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THE EFFECT OF BACTERIAL DNA GYRASE INHIBITORS ON DNA SYNTHESIS IN MAMMALIAN MITOCHONDRIA

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Using isolated rat liver mitochondria, which have previously been shown to carry out true replicative DNA synthesis, we have obtained results which are in accord with the presence and functioning of a DNA gyrase in this organelle. The effects of the *Escherichia coli* DNA gyrase inhibitors, novobiocin, coumermycin, nalidixic acid and oxolinic acid, upon mtDNA replication suggest the involvement of the putative mitochondrial enzyme in various aspects of this process. First, the preferential inhibition of [^{3}H]dATP incorporation into highly supercoiled DNA together with the appearance of labeled, relaxed DNA are consistent with the involvement of a gyrase in the process of generating negative supercoils in mature mtDNA. Second, the overall depression of incorporation of labeled dATP into mtDNA, including the reduction of radioactivity incorporated into replicative intermediates, suggests a 'swivelase' role for the putative gyrase, and this hypothesis is further supported by results obtained on sucrose gradient centrifugation of heat-denatured, D-loop mtDNA. Here, the synthesis of the completed clean circles is inhibited while 9 S initiator strand synthesis is not, suggesting that chain elongation is blocked by the gyrase inhibitors.

Introduction

The isolation and study of the enzymes involved in the replication of mtDNA should help clarify the detailed mechanism of this process. Such studies were initiated some years ago with the isolation and purification of a DNA polymerase from rat mitochondria [1–3], an enzyme closely related and probably identical to the more recently identified rat γ-polymerase [4–9]. More recently, we have turned our attention to mitochondrial topoisomerases.

Mammalian mtDNA is a double-stranded molecule occurring predominantly as a closed circular structure. It is most often isolated in superhelical form, the sense of the supercoils always being found to be negative. The replication of such a molecule must occur under severe topological constraints. Thus, in the absence of a strain relief mechanism, the unwinding of Watson-Crick turns during replication of the molecule, while initially energetically favorable because of negative supercoiling, would become increasingly less so owing to the gradual removal of negative superturns. Indeed, a replication-limiting condition would soon

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Abbreviations: 9 S DNA, nominal designation for the short initiator strand in D-loop DNA; high molecular weight mtDNA, high molecular weight mtDNA (27 S + 39 S + small catenanes) as distinguished from the low molecular weight linear duplex DNA molecules sedimenting at about 8 S and from the large complex mtDNA structures which pellet under our conditions; $PrdI_2$, propidium diiodide; Forms I, I_o, I_R, II, III and C mtDNA, respectively, highly supercoiled, completely relaxed, partially relaxed, open circle, linear and catenated mtDNA.

be reached with the generation of highly positively supercoiled DNA.

A swivel generated by the transient breaking and rejoining of one or both strands would relieve such strain and permit replication to continue. Two enzymes, a type I and a type II topoisomerase which, respectively, are capable of catalyzing such reversible scissions have, in fact, been isolated from rat mitochondria [10–12], the former enzyme also having been found to occur in *Xenopus laevis* mitochondria [13]. However, a potentially more proficient swivelase than this nicking-closing topoisomerase would be a DNA gyrase. In addition to generating a swivel, the gyrase could continually introduce negative superturns into replicating mtDNA, avoiding the possibility of any appreciable decrease in negative supercoiling and thus maintaining the most energetically favorable conditions for the unwinding of Watson-Crick turns during replication. Moreover, maintenance of the DNA in highly supercoiled state could facilitate its transcription as well.

Preliminary experiments in our laboratory showed that all four of the bacterial gyrase inhibitors, novobiocin, coumermycin, nalidixic acid and oxolinic acid, inhibit DNA replication in isolated mitochondria [14]. In addition, it was shown that coumermycin treatment leads to the synthesis of relaxed mtDNA and the concomitant inhibition of synthesis of the superhelical form [14]. These preliminary results suggested that a functioning gyrase might be present in mitochondria. The results of further drug studies, presented here, strengthen this suggestion and offer support for two possible roles for the putative enzyme.

Experimental procedures

Materials

The sources of the gyrase inhibitors and the preparation of these solutions have been described previously [14]. Unlabeled deoxyribonucleoside triphosphates and ethidium bromide were purchased from Sigma Chemical Co. [^{3}H]dATP was obtained from New England Nuclear. Supercoiled plasmid pBR322 was purified as described [15]. Salmon sperm DNA was obtained from Calbiochem. DNA-cellulose was prepared using a mixture of single-stranded and double-stranded salmon sperm DNA according to Alberts and Herrick [16].

Methods

Enzyme assays. Assays for mitochondrial type I topoisomerase (nicking-closing enzyme) were performed with mitochondrial extract prepared by alumina grinding as described [2]. The extract was subsequently fractioned on Sephadex G200 in buffer A (10% (v/v) glycerol/1 mM Na_2·EDTA/2 mM dithiothreitol/150 mM Tris-acetate, pH 8.3) containing 50 mM NaCl. Fractions containing DNA-relaxing activity, which eluted soon after the void volumne, were pooled and used for subsequent inhibitor studies. The assay conditions, electrophoretic separation, visualization of the products and analyses were as described [14].

Partially purified mtDNA polymerase was prepared from alumina-ground mitochondrial extract [2] which was further fractionated on DNA-cellulose (C. Polz-Tejera and M.V. Simpson, unpublished data). Active fractions, eluted by a 0.6 M NaCl step, were combined and used in subsequent drug studies. The assay conditions were as described previously [2].

Isolation of mtDNA. The rats used, as well as the procedures for isolating and incubating liver mitochondria, have been described [17] except that the final volume of the incubation mixture was 10 ml and, in the work reported here and previously [14], a tight homogenizer was used to disrupt the liver (see Results) and the concentration of the nonradioactive deoxynucleotide precursor was 15 μM (not 15 mM as erroneously reported in Ref. 14). After a 45- or 60-min incubation period, a sample was removed, precipitated with trichloroacetic acid, washed and counted [2]. The remaining mitochondria were collected by centrifugation and the DNA was extracted and analyzed by sucrose density gradient centrifugation [14]. In some experiments, samples were removed from the sucrose gradient fractions and further studied by agarose electrophoresis (see below). For the study of the 9 S strand of D-loop DNA, the fractions in the high molecular weight region of the gradient were combined, the mtDNA was heat denatured for 60 s at 100°C [17], and was then subjected to a second sucrose density gradient centrifugation.

Gel electrophoresis. mtDNA samples were

analyzed by electrophoresis in 0.7% agarose prepared in several buffers. Most gels were run un E buffer (40 mM Tris acetate, pH 8.3/20 mM sodium acetate/2 mM $Na_2 \cdot EDTA$). Some gels were run at low temperature (4°C) in E buffer containing 5 mM Mg^{2+} in order to improve the separation between open circular and fully relaxed mtDNA [18]. For the same reason, samples were occasionally electrophoresed in 0.7% agarose in E buffer containing 0.5 μg/ml ethidium bromide. After staining the gels in 1.0 μg/ml ethidium bromide, the bands were visualized by ultraviolet illumination, and were photographed and quantitated by scanning the film with a Joyce-Loebl microdensitometer.

When the distribution of radioactivity among the various forms of mtDNA was to be determined, the agarose gels were photographed as described above, destained and then soaked for 1 h in freshly prepared 1 M sodium salicylate. The gels were dried without heating on a Biorad gel dryer for 1 h, transferred to a fresh piece of Whatman 3MM paper and further dried for several hours. The radioactive bands were visualized by fluorography on Kodak SB5 film after exposure for more than 1 week using a Dupont Cronex Hi-plus intensifying screen. Quantitation was achieved using the Joyce-Loebl microdensitometer.

Results

Inhibitionof mtDNA synthesis by each class of gyrase inhibitor

There are four *E. coli* gyrase inhibitors usually used in studies on this enzyme and they can be divided into two classes [19–21]. The related drugs novobiocin and coumermycin comprise one class which affects the energy transducing function of the bacterial enzyme by competitively inhibiting the interaction of ATP with gyrase subunit B. The nalidixic acid and oxolinic acid pair constitute the second class which affects the breaking-rejoining function of gyrase by inhibiting the gyrase A subunit. Fig. 1 shows a sucrose density gradient analysis of mtDNA labeled with [^{3}H]dATP in the presence of increasing concentrations of one gyrase inhibitor of each class.

The addition of novobiocin to the isolated

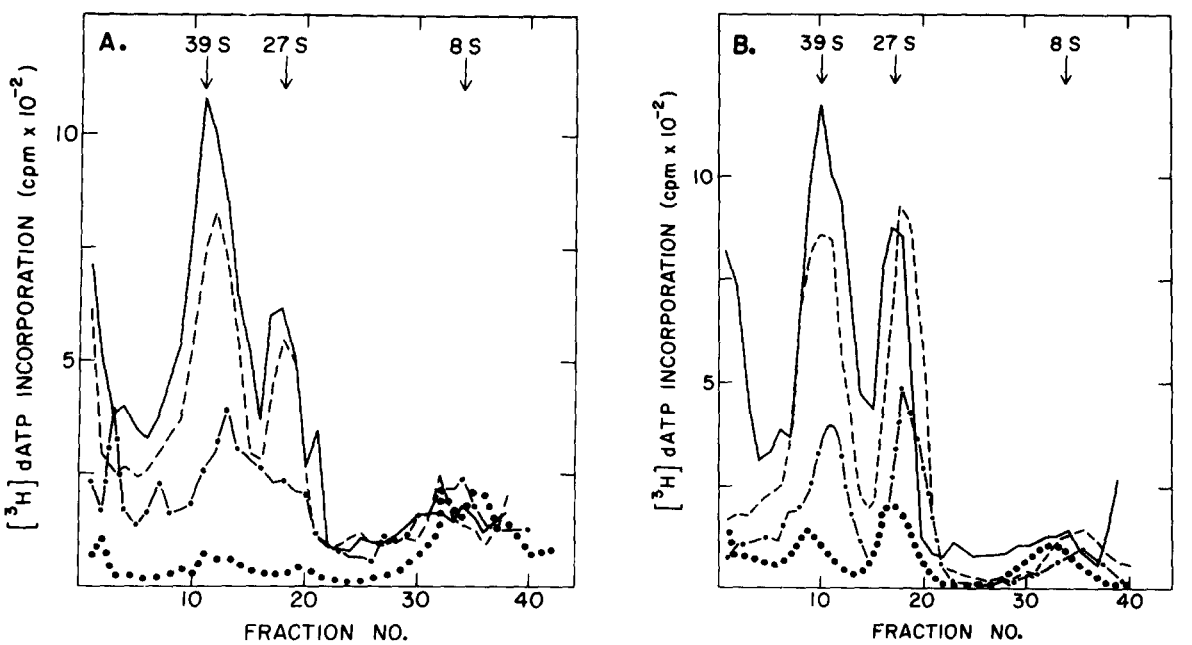

Fig. 1. Sucrose gradient centrifugation of mtDNA synthesized in the presence of novobiocin and nalidixic acid. Incubation of mitochondria was as described in Methods. A, Novobiocin: ———, 0 μg/ml; ------, 50 μg/ml; ·–·–·, 300 μg/ml; ·····, 500 μg/ml. B, Nalidixic acid; ———, 0 μg/ml; ------, 100 μg/ml; ·–·–·, 300 μg/ml; ·····, 500 μg/ml.

mitochondrial system results in a general depression of the incorporation of radioactivity into high molecular weight mtDNA (Fig. 1A). We find that 50% inhibition is achieved at 160 μg/ml novobiocin, a drug concentration comparable to that which inhibits DNA replication 50% in *E. coli* [14]. While coumermycin produces a similar inhibitory effect [14], the novobiocin results show some additional features. As the concentration of this drug increases, the 39 S peak shifts to lower *s* values along with a broadening of the peak, particularly at a drug concentration of 300 μg/ml. This phenomenon could result from the appearance of form I_o DNA, i.e., completely relaxed DNA (whose *s* value is 27 S), or to a decrease in superhelicity of form I DNA, or both. These effects could be brought about by a partial inhibition of a mtDNA gyrase.

The inhibitory effect of nalidixic acid on the various forms of mtDNA is shown in Fig. 1B. In addition to the inhibition of all the high molecular weight mtDNA forms, a shift of the 39 S peak toward 27 S can be seen at the 300 μg/ml concentration, although it is not as pronounced as the shift effected by novobiocin (Fig. 1A).

Preferential inhibition of the synthesis of supercoiled mtDNA

The most dramatic effect of nalidixic acid, however, is its apparent preferential inhibition of synthesis of highly supercoiled mtDNA relative to more relaxed forms. As seen in Table I, the ratio of 39 S:27 S DNA (representing the relative amounts of highly supercoiled to highly-relaxed mtDNA) is 2.06 in the absence of nalidixic acid but decreases to 0.64 at high concentrations (500 μg/ml), indicating a 3-fold greater reduction in 39 S DNA relative to 27 S material. This differential effect on the sucrose gradient peaks is also observed to varying extents, as shown quantitatively in Table I, with the other gyrase inhibitors, namely novobiocin (density gradient profile in Fig. 1A), coumermycin (see profile in Ref. 14), and oxolinic acid (profile not shown).

Effect of nalidixic acid on the synthesis of highly supercoiled mtDNA

The results of our preliminary experiments [14] showed that in the presence of coumermycin, DNA precursor incorporation into highly supercoiled DNA is preferentially inhibited and a new form of DNA appears. This new DNA species was detected on CsCl-propidium diiodide isopycnic centrifugation as a new band, below the usual lower band, in a position which corresponds to that of relaxed mtDNA, but was not further studied. Inasmuch as the identification of this material is important to the problem of implicating a mitochondrial gyrase as the target of the bacterial gyrase inhibitors, we wished to confirm its nature using an independent method; here we have used agarose gel electrophoresis. Moreover, we have substituted nalidixic acid for coumermycin in order to determine whether an inhibitor of a different class would give the same results. Inasmuch as the studies we have reported so far deal solely with labeled, i.e., newly synthesized, DNA and, for the purposes of this experiment, we wished to so confine the gel electrophoresis study, we used fluorography rather than ethidium bromide staining to detect the bands in the gel.

The results show that in the absence of inhibitor (Fig. 2A), fractions from the 39 S peak (wells

TABLE I

RELATIVE EFFECT OF BACTERIAL GYRASE INHIBITORS ON THE SYNTHESIS OF 39 S AND 27 S mtDNA

Drug	Concentration	39 S/27 S [a]
	μg/ml	
Nalidixic acid	0	2.06
	100	1.34
	300	1.26
	500	0.64
Novobiocin	0	2.93
	50	2.44
	300	[b]
	500	[b]
Coumermycin	0	2.43
	5	2.20
	10	1.43
	100	[b]
Oxolinic acid	0	2.05
	100	1.76
	300	1.09

[a] The ratio is calculated from the areas under the 39 S and 27 S sucrose gradient peaks of labeled mtDNA.

[b] The ratio could not be determined accurately because the 39 S and 27 S peaks were not resolvable.

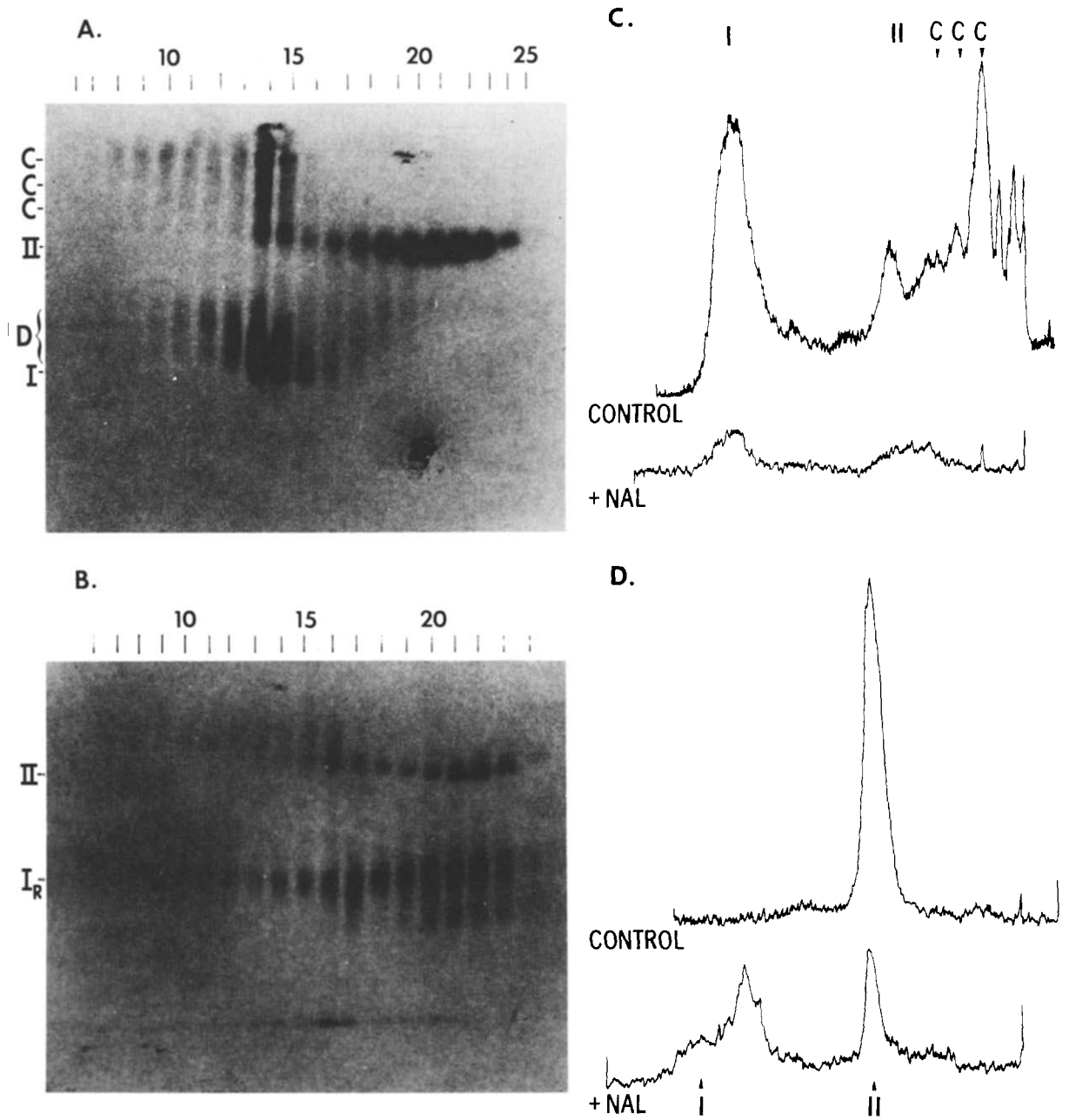

Fig. 2. Gel electrophoresis of sucrose density gradient fractions of mtDNA synthesized in the presence of nalidixic acid. Gradient fractions 6–17 comprise the 39 S peak and fractions 18–24 the 27 S peak. Radioactive bands were detected by fluorography. A, control; B, 150 μg/ml nalidixic acid; C, densitometric tracing of 39 S fraction 14 from control and inhibited samples; D, densitometric tracing of 27 S fraction 21 from control and inhibited samples. See Abbrevatiations for definition of labels for the DNA bands.

6–17) contain a large proportion of radioactivity in the form of highly supercoiled mtDNA and in slower migrating catenated mtDNA forms (dimeric and small multimeric catenanes). There is also some form II DNA, especially in wells 14 and 15, and a diffuse band of D-loop DNA. The con-

trol 27 S material, wells 18–24, is almost entirely form II DNA. The effect of the presence of nalidixic acid is shown in Fig. 2B. Two points to note are the following: first, as expected, less radioactivity is incorporated into all mtDNA forms: second, there are new species of mtDNA in the 27 S fractions which have greater electrophoretic mobility than form II DNA . In passing, it is also of interest to note that the incorporation of labeled precursor into the slowly migrating catenanes is also strongly inhibited.

A quantitative measure of these effects is possible from the densitometric traces of wells 14 and 21 from control and inhibited samples. From Fig. 2C, it can be calculated that highly supercoiled material is inhibited 86% (and incorporation into complex forms is inhibited 94%). Fig. 2D shows that the radioactivity in 27 S open circle mtDNA decreases by 38% after drug treatment. Concomitantly, material of greater electrophoretic mobility than form II DNA, but with fewer supercoils than 39 S mtDNA, appears with 2.3-times the radioactivity that is contained in the form II peak.

Thus, the combined results of the sucrose gradient and the gel electrophoresis experiments show that the presence of nalidixic acid results in the appearance of fully relaxed as well as of partially supercoiled DNA molecules. While the latter species resemble fully relaxed mtDNA by appearing in the 27 S peak, their mobility on the agarose gels indicates that they are only partially relaxed.

The effect of the gyrase inhibitors upon complex mtDNA multimers

In addition to relatively simple catenanes composed of a few DNA circles, rat liver mitochondria have been shown to contain fairly large DNA structures which possess cores [22] and which appear to be at least partly catenated (Ref. 12 and Castora, F.J., Sternglanz, R. and Simpson, M.V., unpublished data). Complex mtDNA structures have also been studied by Van Tuyle [23,24]. The *s* values of these structures are much greater than 39 S and under the conditions of our sucrose density gradient centrifugations used to resolve the monomeric forms, these complexes pellet. Thus, in conjunction with our previously described inhibitor experiments on mtDNA monomers, we took the opportunity to do preliminary studies on the pelleted material. The results show (Table I) that precursor incorporation into the large structures does occur although to a much smaller extent than into the high molecular weight mtDNA and that it is inhibited by coumermycin to an equal or slightly greater extent than into the monomers. The significance of the relatively small amount of radioactivity present in the pellet is not clear at this time inasmuch as specific activity and other data are not yet available.

Role of putative gyrase in mtDNA chain elongation

The preferential inhibition by bacterial gyrase inhibitors of the formation of supercoiled DNA accompanied by the appearance of relaxed molecules as well as those of lower than normal superhelicity, suggests that a DNA gyrase may be functioning in the terminal steps of mtDNA replication. Thus, the gyrase would be acting to introduce negative supercoils into mature form I_o DNA. In addition, however, the decrease brought about by the gyrase inhibitors in the labeling of high molecular weight mtDNA and, in particular, in the synthesis of replicative intermediates, i.e., expanded D-loops and gapped circles, suggests the involvement of the inhibitor target in the process of chain elongation. The possible 'swivelase' role of a gyrase, discussed in the Introduction, would

TABLE II

THE EFFECT OF COUMERMYCIN ON [^{3}]dATP INCORPORATION INTO COMPLEX mtDNA STRUCTURES PELLETED BY SUCROSE GRADIENT CENTRIFUGATION

Coumermycin (μg/ml)	High molecular weight mtDNA [a] (cpm)	Inhibition (%)	Pellet [b] (total cpm)	Inhibition (%)
0	2937	0	352	0
5	2086	29	246	30
10	1794	39	171	51
25	1698	42	143	59

[a] Fractions ≥ 27 S were combined and the radioactivity measured after trichloroacetic acid precipitation, as described [2].

[b] The material at the bottom of the centrifuge tube after the sucrose gradient sedimentation was suspended in 1 ml STE and an aliquot precipitated with trichloroacetic acid and counted [2].

explain such an involvement.

To gain support for this suggestion, it was necessary to show that the inhibition of labeling of high molecular weight mtDNA by the gyrase inhibitors results from a decreased incorporation of label into clean circles rather than a decreased synthesis of the 9 S initiation strand of D-loop DNA. Such an experiment is particularly important in the rat liver system since neither the 39 S nor the 27 S regions of the gradient is free of D-loop DNA. (In rat liver, unlike in L-cells, most of the D-loop DNA is found in the 39 S region of the gradient [17]; the remainder is present in the 27 S peak). Thus, after the incubation of mitochondria, the labeled high molecular weight DNA was isolated by sucrose gradient centrifugation, the 9 S initiator strand was liberated by thermal denaturation [17], and the sample was resubjected to sucrose gradient sedimentation.

The results of such an experiment are shown in Fig. 3. Here, the mitochondrial system was treated with nalidixic acid, but similar profiles have been obtained with other drugs as well. The important point to note is that, although inhibition with 100 or 150 μg/ml nalidixic acid leads to a decrease in overall incorporation of radioactivity, the synthesis of 9 S DNA remains the same or increases while the radioactivity in newly synthesized high molecular weight mtDNA is greatly reduced.

This phenomenon is shown more quantitatively in Table III where the results are given for all four gyrase inhibitors. There appears to be a several-fold decrease in high molecular weight mtDNA synthesis relative to initiator strand synthesis with any of the inhibitors studied.

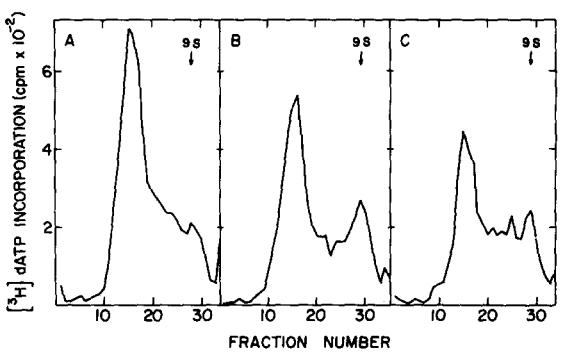

Fig. 3. Sucrose gradient centrifugation of heat-denatured mtDNA labeled is the presence of nalidixic acid. A, control. B, 100 μg/ml nalidix acid. C, 150 μg/ml nalidixic acid.

TABLE III

EFFECT OF BACTERIAL GYRASE INHIBITORS ON THE SYNTHESIS OF THE 9 S INITIATOR STRAND OF D-LOOP DNA

Drug concentration (μg/ml)		[3H]dATP Incorporation into high molecular weight mtDNA [a] (cpm)	9 S mtDNA	High molecular weight/9 S mtDNA [b]
Nalidixic acid	0	1184	330	3.59
	100	933	474	1.97
	150	633	386	1.64
Oxolinic acid	0	1152	579	1.99
	100	781	799	1.16
	500	166	359	0.46
Novobiocin	0	222	40	5.55
	25	178	73	2.44
	100	81	48	1.70
Coumermycin	0	386	174	2.21
	5	297	279	1.06
	25	76	75	1.01

[a] The high molecular weight mtDNA represents the label in fractions ≥ 27 S after sucrose gradient centrifugation of heat-denaturated mtDNA.

[b] For the purposes of this experiment, the absolute values for extent of precursor incorporation are more important than the relative values given in this column. However, the latter values serve somewhat to correct for small differences between samples in the recovery of mtDNA in the phenol extraction procedure and the sucrose density gradient run.

Thus, these results indicate that inhibition of the presumed mtDNA gyrase does not interfere with production of 9 S DNA but it does block elongation of the growing daughter strands, further supporting the postulated role of a mitochondrial gyrase acting as a swivelase.

What is the mitochondrial target enzyme of the bacterial gyrase inhibitors?

While the results presented thus far are consistent with the view that the target of the bacterial gyrase inhibitors is a mitochondrial gyrase, DNA replication systems are known to be complex. Thus, the effects we have observed could be indirect, possibly resulting from some influence of the drugs on some other enzyme or protein involved in mtDNA replication. For example, DNA polymerase γ and yeast nuclear type I topoisomerse

show some inhibition by coumermycin [25] and novobiocin [26], respectively, albeit at extremely high drug concentrations. Perhaps more to the point is the novobiocin inhibition [25] of DNA polymerase α at drug concentrations only slightly higher than those used by us in intact mitochondria. Thus, it was crucial to test the effects of the gyrase inhibitors on any enzymes thought to play a role in mtDNA replication. Three enzymes which probably play such a role are mtDNA polymerase [1–9], mitochondrial type I topoisomerase [10] and mitochondrial catenating enzyme (a type II topoisomerase) [11,12], the source for all three being rat liver. It was therefore possible to test these enzymes for sensitivity to the four drugs. Drug concentrations were chosen which result in about a 50% inhibition of DNA synthesis in isolated mitochondria. The results (Table IV) do not indicate inhibition of any of the mitochondrial enzymes by any of the drugs.

The effect of homogenizer tightness on extent of inhibition by the gyrase inhibitors

In the early phase of these studies, we were perturbed by the high levels of some of the drugs needed to produce 50% inhibition of synthesis of total mtDNA. Removal of the 8 S DNA fraction, which is not inhibited very much by the gyrase inhibitors, resulted in somewhat lower drug levels. However, we wondered whether the major block was one of drug penetration. To disrupt the mitochondrial membranes slightly, we substituted a tight-fitting all glass homogenizer for our normally used loose-fitting one. As seen in Table V, use of the tight homogenizer leads to only a slight reduction of incorporation of precursor nucleotide but a 10–45-fold greater inhibition by coumermycin. Similar (but less dramatic) results were obtained with oxolinic acid (4–5-fold greater inhibition) and nalidixic acid (2–3-fold greater inhibition). Consequently, all experiments reported here as well as our preliminary experiments [14] were done using the tight homogenizer.

An important experimental consequence of the resistance of intact mitochondria to penetration by these classes of gyrase inhibitors is that it may prove difficult to use these drugs successfully in studies employing whole cells. For example, L-cells and Hela cells are often used in mitochondrial biogenesis studies. An additional consequence is

TABLE IV

EFFECT OF BACTERIAL GYRASE INHIBITORS ON DNA POLYMERASE, NICKING-CLOSING ENZYME, AND CATENATING ENZYME FROM RAT LIVER MITOCHONDRIA

Drug	concentration (μg/ml)	Inhibition of			
		Intact mitochondria [a] (%)	DNA polymerase [b] (%)	Nicking-closing enzyme [c] (%)	Catenating enzyme (%)
Novobiocin	50	27	0	0	
	200	55	1	5	0
Coumermycin	5	40	0	0	[e]
	50	100	[d]	[d]	–
Nalidixic acid	50	14	−6	−10	
	200	50	−13	−10	0
Oxolonic acid	50	12	2	0	
	200	35	4	0	0

[a] Average values based on sucrose density gradient profiles from a number of previous experiments on intact mitochondria.

[b] Assayed as described [2].

[c] Assayed as described [10].

[d] Coumermycin, tested at 50 μg/ml, a concentration well in excess of that required for complete inhibition of DNA replication in isolated mitochondria, inhibits somewhat but this is caused mainly by its solvent, dimethylsulfoxide.

[e] Coumermycin could not be tested validly because of certain effects of its solvent, dimethylsulfoxide [12].

TABLE V

EFFECT OF HOMOGENIZER TIGHTNESS ON EFFECTIVESS OF COUMERMYCIN IN INHIBITING mtDNA REPLICATION [a]

Coumermycin (μg/ml)	Tight homogenizer		Loose homogenizer	
	[^{3}H]dATP Incorporation (cpm)	Inhibition [b] (%)	[^{3}H]dATP Incorporation (cpm)	Inhibition (%)
0	1438	0	1612	0
5	1271	12	1595	1
10	788	46	1600	1

[a] Livers were diced and pieces were selected randomly for homogenization by all glass homogenizers, either tight or loose (tolerances 105 and 175 mm, respectively). Results are the average of several experiments.

[b] Because trichloroacetic acid precipitation of bulk mtDNA was used rather than isolation of high molecular weight mtDNA by sucrose gradient analysis, the % inhibition observed is lower than usual.

that the extent of inhibition for a given drug level varies somewhat from experiment to experiment.

Discussion

Isolated rat liver mitochondria appear to be both a valid and a valuable system for studying the replication of mtDNA, and have been used extensively in a number of laboratories. The incorporation of labeled precursor has been shown to reflect true replication rather than repair [27–29] and precursor has been shown to be incorporated both into the 9 S D-loop strand, into later stage intermediates and into final daughter molecules (i.e., clean superhelical circles) [17]. We have described here drug studies using this system in an attempt to discover whether a DNA gyrase functions in mtDNA replication.

We have shown here and in a previous preliminary communication [14] that addition of inhibitors of bacterial gyrase to isolated mitochondria during mtDNA synthesis reduces the synthesis of all forms of high molecular weight mtDNA, with a strong preference for the inhibition of the synthesis of form I DNA. Accompanying this relative decrease in the formation of highly supercoiled DNA is a shift in the sucrose density gradient profile suggesting the formation of DNA species with less than the normal extent of supercoiling. This view was confirmed by banding the mtDNA in CsCl/PrdI$_2$ [14] and by agarose gel electrophoresis, techniques which also revealed that the presence of the bacterial gyrase inhibitors had completely prevented the supercoiling of some of the newly synthesized mtDNA. These results are in accord with the functioning of a mtDNA gyrase at the final stages of formation of supercoiled mtDNA. The observations of Bogenhagen and Clayton [30] that fully relaxed closed circles exist as immediate precursors of superhelical DNA in mouse L-cell mitochondria lends support to this view.

A second possible function of the putative gyrase is also suggested by the results. In addition to inhibiting mtDNA supercoiling, all the bacterial gyrase inhibitors exert a general inhibition on the synthesis of all forms of mtDNA; the entire sucrose density gradient profile of mtDNA is decreased. Moreover, when the CsCl-PrdI$_2$ profile was examined (in this case, the inhibitor was coumermycin), it was evident from the inhibition of precursor incorporation into intermediate density DNA, that expanded D-loop and gapped DNA were included in the general inhibition of mtDNA synthesis [14]. An explanation of this general inhibition could be in the possible swivelase role of the putative gyrase, discussed in the Introduction. This view is supported by the finding that the synthesis of the 9 S initiation strand, derived from D-loop DNA by denaturation, is not curtailed by gyrase inhibitor (nalidixic acid, in this experiment), whereas the synthesis of the remaining clean circles is strongly inhibited. The 9 S strand is only about 680 nucleotides in length [31] and its synthesis would

remove approx. 68 negative superturns from form I DNA . Thus, a swivel might well not be required, which would account for the absence of any effect by gyrase inhibitors. Indeed, when L-cell D-loop mtDNA was incubated with DNA polymerase β, synthesis of the 9S initiation strand could occur without the introduction of a swivel [32].

While the results obtained in this and in our preliminary studies are consistent with the presence of a functioning DNA gyrase in rat liver mitochondria and, indeed, lend support to this view, the evidence obtained cannot be said to constitute unequivocal proof. As is true for most drug studies performed in in vivo and in other complex systems, indirect effects are possible. While we have tried, insofar as we were able, to control such possible indirect effects by varying the type of inhibiting drug used and by showing that the drugs do not inhibit various other relevant non-gyrase targets (e.g., mtDNA polymerase and mitochondrial type I and type II topoisomerases), one cannot eliminate all possible indirect effects in this manner. Unequivocal proof of the presence and functioning of a DNA gyrase in mitochondria must come in great part from experiments on the isolated enzyme. Attempts to detect a gyrase-like type II topoisomerase in mitochondria have led to the isolation and partial purification of a type II topoisomerase with catenating, knotting and decatenating activity but, thus far, without the ability to catalyze the supercoiling of relaxed DNA [12]. Further attempts to elicit supercoiling activity from mitochondrial extracts and from the type II topoisomerase are in progress.

Acknowledgements

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