Biochem. J. (1980) **186**, 325–329
Printed in Great Britain

The Effect of Nitrofurantoin on Deoxyribonucleic Acid Synthesis in Plant Mitochondria

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(*Received* 17 *July* 1979)

Nitrofurantoin (1-{[(5-nitrofuran-2-yl)methylene]amino}imidazolidine-2,4-dione), a widely used drug and also a well-known bacterial mutagen, inhibits DNA synthesis in mitochondria from 48h etiolated seedlings of *Vigna sinensis* (Linn.) Savi (snake bean). The effect appears at the level of the uptake of radioactive deoxynucleoside triphosphates by the plant mitochondria. Nitrofurantoin does not inhibit DNA synthesis *in vitro* by homogeneous *Escherichia coli* DNA polymerase I and DNA polymerase from avian-myeloblastosis virus. No specific nitroreductase activity could be detected in mitochondria.

The use of inhibitors as tools in the investigation of cellular processes has been particularly useful in the characterization of different biosyntheses in the cells. Nitrofurantoin (1-{[(5-nitrofuran-2-yl)methylene]amino}imidazolidine-2,4-dione), an antibacterial drug in extensive use, is a potent inhibitor of bacterial growth and also mutagenic to certain bacteria (Dodd & Stillman, 1944; Goodman & Gilman, 1975; Roschenthaler *et al.*, 1970). However, its use as a tool in the understanding of cellular biosynthetic processes has so far been very limited because its mode of action is poorly understood. It has been reported very recently that nitrofurantoin interferes with gene expression in a highly specific manner by inhibiting specifically the expression of one class of genes in translation, particularly at lower doses (Herrlich & Schweiger, 1976).

It is now well established that, besides containing a prokaryotic type of protein-synthesizing machinery, mitochondria from divergent sources contain a circular DNA genome, DNA-replication machinery and RNA polymerase activity (Schatz & Mason, 1974; Ashwell & Work, 1970). We have already reported that nitrofurantoin inhibits the incorporation of amino acid into proteins synthesized by the isolated mitochondria from a variety of sources (Goswami *et al.*, 1974). In the present investigation we have studied the effect of nitrofurantoin on DNA synthesis *in vitro* by the isolated mitochondria from 48h etiolated seedlings of *Vigna sinensis* (Linn.) Savi (snake bean), and also examined its effect on DNA synthesis *in vitro* by the homogeneous *Escherichia coli* DNA polymerase I and DNA polymerase from AM virus, the two most widely studied DNA polymerases.

Abbreviation used: AM virus, avian-myeloblastosis virus; DNAase, deoxyribonuclease.

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Materials and Methods

Radioactive deoxynucleoside triphosphates (dNTP) were purchased from New England Nuclear Corp., Boston, MA, U.S.A., or The Radiochemical Centre, Amersham, Bucks., U.K. *Escherichia coli* DNA polymerase I, AM-virus DNA polymerase, antisera against *E. coli* DNA polymerase I and radioactive deoxynucleoside triphosphates were generously given by Dr. L. A. Loeb, University of Washington, Seattle, WA, U.S.A Homogeneous *E. coli* DNA polymerase I was purified by the method of Jovin *et al.* (1969) and AM-virus DNA polymerase was purified as described by Kacian & Spigelman (1974). Nitrofurantoin was purchased from Sigma.

Germination of seeds and isolation of mitochondria

Germination of seeds of *V. sinensis* (purchased from local market) and isolation of mitochondria were carried out as reported by Goswami *et al.* (1973).

0306-3283/80/010325-05 $1.50/1

Incorporation of radioactive dNTP by isolated mitochondria

The incubation mixture and reaction conditions are given in the legends to the Tables and Figures.

Determination of radioactivity incorporated into mitochondrial DNA

The incubation was stopped by the addition of 1 ml of ice-cold 10% $HClO_4$ containing 20 mM-sodium pyrophosphate and was chilled for 10 min. The incubation mixture was then centrifuged at 3000*g* for 10 min at 4°C. The supernatant was removed and the pellet was dissolved in 0.5 ml of 0.2 M-NaOH and heated in a boiling-water bath for 30 min. The tubes were then chilled for 15 min, and 5 ml of 10% $HClO_4$ containing 20 mM-sodium pyrophosphate was added to it for precipitation. The precipitate was collected on a glass-fibre filter disc and radioactivity was determined as described elsewhere in a liquid-scintillation spectrometer (Dube *et al.*, 1977).

Uptake of radioactive dNTP by the isolated plant mitochondria

The incubation mixture and reaction conditions are given in the legend of Fig. 1. The reaction was stopped by the addition of a large excess of unlabelled dNTP (corresponding to the radioactive dNTP used) and by the addition of 5 ml of ice-cold buffer A (50 mM-Tris/HCl, pH 7.4, containing 0.25 M-sucrose). The incubation mixture was rapidly filtered through a Millipore filter disc (0.45 μm) and was further washed with 15 ml of the same buffer (ice-cold). The filter paper discs were then dried and radioactivity was determined by the method described elsewhere (Dube & Loeb, 1976), in a liquid-scintillation spectrometer.

Determination of protein and DNA

Protein was determined by the method of Lowry *et al.* (1951) and the DNA was measured by the diphenylamine method of Burton (1956).

Results and Discussion

When incubated with radioactive dGTP, isolated mitochondria from germinating seeds of *V. sinensis* incorporated the radioactivity into acid-insoluble material that was resistant to alkali but was hydrolysed into an acid-soluble product by hot trichloroacetic acid or $HClO_4$ and was also hydrolysed by pancreatic DNAase (Dipak K. Dube, unpublished work). The incorporation of radioactive dAMP into acid-insoluble material is increased with the increase in time of incubation (Fig. 1*b*), and hence truly represents DNA synthesis *in vitro* by plant mitochondria. The optimum conditions for mitochondrial DNA synthesis were also determined (results not shown).

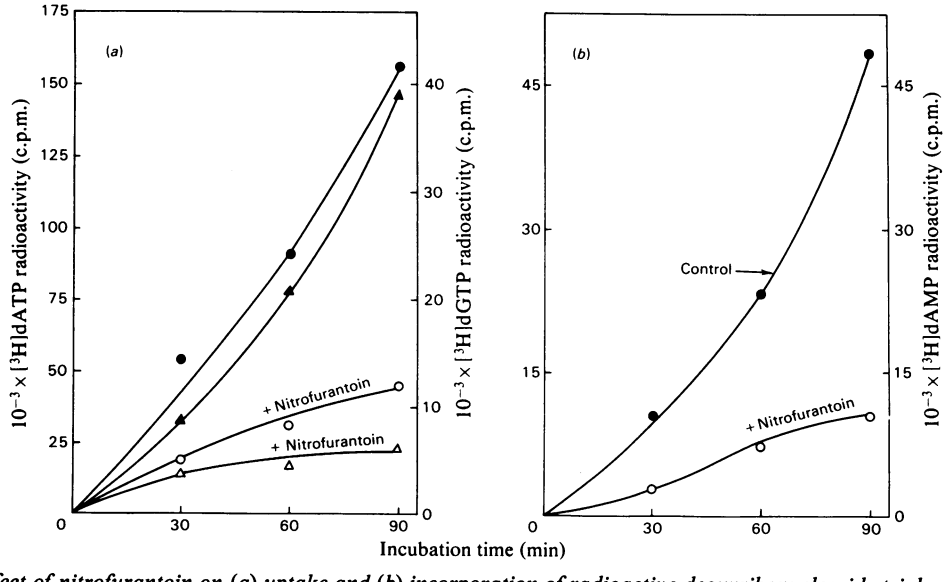

Fig. 1. *Effect of nitrofurantoin on (a) uptake and (b) incorporation of radioactive deoxyribonucleoside triphosphates into DNA by plant mitochondria*

(*a*) The reaction mixture and incubation conditions are the same as given in the legend of Table 1. Portions (0.2 ml) were removed at different times of incubation. The reaction was stopped by the addition of excess unlabelled dGTP or dATP and 1 ml of ice-cold buffer A. Radioactivity was determined as described in the Materials and Methods section. ●, ○, uptake of [^{3}H]dGTP; ▲, △, uptake of [^{3}H]dATP. (*b*) Reaction conditions are the same as given above. Acid insoluble radioactivity was determined as described in the Materials and Methods section.

Nitrofurantoin inhibits the incorporation of radioactive dNTP into mitochondrial DNA (Table 1 and Fig. 1*b*). Even at low concentration (10 μg/ml), nitrofurantoin inhibits about 75% of the mitochondrial DNA synthesis.

Many carcinogens and/or mutagens may bind with DNA templates and subsequently inhibit DNA synthesis *in vitro* and decrease the fidelity of the process (Sirover & Loeb, 1974; Loeb *et al.*, 1977). In order to examine if nitrofurantoin, a well known bacterial mutagen, inhibits DNA synthesis *in vitro* in this manner, we incubated activated calf-thymus DNA with nitrofurantoin for different time periods before the incorporation studies. DNA synthesis *in vitro* by AM-virus DNA polymerase, devoid of any DNAase activity (Battula *et al.*, 1975), is not affected by preincubation of the template, which suggests that nitrofurantoin does not combine with DNA (Table 2). The inhibition of mitochondrial DNA synthesis by nitrofurantoin may be due to the inhibition of dNTP uptake or due to the inhibition of mitochondrial DNA polymerase activity or both. In order to clarify this point, we examined the effect of the drug on the uptake of radioactive dNTP, namely [^{3}H]dATP, [^{3}H]dATP and [^{3}H]dGTP, by plant mitochondria. The results shown in Fig. 1(*a*) indicate that nitrofurantoin inhibits drastically the uptake of both [^{3}H]dATP and [^{3}H]dGTP. Moreover, the effect appears at the early stage of incubation. However, the inhibition of dNTP uptake is not specific for dNTP. Nitrofurantoin also inhibits the incorporation of [^{3}H]uridine into mitochondrial RNA and its uptake by plant mitochondria (Fig. 2). Addition of ATP in the incubation system does not reverse the inhibitory effect of nitrofurantoin. Hence it appears that inhibition by nitrofurantoin of the synthesis of RNA and DNA in isolated plant mitochondria is primarily due to the inhibition of uptake of nucleic acid precursors within the mitochondria. To understand more about the mode of action of nitrofurantoin, we preincubated mitochondria with the drug (50 μg/ml) at 37°C for 10 min. The pretreated mitochondria were then washed with iso-osmotic buffer, and the washed mitochondria were subsequently used for incorporation and uptake studies. The results given in Fig. 3 indicate that the inhibitory effect of nitrofurantoin is reversed, due to the washing of pretreated mitochondria. From these results it may be suggested that the presence of nitrofurantoin in the incubation mixture is an essential prerequisite for its inhibitory effect. From these data one can also rule out the possibility of the presence of specific nitroreductase activity in mitochondria that can convert nitrofurantoin into its active metabolite (McCann & Ames, 1976; McCalla *et al.*, 1971). This conclusion is further substantiated by the results given in Table 3,

Table 1. *Effect of nitrofurantoin on the incorporation of [^{3}H]dGTP into DNA by plant mitochondria in vitro*

Assays were carried out in duplicate in a reaction mixture (total vol. 0.2 ml) containing: 50 mM-Tris/HCl, pH 7.4, 5 mM-$MgCl_2$, 0.25 mM-phosphoenolpyruvate, 0.25 mM-ATP, 12.5 μM-dATP, -dTTP and -dCTP, 0.25 μM-[^{3}H]dGTP (7000–8000 c.p.m./pmol), 0.25M-sucrose and 0.8–1 mg of mitochondrial protein. The reaction mixture was incubated for 90 min at 37°C with constant shaking. The reaction was stopped by the addition of 1 ml of (ice-cold) 10% $HClO_4$ containing 20 mM-sodium pyrophosphate.

Addition	Amount (μg)	Incorporation (pmol of [^{3}H]dGMP/mg of DNA)
None	—	0.61
Nitrofurantoin	2	0.126
	5	0.107
	10	0.006

Table 2. *Effect of nitrofurantoin on DNA synthesis in vitro by homogeneous E. coli DNA polymerase I and AM-virus DNA polymerase*

Assays were carried out in duplicate in a reaction mixture (total vol. 0.1 ml) containing: 50 mM-Tris/HCl, pH 7.4, 5–10 mM-$MgCl_2$, 1 mM-dithiothreitol, 25 μM-dATP, -dCTP and -dTTP, 20 μM-[^{3}H]dGTP (50–100 c.p.m./pmol), 20 μg of 'activated' calf-thymus DNA and DNA polymerase enzyme (0.4 unit of DNA polymerase I or 0.2–0.4 μg of AM-virus DNA polymerase). The reaction was incubated for 30 and 50 min with DNA polymerase I (Pol. I) and AM-virus DNA polymerase respectively at 37°C. Radioactivity in the acid-insoluble material was determined as described in Materials and Methods section.

		Amount of [^{3}H]dGMP incorporated (pmol)	
Addition*	Concn. (μg/ml)	Pol. I	AM-virus DNA polymerase
None		53	131
Nitrofurantoin	50	54	113
	200	40	103
	400	49	98

* Activated DNA and nitrofurantoin were preincubated for 30 min at 25°C.

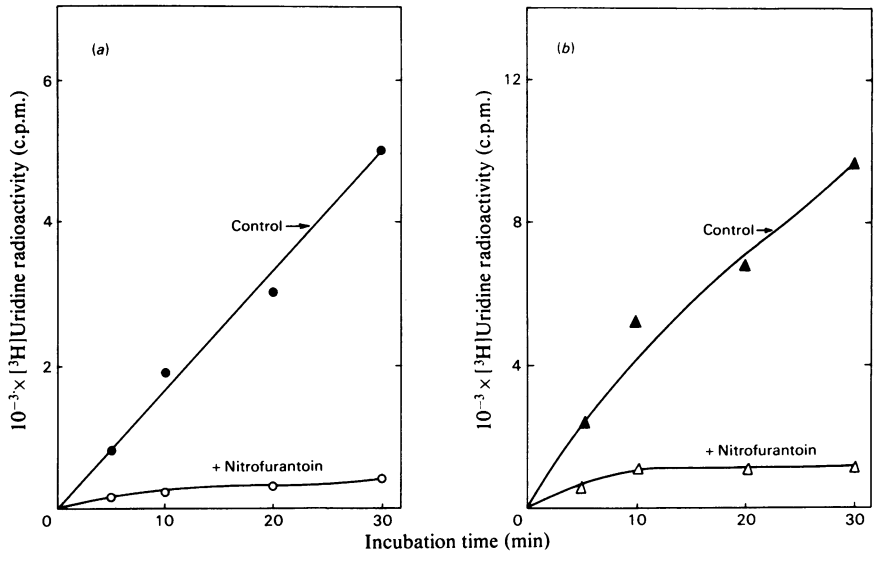

Fig. 2. *Effect of nitrofurantoin on the (a) incorporation and (b) uptake of [^{3}H]uridine by plant mitochondria*
(*a*) The complete incubation system contained, in a total volume of 0.5 ml, 1 μmol of ATP, 5 μmol of $MgCl_2$, 125 μmol of sucrose, 25 μmol of Tris/HCl buffer, pH 7.4, 0.5 μCi of uridine (sp. radioactivity 6200 mCi/mmol), and 800 μg of mitochondrial protein; 50 mM-KCl and 0.5 mM-GTP and -CTP were added as indicated. The reaction was incubated for 1 h at 37°C with constant shaking and was stopped by the addition of equal volume of ice-cold 10% (w/v) trichloroacetic acid and the precipitated material was collected on membrane filters (Millipore, 25 mm diam., 0.45 μm pore size). After being washed with a further 25 ml of ice-cold 5% (w/v) trichloroacetic acid, the filter papers were dried under an i.r. lamp and counted for radioactivity in a liquid-scintillation spectrometer. ●, Control; ○, +nitrofurantoin (20 μg). (*b*) The reaction conditions are as described in the legend to Fig. 2(*a*). After the specified time, 2 ml of ice-cold medium A was added and the contents filtered rapidly through Millipore membrane filters. After being washed rapidly with a further 25 ml of ice-cold medium A, the filter papers were dried under an i.r. lamp and counted for radioactivity as described in the Materials and Methods section.

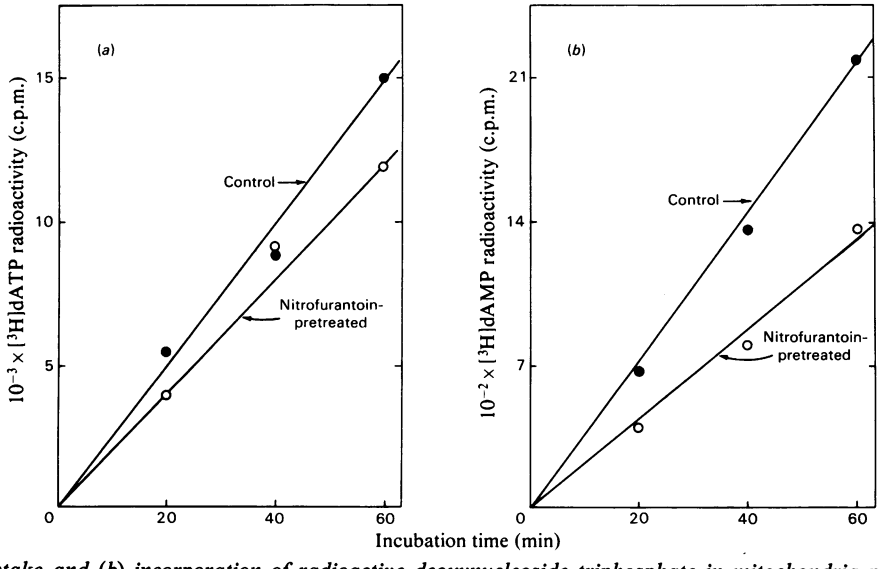

Fig. 3. (*a*) *Uptake and* (*b*) *incorporation of radioactive deoxynucleoside triphosphate in mitochondria pretreated with nitrofurantoin*
The reaction mixture and incubation conditions are given in the Materials and Methods section. The enzyme was pre-incubated with nitrofurantoin at 20°C for different time periods as indicated.

Table 3. *DNA synthesis in vitro by E. coli DNA polymerase I using 'activated' calf-thymus DNA pretreated with nitrofurantoin*

(*a*) Mitochondria (10mg of protein/ml) in a 1ml suspension was lysed with 0.25% detergent NP-40, 1M-KCl, 1mM-dithiothreitol and 10mM-Tris/HCl, pH 7.4. The suspension was kept at 4°C for 30min and was then diluted with an equal volume of 10mM-Tris/HCl buffer, pH 7.4. A portion (20μl) of the lysed suspension (100μg of protein) was incubated in a total volume of 80μl with 40μg of nitrofurantoin at 37°C for 10min. *E. coli* DNA polymerase I and other constituents of the reaction mixture were then added. The incubation system and reaction conditions are the same as given in the legend of Table 2. (*b*) In a separate experiment, lysed mitochondrial extract (i.e. the supernatant resulting from centrifugation at 10000*g* for 30min of the lysed mitochondrial preparation above) was used for the preincubation of 'activated' calf-thymus DNA. The preincubation system in a total volume of 70μl contained the following: 10mM-glucose 6-phosphate; 20μg of NADPH; 0.5 unit of glucose 6-phosphate dehydrogenase; and 40μg of nitrofurantoin. After preincubation for 10min. *E. coli* DNA polymerase I and the reaction mixture stated above were added.

Additions	Amount of [^{3}H]dGMP incorporated (pmol)	
	(*a*) Lysed mitochondria	(*b*) Extract
Control	33	123.3
−DNA polymerase	5	2.2
−NADPH-generating system + nitrofurantoin (40μg)	—	142.3
−Nitrofurantoin (40μg)	37	136.1
Antisera against DNA polymerase I (20μl)	1.4	—

where we preincubated activated DNA either with lysed mitochondria or with the mitochondria extract and NADPH and its generating system in the presence of nitrofurantoin before the use of this treated DNA as the template for DNA synthesis by DNA polymerase I. As there is no inhibition of DNA synthesis with this treated DNA as template, it is tempting to conclude that no specific nitroreductase activity is present in the mitochondria from germinating seed of *V. sinensis*.

We acknowledge unstinting co-operation from the Department of Physics, University of Calcutta, India, and from Professor K. L. Mukherjee, S.S.K.M. Hospital, Calcutta, India, for providing facilities for liquid-scintillation counting. Thanks are due to Dr. L. Rovis and Mrs. S. Williams, International Laboratory for Researches on Animal Diseases (ILRAD), Nairobi, Kenya, for their critical comments on the manuscript. Thanks are also due to Ms. Catherine Munyua, ILRAD, Nairobi, Kenya, for typing the manuscript. The work was sponsored by the University Grants Commission, New Delhi, India.

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