MITOCHONDRIAL VOLUME CHANGES INDUCED BY THE ANTIOBIOTIC SHOWDOMYCIN

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Following Pressman's pioneer findings [Moore and Pressman (1964), Pressman (1965)] antibiotics have been used extensively to induce mitochondrial ion uptake and volume changes in the hope of elucidating the mechanism of ion transport and oxidative phosphorylation. The relationship between antibiotic structure and activity has been an important aspect of this work [Pressman (1965), (1967); Lardy et al (1967)]. These groups have noted the characteristic large neutral rings in the active antibiotics with appropriate D and L sequences of the ring components which form peptide or ester bonds e.g. valinomycin, the enniatins and the nonactins.

On the other hand, the neutral linear polypeptide Gramicidin whose N and C ends are combined through peptide bonds to formyl and ethanolamine groups respectively [Sarges and Witkop (1965) revised the original cyclic structure] also induced mitochondrial ion uptake and volume changes [Presman (1965), Chappell and Crofts (1965)]. The terminal hydroxyl group in the ethanolamine moiety of Gramicidin was identified as a functional nucleophile which reacts with a mitochondrial electrophilic center, associated with oxidative phosphorylation [Falcone and Hadler (1968a), (1968b), Hadler and Falcone (1968)]. These authors also noted that the mercurial thiol reagent p-hydroxymercuribenzoate in the presence of antimycin induced mitochondrial volume changes. Brierley (1966) previously reported that mercurial thiol reagents stimulate the uptake of Mg^{++} by mitochondria and recently observed (1967) that mercurial thiol reagents activate mitochondrial volume changes associated with K^{+} uptake.

In a search for new functional groups in antibiotics which induces mitochondrial ion uptake and volume changes, we now report mitochondrial volume changes induced by the antibiotic Showdomycin [3-(β-D-ribofuranosyl) maleimide, Kano et al (1967), Darnall et al (1967), Nishirmura et al (1964)]. Showdomycin, a relatively simple molecule with no alternating D and L configurations, has a functional group similar to the non mercurial thiol reagent N-ethylmaleimide. Furthermore our data suggests that Showdomycin reacts with a mitochondrial thiol group so located that the normal electrophilic acceptor for the thiol group is generated by cyclical reactions which mesh with the respiratory chain.

It is cogent to note that Showdomycin is active against Streptococcus hemolyticus, Streptoccus pyogenes, Ehrlich mouse ascites tumor (in vivo) and cultured HeLa cells [Nishumura et al (1964), Nishumura (1964), Matsuura et al (1964)].

METHODS

These have been described previously in detail. [Falcone and Hadler (1968a), (1968b), Hadler and Falcone (1968)]. Incubations were at 27° in standard rectangular quartz cuvettes with a 1 cm. light path. The final reaction mixture had a volume of 3 ml. and contained 1.5 mg. of mitochondrial protein and 3.33×10^{-1} mM Tris ATP (Adenosine-5'-triphosphate) which was added in 0.05 ml by means of the adding-mixing device as indicated by an arrow on the diagrams. Except in the experiments of fig. 5 and fig. 6 the final reaction mixture contained 75 mM sucrose and 75 mM trischloride at the pH indicated by the figures and legends. 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. The figures and their legends provide further experimental details. We are grateful to Dr. F. J. Wolf of Merck and Company, Rahway, New Jersey, U. S. A., and Dr. Ken'ichi Takeda of Shionogi and Company, Osaka, Japan for providing the Showdomycin used in this work.

RESULTS

At pH 8.2 (fig. 1) the prompt initiation of a swelling and incipient contraction cycle energized by ATP requires not only Showdomycin but also the respiratory chain inhibitor Antimycin. Addition of L malate enhances the phenomena slightly. On the other hand at pH 7.4 the scope of the phenomena is diminished (fig. 2) and there is complete dependence on L malate. The sensativity of the phenomena to Showdomycin increases as the pH is raised (fig. 3). In the presence of $5x10^{-2}$ mM Showdomycin at pH 7.4 there is no prompt swelling phase however at pH 8.2 a prompt swelling phase ensues. When the concentration of Showdomycin is increased six-fold to $3x10^{-1}$ mM there is a prompt swelling phase at pH 7.4 and a complete swelling contraction cycle at pH 8.2.

The response of the Gramicidin system, (fig. 4) previously studied at pH 7.4, [Falcone and Hadler (1968a), (1968b), Hadler and Falcone (1968)] also was accentuated by raising the pH to 8.2. However the effect of raising the pH was not as pronounced in the Gramicidin system as in the Showdomycin system. This data agrees with the postulated reaction between a non ionizable hydroxyl group on Gramicidin and a non ionizable electrophylic center in the mitochondria.

The Showdomycin mitochondrial system (fig. 5), just like the previously reported Gramicidin mitochondrial membrane system, responds more as the ratio of trischloride to sucrose is increased, at constant osmolarity. In agreement with the Gramicidin mitochondrial membrane system the response of the Showdomycin mitochondrial membrane system is diminished by increasing the osmolarity of the medium, at a constant ratio of trischloride to sucrose (fig. 6).

At pH 7.4 (data not shown, see fig. 2 for conditions) a variety of 2mM dibasic anions were substituted for malate. Oxalate, malonate, succinate, glutarate, sulfate, L malate, D malate behave as permeant anions. Non permeant anions are adipate, pimelate, fumarate, maleate, phthalate, pyrophosphate. Monobasic acetate is non permeant at 2 and 4 mM. In the presence of

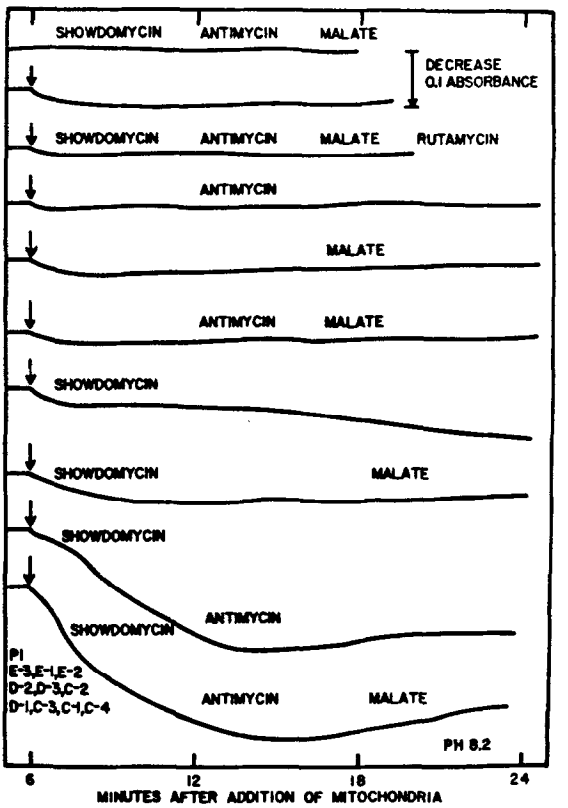

Fig. 1. Requirements at pH 8.2. Concentrations, Antimycin A 1 µg per 3 ml, L malate 5 x 10^{-1} mM.

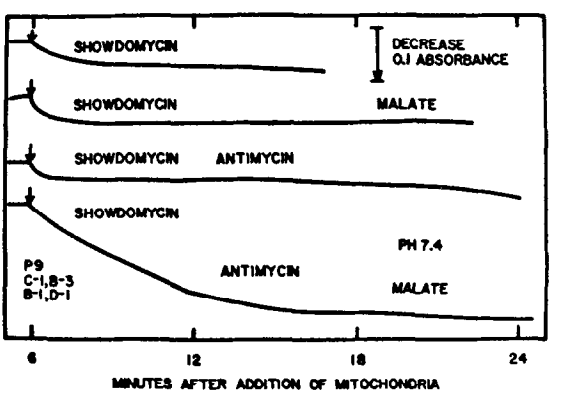

Fig. 2. Requirements at pH 7.4. Concentrations, Antimycin A 1 µg per 3 ml, L malate 5 x 10^{-1} mM, Showdomycin 1.5 x 10^{-1} mM. Other controls not shown agreed with Fig. 1.

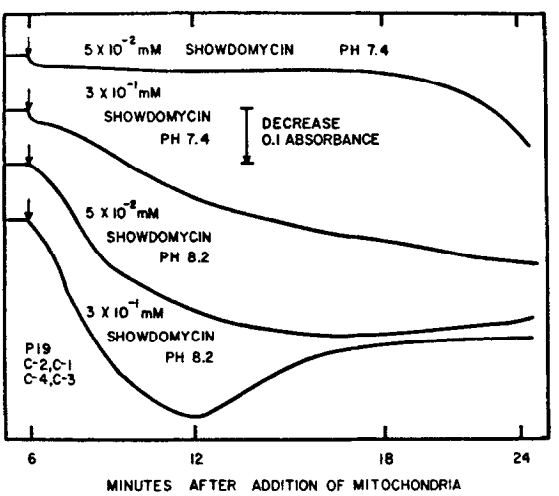

Fig. 3. Effect of pH. Concentrations, in each cuvette. Antimycin A 1 µg per 3 ml, L malate 5 x 10^{-1} mM.

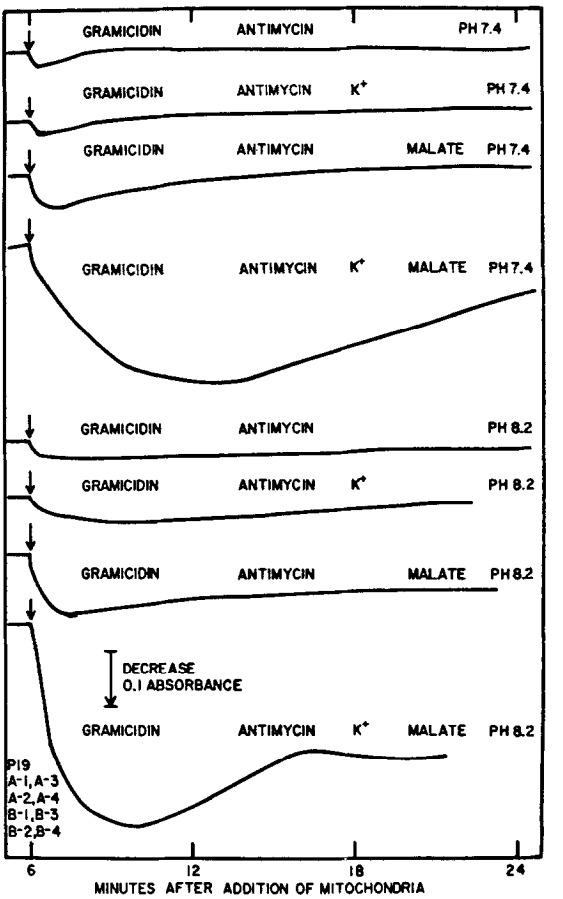

Fig. 4. Effect of pH on Gramicidin Concentrations, Antimycin A, 1 µg per 3 ml, Gramicidin 5 x 10^{-1} nM, L malate 2 mM, K^+ 2mM.

2 mM L malate prompt expansion is not inhibited by 2 mM non permeant anions pimelate and phthalate but is inhibited by 2 mM non permeant anions pyrophospate and maleate. In a similar type of experiment with Gramicidin [(Falcone and Hadler (1968a)] maleate was permeant and the dibasic anions larger than succinate were not permeant. All the non permeant dibasic anions tested were also inhibitory. These experiments with anions suggest that Showdomycin reacts with a mitochondrial site related to oxidative phosphorylation but less specifically than Gramicidin.

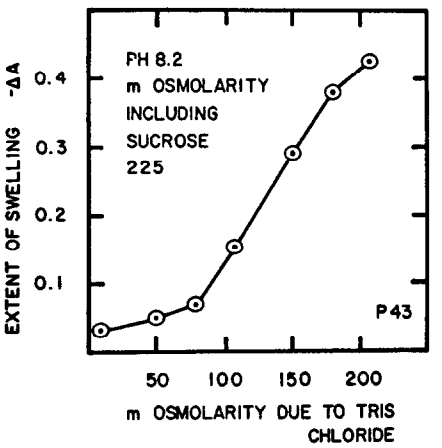

Fig. 5. Effect of ratio of trischloride to sucrose. Concentrations in each cuvette, Antimycin A 1 µg per 3 ml, Showdomycin 5 x 10^{-2} mM, L malate 5 x 10^{-1} mM.

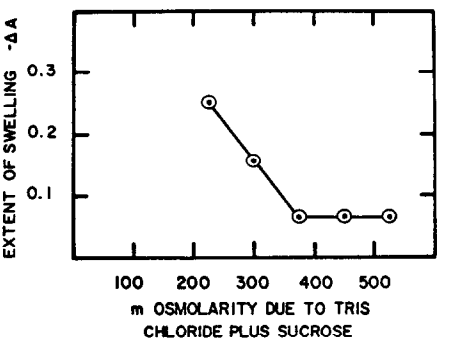

Fig. 6. Effect of osmolarity. Concentrations in each cuvette, Antimycin A 1 µg per 3 ml, Showdomycin 5 x 10^{-2} mM, L malate 5 x 10^{-1} mM, pH 8.2.

The addition of 1 mM potassium chloride to the experiment described in Fig. 3 did not alter the observations. Thus another experimental technique is necessary to determine the nature of the permeant cation.

DISCUSSION

The ATP energized volume change phenomena is attributed to a chemical reaction between Showdomycin and a mitochondrial thiol group normally involved in oxidative phosphorylation. Such a thiol group has been postulated [e.g. Falcone (1966), Hadler (1961)]. The thiol behaves as a nucleophile and adds to the open β electrophilic center of the α,β unsaturated carbonyl system of Showdomycin. The swelling contraction response is thus enhanced either by increasing the concentration of Showdomycin at constant pH (fig. 3) or by increasing the ionization of the thiol group by raising the pH (fig. 3).

The thiol group reacts with a normal electrophilic acceptor which meshes with the respiratory chain by means of an as yet unknown cyclical reaction sequence involving reduction and oxidation in at least two steps when coupled oxidative phosphorylation is intact. In the absence of antimycin the thiol

group preferentially conjugates with its normal electrophilic acceptor and hence is unavailable for reaction with Showdomycin (fig. 1, fig. 2). The inhibition of a portion of the respiratory chain by the addition of antimycin interrupts the cyclical regeneration of the normal electrophilic acceptor. This interruption lowers the concentration of the normally conjugated thiol group and the resultant unconjugated thiol group becomes available for reaction with the foreign electrophile, Showdomycin.

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