THE INDUCTION OF ATP ENERGIZED MITOCHONDRIAL VOLUME CHANGES BY SHOWDOMYCIN WHEN COMBINED WITH 4′,8′-DIHYDROXY-1,2,5,6-DIBENZ-9,10-ANTHRAQUINONE, A METABOLITE OF THE CARCINOGENIC POLYNUCLEAR HYDROCARBON DIBENZ(A, H) ANTHRACENE *,**,***

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The hydroxy quinone DI-OH-DBAQ [4′,8′-dihydroxy-1,2,5,6-dibenz-9,10-anthraquinone] was shown to be a metabolite of the carcinogenic polynuclear hydrocarbon DBA [dibenz(a, h)anthracene] in 1953 by HEIDELBERGER, HADLER and WOLF. DI-OH-DBAQ induces an ATP energized mitochondrial volume change in combination with the nonmercurial thiol reagent, showdomycin, [an antibiotic and an antitumor agent]. The parent carcinogen DBA when combined with showdomycin does not induce an ATP energized mitochondrial volume change. An appropriate concentration of DI-OH-DBAQ inhibits the ATP energized mitochondrial volume change induced by gramicidin in the presence of the permeant ions potassium and malate. The further addition of showdomysin reinstates the effect of gramicidin. Thus DI-OH-DBAQ exposes the strategically located pivotal mitochondrial thiol group which occupies the position between the cycle which meshes with the respiratory chain and a cycle which meshes with ATP. In these *in vitro* studies DI-OH-DBAQ bears the same relationship to DBA that N-hydroxy-N-acetyl-2-aminofluorene bears to N-acetyl-2-aminofluorene an aromatic amide which is also a carcinogen. DI-OH-DBAQ is an uncoupling agent rather than a

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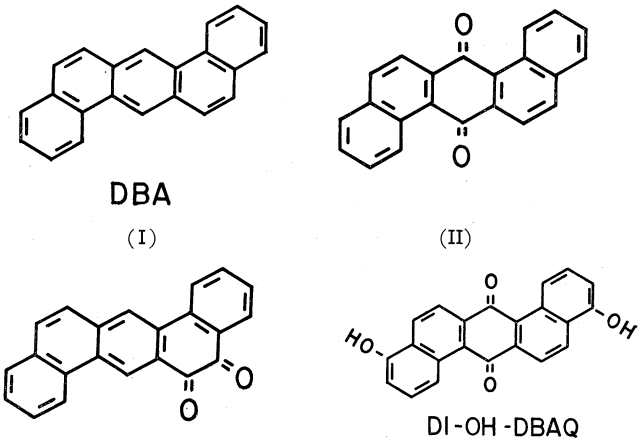
*** Abbreviations used :

ATP	adenosine-5′-triphosphate
ADP	adenosine-5′-diphosphate
Pi	inorganic phosphate
DNP	2,4-dinitrophenol
PHMB	*p*-hydroxymercuribenzoate
pFCCP	carbonyl cyanide *p*-triflurormethoxyphenylhydrazone
NEMI	N-ethylmaleimide
DBA	dibenz(a,h)anthracene
DI-OH-DBAQ	4′,8′-dihydroxy-1,2,5,6-dibenz-9,10-anthraquinone also known as 4,11-dihydroxy-dibenz(a,h)anthracene-7,14-dione
AAF	N-acetyl-2-aminofluorene
N-OH-AAF	N-hydroxy-N-acetyl-2-aminofluorene
Mw	Mitochondria

respiratory inhibitor as DI-OH-DBAQ stimulates mitochondrial respiration and induces the mitochondrial hydrolysis of adenosine-5′-triphosphate [ATPase activity]. These observations broaden the support for our hypothesis of oxidative phosphorylation (1961), the experimental confluence of oxidative phosphorylation with carcinogenesis and our unitary hypothesis for carcinogenesis (1971).

HEIDELBERGER, HADLER and WOLF[1] reported in 1953 that the carcinogenic polynuclear hydrocarbon DBA (I) was converted metabolically *in vivo* by the mouse into the L-region quinone (II), the K-region quinone (III) and a hydroxyquinone, DI-OH-DBAQ (IV). The latter compound was first thought of as a metabolite by DAUBEN and TANABE[2] and implicated as a metabolite by the work of WIEST and HEIDELBERGER[3].



In 1961 HADLER[4] published an hypothesis for oxidative phosphorylation. In this paper a catalytic role was assigned to quinones, hydroquinones, hydroxyquinones, and thiol derivatives of these compounds. It was also noted that the hydroxyquinone, DI-OH-DBAQ, was a metabolite of a carcinogen.

For some time now[5~12], antibiotics including the non mercurial thiol reagent showdomycin[13] and other agents of known structure have been used to identify mitochondrial functional groups participating in oxidative phosphorylation and the related processes of ion transport and energized mitochondrial volume change. Studies have shown that the exposed thiol group occupies a pivotal position betwen two catalytic cycles[10]. One cycle meshes with the respiratory chain and the other cycle meshes with ATP, ADP and Pi. These ideas have recently been collected[14].

A consideration of our hypothesis for oxidative phosphorylation and our rationalization regarding the pivotal mitochondrial thiol group suggested that a foreign hydroxyquinone might compete with the endogenous hydroxyquinone and thus interfere with the cycle which meshes with the respiratory chain. Should such an interaction occur, the foreign hydroxyquinone would be expected to expose the pivotal mitochondrial thiol group. The exposed pivotal mitochondrial thiol group could then be detected as before by inducing an ATP energized mitochondrial volume change on the further addition of a thiol reagent, such as showdomycin. These considerations led to the successful demonstration[11] that the hydroxyquinone lapachol (an antitumor agent) when combined with showdomycin induced an ATP energized mitochondrial volume change.

A significant extension of these experiments would be to learn whether the hydroxyquinone, DI-OH-DBAQ would also expose a mitochondrial thiol group. Such an interaction could be detected by inducing an ATP energized mitochondrial volume change by the combination of DI-OH-DBAQ with the thiol reagent showdomycin.

In order to evaluate the relationship of such a possible observation to the process of carcinogenesis, the metabolism of other carcinogens distinctly different from the carcinogenic polynuclear hydrocarbons were also considered. The acidic nature of hydroxyquinones (they are vinylogues of carboxylic acids and should not be confused with hydroquinones)

and other agents such as DNP, antimycin, pFCCP, and dicoumarol, which interacted with the cycle which meshes with the respiratory chain, directed our attention to acidic metabolites of other carcinogens. From this point of view the metabolite of choice derived from the well studied carcinogen AAF was the hydroxamic acid, N-OH-AAF. We have recently reported that N-OH-AAF does indeed expose a pivotal mitochondrial thiol group[14]. This observation suggested to us a confluence of experimentation in oxidative phosphorylation and carcinogenesis.

In order to bridge the gap between viral carcinogenesis and chemical carcinogenesis we proposed a unitary hypothesis of carcinogenesis[14]. According to this hypothesis, as a consequence of a subtle disturbance of the machinery of oxidative phosphorylation, mitochondrial genetic material may leak through the mitochondrial membrane. The released genetic material could then act like an oncogenic entity such as an oncogenic virus and enter the genome of the cell. The symbiotic relationship established through evolution between mitochondria and the host cell would thus be disrupted and *evolution would be partially reversed.* Our unitary hypothesis is compatible with the suggestion of HUEBNER and TODARO[15] that chemical carcinogens activate a latent oncogenic virus present in every normal cell. Others have also suggested that chemical carcinogens activate a latent virus, as mentioned in HEIDELBERGER's review[16].

It is shown in this investigation that DI-OH-DBAQ exposes the mitochondrial thiol group. This not only broadens the support for our hypothesis of oxidative phosphoryla-

Fig. 1. DI-OH-DBAQ combined with showdomycin and effect of oligomycin.

The cuvettes contained the basic medium plus the common components and oligomycin (when noted) prior to the addition of mitochondria. The addition of showdomycin (in water) or the control amount of water was made (adding-mixing device) five minutes after the mitochondria and the DI-OH-DBAQ (in dimethylsulfoxide) or the control amount of dimethylsulfoxide were added (adding-mixing device) 9 minutes after the addition of mitochondria.

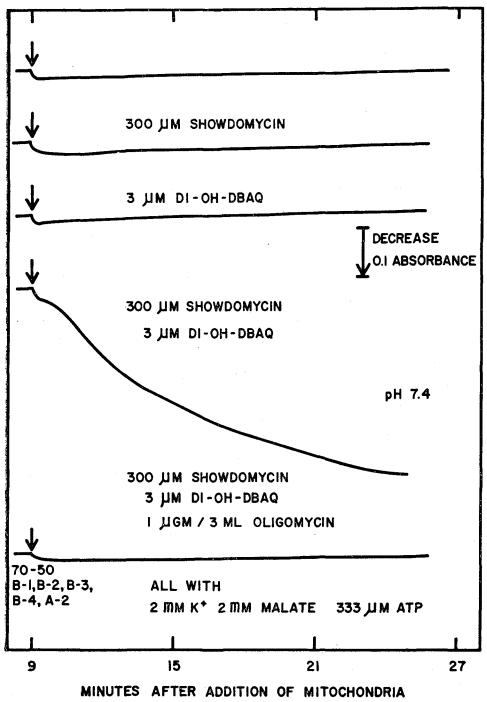
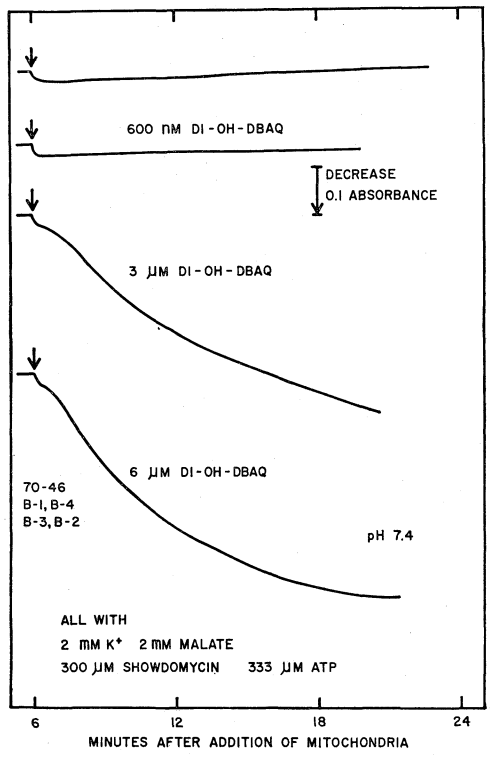


Fig. 2. Various concentrations of DI-OH-DBAQ at pH 7.4.

The cuvettes contained the basic medium plus the common components prior to the addition of mitochondria. DI-OH-DBAQ (in dimethylsulfoxide) or the control amount of dimethylsulfoxide were added (adding-mixing device) 6 minutes after the mitochondria.



tion, but also lends more substance to our unitary hypothesis for carcinogenesis and adds to the generality of the experimental confluence of oxidative phosphorylation with carcinogenesis.

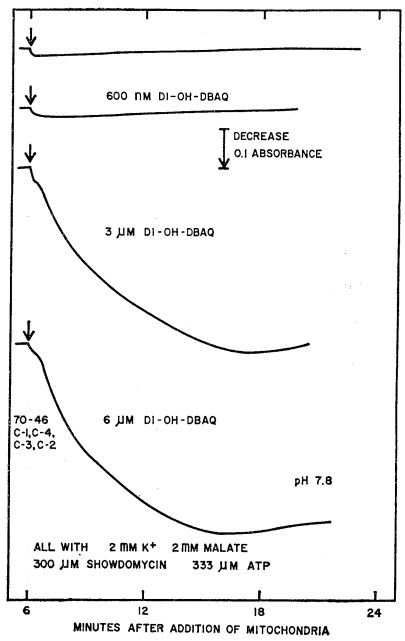
Methods

The general procedures, methods, and purification of the water have been previously described, for the mitochondrial volume change experiments[5,9]. The pH of the trischloride buffer is indicated on the figures. Incubation was at 27°C in standard rectangular glass 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 0.75 mg mitochondrial protein (prepared from rat liver); 75 mM sucrose; and 75 mM trischloride buffer. A decrease in absorbancy at 620 nm rather than at 520 nm was taken as a measure of mitochondrial swelling because DI-OH-DBAQ is a colored compound with slight absorbance at 520 nm. A model 2,000 automatic spectrophotometer manufactured by Gilford Instrument Laboratories Incorporated, Oberlin, Ohio was used. All cations were added in the form of chloride salt and anions were added in the form of tris salts neutralized to pH 7.4. Solutions of DBA and DI-OH-DBAQ were prepared in dimethyl sulfoxide (distilled *in vacuo*) and added to the incubation mixture either in a volume of 0.06 ml or 0.03 ml by means of the adding-mixing device. Gramicidin dissolved in dimethylsulfoxide (0.02 ml) was added. All controls contained the appropriate amount of dimethyl sulfoxide. The figures and legends provide additional experimental details.

Oxygen consumption was measured in an oxygen monitor which utilized a CLARK electrode. The instrument (model 53 SA) was manufactured by Yellow Springs Instrument Company, Yellow Springs, Ohio, U.S.A. A 10-inch recorder (model 1005) manufactured by Beckman Instruments, Fullerton, California, U.S.A. was used in conjunction with the oxygen monitor.

Fig. 3. Various concentrations of DI-OH-DBAQ at pH 7.8.

The cuvettes contained the basic medium plus the common components prior to the addition of mitochondria. DI-OH-DBAQ (in dimethylsulfoxide) or the control amount of dimethylsulfoxide were added (adding-mixing device) 6 minutes after the mitochondria.



In the ATPase experiments orthophosphate was measured as previously described[5]. Samples (1 ml) were removed at the appropriate time from the incubation tubes (18×150 mm) kept at 27°C without shaking by means of a spring loaded Cornwall glass syringe fitted with a 6″, 20 gauge stainless steel needle. DI-OH-DBAQ in 0.12 ml of dimethyl sulfoxide was added by means of a large mixing-adding device. In the controls the appropriate amount of dimethylsulfoxide was added. The final volume of the system was 6 ml.

Commercial gramicidin, obtained from Mann Research Laboratory Incorporated, New York, N. Y., U.S.A., was used. The activity of such a mixture of gramicidin has been previously shown to be indistinguishable from gramicidin A in related systems[7]. The molecular weight of the mixture was arbitrarily taken as 1,870. We wish to thank Dr. KEN'ICHI TAKEDA of Shionogi and Company, Osaka, Japan, for the showdomycin used in this work. DBA was purchased from Mann Research Laboratory Inc., New York, N.Y., U.S.A. It was chromatographed on Florisil (the solvent system has been described)[1] crystallized several times from cyclohexane and then sublimed

Fig. 4. Effect of pH.

The cuvettes contained the basic medium, the common components, ATP (when noted) and showdomycin (when noted) prior to the addition of mitochondria. DI-OH-DBAQ (in dimethylsulfoxide) or the control amount of dimethylsulfoxide were added (adding-mixing device) 6 minutes after the mitochondria.

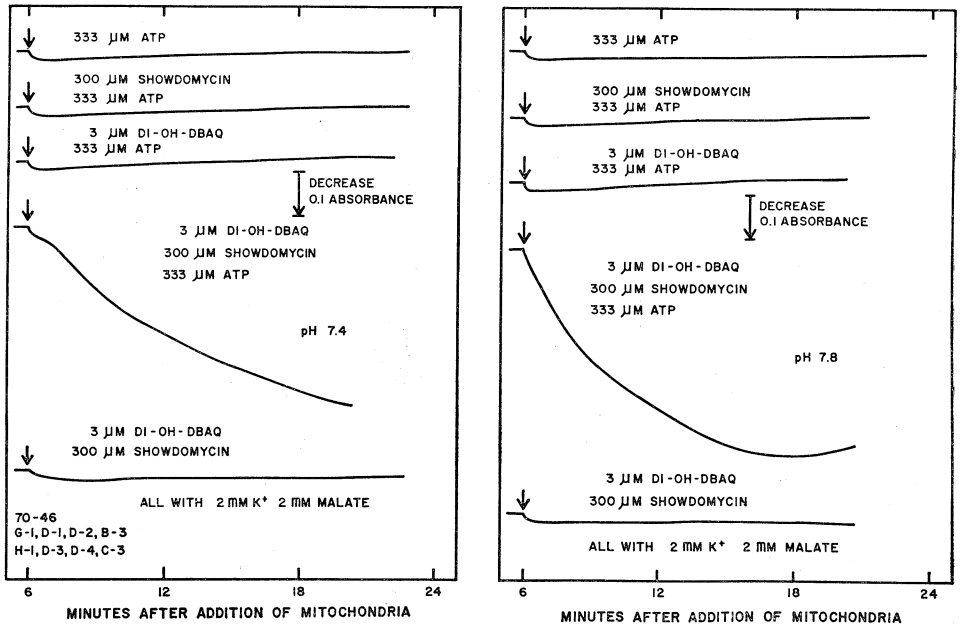
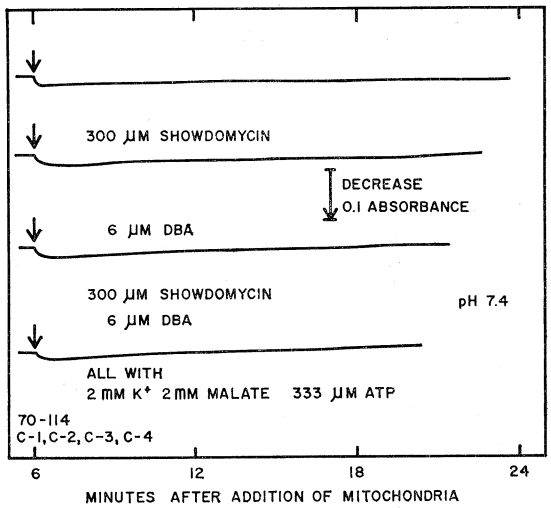


Fig. 5. DBA combined with showdomycin.

The cuvettes contained the basic medium, the common components and showdomycin (when noted) prior to the addition of mitochondria. DBA (in dimethylsulfoxide) or the control amount of dimethylsulfoxide were added (adding-mixing device) 6 minutes after the mitochondria.



[250°C, 0.05 mmHg]. DI-OH-DBAQ was synthesized by the procedure described in the literature[17]. In order to assure the purity of the final product, the two quinone intermediates *viz.*, the L region quinone (II), and 4′, 8′-diacetoxy-1, 2, 5, 6-dibenz-9, 10-anthraquinone were carefully chromatographed on Florisil by means of the solvent systems previously described[1]. In latter preparations 100 % benzene was conveniently replaced by *o*-dichlorobenzene when chromatographing the rather insoluble immediate precursor of DI-OH-DBAQ. The final product, DI-OH-DBAQ, was sublime α (300°C, 0.05 mmHg).

Analytical grades of organic solvents were processed as follows before use. Benzene, cyclohexane, *o*-dichlorobenzene and ethylene dichloride were shaken for several days at room temperature with daily changes of concentrated sulfuric acid until the acid layer remained colorless. The acid was washed out with water, the solvent was then dried with anhydrous sodium sulfate, filtered and distilled in an all-glass apparatus. Acetone was dried with anhydrous sodium sulfate, filtered, refluxed briefly with a small amount of potassium

Fig. 6. Role of ions at pH 7.4.

The cuvettes contained the basic medium, potassium ion (when noted), malate ion (when noted) and the common components except DI-OH-DBAQ prior to the addition of mitochondria. DI-OH-DBAQ (in dimethylsulfoxide) or the control amount of dimethylsulfoxide were added (adding-mixing device) 6 minutes after the mitochondria.

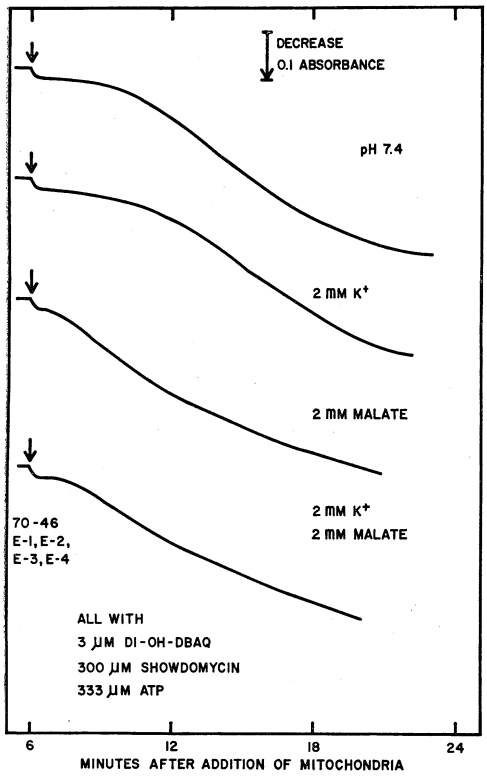
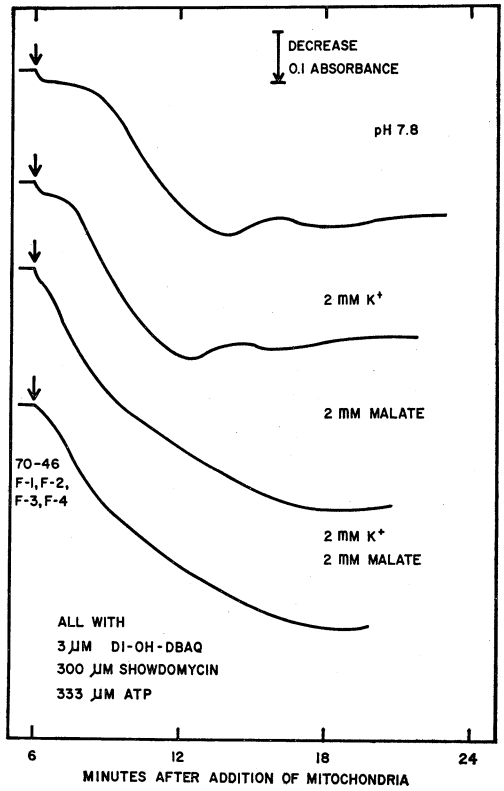


Fig. 7. Role of ions at pH 7.8.

The cuvettes contained the basic medium, potassium ion (when noted), malate ion (when noted) and the common components except DI-OH-DBAQ prior to the addition of mitochondria. DI-OH-DBAQ (in dimethylsulfoxide) or the control amouut of dimethylsulfoxide were added (adding-mixing device) 6 minutes after the mitochondria.



permanganate and then distilled in an all-glass apparatus.

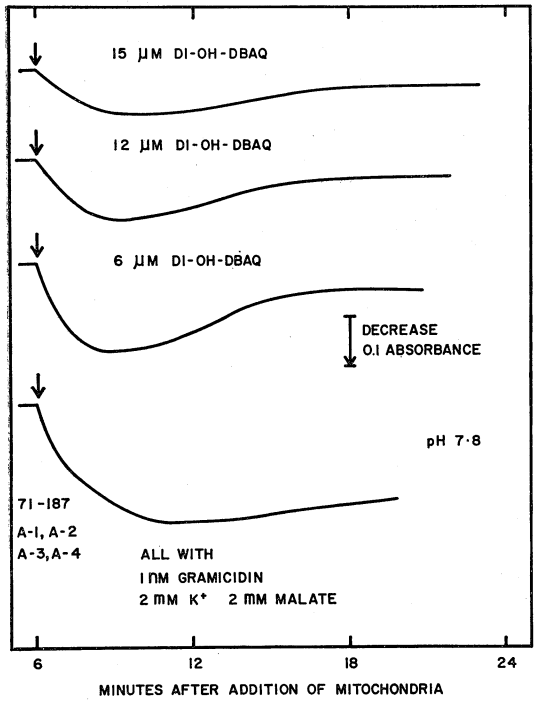
Glassware and cuvettes were cleaned in a warm acid bath (90 % concentrated sulfuric acid, 10 % concentrated nitric acid). Plastic ware were cleaned in warm detergent (ALCOJET). Precautions were taken to prevent the plastic ware from absorbing organic material. We have found these precautions to be critical.

Results

It is seen in Fig. 1 that while neither 300 μM showdomycin nor 3 μM DI-OH-DBAQ by themselves induce an ATP energized mitochondrial volume change the combination of these two agents induce an ATP energized mitochondrial volume change. The further addition of oligomycin inhibits the effect induced by the combination of agents. At pH 7.4 (Fig. 2) and at pH 7.8 (Fig. 3) the effect in the presence of 300 μM showdomycin is enhanced when the concentration of DI-OH-DBAQ is increased from 600 ηM to 6 μM. It is seen in Fig. 4 that the phenomena is increased by raising the pH from 7.4 to 7.8. Also if ATP is deleted no volume change is induced by the combination of 3 μM DI-OH-DBAQ with 300 μM showdo-

Fig. 8. Inhibition of the gramicidin system by DI-OH-DBAQ.

The cuvettes contained the basic medium, the common components plus 333 μM ATP prior to the addition of mitochondria. The gramicidin (in dimethylsulfoxide) the DI-OH-DBAQ (in dimethylsulfoxide) or the control amount of dimethylsulfoxide were added (adding-mixing device) 6 minutes after the addition of the mitochondria.



mycin. The parent carcinogen DBA (6 μM) unlike its metabolite DI-OH-DBAQ when combined with 300 μM showdomycin did not induce an ATP energized mitochondrial volume change (Fig. 5). The characteristic effect of potassium ion and malate ion at pH 7.4 is not too apparent (Fig. 6) however at pH 7.8 (Fig. 7) the addition of potassium has only a very slight effect while the addition of malate increases the period and amplitude of the oscillation in the usual manner[9]. It is seen in Fig. 8 that as the concentration of DI-OH-DBAQ is increased there is progressive inhibition of the ATP energized mitochondrial volume change induced by gramicidin. The inhibition of the gramicidin system by DI-OH-DBAQ is relieved by the further addition of 300 μM showdomycin (Fig. 9). Also the relieved system which contains gramicidin plus DI-OH-DBAQ plus showdomycin is stimulated by the addition of potassium ion in agreement with previous observations made with similar systems[10,11].

In all instances DI-OH-DBAQ (in dimethylsulfoxide) was added by means of an adding-mixing divice to a suspension of mitochondria in a cuvette. If the DI-OH-DBAQ was added to the cuvette before the mitochondria the DI-OH-DBAQ was much less active, likely because of insolubility. The mitochondria thus possesses the ability to absorb DI-OH-DBAQ under appropriate conditions.

An increase in the rate of mitochondrial respiration on the addition of DI-OH-DBAQ is seen in Fig. 10. This increased rate corresponds well to that characteristic of the state of active phosphorylation induced by the previous addition of ADP. The oxygen uptake is inhibited by the further addition of the respiratory inhibitor antimycin. Thus DI-OH-DBAQ is an uncoupling agent rather than a respiratory inhibitor.

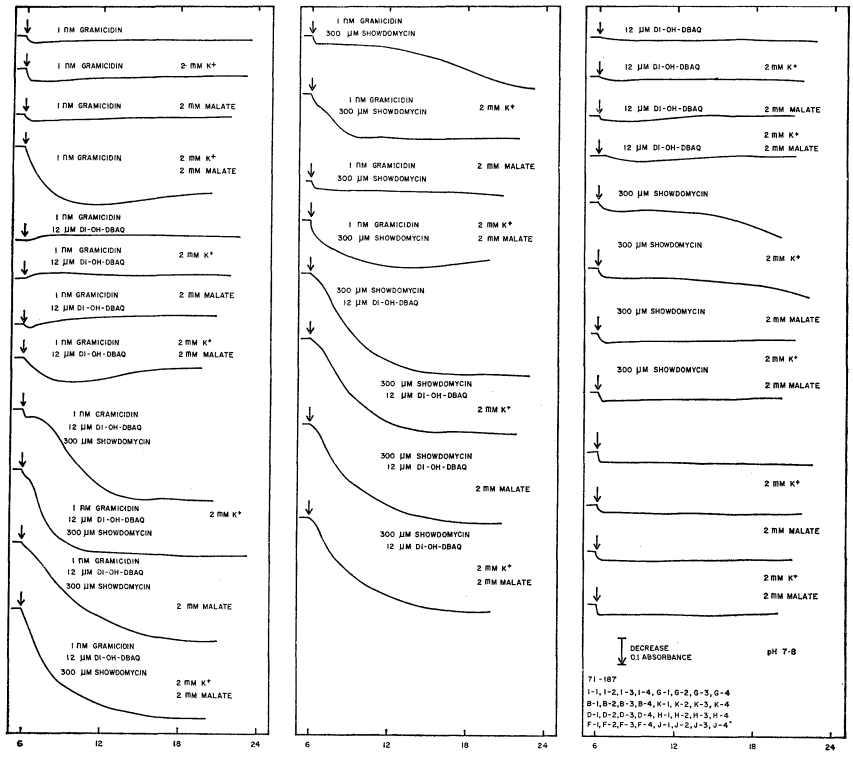
The addition of DI-OH-DBAQ to a mitochondrial system, without showdomycin, induces ATPase activity which is inhibited by oligomycin (Fig. 11).

Discussion

DI-OH-DBAQ like a respiratory inhibitor such as antimycin[8] or an uncoupling agent such as DNP[9] exposes the mitochondrial thiol group which can conjugate with the

Fig. 9. Reinstated system and role of ions.

The cuvettes contained the basic medium, 333 μM ATP, potassium ion (when noted), malate ion (when noted) and showdomycin (when noted) prior to the addition of mitochondria. The gramicidin (in dimethylsulfoxide), the DI-OH-DBAQ (in dimethylsulfoxide) or the control amount of dimethylsulfoxide were added (adding-mixing device) 6 minutes after the addition of the mitochondria.



non-mercurial thiol reagent showdomycin and induce an ATP energized mitochondrial volume change. The induced effect is enhanced by increasing the concentration of DI-OH-DBAQ and also by raising the pH, in keeping with the postulated role for an ionizable nucleoplic thiol group. The ATP energized phenomena is inhibited by oligomycin thus demonstrating the involvement of the machinery of oxidative phosphorylation.

The parent carcinogen DBA does not expose a mitochondrial thiol group, as DBA by itself or in combination with showdomycin does not induce an ATP energized mitochondrial volume change. Clearly, the exposure of a mitochondrial thiol group is a property of DI-OH-DBAQ and not a property of DBA.

The mitochondrial thiol group exposed by DI-OH-DBAQ is capable of inhibiting the ATP energized mitochondrial volume change induced by gramicidin. When this exposed mitochondrial thiol group has the opportunity to react with showdomycin, the gramicidin system is reinstated. In the reinstated gramicidin system, that is, the system composed of gramicidin plus DI-OH-DBAQ plus showdomycin, potassium ion has a stimulatory effect. Based upon our previous rationalization[10], DI-OH-DBAQ exposes a strategically located mitochondrial thiol group which occupies a pivotal position between the cycle which meshes with the respiratory chain and the cycle which meshes with ATP, ADP and Pi.

Studies involving mitochondrial respiration were necessary in order to determine whether DI-OH-DBAQ was a respiratory inhibitor such as antimycin, or an uncoupling agent such as DNP, as the ATP energized mitochondrial volume change data does not distinguish between these two possibilities. DI-OH-DBAQ stimulates mitochondrial

Fig. 10. Effect of DI-OH-DBAQ on mitochondrial respiration.

The final concentrations were sucrose 0.25 M; KCl 20 mM; $MgCl_2$ 5 mM; potassium phosphate buffer (pH 7.2) 10 mM; Tris chloride (pH 7.4) 20 mM; β hydroxybutyric acid 13 mM; DI-OH-DBAQ was added (syringe) in 0.06 ml of dimethylsulfoxide. There were 2.5 mg of mitochondrial protein. The dimethylsulfoxide in other control experiments did not affect the rate of respiration. The antimycinwas added (syringe) in 0.02 ml of 95% ethanol. The final volume was 3 ml.

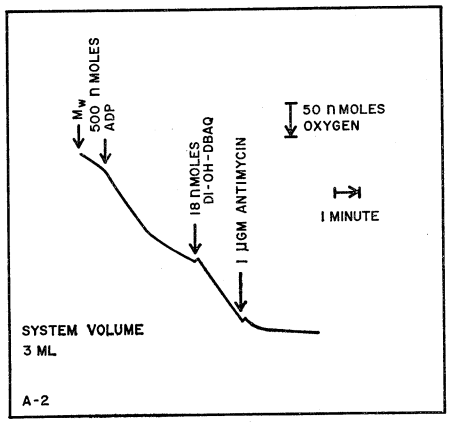
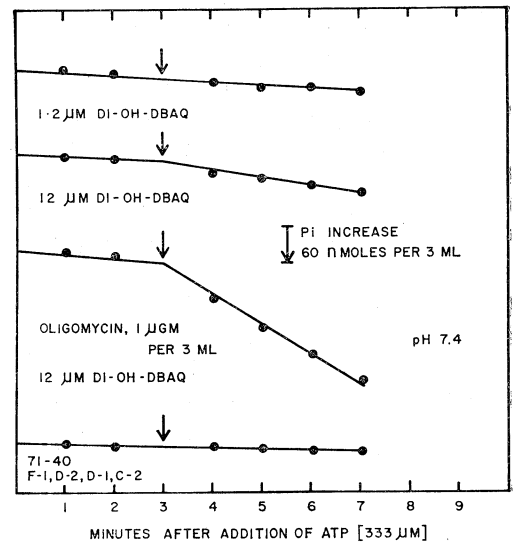


Fig. 11. Induction of ATPase activity by DI-OH-DBAQ and effect of oligomycin.

The test tube cotained the basic medium, plus 2 mM potassium ion, 2 mM malate, and oligomycin (in 95% ethanol when noted) or the control amount of 95% ethanol prior to the addition of the mitochondria. After the tubes were kept at 27℃ for 4 minutes the mitochondria were added. The ATP (333 µM final concentration) was added 1 minute after the mitochondria.



respiration; thus DI-OH-DBAQ is categorized as an uncoupling agent. Like DNP[19], DI-OH-DBAQ also induces a mitochondrial ATPase activity. Further evidence that these two activities induced by DI-OH-DBAQ were related to oxidative phosphorylation was obtained when, a) the respiration stimulated by DI-OH-DBAQ was inhibited by antimycin, an agent established to interact with the respiratory chain[19], b) the ATPase activity induced by DI-OH-DBAQ was inhibited by oligomycin, an agent established to interact with the phosphoryl transfer site of oxidative phosphorylation[18].

This role for DI-OH-DBAQ is in harmony with our previous hypothesis for oxidative phosphorylation[4] which implied that a mitochondrial thiol group could be exposed when the cycle meshing with the respiratory chain is disturbed by a foreign hydroxyquinone which competes with the endogenous hydroxyquinone. The ability of a bipolar agent such as DI-OH-DBAQ (with two acidic hydroxyl groups) to interact with the machinery of oxidative phosphorylation and hence to have penetrated the mitochondria is noteworthy in view of the recent report by REED and LARDY[20] who observed that monopolar indole-derivatives such as 5-methoxyindole-2-carboxylic acid and indole-2-carboxylic acid were absorbed by mitochondria while a bipolar derivative such as 5-hydroxyindole-2-carboxylic acid was not absorbed. Possibly, the phenomena of keto-enol tautomerism, which is a significant phenomena in polycyclic hydroxyquinones[21] and important to our hypothesis of oxidative phosphorylation[4] converts one or both of the acidic hydroxyl groups to neutral carbonyl groups at the appropriate stage of, entry through or interaction with, mitochondria.

In our mitochondrial system the activity of DI-OH-DBAQ a metabolite of DBA resembles the activity of N-OH-AAF which is a metabolite of the carcinogen AAF. While the two carcinogens, DBA and AAF, do not expose a pivotal mitochondrial thiol group, their two appropriate metabolites, each exposes a pivotal mitochondrial thiol group in spite of the otherwise dicotomous behavior of the metabolites on mitochondrial

respiration. The metabolite of the hydrocarbon is a uncoupling agent and stimulates respiration while the metabolite of the amide is a respiratory inhibitor and hinders respiration. The two distinctly different carcinogens have two distinctly different metabolites which have as their intracellular target the same intracellular organelle, namely, the mitochondria. Both sets of observations are encompassed by our unitary hypothesis for carcinogenesis[14]. We therefore suggest that DI-OH-DBAQ which is a metabolite of the carcinogenic hydrocarbon DBA is significant in the carcinogenic process engendered by DBA and that DI-OH-DBAQ bears the same relationship to DBA that N-OH-AAF bears to AAF.

It is now appropriate to search for other hydroxyquinones which may be metabolites of other carcinogenic polynuclear hydrocarbons.

We plan to present in the future additional data dealing with the relationship of our *in vitro* mitochondrial system to the mechanism of carcinogenesis. The concept of the release of intraorganelluar genetic material[14] may be a useful vantage point for the examination of aging and other degenerative conditions of complex organisms. This suggestion is compatible with that made by HUEBNER and TODARO[15]. Environmental assaults have been implicated in the process of aging[22].

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