The Mechanism of Action of Nitro-heterocyclic Antimicrobial Drugs. Metabolic Activation by Micro-organisms

By BETH P. GOLDSTEIN, RAMON R. VIDAL-PLANA,*
BRUNO CAVALLERI, LUIGI ZERILLI, GIULIANA CARNITI
AND LUIGI G. SILVESTRI†

Direzione Laboratori di Chemioterapia, Gruppo Lepetit S.p.A., Milan, Italy

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SUMMARY

Although the target of the antimicrobial drug 1-methyl-2-nitro-5-vinylimidazole (MEV) has been shown to be DNA (Goldstein *et al.*, 1977) the drug was ineffective in cell-free systems because it was not activated. Both the rate of metabolic activation of MEV and its antibacterial activity were increased when bacteria were grown in limiting oxygen. Mutants of *Escherichia coli* which were conditionally resistant to nitroimidazoles and nitrofurans were defective in drug activation. The activities of these drugs against *E. coli* correlated with their rates of metabolism. The antimicrobial spectrum of the drugs appeared to be related to their reducibility by different species.

INTRODUCTION

1-Methyl-2-nitro-5-vinylimidazole (MEV) (Cavalleri *et al.*, 1973) belongs to a class of drugs (including nitrofurans as well as nitroimidazoles) whose antimicrobial activity depends on the presence of the nitro group (Dann & Möller, 1947; Grunberg & Titsworth, 1973). Although members of this class of drugs differ in a number of properties including their spectrum of antimicrobial activity (Grunberg & Titsworth, 1973; Edwards, Dye & Carne, 1973), studies of the mechanism of action of nitrofurazone (McCalla, Reuvers & Kaiser, 1970), metronidazole (Ings, McFadzean & Ormerod, 1974) and MEV (Goldstein *et al.*, 1977) suggest that the effective target of all of these drugs is DNA.

It has been known for some time that nitro-organic compounds are reduced by microbial systems (Dann & Möller, 1947) and, recently, reduced derivatives were shown to be reactive and to bind to DNA and to proteins (McCalla *et al.*, 1970; Ings *et al.*, 1974; Tu & McCalla, 1975). In this paper, we show that metabolic activation (reduction of the nitro group) necessarily accompanies the bactericidal effects of MEV. We also provide evidence that the spectra of activity of nitroimidazoles and nitrofurans are determined, in part, by whether the target organism grows at a redox potential consistent with the generation of reactive reduction products.

METHODS

Antibiotics and chemotherapeutic agents. The nitroimidazoles and aminoimidazoles listed in Table 1 were synthesized at Lepetit by the methods cited. Metronidazole and streptomycin were obtained from Farmitalia (Milan, Italy); nitrofurazone, nitrofurantoin, nitro-

* Present address: Rotta Research, San Fruttuoso di Monza (Milan), Italy.

† Present address: Cattedra di Microbiologia, Università di Roma, Italy.

Table I. Structures of nitroimidazoles and aminoimidazoles studied

Structure				
Compound	R	R'	R''	References
MEV	CH ₃	NO ₂	H ₂ C=CH	Cavalleri, Ballotta & Lancini (1972)
Azomycin	H	NO ₂	H	Lancini & Lazzari (1965)
L5553	CH ₃	NO ₂	CH ₃	Lancini <i>et al.</i> (1969)
L6347	CH ₃	NO ₂	iso-C ₃ H ₇	Lancini <i>et al.</i> (1969)
L6678	HOCH ₂ CH ₂	NO ₂	CH ₃	Lancini <i>et al.</i> (1969)
L7373	CH ₃	NO ₂	COOC ₂ H ₅	Cavalleri <i>et al.</i> (1972)
L8711	CH ₃	NO ₂	CHO	Cavalleri <i>et al.</i> (1972)
L9451	CH ₃	NO ₂	CH=N-CH ₃ ↓ O	Cavalleri, Ballotta & Arioli (1975)
L10997	CH ₃	NO ₂	CH=N-NH-	Cavalleri, Arioli & Lancini (1974)
L12134	CH ₃	NO ₂		Assandri <i>et al.</i> (1976)
L12375	CH ₃	CHO	NO ₂	Henry & Hoff (1965)
L12376	CH ₃	CH=N-CH ₃ ↓ O	NO ₂	Bambury <i>et al.</i> (1973)
L12407	CH ₃		NO ₂	Hoffer & MacDonald (1972)
L12415	CH ₃	H ₂ C=C- CH ₃	NO ₂	Hoffer & MacDonald (1972)
L6602.HCl	CH ₃	NH ₂	iso-C ₃ H ₇	Lancini <i>et al.</i> (1969)
L6721.HCl	HOCH ₂ CH ₂	NH ₂	CH ₃	Lancini <i>et al.</i> (1969)
L5541.HCl	CH ₃	NH ₂	CH ₃	Lancini <i>et al.</i> (1969)
L5130.HCl	H	NH ₂	H	Lawson (1956)

furaltadone, furazolidone, nalidixic acid, tetracycline and kanamycin from Prodotti Gianni (Milan, Italy); and mitomycin C from Sigma. Chloramphenicol and rifampicin are produced by Lepetit (Milan, Italy).

Bacteriophages and bacteria. Coliphage λ strains, T4b05 and ϕ 80psuIII⁺ (which carries the *coli* amber suppressor *tyrT*) were obtained from W. F. Dove. P1 *Cm* was from M. Iaccarino and T6 from N. Willets. *Escherichia coli* strain L47 (= SKF12140) was obtained from Smith, Kline & French Laboratories (Philadelphia, U.S.A.). *Escherichia coli* K12

Table 2. *Strains of E. coli* K12 used

Lepetit no.	Common name	Relevant genotype	Comments	Source
L333	AT915	Hfr KL16		B. Bachmann
L674	AT978	Hfr KL16		B. Bachmann
L354	JC2926	<i>recA13</i>		G. Sironi
L694	JC2879	F ⁻ <i>leu proA lac gal his tsx supE str</i>	<i>rec</i> ⁺ ; otherwise isogenic to L354	G. Sironi
L699		L694 <i>thy</i>		Goldstein <i>et al.</i> (1977)
L490	R901	<i>chlA chlD</i>		A. Campbell
L714	Puig442	<i>chlB2</i>		B. Bachmann
L716	C26	<i>chlE5</i>		B. Bachmann
L725	Puig426	<i>chlC3</i>		B. Bachmann
L620	N1071	<i>trp_{am} galK_{am} galE</i>	Leaky Gal	J. Pulitzer
L523	KL1699	Hfr KL16 <i>recA1 thy</i> ⁺		A. Campbell
L737		L620 (ϕ 80 ψ suIII ⁺)	Phenotypically Trp ⁺ , Gal non-leaky	This work
L710		L699 (λ h)		This work
L730		L710 <i>ndr-3</i>	Selected by method 2; m.i.c. 1.3 mM-MEV	This work
L740		L710 <i>recA1 thy</i> ⁺ , non-lysogen	Conjugation L710 $\times$ L523; cured of λ	This work
L744		L730 <i>recA1 thy</i> ⁺ , non-lysogen	Conjugation L730 $\times$ L523; cured of λ	This work
L739		L730 non-lysogen	Cured of λ	This work
L691		L333 <i>ndr-101</i>	Selected by method 1; m.i.c. 0.7 mM-MEV	This work
L700		L694 <i>pro⁺ lac⁺ gal⁺ sup⁺ tsx⁺ ndr-101</i>	Conjugation L694 $\times$ L691	This work
L711		L700 (P1 Cm)	Constructed according to Miller (1972)	This work
L726		L674 <i>ndr-150</i>	Selected by method 1; m.i.c. 1.0 mM-MEV	This work

strains used are described in Table 2. Strains cured of λ were obtained by screening survivors after λ imm21b2 infection of lysogens.

Growth conditions. Unless otherwise indicated, *E. coli* strains were cultured and treated in EM9 medium (Clark *et al.*, 1966) at 37 °C.

Drug sensitivity. Minimal inhibitory concentrations (m.i.c.) for *E. coli* K12 strains were determined as described by Goldstein *et al.* (1977). For strain L47, the same procedure was used except that bacteria were grown in Penassay broth (Difco). M.i.c. for *Trichomonas vaginalis* (strain obtained from I. De Carneri) were determined as described by Cavalleri, Ballotta & Arioli (1971).

DNA synthesis in toluene-treated bacteria. Bacteria were treated with toluene and assayed as described by Kohiyama & Kolber (1970). [³H]dTTP (specific activity 28 Ci mmol⁻¹) was purchased from The Radiochemical Centre, Amersham. Unlabelled deoxynucleoside and nucleoside triphosphates were from Sigma. ATP was present in the incubation mixture.

Metabolism of drugs. Spectrophotometric measurements of nitro-drugs were made on extracellular medium obtained by filtration (Millipore, HAWP) or by centrifugation of treated cultures. The ultraviolet (u.v.) absorption maxima for the drugs were: MEV, 365 nm; nitrofurazone, 375 nm; azomycin, 370 nm; and metronidazole, 320 nm.

[2-¹⁴C]MEV (specific activity 0.37 Ci mol⁻¹) was synthesized by G. Sartori. Its uptake by *E. coli* was estimated from the radioactivity retained by Millipore filters. Extracellular radioactivity was measured in the supernatant fluids after centrifugation. Samples of cultures

were also precipitated in cold 5% (w/v) trichloroacetic acid (TCA) and assayed for precipitable (retained by Sartorius SM13400 glass fibre filters) and for soluble (supernatant fluid after centrifugation) radioactivity. The radioactivities of the supernatant fluids (1 ml samples) and Millipore filters were measured in 10 ml Dimilume (Packard); and those of glass fibre filters were measured in 10 ml toluene-based scintillation mixture [5.5 g Perma-blend I (Packard) per 1 toluene].

The extracellular medium was further analysed by thin-layer chromatography. Samples (1 ml) were lyophilized, resuspended in 0.2 ml distilled water, and applied to 0.25 mm thick silica gel F254 plates (Merck). They were run for approximately 18 cm in solvent system A (chloroform/methanol, 9:1, v/v). In some cases, material remaining at the origin was scraped off, extracted with 3 ml methanol/water (1:1, v/v), lyophilized, dissolved in 0.1 ml water and re-run in solvent system B (methanol/ acetic acid, 95:1, v/v). The radiochromatograms were scanned on a Packard Model 7201 radioscanner at a rate of 0.5 cm min⁻¹.

Effect of growth conditions on MEV activity. The bactericidal effect of MEV, and DNA degradation induced by MEV, were determined as described by Goldstein *et al.* (1977). Redox potentials of cultures (relative to a standard calomel electrode) were followed with an Amel pH meter which, when equipped with a platinum electrode, functioned as a potentiometer.

Selection of MEV-resistant (ndr) mutants. Method 1 (training). Subcultures in NB (Oxoid no. 2 nutrient broth) containing different concentrations of MEV were grown with vigorous aeration to apparent saturation and observed microscopically. The culture with the highest drug concentration which had good growth and a majority of normal sized (non-filamentous) cells was used to inoculate broths containing higher concentrations of MEV. Bacteria were never stored in the presence of the drug. When the desired level of resistance was obtained, cultures were cloned and the m.i.c. of the isolates were checked.

Method 2. Bacteria lysogenic for λ (strain L710) were incubated in TM buffer (0.01 M-Tris plus 0.01 M-MgSO₄, pH 8.0) for 10 min with 13 mM-MEV. The bacteria were washed and diluted 20-fold (to about 10⁷ bacteria ml⁻¹) in Tryptone broth (Difco) supplemented with 25 μ g thymine ml⁻¹ and grown to saturation. The culture was challenged with λ cI72, to eliminate non-lysogens, and cloned on NB agar containing thymine and 0.07 mM-MEV.

Mapping of ndr mutants. Mutations selected in the Hfr strains L333 and L674 were mapped by conjugation with L694; *ndr-3* which was selected in an F⁻ strain (L710) was conjugated with L674. Pro⁺Str recombinants were selected on Davis agar (Davis & Mingioli, 1950) containing 0.1 mg streptomycin ml⁻¹. Recombinants were scored for the unselected markers *his*, *gal*, *lac*, *leu* and *ndr* by replicating to appropriate media and for *tsx* and *sup* by spot tests with bacteriophages T6 and λ susO29.

A P1 lysate, obtained by heat induction of L711 (*ndr-101*), was used to transduce L737. Gal⁺ recombinants were selected and scored for *ndr*. Conjugations and transductions were performed as described by Miller (1972).

Polarographic analysis. We prepared solutions containing approximately 40 μ g drug ml⁻¹ in methanol/1 M-sodium acetate buffer pH 5.4 (1:1, v/v). The solution, at 25 °C, was placed in a polarographic cell, which consisted of a mercury drop electrode, a saturated calomel electrode and a saturated KNO₃ bridge. Oxygen was expelled by bubbling with nitrogen for about 10 min. The polarogram was recorded from 0.0 to -1.7 V with a Beckman Electroscan 30 polarograph. The reduction waves were measured by the method of tangents. The $E_{\frac{1}{2}}$ values reported are due to the first reduction wave of the nitro group and represent the potential at which reduction to the hydroxylamine is 50% complete (Kolthoff & Lingane, 1952; Brezina & Zuman, 1958).

Fig. 1. Effect of MEV on DNA synthesis in toluene-treated *E. coli*. Strain L694 was treated with toluene and incubated in the presence of various concentrations of MEV for 10 (○) or 20 (●) min.

Table 3. *Metabolism and uptake of [¹⁴C]MEV by E. coli*

The experiment was done using a culture of strain L694 which had an extinction (E_{390}) of 0.9 at time 0.

Time after MEV addition (min)	E_{365}		Radioactivity [c.p.m. (ml culture) ⁻¹] in:			
	Medium	TCA-soluble	Cells	Medium	TCA-precipitable	TCA-soluble
0	0.459	0.406	742	23 800	126	22 300
15	0.335	0.306	1010	20 800	364	22 200
30	0.234	0.212	884	20 800	463	22 400
45	0.184	0.152	810	19 900	527	21 000
60	0.113	0.094	706	23 100	549	22 200
90	0.078	0.034	800	23 500	585	21 700

RESULTS AND DISCUSSION

Inactivity of MEV in cell-free systems

We have demonstrated that the antibacterial effect of MEV is due to induction of damage in DNA (Goldstein *et al.*, 1977). Concentrations as low as 3 μM were bactericidal for *recA* strains of *E. coli* and 10-fold higher concentrations induced prophage λ and mutations in wild-type strains (Goldstein *et al.*, 1977). However, extracellular bacteriophage T4b05 (a highly permeable mutant, obtained from S. Benzer) was completely resistant to incubation with up to 3.3 mM-MEV for 1 h (data not shown). DNA from *Bacillus subtilis* had 100% transforming ability after incubation *in vitro* with 0.33 mM-MEV for 30 min, while DNA extracted from treated bacteria was only 13% as efficient as an equivalent amount of DNA from untreated bacteria (Mastromei & Riva, personal communication).

RNA synthesis in intact *E. coli* was blocked within 30 min by 0.33 mM-MEV, while 6.7 mM-MEV did not inhibit cell-free RNA synthesis, assayed by the method of Burgess (1969). In a non-agitated culture, 0.03 mM-MEV inhibited DNA synthesis by 40 to 80% over the first 20 min of treatment (unpublished data). However, [³H]dTTP incorporation by toluene-treated bacteria of the same strain was not inhibited by up to 0.67 mM-MEV (Fig. 1). Saeki *et al.* (1974) had observed that the related drug azomycin likewise inhibited polynucleotide synthesis only in intact cell systems.

A possible explanation for the lack of activity of MEV in cell-free systems is that it must

Fig. 2

Fig. 2. Chromatography of cell-free medium in solvent system A. (a) Control, MEV in EM9 medium; (b to g) after MEV had been in contact with *E. coli* for (min): (b) 0; (c) 15; (d) 30; (e) 45; (f) 60; (g) 90.

Fig. 3

Fig. 3. Chromatography of cell-free medium in solvent system B. (a) Control, MEV in EM9 medium; (b) rechromatography of material at the origin in Fig. 2 (g).

be activated by a metabolic process. McCalla *et al.* (1970) found that the inactivity of nitrofurazone in cell-free systems was remedied by enzymic or electrochemical reduction of the drug. As several nitro-heterocyclic compounds can be reduced by micro-organisms (Beckett & Robinson, 1956, 1959; Cartwright & Cain, 1959; McCalla *et al.*, 1970) this represents a probable pathway for metabolic activation.

Fig. 4. Effect of agitation on MEV metabolism and activity. A culture of *E. coli* strain 1.694 was grown, with agitation, to an extinction (E_{580}) of 0.5 and divided. Growth was continued either with ($\circ$, $\bullet$) or without (∇ , $\blacktriangledown$) agitation, in the presence ($\bullet$, $\blacktriangledown$) or absence ($\circ$, ∇) of 0.2 mM-MEV. (a) Amount of MEV metabolized per 10^9 bacteria. (b) Bactericidal effect of MEV.

Metabolism of MEV by *E. coli*

As shown in Table 3, when [^{14}C]MEV was added to a culture of *E. coli*, 80% of the drug (measured spectrophotometrically at 365 nm, the absorption maximum when the nitro group is present) disappeared from the medium and from the cold TCA-soluble fraction in 90 min. This was not due to uptake of the drug by the bacteria as almost all of the radioactivity remained in the TCA-soluble and extracellular fractions. At all times, approximately 4% of the labelled material appeared to be cell-associated (retained by Millipore filters). TCA-precipitable radioactivity increased with time to a maximum of about 3%.

Chromatography of the medium showed loss of radioactivity from the MEV peak and the appearance of new, more polar radioactive compounds. In solvent system A, MEV had an R_F value of 0.87. In the presence of EM9 medium, about 30% remained at the origin (Fig. 2, trace a). On incubation with *E. coli*, material was progressively lost from the MEV peak (traces b to g). In solvent system B, the presence of EM9 did not affect the migration of MEV, which had an R_F value of 0.66 (Fig. 3, trace a). We therefore re-ran the material at the origin of the chromatogram in Fig. 2(g) (90 min of contact with bacteria) in system B (Fig. 3, trace b). The results confirmed that MEV had essentially disappeared by this time and had been replaced by three compounds with R_F values of 0.0, 0.21 and 0.56. This behaviour was consistent with the properties of reduced nitroimidazoles which, in our experience, remain at the origin in solvent system A but run in solvent B.

Evidence that MEV metabolism is required for activity

Effect of growth conditions on MEV metabolism and activity. MEV disappeared from the medium only in conditions in which it was effective. We never detected spontaneous disappearance of MEV from bacteria-free medium; nor was it metabolized by toluene-treated bacteria, whose reducing capacity is reportedly defective (Brown, Wissemann & Matsushita,

Fig. 5. Effect of cell density of cultures of *E. coli* on MEV metabolism. Two cultures of strain L694, one with $E_{590} = 0.2$ (x) and the other with $E_{590} = 0.4$ (○), were divided: one half of each culture was treated with 0.1 mM-MEV (2, 4); the other halves were left untreated (1, 3). All cultures were placed under a layer of liquid Vaseline and stirred magnetically with the minimum agitation required to ensure uniformity of the cultures. (a) E_{590} ; (b) redox potentials of the cultures; (c) MEV metabolism in the two treated cultures.

1972). In *E. coli* cultures, the rate of MEV metabolism varied inversely with the degree of aeration. Figure 4(a) shows that a culture of *E. coli* incubated without agitation metabolized MEV more rapidly than an agitated culture. The bactericidal effect of MEV was much more pronounced in the non-agitated culture (Fig. 4b).

The observed difference between agitated and non-agitated cultures seemed to reflect the availability of oxygen, as the extinction of the cultures also influenced the response to MEV treatment. Two cultures, differing only in extinction, each began to metabolize MEV when the redox potential of the medium dropped to about -100 mV. As the redox potential dropped more rapidly in the denser culture, MEV metabolism began sooner in this culture (Fig. 5). The onset of the bactericidal effect corresponded in time with the onset of MEV metabolism (data not shown). The presence of MEV retarded the drop in redox potential of treated cultures (Fig. 5b); this phenomenon was observed by Cramer (1947) for nitrofurazone and was called 'poising'.

Besides its bactericidal effect, other effects of MEV were more pronounced under conditions of reduced aeration: (i) 0.17 mM-MEV, when added to a dilute agitated culture, blocked DNA synthesis after a lag of 30 min; dense non-agitated cultures did not show this lag (data not shown). (ii) [^{14}C]MEV uptake into cold TCA-precipitable material was greater in non-agitated than in agitated cultures (Fig. 6a). (iii) DNA was degraded more rapidly, after MEV addition, in denser cultures (Fig. 6b).

Fig. 6. Effect of aeration on MEV activity in *E. coli*. (a) Incorporation of [¹⁴C]MEV (0.03 mM added) into TCA-precipitable material in strain L694, grown with (●) or without (▲) agitation, and in strain L700 (= L694 *ndr-101*) grown with vigorous agitation (■). E_{590} at time zero was 0.25 in all cultures. (b) A culture of strain L694, prelabelled with [³H]thymidine, was grown with moderate agitation. Samples were treated with 0.2 mM-MEV when the extinction (E_{590}) reached 0.05 (○) and 0.25 (●). DNA degradation was calculated from the radioactivity in the TCA-soluble fraction.

Fig. 7. Induction of MEV reductase by substrate. *Escherichia coli* strain L694 was pretreated for various times, with agitation, with either 100 µg chloramphenicol ml⁻¹ (○) or with 0.1 mM-MEV (×), and then incubated in static conditions with 0.1 mM-MEV and 100 µg chloramphenicol ml⁻¹ for 100 min. MEV metabolism was normalized to cell density.

The close correlation between the extent of metabolism of MEV and its activity, suggest that the drug must be activated by cellular metabolism in order to be effective. The apparent inhibition and/or repression by oxygen of MEV metabolism is consistent with its activation by reduction. Inorganic nitrate reductase activities of bacteria are similarly affected by oxygen (Wimpenny & Cole, 1967). As is the case for inorganic nitrate (Wimpenny & Cole, 1967), MEV metabolism was also induced by substrate. As shown in Fig. 7, pre-incubation

Fig. 8. Effect of culture conditions on expression of *ndr*. *Escherichia coli* strains L694 (sensitive strain; dashed lines and open symbols) and L700 (= L694 *ndr-101*; solid lines and symbols) were incubated; (a) at high cell density with limited aeration provided by a magnetic stirrer; (b) at low cell density without aeration; and (c) at high cell density without aeration. ■, □, Untreated or zero-time controls; ●, ○, addition of 0.2 mM-MEV; ▼, ▽, addition of 0.8 mM-MEV.

of bacteria with MEV (aerated culture) for 4 min or longer led to an increased rate of MEV metabolism on subsequent incubation in the presence of chloramphenicol without agitation. As this experiment was performed with whole cells, the increase in activity could represent induction of either a permease or a reductase.

Characteristics of resistant mutants. MEV-resistant (*ndr*) mutants were difficult to select. Direct plating on various concentrations of the drug yielded mutants with levels of resistance only about three times that of the wild type; small colonies which appeared at higher concentrations were non-viable. In liquid cultures containing moderate levels of the drug and high cell inocula, growth of surviving sensitive cells occurred, apparently after MEV had been completely metabolized to an inactive form. At drug concentrations high enough to prevent this, there were no survivors.

Mutants with high levels of resistance (10 to 40 times the parental m.i.c. of 0.03 mM) were obtained in one of two ways: by serial subculture in broth containing increasing MEV concentrations (method 1) or by brief exposure of a λ -lysogen to high levels of MEV (method 2). Method 2 took advantage of the fact that sublethal doses induced λ (Goldstein *et al.*, 1977); thus, enrichment for highly resistant lysogens could be achieved by relatively mild treatment and the mutants could be selected by plating on low levels of MEV. The mutants obtained by both procedures were conditionally resistant. While they showed a survival advantage on MEV treatment in agitated cultures (Fig. 8a) or at low cell density (Fig. 8b), they were barely distinguishable from the parental strains when treated at high

Table 4. Resistance of an *ndr* strain to nitroimidazoles, nitrofurans and other drugs

Strain L691 is an *ndr* derivative (*ndr-101*) of strain L333. Similar results were obtained with other *ndr* mutants.

Nitro-drug	M.i.c. L691	Other drug	M.i.c. L691
	M.i.c. L333		M.i.c. L333
MEV	20	Nalidixic acid	1
Nitrofurazone	10	Mitomycin C	1
Nitrofurantoin	10	U.v. light	1
Furazolidone	10	Tetracycline	1
Nitrofuraltadone	> 5	Kanamycin	1
Azomycin	5	Rifampicin	1
		Chloramphenicol	1

Fig. 9

Fig. 10

Fig. 9. Activation of MEV by sensitive and *ndr* strains of *E. coli*. ●, L694 (sensitive); ×, L700 (*ndr-101*). The concentration of MEV remaining in the medium was determined spectrophotometrically.

Fig. 10. Metabolism of nitroimidazoles and nitrofurans by *E. coli*. Strain L694, growing without agitation, was exposed to 0.1 mM nitrofurazone (▽), MEV (●), azomycin (▼) or metronidazole (○). At the time of drug addition E_{590} was 0.2.

cell density in non-aerated cultures (Fig. 8c). The need for cycles of enrichment (method 1) or for indirect selection (method 2) in the isolation of *ndr* mutants, is presumably due to the low selective advantage of the *ndr* genotype in the dense cultures normally used when looking for rare mutants.

When grown with vigorous aeration (permitting full expression of resistance) *ndr* mutants did not metabolize MEV (Fig. 9). This suggests that the mutations might affect either regulation of nitro-organic metabolism, or the activity of one of several enzymes which catalyse

Table 5. *Map location of ndr-150*

Data are from a conjugation between strain L726 (Hfr KL16 *ndr-150*) and strain L694 (F⁻ *proA lac tsx supE gal his str*); selection was for Pro⁺Str. Recombinant classes are ordered according to the distance of the unselected markers from *proA* (Taylor & Trotter, 1972).

Recombinant class	Number	Percentage that were <i>ndr</i>
<i>pro</i> ⁺	103	20
<i>pro</i> ⁺ <i>lac</i> ⁺	81	21
<i>pro</i> ⁺ <i>tsx</i> ⁺	56	25
<i>pro</i> ⁺ <i>sup</i> ⁺	31	48
<i>pro</i> ⁺ <i>gal</i> ⁺	24	63
<i>pro</i> ⁺ <i>ndr</i>	21	—
<i>pro</i> ⁺ <i>his</i> ⁺	33	36

the reduction under different physiological conditions. The *ndr* mutants were resistant to a variety of nitroimidazoles and nitrofurans (Table 4), indicating that these drugs are probably activated by the same system. The mutants had normal sensitivities to structurally unrelated drugs, including mitomycin C and nalidixic acid, and to u.v. light which interfere with DNA synthesis (Table 4). By m.i.c. determination, we found strain L744 (*ndr-3 recA1*) to be 100 times more sensitive to MEV than strain L739 (isogenic *ndr-3 rec*⁺) and 40 times more resistant than strain L740 (*ndr*⁺*recA1*). The u.v.-sensitivities of these strains correlated only with their *rec* genotypes, while MEV activation correlated only with their *ndr* genotypes.

Mutants of *E. coli* conditionally resistant to nitrofurazone have also been reported (Asnis, Cohen & Gots, 1952). These mutants are probably similar to *ndr*, as they were deficient in nitrofurazone reduction. Another class of nitrofurant-resistant mutants was isolated in *E. coli* B (Szybalski & Nelson, 1954; McCalla, 1965) and in *E. coli* s (Woody-Karrer & Greenberg, 1963); these behaved like u.v.-resistant derivatives of the hypersensitive parental strains. We observed that MEV was a suitable selective agent for Rec⁺ revertants of strain L354. Derivatives of *E. coli* B/r that showed higher resistance to nitrofurant (Payne *et al.*, 1956; McCalla *et al.*, 1970) could be similar to *ndr*.

Mapping of *ndr* mutations was complicated by the necessity for indirect selection of resistant recombinants and by the absence of easily selected nearby markers. Six *ndr* mutations (*ndr-101*, *ndr-150*, *ndr-3* and three mutants with low resistance selected by direct plating of L674 on NB agar containing 0.13 mM-MEV) were mapped in the *gal* region by conjugation. However, we did not observe Pr-mediated cotransduction of *ndr* with *gal*. Data for the mapping of *ndr-150* is shown in Table 5.

Among the known *E. coli* markers in the *gal* region are several of the *chl* series which control inorganic nitrate reduction (Venables & Guest, 1968). Our *ndr* mutants were all *chl*⁺ by the plate test of Glaser & DeMoss (1971), and several *chl* mutants tested (strains L490, L714, L716 and L725) metabolized MEV normally and had wild-type sensitivity to nitrodrugs. Thus, inorganic nitrate and nitro-organic reduction have at least partially independent enzymic pathways in *E. coli*.

Correlation of antimicrobial activity of nitroimidazoles and nitrofurans with their reducibility

Different nitro-heterocyclic antimicrobial drugs appear to have the same mechanism of action as MEV, as shown by the cross-resistance of strains selected for resistance to MEV (Table 4) and the hypersensitivity of *recA* strains to these drugs (Yahagi *et al.*, 1974; Gold-

Table 6. Relationship between E_h and spectrum of antimicrobial activity of nitro-drugs

Values of E_h were measured relative to a saturated calomel electrode. Where lower limits of activity are given, insolubility of the drug was the usual reason for failure to test higher concentrations.

Drug	E_h (mV)	M.i.c. (mm)		(1)/(2)
		<i>E. coli</i> L47 (1)	<i>T. vaginalis</i> (2)	
L10997	-165	0.2	> 0.4	< 0.5
L7373	-195	0.1	> 0.5	< 0.2
L9451	-210	0.05	0.5	0.1
L8711	-230	0.1	0.6	0.17
Furazolidone	-275	0.004		
Nitrofurazone	-278	0.01		
L12376	-345	0.3	0.05	6
MEV	-355	0.03	0.007	4
L12134	-374	2	0.03	67
L12415	-400	> 0.6	0.001	> 600
L6347	-400	3	0.06	50
L6678	-435	> 2	0.1	> 20
L5553	-443	> 1	0.04	> 25
Azomycin	-470	0.2	0.004	50
L12407	-470	> 0.5	0.003	> 160
L12375	-497	2	0.1	20
Metronidazole	-500	4	0.003	1200

Table 7. Comparative activities of nitroimidazoles and aminoimidazoles

Compound	Structure (see Table 1)			M.i.c. (mm) for <i>T. vaginalis</i>
	R	R'	R''	
L6347	CH ₃	NO ₂	iso-C ₃ H ₇	0.06
L6602.HCl	CH ₃	NH ₂	iso-C ₃ H ₇	> 13
L6678	HOCH ₂ CH ₃	NO ₂	CH ₃	0.1
L6721.HCl	HOCH ₂ CH ₂	NH ₂	CH ₃	> 14
L5553	CH ₃	NO ₂	CH ₃	0.04
L5541.HCl	CH ₃	NH ₂	CH ₃	> 20
Azomycin	H	NO ₂	H	0.004
L5130.HCl	H	NH ₂	H	> 24

stein *et al.*, 1977). The abilities of different nitro-heterocyclic drugs to inhibit the growth of *E. coli* correlated with their rates of metabolic conversion by this organism. Figure 10 compares the rates of disappearance of four different drugs from *E. coli* cultures. Nitrofurazone was the most rapidly metabolized of the four, followed by MEV, azomycin and metronidazole. As can be seen from Table 6, their rates of metabolism correlated with their effectiveness against *E. coli*. The values of E_h (Table 6) correlated with both of these parameters; the lower the E_h of a drug, the less rapidly was it metabolized by *E. coli* and the less effective it was. Metronidazole, which has a very low E_h , was neither metabolized by *E. coli* (Fig. 10) nor active against this organism (Table 6).

We assayed a number of nitro-drugs for their anti-*coli* and anti-trichomonal activity. When these compounds were ordered by increasingly negative E_h (Table 6), we observed that those drugs whose E_h was more negative than -370 mV (with the sole exception of azomycin) showed little activity against *E. coli*, a facultative anaerobe. However, all of the

drugs were quite active against two obligate anaerobes, *T. vaginalis* (Table 6) and *Clostridium perfringens* (data not shown). This relationship is more clearly illustrated by limiting comparison to three isomeric pairs of nitroimidazoles in which the 2- and 5-substituents are interchanged (L9451/L12376; L8711/L12375; L12134/L12407; see Table 1). The 5-nitroimidazoles all had lower E_1 than the corresponding 2-nitro isomers; the former were less active against *E. coli* and more active against *T. vaginalis* than their isomers.

From these data, one may conclude that a necessary (if not sufficient) condition for the activity of nitro-drugs is that they be reduced at the redox potential present in cultures of the target organisms, as already suggested by Dann & Möller (1949) for nitrofurans and nitrothiophenes. However, certain discordant observations must be explained: the culture medium of MEV-treated *E. coli* had an antimicrobial titre equal to the remaining unreduced MEV (data not shown); bacteriophage T4b05 was not inactivated by incubation in the presence of T4-resistant bacteria (incapable of adsorbing the phage) which were metabolizing the drug (data not shown); compounds with less negative E_1 were relatively inactive against *T. vaginalis* (Table 6).

The explanation for these observations may be due to the final reduction products of nitro-drugs (the amino-derivatives) being inactive. This is illustrated in Table 7, in which the activities of four pairs of nitro- and aminoimidazoles are compared. Dann & Möller (1947, 1949) had similarly observed that aminothiophenes and aminofurans were inactive. It is probable that the reactive forms of all of these drugs are partially reduced intermediates which are highly unstable; likely candidates are the hydroxylamino- or nitroso-derivatives (Dann & Möller, 1949; Paul *et al.*, 1960; McCalla *et al.*, 1970). Thus, the metabolites of MEV in the medium of treated cells were probably not the reactive compounds. We consider that this accounts for the behaviour of all the nitro-drugs studied, with the possible exception of azomycin, which may have a second mechanism of action (unpublished observations).

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