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On the Capacity of Nitroheterocyclic Compounds to Elicit an Unusual Axial Asymmetry in Cultured Rat Embryos[1]

JEAN C. GREENAWAY, ALAN G. FANTEL,[2] AND MONT R. JUCHAU[2,3]

Departments of Pediatrics and Pharmacology, School of Medicine, University of Washington, Seattle, Washington 98195

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On the Capacity of Nitroheterocyclic Compounds to Elicit an Unusual Axial Asymmetry in Cultured Rat Embryos. GREENAWAY, J. C., FANTEL, A. G., AND JUCHAU, M. R. (1986). *Toxicol. Appl. Pharmacol.* **82**, 307–315. A series of nitroheterocyclic compounds with antimicrobial and radiosensitizing properties was tested for embryotoxicity in cultured Sprague–Dawley rat embryos, and their effects were compared with various other five-membered heterocycles. Nifuroxime, furazolidone, nitrofurazone, niridazole, 2-nitroimidazole, and ronidazole each elicited a striking malformation characterized by asymmetrical, right-sided hypoplasia when coincubated with embryos for 26 hr. Minimum concentrations required to elicit this unusual defect ranged from 0.01 mM with nifuroxime and furazolidone to 0.5 mM with ronidazole and were roughly correlated with single electron redox potentials; i.e., agents with relatively high redox potentials were generally more effective than those with low potentials. Nitrofurantoin failed to elicit this unusual malformation but exhibited an extremely steep dose–response curve for embryolethality. Metronidazole and 4-nitroimidazole, nitroheterocyclic agents with relatively low redox potentials, did not produce the asymmetric abnormality nor were they highly embryotoxic, even at concentrations up to 2 mM. 2-Amino-5-nitrothiazole and 4′-methylniridazole also failed to evoke the asymmetric malformation but produced significant embryotoxicity as manifested by decreased growth parameters and elicitation of other kinds of malformations. Heterocyclic compounds not bearing a nitro group (furosemide, 2-aminothiazole, and 2-aminoimidazole) failed to elicit axial asymmetry at concentrations up to 1.0 mM but produced other signs of embryotoxicity at the highest concentrations tested. The results suggest that the presence of a reducible nitro group is critical for generation of the unusual malformation and that the single-electron redox potential of the nitro group plays a dominant but not exclusive role in the capacity of these chemicals to evoke axial asymmetry in cultured rat embryos.

Several five-membered nitroheterocyclic compounds are currently utilized extensively as therapeutic agents in the treatment of microbial and parasitic infections and as radiosensitizers in the treatment of neoplasms. Such agents are known to exhibit mutagenicity in various test systems and many have also been identified as cytotoxins, carcinogens, and immunosuppressants (Moreno *et al.*, 1984; Yeung *et al.*, 1983; Lu *et al.*, 1984). On the basis of these biological effects, one would expect that they might also produce embryotoxicity of varying degrees. However, little ap-

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[2] Affiliates of the Child Development and Mental Retardation Center, University of Washington, Seattle, Wash.

[3] To whom all correspondence and reprint requests should be addressed.

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parent research effort has been expended in evaluating this potentially important facet of their toxicologic spectrum.

We recently reported that niridazole, a widely used antischistosomal nitrothiazole, produced a highly unusual axial asymmetry as the predominant malformation when added to whole embryo cultures (Fantel *et al.*, 1984; Greenaway *et al.*, 1985). No exogenous enzyme source was required and the effect was inhibited by carbon monoxide but not by varying concentrations of *N*-acetylcysteine. The 4′-methyl analog of niridazole, which does not undergo significant nitro group reduction in various biological systems (Tracy *et al.*, 1983), failed to produce the unusual axial asymmetry. On the basis of those preliminary observations, we postulated that the embryos themselves contained the necessary components to catalyze the reduction of niridazole (but not 4′-methylniridazole) to reactive intermediates capable of evoking embryonic axial asymmetry.

In this investigation, we have further tested this hypothesis by examining a series of heterocyclic chemicals structurally related to niridazole. We have utilized a whole embryo culture system to evaluate the effects of these chemicals because it avoids the disadvantages of complicating maternal factors (dispositional, hormonal, etc.) and thus permits a better approach to questions concerning mechanisms. We report that several five-membered nitroheterocycles with relatively high redox potentials can produce the same asymmetric malformation as niridazole. Nitroheterocyclic agents with relatively low single electron redox potentials or heterocyclic compounds not bearing a nitro group failed to elicit axial asymmetry at millimolar concentrations. As described elsewhere, the capacity to produce the unique malformation was lessened at higher oxygen tensions (Greenaway *et al.*, 1985). We postulate that embryonic tissues contain the necessary enzymes and/or other catalytic components to catalyze the reduction of nitroheterocyclic chemicals to radical intermediates that can preferentially damage those embryonic cells with the lowest O_2 tension.

METHODS

Chemicals. The generic and/or chemical names and structures of the heterocyclic chemicals investigated are given in Fig. 1. With the exception of 4′-methylniridazole, each of these chemicals was purchased from Aldrich Chemical Company (Milwaukee, Wisc.). 4′-Methylniridazole was synthesized and purified according to methods described by Wilhelm *et al.* (1966) and Tracy *et al.* (1983). All chemicals were dissolved in redistilled dimethyl sulfoxide immediately prior to use. For range finding of concentrations to be tested, preliminary experiments were conducted with 0.05 and 0.5 mM (initial) concentrations of each chemical. Subsequent concentrations examined were dependent upon observations with the two range-finding concentrations. Purity of the chemicals was checked by analyses of melting points which were in agreement with the literature values. All other reagents and chemicals utilized were of the highest purity commercially available.

Experimental animals. Virgin Sprague–Dawley (Wistar-derived) rats weighing 250–300 g were mated by and obtained from Tyler Laboratories (Bellevue, Wash.). Mating was checked by the presence of vaginal copulatory plugs on the morning after mating. The morning following overnight mating was designated as Day 0 of gestation. The pregnant animals were received on Day 3 of gestation and were housed two to three per cage in the animal facility of the Central Laboratory for Human Embryology. They were placed on crushed corncob bedding material (Sanicel), received Purina Rat Chow and water *ad libitum*, and were exposed to cycles of 14 hr light/10 hr dark each day.

Explantation and evaluation of cultured embryos. Embryos were explanted on the morning of Day 10 of gestation when they have 8–12 (10 ± 2 SD) somites. They were grown in a semisynthetic medium consisting of 50% Waymouth medium 752/1 (GIBCO), 25% rat serum, and 25% human serum. All sera were centrifuged immediately following blood withdrawal and were subsequently heated at 56°C for 20 min. Culture media were placed in 125-ml glass roller bottles and equilibrated with O_2:CO_2:N_2 (5:5:90) before the addition of embryos and heterocyclic chemical. The lower (5%) O_2 tensions were maintained because previous experiments with niridazole (Fantel *et al.*, 1984; Greenaway *et al.*, 1985) indicated that higher (20%) O_2 tensions were associated with decreased embryotoxicity, presumably due to decreased nitroreduction. After 21 hr of culture at 37.5°C in total darkness, the gas mixture was replaced with O_2:CO_2 (95:5) and the embryos were permitted to grow for an additional 5 hr under otherwise identical conditions.

After approximately 26 hr in culture medium, embryos were removed and immediately examined under a dis-

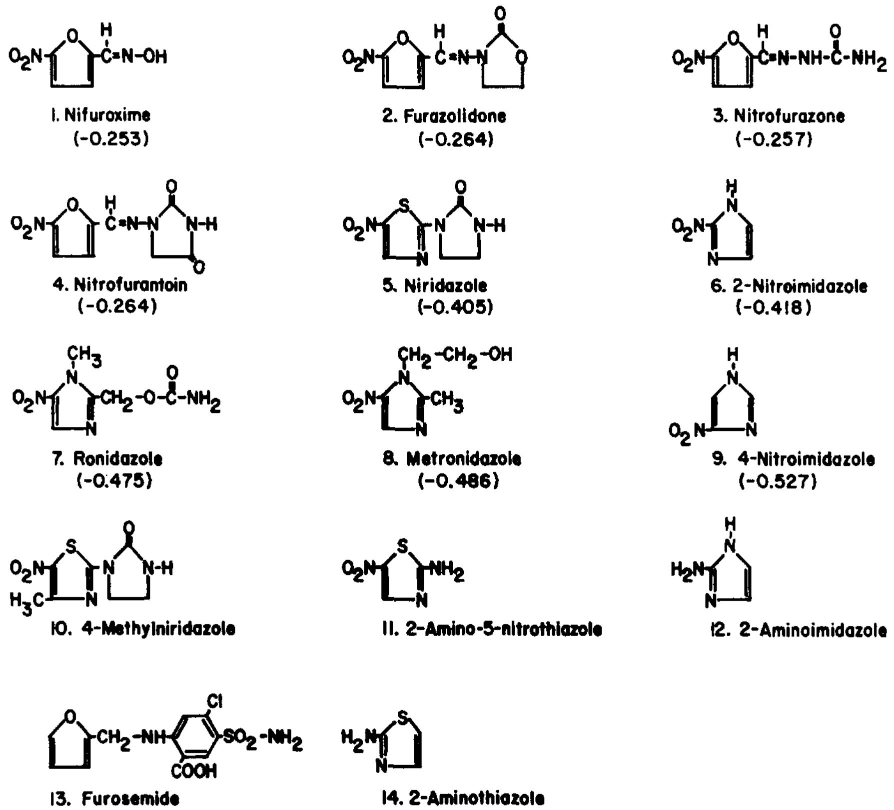

FIG. 1. Structures and single electron redox potentials (E_7^1) of the heterocycles investigated. Information on the redox potentials of 4'-methylniridazole and 2-amino-5-nitrothiazole was not available. Redox potentials were taken from the literature (Wardman, 1977; Chin *et al.*, 1978; Olive, 1979a,b; Greenstock *et al.*, 1976; Reynolds, 1981).

secting microscope. All embryos were examined and scored without knowledge of treatment. To eliminate postmortem artifacts, embryos without heartbeat and/or yolk sac circulation were considered nonviable and were not examined further. Viable embryos were examined in saline and record was made of the greatest diameter of the flexed embryo (embryonic length) within the intact yolk sac. The visceral yolk sac, amnion, and ectoplacental cone were then removed and embryos were further evaluated in terms of numbers of somite pairs and detectable malformations. The embryos were rinsed twice with normal saline and frozen in potassium phosphate buffer (0.1 M, pH 7.4) for later protein determinations (Bradford, 1976). Other details of procedures utilized in the explanation and evaluation of embryos have been described previously (Greenaway *et al.*, 1982).

RESULTS

Six of the fourteen chemicals investigated exhibited a concentration-dependent capacity to produce asymmetric malformations in cultured embryos. Each of the six possesses an aromatic nitro group; the embryotoxicity of these agents is presented in Table 1. The nitrofurans (except nitrofurantoin) elicited embryotoxic effects at relatively low concentrations and were capable of producing a relatively high proportion of asymmetric malformations. Of 57 embryos malformed by the nitrofurans, 22 (39%) exhibited axial asymmetry.

A typical asymmetrically malformed embryo is illustrated in Fig. 2. The severity of the malformation appeared to be greatest in the cephalic region. A hypoplastic mesencephalon, dilated rhombencephalon, open and/or distorted otic vesicles, and micro- or anopthalmia were also frequently observed. The axial

TABLE 1

COMPARATIVE EMBRYOTOXIC EFFECTS OF FIVE-MEMBERED HETEROCYCLES CAPABLE OF ELICITING AXIAL ASYMMETRY IN CULTURED RAT EMBRYOS

Chemical	Initial concentration (mM)	Cultured/ viable	Axial asymmetry (%)	Total malformations (%)	Embryonic protein (μg/ embryo)	Embryonic length (mm)[a]	Somite number
Nifuroxime	0.01	8/8	13	13	248 ± 36	3.0 ± 0.2	20.1 ± 2.0
	0.02	7/6	17	67	215 ± 52	2.9 ± 0.2	20.4 ± 0.9
	0.05	14/14	21	100	184 ± 45	2.6 ± 0.3	18.9 ± 1.5
	0.10	8/0	—[b]	—	—	—	—
	Control	15/15	0	0	308 ± 66	3.1 ± 0.2	21.5 ± 1.7
Furazolidone	0.01	5/5	20	80	236 ± 71	2.9 ± 0.7	21.0 ± 2.0
	0.02	14/11	64	91	129 ± 39	2.6 ± 0.3	20.2 ± 0.8
	0.05	8/0	—	—	—	—	—
	0.10	7/0	—	—	—	—	—
	Control	22/21	0	0	271 ± 81	3.1 ± 0.2	21.6 ± 1.5
Nitrofurazone	0.02	7/7	14	14	282 ± 47	3.0 ± 0.3	21.3 ± 0.5
	0.05	17/14	14	64	199 ± 66	2.8 ± 0.2	19.2 ± 1.1
	0.10	18/14	21	79	125 ± 36	2.3 ± 0.2	18.9 ± 1.7
	0.20	9/4	100	100	73 ± 31	1.9 ± 0.2	17.8 ± 1.5
	0.50	14/0	—	—	—	—	—
	Control	16/14	0	7	297 ± 50	3.2 ± 0.2	22.6 ± 1.2
Niridazole	0.05	9/9	0	0	289 ± 38	3.1 ± 0.3	21.8 ± 1.0
	0.10	30/30	10	40	251 ± 27	3.0 ± 0.2	21.2 ± 0.7
	0.20	15/14	43	83	227 ± 44	2.8 ± 0.1	20.6 ± 1.2
	0.30	9/2	50	100	148 ± 51	2.6 ± 0.2	19.4 ± 0.9
	0.40	8/0	—	—	—	—	—
	Control	18/18	0	6	291 ± 41	3.2 ± 0.2	21.9 ± 1.2
2-Nitroimidazole	0.10	8/8	0	25	231 ± 43	3.0 ± 0.2	20.9 ± 0.7
	0.20	12/10	17	100	198 ± 24	2.8 ± 0.3	20.1 ± 0.8
	0.30	13/12	80	100	172 ± 27	2.7 ± 0.1	19.0 ± 0.3
	0.50	8/0	—	—	—	—	—
	Control	13/13	0	8	243 ± 45	3.1 ± 0.3	21.5 ± 1.3
Ronidazole	0.33	13/11	0	0	232 ± 35	3.3 ± 0.7	21.6 ± 1.0
	0.50	24/16	6	25	192 ± 70	3.0 ± 0.2	21.1 ± 1.5
	1.00	16/12	67	75	163 ± 34	2.9 ± 0.3	20.3 ± 0.7
	1.33	7/7	57	57	183 ± 24	2.9 ± 0.2	20.1 ± 0.9
	Control	25/24	0	0	303 ± 59	2.8 ± 0.3	21.1 ± 0.5

[a] Embryonic length measured as described under Methods.
[b] Dashes indicate that no evaluations were made because all embryos cultured displayed a lack of heartbeat and/or yolk sac circulation.

asymmetry consisted of a right-sided hypoplasia which involved microphthalmia or anophthalmia of the same side. Scoliosis with hyperflexion of the head toward the right side was frequently associated with the observed defect.

Nitrofurantoin exposure was associated with cephalic hypoplasia at 0.2-mM concen-

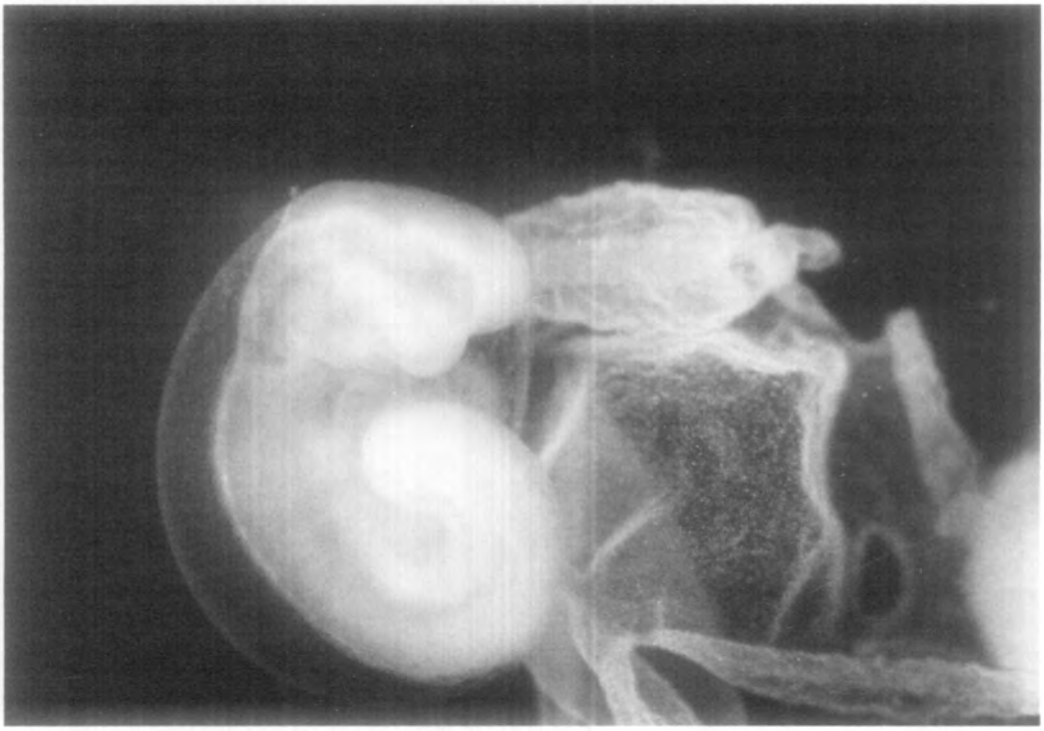

FIG. 2. A typical malformation elicited by the nitroheterocycles. Severe hypoplasia of the right side of the prosencephalon and hypoplasia of the mesencephalon are evident. This embryo was exposed to an initial concentration of 0.02 mM furazolidone. Membranes were left attached to enable positioning of the embryo for photography.

trations and was embryolethal to 100% of nine embryos tested at 0.25 mM. A steep concentration effect in terms of embryolethality may have prevented discernment of the capacity of this chemical to produce axial asymmetry.

Embryotoxicity of the compounds that did not appear capable of evoking the asymmetric abnormality is presented in Table 2. All of those compounds with nitro groups (again, with the exception of nitrofurantoin) have relatively low single-electron redox potentials (Fig. 1). Although the measured redox potentials for 4′-methylniridazole and 2-amino-5-nitrothiazole were not available, it is evident from a comparison of structures that their redox potentials would be relatively low.

Chemicals without nitro groups did not elicit asymmetric malformations. At 1.0 mM concentrations or less, the only abnormality noted in embryos exposed to furosemide, a furan without a nitro group, was necrosis in the mandibular arch. The highest tested concentrations of 2-aminothiazole (1.0 mM) produced disrupted somites and caudal edema but no other detectable malformations. The principal malformation observed following exposure to 2-aminoimidazole was bilateral hypoplasia of the prosencephalon. Smaller mandibular arches were also noted.

Metronidazole, a nitroimidazole with a low single electron redox potential, did not cause considerable embryotoxicity, even at 2.0 mM concentrations. Only one abnormality was observed at that concentration and this was characterized as an abnormal rotation of flexure. The embryotoxicity of 4-nitroimidazole was also quite low although greater than that of metronidazole. This imidazole produced no detectable morphologic abnormalities, even at 2.0-mM concentrations. Growth parameters,

TABLE 2

EMBRYOTOXIC EFFECTS OF FIVE-MEMBERED HETEROCYCLIC CHEMICALS THAT DID NOT ELICIT AXIAL ASYMMETRY AT THE HIGHEST NONLETHAL CONCENTRATIONS TESTED[a]

Chemical	Concentration (mM)	Cultured/ viable	Malformation incidence[b] (%)	Protein content (μg/ embryo)	Embryonic length (mm)	Somite number
Nitrofurantoin	0.20	15/12	100	205 ± 47	2.8 ± 0.3	18.8 ± 3.5
Metronidazole	2.00	12/11	9	262 ± 90	2.9 ± 0.2	20.4 ± 0.5
4-Nitroimidazole	2.00	7/7	0	173 ± 31	2.7 ± 0.1	19.3 ± 0.5
4'-Methylniridazole	0.50	12/12	50	187 ± 27	2.7 ± 0.2	20.1 ± 0.8
2-Amino-5-Nitrothiazole	1.00	7/6	100	156 ± 30	2.7 ± 0.1	20.7 ± 0.5
2-Aminoimidazole	1.00	12/8	100	143 ± 20	2.5 ± 0.1	18.4 ± 0.9
Furosemide	1.00	7/7	71	252 ± 37	3.0 ± 0.2	21.5 ± 1.5
2-Aminothiazole	1.00	7/7	57	221 ± 28	3.0 ± 0.2	21.2 ± 0.8

[a] At higher tested concentrations (see Methods), embryolethality exceeded 50%. Controls were run in each experiment and yielded values very similar to those presented in Table 1. In none of these experiments were axial asymmetries observed.

[b] Malformations observed included prosencephalic hypoplasia, abnormal axial rotations, disrupted somites, caudal edema, and (rarely) abnormally open neural tubes.

however, were significantly decreased as compared with controls. 4'-Methylniridazole produced a variety of observable defects including abnormally open and dilated neural tubes and abnormal flexure rotation, but did not elicit asymmetric malformations. Of the six abnormal embryos associated with exposure to 2-amino-5-nitrothiazole, five exhibited bilateral prosencephalic hypoplasia and one exhibited abnormal rotation of flexure.

Because the results suggested an apparent relationship between single electron redox potentials and the capacity of nitroheterocycles to evoke the unusual asymmetric abnormality, we analyzed this relationship more carefully. As illustrated in Fig. 3, regression analyses indicated that the capacity of the agents to cause the malformation varied with the logarithm of the concentration. The correlation coefficient calculated for this relationship was +0.84 and was significant at $p < 0.001$.

DISCUSSION

Of 11 nitroheterocyclics studied, five did not exhibit the capacity to produce axial asymmetry in cultured rat embryos. Of these five, two (metronidazole and 4-nitroimidazole) are known to have single electron redox potentials less than −0.480. Another two (4'-methylniridazole and 2-amino-5-nitrothiazole) also would be expected to have low redox potentials on the basis of structural considerations, i.e., the electron-releasing properties of substituted methyl and amino groups. However, nitrofurantoin, a nitroheterocycle with a relatively high redox potential (−0.264) also failed to evoke the unusual, asymmetric malformation. At present, we are unable to provide a definitive reason for this lack of effect although several explanations are possible. These include questions of penetration of nitrofurantoin to critical target cells at sufficiently high concentrations, of the capacity of embryonic target tissues to bioactivate nitrofurantoin to embryotoxic intermediates, and of the capacity of reactive intermediates to persist at critical target sites for sufficient time to produce embryotoxicity. Because nitrofurantoin is a systemically effective antibacterial agent, one would not expect penetration to be a major factor although the octanol/water partition coefficient of nitrofurantoin is nearly sixfold

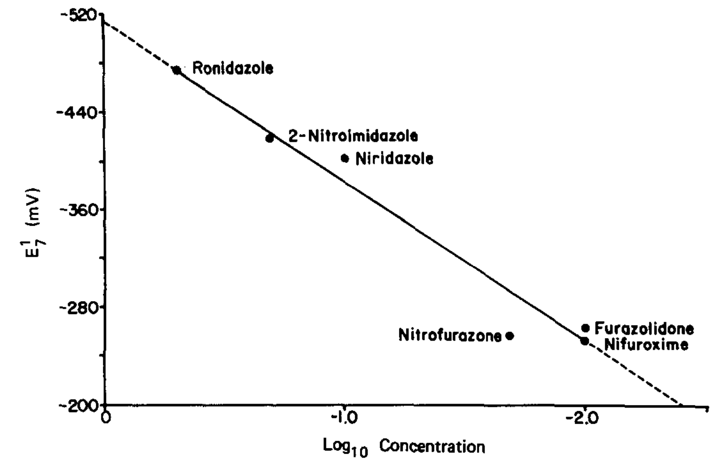

FIG. 3. Correlation between single-electron redox potentials and the capacity of compounds listed in Table 1 to elicit embryonic axial asymmetry. On the abscissa are plotted the logarithms of the lowest concentrations (mM) observed to produce the asymmetric abnormality. Correlation coefficient was calculated at +0.84 and was significant at $p < 0.001$.

lower than that of nitrofurazone (Chin *et al.*, 1978) which possesses a very similar redox potential.

All of the chemicals tested which exhibited the capacity to produce axial asymmetry were nitroheterocycles. Three five-membered heterocyclic compounds that do not bear a nitro group, 2-aminothiazole, 2-aminoimidazole, and furosemide, did not exhibit this capacity. Since 1979, we have studied numerous other chemicals in the whole embryo culture system (recently reviewed by Shepard *et al.*, 1983). In our experience, none has produced this unique kind of malformation, and we are unaware that other investigators have observed a similar malformation. On the basis of these observations, we suggest that the aromatic nitro group is essential for the unusual form of embryotoxicity observed. We also consider it likely that embryonic components catalyze reduction of the nitro group to one-electron- or three-electron-reduced radical intermediates responsible for the defect. The relatively strong relationship between redox potential and capacity to produce the defect is the major reason for presuming that reduction of the nitro group is essential for activity. The fact that nitroreduction is essential for various other biological effects of nitroheterocycles (Moreno *et al.*, 1984; Yeung *et al.*, 1983; Lu *et al.*, 1984) also provides support for this postulate. 4′-Methylniridazole, a nitroheterocycle which does not undergo significant nitro group reduction in biological systems (Tracy *et al.*, 1983), also fails to evoke many of the biological effects produced by niridazole. The methyl group, in addition to being a relatively strong electron donor and which reduces the redox potential of the adjacent nitro group, may also tend to prevent reduction via a steric effect. The failure of this compound to produce axial asymmetry also provides strong support for the hypothesis. The dose–response data of Table 1 (incidence of axial asymmetry vs incidence of total malformations) suggest the possibility that expression of axial asymmetry could be partially masked by the occurrence of other malformations in certain cases.

An interesting question which arises as the result of our observations deals with the mechanism for the specificity of the observed defect. Right-sided defects have been observed with acetazolamide and it has been shown recently that such defects can be elicited by exposure of mice *in vivo* to 20% CO_2 during a critical period of gestation (Weaver and Scott, 1984a,b). Both increased CO_2 and decreased pH appeared involved in the induction of the defect and the authors have suggested that the mechanisms whereby acetazolamide produces the defect could be linked to its capacity to increase tissue pCO_2. However, the malformation is described as a right-sided, forelimb, postaxial ectrodactyly and is primarily a limb-reduction defect; whereas the defect observed in these experiments was primarily encephalic. Thus, it seems clear that the nitroheterocycles elicit a defect that is clearly distinguishable from that produced by acetazolamide or CO_2. Nevertheless, the reason(s) why these chemicals produce defects specifically on the right side of the embryo is not clear. At present, there is no known embryologic basis for the development of the asymmetric malformation that we observed. This is an intriguing aspect of the findings but is one whose interpretation must await much more detailed investigations.

Reduction of nitro groups can lead to the formation of nitro anion radicals via one-electron reduction or of nitroso anion radicals via three-electron reduction (Wardman, 1977). Although auto-oxidation of these radicals generates superoxide anions and, potentially, highly reactive hydroxyl radicals, this does not appear to be the mechanism whereby nitroheterocyclic chemicals elicit axial asymmetry. Otherwise, one would expect increasing oxygen concentrations to exacerbate the effect. Rather, the effect is accentuated at low oxygen tension (Fantel *et al.*, 1984; Greenaway *et al.*, 1985), suggesting the possibility that free radical intermediates may interact directly with critical macromolecules to disrupt normal developmental processes. Conceivably, this could be via initiation of lipid peroxidation or covalent binding to nucleic acids and/or proteins. Other possible mechanisms include a localized loss of reducing equivalents such as NADPH, NADH, or GSH, via futile redox recycling in the presence of O_2 or a localized depletion of molecular oxygen under conditions of low oxygen tension. Elucidation of the mechanism presents a formidable challenge and should be of considerable interest in future investigations.

The conclusions that may be drawn from these investigations are as follows: (1) Several nitroheterocycles are capable of eliciting a highly unusual asymmetric defect in cultured embryos. (2) The capacity of these agents to elicit the defect appears to be at least partially a function of the single-electron redox potential of the aromatic nitro group. (3) Chemicals without aromatic nitro groups or chemicals possessing nitro groups with relatively low single-electron redox potentials are not likely to elicit the defect. (4) Nitrofurantoin, although possessing a relatively high redox potential, did not produce axial asymmetry for reasons that are currently unexplained. (5) We postulate that the explanted embryos are capable of converting several nitroheterocycles to radical intermediates which are ultimate embryotoxins.

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