Effect of the Bacterial DNA Gyrase Inhibitors, Novobiocin, Nalidixic Acid, and Oxolinic Acid, on Oxidative Phosphorylation*

(Received for publication, April 14, 1986)

Maureen Gallagher, Robin Weinberg, and Melvin V. Simpson
From the Department of Biochemistry, State University of New York, Stony Brook, New York 11794

When incubated with isolated intact rat liver mitochondria, novobiocin and nalidixic acid act as uncouplers of oxidative phosphorylation; they stimulate oxygen uptake and inhibit ATP synthesis. Novobiocin is about as powerful an uncoupler as is 2,4-dinitrophenol, nalidixic acid is somewhat less powerful, and oxolinic acid exerts no inhibition whatsoever at the concentrations used. The three inhibitors are without effect on oxidative phosphorylation in Escherichia coli nor does novobiocin affect this process in a novobiocin-permeable mutant of yeast. While it would appear that oxolinic acid may be a relatively specific tool for the manipulation of the superhelicity of DNA in complex systems such as mammalian mitochondria and intact mammalian cells, the specificity of each of these inhibitors may depend upon the particular conditions and species used and such experiments require adequate controls on oxidative phosphorylation.

Topological considerations of the mechanism of replication of mammalian mtDNA, a closed circular negatively superhelical molecule, lead to the expectation that mitochondria will contain enzymes which can generate swivels, can separate replicated daughter circles from each other, and can introduce negative supercoils into these circles. A type I topoisomerase from rat liver (1, 2) and Xenopus (3) mitochondria and a type II enzyme from rat liver mitochondria (4) have in fact been isolated. However, with respect to a mitochondrial gyrase, only indirect evidence based on drug studies has been available (5, 6). Thus, addition to isolated mitochondria of bacterial gyrase inhibitors, namely novobiocin, coumermycin, oxolinic acid, or nalidixic acid, leads at appropriate concentrations to the inhibition of DNA replication, to the preferential inhibition of replication of the supercoiled form, and to the concomitant appearance of the relaxed form (5, 6). However, despite intensive efforts, we have been unable to detect supercoiling activity in crude or fractionated mitochondrial extracts or in reconstituted fractions.

As we have noted previously (5, 6), the presence of a gyrase in mitochondria is not proved until it is detected directly and isolated. Thus, our continued inability to directly identify the enzyme combined with the fact that drug studies suffer from the well known hazard that the target aimed at may not be the sole target hit prompted us to seek other possible targets of these inhibitors. Such a study could also prove important to research on other systems and problems. For example, inhibitors of DNA gyrase have been used in Escherichia coli in many studies on the role of the enzyme in DNA supercoiling, replication, and recombination (7, 8), as well as on the role of DNA supercoiling on transcription, including promoter selection and usage (7, 8). Moreover, with the finding that eucaryotic type II topoisomerases are inhibited by one or more of the gyrase inhibitors (7, 9), these drugs have been used in studies on intact eucaryotic cells, e.g. those of yeast (10), Drosophila (11), Xenopus (12), and chicken (13). In our own laboratory, using isolated mitochondria, we have initiated studies (14) on the role of superhelicity on aspects of transcription and have used these drugs to manipulate mtDNA superhelicity.

Our previous investigations had already eliminated some possible targets. At the concentrations used in DNA replication studies, these drugs had no influence on the mtDNA polymerase (DNA polymerase γ) (6), or on the type I (2) or type II (4) mitochondrial topoisomerases, although polymerase γ is subject to inhibition at higher concentrations of novobiocin and coumermycin (15). We began the investigation (16) reported here asking whether these drugs might have a general "cytotoxic" effect on mitochondria and therefore we studied their effect on oxygen uptake in rat liver mitochondria and then on oxidative phosphorylation in this organelle as well as in E. coli and Saccharomyces cerevisiae. We have chosen not to use coumermycin in these studies because of the anomalous effects of its solvent, dimethyl sulfoxide, on mitochondria and possibly on intact cells.

EXPERIMENTAL PROCEDURES

Assays—Mitochondria were prepared from rat liver as described previously (17). Oxygen uptake was measured in a YSI Model 52 Biological Oxygen Monitor at 37 °C for 5 min during which time uptake was linear. Measurement of oxidative phosphorylation in isolated mitochondria was performed by measuring the uptake of 32P-labeled P, according to the method of Berger (19) in which L-[U-14C]glutamine transport is followed. In the case of intact yeast cells, oxidative phosphorylation was assessed by measuring the concentration of ATP in the cell extracts after incubation of the cells with novobiocin. ATP was measured spectrophotometrically by a coupled enzyme reaction (20). Added glyceraldehyde-3-P is converted to glyceraldehyde-3-diphosphate by added phosphoglycerate kinase, the diphosphate then being reduced to glyceraldehyde-3-phosphate by added NADH and glyceraldehyde-3-phosphate dehydrogenase.

Inhibitor Solutions—Nalidixic acid was purchased as the water-soluble sodium salt. Oxolinic acid, purchased as such, was dissolved in an exact stoichiometric amount of NaOH, an appreciable excess of which has a small inhibitory effect on oxidative phosphorylation. The range of inhibitor concentrations used here includes those used in published studies both from our laboratory and from laboratories studying gyrase or transcription.

RESULTS

Terminal Electron Transport—The effects of the three drugs on oxygen uptake by isolated rat liver mitochondria are shown in Fig. 1. At low concentrations, both novobiocin and nalidixic acid exert an appreciable stimulatory effect, no less
Inhibition of Oxidative Phosphorylation by Gyrase Inhibitors

FIG. 1. The effect of DNA gyrase inhibitors on oxygen uptake by isolated rat liver mitochondria. The reaction mixture consisted of 6.67 mM KCl, 8.33 mM K$_2$HPO$_4$, 10 mM sodium pyruvate, 0.833 mM sodium malate, 10 mM sodium succinate, 4.0 mM MgCl$_2$, 0.1 mM each of GTP, CTP, UTP, and ATP, and 1.5 ml of mitochondrial suspension (containing 0.5 mg of protein). The mixture was adjusted to pH 7.4, and the final volume was 3.0 ml. Samples were preincubated for 2 min prior to incubation which was for a period of 5 min at 37.5°C. DNP, 2,4-dinitrophenol; NAL, nalidixic acid; OXO, oxolinic acid; NOVO, novobiocin.

FIG. 2. The effect of DNA gyrase inhibitors on oxidative phosphorylation by isolated rat liver mitochondria. Reaction conditions and analytical procedures were those of the 32P assay of Nielsen and Lehninger (18). Incubation was for 15 min at 37°C. The control sample incorporated 57,956 cpm into ATP; the zero time control incorporated 244 cpm. DNP, 2,4-dinitrophenol; NAL, nalidixic acid; OXO, oxolinic acid; NOVO, novobiocin.

The standard Nielsen and Lehninger (18) assay conditions for oxidative phosphorylation (used for Fig. 2) call for a mitochondrial concentration which is about 3% that normally used in our mtDNA replication (5, 6, 17) or our transcription experiments, and a few other minor conditions differ as well. However, measurement of the effects of the drugs on ATP synthesis under our DNA replication or transcription conditions (except that no nucleotide precursors were added), yielded results virtually identical to those obtained under the Nielsen and Lehninger conditions.

Effect of Gyrase Inhibitors on Oxidative Phosphorylation in Intact E. coli Cells—A convenient method for detecting an inhibition of ATP synthesis in intact E. coli cells is to follow the transport of glutamine into the cell, since glutamine transport with lactate as the substrate is directly and specifically dependent on the synthesis of ATP. For this purpose, intact cells are first incubated with the uncoupler. For this purpose, intact cells are first incubated with the uncoupler. After 5 min at 37°C, the cells are collected, washed, and resuspended in fresh media. The cells are then incubated with [14C]glutamine, and the amount of [14C]glutamine taken up by the cells is measured by liquid scintillation counting. Oxolinic acid is an effect on oxidative phosphorylation or is a direct effect on glutamine transport.

Effect of Gyrase Inhibitors on Oxidative Phosphorylation in Intact Yeast Cells—The effect of uncouplers of oxidative phosphorylation in yeast (S. cerevisiae) can be assessed directly by examining the steady state level of ATP (20) after incubation of intact cells with the uncoupler. For this purpose, we were fortunate in having available to us a novobiocin-impermeable strain. The results show (Table II) that concentrations of novobiocin up to 4-fold higher (0.8 mM) than that which gives 95% inhibition of ATP synthesis in rat liver mitochondria have no effect on intracellular ATP concentration in the novobiocin-permeable strain, KW114, a strain which is killed at 0.16 mL novobiocin. The wild type, novobiocin-impermeable strain, A364A, was not expected to show a novobiocin effect and did not.
**Inhibition of Oxidative Phosphorylation by Gyrase Inhibitors**

**TABLE I**

_E. coli_ strains JM103 and W3110 were used. [³⁵S]Glutamine transport was assayed as described (19). Glutamine transport in _E. coli_ has been shown to be directly dependent on ATP synthesized via oxidative phosphorylation under the conditions (substrate, lactate) of this experiment. DNP, 2,4-dinitrophenol; Novo, novobiocin; Oxo, oxolinic acid; NaI, nalidixic acid.

**[³⁵S]Glutamine transported**

<table>
<thead>
<tr>
<th>Additions</th>
<th>JM103</th>
<th>W3110</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1212</td>
<td>1087</td>
</tr>
<tr>
<td>DNP, 0.5</td>
<td>125</td>
<td>259</td>
</tr>
<tr>
<td>Novo, 0.04</td>
<td>1135</td>
<td>1011</td>
</tr>
<tr>
<td>Novo, 0.08</td>
<td>1260</td>
<td>1130</td>
</tr>
<tr>
<td>Novo, 0.16</td>
<td>1139</td>
<td>1011</td>
</tr>
<tr>
<td>Novo, 0.28</td>
<td>1135</td>
<td>1013</td>
</tr>
<tr>
<td>Novo, 0.40</td>
<td>1178</td>
<td>1054</td>
</tr>
<tr>
<td>Novo, 0.56</td>
<td>1053</td>
<td>924</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The results show that when used with intact mitochondria, novobiocin and nalidixic acid are effective uncouplers of oxidative phosphorylation, with novobiocin as strong an uncoupler as 2,4-dinitrophenol. On the other hand, even elevated concentrations of oxolinic acid seems to have no discernible uncoupling effect as measured either by stimulation of respiratory or by ATP synthesis. Surprisingly, in a strain of yeast which is permeable to novobiocin, no effect on oxidative phosphorylation could be observed. It is conceivable, however, that mitochondria in situ are impermeable to this drug and become permeable upon their isolation, usually under conditions under which some swelling has occurred. Alternatively, oxidative phosphorylation in yeast may not be sensitive to these drugs.

The absence of an uncoupling effect in _E. coli_ induced by the gyrase inhibitors is not surprising in view of the studies on Gyr A and Gyr B mutants. These show great increase resistance to nalidixic acid and novobiocin class drugs, respectively, because of the resistance conferred on the DNA gyrase (21, 22). The fact that such cells continue to grow in the presence of these gyrase inhibitors points to an absence of an inhibition of oxidative phosphorylation.

The results presented here suggest the exercise of caution in the interpretation of the results of drug studies using novobiocin, nalidixic acid and, by extension, probably coumermycin. While we have found no inhibitory effects whatsoever by oxolinic acid on oxidative phosphorylation in rat mitochondria, this may not be true in other species, and caution should be exercised here as well. It is of interest that, recently, the use of novobiocin in transcription studies has shown novobiocin to strike yet another target, as yet not precisely identified (27).

The only mammalian mitochondrial system we have used thus far to test the effect of the gyrase inhibitors on oxidative phosphorylation is isolated intact mitochondria. We plan to test the effect of these drugs _in vivo_ on oxidative phosphorylation in a variety of intact cultured cell lines.

**REFERENCES**


---

**TABLE II**

_S. cerevisiae_ cells, strains KW114 (novobiocin-permeable) and A364A (novobiocin-impermeable) were grown in rich glucose medium (25). After washing, 500 mg of cells were incubated for 20 min at 30 °C in 20 mM Tris citrate buffer, pH 4.5 (26), final volume 2.5 ml. After the incubation, the cells were washed twice in 10 volumes of water at 0 °C, the final pellet was taken up in 1 ml of H₂O, and the suspension was brought to 0.6 M perchloric acid. After standing for 10 min, the precipitate was centrifuged, and the clear supernatant was neutralized with KOH. ATP was assayed as described in the text. DNP, 2,4-dinitrophenol; Novo, novobiocin.

**ATP present**

<table>
<thead>
<tr>
<th>Additions</th>
<th>KW114</th>
<th>A364A</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>20.4</td>
<td>15.2</td>
</tr>
<tr>
<td>2,4-DNP, 0.5</td>
<td>6.5</td>
<td>6.1</td>
</tr>
<tr>
<td>Novo, 0.2</td>
<td>17.2</td>
<td>11.6</td>
</tr>
<tr>
<td>Novo, 0.4</td>
<td>22.7</td>
<td>17.1</td>
</tr>
<tr>
<td>Novo, 0.6</td>
<td>27.2</td>
<td>11.7</td>
</tr>
<tr>
<td>Novo, 0.8</td>
<td>18.6</td>
<td>15.9</td>
</tr>
</tbody>
</table>

**Acknowledgments**—We are indebted to Dr. Rolf Sternglanz for a gift of the yeast strains and, in particular, his novobiocin-permeable strain. We thank Dr. Vincent Cirillo and Rita Ongjoco for doing the yeast ATP assays.