A New Inhibitor of Coupled Oxidative Phosphorylation, 5-Hydroxynaphthalenedicarboxylic Anhydride, a Derivative of a Carcinogenic Polynuclear Hydrocarbon†

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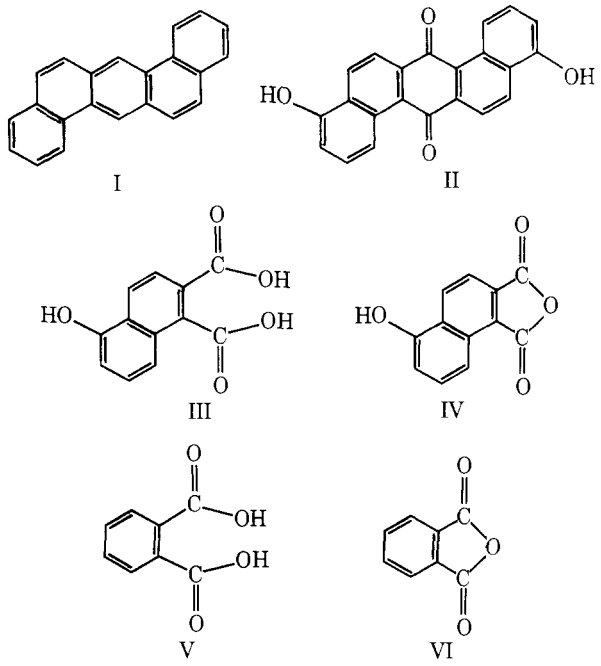
ABSTRACT: 5-Hydroxy-1,2-naphthalenedicarboxylic anhydride is closely related to its precursor dibasic acid which is a metabolite of the carcinogenic polynuclear hydrocarbon dibenz[*a,h*]anthracene. The anhydride inhibited respiration of coupled mitochondria. This inhibition was relieved by 2,4-dinitrophenol. Several mitochondrial volume change processes energized by ATP were also inhibited by the anhydride. Both the mitochondrial ATPase activity induced by 2,4-dinitrophenol and the ATPase activity of submitochondrial particles induced by magnesium ion were inhibited by the anhydride. The spectrum of inhibitory activity was not associated with acetic anhydride, succinic anhydride, or phthalic anhydride. The data indicate that 5-hydroxy-1,2-naphthalenedicarboxylic anhydride inhibits the machinery of oxidative phosphorylation in a manner similar to rutamycin. 5-Hydroxy-1,2-naphthalenedicarboxylic anhydride is the first molecule derived from a carcinogen with such inhibitory properties.

We have examined the action of diverse agents on oxidative phosphorylation by means of a convenient volume change assay (Hadler et al., 1970), ATPase and oxygen uptake studies. The agents examined have included the carcinogens *N*-acetyl-2-aminofluorene (Hadler et al., 1971a), *N*-acetyl-4-aminobiphenyl (Hadler and Daniel 1972), and dibenz[*a,h*]anthracene (I) (Hadler et al., 1971b) and their respective acidic metabolites *N*-hydroxy-*N*-acetyl-2-aminofluorene, *N*-hydroxy-*N*-acetyl-4-aminobiphenyl, and 4,11-dihydroxydibenz[*a,h*]anthracene-7,14-dione (II). Only the acidic metabolites disturbed oxidative phosphorylation. These and other data (Hadler and Daniel 1973) (Hadler and Demetriou, 1975) led to an experimental confluence between oxidative phosphorylation and chemical carcinogenesis.



We have been aware of certain hitherto unrelated facts. Firstly phthalic acid (V) inhibited the ATP-energized mitochondrial volume change induced by gramicidin (Falcone and Hadler, 1968), likely by blocking ion translocation, essential for the gramicidin dependent effect. Secondly HNDCA (III) was a metabolite of I (Heidelberger and Wiest, 1951), and supposedly was derived metabolically from II (Heidelberger et al., 1953). These considerations suggested that HNDCA might mimic the behavior of phthalic acid and interfere with the flux of energy in mitochondria and thus further substantiate an experimental confluence between oxidative phosphorylation and chemical carcinogenesis. While carrying out this investigation it was observed that the anhydride of HNDCA namely HNA (IV) was a much more effective mitochondrial reagent than HNDCA. This report shows that HNA, a fairly small molecule, mimics the behavior of the much used antibiotic rutamycin (a member of the oligomycin family) which is a specific inhibitor of coupled phosphorylation (Lardy et al., 1965). This represents the first derivative of a carcinogen, with such properties.

Materials and Methods

General procedures, methods, and purification of water and preparation of mitochondria have been described (Falcone and Hadler, 1968; Hadler and Falcone, 1968; Hadler et al., 1971b) for the mitochondrial volume change experiments. Incubation was at 27° in standard rectangular glass

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[1] Abbreviations and nomenclature: dibenz[*a,h*]anthracene (I) is known in the older literature as 1,2,5,6-dibenzanthracene; 4,11-dihydroxydibenz[*a,h*]anthracene-7,14-dione (II) is known in the older literature as 4′,8′-dihydroxy-1,2,5,6-dibenz-9,10-anthraquinone; HNDCA, 5-hydroxy-1,2-naphthalenedicarboxylic acid (III), is known in the older literature as 5-hydroxy-1,2-naphthalic acid; HNA, 5-hydroxy-1,2-naphthalenedicarboxylic anhydride (IV), is known in the older literature as 5-hydroxy-1,2-naphthalic anhydride; DNP, 2,4-dinitrophenol.

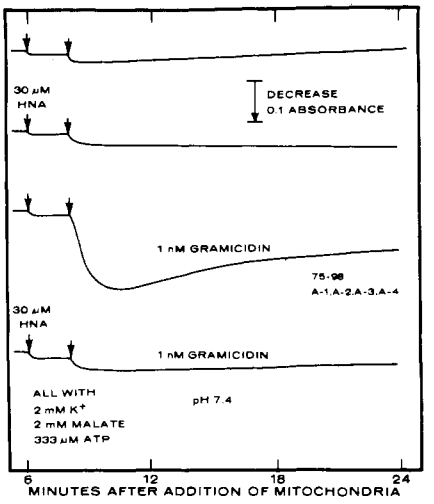

FIGURE 1: HNA inhibition of gramicidin induced mitochondrial swelling. The basic medium (see Materials and Methods) was used. HNA (in acetone) was added where indicated in a volume of 0.03 ml by means of the adding-mixing device as indicated by first arrows. Traces with unmarked first arrows are controls with the addition of solvent. ATP (Tris salt) was added to all cuvettes as indicated by second arrows. Gramicidin, where indicated, was present in the cuvette before the mitochondria were added. Potassium ion and malate were present in all cuvettes before the mitochondria were added.

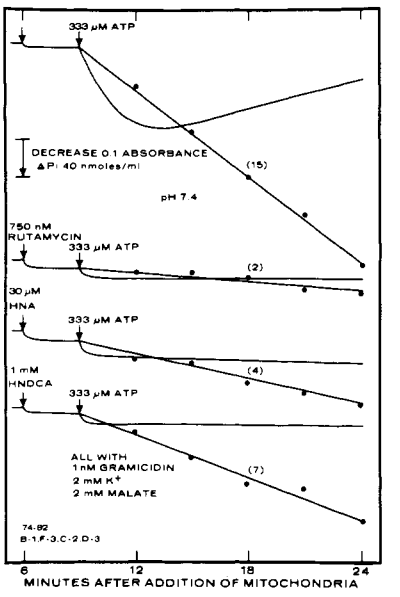

FIGURE 2: HNA, HNDCA, and rutamycin inhibition of gramicidin induced ATPase and mitochondrial swelling in the presence of potassium ion and malate. See legend of Figure 1 for basic conditions. Either HNA (in acetone) or rutamycin (in acetone) or an aqueous solution of Tris-HNDCA was added in a volume of 0.03 ml by means of the adding-mixing device as indicated by first arrows. The adding-mixing device also carried 0.03 ml of acetone when Tris-HNDCA was added. Gramicidin was present in the cuvette before the mitochondria were added. The numbers above the ATPase traces are nmol of P_i per ml per min derived from the slopes.

cuvettes with a 1-cm light path. The basic reaction mixture for the volume change experiments had a final volume of 3 ml and contained 0.25 mg/ml of mitochondrial protein (prepared fresh daily from male rat liver), 75 m*M* sucrose, and 75 m*M* Tris-HCl buffer of pH 7.4. A decrease in absorbancy at 520 nm was taken as a measure of mitochondrial swelling (Falcone and Hadler, 1968). A Model 2000 automatic spectrophotometer manufactured by Gilford Instrument Laboratories, Inc., Oberlin, Ohio, was used. All cations were added in the form of chloride salts and anions were added in the form of Tris salts neutralized to pH 7.4. HNA and the other anhydride used were dissolved in acetone (dried with sodium sulfate and distilled from potassium permanganate) when added to the incubation mixture. All controls contained the appropriate amount of carrier solvent. The adding-mixing device has been described (Hadler and Falcone, 1968). The charts and legends provide additional experimental details.

Submitochondrial particles were prepared by the disintegration of isolated rat liver mitochondria by sonic oscillation employing a Bronwill Biosonik III as described by Howland et al. (1973). The submitochondrial particles were suspended in 250 m*M* sucrose.

When ATPase was assayed simultaneously with the mitochondrial volume change experiments specially designed optical cuvettes (Falcone and Hadler, 1968) were used, otherwise the ATPase reaction was carried out in 16 × 150 mm test tubes in a 27° water bath without shaking. Orthophosphate was measured as previously described (Falcone and Hadler, 1968; Hadler et al., 1971b). Aliquots of 1 ml were removed at the appropriate time by means of a spring loaded Cornwall glass syringe fitted with a 6 in., 20 gauge stainless steel needle.

Oxygen consumption was measured at 27° in an oxygen monitor which utilized a Clark electrode. The instrument (Model 53SA) was manufactured by Yellow Springs Instrument Company, Yellow Springs, Ohio. A 10-in. recorder (Model 1005) manufactured by Beckman Instruments, Fullerton, Calif., was used in conjunction with the oxygen monitor.

HNA was synthesized according to the procedure of Dauben and Tanabe (1949). Our sample had a melting point of 271–272° (cor hot stage) which is identical with that in the original report. This sample gave a single spot, R_f 0.37, on thin-layer chromatography (silica gel plates, solvent system 5% acetone–95% benzene). An aqueous solution at pH 7.4 of the Tris salt of HNDCA was obtained by suspending HNA in water and adding with stirring pure Tris until a clear solution was obtained. Hydrochloric acid was added to adjust the pH.

Results

The ATP energized mitochondrial volume change induced by gramicidin in the presence of the permeant cation potassium and the permeant anion malate was totally inhibited by 30 μM HNA (Figure 1). Partial inhibition was observed at a level as low as 1 μM HNA and total inhibition was still evident at a level of 15 μM HNA (data not shown). The inhibitory effect of 30 μM HNA, 1 m*M* HNDCA, and 750 n*M* rutamycin on the ATP energized mitochondrial volume change and ATPase activity induced by 1 n*M* gramicidin in the presence or absence of 2 m*M* of potassium ion and 2 m*M* malate is seen in Figure 2 and Table I. While 1 n*M* gramicidin alone (i.e., without potassium ion or malate ion) did not induce a mitochondrial volume change there was significant ATPase activity. This ATPase activity was inhibited by either 30 μM HNA or 750 n*M* rutamycin but not by 1 m*M* HNDCA. The addition of 2 m*M* potassium ion raised the ATPase activity induced by gramicidin. Either HNA or rutamycin again inhibited the ATPase activity completely while HNDCA only eliminted the additional ATPase activity introduced by po-

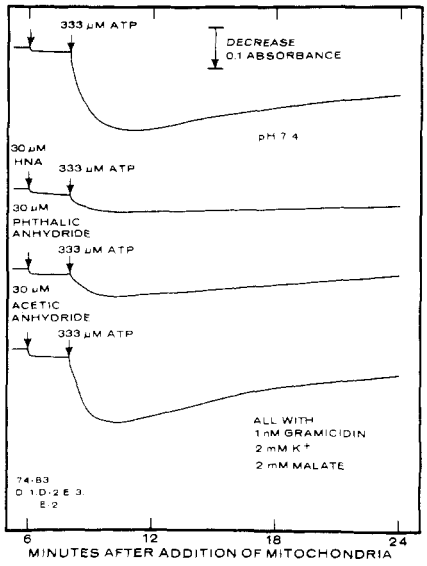

FIGURE 3: Comparison of various anhydrides on gramicidin induced mitochondrial swelling. See legend of Figure 1 for basic conditions.

Table I: Inhibition of Gramicidin Induced ATPase.[a]

	Additions			
	None	Rutamycin	HNA	HNDCA
Gramicidin	9	2	4	8
Gramicidin plus potassium ion	16	2	4	8
Gramicidin plus malate	9	2	4	8
Gramicidin plus potassium ion and malate	15	2	4	7

[a] A summary of experiments similar to those shown in Figure 2. The amounts of the various substances and conditions are identical. The numbers are nmoles of P_i per ml, per min derived from the various slopes. No swelling was observed except that shown in Figure 2.

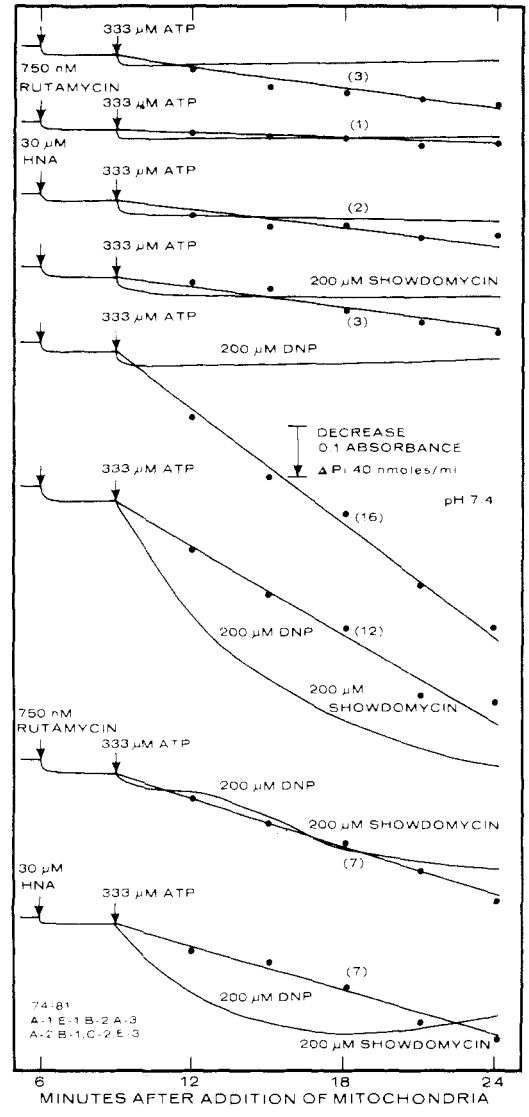

FIGURE 4: HNA and rutamycin inhibition of DNP plus showdomycin induced ATPase and mitochondrial swelling. See legend of Figure 1 for basic conditions. DNP and showdomycin, where indicated, were present in the cuvette before the mitochondria were added. The numbers above the ATPase traces are nmol of P_i per ml per min derived from the slopes.

tassium ion. The inclusion of malate had little added effect upon the ATP energized system and the results are almost identical with that of the gramicidin alone series. When 1 n*M* gramicidin is present with both 2 m*M* potassium ion and 2 m*M* malate there was not only an ATPase reaction comparable to gramicidin plus potassium ion but also a large increase in mitochondrial volume. HNA and rutamycin totally inhibited not only the ATPase reaction but also the mitochondrial volume change. HNDCA also totally inhibited the swelling but again only eliminated the additional ATPase activity due to the introduction of potassium ion.

It is seen in Figure 3 that 30 μM phthalic anhydride partially inhibited the ATP energized gramicidin induced mitochondrial swelling while 30 μM acetic anhydride had no effect. The inhibition by 30 μM HNA, as before (Figure 1), was total.

The ATP energized DNP plus showdomycin induced mitochondrial swelling system which does not require additional ions (Figure 4) was markedly inhibited by 30 μM HNA. There was only slight inhibition of this system by 1 μM HNA (data not shown). It was also observed that 30 μM phthalic anhydride and 30 μM acetic anhydride had no inhibitory effect on the swelling of this system (data not shown). The inhibitory effect of 30 μM HNA was comparable to that of 750 n*M* rutamycin on the ATPase activity of the DNP plus showdomycin system (Figure 4). In Figure 5 it is seen that 30 μM HNA completely inhibited the ATPase activity induced by DNP (in a range from 2 to 200 μM) and this inhibition was again comparable to that of 750 n*M* rutamycin.

The ATPase activity induced by magnesium ions acting on submitochondrial particles was inhibited to a comparable degree by either 30 μM HNA or 750 n*M* rutamycin. On the other hand, atractyloside, a known inhibitor of the translocation of ATP across intact inner mitochondrial membranes, did not inhibit the reaction (Figure 5).

Increasing amounts of HNA (from 1 to 30 μM) progressively inhibited state 3 (coupled) respiration (data not shown). In Figure 7 it is seen that 30 μM HNA inhibited state 3 respiration but not quite as well as 750 n*M* rutamycin and that 30 μM HNA inhibited slightly DNP uncoupled respiration while 750 n*M* rutamycin has no effect on

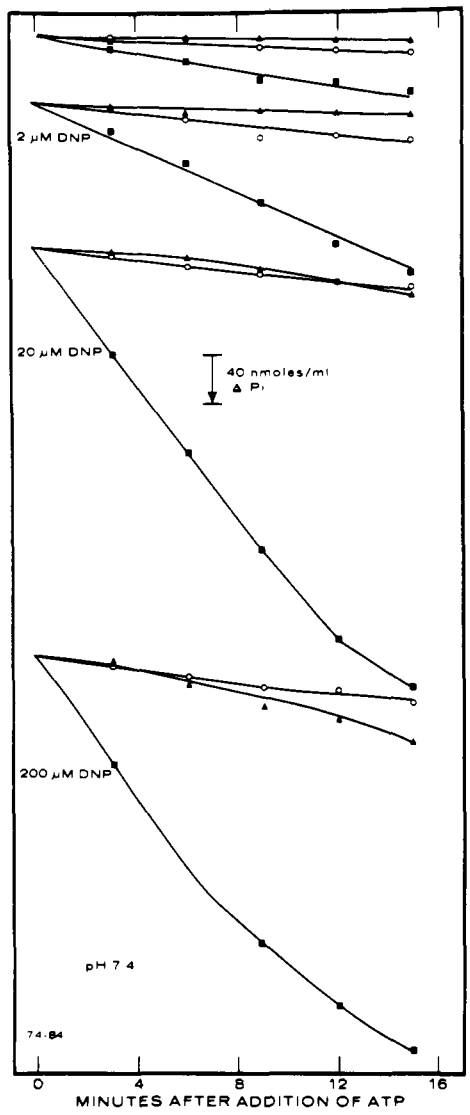

FIGURE 5: HNA and rutamycin inhibition of DNP induced ATPase. See legend of Figure 1 for basic conditions. (○) HNA, (▲) rutamycin, or (■) acetone was added 2 min before the addition of ATP. The mitochondria were added 8 min before the addition of ATP.

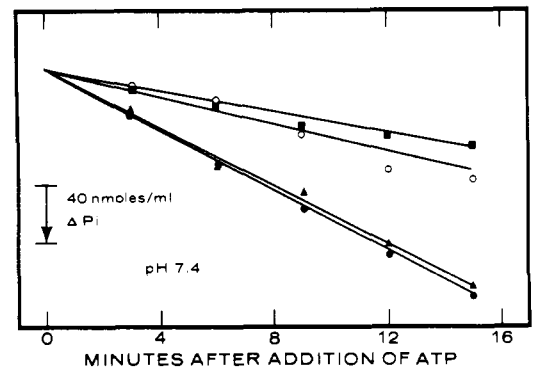

FIGURE 6: HNA, rutamycin, and atractyloside inhibition of magnesium ion induced ATPase in submitochondrial particles. The basic medium (see Materials and Methods) was used plus 1 m*M* magnesium ion. (○) HNA, (■) rutamycin, (▲) atractyloside, or (●) acetone control was added 2 min before the addition of ATP. Submitochondrial particles (1.20 mg of protein) were added 8 min before the addition of ATP. Final volume was 6 ml.

this mitochondrial state. Phthalic anhydride, acetic anhydride, and succinic anhydride at levels of 30 μM did not affect state 3 respiration (data not shown).

Discussion

The oligomycin family of antibiotics which includes rutamycin (Lardy et al., 1965) has been established as unique inhibitors of coupled oxidative phosphorylation (Lardy et al., 1958). The site of action of rutamycin has been located to be between the mitochondrial target of DNP and the locale of phosphorylation (Henderson and Lardy, 1970). HNA at a level as low as 15 μM behaved like rutamycin and inhibited the ATP energized mitochondrial volume change induced by gramicidin in the presence of appropriate permeant ions (Figures 1 and 2). In the absence of either one or both of the permeant ions (potassium ion and malate) the ATPase activity induced by gramicidin was totally inhibited by 30 μM HNA or 750 nM rutamycin (Figure 2 and Table I). This included the added ATPase activity introduced by potassium ion.

HNA at a level of 30 μM resembled the inhibitory effect of rutamycin on the ATP energized mitochondrial volume change and associated ATPase activity induced by DNP plus showdomycin (Figure 4). This phenomenon is clearly a partial reaction of oxidative phosphorylation located on the ATP side (i.e., not on the respiratory chain side) of the site of action of DNP (Hadler et al., 1968).

Both the mitochondrial ATPase engendered by DNP and the ATPase induced by magnesium ion on submitochondrial particles were inhibited to the same extent by 30 μM HNA and 750 nM rutamycin (Figures 5 and 6). The result with the submitochondrial particles clearly distinguished between the mode of action of HNA and atractyloside as the latter substance was as expected (Löw et al., 1963, Henderson and Lardy, 1969) ineffective (Figure 6) in this system.

While 30 μM HNA was an inhibitor of state 3 (coupled) respiration (Figure 7) its action was not quite as specific as 750 nM rutamycin as the DNP relieved respiration was slightly inhibited by HNA and not at all inhibited by 750 nM rutamycin.

The inhibitory properties of HNA were not exhibited by comparable levels of phthalic anhydride or acetic anhydride either on the gramicidin system (Figure 3), on the DNP plus showdomycin system, or on state 3 (coupled) respiration. In addition 30 μM succinic anhydride did not affect state 3 respiration.

The ATP energized mitochondrial volume change induced by gramicidin in conjunction with appropriate permeant ions was inhibited by a relatively higher level (1 mM) of HNDCA (Figure 2). Unlike HNA, HNDCA inhibited the "added" ATPase associated with potassium ion (Figure 2 and Table I) and hence may be associated with a blockage of the transport of potassium ion. The decided inhibition of the ATP energized gramicidin system exhibited by 30 μM phthalic anhydride (Figure 3) may also be related in part to potassium ion transport phenomena.

Dicyclohexylcarbodiimide also has inhibitory properties resembling oligomycin (Beechey et al., 1966, 1967). There may be a relationship between the mode of action of HNA and dicyclohexylcarbodiimide as the latter compound is known to convert carboxylic acids to anhydrides (Khorana, 1961). The structural specificity noted above for the action of HNA may eventually provide a clue as to the nature of its mode of action.

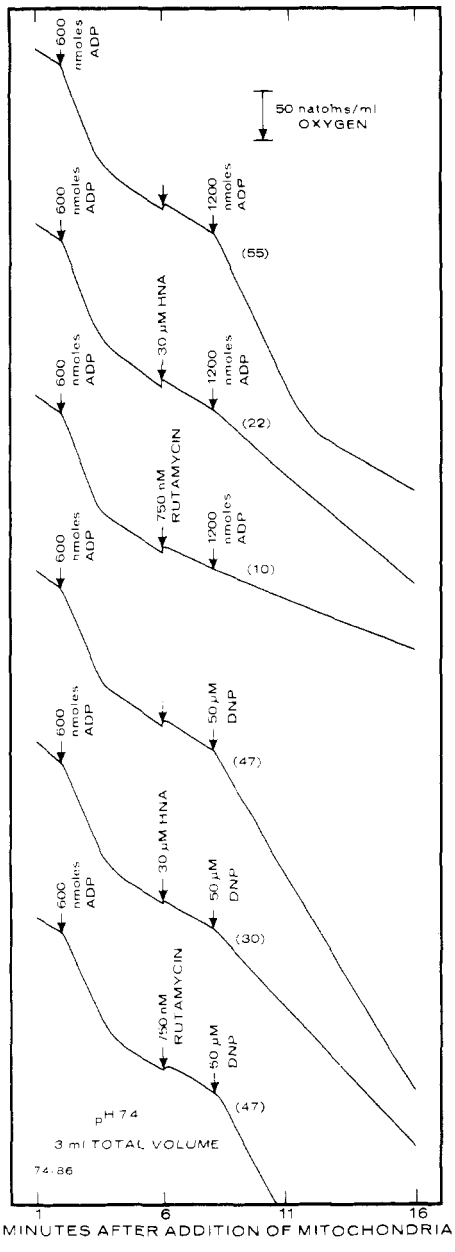

FIGURE 7: The effects of HNA and rutamycin upon state III and DNP uncoupled respiration. The final concentrations were: 135 m*M* sucrose, 75 m*M* Tris-chloride (pH 7.4), 2 m*M* Tris-phosphate (pH 7.4), 12 m*M* Tris β-hydroxybutyrate (pH 7.4). ADP or HNA (in acetone) was added in a volume of 0.02 ml via a micro syringe fitted with a stainless steel needle (20 gauge) as indicated by arrows. The final volume of the system was 3 ml. The mitochondrial protein concentration was 1.0 mg/ml. DNP, where indicated, was added as Tris salt (pH 7.4) in a volume of 0.02 ml.

HNA is the first substance closely related to the metabolism of a carcinogen which blocks the flux of energy in the process of oxidative phosphorylation at a site associated with the unique action of the oligomycin group of inhibitors.

HNA has been reported to be inactive as a carcinogen (Heidelberger and Wiest, 1951). This does not negate a role for HNA in carcinogenesis as administering HNA subcutaneously does not mean that HNA has been delivered to the appropriate target inside the cell. Indeed one way of circumventing the uncertainty of delivery is to assemble correlative in vitro data such as that which initiated this study.

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