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THE INVOLVEMENT OF THE GASTRO-INTESTINAL MICROFLORA IN NITRO-COMPOUND-INDUCED METHAEMOGLOBINAEMIA IN RATS AND ITS RELATIONSHIP TO NITROGROUP REDUCTION

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Abstract—The present study describes investigations into the methaemoglobinaemia-inducing capacity of three mono- and five dinitro-compounds in both normal and antibiotically pretreated rats *in vivo*, and *in vitro* on incubation with rat blood. The results obtained following administration of certain nitro-compounds indicated that the induction of methaemoglobinaemia and nitroreduction *in vivo* could occur independently of the gastro-intestinal microflora. The *in vitro* studies suggest that in some cases the conversion of haemoglobin to methaemoglobin could occur possibly as a result of a direct interaction between unchanged nitro-compounds and haemoglobin.

It is widely held that methaemoglobinaemia induced by the nitrobenzenes is associated with the formation of reactive intermediates of nitroreduction viz the nitrobenzenes and phenylhydroxylamines [1]. This follows from the observations that nitroso- and hydroxylamino-compounds have been shown to be potent methaemoglobin (metHb) producers *in vivo* and *in vitro*, while none of the corresponding arylamines nor the nitrobenzenes investigated were capable of directly oxidising haemoglobin (Hb) *in vitro* [2–4]. However it has been claimed by Kusumoto and Nakajima [5] that nitrobenzene incubated *in vitro* in the presence of isolated native Hb increased the production of metHb in 5 hr by proximately 10 per cent over a control lacking nitrobenzene. These workers proposed that nitrobenzene could act directly on Hb *in vitro* by causing a transconformation to an unstable structure, and that

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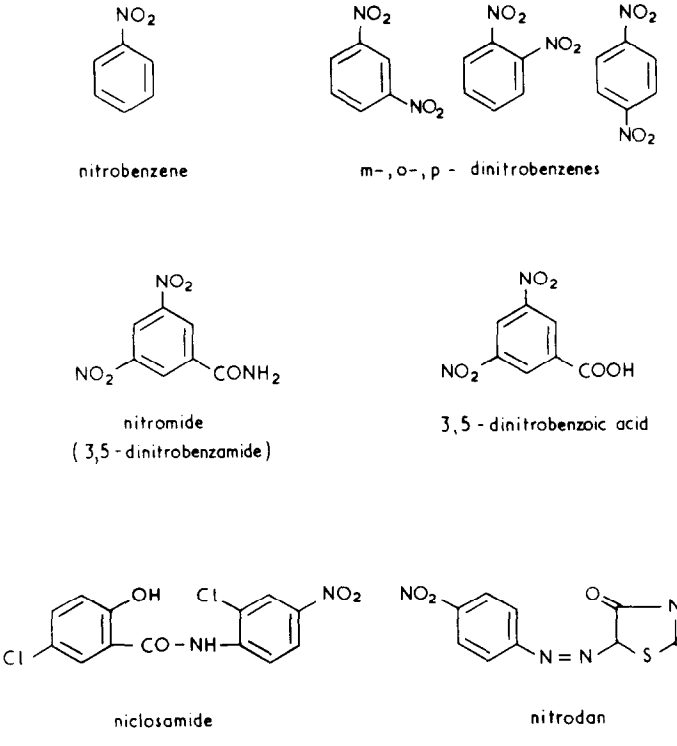

Fig. 1. Structures of nitrocompounds.

as a consequence heme iron is oxidised to the ferric state by atmospheric oxygen resulting in metHb formation. More recently it has been claimed by Reddy *et al.* [6] that the *in vivo* induction of methamoglobinaemia by nitrobenzene is dependent on the reduction of the compound to active intermediates and reduced metabolites by the gastro-intestinal (g.i.) microflora.

A study was therefore undertaken to investigate the relationship between reduction of nitrogroups and induction of methaemoglobinaemia and the involvement of the g.i. microflora in both these processes using a range of mono- and dinitro-compounds (Fig. 1). Of the compounds used, *m*-dinitrobenzene [7], niclosamide [8], nitromide(3,5-dinitrobenzamide) [9] and nitrobenzene [10] are known to be extensively reduced to aminometabolites *in vivo* although the metabolism of 3,5-dinitrobenzoic acid and *o*- and *p*-dinitrobenzene has not been investigated. Nitrodan is not reduced *in vivo* [11].

MATERIALS AND METHODS

Animals. Male albino rats of the Wistar strain (250 g) were used in the study. Nitrocompounds were administered by intraperitoneal injection (i.p.) as a homogenized suspension in corn oil. Animals were treated with antibiotics (neomycin sulphate (100 mg), tetracycline hydrochloride (50 mg) and bacitracin (50 mg)), twice daily for 2 days before the administration of the test compound and twice on the day of the experiment. Blood samples were obtained from an incision made in the tail vein at 1, 2.5, 5 and 8 hr following the administration of the test compound. Appropriate control blood samples were obtained from animals receiving corn oil alone.

Chemicals. All nitro-compounds were obtained from commercial sources and shown to be chromatographically pure, free of reduced metabolites. At the onset of the investigation mononitro-compounds and dinitro-compounds were administered at a dose level of 200 and 100 mg/kg/body weight respectively. However, as the study progressed doses were lowered to reduce fatalities: niclosamide and nitrobenzene (200 mg/kg body weight), 3,5-dinitrobenzamide and 3,5-dinitrobenzoic acid (80 mg/kg body weight), *o*- and *m*-dinitrobenzene (60 mg/kg body weight), *p*-dinitrobenzene (50 mg/kg body weight) and nitrodan (30 mg/kg body weight).

In vitro *incubations with rat blood.* Fresh oxalated blood (1 ml) which was obtained by severing the jugular vein of a rat was incubated with 0.9 cm^3 of saline and 0.1 cm^3 of ethanolic substrate solution to give an overall substrate concentration of 1×10^{-3} M. Two controls were routinely set up, one in which the substrate was replaced by 0.1 cm^3 of ethanol, and a second in which the blood was replaced by saline. Incubations were carried out for periods of 1, 2.5, 5, 6.5 and 8 hr at 37° in stoppered tubes.

Methaemoglobin determinations. Methaemoglobin levels and spectral analysis of blood samples were determined using a Pye Unicam Sp 1800 spectrophotometer according to the method of Evelyn and Malloy [12]. A pronounced peak at 635 nm which is observed on scanning diluted blood samples (0.1 cm^3 of blood in 10 cm^3 of M/60 phosphate buffer pH 6.6) and which disappears on addition of neutralized cyanide solution was taken as evidence of the presence of methaemoglobin.

Extraction of metabolites from blood samples. Blood incubates were extracted after 8 hr by a modification of the Herr and Kiese method [13]. To both control and test blood samples (volumes up to 10 cm^3) were added 0.1 cm^3 of 10% potassium ferricyanide, 10 cm^3 of deionised H_2O and the mixture shaken for 10 min. An equal volume of acetone was added with a further 10 min shaking. The precipitated proteins were centrifuged down. The supernatant was reduced in volume in a vacuum oven, and made up to 2 cm^3 with methanol for chromatography.

Chromatography. All chromatography was carried out on Whatman 3MM chromatography paper. All chromatograms were developed by the ascending method in the following solvents: (A) Toluene : ethyl formate : formic acid (5:7:1 by volume); (B) Butan-1-ol : acetic acid : water : benzene (6:2:6:1 by volume); (C) Propan-2-ol : ethanol : ammonia (2:2:1 by volume); (D) Butan-1-ol : ethanol : water : ammonia (10:10:4:1 by volume); (E) Butan-1-ol : ethanol : water : acetic acid (30:10:10:1 by volume).

Detection reagents. (A) 4-Dimethylaminocinnamaldehyde reagent [14] for the detection of aromatic amines and their conjugates. (B) Titanium trichloride reagent [15] used to convert nitrocompounds to the corresponding amine, thus permitting their detection with reagent A. (C) Trisodium pentacyanoamino-ferroate [16] for the detection of hydroxylamino and nitroso derivatives. (D) Picryl chloride/ammonia [17] used to detect hydroxylamino derivatives. (E) 5% Silver nitrate in 0.88 ammonia [18] used to detect reducing substances.

RESULTS

In vivo *study*

The results pertaining to the induction of methaemoglobinaemia by the nitro-compounds under investigation are summarized in Table 1.

Nitrobenzene was found to induce methaemoglobinaemia to significant levels in normal animals but not in antibiotic-treated animals. Similarly, scanning of blood (sampled at 2.5 hr) from nitrobenzene dosed animals revealed the presence of the metHB peak only in the blood obtained from normal animals. These results were in good agreement with those obtained by Reddy and his co-workers [6] and fully validated the antibiotic pretreatment.

All the dinitro-compounds investigated were capable of inducing methaemoglobinaemia in both normal and antibiotically pretreated rats to comparable levels. Similarly scanning of blood samples taken 2.5 hr after dosing from both normal and antibiotically pretreated rats revealed the presence of the metHb peak at 635 nm.

Nitrodan was initially administered at a level of 200 mg/kg of body weight (b.w.) but produced 100% fatality within 1–2 hr. Investigations of blood prior to death and even immediately *post mortem* failed to detect metHb. The dose was eventually reduced

Table 1. Methaemoglobin induction *in vivo* after intraperitoneal administration of nitro-compounds to normal and antibiotically pretreated animals (AB) expressed as percentage of haemoglobin converted to methaemoglobin

Time (hr)	NM	NM + AB	NB	NB + AB
1	63.6 ± 4.6	56.1 ± 2.6	18.2 ± 5.0	1.7 ± 0.4
2.5	65.5 ± 3.4	59.7 ± 5.3	24.7 ± 4.2	2.1 ± 0.2
5.0	43.7 ± 6.9	37.9 ± 1.4	32.7 ± 5.0	1.9 ± 0.4
8.0	22.0 ± 0.1	16.0 ± 0.6	9.9 ± 2.3	0.4 ± 0.1
	DNBA	DNBA + AB	*o*-DNB	*o*-DNB + AB
1	74.0 ± 3.8	46.1 ± 2.5	58.6 ± 6.6	62.5 ± 18.6
2.5	83.4 ± 2.2	86.7 ± 13.9	46.0 ± 5.2	42.8 ± 6.7
5.0	50.7 ± 9.5	39.2 ± 7.2	3.9 ± 0.9	13.7 ± 4.5
8.0	20.4 ± 4.9	6.6 ± 3.1	1.6 ± 0.1	0.0 ± 0.0
	p-DNB	*p*-DNB + AB	*m*-DNB	*m*-DNB + AB
1	84.2 ± 13.0	78.9 ± 10.3	41.1 ± 6.9	38.6 ± 0.3
2.5	43.6 ± 10.9	47.6 ± 8.3	56.6 ± 7.5	55.3 ± 2.80
5.0	37.6 ± 12.2	43.0 ± 3.2	62.0 ± 3.3	54.8 ± 1.5
8.0	5.2 ± 1.7	21.0 ± 3.5	45.1 ± 10.1	33.8 ± 2.0
		ND	NC	
1		0.0	0.0	
2.5		0.0	0.0	
5.0		0.0	0.0	
8.0		0.0	0.0	

All results are ± S.E.M. of three determinations.
NM = animals which received 3,5-dinitrobenzamide (80 mg/kg body wt).
NB = animals which received nitrobenzene (200 mg/kg body wt).
NC = animals which received niclosamide (200 mg/kg body wt).
o-DNB = animals which received *o*-dinitrobenzene (60 mg/kg body wt).
m-DNB = animals which received *m*-dinitrobenzene (60 mg/kg body wt).
p-DNB = animals which received *p*-dinitrobenzene (50 mg/kg body wt).
ND = animals which received nitrodan (30 mg/kg body wt).
DNBA = animals which received 3,5-dinitrobenzoic acid (80 mg/kg body wt).
AB = animals which had been treated with antibiotics.

to 30 mg/kg of body weight but again no metHb was detected.

Niclosamide did not adversely effect the animals to which it had been administered and over the 0–8 hr sampling time metHb was not detected.

In vitro *studies*

Methaemoglobin formation. The results pertaining to the formation of metHb by nitro-compounds *in vitro* are summarized in Table 2.

All dinitro-compounds investigated were found to

Table 2. Formation of methaemoglobin *in vitro* on incubation of nitro-compounds with oxalated blood expressed as percentage of haemoglobin converted to methaemoglobin

Time (hr)	CB	NC	NB	NM	
1.0	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	22.5 ± 5.7	
2.5	0.9 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	32.9 ± 2.0	
5.0	0.6 ± 0.4	0.2 ± 0.0	1.5 ± 0.9	47.3 ± 1.0	
6.5	1.5 ± 0.8	0.0 ± 0.0	0.4 ± 0.3	42.7 ± 0.1	
8.0	0.8 ± 0.5	0.4 ± 0.1	0.5 ± 0.3	45.9 ± 3.1	
	DNBA	*m*-DNB	*o*-DNB	*p*-DNB	ND
1.0	27.2 ± 4.6	24.4 ± 0.1	59.9 ± 0.4	86.7 ± 11.1	00.0 ± 0
2.5	35.0 ± 5.9	37.2 ± 3.0	75.3 ± 2.9	55.1 ± 7.4	0.0 ± 0
5.0	37.3 ± 2.9	44.0 ± 5.4	76.2 ± 3.7	44.9 ± 7.6	0.0 ± 0
6.5	31.9 ± 3.3	39.7 ± 4.2	76.6 ± 0.4	36.8 ± 2.5	0.0 ± 0
8.0	36.2 ± 5.6	38.0 ± 4.6	80.3 ± 3.3	41.4 ± 8.0	0.0 ± 0

Results are ± S.E.M. of three determinations.
CB = control blood; DNBA = 3,5-dinitrobenzoic acid; NC = Niclosamide; *o*-DNB = *o*-dinitrobenzene; NB = Nitrobenzene; *p*-DNB = *p*-dinitrobenzene; NM = 3,5-dinitrobenzamide; *m*-DNB = *m*-dinitrobenzene; NB = nitrodan. The nitrocompounds were incubated with saline (0.9 cm^3) and oxalated blood (1.0 cm^3) to give an overall concentration 2×10^{-3} M at 37°.

induce metHb formation to significant levels *in vitro*. These incubates showed the brown pigmentation characteristic of metHb. Scanning of these blood incubates revealed a pronounced peak at 635 nm.

Niclosamide, nitrodan and nitrobenzene were shown to be incapable of converting Hb of the red blood cell (r.b.c.) to metHb *in vitro*. In contrast to the results of Kusumoto and Nakajima [5] who have reported formation of metHb on incubation of nitrobenzene with isolated Hb, no significant formation of metHb was detected on incubation of this nitrocompound with *intact* blood cells.

Chromatographic investigation. Extracts of the 8 hr blood incubates containing dinitro-compounds in which the presence of metHb had been confirmed were subjected to paper chromatography in the solvents listed above, and sprayed with reagents specific for amino-, nitroso- and hydroxylamino-metabolites. This screening for the presence of reduced metabolites was undertaken because a small but detectable nitroreductase activity has earlier been attributed to the red blood cell (r.b.c.) [19], which might prove capable of reducing sufficient nitro-compound to metabolites capable of oxidising Hb to metHb. Chromatography revealed the presence of the unchanged parent compound but no evidence for the presence of nitroso- or hydroxylamino-metabolites was found. Trace amounts only of amino compounds were detected and these were present to a similar extent in control incubations.

These results would appear to imply direct interaction *in vitro* between the parent nitro-compound (without prior reduction) and Hb resulting in the oxidation of the latter to metHb by a mechanism analogous to that suggested by Kusumoto and Nakajima [5].

DISCUSSION

The fact that dinitro-compounds such as 3,5-dinitrobenzamide induce metHb in both normal and antibiotically pretreated rats, while nitrobenzene is capable only of inducing the condition in the former indicates that more than one mechanism may be involved in the induction of methaemoglobinaemia *in vivo* by nitro-compounds. Certainly the findings of Reddy *et al.* [6] relating metHb formation to nitro-reduction mediated by the g.i. microflora should be seen as valid only for the nitro-compound they investigated, namely nitrobenzene. MetHb formation *in vivo* by dinitro-compounds has in the current study been shown to be independent of g.i microfloral action. Although the observations of Bray *et al.* [20] and Flynn and Kohl [21] suggest that tissue nitro-reductases may be active in the reduction of some nitro-compounds our *in vitro* results suggest that the dinitro-compounds investigated can react directly with Hb *in situ* in the r.b.c., resulting in oxidation of the latter to metHb without significant biological reduction of the parent nitro-compound to nitroso- and hydroxylamino intermediates or amino metabolites. The induction of metHb by these dinitro-compounds *in vivo* may therefore, not only occur independently of the g.i. microflora, but also independently of tissue mediated nitroreduction.

However, there still remains to be explained the mechanism by which the dinitro-compounds studied but not nitrobenzene, niclosamide and nitrodan induce oxidation of erythrocyte Hb to metHb *in vitro*. Furthermore, an explanation must be found as to why dinitro-compounds are capable of inducing metHb formation in antibiotically pretreated rats, while nitrobenzene is not. The question also remains why nitrobenzene employed in our *in vitro* study employing intact blood cells was incapable of producing metHb, although increased nitrobenzene-induced metHb formation from isolated Hb has been claimed by Kusumoto and Nakajima [5]. It appears possible that the mechanism by which a nitro-compound or a metabolite induces the oxidation of Hb to metHb *in vivo* or *in vitro* may be largely determined by the ability of the compound to penetrate the r.b.c. membrane. Indeed failure of nitrobenzene to induce the formation of metHb in blood cell incubates, despite its ability to increase the conversion of purified isolated Hb *in vitro* to metHb [5] suggests that nitrobenzene is unable to penetrate the r.b.c. membrane to catalyse the atmospheric oxidation of Hb to metHb. If on the other hand a nitro-compound is able to penetrate the r.b.c. membrane then it may bind to Hb and precipitate the sequence of reactions leading to metHb, Heinz body formation and eventual haemolysis leading to even greater atmospheric oxidation of any liberated Hb. If the nitro-compound is unable however to penetrate the r.b.c. membrane or bind to Hb once in the cell, it may require metabolic conversion to active intermediates i.e. nitroso- and hydroxylamino-compounds. Thus this hypothesis would explain the discrepancy between our *in vitro* nitrobenzene results utilizing erythrocytes and those obtained by Kusumoto and Nakajima [5] employing isolated Hb. The hypothesis is also applicable to the *in vivo* situation, where in normal animals, nitrobenzene which is incapable of reacting with Hb directly, because it is unable to penetrate the r.b.c. membrane, is reduced to active intermediates which induce the condition. Both nitrosobenzene and phenylhydroxylamine are capable of reacting with Hb of the r.b.c. *in vitro* oxidising it to metHb. In the antibiotically pretreated rats, no metHb is produced by nitrobenzene because there is no reduction by the g.i. microflora to metabolites capable of inducing the condition, tissue reduction of nitrobenzene does not occur, and nitrobenzene itself is unable to penetrate the r.b.c. membrane to react with Hb. Our observations suggest that dinitro-compounds are capable of penetrating the r.b.c. membrane and can catalyse the oxidation of Hb directly. Niclosamide however, although extensively reduced *in vivo* does not produce metHb. This may be attributable to failure of the reduced metabolites to penetrate the r.b.c. membrane or to bind the Hb once within the cell; certainly the parent compound itself is incapable of oxidising Hb of the r.b.c. Similarly nitrodan which is not reduced *in vivo* was not observed to elevate metHb *in vivo* or *in vitro* suggesting non-penetration of the r.b.c. or inability to bind Hb once within the cell. On the basis of the results obtained in this and other studies, nitro-compounds may be tentatively grouped into four categories, I–IV, in relation to their metabolism and ability to induce methaemoglobinaemia.

Type I nitro-compounds which resemble nitrobenzene and are extensively reduced *in vivo* to amino-metabolites prior to the induction of methaemoglobinaemia. For these compounds the reduction of the nitrogroup and the induction of methaemoglobinaemia *in vivo* require viable gut microflora. Type I nitro-compounds are also incapable of direct interaction with Hb of the r.b.c. *in situ* either *in vivo* or *in vitro*.

Type II compounds (which include the dinitrocompounds investigated) induce methaemoglobinaemia in both normal and antibiotically pretreated animals. For type II nitro-compounds, the induction of methaemoglobinaemia is independent of the gut microflora. For 3,5-dinitrobenzamide it can also be stated that reduction to amino metabolites is also independent of the gut microflora, since reduction of the compound cannot be prevented in animals administered antibiotics, and furthermore this reduction can be achieved *in vitro* by liver homogenates under aerobic conditions [9]. Furthermore these compounds are capable of direct interaction with the Hb of the r.b.c. *in vitro*. This suggests that metHb formation in antibiotically pretreated rats following the i.p. administration of these dinitro-compounds may be a product of the interaction of Hb with both the parent nitro-compound and with reactive intermediates produced by tissue reduction.

Type III compounds are extensively reduced *in vivo* to amino metabolites yet are incapable of inducing methaemoglobinaemia in experimental animals, e.g. niclosamide [22] and pentachloronitrobenzene [23, 24]. For niclosamide it has been established that nitro-reduction is effected by the g.i. microflora, yet the compound will not induce metHb formation following either oral or i.p. administration. Furthermore niclosamide is incapable of *in vitro* reaction with Hb of the r.b.c.

Type IV compounds are not reduced *in vivo* to amino metabolites, nor are they capable of a direct reaction with Hb of the r.b.c. (as shown by the non-production of metHb following the addition of nitrodan to blood incubates) consequently methaemoglobinaemia is not produced *in vivo*.

This study indicates that the mechanism of induction of methaemoglobinaemia varies with different nitro-compounds and that although the induction of methaemoglobinaemia by nitrobenzene is dependent upon the presence of a viable microflora as shown by Reddy *et al.* [6], induction by other nitro-compounds may proceed independently of the intestinal microflora.

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