THE MODE OF ACTION OF DINITROPHENOL REVEALED BY MITOCHONDRIAL VOLUME CHANGES REQUIRING ROTENONE OR ANTIMYCIN OR DINITROPHENOL WITH SHOWDOMYCIN

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Mitochondrial volume changes induced by the antibiotic showdomycin and energized by ATP require the addition of the respiratory inhibitor antimycin A [HADLER, H. I.; B. E. CLAYBOURN and T. P. TSCHANG ; Biochem. Biophys. Res. Comm., 31 : 25 (1968)]. The requirement for the antibiotic antimycin A may be replaced by the uncoupling agent 2, 4-dinitrophenol. Indeed, damped oscillatory ATP energized mitochondrial volume changes may be induced by the combination of showdomycin plus dinitrophenol. It is proposed that both antimycin A and dinitrophenol expose the same strategically located mitochondrial thiol group which conjugates with the maleimide moiety present in showdomycin. This unexpected flux of useful chemical energy dependent upon dinitrophenol is contrary to several suggestions in the literature regarding the mode of action of dinitrophenol. The postulated high energy intermediate cleaved or not formed because of the action of dinitrophenol [LARDY, H. A. and C. A. ELVEHJEM ; Ann. Rev. Biochem. 14 : 1 (1945), SLATER, E. C. ; Proc. Int. Congr. Biochem. 5th Moscow, 1961, 5 : 325, (1963)] is now indicated to be a thiol conjugate which meshes with the respiratory chain.

The use of antibiotics of know structure but with widely differing functional groups has been initiated in order to identify, in the intact mitochondrion, functional groups which participate in oxidative phosphorylation, ion transport and mitochondrial volume changes. The action of the antibiotic gramicidin a neutral linear modified polypeptide has been attributed to the reaction of its terminal hydroxyl group with an electrophilic center in the mitochondrion[1,2,3,4]. The action of the antibiotic showdomycin has been attributed to the addition of a mitochondrial thiol group to the maleimide moiety of showdomycin[4]. The latter report also established that the antibiotic antimycin exposed a mitochondrial thiol group for reaction with showdomycin.

Showdomycin is only effective in the presence of the respiratory chain inhibitor antimycin[4]. In sharp contrast we now report conditions whereby ATP (adenosine-5′-triphosphate) energized mitochondrial volume changes induced by showdomycin are dependent upon DNP (2,4-dinitrophenol). It is well known that this classical uncoupling agent accelerates respiration and simultaneously deters the flux of useful chemical energy from respiration to phosphorylation[5,6]. As a consequence of their common effect when combined with showdomycin we propose that DNP and antimycin

expose the same mitochondrial thiol group to showdomycin. The uncoupling action of DNP accordingly is due to the inability of this strategically located mitochondrial thiol group to conjugate with or remain conjugated with its normal electrophilic acceptor which is generated by cyclical reactions meshing with the respiratory chain. In further support of our thesis we also report that the antibiotic showdomycin may be replaced by the mercurial thiol reagent PHMB (para hydroxymercuribenzoate) *i. e.*, ATP energized mitochondrial volume changes are induced by PHMB combined with antimycin or PHMB combined with DNP but not by PHMB alone.

Methods

The procedures were those which have been previously described[1,2,3,4] except that house distilled water was passed through a mixed bed resin and then distilled again from potassium permanganate. Incubations were at 27°C in standard rectangular quartz curvettes with a 1-cm light path. The final basic reaction mixture had a volume of 3 ml and contained 1.5 mg of mitochondrial protein (prepared from rat liver[1]); 333 μM tris ATP which was added in 0.05 ml by means of the adding-mixing device[2] as indicated by an arrow on the diagrams; 75 mM sucrose; and 75 mM trischloride at pH 8.2. A decrease in absorbancy at 520 mμ was considered to be a measure of mitochondrial swelling. A model 2000 automatic spectrophotometer manufactured by Gilford Instrument Laboratories, In-

Fig. 1. Requirements for respiratory inhibitors or DNP.
Concentrations, antimycin A, 1 μg per 3 ml, L malate 2 mM, basic medium (see methods)

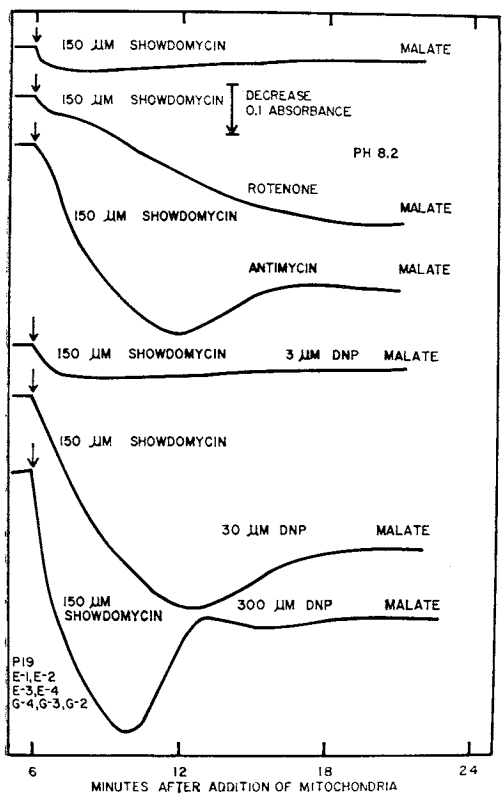
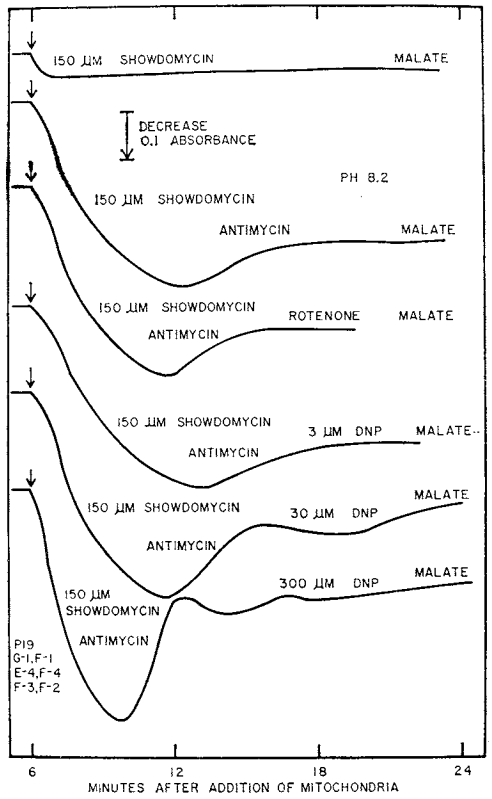


Fig. 2. Combination of inhibitors and DNP.
Concentrations, antimycin A, 1 μg per 3 ml, rotenone 3.3 μM, L malate 2 mM, basic medium (see methods)



corporated, Oberlin, Ohio, was used. All cations were added in the form of chloride salts and all anions were added in the form of tris salts neutralized to pH 7.4[1]. The figures and their legends provide further experimental details.

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Results

Mitochondrial volume changes energized by ATP at pH 8.2 require in addition to malate plus showdomycin either the respiratory inhibitors rotenone or antimycin, or an adequate amount of DNP (Fig. 1). Antimycin is more effective than rotenone and rotenone plus antimycin resembles antimycin (Fig. 2). The inclusion of increasing amounts of DNP to a system already containing antimycin markedly enhances the phenomenon and at a level of 300 μM DNP damped oscillations develop. At this level of DNP the presence of antimycin does not significantly affect the data (Fig. 1, Fig. 2). The phenomenon is progressively enhanced either by the graded increase in the concentration of showdomycin when the concentration of DNP is kept constant (Fig. 3) or by the graded increase in the level of DNP when the concentration of showdomycin is kept constant, with or without antimycin (Fig. 1, Fig. 2). ATP energized mitochondrial volume changes in the presence of 150 μM showdomycin occur with

Fig. 3. Effect of showdomycin concentration. Concentration, L malate 0.5 mM, basic medium (see methods)

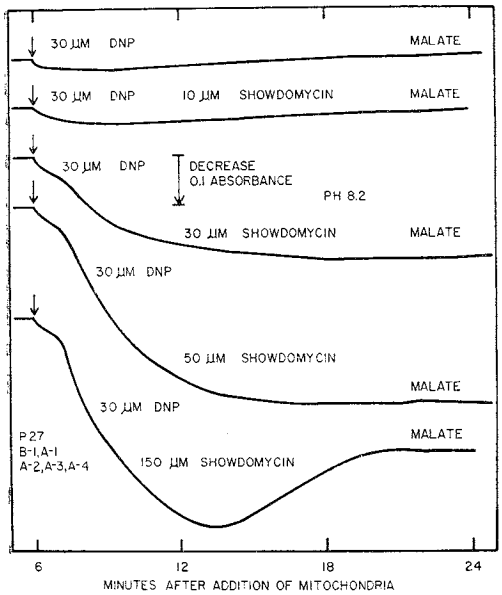


Fig. 4. Effect of malate. Cencentrations, L malate 0.5 mM, basic medium (see methods)

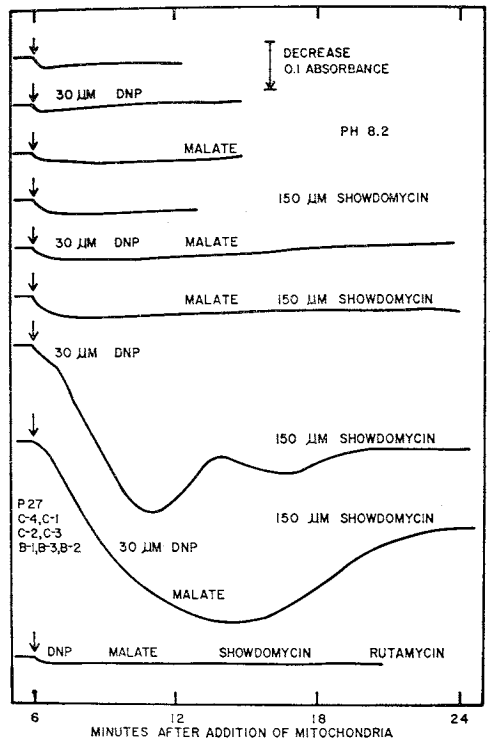


Fig. 5. Effect of malate plus antimycin.

Concentrations, antimycin A, 1 μg per 3 ml, rutamycin 1 μg per 3 ml, L malate 0.5 mM, basic medium (see methods)

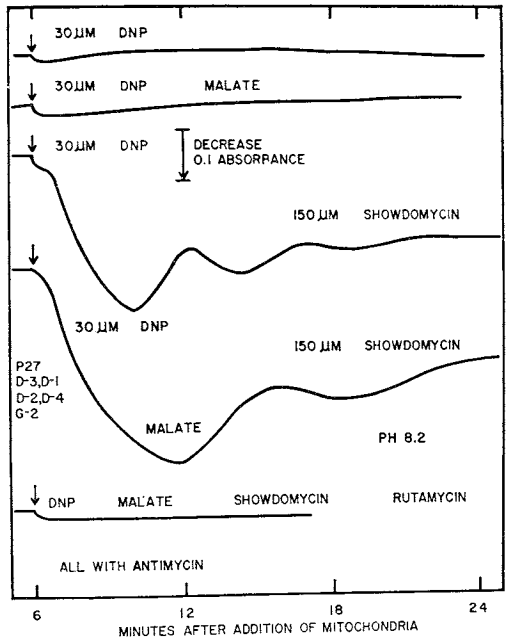
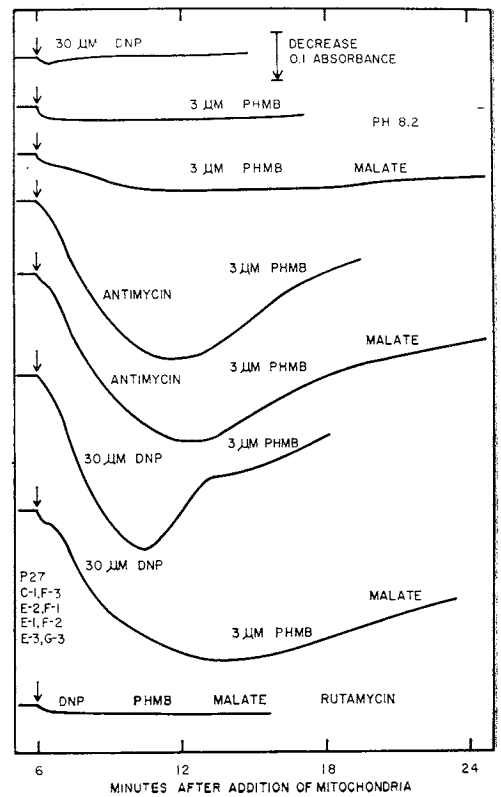


Fig. 6. Role of PHMB.

Concentrations, L malate 0.5 mM, rutamycin 1 μg per 3 ml, basic medium (see methods)



only the further addition of 30 μM DNP (Fig. 4). These conditions produce damped oscillations. The inclusion of L malate increases the period of oscillation and increases the extent of oscillation. Similar unexpected and as yet unexplained data is obtained with L malate in the presence of antimycin (Fig. 5). In other related experiments (data not shown) D malate behaves like L malate. Rutamycin inhibits this ATP energized system in the absence or presence of antimycin (Fig. 4, Fig. 5).

The mercurial thiol reagents PHMB serves as a replacement for showdomycin (Fig. 6). The ATP energized phenomenon again is dependent upon combinations of a thiol reagent with either antimycin or DNP. Malate as before damps oscillations and rutamycin again is inhibitory.

Discussion

We have previously attributed the ATP energized mitochondrial volume change induced by showdomycin to the conjugation of showdomycin with a mitochondrial thiol group normally involved in oxidative phosphorylation. It was also pointed out that under normal conditions this thiol group is unavailable for reaction with the foreign electrophile, showdomycin, and conjugates with an electrophilic acceptor which meshes with the respiratory chain by means of a cyclical sequence of reactions which includes at least one reduction and one oxidation step[4], as one reduction step and one oxidation step must be present in a single coupling site of the respiratory chain,

The data in this report is also consistent with the above rationalization. The mitochondrial volume change phenomenon again is enhanced by increasing the extent of conjugation between thiol group and showdomycin. This is accomplished either by raising the concentration of showdomycin or by raising the concentration of DNP (Fig. 1, Fig. 2, Fig. 3). DNP thus makes the thiol group available for reaction with showdomycin by cleaving the thiol from the normal conjugate or by preventing the conjugation of the thiol group with its normal electrophilic acceptor. These data and considerations are in harmony with our hypothesis for oxidative phosphorylation and our remarks concerning the role of DNP[7]. The current extension of the suggestion of LARDY and ELVEHJEM[5], that DNP either cleaves a nonphosphorylated high energy intermediate or prevents this high energy intermediate from forming[6], is now further advanced by identifying the nonphosphorylated high energy intermediate as a thiol conjugate which meshes with the respiratory chain.

The progressive effectiveness in our experimental system of respiratory chain inhibitors which act at the first and second coupling site of the respiratory chain (rotenone and antimycin respectively[8]), and the uncoupling agent DNP (Fig. 1, Fig. 2) indicates that the thiol group is strategically located at the three coupling sites of respiratory chain linked oxidative phosphorylation and that DNP is able to act at all three coupling sites. The ineffectiveness of adding rotenone to antimycin and antimycin to an optimal concentration of DNP conforms with this view (Fig. 1, Fig. 2).

The postulated existence of a *chemical cycle* involving the normal electrophilic acceptor of the thiol group and at least one reduction step and one oxidation step which mesh with the respiratory chain provides a consistent explanation for the same phenomenological result being achieved by the respiratory chain inhibitors, rotenone and antimycin and a dicotomous agent such as DNP which stimulates respiration. The finding that PHMB emulates the behavior of showdomycin adds much credence to our basic tenet that showdomycin conjugates with the strategically located mitochondrial thiol group involved in oxidative phosphorylation.

The isolation of a conjugate between a mitochondrial group which participates in the control of membrane permeability and showdomycin or PHMB may now be considered for future investigation. The vital need for the development of methods of recognizing and labelling components of transport systems while the transport system is intact has been stressed by KENNEDY[9]. The possibility that a mitochondrial group other than thiol is responsible for the volume changes induced by the mercurial thiol reagent PHMB and the non-mercurial antibiotic showdomycin may persist until the appropriate conjugate is isolated and characterized.

The role of a thiol group in the coupling mechanism of oxidative phosphorylation has also been stressed by others (KIELLEY[10], FLUHARTY and SANADI[11], FALCONE[12], FONYO and BESSMAN[13], GAUTHERON *et al.*[14], BRIERLEY *et al.*[15]). More recently BRIERLEY *et al.*[16] withdrew this view.

Our report describes a *flux of useful chemical energy* which depends upon DNP plus a thiol reagent and thus differs significantly from the majority of the mitochondrial studies with DNP or thiol reagents.

MITCHELL[17] with recent support from HOPFER *et al.*[18] has proposed that a proton gradiant is the source of chemical potential in mitochondrial processes. The view and supporting data has been presented that DNP acts as an uncoupling agent by increasing proton conductance across the cristae membrane of the mitochondrion[19]. Accordingly, useful chemical work such as the mitochondrial volume changes which we have observed should not be dependent upon DNP. Clearly, either these views require revision or the data of MITCHELL and MOYLE[19] does not support the proton gradiant hypothesis.

PACKER *et al.*[20] ascribed mitochondrial volume oscillations to a progression of coupled and uncoupled states. This suggestion is incompatible with our data (Figs. 1, 2, 4 and

5) as 30 μM and 300 μM DNP are present during mitochondrial volume oscillations and thus recoupling in the classical sense used by PACKER *et al.* would be precluded.

BLAIR and SOLLARS[21] have proposed that DNP specifically inhibits mitochondrial volume changes. Our data is contrary.

VAN DAM and SLATER[22] proposed that DNP is an uncoupling agent because it inhibits the passage of anions through the mitochondrial membrane. Our data with DNP which we presume involves an as yet unidentified permeant anion (note unexplained influence of D and L malate) during the volume change cycles does not support the proposal of VAN DAM and SLATER. It is cogent to point out that our rationalization accommodates the respiratory chain in the uncoupled and inhibited states (a requirement stressed by VAN DAM and SLATER[22]) and in the oxidized and reduced states.

Complete control are now essential when studying the effect of DNP on ATP energized systems especially when lack of inhibition by DNP seems apparent. The possibility of a DNP dependent process must be considered.

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