The Selective Inhibition of Ribosomal RNA Synthesis in *E. coli* by 2,4-Dinitrophenol

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SUMMARY

2,4-Dinitrophenol (DNP), when added to cultures of *Escherichia coli* in concentrations not greatly in excess of those required to inhibit growth, exhibits marked inhibition of RNA synthesis, while little effect on DNA and protein synthesis is seen. The selectivity disappears rapidly with increasing concentrations of DNP. Evidence is presented which demonstrates that low concentrations of DNP inhibit primarily the synthesis of ribosomal RNA with relatively slight effects on the synthesis of messenger and transfer RNA's. At the concentrations at which selectivity is observed, neither oxidative phosphorylation nor transport of uracil across the cell membrane appears to be affected appreciably by DNP. The similarities and differences between the modes of action of levorphanol and of DNP are discussed.

INTRODUCTION

Our findings on the selective inhibition of the synthesis of ribosomal RNA by levorphanol (1, 2) prompted us to investigate the effects of other compounds on RNA synthesis. 2,4-Dinitrophenol (DNP) was chosen because it was expected to exert its effect as a result of its well known action as an uncoupler of oxidative phosphorylation. A rather nonspecific inhibition of all macromolecular syntheses was, therefore, anticipated. Reports by Gros and his collaborators (3–5), which appeared while these studies were in progress, seemed to lend support to this hypothesis. They showed that treatment of *E. coli* with large concentrations of DNP inhibited virtually completely both RNA and protein synthesis.

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The present studies indicate that, contrary to expectations, treatment of *E. coli* with DNP at levels just sufficient to inhibit growth results in a highly specific inhibition of RNA synthesis, and that this effect is predominantly on the synthesis of ribosomal RNA. This action of DNP is probably not the result of uncoupling of oxidative phosphorylation.

MATERIALS AND METHODS

Bacterial and phage strains. E. coli K-13 was provided by Dr. Norton Zinder of the Rockefeller Institute. The methionine auxotroph of *E. coli* K-12, strain 58-161, with relaxed control of RNA synthesis, and strain 15 $T^-A^-U^-$ were obtained from the stocks of the Department of Microbiology, New York University School of Medicine. *E. coli* B and bacteriophage T2 were provided by Dr. R. Hausmann.

Media. All experiments except those involving T2-infected cells were performed

in the low phosphate medium, buffered with triethanolamine (TEA) at pH 8.0, described previously (1), with 0.5% sodium succinate and 0.2% Difco vitamin-free casamino acids as the sources of carbon. The medium used to grow *E. coli* B for T2 production was that described by Gold *et al.* (6).

Materials. 2,4-Dinitrophenol was purchased from Mann Research Laboratories. All radioactive chemicals were obtained from New England Nuclear Corporation.

Methods. Determinations of bacterial growth, acid-precipitable radioactivity, β-galactosidase, RNA, and protein, as well as the isolation procedure for sRNA for methylation experiments and of total RNA for sucrose density gradient fractionation have been described previously (1, 2). Experiments were carried out on cultures of *E. coli* in the logarithmic phase of growth. The initial optical density at 550 mμ was generally 0.05–0.08, read on a Lumetron colorimeter in tubes of 15 mm diameter (except as described in the next section).

DNA methylase in T2-infected cells. Infection of *E. coli* B with T2, preparation of cell-free extracts, and enzyme assays were carried out as described by Gold *et al.* (6). The initial pH of the growth medium was 7.2 but dropped to about 6.7 in the course of the experiment. DNP was added to the cultures at optical density of 0.65 (Beckman DU Spectrophotometer at 650 mμ). Samples were removed for measurement of uracil-^{14}C incorporation into RNA in uninfected cells. Cultures were infected with T2 at a multiplicity of about 5 phages per cell 2 min after treatment with DNP. Samples for enzyme assay were removed at 5 min and 60 min after infection. Uracil-^{14}C (18 μg of uracil and 0.05 μC/ml of culture) was added to the uninfected cells 3 min after the DNP. Samples for measurement of acid precipitable radioactivity were taken at 15 min and 45 min after the addition of isotope.

RESULTS

Inhibition of Growth of E. coli

The growth of *E. coli* was inhibited by DNP. At pH 8 there was little or no increase in absorbancy at 550 mμ or viable cell count when cultures were treated with $0.8–1.2 \times 10^{-3}$ M DNP. As the pH of the medium was lowered, the effectiveness of DNP increased. At pH 7 growth inhibition was achieved at $1.5–2 \times 10^{-4}$ M DNP. The inhibition is completely reversible. Even at the highest concentration of DNP used (2.5×10^{-3} M at pH 8) there was virtually no decrease in viable cell count after exposure of the bacteria to the drug for 2–3 hr. Since it was planned to compare the action of DNP with that of levorphanol, cultures

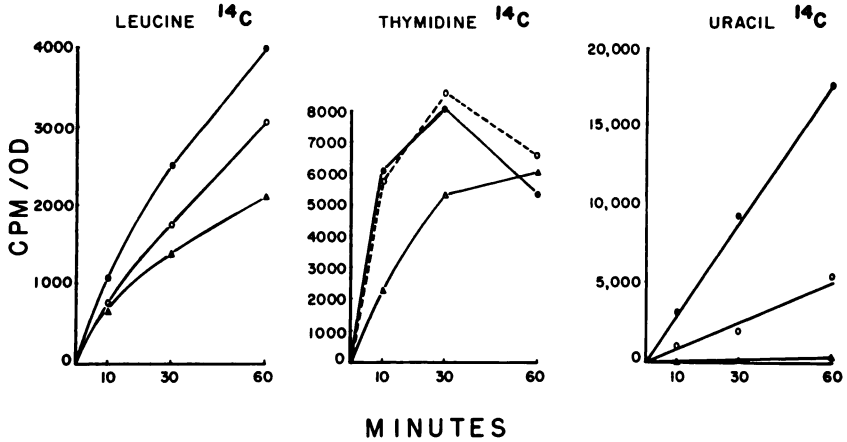

FIG. 1. *Effect of DNP on protein, DNA, and RNA synthesis in E. coli K-13*
●——● Normal cells; ○——○ 9×10^{-4} M DNP; △——△ 1.5×10^{-3} M DNP.

were grown in the triethanolamine-buffered medium at pH 8, used for most of the levorphanol experiments.

Effect of DNP on the Synthesis of Macromolecules

In agreement with reports from other laboratories (3, 7), DNP was found to be a potent inhibitor of RNA synthesis. However, at concentrations just sufficient to inhibit growth, we have found this inhibition to be very selective. Figure 1 shows that at a concentration of 10^{-3} M DNP (at pH 8) incorporation of uracil into the acid precipitable fraction was inhibited by 75%, while thymidine incorporation into DNA was unaffected, and protein synthesis proceeded at 70% of the rate of the growing control culture. At 1.5×10^{-3} M DNP, RNA synthesis was inhibited 95% while inhibition of DNA and protein synthesis was less than 50%. At 2.0×10^{-3} M DNP or higher, inhibition of the synthesis of RNA as well as of DNA and protein was virtually complete. The experiments shown in Fig. 1 were carried out with *E. coli* K-13. Similar results were obtained with strains 15 T⁻A⁻U⁻, B, and 58-161.

Inhibition of RNA Synthesis in the Absence of Growth and Protein Synthesis

Experiments were performed to determine whether growth and protein synthesis are required for the effect of DNP on RNA synthesis. Strain 58-161, a methionine requiring "relaxed" mutant of *E. coli* K-12 was shown by Borek *et al.* (8) to continue synthesis of RNA for 2–3 hr in the absence of methionine. Figure 2 shows that in such a starved culture which exhibits no growth or protein synthesis, the formation of RNA was inhibited by DNP. A similar result was obtained in cultures in which protein synthesis was inhibited by chloramphenicol.

Effect of Magnesium Concentration on the Effectiveness of DNP

The inhibition of RNA synthesis by levorphanol was shown to become more effective as the magnesium concentration of the medium was decreased (9). The inhibitory effectiveness of DNP, on the other hand, was unaffected by magnesium concentration when varied between 10^{-3} M and 10^{-6} M as shown in Table 1.

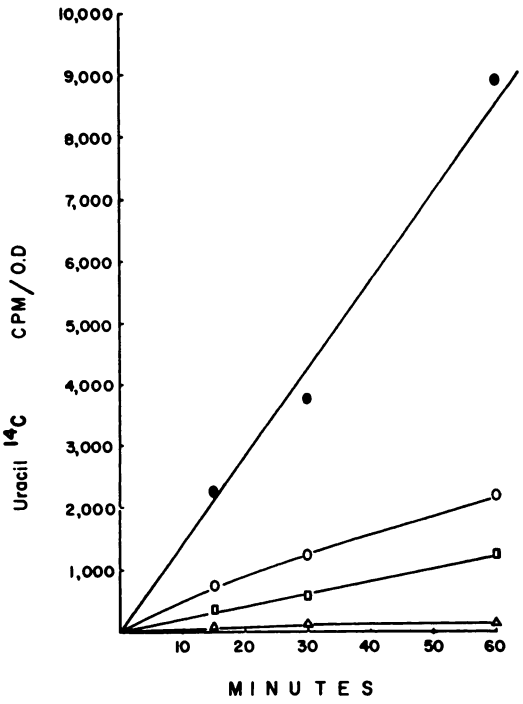

FIG. 2. *Effect of DNP on RNA synthesis in methionine-starved cultures of E. coli 58-161*

●——● Control cells; ○——○ 8.2×10^{-4} M DNP; □——□ 1.0×10^{-3} M DNP; △——△ 1.4×10^{-3} M DNP.

TABLE 1

Inhibition of RNA synthesis by DNP at high and low magnesium concentrations

E. coli K-13 was grown in TEA-buffered minimal medium, pH 7.8, using sodium lactate and a synthetic mixture of amino acids as sources of carbon. Uracil-^{14}C (0.6 μC and 73 μg per 10 ml of culture) was added 5 min after addition of DNP. The values shown represent samples taken after 45 min of incubation with uracil-^{14}C.

Concentration of DNP (M × 10^4)	% Inhibition of uracil-^{14}C incorporation	
	10^{-3} M Mg	10^{-6} M Mg
0	0	0
4	71	73
6	85	85
8	95	92

Effect of DNP on Uracil Transport

DNP has been found to be an inhibitor of bacterial permeases (10). Although the induction experiments, described in the next section, demonstrate that there is no detectable inhibition of β-galactoside permease at the concentrations of DNP used, the possibility was considered that the observed inhibition of uracil incorporation into RNA by DNP was the result of a selective effect on uracil transport. Evidence to rule out this possibility was provided by the following observations: (a) It was shown by chemical determinations that net synthesis of RNA is inhibited by DNP. Figure 3 shows a semilogarithmic plot of acid-precipitable orcinol active material (expressed as millimicromoles of ribose per milliliter) vs. time. At the time indicated by the arrow, DNP was added to a portion of the culture. A marked reduction in the net synthesis of RNA was observed in the DNP-treated bacteria. The increase in total RNA in the inhibited cells during the 2-hr period was about 17% of that in the control culture. During the same period the increase in DNA and in viable cells was 30% and the increase in absorbancy at 550 mμ was 40% as compared to the control culture. (b) The incorporation of uracil-^{14}C and of $H^{32}PO_4^{--}$ into RNA were inhibited to the same extent. These precursors are unrelated structurally and are, therefore, most likely handled by separate permeases. (c) The uptake of uracil-^{14}C into the acid-soluble cell pool was inhibited only slightly. *E. coli* 15 $T^-A^-U^-$ starved of arginine and thymidine was used for these studies. Since this strain exhibits stringent control of RNA synthesis, little nucleic acid is made during starvation, so that the acid-soluble pool constitutes the major portion of cellular radioactivity after incubation of the starved culture with uracil-^{14}C. The results of such an experiment are shown in Table 2.

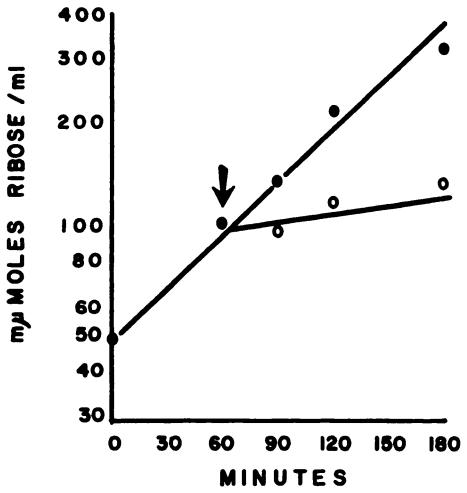

FIG. 3. *Effect of DNP on the net synthesis of RNA in E. coli K-13*

The drug was added at the time indicated by the arrow. RNA was determined by the orcinol reaction performed on the acid-precipitable fraction of the cells. ●——● Normal cells; ○——○ Cells treated with 1×10^{-3} M DNP.

TABLE 2

Uptake of uracil-^{14}C in arginine and thymidine-starved E. coli 15 $T^-A^-U^-$

E. coli 15 $T^-A^-U^-$ was grown in TEA-buffered minimal medium at pH 7.9, supplemented with thymidine (10 μg/ml), arginine (50 μg/ml) and uracil (20 μg/ml) with 0.5% sodium lactate as the carbon source. The log phase culture was filtered (Millipore HA membrane) and washed with medium free of thymidine, arginine, and uracil. DNP was added after 15 min of starvation, followed 5 min later by uracil-^{14}C (2 μC and 7 μg per 10 ml of culture). Thymidine and arginine were re-added to a portion of the culture for measurement of inhibition of RNA synthesis by DNP in growing cells (see text).

Concentration of DNP (M)	Acid-soluble pool (cpm/OD)			Acid-precipitable fraction (cpm/OD)		
	1 min	5 min	20 min	1 min	5 min	20 min
0	5334	8900	11680	756	2180	4340
8×10^{-4}	5418	6950	9490	402	1670	3410
1.2×10^{-3}	4860	5740	7090	380	1040	3210

The uracil uptake into the acid-soluble pool of starved cultures was inhibited only 20–40% at concentrations of DNP that inhibited RNA synthesis in growing cultures of the same strain 80–90%.

The incorporation of uracil-^{14}C into the small amount of RNA made during starvation is also shown in Table 2. Inhibition of uracil incorporation into this fraction is seen not to exceed 50%. Since it has been found by Stern *et al.* (11), that the RNA made in starved cultures of *E. coli* 15 T$^-$A$^-$U$^-$ is predominantly messenger RNA, these data provide some evidence for the relative insensitivity of messenger RNA synthesis to the action of DNP. Further evidence for this will be presented in the following section.

Evidence for Selective Inhibition of Ribosomal RNA Synthesis

Synthesis of messenger RNA (mRNA) in the presence of DNP. The high level of protein synthesis observed at concentrations of DNP which effectively inhibit RNA synthesis caused us to investigate the effect of DNP on mRNA production. To study the effect of the drug on the production of the mRNA for a functional protein, β-galactosidase induction was measured at various concentrations of DNP. Incorporation of uracil-^{14}C was measured in the same cultures of *E. coli* K-13. The results of these experiments are summarized in Table 3. At 1×10^{-3} M DNP enzyme induction was virtually unaffected, while RNA synthesis was inhibited 85%. As the concentration of DNP was raised, there was a rapid decrease in β-galactosidase induction until selectivity disappeared completely at 2×10^{-3} M DNP, at which concentration both RNA synthesis and enzyme induction were completely inhibited.

To ascertain that the β-galactosidase messenger is not in some way uniquely resistant to the action of DNP, the induction of an unrelated enzyme, the phage-induced enzyme DNA methylase, synthesized in the early phase of infection of *E. coli* B with bacteriophage T2 was investigated. Induction of this enzyme was measured in the presence of various concentrations of DNP and compared to inhibition of RNA synthesis in the same but uninfected cultures. Table 4 shows that enzyme induction is much less sensitive to inhibition by DNP than RNA synthesis in uninfected cells. DNA methylase is made at the normal rate in the presence of 1×10^{-4} M DNP which causes 76% inhibition of RNA synthesis; it is still made at 57% of control rate when RNA synthesis in uninfected cells is inhibited 97%. The fact that phage yield is more sensitive to DNP than early enzyme production may indicate that the drug affects other steps in the reproduction of phage T2.

Synthesis of transfer RNA (sRNA) in the presence of DNP. E. coli 58-161, a *"relaxed"* mutant, was treated with various concentrations of DNP during 2 hr of methionine starvation. Uracil incorporation was measured in aliquots of these cultures. sRNA isolated from the cultures as described previously (2) was incubated with the enzyme which specifically methylates uracil residues in sRNA. The data shown in Table 5 indicate that the production of total RNA is inhibited to a much greater extent than the formation of sRNA during this period. Thus, at 7×10^{-4} M DNP, the concentration of newly synthesized sRNA was the same as in the control culture while overall RNA synthesis was inhibited 61%.

TABLE 3

Effect of 2,4-dinitrophenol on RNA synthesis and β-galactosidase induction in E. coli K-13

The inducer, IPTG (1×10^{-3} M), and uracil-^{14}C (126 μg and 1.6 μC per 30 ml of culture) were added 5 min after treatment with DNP. Values shown are for samples taken after 30 min of incubation.

Concentration of DNP (M × 10^3)	RNA synthesis		β-galactosidase induction	
	cpm/OD	% of control	Units/OD	% of control
0	7575	100	460	100
1.0	1150	15	430	93
1.2	472	6	190	41
1.4	236	3	110	23
2.0	22	0.3	0	0

TABLE 4
Early enzyme formation in T2-infected E. coli B in the presence of 2,4-dinitrophenol

Concentration of DNP (M)	Burst size		DNA methylase 5 min after infection		DNA methylase 60 min after infection		Uracil incorporation (15 min) uninfected cells	
	Phages per cell	% of control	mμMoles per mg protein[a]	% of control	mμMoles per mg protein[a]	% of control	Cpm	% of control
None	33	100	7.5	100	19.3	100	8830	100
1×10^{-4}	24	73	7.9	100	19.0	100	2140	24
2×10^{-4}	3.3	10	5.1	68	19.0	100	885	10
4×10^{-4}	1.3	4	4.3	57	20.7	100	302	3

[a] Millimicromoles of ^{14}C-methyl groups from ^{14}C-S-adenosylmethionine incorporated per milligram of protein in 30 min into methyl-deficient *E. coli* DNA.

TABLE 5
Methylation of "starved" sRNA from DNP-treated and control cells

Cultures of *E. coli* 58-161 were incubated for 2 hr in the absence of methionine with DNP in the concentrations shown. Aliquots (10 ml) of the same cultures were used to study uracil-^{14}C incorporation.

Concentration of DNP (M)	Uracil incorporation at 120 min		Methylation of sRNA	
	Cpm	% of control	^{14}C-methyl incorporation[a]	% of control
0 (control)	11000	100	0.240	100
7×10^{-4}	4300	39	0.232	97
1×10^{-3}	3000	27	0.184	77
1.2×10^{-3}	1400	13	0.147	61
1.6×10^{-3}	500	5	0.083	35

[a] Millimicromoles of ^{14}C-methyl groups incorporated in 60 min when sRNA (500mμmoles of nucleotides) was incubated with ^{14}C-methyl-S-adenosylmethionine and the enzyme which converts uracil to thymine in sRNA.

Sucrose gradient fractionation experiments supported this conclusion.

DISCUSSION

DNP has been the classical uncoupling agent for oxidative phosphorylation in mitochondrial systems for many years. Even in bacteria, inhibition of a process by DNP has been widely regarded as evidence that the process is dependent on metabolic energy derived from ATP. Thus, inhibition of the uptake of metabolites by cells has often been used as evidence for transport dependent on energy metabolism (10), and as early as 1944 inhibition of enzyme induction was cited as evidence that this process requires energy (12).

The results reported here show that levels of DNP just sufficient to inhibit bacterial growth exert a selective inhibition on the synthesis of RNA and, in particular, of ribosomal RNA. The selectivity observed makes it appear unlikely that the effect is a result of uncoupling of oxidative phosphorylation. A situation in which inhibition of macromolecular synthesis occurred as a consequence of disruption of energy metabolism has been reported by Levinthal and Levinthal (personal communication). These authors observed that the toxic effects of colicine on *E. coli* appear to be due to its inhibition of ATP synthesis. In this case, however, the effect on macromolecular synthesis was completely nonselective. DNA, RNA, and protein synthesis were inhibited at the same time and to the same extent. Furthermore, it was possible to protect the bacteria against the toxicity of colicine by anaerobiosis. This is in contrast to some preliminary findings in our laboratory indicating that the growth-inhibitory concentrations of DNP are the same whether the bacteria are grown with vigorous aeration or anaerobically (in an atmosphere of nitrogen without agitation). Further support for the relative insensitivity of oxida-

tive phosphorylation in bacteria to DNP comes from the work of Kashket and Brodie (13), who report that they were unable to uncouple oxidative phosphorylation in extracts of *E. coli* by DNP.

The present results are not in conflict with earlier published data reporting the inhibition of RNA synthesis including production of mRNA by DNP (3). These investigators used high concentrations of DNP (usually 5×10^{-3} M at pH 7.4), at which we also observe complete cessation of RNA as well as of DNA and protein synthesis. Gros and his collaborators (3, 4) used high concentrations of DNP for a study of the half-life of mRNA in *E. coli*.

Much like levorphanol, low concentrations of DNP inhibit RNA synthesis without inhibiting DNA synthesis and with relatively little effect on protein synthesis. The formation of ribosomal RNA is inhibited to a much greater degree than the production of sRNA and mRNA. Two functional messengers, those for β-galactosidase and for T2-induced DNA methylase, were found to be made normally at DNP concentrations which inhibit ribosomal RNA synthesis profoundly. The experiments involving the methylation of uracil residues in sRNA, clearly demonstrated that *de novo* synthesis of sRNA is also continued.

Effective arrest of ribosomal RNA synthesis can also be accomplished by the shift-down procedure which involves the transfer of cultures from a rich growth medium to a poorer one. This treatment has been shown to stop effectively the synthesis of both ribosomal and transfer RNA (14). Treatment with either DNP or levorphanol, on the other hand, has little effect on the synthesis of sRNA. It is, therefore, unlikely that the mechanism of action of these drugs is identical to that of the inhibition produced by shift-down.

Two differences have been observed between the action of DNP and levorphanol. One is the rapid loss of selectivity seen with increasing drug concentration, which is evident with DNP but not with levorphanol. The other is the effect of lowering the magnesium concentration of the medium which has been shown to increase the effectiveness of levorphanol (9). The effectiveness of DNP is unaffected by the magnesium concentration even when it is varied over several orders of magnitude. It will be of considerable interest to investigate whether the two compounds exert their selective inhibition on ribosomal RNA production by the same or by different mechanisms.

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