THE DIRECT *IN VITRO* ACTION OF GLIOTOXIN ON RAT LIVER MITOCHONDRIA*,**

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Gliotoxin by itself induced a mitochondrial volume change believed to be respiration energized as the effect was blocked by either antimycin or 2,4-dinitrophenol. It has been postulated that the pivotal mitochondrial thiol group interacted with the -S-S- bond in gliotoxin to form a new -S-S- bond and a new extended pivotal mitochondrial thiol group. The behaviour of gliotoxin with mitochondria was clearly differentiated from that of ELLMAN's reagent, showdomycin and N-ethylmaleimide, and could account for the antibiotic and antitumor properties of gliotoxin and with certain qualifications the antiviral properties of gliotoxin.

Many combinations of either a respiratory inhibitor or an uncoupling agent with a thiol reagent have induced an ATP-energized mitochondrial volume change.[1,2,3] This phenomena has been attributed to the exposure by the respiratory inhibitor or uncoupling agent of a nucleophilic pivotal mitochondrial thiol group which reacted with the electrophilic moiety of the thiol reagent. We have recently extended the structures of the thiol reagent effective in the ATP-energized effect to include compounds which have -S-S- bonds, *viz.*, ELLMAN's[4] reagent and gliotoxin[5] (an antibiotic, an antitumor agent and an antiviral agent).

While studying the ATP-energized effect which involved gliotoxin we observed that under appropriate conditions when ATP, DNP, and antimycin were deleted from the incubation mixture, gliotoxin alone induced a mitochondrial volume change. This report concerns itself with this phenomena and its relationship to the postulated cycles[6] which mesh with the respiratory chain and with Pi, ADP and ATP. The mode of action by which gliotoxin acts as an antibiotic, an antitumor agent and an antiviral agent is also considered.

Methods

General procedures, methods, and purification of the water have been previously described[6], for the mitochondrial volume-change experiments. The pH of the tris-chloride

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** Abbreviations used : ATP : Adenosine-5′-triphosphate ADP : Adenosine-5′-diphosphate Pi : Inorganic phosphate DNP : 2,4-Dinitrophenol NEMI : N-Ethylmaleimide DNA : Deoxyribonucleic acid RNA : Ribonucleic acid ELLMAN : ELLMAN's reagent, *i.e.* 5,5′-dithio-bis-(2-nitrobenzoic acid)

buffer is indicated on the charts. 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 tris-chloride buffer. A decrease in absorbance at 520 nm was taken as a measure of mitochondrial swelling. A Model 2000 automatic spectrophotometer manufactured by Gilford Instrument Laboratories, Inc., Oberlin, Ohio, was used. All cations were added in the form of chloride salts, and anions were added in the form of Tris salts neutralized to pH 7.4. Solutions of gliotoxin, antimycin and oligomycin were prepared in 95% ethanol (distilled). All controls contained the appropriate amount of 95% ethanol. A cuvette never contained more than 0.06 ml of 95% ethanol. The adding-mixing device was used in making additions after the mitochondria were added to the cuvette. In all experiments gliotoxin was added after the mitochondria as the final component. The charts and legends provide additional experimental details.

Oxygen consumption was measured in an oxygen monitor which utilized a CLARK electrode. The instrument (Model 35 SA) was manufactured by Yellow Springs, Ohio, U.S.A. A 10″ recorder, Model 1005, manufactured by Beckman Instruments Fullerton, California, was used in conjunction with the oxygen monitor.

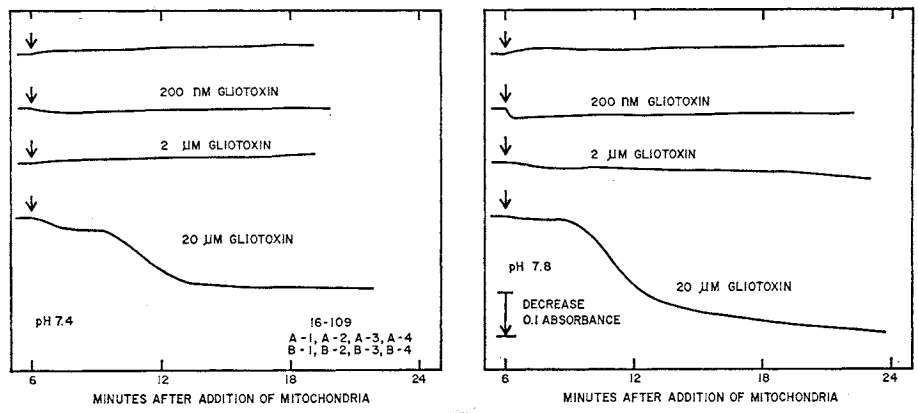
Samples of gliotoxin were generously provided by Dr. P. W. TROWN, Lederle Laboratories, American Cyanamid Co., Pearl River, New York 10965, U.S.A. and by Dr. N. M. LARIN, Pfizer Ltd., Ramsgate Road, Sandwich, Kent, England.

Results

It is seen in Fig. 1 that following the addition of an adequate level of gliotoxin (20 μM) a mitochondrial volume change was induced following a brief induction period. This induction period may be due to the time required for gliotoxin to penetrate the mitochondria and seek out the reactive center in a mitochondrion as noted previously[5]. The effect was enhanced by raising the pH thus implicating that a reaction took place between a nucleophilic mitochondrial thiol group and the electrophilic -S-S- bond of gliotoxin[1,2]. The addition of potassium ion had neglible effect while the addition of malate ion caused a diminuation of the extent of the volume change. This decrease was most noticeable at the higher pH of 7.8 (Fig. 2).

The effect induced by gliotoxin was inhibited by an adequate level of ATP (333 μM) and was very slightly inhibited or altered by oligomycin. The inhibition by ATP

Fig. 1. The effect of gliotoxin.
Basic medium see methods.



persisted in the presence of oligomycin; that is, oligomycin did not block the inhibition by ATP (Fig. 3). When the pH was raised from 7.4 to 7.8 (Fig. 4) the results were similar but more pronounced. ATP inhibited the mitochondrial volume change induced by gliotoxin. Oligomycin had a minimal effect and the inhibition by ATP

Fig. 2. Role of ions.
Basic medium see methods.

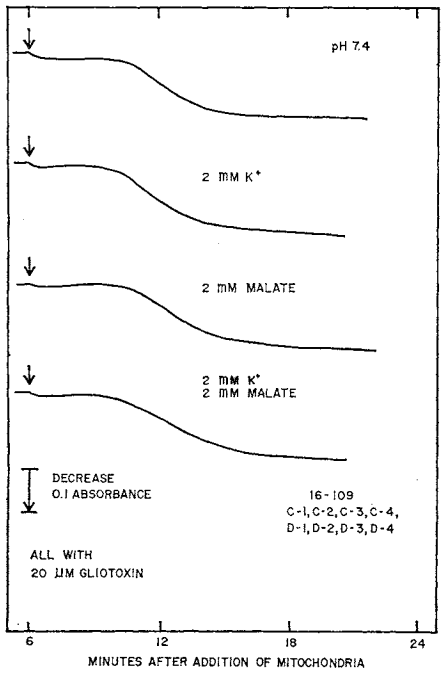


Fig. 3. Effect of ATP at pH 7.4.
Basic medium see methods.

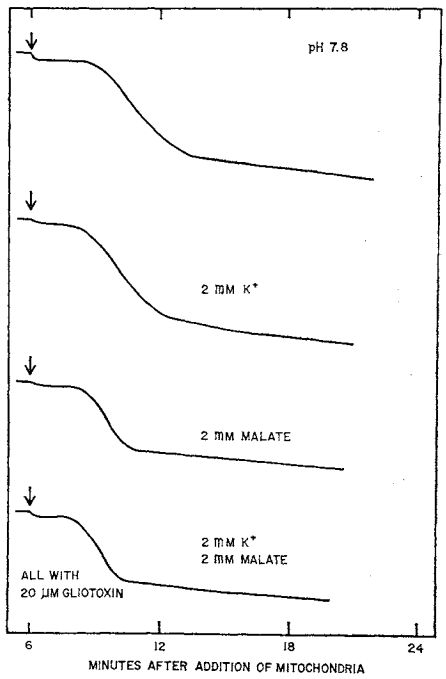


Fig. 4. Effect of ATP at pH 7.8.
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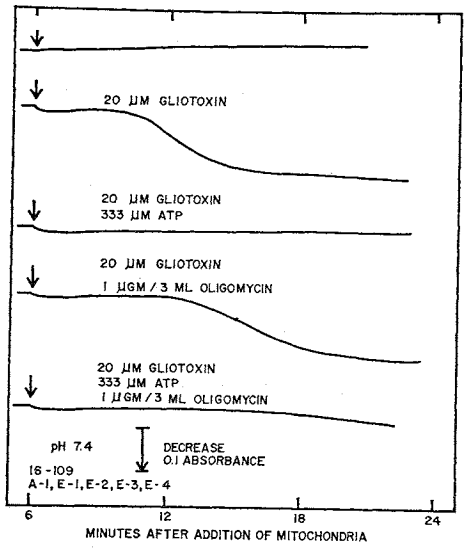
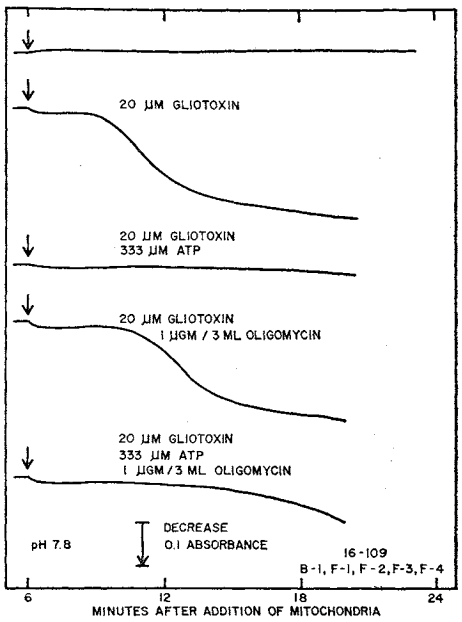




was somewhat better blocked by oligomycin.

The mitochondrial volume change induced by gliotoxin was inhibited by the respiratory inhibitor antimycin, by the uncoupling agent DNP and almost completely inhibited by the thiol reagent showdomycin (Fig. 5). Similar but more pronounced results were obtained when the pH was raised from 7.4 to 7.8 (Fig. 6).

It was possible to induce at a pH of 7.8 a marked mitochondrial volume change with 20 μM gliotoxin and, incipient effects with 300 μM ELLMAN's reagent and 200 μM NEMI (Fig. 7). The effect induced by these various thiol reagents were compared. In addition, the effect induced by gliotoxin at a level of 200 μM was also examined (Fig. 8). It was noted that 200 μM gliotoxin induced an effect sooner than 20 μM gliotoxin. The effect induced by 20 μM gliotoxin as noted previously was inhibited by antimycin, DNP and ATP, and almost completely inhibited by showdomycin. The effect induced by 300 μM ELLMAN's

Fig. 5. Various inhibitors at pH 7.4.
Basic medium see methods.

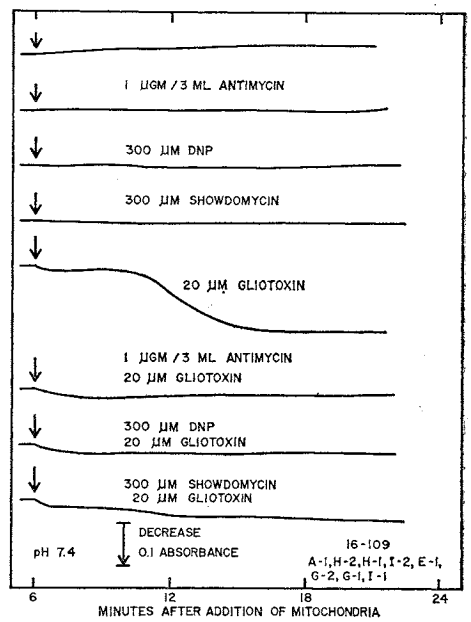


Fig. 6. Various inhibitors at pH 7.8.
Basic medium see methods.

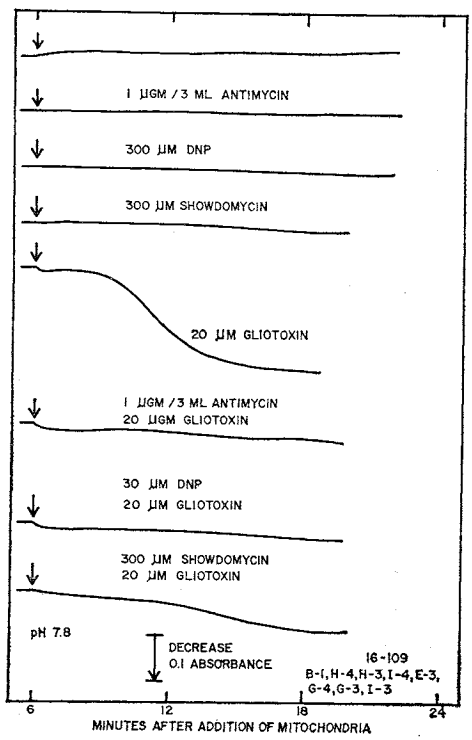


Fig. 7. Various thiol reagents.
Basic medium see methods.

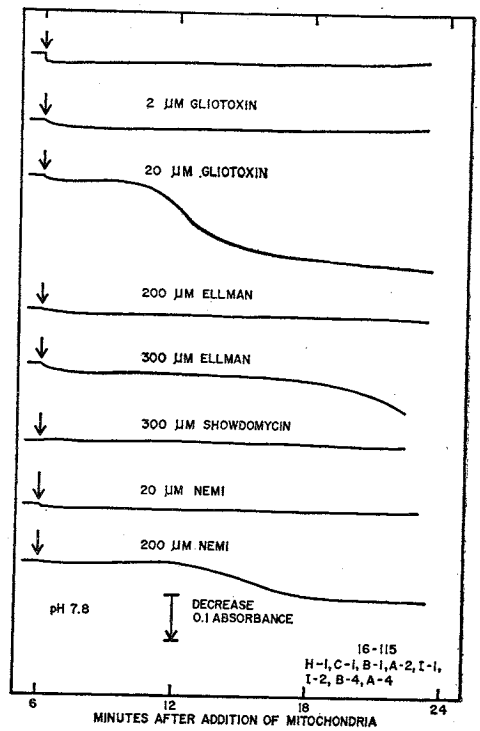
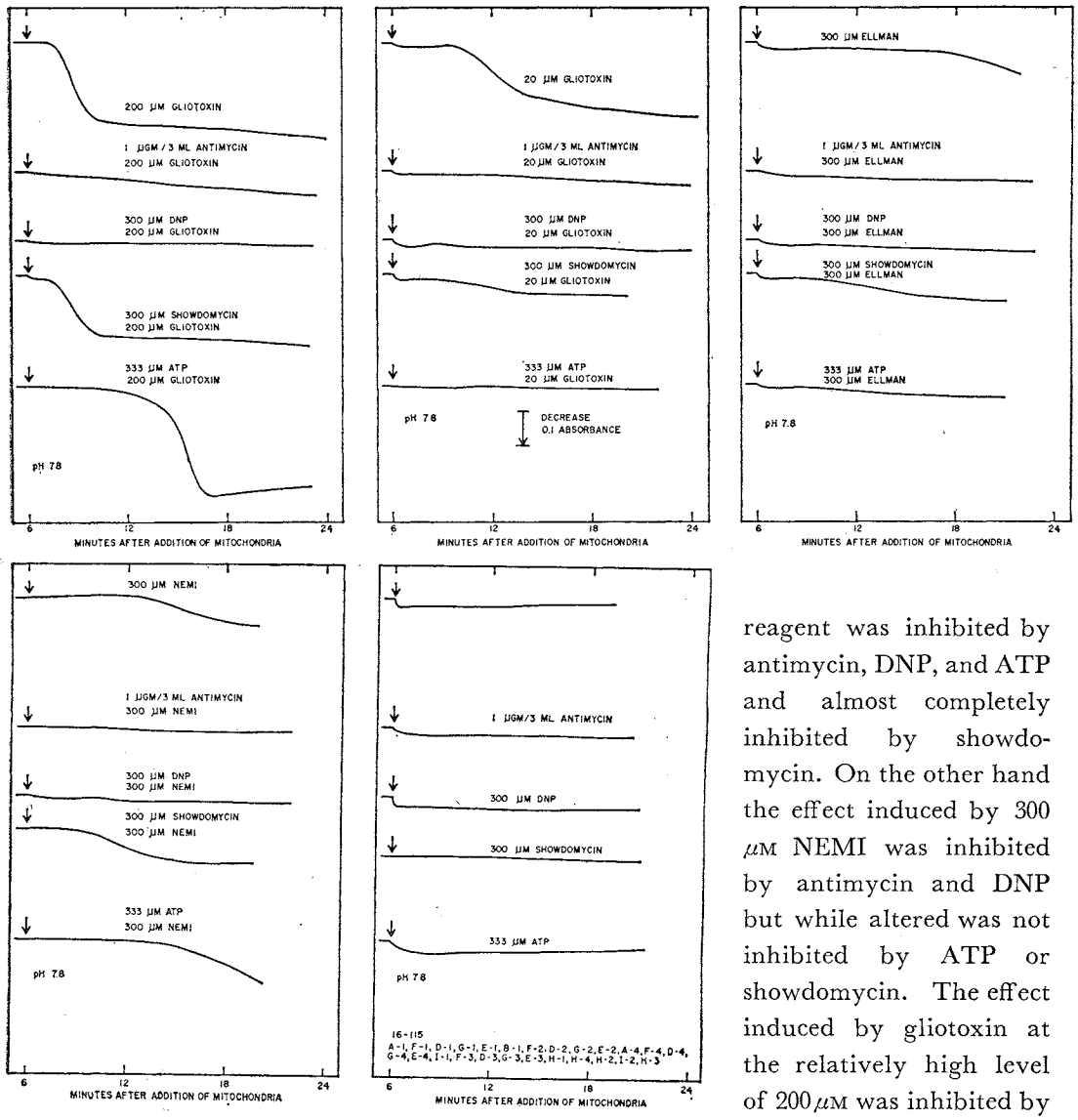


Fig. 8. Various inhibitors and various thiol reagents.
Basic medium see methods.



reagent was inhibited by antimycin, DNP, and ATP and almost completely inhibited by showdomycin. On the other hand the effect induced by 300 μM NEMI was inhibited by antimycin and DNP but while altered was not inhibited by ATP or showdomycin. The effect induced by gliotoxin at the relatively high level of 200 μM was inhibited by DNP, almost completely inhibited by antimycin, partially inhibited by showdomycin and altered and increased by ATP.

No effect was induced by showdomycin at a level of 300 μM.

In cursory experiments we were unable to observe an alteration in the rate of oxygen consumption (measured by an oxygen electrode) when we added gliotoxin to mitochondria under a variety of conditions.

Discussion

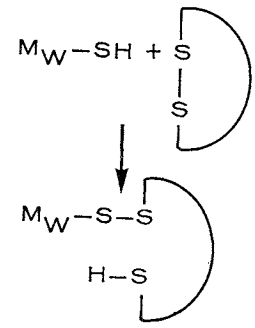
Unlike the other thiol reagents previously studied (showdomycin, NEMI and ELLMAN's reagent) gliotoxin at a low concentration, (20 μM) induced a mitochondrial volume change without requirements for ATP, or a respiratory inhibitor or an uncoupling agent (Fig. 1). As this volume change was inhibited by the addition of either the respiratory inhibitor

antimycin or the uncoupling agent DNP (Figs. 5 and 6) we propose that the volume change induced solely by gliotoxin was energized by respiration.

The volume change was enhanced by raising the pH (Fig. 1) in agreement with a decisive reaction between a nucleophilic and ionizable mitochondrial thiol group and gliotoxin acting as an electrophilic thiol reagent. Malate ion, which consistently has influenced the ATP-energized mitochondrial volume changes involving various[1~6] thiol reagents including gliotoxin[5] when they were combined with either antimycin or DNP, also influenced the respiration-energized mitochondrial volume change induced by gliotoxin (Fig. 2). As in the ATP-energized system, potassium ion had negligible effect on the respiration-energized gliotoxin induced mitochondrial volume change (Fig. 2). Thus there are characteristic similarities between the respiration and ATP-energized mitochondrial volume changes dependent upon gliotoxin.

The experimental plan may be understood and the results collated if the assumption is made that gliotoxin because of its -S-S- group and unique structure is able to interact with the pivotal mitochondrial thiol group[6] without the assistance of a respiratory inhibitor such as antimycin or an uncoupling agent such as DNP. Such an interaction would generate a new -S-S- bond between gliotoxin and the mitochondria and also generate a new -SH bond attached to the gliotoxin residue. In essence the pivotal mitochondrial thiol group would be extended (Fig. 9). According to this picture there could be a flux of energy transmitted from the upper cycle which meshes with the respiratory chain to the lower cycle which meshes with Pi, ADP and ATP by means of a pivotal cycle[6] involving an extended pivotal mitochondrial thiol group. The rotation of the cycles would be in the directions associated with respiration-energized ATP formation.

Fig. 9.



On the addition of ATP there would be a tendency to stop and reverse the direction of the lower cycle. The pivotal cycle would no longer be able to transmit energy from the upper to lower cycle as the lower cycle would be prevented from rotating in the direction associated with respiration-energized ATP formation. Thus the addition of ATP could as observed (Figs. 3 and 4) inhibit the induced respiration-energized mitochondrial volume change.

As in other respiration energized mitochondrial volume changes[7], oligomycin was not inhibitory (Figs. 3 and 4); however the ATP inhibition of the respiration-energized effect was not appreciably blocked by oligomycin. One must therefore conclude that there must be a means of communication between ATP and the extended pivotal mitochondrial thiol group which circumvents the oligomycin-sensitive site in the presence of gliotoxin. It is important to point out that in the previously reported ATP-energized mitochondrial volume change involving gliotoxin and DNP[5], oligomycin was not as effective as usual, also in the system involving ELLMAN's reagent ATP and DNP[4], there was an incipient inffectiveness of oligomycin. Thus, there is a tendency to vitiate the effectiveness of oligomycin by -S-S- compounds.

The picture involving an extended pivotal mitochondrial thiol group suggested that the further addition of a thiol reagent such as showdomycin could tie up the extended -SH group and thus block the flux of energy from the upper to the lower cycle. We have observed in agreement with these considerations that showdomycin almost completely inhibited the respiration-energized volume change induced by gliotoxin (Figs. 5 and 6).

An incipient mitochondrial volume change was induced by a relatively high level of ELLMAN's reagent (300 μM) and NEMI (200 μM) (Fig. 7) without the assistance of ATP or antimycin or DNP. According to the proposed rationalization the incipient effect induced by the -S-S- compound ELLMAN's reagent could resemble that induced by gliotoxin at the

20 μM level while the effect induced by 200 μM NEMI which could tie up an -SH group might not necessarily resemble that induced by 20 μM gliotoxin. Such, indeed, was the case. The effect induced by ELLMAN's reagent was respiration-energized as it was inhibited by antimycin and DNP. This effect, like that of 20 μM gliotoxin, was inhibited by ATP and to a noticeable extent by showdomycin. The effect induced by 200 μM NEMI was also respiration-energized as it was inhibited by antimycin and DNP. This effect however was not inhibited by either ATP or showdomycin.

Showdomycin at the level of 300 μM was remarkable in that it did not induce any significant volume change. Showdomycin when compared with NEMI, ELLMAN's reagent and gliotoxin thus reacted directly to the least extent with mitochondrial thiol groups possibly involved in the machinery of oxidative phosphorylation.

NEMI was noticeably random in its interaction with mitochondria in agreement with the previously optimum concentration of 30 μM observed when NEMI was used as a thiol reagent in combination with DNP or antimycin in an ATP-energized mitochondrial volume change system[3].

When the levels of gliotoxin was increased from 20 μM to 200 μM the interaction between gliotoxin and the pivotal mitochondrial thiol group was no longer a subtle, discriminating reaction. The reaction took on characteristics which resembled to a considerable extent those associated with 300 μM NEMI. This reaction was respiration-energized as it was almost entirely inhibited by antimycin and completely inhibited by DNP, but was only partially inhibited by showdomycin and not inhibited indeed if anything extended by ATP.

The effect of 20 μM gliotoxin may be viewed as a subtle diversion of respiratory energy along an alternative route and could, but not necessarily, be associated with an altered rate of respiration; however, our attempts to detect such a change were unsuccessful. This could be due to the lack of sensitivity of our measuring system. These findings with the oxygen electrode are in complete agreement with the report by LARDY *et al.*[8] that gliotoxin did not effect the respiration or phosphorylation of rat liver mitochondria in Warburg vessels. It is highly unlikely however that gliotoxin was added after the mitochondria in the survey conducted by LARDY *et al*[8].

The concept of a strategically located pivotal mitochondrial thiol group[6] has suggested fruitful experiments with gliotoxin and served as a unifying concept in the interpretation of the results. It was possible to show that there was a gradation in properties between showdomycin, NEMI, ELLMAN's reagent and gliotoxin. There is no question that a level of 20 μM gliotoxin by itself had the ability to interact in a unique, subtle, discriminating fashion with the machinery of oxidative phosphorylation. Now that such an interaction has been established, it is hoped that these results and conclusions will be examined by means of experimental techniques other than those presently being reported.

The reaction of gliotoxin with the machinery of oxidative phosphorylation without the assistance of a respiratory inhibitor or an uncoupling agent may indeed be related to the interesting biological properties of gliotoxin. Such an interaction may explain the growth inhibition of bacteria, fungi, and experimental tumors and the toxicity which limits the use of related compounds as antiviral agents[9].

MAXIA, *et al.*[10] proposed that the antiviral activity of gliotoxin was related to the ability of gliotoxin to bind to an unknown cell structure. The binding conld occur even before the cell was infected with the virus. According to this view our results with gliotoxin could be related to the antiviral activity of gliotoxin. It is difficult to see, if one applies the current knowledge of viral replication, how a subtle diversion of respiratory energy could selectively inhibit the synthesis of viral RNA without inhibiting simultaneously the synthesis of viral protein and cellular RNA as reported by MILLER, *et al.*[11] (see also[9]). The report by KARA, *et al.*[12] that the replication of the ROUS sarcoma virus took place in the mitochondria of the tumor tissue could relate our data directly to the antiviral properties of gliotoxin should the findings fo KARA, *et al.* become applicable to viral

replications which are sensitive to gliotoxin. With respect to both the antitumor and antiviral activity of gliotoxin it is of cogent interest that the synthesis of mitochondrial DNA in host cultures of cells was induced by infection with Polyoma virus[13] and Simian Virus 40[14].

The *in vitro* action of gliotoxin with mitochondria has been ascribed to its -S-S- moiety, a group also demonstrated to be essential for the biological activity of gliotoxin and related compounds[9,15~18].

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