FCCP to relieve such inhibition of NADH oxidation under these conditions of inhibition, and the constancy of the I + GlyOMe effect after gel filtration on Sephadex suggest that free carboxyl groups may be involved in the I-induced inhibition of NADH oxidation (Hoare and Koshland, 1966, 1967). The greater effectiveness of nonpolar carbodiimides as compared to water-soluble carbodiimides suggests that the binding site for carbodiimides resides in hydrophobic regions of the inner mitochondrial membrane. If, in fact, carboxyl groups in such hydrophobic regions are responsible for the observed inhibition, it is possible that an irreversible conformational alteration ensues as a result of covalent binding of the carbodiimide preventing the altered electron-transfer carrier from accepting electrons from $FeNH_N$. Such a mechanism is consistent with the suggestions of Abrams and Baron (1970) and Godin and Schrier (1970) derived from studies on membrane ATPases, and Azzi and Santato (1970) working with energy-dependent interactions between I and the mitochondrial inner membrane. It should be noted, however, that to date such evidence for the nature of carbodiimide interaction with mitochondrial membranes is indirect. The possibility that carbodiimides may react with sulfhydryl groups (Carraway and Triplett, 1970) and tyrosine residues (Carraway and Koshland, 1968), as well as with a variety of nucleotides (Todd, 1959), should not be overlooked. The latter possibility, *i.e.*, that hydrophobic carbodiimides might react to yield dimeric or polymeric nucleotides from adenine nucleotides bound within the inner mitochondrial membrane (Beyer, 1968), does not appear to have received consideration.

Acknowledgments

We are grateful to Dr. Graham Palmer for his participation in the experiments requiring use of the epr spectrometer and to Dr. Hiroshi Ikuma for participation in preliminary experiments on the effects of inhibitors on the Mung bean system. Ms. Janet MacDonald provided excellent technical assistance.

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Differentiation of Avian Keratinocytes. Characterization and Relationships of the Keratin Proteins of Adult and Embryonic Feathers and Scales†

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ABSTRACT: The keratin proteins of embryonic and adult feathers and scales of the chicken were investigated, with the aim of providing a basis for the study of cellular differentiation in these tissues at a molecular level. Results from gel electrophoresis, isoelectric focusing, immunodiffusion, and amino acid analysis have demonstrated that the proteins of feathers are different from those of scales. Differences also occur between adult and embryonic tissues. The results suggest that in each cell line which originates from embryonic epidermis, a different and restricted set of structural genes for keratin proteins is selected from a larger set.

In developing embryonic chick skin, inductive events originating from the dermis result in the transformation of epidermal cell groups into feather and scale primordia (Wessells, 1962–1965; Rawles, 1963; Bell, 1963, 1964; Sengel, 1971). These primordia develop until they reach a form approximating the definitive organ. Tissue specific proteins (keratins) then become detectable by histological criteria and their synthesis continues rapidly until the cells fill with keratin (Bell and Thathachari, 1963). In suitable culture systems the developmental events can be experimentally investigated by recombining isolated epidermis and dermis from various regions (see Rawles, 1963). Such studies have shown that the developmental fate of the epidermis is determined by the nature of the underlying dermis. For example, epidermis from a prospective scale region (anterior tarsometatarsus) can be experimentally induced to form feathers in the presence of dermis from a prospective feather region (Rawles, 1963).

Studies on the molecular events occurring in response to these inductive influences have been hampered by lack of a suitable assay system for the keratin proteins which the tissues synthesize during their subsequent development. The component proteins of embryonic feathers and scales have not previously been identified. It was consequently not known whether the inductive events described above, control only tissue morphology or result as well in the synthesis of different tissue-specific proteins. Identification and characterization of the component keratin proteins of embryonic feathers and scales is a prerequisite in order to answer this question and to provide a quantitative and qualitative basis for the study of specific gene action during the differentiation of these tissues.

Malt and Bell (1965) and Ben-Or and Bell (1965) studied the proteins of embryonic feathers by chemical and immunological methods, but did not identify any individual keratin proteins. Harrap and Woods (1964a,b, 1967) and Woods (1971) extended the earlier studies of Ward *et al.* (1946), Woodin (1954, 1956), and Schroeder and Kay (1955) on the proteins of adult feathers. They prepared soluble proteins from adult feathers by reduction and S-carboxymethylation and showed that each of the morphological parts of the adult feather (Schroeder and Kay, 1955) contains several proteins. These were all of the same molecular weight (10,500) but were electrophoretically and chromatographically distinguishable.

In the present paper, soluble SCM[1]-proteins of adult and embryonic feathers and scales from the chicken have been investigated by gel electrophoresis, isoelectric focusing, peptide mapping, and immunological methods. The proteins of feathers are different from those of scales. Differences occur between the proteins of each tissue in their adult and embryonic states.

Materials and Methods

Preparation of Reduced S-Carboxymethylkeratins. Adult feathers and leg scales (from anterior tarsometatarsus) were obtained from approximately 1-year-old White Leghorn chickens, strain Para 3, a pure-bred strain obtained from Parafield Poultry Station, Parafield, South Australia. The so-called "embryonic" (down) feathers and scales were from newly hatched (21 day) chickens of the same strain. Adult feathers were separated into their four morphological parts, namely, rachis, calamus, barbs, and medulla (Schroeder and Kay, 1955). All tissues were washed, reduced and carboxymethylated essentially as described by Harrap and Woods (1964a) except that the conditions for reduction were altered to 3 hr at 37° in a solution containing 8 M urea, 0.5 M ethanolamine, and 0.1 M mercaptoethanol (pH 10.5).

The extracted SCM-proteins were dialyzed exhaustively against glass-distilled water and freeze-dried. Amino acid analysis demonstrated that the reduction and carboxymethylation reactions had gone to completion. Yields of freeze-dried material were greater than 70% of the dry weight of the start-

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[1] Abbreviation used is: SCM, *S*-carboxymethyl.