THE PIVOTAL POSITION OF THE MITOCHONDRIAL THIOL GROUP EXPOSED BY DINITROPHENOL LOCATED BY MEANS OF ATP ENERGIZED MITOCHONDRIAL VOLUME CHANGES REQUIRING GRAMICIDIN, SHOWDOMYCIN, AND DINITRIPHENOL

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An appropriate concentration of DNP (2, 4-dinitrophenol) is capable of inhibiting the ATP energized mitochondrial volume change induced by gramicidin in the presence of the permeant ions potassium and L-malate. The further addition of the non-mercurial thiol reagent, showdomycin reinstates, an effect of gramicidin which is dependent upon potassium ion. Thus the strategically located nucleophilic mitochondrial thiol group exposed by DNP occupies a pivotal position between two cycles. One of the cycles meshes with the respiratory chain and the other cycle meshes with ATP. This latter cycle contains the site sensitive to gramicidin.

The chemical description of the complex sequence of reactions encompassed by the term oxidative phosphorylation has been a major goal in biochemical research for the last quarter of a century. While there has been many general approaches to the problem, perhaps the most successful to date, has been that using intact mitochondria. LARDY *et al.*[1] began the systematic study of toxic antibiotics with mitochondria and pointed out the advantages of using for the study of oxidative phosphorylation a mitochondrial system which still retains part of the structural organization associated with the whole cell. A most significant advance in this approach was the finding in PRESSMAN's laboratory[2,3] that certain antibiotics induced energized ion dependent mitochondrial volume changes with related concomitant ion translocation. Mitochondria now become a reproducible experimental system for the study of organized enzymes, oxidative phosphorylation, and membrane phenomena.

In order to identify in intact mitochondria functional groups which participate in the related phenomena of oxidative phosphorylation, ion transport and mitochondrial volume changes, the use of antibiotics and other agents of known structure but with widely different functional groups was commenced. The action of gramicidin, a neutral linear modified polypeptide [whose N and C ends form peptide bonds, respectively with formyl and ethanol-amine groups] was attributed to the reaction of its nucleophilic terminal hydroxyl group with an electrophilic center in the mitochon-

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dria[4,5,6]. This interaction induced a new catalytic cycle which was linked to ATP[6]. These studies with gramicidin also brought to light a role for mercurial thiol reagents in the process of energized mitochondrial volume changes associated with ion transport. Further investigation[7,8] showed that showdomycin, an expected non-mercurial thiol reagent, induced mitochondrial volume changes when combined with either a respiratory inhibitor, antimycin, or an uncoupling agent, DNP (2, 4-dinitrophenol). The action of showdomycin was attributed to the 1, 4 addition of a mitochondrial thiol group to the α, β conjugated system of the maleimide moiety present in showdomycin. The mitochondrial thiol group was made available for conjugation with showdomycin by antimycin or DNP. A mechanism which rationalized the behaviour of both the respiratory inhibitor antimycin and the uncoupling agent DNP was proposed. According to this rationalization there is a chemical cycle which meshes with at least one reduction step and one oxidation step of a coupling site in the respiratory chain and in this chemical cycle there is a normal electrophilic receptor for the mitochodrial thiol group. Antimycin prevents the cyclic regeneration of the normal electrophilic acceptor, while DNP either prevents the conjugation of the mitochondrial thiol group with the normal electrophilic acceptor or catalyzes the cleavage of the conjugate between the mitochondrial thiol group and the normal electrophilic acceptor.

As it has been known for some time that mitochondrial volume changes and concomitant ion transport may be energized by respiratory energy in the presence of rutamycin (a classical inhibitor of phosphorylation associated with oxidative phosphorylation)[1], it follows that mitochondrial volume changes are energized by non-phosphorylated high energy intermediates located between ATP and the respiratory chain[5,9,10]. It was also previously observed that a mercurial thiol reagent enhanced the effect of gramicidin, accordingly it was postulated that there was a nucleophilic mitochondrial thiol group which competed with the nucleophilic hydroxyl group of gramicidin for the same electrophilic center of a non-phosphorylated high energy intermediate[4,5,6]. In view of our data with DNP there is a question whether the thiol group exposed by DNP is identical with the thiol group which competes with gramicidin. It is the purpose of this report to show that indeed the same thiol group is involved in the two instances.

Methods

The procedures and methods have been previously described[8], however, the pH of the tris-chloride buffer is indicated on the diagrams. Incubations were at 27°C in standard rectangular quartz cuvette with a 1-cm light path. The basic reaction mixture had a final volume of 3 ml and contained 1.5 mg of mitochondrial protein (prepared from rat liver[4]); 333 μM ATP (tris salt) which was added in 0.05 ml by means of the adding-mixing device[5] as indicated by an arrow on the diagrams; 75 mM sucrose; and 75 mM tris-chloride buffer. 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, Incorporated, 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[4]. The figures and legends provide further experimental details.

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[4,5,6]. The molecular weight of the mixture was arbitarily taken as 1,870.

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Results

The ATP energized mitochondrial volume change induced by gramicidin in the presence of a permeant cation such as potassium and a permeant anion such as L-malate was inhibited in the range of pH from 7.4 to 8.2 by 300 μM DNP. At a higher pH, *viz.* 8.6, the effect of gramicidin was diminished and the inhibitory effect of 300 μM DNP was abolished. The data obtained with DNP at a level of 75 μM DNP at pH 7.4, 7.8, and 8.2 clearly indicate that the inhibitory effect of DNP was diminished as the pH was increased (Fig. 1).

If to the combination of gramicidin with an inhibitory concentration of DNP there is added a concentration of 50 μM showdomycin, the ATP energized mitochondrial volume change is reinstituted as indicated by the observed oscillatory phenomena (Fig. 2). The ATP energized mitochondrial volume changes induced by the combination of gramicidin with showdomycin and by the combination of DNP with showdomycin are also shown (Fig. 2).

In Fig. 3 the effects of the addition of either potassium ion or malate on systems comprised of various combinations of gramicidin, showdomycin, and DNP are shown. The effect of the combination gramicidin, DNP, plus showdmycin is markedly enhanced by the addition of potassium ion. The combination of gramicidin, plus shodomycin is markedly enhanced by the addition of potassium ion. Gramicidin by itself is stimulated by potassium ion, to a definite but

Fig. 1. The inhibition of the gramicidin system by DNP.
Basic medium (see methods)

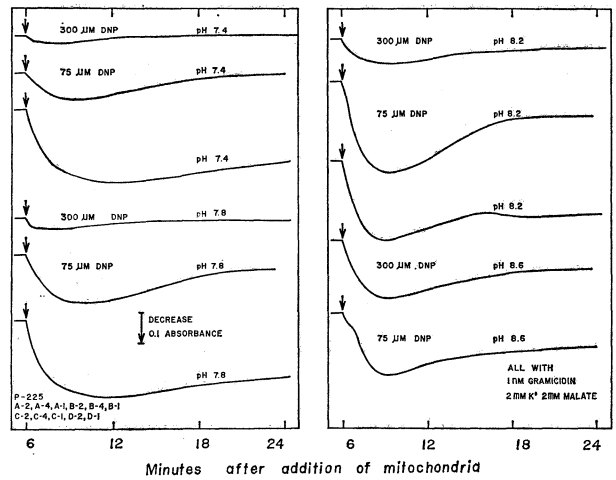
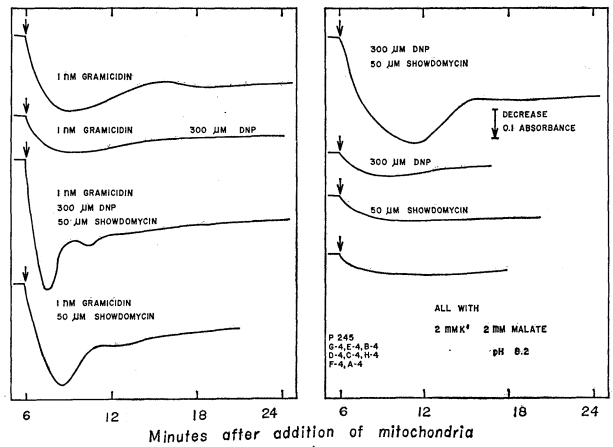


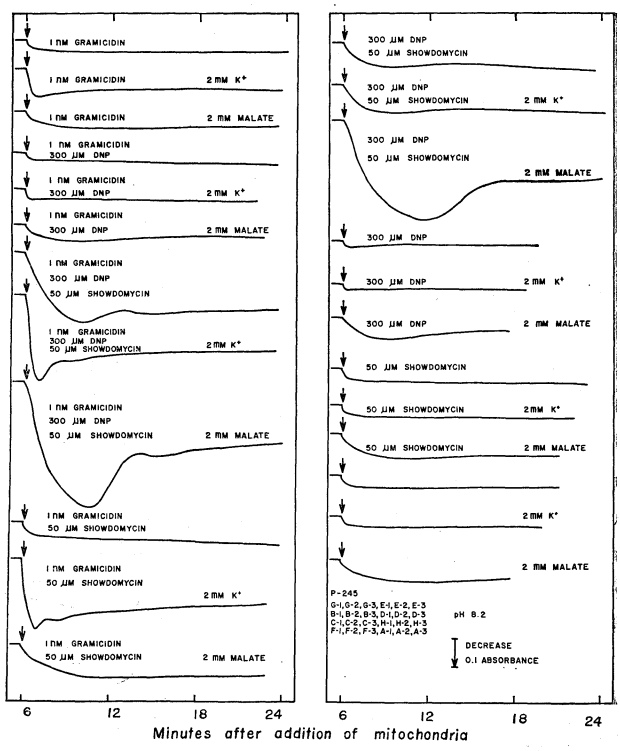
Fig. 2. Showdomycin plus the inhibited system.
Basic medium (see methods)



minor extent, while the combination of DNP, plus showdomycin is not affected by the addition of potassium ion. Neither DNP or showdomycin by itself is affected by the addition of potassium ion. Thus it is clear that potassium ion only stimulates the non-inhibited systems which contain gramicidin.

It is also apparent from Fig. 3 that the conditions are such that DNP plus showdomycin require L-malate for a marked ATP energized mitochondrial volume change. The addition of L-malate has negligible effect on ; showdomycin by itself, DNP by itself, gramicidin by itself, and the combination of gramicidin plus showdomycin. However, in the only other system in which there is a combination of DNP with showdomycin, namely the gramicidin, DNP plus showdomycin system, the addition of L-malate does have an effect in that the period and amplitude of the oscillation are increased. Thus malate only affects the systems in which there is included a mixture of DNP and showdomycin. All the data in Fig. 3 are at pH 8.2 in order to minimize the concentration of showdomycin[7].

Fig. 3. The roles of potassium ion and L-malate. Basic medium (see methods)



Discussion

Conditions have been found whereby DNP inhibits completely the ATP-energized mitochondrial volume change induced by gramicidin in the presence of potassium ion and L-malate (Fig. 1). We attribute this inhibition to the interaction of the nucleophilic thiol group exposed by DNP[8] with the mitochondrial electrophilic center subject to nucleophilic attack by gramicidin. When the pH of the system is increased, the inhibitory effect of DNP and consequently the interaction of the exposed thiol group is diminished (Fig. 1). This could not be due to a decrease in the reactivity of the thiol group as the thiol group becomes more ionized and more neucleophilic as the pH is raised. Accordingly it must mean that as the pH is raised, the geometry of the interaction is such that it is no longer possible for the exposed thiol group to reach and interact with the mitochondrial electrophilic center which is the target of gramicidin.

If the gramicidin system is inhibited by the mitochondrial thiol group exposed by DNP, then removal of this exposed mitochondrial thiol group by reaction with the thiol reagent showdomycin should reinstate the gramicidin system. The addition of showdomycin to the gramicidin system inhibited by DNP reinstitutes an ATP-energized mitochondrial volume change phenomenon (Fig. 2). However, since the combination of showdomycin plus DNP

also induces an ATP-energized mitochondrial volume change phenomenon the proof of the reinstatement of the gramicidin dependent phenomena requires further evidence.

The addition of potassium ion to the triple combination of showdomycin, DNP and gramicidin produces a marked acceleration of the ATP-energized mitochondrial volume change phenomena, while the addition of potassium ion is without detectable effect on the double combination of showdomycin and DNP (Fig. 3). Clearly the potassium ion dependent gramicidin system[4,5,6] (Fig. 3) is still intact in the reinstituted mitochondrial volume change system of gramicidin, DNP plus showdomycin.

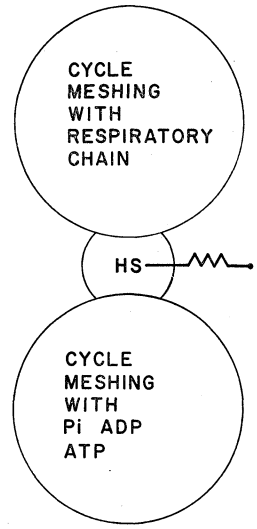
The addition of L-malate to the triple combination of gramicidin, DNP and showdomycin produces the characteristic increase in the period and amplitude as in the case of the showdomycin plus DNP system[8] (Fig. 3). Thus the reistituted system of gramicidin, DNP plus showdomycin has properties common to its two constituent systems *viz.* (a) the gramicidin system and (b) the showdomycin plus DNP system.

The data reveals that the mitochondrial thiol group exposed by DNP is able to react with the same electrophilic center that gramicidin does. We wish to project this observation to the normal mitochondria. Clearly there is a pivotal mitochondrial thiol group strategically located between two cycles, one of the cycles meshes with the respiratory chain[7,8] and the other meshes with ATP[6]. This strategically located pivotal nucleophilic mitochondrial thiol group also must communicate with an electrophilic center in each of the two cycles (Fig. 4). These electrophilic centers are located between the respiratory chain and the site of action of rutamycin (see introduction) and hence are non-phosphorylated. These new findings and conclusions extend and are in complete harmony with the data and conclusions previously published in partnership with FALCONE[4,5,6].

The recent observations by CASWELL and PRESSMAN[11] that in the presence of an appropriate uncoupling agent, translocation of potassium ion is not correlated with the state of the respiratory chain provides support for our location of the gramicidin sensitive site (which has a requirement for potassium ion) in the lower cycle of Fig. 4. Thus potassium ion translocation may be disengaged from the respiratory chain by an appropriate uncoupling agent.

A few more conclusions may be drawn from the data. As gramicidin activity[4,5,6] requires both a permeant cation such as potassium ion and a permeant anion such as malate there is no significant activity induced by the combination of gramicidin plus 50 μM showdomycin in the presence of 2 mM malate (Fig. 3). Since there is significant activity induced by the combination of gramicidin, 50 μM showdomycin, and 2 mM potassium ion (Fig. 3) it follows that during the preincubation period of gramicidin, 50 μM showdomycin plus 2 mM potassium ion (Fig. 3), some permeant anion must have gone from the inside of the mitochondria to the outside of the mitochondria. A similar leakage of a permeant anion from the inside of the mitochondria to the outside of the mitochondria must also have occurred during the preincubation of gramicidin, DNP plus 50 μM showdomycin in the presence of 2 mM potassium ion, as again there was an effect due to the addition of 2 mM potassium ion (Fig. 3). Previous work[8] indicated that if the concentration of showdomycin is increased, to 150 μM, the requirement of L-malate is not apparent at pH 8.2 in the system with 30 μM DNP. Accordingly under these previous conditions, there must have been leakage of a permeant anion from the inside of the mitochondria to the outside of the mitochondria. Finally the requirement of L-malate for the ion dependent mitochondrial volume change induced by 300 μM DNP and 50 μM showdomycin and energized by ATP (Fig. 3) is completely contrary to the

Fig. 4. The pivotal thiol group.



proposal of VAN DAM and SLATER[12] that DNP is an uncoupling agent because it inhibits the passage of anions through the mitochondrial membrane.

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