# COMBINATIONS OF AGENTS WHICH INDUCE ATP ENERGIZED MITOCHONDRIAL VOLUME CHANGES*

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The ATP energized mitochondrial volume change phenomena employing the antibiotic and antitumor agent showdomycin has visualized the interaction with mitochondria of a respiratory inhibitor antimycin and an uncoupling agent 2, 4-dinitrophenol. These studies have been extended to other well known agents which interact with mitochondria. Dicoumarol, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and heat treatment mimicked 2, 4-dinitrophenol. N-Ethylmaleimide resembled showdomycin. Valinomycin behaved like gramicidin. Our previous concept of a strategically located pivotal mitochondrial thiol group rationalized the behavior of all the above agents and hence this concept is of general significance.

Antibiotics and other agents of known structure have been used to identify the functional groups of mitochondria involved in the related phenomenae of oxidative phosphorylation, ion transfer, and energized mitochondrial volume change.[1,2,3,4,5,6] The interaction of an exogenous agent with a functional mitochondrial group was implicated when an ATP** energized mitochondrial volume change occurred. It was deduced that a pivotal mitochondrial thiol group was strategically located between two catalytic cycles[6]. One of the cycles meshed with the respiratory chain and the other cycle meshed with ATP, ADP, and Pi. The pivotal thiol group was exposed (*i. e.* made reactive) either by the addition of an uncoupling agent such as DNP, or by the addition of a respiratory inhibitor such as antimycin. When the exposed pivotal thiol group became conjugated with a foreign thiol reagent such as the antibiotic showdomycin, or the mercurial thiol reagent PHMB an ATP energized mitochondrial volume change was induced. Data was also presented to show that the antibiotic gramicidin interacted with the cycle which engaged ATP, ADP, and Pi.[1,2,3,6]

The salient observations were as follows: (1) Thiol reagents such as showdomycin or PHMB by themselves produced no effect[4,5]. (2) Respiratory inhibitors such as antimycin or rotenone by themselves produced no effect[4,5]. (3) Uncoupling agents such as DNP[5] or lapachol[7] by themselves produced no effect. (4) A thiol reagent in combination either with a respiratory inhibitor or an uncoupling agent induced an

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** The following abbreviations have been used,
ATP: Adenosine-5'-triphosphate, ADP: adenosine-5'-diphosphate, DNP: 2,4-Dinitrophenol,
PHMB: *p*-Hydroxymercuribenzoate, pFCCP: Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone,
NEMI: N-Ethylmaleimide.

ATP energized mitochondrial volume change.[4,5,7] (5) The ATP energized effect induced by gramicidin was inhibited by an adequate level of an uncoupling agent such as DNP or lapachol[6]. (6) The above inhibition (item 5) was relieved by the further addition of a thiol reagent. (7) In the complex relieved system comprising of gramicidin, an uncoupling agent, and a thiol reagent, a unique stimulatory effect was produced by potassium ion. The thiol reagent of choice has been the antibiotic showdomycin.

It is the purpose of this report to show that all these observations which have been rationalized by the concept of a pivotal mitochondrial thiol group are in harmony with the qualitative behavior of a variety of well established agents[8] known to interact with mitochondria. The following agents have been examined; dicoumarol, pFCCP, heat treatment, the classical thiol reagent NEMI, and the antibiotic valinomycin.

## Methods

The procedures and methods have been previously described,[1,2,3,5] however, the pH of the trischloride buffer is indicated on the diagrams. Incubations were at 27°C in standard rectangular quartz cuvettes with a 1-cm light path. The basic reaction mixture has a final volume of 3.0 ml and contained 1.5 mg of mitochondrial protein (prepared from rat liver[1]); 333 $\mu$M ATP (tris salt) 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 buffer. A decrease in absorbancy at 520 m$\mu$ was considered to be a measure of mitochondrial swelling. A model 2000 automatic spectrophotometer manufactured by Gilford Instrument Laboratories, Incorporated, Oberlin, Ohio, U. S. A. 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 legends provide further experimental details.

Fig. 1. The combination of showdomycin with dicoumarol.
Basic medium, see methods.

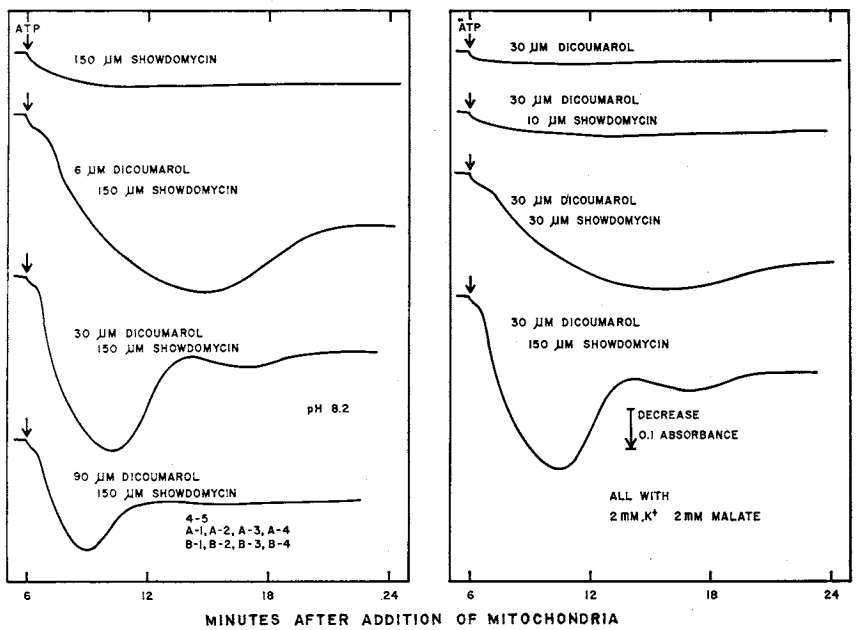


Fig. 2. The effect of pH on the combination of showdomycin with dicoumarol.
Basic medium, see methods.

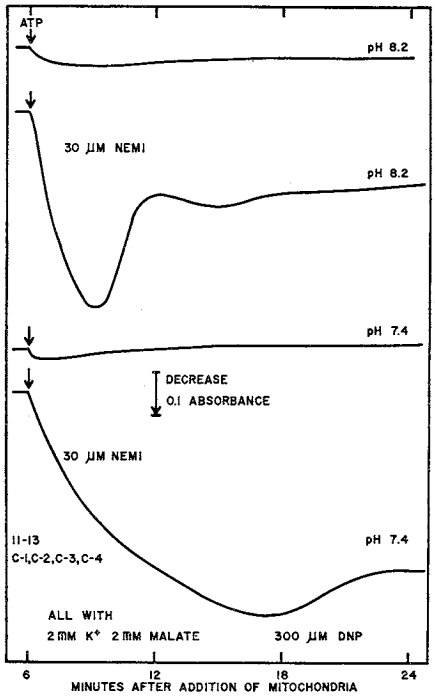



Fig. 3. The inhibition of the gramicidin effect by dicoumarol.
Basic medium, see methods.

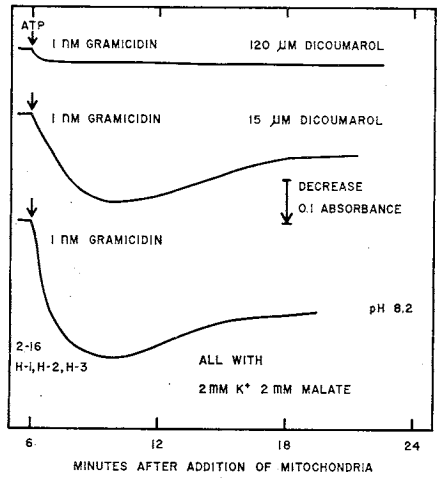
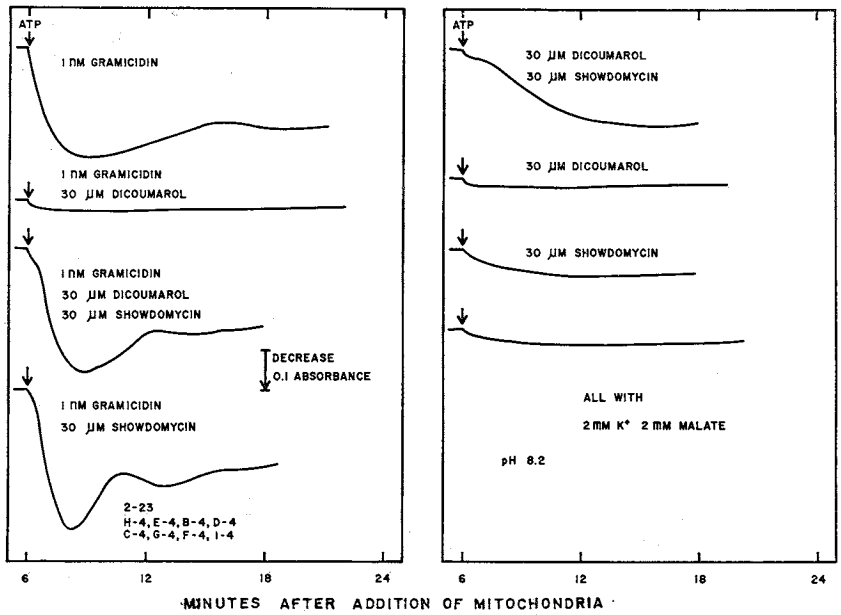



Fig. 4. Various combinations of gramicidin, dicoumarol and showdomycin.
Basic medium, see methods.

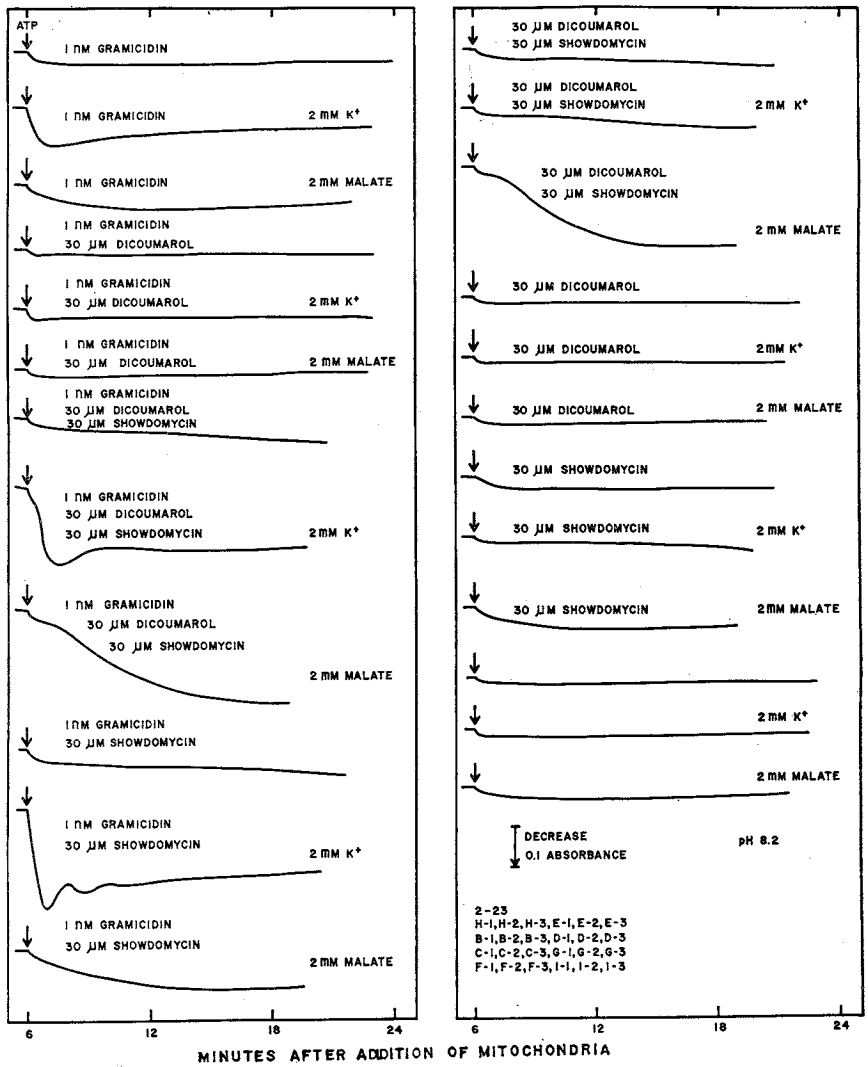


Commercial gramicidin, obtained from Mann Research Laboratories, Inc., was used. Such a mixture of gramicidins had been previously shown to be indistinguishable from gramicidin A[1,2,8]. The molecular weight of the mixture was arbitrarily taken as 1,870.

## Results

Neither dicoumarol nor showdomycin by themselves (Fig. 1) induced an ATP energized mitochondrial volume change. An appropriate combination of these two agents however, did induce a marked effect. When the pH was raised from 7.4 to 8.2 as illustrated in Fig. 2, there was decided enhancement of the ATP energized mitochondrial volume change induced by the combination of dicoumarol plus showdomycin. These observations are in agreement with the increased reactivity of a thiol group as the pH is raised and resembles the data previously obtained with the com-

Fig. 5. The role of ions with various combinations of gramicidin, dicoumarol and showdomycin.
Basic medium, see methods.



bination of DNP plus showdomycin[5]. An adequate level of dicumarol behaved like DNP and inhibited the ATP energized mitochondrial volume change induced by gramicidin (Fig. 3). It is seen (Fig. 4) that the effect of gramicidin which had been inhibited by dicoumarol was reinstated by the further addition of showdomycin. These observations parallel the previous result obtained with various combinations of gramicidin, DNP, and showdomycin[6]. A uniquely characteristic stimulatory effect of potassium ion on the combination of gramicidin plus dicoumarol, plus showdomycin was observed (Fig. 5). This characteristic effect of potassium ion was only evident in an active system containing gramicidin. Malate was significant when added to the combination of

Fig. 6. The combination of showdomycin with pFCCP.
Basic medium, see methods.

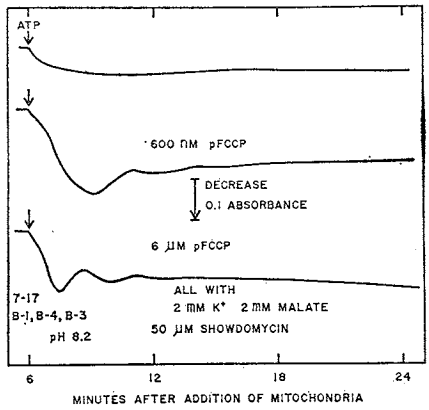



Fig. 7. The inhibition of the gramicidin effect by pFCCP.
Basic medium, see methods.

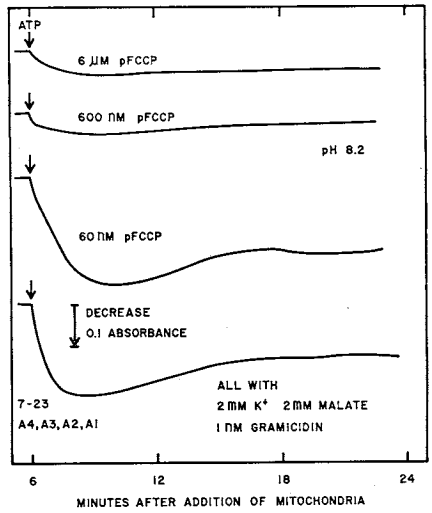
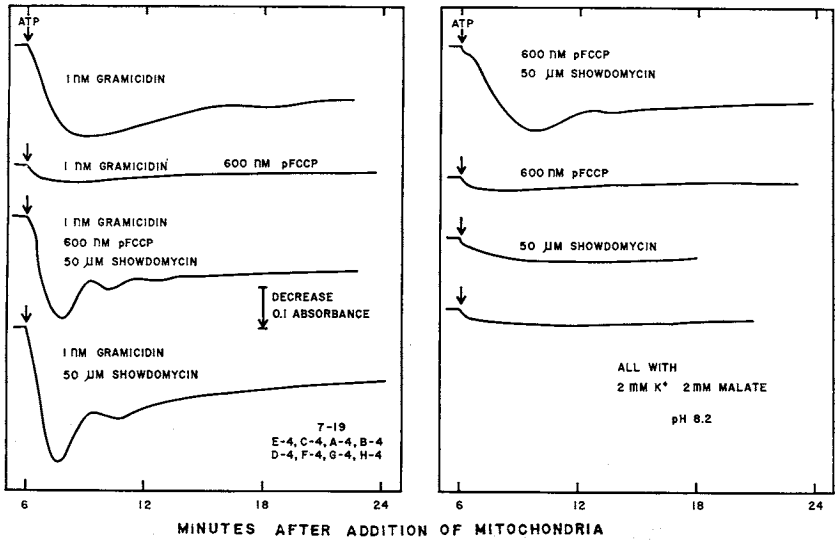



Fig. 8. Various combinations of showdomycin, gramicidin and pFCCP.
Basic medium, see methods.



dicoumarol plus showdomycin, while the addition of potassium ion was without effect when added to this combination. All these observations agree qualitatively wih the previous observations obtained with various combinations of gramicidin, DNP and showdomycin[6].

The uncoupling agent pFCCP behaved like DNP[5] and induced an ATP energized mitochondrial volume change when combined with showdomycin (Fig. 6). There was an optimum concentration of pFCCP (600 nM). The ATP energized effect induced by gramicidin (Fig. 7) was inhibited by the optimum concentration of pFCCP. The inhibition was relieved by the further addition of showdomycin (Fig. 8). Again there was a stimulatory effect associated with the addition of potassium ion to the complex relieved system (Fig. 9). Malate was significant when added to the combination of pFCCP plus showdomycin (Fig. 9), pFCCP clearly resembled DNP[6].

Heat treated mitochondria also resembled mitochondria to which DNP had been added[6] (Fig. 10). The effect induced by gramicidin was inhibited by heat treatment. The further addition of showdomycin (Fig. 10) relieved the inhibition. The relieved

Fig. 9. The role of ions with various combinations of showdomycin, gramicidin and pFCCP.
Basic medium, see methods.

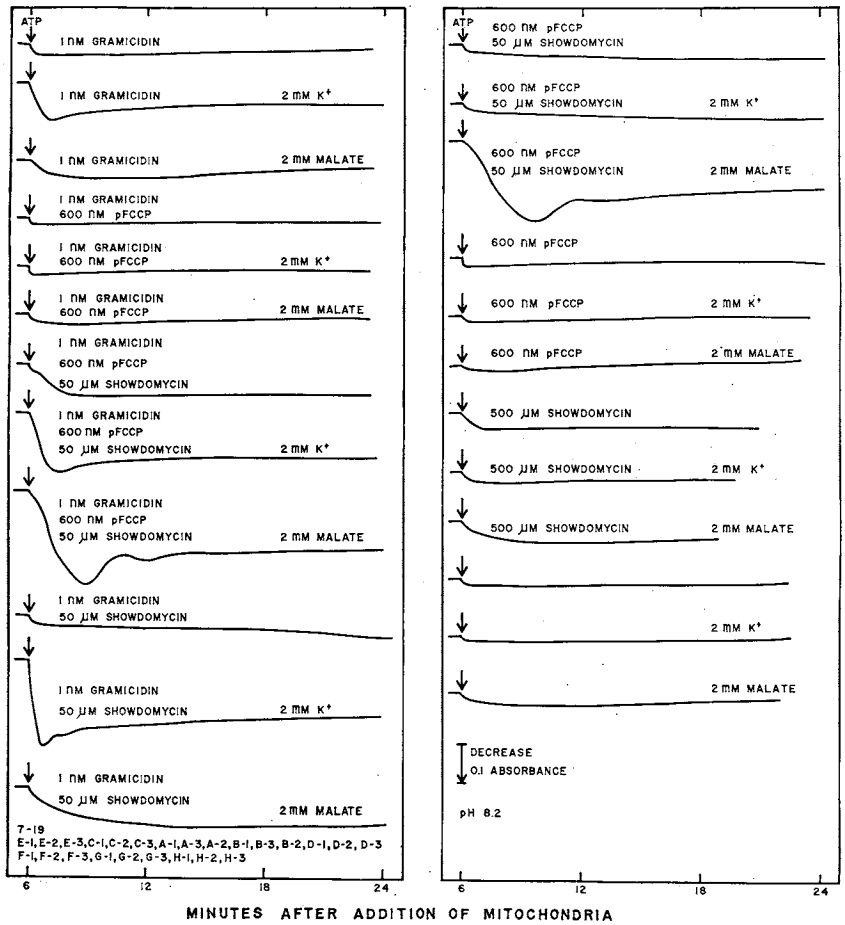


Fig. 10. The inhibition of the gramicidin effect by heat treatment.

The final incubation mixture corresponded to the basic medium, see methods. Prior to the addition of ATP the usual amount of mitochondria (0.06 ml) was added to the usual amount of sucrose and trischloride to give a volume of 2.50 ml. This mixture which was at pH 8.2 was kept at 35° for 20 minutes, then at 0° for 5 minutes followed by 6 minutes at 25℃. The mixture (2.5 ml) was added to a cuvette containing all components except ATP. After six minutes at 27℃, ATP was added in the usual manner. The left portion of the figure utilized non heat treated mitochondria. The right portion of the figure utilized heat treated mitochondria.

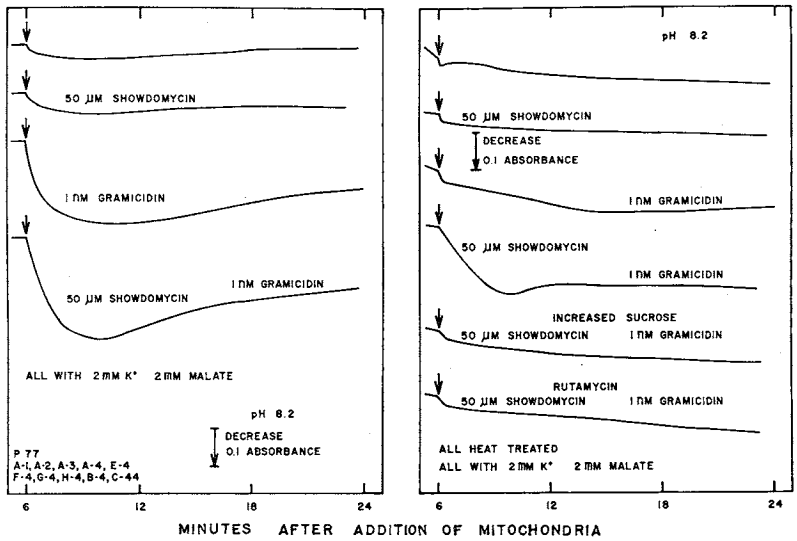



Fig. 11. The combination of NEMI and antimycin.

Basic medium, see methods.

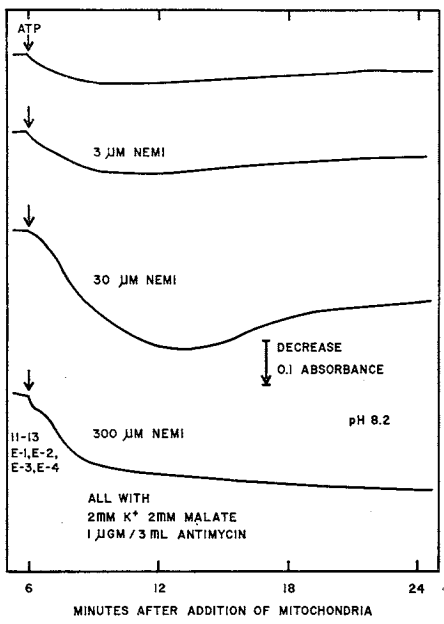



Fig. 12. The combination of NEMI and DNP.

Basic medium, see methods.

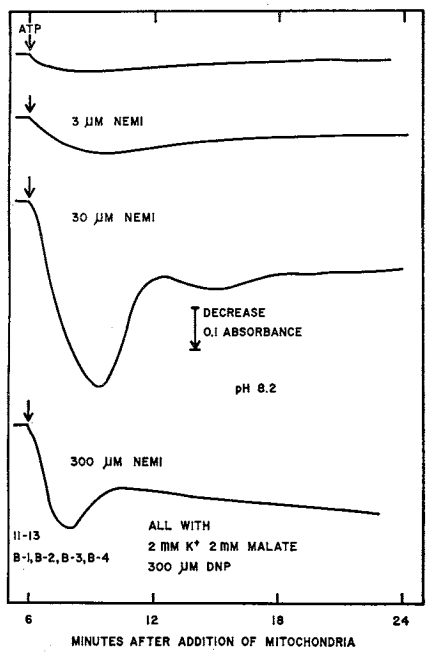


Fig. 13. Effect of pH on the combination of NEMI and antimycin.

Basic medium, see methods.

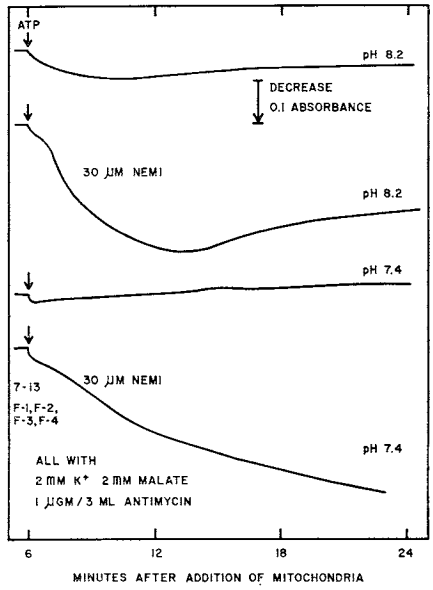
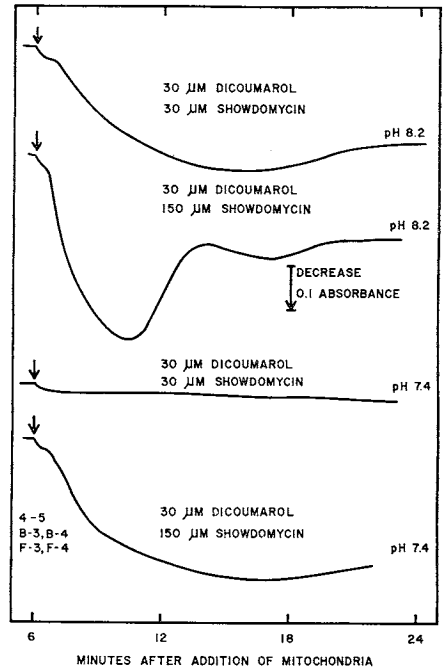



Fig. 14. The effect of pH on the combination of NEMI and DNP.

Basic medium, see methods.



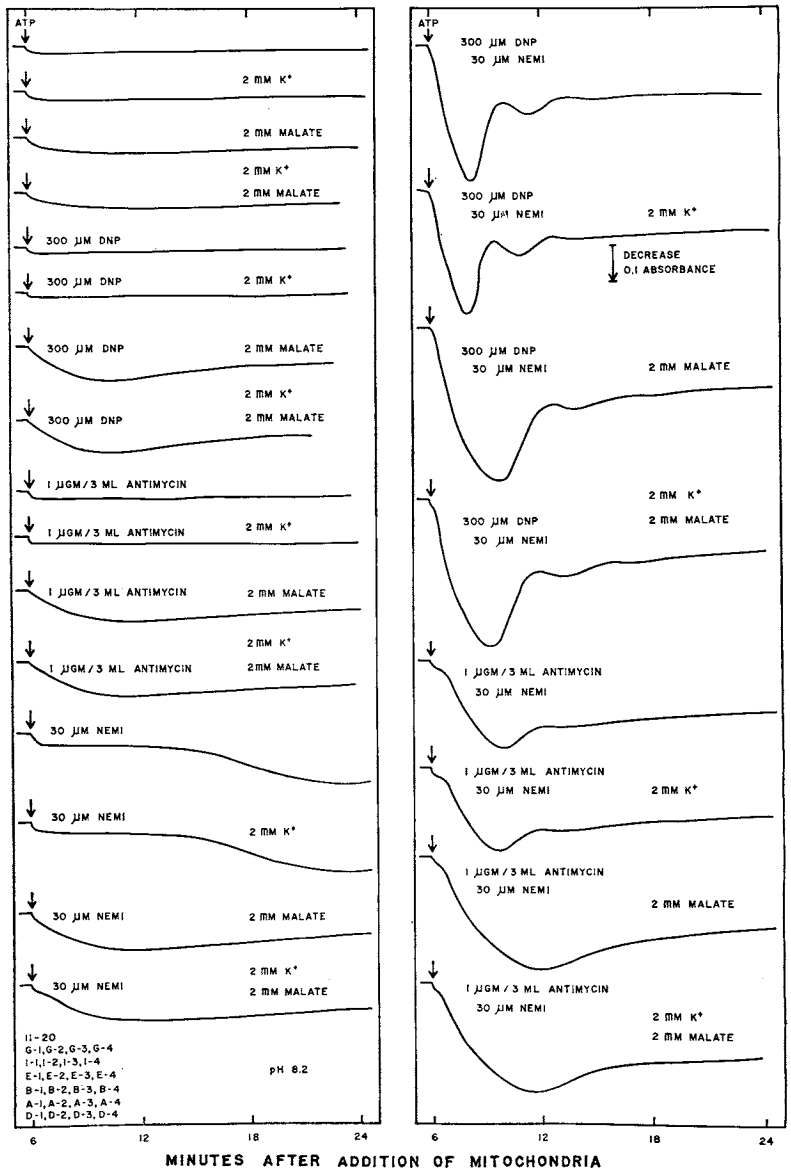

system was inhibited by increasing both the osmolarity of the medium and the ratio of sucrose to trischloride by adding sucrose to the standard medium, thus indicating that a membrane phenomena was still operative in the heat treated mitochondria (see Fig. 6, reference 4). The relieved system was also inhibited by the antibiotic rutamycin. As heat treated mitochondria did not respond as sharply as did mitochondria to which DNP had been added extensive experimentation with heat treated mitochondria was not carried out.

The combination of NEMI plus antimycin induced an ATP energized mitochondrial volume change (Fig. 11). There was an optimum concentration of NEMI (30 $\mu$M). The combination of NEMI plus DNP induced a marked oscillatory ATP energized mitochondrial volume change at the above optimum concentration of NEMI (Fig. 12). When the pH was increased from 7.4 to 8.2 (Fig. 13) the effect induced by the combination of NEMI and antimycin was markedly enhanced. The effect induced by the combination of DNP plus NEMI was also markedly increased when the pH was raised from 7.4 to 8.2 (Fig. 14). All possible controls and the role of potassium ions and malate ions are shown in Fig. 16. It is clear that a marked effect is produced only when NEMI is combined with either DNP, or with antimycin. Potassium plays an insignificant role, while malate increased the amplitude and period of oscillation. An optimum concentration of the non-mercurial thiol reagent, NEMI, resembled the previously reported action of the antibiotic showdomycin[4,5].

An appropriate concentration of the antibiotic valinomycin (Fig. 16) induced a marked ATP energized mitochondrial volume change in the presence of the permeant

Fig. 15. The role of ions with the combination of NEMI with antimycin and with DNP.

Basic medium, see methods.




ions potassium and malate. The effect induced by valinomycin was inhibited by the addition of an adequate concentration of DNP (Fig. 17). The inhibitory effect of DNP was neutralized by the further addition of the thiol reagent showdomycin-relieved system. Potassium ions produced a stimulatory effect (Fig. 18) when added to the showdomycin. Accordingly the activity of valinomycin was parallel to the previously reported activity of gramicidin[6].

Fig. 16. The effect of valinomycin.
Basic medium, see methods.

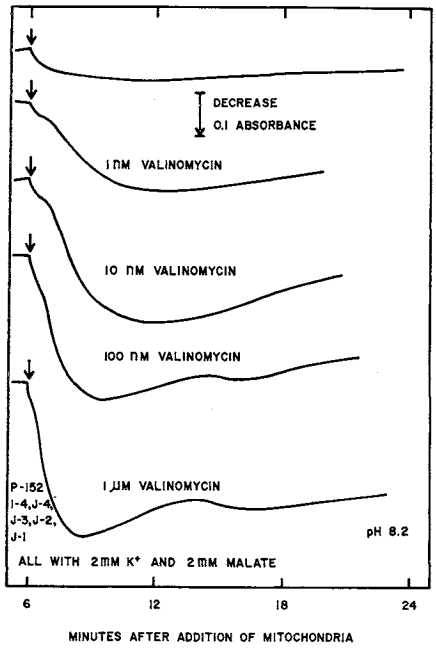
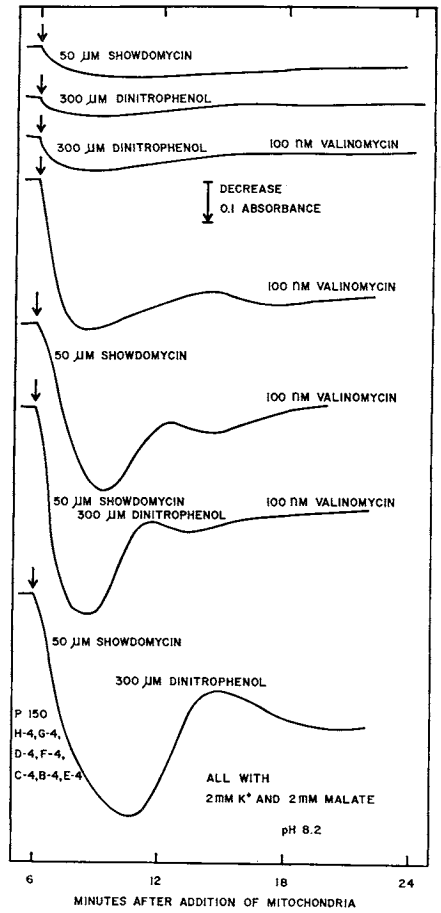



Fig. 17. Various combinations of showdomycin, DNP and valinomycin.
Basic medium, see methods.




## Discussion

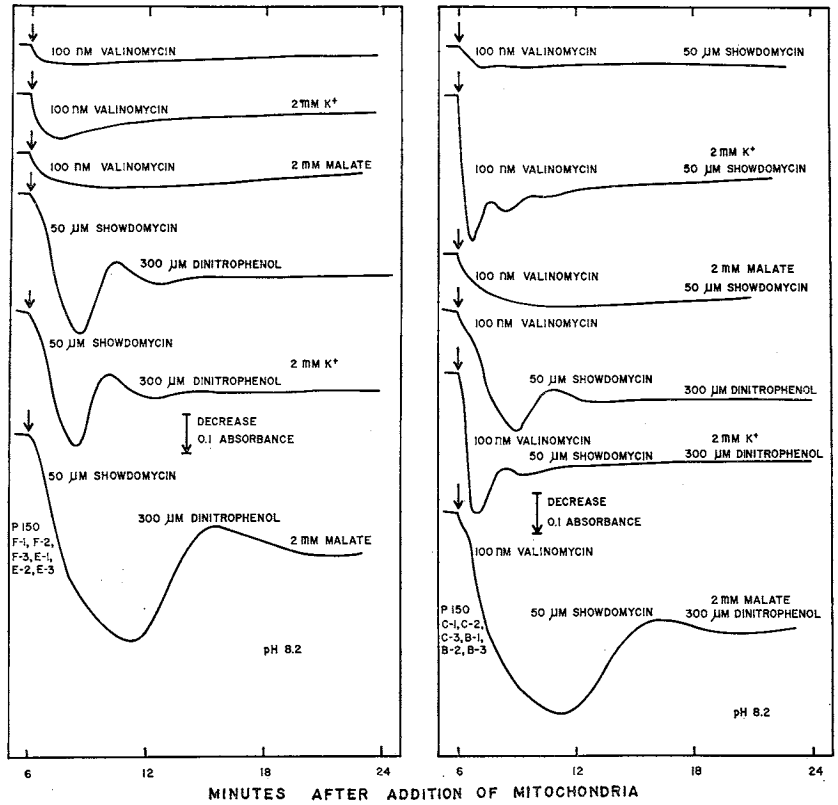
Dicoumarol, and pFCCP induced an ATP energized mitochondrial volume change when combined with showdomycin. These two well recognized uncoupling agents inhibited the ATP energized mitochondrial volume change induced by gramicidin. The inhibition was relieved by the further addition of showdomycin. The relieved system responded positively to the addition of potassium ion. In accordance with our previous rationalization dicoumarol and pFCCP, like DNP[5,6], exposed the strategically located pivotal mitochondrial thiol group.

Heat treatment of mitochondria inhibited the ATP energized mitochondrial volume change induced by gramicidin. The inhibition was relieved by showdomycin. The relieved heat treated system was inhibited by increasing the osmolarity of the medium, and also by the addition of rutamycin. Rutamycin is a recognized inhibitor of the phosphorylating sequence, associated with oxidative phosphorylation[8]. These cursory observations indicated that while heat treatment exposed the pivotal mitochondrial thiol group a mitochondrial membrane system with properties characteristic of oxidative phosphorylation remained intact.

The non-mercurial thiol reagent NEMI induced an ATP energized mitochondrial volume change when combined either with the respiratory inhibitor antimycin, or the

Fig. 18. The role of ions with various combinations of showdomycin, DNP and valinomycin.

Basic medium, see methods.




uncoupling agent DNP. The phenomena was enhanced by raising the pH, thus according to our rationalization NEMI conjugated with the mitochondrial thiol group exposed either by the respiratory inhibitor antimycin, or the uncoupling agent DNP[4,5]. It is cogent to point out that under conditions reported by others that although ion transport was induced by the mercurial thiol reagent PHMB, NEMI was almost inactive[9].

It has been well established by PRESSMAN[10] that valinomycin had activity similar to that of gramicidin, and induced an energized mitochondrial volume change dependent upon the permeant cation, potassium, and a permeant anion[4,6]. In our experiments the effect induced by valinomycin was inhibited by an adequate level of DNP. This inhibition was relieved by the further addition of showdomycin. The addition of potassium ion was stimulatory when added to the complex relieved system which was comprised of valinomycin, plus DNP, plus showdomycin. According to our rationalization the catalytic cycle involving valinomycin was intact in the complex relieved system and the strategically located pivotal mitochondrial thiol group interacted as a nucleophile with an electrophylic site in the catalytic cycle involving valinomycin. The action of valinomycin thus paralleled the activity of gramicidin in similar ATP energized mitochondrial volume change experiments[6].

The concept of a strategically located pivotal mitochondrial thiol group served to explain observations with a large group of agents which interact with mitochondria, namely antimycin, DNP, lapachol, lawsone[7], dicoumarol, pFCCP, heat treatment, showdomycin, PHMB, NEMI, gramicidin, and valinomycin, and thus is of general significance in the

related phenomenae of oxidative phosphorylation, ion transport, and energized mitochondrial volume change.

ATP energized mitochondrial volume changes may be used to detect the cooperative interaction of various agents with mitochondria.

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