Inhibition of Electron and Energy Transfer in Rat Liver Mitochondria by Nordihydroguaiaretic Acid*

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ABSTRACT: Nordihydroguaiaretic acid (NDGA) is an effective inhibitor of mitrochondrial respiration associated with NAD^+-linked substrates. At concentrations which inhibit glutamate–malate oxidation (over 90% inhibition) succinate oxidation is inhibited less than 25%. NDGA inhibits both electron and energy transfer in mitochondria as revealed by (a) the partial release of NDGA inhibition by dinitrophenol, (b) inhibition of succinate-linked reduction of acetoacetate, and (c) inhibition of energy-linked swelling of mitochondria. NDGA binds tightly to mitochondria since washing with sucrose does not remove the inhibition. Addition of NDGA to mitochondria oxidizing NAD^+-linked substrates under state 3 condition shifts the redox level of cytochrome *b* to the oxidized state and that of the pyridine nucleotide to a more reduced state. The lack of effect on the inhibition by washing with sucrose as well as the effect of albumin in reversing the inhibitory effect indicate the involvement of a lipid component in the respiratory chain in NDGA inhibition.

Nordihydroguaiaretic acid (NDGA)[1] is a major chemical principle of the leaves and stem of the plant cresote bush (*Larrea divaricata* Cav Fam. Zygophyllaceae). The chemical formula of this compound is given in Figure 1. It has been used extensively as an antioxidant additive in human foods at levels of 0.01–0.02% (Smart *et al.*, 1969). Various studies have indicated no toxicity in animals and man (Lisanti and Eichel, 1963; Cranston *et al.*, 1947; also see references listed by Smart *et al.*, 1969). However, Burk and Woods (1963) reported that NDGA produced almost complete inhibition of aerobic and anaerobic glycolysis and respiration of Ehrlich carcinoma and L-1210 mouse leukemia, ascite cells, and other cancer tissues both *in vitro* and *in vivo*. More recently, Smart *et al.* (1969) reported the striking regression of malignant melanoma of the right cheek in a patient following continued medication with an aqueous extract of the cresote bush. Very little is known about the mode of action of NDGA. Burk and Woods (1963) believe that the hydroquinone form of NDGA maintains the cellular pyridine nucleotides in the reduced forms, thus inhibiting anaerobic glycolysis due to lack of NAD^+. Pardini *et al.* (1970) studied manometrically the effect of NDGA on mitochondrial electron-transport system and reported their preliminary observation that both NADH-oxidase and succinoxidase systems were inhibited whereas cytochrome oxidase activity was not affected. We present evidence to suggest that NDGA exerts its influence both on the electron and energy-transfer reactions associated with site I phosphorylation of rat liver mitochondria.

Materials and Methods

Preparation of Rat Liver Mitochondria. Liver mitochondria from stock Holtzman rats were prepared in 0.25 M sucrose essentially according to Schneider (1948). The mitochondria were washed once and finally resuspended in 0.25 M sucrose. During washing the loose white fluffy layer sedimenting over the packed brown sediment was removed. Mitochondria used in the swelling experiment and for estimation of the redox states of pyridine nucleotide and cytochrome *b* were subjected to a second washing with a medium (pH 7.4) containing 0.175 M KCl and 0.025 M Tris-HCl. The stock suspensions of mitochondria contained approximately 25 mg of protein/ml.

Measurement of Mitochondrial Respiration. This was performed polarographically at 28° in an oxygraph (G. M. C., Madison, Wis.) with a Clark electrode according to Dakshinamurti *et al.* (1970). Mitochondria (2 mg of protein) were added to 1.5 ml (final volume) of medium saturated with air at 28° and containing 0.23 M mannitol, 0.07 M sucrose, 0.02 M Tris-HCl, 0.02 mM EDTA, and 5 mM potassium phosphate, all at a final pH of 7.2. The following substrates were used in individual experiments: 5 mM glutamate plus 1 mM malate plus 1 mM malonate, 5 mM β-hydroxybutyrate, 5 mM pyruvate plus 1 mM malate, and 5 mM succinate plus 10 μM rotenone. State 3 respiration was initiated by addition of ADP in limiting amounts (440 nmoles). Dinitrophenol (120 μM final concentration) was used as uncoupler of oxidative phosphorylation.

Dual-Wavelength Spectrophotometry. The redox states of pyridine nucleotide and cytochrome *b* were determined with an Aminco-Chance spectrophotometer (American Instruments Co., Silver Spring, Md.) essentially according to Boveris and Stoppani (1970). For the spectrophotometric determinations the medium used contained 0.15 M KCl, 20 mM Tris-HCl, and 5 mM potassium phosphate, at a final pH of 7.4.

Succinate-Linked Reduction of Acetoacetate with Aerobically Generated High-Energy Intermediates. This was performed by incubating tightly coupled mitochondria aerobically at 29° in the system described by Azzone *et al.* (1963). Acetoacetate was determined by the method of Walker (1954).

Measurement of Swelling. The stock suspension of mitochondria was adjusted so that 20 μl (~0.5 mg of protein) diluted to 3.0 ml in 0.15 M KCl containing 0.02 M Tris-HCl

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[1] Abbreviations used are: NDGA, nordihydroguaiaretic acid; DNP, 2,4-dinitrophenol; BSA, bovine serum albumin; PMS, phenazine-methosulfate.

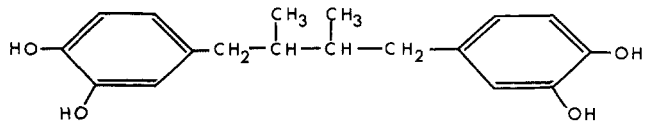

FIGURE 1: Nordihydroguaiaretic Acid.

(pH 7.4, Tris-HCl medium) gave an initial absorbance at 520 mμ of 0.9–1.0. Swelling of mitochondria was followed by continuous recording of the change in absorbance at 520 mμ in a Beckman DB-G spectrophotometer in cells of 1-cm light path at 28° as reported earlier (Bhuvaneswaran and Dakshinamurti, 1970).

Protein Determination. The protein concentration of mitochondrial suspension was determined using the biuret method of Layne (1957) in the presence of 1% sodium deoxycholate.

Chemicals. Crystalline BSA, ATP, ADP, rotenone, succinic acid, DL-β-hydroxybutyrate (sodium salt), pyruvic acid (sodium salt), L-malic acid (monosodium salt), malonic acid, DL-glutamic acid, DNP, PMS, Tris, and L-thyroxine were obtained from Sigma Chemical Co. All other common chemicals were of analytical reagent grade obtained from British Drug House, Merck or Baker Chemicals. We are indebted to Dr. Arthur D. Broom, University of Utah, Salt Lake City, for the gift of nordihydroguaiaretic acid [pyrocatechol (4,4′-(2,3-dimethyltetramethylene)]. All solutions were made in double-distilled water. Rotenone and NDGA were used as ethanolic solutions.

Results

NDGA Inhibition of Glutamate–Malate–Malonate Oxidation by Rat Liver Mitochondria. In Figure 2 the responses of mitochondria oxidizing glutamate–malate–malonate to varying amounts of NDGA is given. The composite curve consists of a series of superimposed records of individual experiments in which the amount of inhibitor added at the indicated time was varied. The maximal inhibition (over 92%) with NDGA occurred at a concentration of 50 μM of the inhibitor. Addition of 2,4-dinitrophenol induced a release of the respiratory inhibition. However, the rate of DNP-stimulated respiration in presence of NDGA was lower than the corresponding value in the absence of the inhibitor. There was a time lag in the DNP effect, although it is evident only at higher concentrations of NDGA.

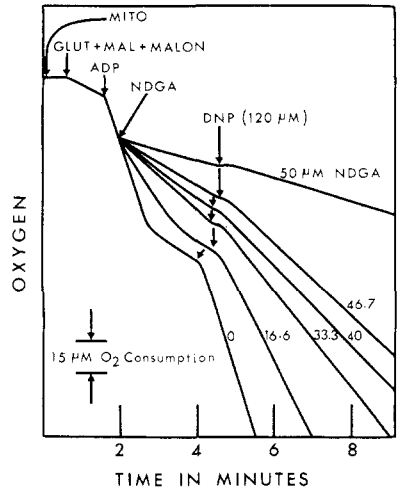

FIGURE 2: Titration of mitochondria with NDGA. The composite curve consists of a series of superimposed records of individual experiments in which the amount of inhibitor added at the indicated time was varied. All other conditions of concentrations and time were maintained constant. Dinitrophenol (DNP) was added at the indicated time to test its effect in releasing the respiratory inhibition. Mitochondria (2 mg of protein) were added to 1.5 ml (final volume) of medium saturated with air at 28° and containing mannitol, 0.23 M; sucrose, 0.07 M; Tris-HCl, 0.02 M; EDTA, 0.02 mM; and potassium phosphate, 5 mM all at a final pH of 7.2. The concentrations for other additions were: glutamate, 5 mM; malate, 1 mM; malonate, 1 mM; ADP, 293 μM; and DNP, 120 μM.

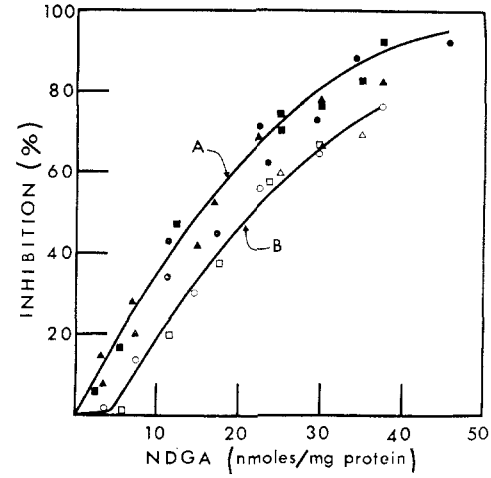

FIGURE 3: Inhibition of glutamate–malate–malonate oxidation by NDGA, as a function of the concentration of mitochondrial protein. Different symbols indicate different experiments. The closed symbols are for values in the absence of any uncoupler and the open symbols are for values in the presence of 120 μM of DNP. Protein concentration ranged between 1 and 4 mg per ml; NDGA concentration ranged between 5 and 70 μM. Experimental conditions as in Figure 2 with glutamate–malate–malonate as substrates. (A) Metabolic state 3; (B) DNP uncoupled.

The extent of inhibition of mitochondrial respiration by NDGA was dependent on the relative amounts of NDGA to mitochondrial protein in the system (Figure 3). Half-maximal effect was produced at 15 nmoles of NDGA/mg of protein. Addition of DNP to NDGA-inhibited mitochondria resulted in a partial release of the inhibition. This was in sharp contrast to the lack of effect by DNP on rotenone-inhibited mitochondria (Table I). The disparity of DNP effect on rotenone inhibition sets the standard to establish the effect of DNP on NDGA-inhibited respiration. At amounts lower than 4 nmoles of NDGA/mg of protein, DNP completely reversed the inhibition. It should be mentioned here that in Figure 3 the inhibition (%) was calculated on the respective controls, *i.e.*, for state 3 the control was state 3 in the absence of inhibitor, and for the DNP-uncoupled state the control was the rate in presence of DNP but in the absence of the inhibitor. While the difference in percentage inhibition between state 3 and DNP uncoupled state was 20% or less the actual respiratory rates were stimulated over 100% in case of NDGA. In similar experiments with rotenone there was no stimulation whatever (Table I). In Figure 4 the titration of NDGA with respect to mitochondrial protein to achieve two levels of inhibition corresponding to 70 and 80% of the control value is presented. The linearity of this curve indicates a stoichiometric relationship between the inhibitor and mitochondrial protein concentration.

Binding of NDGA with Mitochondria. The inhibitor at

TABLE I: Release by DNP of NDGA Inhibition of Mitochondrial Respiration.[a]

Additions	ng-atoms of O per min per mg of Protein				Difference in % Inhibn (State 3 − DNP Uncoupled)	Actual Stimulation (+) or Inhibn (−) of Respiration by DNP over Corresponding State 3 Value
	+ADP	% Inhibn	+DNP	% Inhibn		
None	80		109			+36
NDGA (42.8 μM)	25	68.8	56	48.6	+20.2	+120
NDGA (57.0 μM)	17	78.8	39	64.2	+14.6	+129
NDGA (71.5 μM)	13	83.8	28	74.4	+9.4	+115
Rotenone (17.2 nM)	31	61.3	38	65.2	−3.9	+22.6
Rotenone (32.4 nM)	16	80.0	14	87.2	−7.2	−12.5
Rotenone (42.8 nM)	13	83.8	10	91.1	−7.3	−23.0

[a] Experimental conditions as described under Methods. Mitochondria (4.5 mg of protein) suspended in 1.75 ml of mannitol–sucrose–EDTA medium and the oxygen uptake measured polarographically at 28°. Substrates used were glutamate (10 mM) plus malate (2 mM) plus malonate (2 mM). ADP (220 μM) and DNP (100 μM) were used to obtain respiration in state 3 and the uncoupled state.

three different levels was added to aliquots of mitochondria. The control contained an equivalent amount of ethanol. They were then incubated at 0° for 10 min followed by centrifugation at 10,000*g* for 10 min. The mitochondria were washed once with 0.25 M sucrose and resuspended to their original volume in 0.25 M sucrose. Oxidation of glutamate–malate by the NDGA-treated mitochondria as well as by the control mitochondria to which an equivalent amount of NDGA was added directly prior to oxygen uptake measurement were then compared. The plots of the resulting inhibition of respiration against NDGA levels were very similar (Figure 5) indicating that the inhibitor is bound to mitochondria and is not removed by washing with a sucrose medium.

Reversal of NDGA Inhibition by Albumin. In spite of the binding of NDGA to liver mitochondria, the addition of bovine serum albumin (10 mg) to NDGA-treated mitochondria reversed the respiratory inhibition (Figure 6). Up to a concentration of 40 μM NDGA, the inhibition could be very effectively reversed by albumin. At higher concentrations of the inhibitor, albumin could still reverse the inhibition to a large extent. It did not matter whether albumin was added prior to or after the addition of NDGA.

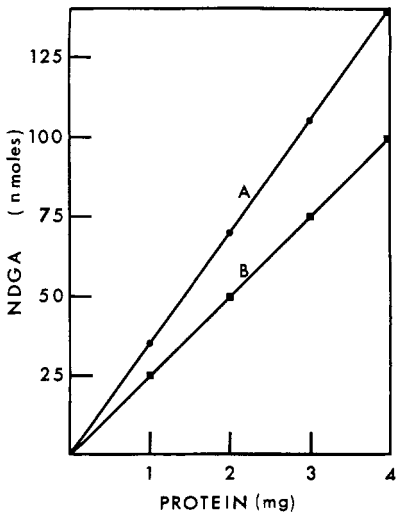

FIGURE 4: Effect of mitochondrial protein concentration on NDGA titer values. Inhibition by NDGA of mitochondrial oxidation (state 3) was monitored using the oxygraph. The substrates used were glutamate, 5 mM, plus malate, 1 mM, plus malonate, 1 mM. State 3 respiration was initiated by adding 293 μM ADP to the system. Mitochondrial proteins ranged between 1 and 4 mg and NDGA concentrations were varied between 0 and 140 nmoles. Inhibition levels were A, 80% and B, 70% of the control value. Other conditions as in Figure 2.

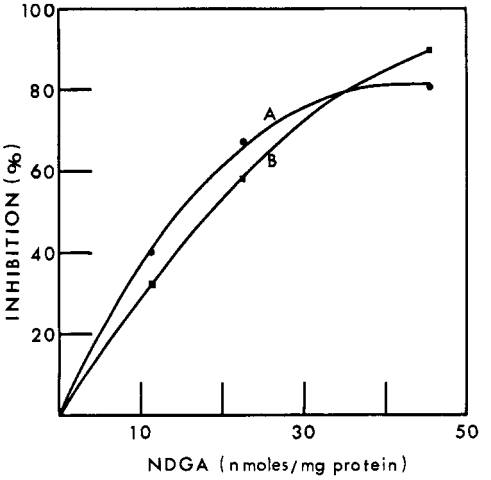

FIGURE 5: Effect of NDGA pretreatment and subsequent washing on glutamate–malate oxidation by mitochondria. NDGA at three different levels (11.25, 22.5, and 45 nmoles per mg of mitochondrial protein) was added to aliquots of mitochondria. The control contained equivalent amounts of ethanol. They were incubated at 0° for 10 min followed by centrifugation at 10,000*g* for 10 min. The mitochondria were washed once with 0.25 M sucrose. Oxygen uptake in state 3 was measured for the NDGA-pretreated mitochondria as well as for the control mitochondria to which an equivalent amount of NDGA have been added. Other conditions as described for Figure 2. (A) Control mitochondria with varying amounts of NDGA; (B) NDGA pretreated and washed mitochondria.

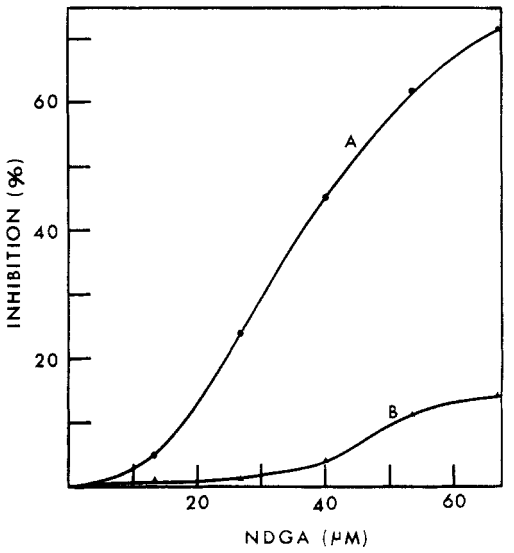

FIGURE 6: Effect of bovine serum albumin on NDGA inhibition of mitochondrial respiration. The conditions are as described under Figure 2. Bovine serum albumin (BSA), 10 mg, was added after addition of NDGA. (A) Control mitochondria; (B) mitochondria plus BSA.

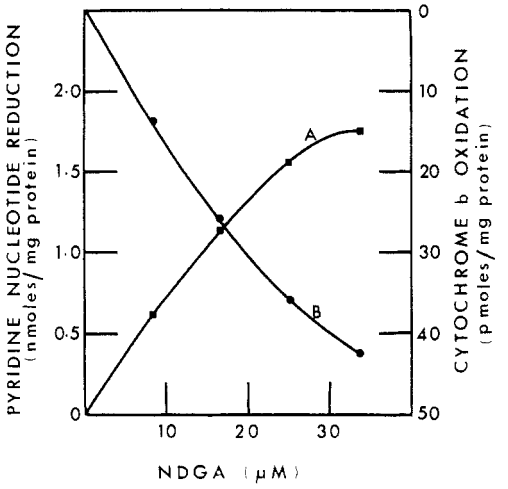

FIGURE 8: Titration of NDGA effect on the redox levels of pyridine nucleotide (curve A) and cytochrome *b* (curve B) (metabolic state 3). Experimental conditions as described under Methods. The reaction medium (3-ml final volume) contained KCl, 150 mM; Tris-HCl, 20 mM; potassium phosphate, 5 mM; glutamate, 5 mM; malate, 1 mM; malonate, 1 mM; and ADP, 293 μM. Pyridine nucleotide was monitored at 350–375 nm; and cytochrome *b* was monitored at 430–410 nm. Mitochondrial protein in the assay system was 0.87 mg/ml.

NDGA Inhibition of Oxidation of Various Substrates. The effect of NDGA on the oxidation of various substrates was tested (Figure 7). Highest inhibition was observed with glutamate–malate system followed closely by β-hydroxybutyrate and pyruvate–malate systems. The least inhibitory effect was observed with succinate indicating that the main site of inhibition of NDGA involves electron transfer through the first phosphorylation site.

Action of NDGA on Redox Steady States of Pyridine Nucleotide and Cytochrome b. Using glutamate–malate as substrate and mitochondria in state 3, addition of NDGA resulted in progressive reduction of pyridine nucleotide whereas cytochrome *b* was progressively oxidized. Figure 8 depicts the titration of NDGA effect on the redox levels of both the mitochondrial components estimated.

Effect of NDGA on Succinate-Linked Reduction of Acetoacetate. Azzone *et al.* (1963) have shown that β-hydroxybutyrate synthesis from acetoacetate can be a measure of the reduction of NAD^+ by succinate. NDGA strongly inhibited the reduction of acetoacetate by succinate when aerobically generated high-energy intermediate served as the energy source. The results are presented in Figure 9.

Effect of NDGA on Phosphate plus Thyroxine Induced Swelling of Liver Mitochondria. Curve 1 in Figure 10 represent swelling mediated by all the three energy-conserving sites in normal untreated mitochondria oxidizing glutamate plus

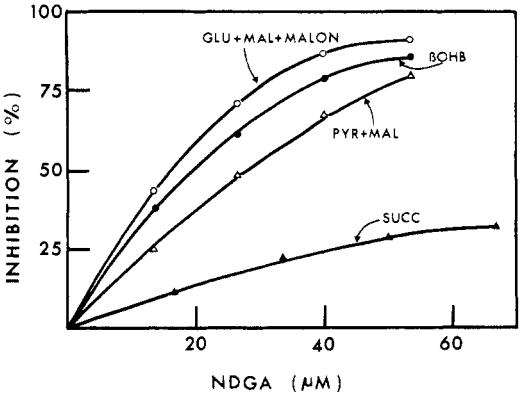

FIGURE 7: Effect of NDGA on oxidation of various substrates by mitochondria. All conditions were as given for Figure 2. The substrates used were: glutamate, 5 mM, plus malate, 1 mM, plus malonate, 1 mM; pyruvate, 5 mM, plus malate, 1 mM; β-hydroxybutyrate, 5 mM; and succinate, 5 mM, plus rotenone, 10 μM.

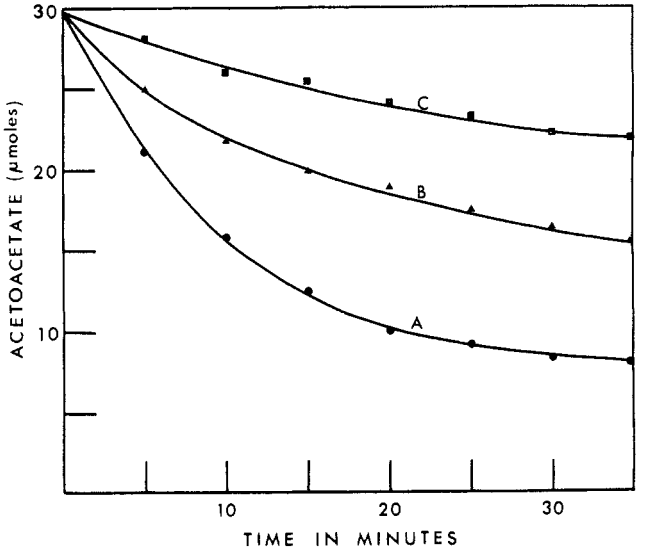

FIGURE 9: Effect of NDGA on succinate-linked acetoacetate reduction in mitochondria with aerobically generated energy. Each vessel contained, in a final volume of 5 ml: mitochondria, 32.2 mg of protein; acetoacetate, 5.96 mM; $MgCl_2$, 8 mM; glycylglycine buffer (pH 7.5), 20 mM; KCl, 50 mM; sucrose, 75 mM; and succinate, 10 mM. Incubation was at 29°. At 5-min intervals 0.2-ml aliquots were taken and fixed with 0.1 ml of 1.5 M perchloric acid. (A) No further additions; (B) same as system A plus NDGA (0.1 mM); (C) same as system A plus NDGA (0.2 mM).

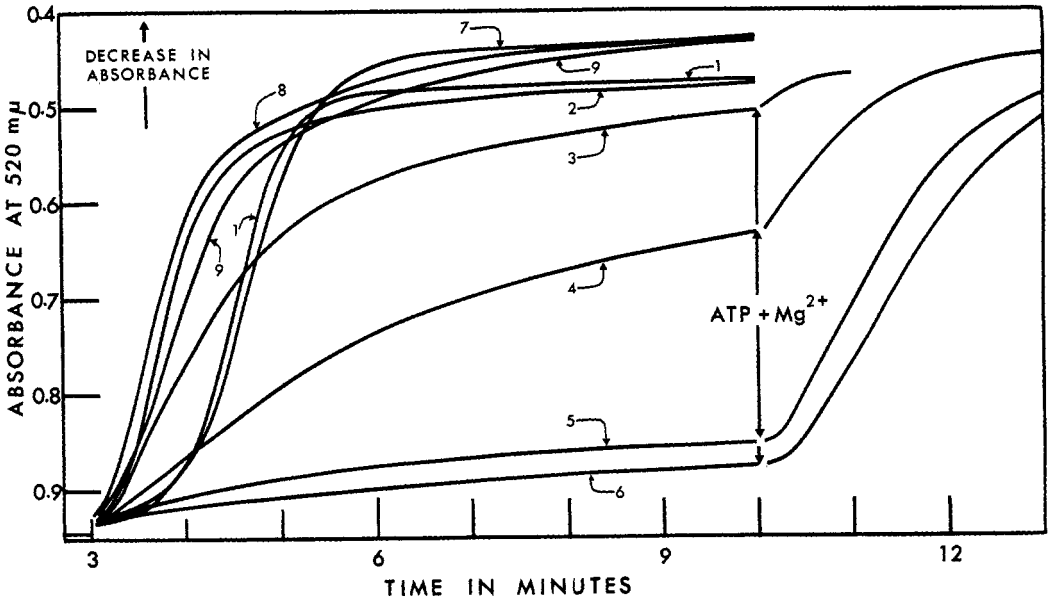

FIGURE 10: Effect of NDGA on phosphate plus thyroxine induced swelling of liver mitochondria. Mitochondria (0.63 mg of protein) were suspended in 3 ml of 0.15 M KCl containing 0.02 M Tris-HCl (pH 7.4) at 28° with either glutamate plus malate plus malonate or succinate plus rotenone as substrates. Swelling by phosphate (2 mM) and thyroxine (12 μM) was started after a 3-min incubation with the following: (1) glutamate (5 mM), plus malate (1 mM), plus malonate (1 mM); curves 2–6 consisted of increasing concentrations of NDGA (8, 12, 16, 20, and 25 μM final concentration) added to system represented by 1; (7), succinate (5 mM), plus rotenone (10 μM); curves 8 and 9, NDGA (20 and 25 μM final concentration) added to system represented by 7. ATP and Mg^{2+} (60 μM of each) was added to systems 2–6 at the end of 7 min after addition of phosphate plus thyroxine.

malate. The swelling was maximal and no further swelling was brought about by addition of 60 μM each of ATP and Mg^{2+}. The progressive inhibition of swelling brought about by addition of increasing concentrations of NDGA (8, 12, 16, 20, and 25 μM final concentration) is represented by curves 2–6. At 25 μM concentration, the swelling was completely abolished. However, addition of 60 μM each of ATP and Mg^{2+} promptly initiated swelling to the maximum extent. Curve 7 represents swelling using succinate plus rotenone as the substrate. Addition of the two highest concentrations of NDGA (20 and 25 μM) did not inhibit this swelling (curves 8 and 9). This indicates a lack of effect of the inhibitor in the succinoxidase system at the NDGA concentrations used here.

Effect of NDGA on Swelling Supported by Energy Conservation at Site I. Addition of antimycin A and cyanide effectively blocked electron transport between cytochromes. *b* and c_1 as well as between cytochrome a_3 and oxygen. Under these conditions, addition of the artificial electron acceptor PMS supported oxidation of NAD^+-linked substrates as well as energy conservation at site I (Bhuvaneswaran and Dakshinamurti, 1970; Scott and Hunter, 1966). This method offered a convenient system to study swelling mediated by energy produced at site I specifically. The results with glutamate–malate–malonate are given in Figure 11. Curve 1 represents the swelling mediated by all the three energy-conserving sites in the absence of any inhibitors. The swelling was maximal and no further swelling was brought about by addition of 60 μM each of ATP and Mg^{2+}. Treatment with 0.15 μM antimycin A and 0.5 mM KCN blocked mitochondrial swelling (curve 7). If, however, 0.1 mM PMS was added to the above system, swelling occurred (curve 2). In the absence of PMS, swelling could be supported by addition of 60 μM each of ATP and Mg^{2+} (curve 7). Curve 7 also represents the swelling obtained in the presence of 8 μM NDGA but without PMS. Addition of ATP and Mg^{2+} (60 μM of each) initiated swelling. Curves 3–6 represent swelling patterns obtained in the presence of 1, 3, 5, and 8 μM NDGA using the system represented by curve 2. There was a progressive inhibition of swelling with increasing concentration of the inhibitor. Addition of ATP and Mg^{2+} (60 μM of each) brought the swelling to the level in the absence of NDGA. Curve 8 represents the swelling in presence of 8 μM NDGA and 0.1 mM PMS but in the absence of the swelling agents phosphate plus thyroxine, indicating that there was no nonspecific swelling. Although not shown here, essentially similar results were obtained using β-hydroxybutyrate as substrate.

Discussion

A large number of compounds have been reported to affect the respiratory activity of mitochondria at specific sites. This list includes such diverse compounds as guanidines (Pressman, 1963a,b; Chappell, 1963; Chance and Hollunger, 1963a; Guillory and Slater, 1965; Schafer, 1969; Bhuvaneswaran and Dakshinamurti, 1970), steriods (Chance *et al.*, 1962; Chance and Hollunger, 1963b; Stoppani *et al.*, 1968; Boveris and Stoppani, 1970, 1971), amytal (Chance and Hollunger, 1963b), rotenone and piericidin A (Ernster and Lee, 1964; Pullman and Schatz, 1967; Chance *et al.*, 1967; Jeng *et al.*, 1968), and rhein (Kean *et al.*, 1970). NDGA can now be added to this list.

Our observations reveal that NDGA inhibits NAD^+-linked substrate oxidation (Figures 2 and 7). At concentrations of the inhibitor that result in 92% of inhibition with NAD^+-linked substrates, succinate oxidation is inhibited to only 25%. The fact that DNP can release the inhibition to some extent (Figures 2 and 3 and Table I) especially at lower concentrations of the inhibitor would indicate that NDGA is also an inhibitor of the energy-transfer process related to the first site of phosphorylation. The simultaneous

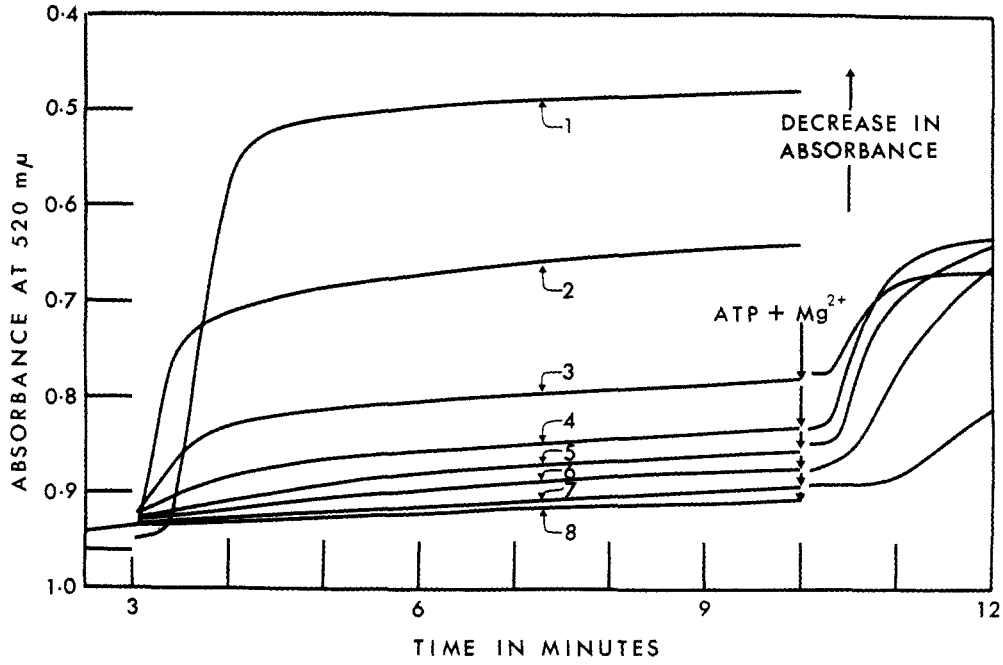

FIGURE 11: Effect of NDGA on swelling supported by energy conservation at site I using glutamate–malate–malonate as substrates. Phosphate (2 mM) plus thyroxine (12 μM) added to mitochondria (0.61 mg of protein) preincubated for 3 min with the following: (1) glutamate (5 mM), malate (1 mM), and malonate (1 mM); (2) antimycin A (0.15 μM), KCN (0.5 mM), and PMS (0.1 mM) added to system 1; curves 3–6 are the same as system 2 with NDGA added to give 1, 3, 5, and 8 μM final concentration; (7) same as system 2 or 6 with PMS omitted; and (8) same as system 2 or 6 with phosphate plus thyroxine omitted. ATP and Mg^{2+} (60 μM of each) was added to system 3–7 at the end of 7 min after addition of phosphate plus thyroxine. Other details as in Figure 10.

inhibition of electron and energy transfer could explain the failure of DNP to restore completely respiration inhibited by NDGA to the level obtained in the absence of the inhibitor. The dissimilar nature of DNP effect on the NDGA- and rotenone-inhibited respiration (Table I) establishes that the DNP effect is significant. The nature of the release by DNP of the NDGA inhibition is similar to that seen in the presence of guanidines (Pressman, 1963a,b) but differs from that in the presence of oligomycin (Lardy *et al.*, 1958; Huijing and Slater, 1961; Bhuvaneswaran and Dakshinamurti, 1970) with respect to time of onset and extent.

Further proof that NDGA is also an inhibitor of energy-transfer process is evident when we compare the concentration of NDGA needed to inhibit swelling when all the three sites are functional (Figure 10) to that needed for inhibition of respiration (Figures 2, 3, and 7). At concentration of 8.2 μM NDGA (curve 2, Figure 10) (the value in nmole of NDGA/mg of protein was 39.6) there was no inhibition of swelling, but respiration was inhibited over 86%. However, when the concentration of the inhibitor was increased, there was a progressive inhibition of swelling. The addition of ATP and Mg^{2+} (60 μM of each) to maximally inhibited mitochondria initiated the swelling again. The possibility that the energy requirement for swelling is less than can be supplied by respiration and only when the respiration is severely inhibited does the energy supply drop below the value required to support swelling also exists. To discern this, mitochondria were inhibited to varying degrees by graded doses of rotenone and the swelling experiments were carried out. The insensitivity of energy transfer toward rotenone is known (Ernster *et al.*, 1963; Boveris and Stoppani, 1970). The results indicated that below 68% inhibition of respiration, the mitochondria did not show any differences in the swelling pattern. But when the mitochondrial respiration was inhibited more than 68%, there was inhibition of swelling. At 80% inhibition of respiration, the swelling was clearly inhibited. If the action of NDGA was restricted solely to the electron-transfer process, then at comparable degree of respiratory inhibition by NDGA, the level of inhibition of mitochondrial swelling should be the same as with rotenone. This was not the case, since in presence of NDGA resulting in more than 86% inhibition of respiration there was no inhibition of swelling. The above facts indicate that NDGA acts in the energy-transfer chain, the site of action being between $C_1 \sim I$ and $I \sim X$ as both $C_1 \sim I$ and $I \sim X$ can be used as energy source for swelling (Bhuvaneswaran and Dakshinamurti, 1970), besides its action on the respiratory chain.

When the effect of NDGA on swelling using energy derived specifically from site I phosphorylation (Figure 11) is compared with that when all the three sites are functional (Figure 10) there is a marked difference in the amounts of the inhibitor needed for maximal inhibition of swelling. Thus maximal inhibition of swelling under conditions where all the sites of phosphorylation are operative was achieved at 95 nmoles of NDGA/mg of mitochondrial protein (curve 5, Figure 10), whereas in the system using site I only, the inhibition was achieved at 39.2 nmoles of NDGA/mg of mitochondrial protein (curve 6, in Figure 11). One possible explanation would be that the hydroquinone form of NDGA is the actual inhibitor and that under conditions where the respiratory chain is intact, the actual concentration of the reduced NDGA may be less. Burk and Woods (1963) observed that the reduced form of NDGA gave considerably greater and sustained inhibition of glycolysis even at lower concentrations. The reduction of NDGA was achieved by addition of the reducing agents like cysteine or glutathione in excess. These authors also found that ferricyanide could not oxidize the reduced form of NDGA. Thus, the value of 15 nmoles of NDGA/mg of mitochondrial

protein observed by us for half-maximal inhibition could be less when expressed in terms of the actual reduced form (Figure 3).

The redox changes observed in the pyridine nucleotides and cytochrome *b* (Figure 8) and also the inhibition produced on the succinate-linked reduction of acetoacetate (Figure 9) by NDGA are consistent with the action of NDGA in the energy- and electron-transfer reactions. The lack of response to washing of NDGA-treated mitochondria (Figure 5) as well as the reversibility of NDGA inhibition by albumin (Figure 6) and the effect of NDGA as a function of mitochondrial protein concentration (Figures 3 and 4) all point to the involvement of a lipid component in this inhibition. These results resemble those reported with 19-norethynyltestosterone acetate (Boveris and Stoppani, 1970) and with rotenone and piericidin A (Horgan *et al.*, 1968). The various sites of inhibition of NDGA are summarized in Figure 12.

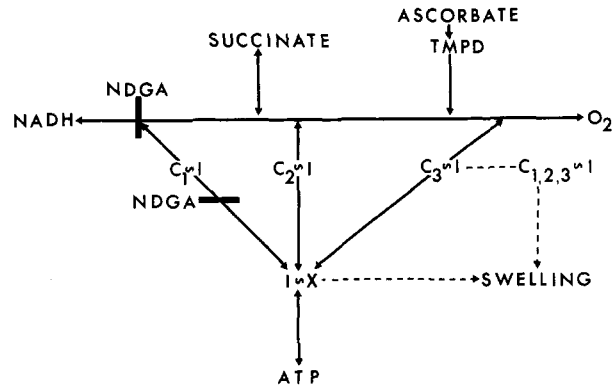

FIGURE 12: Hypothetic scheme of the energy-transfer system linked to the respiratory chain and site of action of NDGA. C_1, C_2, and C_3 indicate electron carries at the energy coupling site I, II, and III; I and X denote hypothetic energy-transfer carriers (see Lee and Ernster, 1966).

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