Biochem. J. (1983) **212**, 599–608
Printed in Great Britain

Formation of N-alkylated protoporphyrin IX in the livers of mice after diethylnitrosamine treatment

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(*Received 21 December 1982/Accepted 24 February 1983*)

1. The administration of di[1-^{14}C]ethylnitrosamine to phenobarbital-pretreated mice resulted in the formation of a radiolabelled green pigment in their livers. Green-pigment concentrations were time- and dose-dependent, maximum levels being reached 1–2h after dosing. There was only a slight decrease in cytochrome *P*-450 levels and accumulation of porphyrins in the liver at this time. 2. Dimethyl- or dipropylnitrosamine also caused an accumulation of similar, though not identical, compounds in the liver. 3. The formation of green pigment was induced by pretreatment of mice with phenobarbital or 3-methylcholanthrene and was inhibited by the acute administration of pyrazole or ethanol. 4. From the absorption spectra, the green pigment methyl esters appeared to be *N*-alkylporphyrins. Analysis of the diethylnitrosamine-induced green pigment by high-pressure liquid chromatography showed it to be more polar than the expected *N*-ethylprotoporphyrin IX, having a retention time similar to that of *N*-hydroxyethylprotoporphyrin IX. Desorption chemical-ionization mass spectrometry gave a protonated molecular ion, *m/z* 635, compatible with *N*-hydroxyethylprotoporphyrin IX. The presence of a free hydroxy group was demonstrated by acetylation with [1-^{14}C]acetic anhydride. 5. No conversion of *N*-ethylprotoporphyrin IX into *N*-hydroxyethylprotoporphyrin IX could be demonstrated *in vivo* or *in vitro*. Little or no *N*-ethylprotoporphyrin IX accumulated in the livers of mice given diethylnitrosamine. It was concluded that *N*-hydroxyethylprotoporphyrin IX is the primary reaction product between an active metabolite of diethylnitrosamine and hepatic haem.

Since the discovery that nitrosamines cause liver cancer in rats (Magee & Barnes, 1956), many have studied the mechanisms involved (for review, see Lai & Arcos, 1980; Michejda *et al.*, 1981). Metabolic activation is required in order for the dialkylnitrosamines to exert their biological effects. Classically, this involves microsomal cytochrome *P*-450-dependent mixed-function oxidases (Dutton & Heath, 1956; Venkatesan *et al.*, 1970; Czygan *et al.*, 1973). More recent evidence implicates monoamine oxidases (Rowland *et al.*, 1980; Lake *et al.*, 1982). Activation converts the nitrosamine into an alkylating species, generally considered to be an alkylcarbonium ion (Lijinsky *et al.*, 1968; Lai & Arcos, 1980).

Rats dosed with dialkylnitrosamines show a loss of hepatic cytochrome *P*-450 and drug-metabolising activities (Smuckler *et al.*, 1967) and an enhanced excretion of porphyrins in their urine (Schoental & Gibbard, 1976, 1979). These latter workers suggested that active metabolites of the nitrosamines might alkylate hepatic haem, though no such reaction products were isolated.

Little is known of the activity of alkylcarbonium ions generated in the liver *in vivo* to alkylate haem. Metabolic activation of the compound 3,5-diethoxycarbonyl-4-ethyl-1,4-dihydro-2,6-dimethylpyridine leads to the alkylation of hepatic haem to give a green pigment, identified as *N*-ethylprotoporphyrin IX (De Matteis *et al.*, 1981; Coffman *et al.*, 1982). Originally it was proposed that the active intermediate involved in this reaction might be a carbonium ion (De Matteis *et al.*, 1981); however, subsequent studies showed the alkylating species to be an ethyl radical (Augusto *et al.*, 1982).

Nitrosamine-mediated alkylation of haem would not presumably be directly associated with the

Abbreviation used: h.p.l.c., high-pressure liquid chromatography.

genotoxic effects of these compounds. However, if such a reaction occurred, a study of the nature of the adduct and the activating mechanisms involved might permit a further insight into the mode of action of these compounds. The results of such a study with diethylnitrosamine are reported in the present paper.

Experimental

Chemicals

Diethylnitrosamine and dimethylnitrosamine both of >99% purity by g.l.c. were from Eastman Organic Chemicals, Kirkby, Liverpool, U.K., and the Aldrich Chemical Co., Gillingham, Dorset, U.K., respectively. Di-n-propylnitrosamine and di-n-butylnitrosamine, $NADP^+$, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Co., Poole, Dorset, U.K. 3,5-Diethoxycarbonyl-4-ethyl-1,4-dihydro-2,6-dimethylpyridine was prepared as described by De Matteis *et al.* (1981). $^{65}ZnCl_2$ (sp. radioactivity 490 Ci/mol), [1-^{14}C]acetic anhydride (10 Ci/mol) and *NN*-di[1-^{14}C]ethylnitrosamine (50 Ci/mol) were from The Radiochemical Centre, Amersham, Bucks., U.K. *N*-Methyl-, -ethyl- and -n-propylprotoporphyrin IX dimethyl esters were prepared by heating 5 mg of protoporphyrin IX dimethyl ester (Sigma Chemical Co.) with 1.0 ml of the appropriate iodoalkane (BDH Chemicals, Poole, Dorset, U.K.) at 100°C under N_2 for 6 or 24 h in a similar manner to that described by De Matteis *et al.* (1981). Excess iodoalkane was removed with an N_2 stream and the alkylated porphyrins were purified by repeated t.l.c. Identities were confirmed by absorption spectroscopy and mass spectrometry.

Animals and dosing

Male C57BL/10 ScSn mice (20–25 g) were from OLAC (1976) Ltd., Bicester, Oxfordshire, U.K. Where indicated, these were pretreated with 0.1% (w/v) sodium phenobarbital in the drinking water for 7 days. Nitrosamines were dissolved in trioctanoylglycerol (trioctanoin; usually 20 mg/ml) and administered intraperitoneally at a dose of 1 mmol/kg body wt., or as indicated. In one experiment mice were given 10 μCi [^{14}C]diethylnitrosamine, diluted with unlabelled material, at a dose of 1 mmol/kg body wt. **Because of the volatile nature and carcinogenicity of nitrosamines, appropriate precautions were taken to avoid human contact.**

For the induction of mixed-function oxidases, control mice were injected intraperitoneally with one of the following compounds once a day for 3 days: 3-methylcholanthrene (2 mg/ml in trioctanoin), 20 mg/kg body wt.; pyrazole (20 mg/ml in 0.14 M-NaCl), 200 mg/kg body wt. Dosing was carried out between 09:00 h and 10:00 h. Diethylnitrosamine was administered 24 h after the last dose of inducer.

Inhibitors of green pigment formation (dissolved in 0.14 M-NaCl and administered intraperitoneally) were given as a single dose to phenobarbital pretreated mice 1 h before dosing with diethylnitrosamine. These were: ethanol (150 mg/ml), 1.5 g/kg body wt.; pyrazole (20 mg/ml), 200 mg/kg body wt.; nicotinamide (20 mg/ml), 200 mg/kg body wt.

The formation of green pigments in phenobarbital-pretreated mice was also investigated after the administration of either 3,5-diethoxycarbonyl-4-ethyl-1,4-dihydro-2,6-dimethylpyridine (20 mg/ml in trioctanoin; 100 mg/kg body wt.; intraperitoneal injection) or ethylene gas (99.9% CP grade; BOC Special Gasses, London, U.K.). Mice were exposed to ethylene/air (1:19) for 18 h using glass-fibre chambers described previously (White, 1978).

Estimation of porphyrins and cytochrome P-450 in liver homogenates

Liver homogenates (10%, w/v) were prepared from mice killed by cervical dislocation in ice-cold 0.25 M-sucrose. Porphyrins were estimated in the supernatant using the fluorimetric procedure of Abbritti & De Matteis (1971). Cytochrome *P*-450 was estimated in liver homogenates from the CO-reduced versus the CO-oxidized difference spectrum (McLean & Day, 1974).

Preparation of liver microsomes: assay of cytochrome P-450 destruction in vitro

Washed microsomal fractions were prepared as described previously (White, 1978). The final pellet was made up in 0.25 M-sucrose so that 1 ml was equivalent to 0.5 g of liver wet wt. Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Reaction mixtures (3 ml) in screw-capped flasks fitted with Teflon septa contained: potassium phosphate buffer, pH 7.4, 0.1 M; $MgCl_2$, 2 mM; EDTA, 0.5 mM; $NADP^+$, 0.5 mM; glucose 6-phosphate, 5 mM; glucose 6-phosphate dehydrogenase, 2 units; microsomal fraction (3–4 mg of protein), 0.2 ml. Reactions were started by the addition of nitrosamine dissolved in dimethyl sulphoxide (10 μl) to give a final concentration of 20 mM unless indicated otherwise. In some instances nitrosamine was omitted or replaced with *N*-ethylprotoporphyrin IX or *N*-hydroxyethylprotoporphyrin IX dissolved in dimethyl sulphoxide and added in a volume of 10 μl to give a final concentration of 1 μM. After incubation for various times as indicated at 37°C in a shaking water bath in the dark, reactions were stopped by placing the flasks on an ice/salt mixture. Green pigments were estimated by transferring 2 ml of the reaction mixture to 40 ml

of ice-cold 5% (v/v) H_2SO_4 in methanol. After 18h at 4°C in the dark the esterified products were extracted into chloroform (White & Muller-Eberhard, 1977) and subjected to h.p.l.c. as described below. Cytochrome *P*-450 was estimated from the CO-reduced versus reduced difference spectra (Omura & Sato, 1964).

Total haem was determined as the pyridine haemochromogen from the dithionite reduced versus the $K_3Fe(CN)_6$ oxidized difference spectrum (Omura and Sato, 1964). Results for cytochrome *P*-450 and haem concentrations were expressed relative to control mixtures containing no nitrosamine to compensate for destruction of cytochrome *P*-450 caused by NADPH-catalysed lipid peroxidation.

H.p.l.c. of green pigments

H.p.l.c. of green pigments was carried out on silica columns (25cm × 0.47cm; Machery Nagel Nucleosil 50:5; Camlab, Cambridge, U.K.). The solvent (flow rate 1.5ml/min) was cyclohexane/chloroform/methanol (4:2:1, by vol.) containing 0.2% (v/v) acetic acid. The spectrophotometric detector was set at 417nm. Peak areas were computed by using a Pye–Unicam DP88 integrator. Samples were injected via a Rheodyne 7125 loop injector.

Purification of and characterization of green pigments

N-Alkylprotoporphyrin IX was extracted from the pooled liver homogenate from 25–30 mice and chromatographed on Sephadex LH20 columns by the procedure of Tephly *et al.* (1979). Esterification of the green pigments was carried out by refluxing for 30min with 14% (w/v) BF_3 in methanol (Smith & Francis, 1979). The methyl esters were extracted into chloroform, washed with water, dried (anhydrous Na_2SO_4) and then subjected to silica-gel t.l.c. (Merck silica-gel 60 t.l.c. plates; layer thickness 0.25mm). The solvent system was chloroform/methanol (17:3, v/v). The green-coloured band (R_F 0.34) was eluted with chloroform/methanol (1:1, v/v) and rechromatographed twice using the same solvent system.

Optical spectra in chloroform were recorded on a Varian DMS 90 spectrophotometer. Wavelength accuracy was relative to a didydium standard. Dicationic spectra were obtained after the addition of 10μl of trifluoroacetic acid to the 3ml cuvettes (De Matteis & Cantoni, 1979).

Mass spectrometry

Samples were studied by chemical-desorption mass spectrometry using a VG 70-70F mass spectrometer. Ammonia was used as the chemical-ionization reagent gas with a mass-spectrometer source temperature of 200°C. Spectra were processed with a VG 2035 Data System. The desorption probe current was initially kept at zero until all of the solvent-derived impurities in the sample had been ionized and the current was then increased to 1.5A. The protonated molecular ion for the porphyrin was observed transiently (over one to three spectra); subsequent spectra contained a much greater total ion current but did not show any high-mass fragments.

Reaction of green pigment with [1-^{14}C]acetic anhydride

This was carried out by using the procedure briefly described by De Matteis *et al.* (1980). The green pigment (30nmol) from diethylnitrosamine-treated mice, purified as described above, was dissolved in 0.9ml of dry pyridine. [1-^{14}C]Acetic anhydride (0.1ml) was added and the mixture left under N_2 in the dark at room temperature for 18h. Water (2ml) was added and the acetylated products extracted into diethyl ether. The diethyl ether extracts were washed with water, dried (anhydrous Na_2SO_4) and purified by t.l.c. and h.p.l.c. as described above. ^{14}C radioactivity in fractions from the h.p.l.c. detector was determined in a Searle scintillation counter using Picofluor scintillant (Packard Instrument Co.).

Reaction of green pigments with $^{65}ZnCl_2$

To 30nmol of purified green pigment in chloroform/methanol (2:1, v/v) was added 2μmol of $ZnCl_2$ (10μl) in water. The spectrum of the mixture was recorded before and after the addition of the $ZnCl_2$ (De Matteis *et al.*, 1982). When the reaction was complete, as judged from the bathochromic shift in the Soret band absorption maximum from 417 to 431nm, the green pigments were washed with water and then subjected to t.l.c. The zinc complex (R_F 0.7) was eluted with chloroform/methanol and the absorption in chloroform at 431nm was recorded. The radioactivity of the sample was determined in a Packard Auto-gamma counter.

Results

Diethylnitrosamine-mediated loss of cytochrome P-450 and accumulation of porphyrins in the livers of mice in vivo

The intraperitoneal administration of diethylnitrosamine (1mmol/kg body wt.) to phenobarbital-pretreated mice resulted in a slow time-dependent loss of cytochrome *P*-450, first evident 2–4h after dosing and reaching 58% of control values by 24h.

Porphyrin concentrations also rose slightly in the liver to twice control values by 4h after dosing. This was followed by a slow return to control values by 24h.

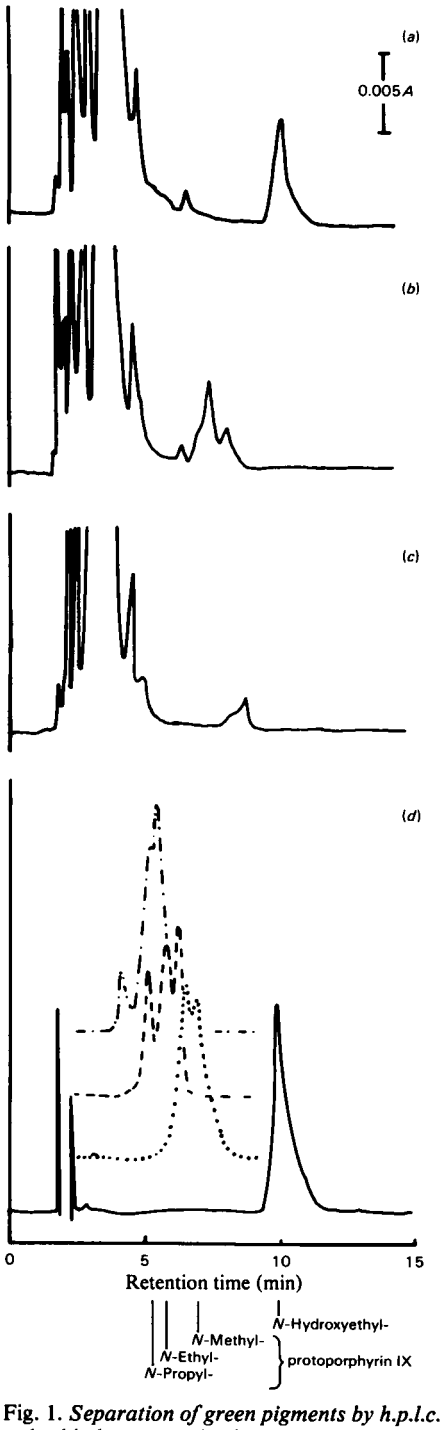

Fig. 1. *Separation of green pigments by h.p.l.c.* Phenobarbital-pretreated mice were dosed with (*a*) diethyl-, (*b*) dimethyl- and (*c*) dipropyl-nitrosamine intraperitoneally (1 mmol/kg body wt.) and killed 2h after dosing. Livers were homogenized in methanol/H_2SO_4 and the concentrated chloroform extracts were subjected to h.p.l.c. Ordinates represent relative absorbance at 417nm. Trace (*d*) represents a composite chromatogram showing the elution profile of *N*-alkylporphyrin standard. ----, *N*-Ethylprotoporphyrin IX; ——, *N*-hydroxyethylprotoporphyrin IX; ····, *N*-methylprotoporphyrin IX; -·-·-, *N*-propylprotoporphyrin IX.

T.l.c. of the esterified liver extracts of mice dosed 2h earlier with 1mmol of diethylnitrosamine/kg body wt. showed the presence of a green band (R_F 0.34) that fluoresced red under u.v. light. Liver extracts subjected to h.p.l.c. also showed a component, retention time 10.0min (Fig. 1*a*). This was not present in extracts of control mouse liver. The green pigment (R_F 0.34) eluted from t.l.c. plates co-chromatographed with this h.p.l.c. peak. Although by t.l.c. the R_F values of diethylnitrosamine green pigment and *N*-ethylprotoporphyrin IX were similar, the two were shown to be different by h.p.l.c. The retention time of the diethylnitrosamine green pigment was considerably greater than *N*-ethylprotoporphyrin IX and the same as that of *N*-hydroxyethylprotoporphyrin (Figs. 1*a* and 1*d*). (The synthetic *N*-alkylporphyrins show more than one component after h.p.l.c., owing to partial resolution of the different isomeric forms.) When di[1-^{14}C]ethylnitrosamine was administered to rats, radioactivity remained associated with the purified green pigment after h.p.l.c. (Figs. 2*a* and 2*b*).

Treatment of mice with dimethyl- or dipropyl-nitrosamine also resulted in the formation of green pigments. With dimethylnitrosamine these had a similar retention time to *N*-methylprotoporphyrin IX (Fig. 1*b*) and similar spectral characteristics (Table 1). Treatment of mice with dipropylnitrosamine resulted in the formation of green pigments with a retention time greater than that of synthetic *N*-propylprotoporphyrin IX (Fig. 1*c*).

Optical spectra of nitrosamine-induced green pigments

In order to differentiate the green pigments produced by different nitrosamines, their optical spectra were compared (Table 1). The green pigments produced by dimethyl- and dipropyl-nitrosamines all exhibited typical aetio-type neutral spectra. There was a clear bathochromic shift in the wavelength maximum of the Soret band of the green pigments (both neutral and dicationic species) in the order dipropyl->diethyl->dimethyl-nitrosamine. It was not possible to distinguish the absorption spectra of diethylnitrosamine green pigments and authentic *N*-ethylprotoporphyrin IX or *N*-hydroxyethylprotoporphyrin IX.

Mass spectrometry

We have previously observed that electron-impact mass spectrometry of *N*-alkylated porphyrins is often beset with problems caused by extensive

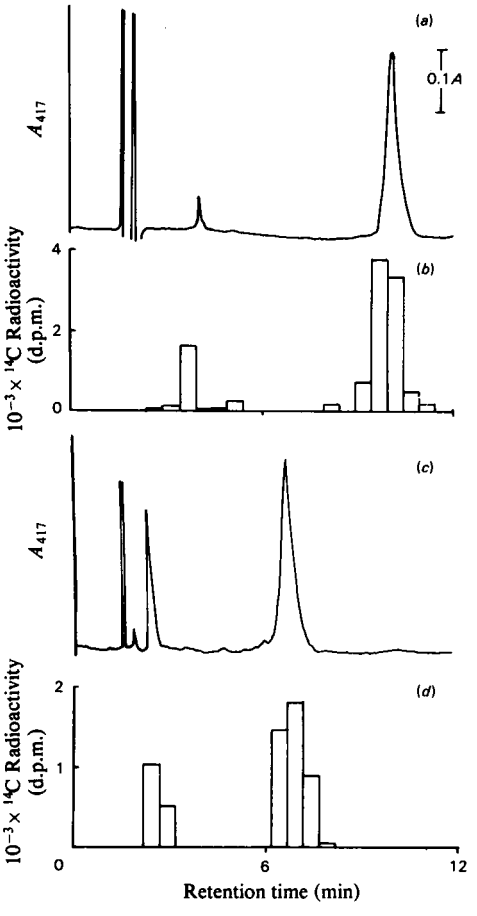

Fig. 2. *H.p.l.c. elution profiles of diethylnitrosamine green pigment: effect of reaction with acetic anhydride*
Green pigment extracted from the livers of mice given diethylnitrosamine (1 mmol/kg body wt.) was subjected to Sephadex LH20 chromatography, esterification with methanolic BF_3, t.l.c. and h.p.l.c. using procedures described in the Experimental section. In one experiment (*a* and *b*), mice were injected with di[1-^{14}C]ethylnitrosamine and the purified green pigment subject to h.p.l.c. In a separate experiment (*c* and *d*), mice were given unlabelled diethylnitrosamine and the purified green pigment treated with [1-^{14}C]acetic anhydride. Ordinates in (*a*) and (*c*) represent relative absorbances at 417nm; (*b*) and (*d*), ^{14}C radioactivity in 0.75ml fractions collected from the detector outlet in (*a*) and (*c*) respectively.

thermal decomposition of the sample and its degradation to the dealkylated material (Smith & Farmer, 1981). The technique of ammonia chemical desorption was therefore used and this showed protonated molecular ions for a short time period before decomposition of the sample occurred. The use of a chemical-desorption probe under electron impact conditions also gave satisfactory spectra.

Thus desorption chemical-ionization mass spectrometry of the purified diethylnitrosamine-induced green pigment dimethyl ester gave a weak spectrum containing a protonated molecular ion at *m/z* 635 (24.3% of base peak), compatible with the compound being *N*-hydroxyethylprotoporphyrin IX. After acetylation with acetic anhydride the R_F value after t.l.c. increased from 0.34 to 0.64, and the retention time after h.p.l.c. decreased from 10.0 to 4.6min (Figs. 2*a* and 2*c*). When acetylation was carried out using [1-^{14}C]acetic anhydride, radioactive label remained associated with the green pigment peak (Fig. 2*d*). Mass spectrometry of the acetylated green pigment gave a protonated molecular ion at *m/z* 667 (base peak) and a fragmentation peak at *m/z* 617 ([$M-CH_3COO$], 29.7% of base peak).

Table 1. *Spectral characteristics of green pigments isolated from the livers of mice given dialkylnitrosamines*
Phenobarbital-pretreated mice were dosed with dialkylnitrosamine intraperitoneally (1 mmol/kg body wt.) and killed 2h after dosing. Green pigment was extracted from the liver and subjected to Sephadex LH20 column chromatography, esterification with methanolic BF_3 and t.l.c. The purified pigment was dissolved in chloroform (3ml). Neutral spectra were obtained after the addition of 10μl of *NN*-di-isopropylethylamine to the cuvettes, dication spectra after the addition of 10μl of trifluoroacetic acid. *N*-Hydroxyethylprotoporphyrin IX was prepared in a similar manner from mice exposed to ethylene. *N*-Ethylprotoporphyrin IX was prepared synthetically as described in the Experimental section.

	Wavelength maxima of green pigments (nm)							
	Neutral spectrum					Dication spectrum		
Green pigment isolated from mice treated with:								
Dimethylnitrosamine	415	511	542	583	651	412	558	596
Diethylnitrosamine	417	513	546	593	650	417	564	607
Dipropylnitrosamine	419	515	546	596	652	420	568	611
Green pigment standards:								
N-Ethylprotoporphyrin IX	418	514	547	593	648	417	563	608
N-Hydroxyethylprotoporphyrin IX	418	514	547	595	652	417	564	607

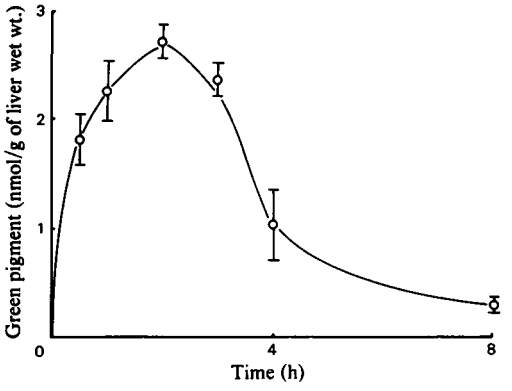

Fig. 3. *Dependence of hepatic green-pigment formation in vivo on times of dosing with diethylnitrosamine*
Phenobarbital-pretreated mice were given diethylnitrosamine intraperitoneally (1 mmol/kg body wt.). At various times after dosing, animals were killed and livers were homogenized in methanol/H_2SO_4. Concentrations of green pigment were estimated by h.p.l.c. as described in the Experimental section. Results are means ± S.E.M. (represented by the bars) for four determinations.

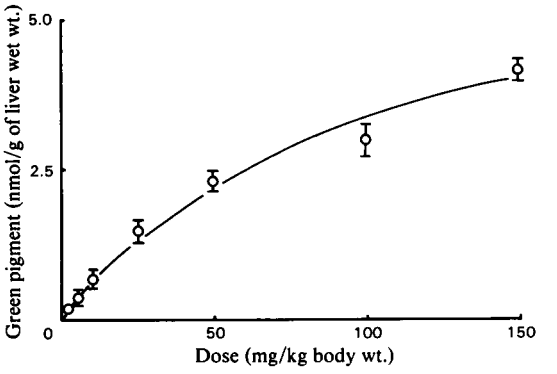

Fig. 4. *Dose–response curve of green-pigment formation in mouse liver caused by diethylnitrosamine*
Phenobarbital-pretreated mice were given various doses of diethylnitrosamine intraperitoneally and killed 2 h later. Portions of liver were homogenized in methanol/H_2SO_4 and concentrations of green pigment estimated by h.p.l.c. Results are mean ± S.E.M. (represented by the bars) for four determinations.

Accumulation of green pigments in the livers of mice given diethylnitrosamine

The absorption coefficient of the purified green pigment methyl ester was first determined from optical spectra run before and after the addition of $^{65}ZnCl_2$. On the basis of a binding of the zinc to green pigment in a 1:1 molar ratio, an absorption coefficient in chloroform of 124 litre · mmol · cm^{-1} at 431 nm was obtained for the zinc complex and 113 litre · mmol · cm^{-1} at 417 nm for the metal-free green pigment.

Fig. 3 shows the time course for the accumulation of green pigments in the livers of mice given diethylnitrosamine. Concentrations of green pigment rose rapidly, reaching maximum values 1–2 h after dosing, followed by a more gradual decline. Recoveries of *N*-hydroxyethylprotoporphyrin IX added to control mouse liver was 98.1 ± 1.2% (mean ± S.E.M. of four experiments). With diethylnitrosamine, there was also a dose-dependent relationship for the accumulation of green pigments in the liver measured 2 h after dosing (Fig. 4). A similar time course for the accumulation of green pigments was seen in mice given dimethyl- or dipropylnitrosamine, although the maximum accumulation, measured at 2 h after a dose of 1 mmol/kg body wt. (expressed as *N*-hydroxyethylprotoporphyrin IX equivalents) was less with both compounds 1.2 ± 0.1 and 0.9 ± 0.1 nmol/g of liver wet wt. (mean ± S.E.M. of four experiments) for dimethyl- and dipropylnitrosamine respectively.

Table 2. *Effects of chronic pretreatment of mice with mixed-function oxidase inducers on the formation of green pigments after dosing with diethylnitrosamine*
Mice were pretreated once daily for 3 days with microsomal enzyme inducers as described in the Experimental section. Diethylnitrosamine in trioctanoin was given intraperitoneally at a dose of 1 mmol/kg body wt. 24 h after the last dose of inducer. Controls received trioctanoin only. Animals were killed 2 h after dosing. Cytochrome *P*-450 was estimated in liver homogenates of control animals. Green-pigment formation was determined by h.p.l.c. after portions of liver had been homogenized in methanol/H_2SO_4 and the methylated products extracted into chloroform. Results are means ± S.E.M. for four determinations.

Pretreatment	Cytochrome *P*-450 (nmol/g wet wt.)	Green pigment formed (nmol/g wet wt.)
None	54 ± 1	1.6 ± 0.1
Phenobarbital	157 ± 8	3.3 ± 0.3
3-Methylcholanthrene	111 ± 6	2.2 ± 0.07
Pyrazole	48 ± 5	2.0 ± 0.3

Effects of pretreating mice with mixed-function oxidase inducers on diethylnitrosamine-mediated green pigment formation

Table 2 shows that relative to control mice, pretreatment with either phenobarbital or 3-methylcholanthrene increased the concentration of cytochrome *P*-450 and the subsequent accumulation of hepatic green pigments measured 2 h after dosing

Table 3. *Protective effects of a single dose of ethanol, pyrazole or nicotinamide given 1 h before diethylnitrosamine*

Phenobarbital-pretreated mice were given a single intraperitoneal injection of the compounds shown in the Table at the doses indicated, 1 h before a dose of 1 mmol of diethylnitrosamine/kg body wt. At 2 h after the nitrosamine, the animals were killed. Portions of liver were homogenized in methanol/H_2SO_4 and green pigment in the concentrated chloroform extracts was estimated by h.p.l.c. Results represent the means ± S.E.M. of four experiments.

Pretreatment	Green pigment formed (nmol/g of liver wet wt.)
None	2.9 ± 0.3
Ethanol (1.5 g/kg)	0.16 ± 0.05*
Pyrazole (200 mg/kg)	0.35 ± 0.15*
Nicotinamide (200 mg/kg)	2.4 ± 0.3

* Probability of significance of difference between treated and control groups ($P < 0.001$).

with diethylnitrosamine. The effects of pretreating mice with pyrazole was also investigated since this compound has been reported to be an inducer of dimethylnitrosamine demethylase (Evarts *et al.*, 1982). The present results show that with pyrazole pretreatment, cytochrome *P*-450 was not induced nor was there any significant effect, relative to control mice, on hepatic green pigment accumulation.

Inhibitory effects of a single dose of ethanol, nicotinamide or pyrazole on green-pigment accumulation

Table 3 shows that when given as a single dose, 1 h before the administration of diethylnitrosamine, both ethanol and pyrazole greatly diminished the concentration of green pigments accumulated in the liver. Under similar conditions, nicotinamide was without effect.

Destruction of cytochrome P-450 in vitro caused by diethylnitrosamine

When mouse liver microsomes were incubated with diethylnitrosamine and an NADPH-generating system, there was a time-dependent loss of both cytochrome *P*-450 and haem from the reaction mixture (Fig. 5). Such a loss did not occur in the absence of NADPH. There was no accumulation of a component with $\lambda_{max.}$ 420 nm. The loss of cytochrome *P*-450 measured after 10 min incubation was dependent on the concentration of diethylnitrosamine in the reaction mixture (Fig. 6). No green-pigment formation could be detected in these reaction mixtures *in vitro*. Similar negative results *in vitro* were obtained by using mouse liver homogenates or 10000 *g*-supernatant fractions.

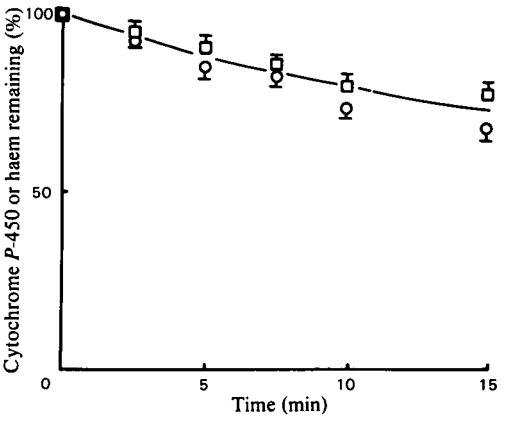

Fig. 5. *Time course for cytochrome P-450 and haem destruction by diethylnitrosamine in vitro*

Liver microsomal fractions from phenobarbital-pretreated mice were incubated for various lengths of time with diethylnitrosamine and an NADPH-generating system as described in the Experimental section. Incubation mixtures of 3 ml contained 10–12 mg of microsomal protein. Reactions were stopped by placing flasks on an ice/salt mixture. Cytochrome *P*-450 (○) was estimated from the CO-reduced versus reduced difference spectrum and total haem (□) as the pyridine haemochromogen. Incubation mixtures originally contained 10 nmol of cytochrome *P*-450 and 34 nmol of haem/3 ml reaction volume.

Metabolism of N-ethylprotoporphyrin IX and N-hydroxyethylprotoporphyrin IX

The present results demonstrated two unexpected findings: (*a*) the formation of *N*-hydroxyethylprotoporphyrin IX instead of the expected *N*-ethylprotoporphyrin IX in diethylnitrosamine-dosed mice; (*b*) the absence of green-pigment accumulation in microsomal systems *in vitro* incubated with diethylnitrosamine. The possibility was investigated that: (*a*) *N*-ethylprotoporphyrin IX once formed might be further metabolised to *N*-hydroxyethylprotoporphyrin IX; (*b*) that *N*-hydroxyethylprotoporphyrin IX might be further metabolised.

N-Ethylprotoporphyrin IX was produced in the livers of mice *in vivo* by the administration of 3,5-diethoxycarbonyl-4-ethyl-1,4-dihydro-2,6-dimethylpyridine (De Matteis *et al.*, 1981). Animals were killed at various times up to 8 h after dosing. H.p.l.c. of the esterified liver extracts showed the major green pigment to co-chromatograph with authentic *N*-ethylprotoporphyrin IX. There was no evidence for the formation of *N*-hydroxyethylprotoporphyrin IX.

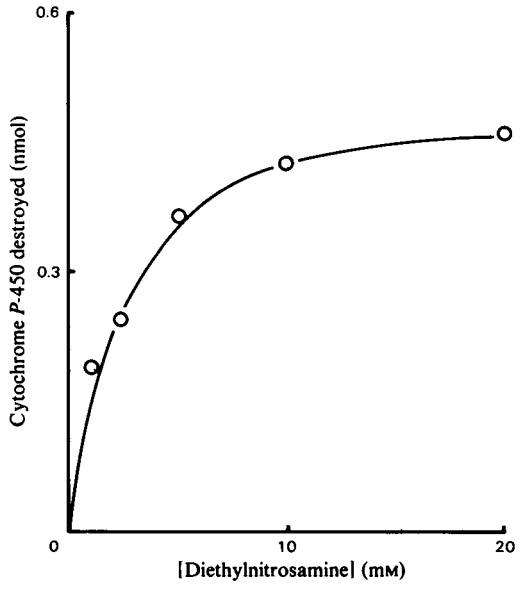

Fig. 6. *Effects of diethylnitrosamine concentrations on the loss of cytochrome P-450 in vitro*
Mixtures of 3 ml volume were incubated with diethylnitrosamine, an NADPH generating system and liver microsomes from phenobarbital-pretreated mice for 15 min at 37°C. Flasks were placed on an ice/salt mixture and cytochrome *P*-450 content was determined. Incubation mixtures originally contained 2.5 nmol of cytochrome *P*-450. Results are means of two experiments.

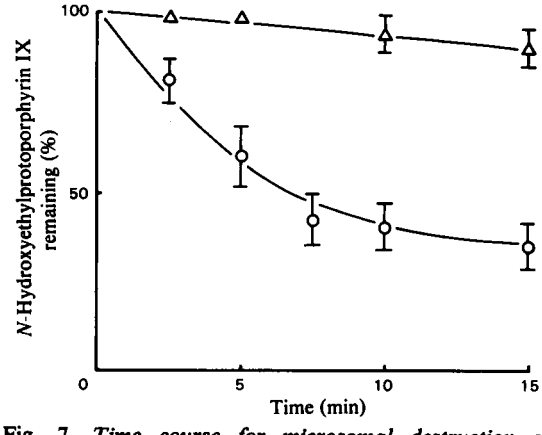

Fig. 7. *Time course for microsomal destruction of N-hydroxyethylprotoporphyrin IX*
Liver microsomes from phenobarbital-pretreated mice were incubated for various lengths of time with *N*-hydroxyethylprotoporphyrin IX (free acid) in the presence (○) or in the absence (△) of an NADPH-generating system. Reactions were stopped with methanol/H_2SO_4 and the concentrations of green pigment estimated by h.p.l.c. Results are means ± S.E.M. (represented by the bars) of four experiments.

Fig. 7 shows that incubation of *N*-hydroxyethylprotoporphyrin IX with NADPH and mouse liver microsomes *in vitro* resulted in a relatively rapid time-dependent loss of this compound. The nature of the degradation products is not known. A similar loss was seen when *N*-ethylprotoporphyrin IX was used (results not shown), but there was no detectable conversion into *N*-hydroxyethylprotoporphyrin IX.

Discussion

Loss of cytochrome P-450 in vivo

The loss of cytochrome *P*-450 *in vivo* after the administration of diethylnitrosamine was slow relative to the accumulation of porphyrins and green pigments. It seems probable that the reduction in the levels of this cyrochrome seen 24 h after dosing represents more an inhibition of protein synthesis by nitrosamine (Magee & Barnes, 1967) than a specific destruction of the haem moiety. Similar long-term reductions in hepatic cytochrome *P*-450 concentrations occur after the administration of other alkylating agents (Litterst, 1981; Wilson *et al.*, 1981).

The identity of the diethylnitrosamine-induced green pigment

A green pigment was formed in the livers of diethylnitrosamine-treated mice, whereas none was seen in control animals. Radioactive label was also present in the green pigment when mice were given di[1-^{14}C]ethylnitrosamine (Fig. 2). These results suggest that active metabolites originating from diethylnitrosamine were capable of alkylating haem. The diethylnitrosamine-induced green pigment had an absorption spectrum that was indistinguishable from that of either *N*-ethyl- or *N*-hydroxyethylprotoporphyrin IX (Table 1), indicating alkylation had taken place at one of the nitrogen atoms of the protoporphyrin IX ring. The magnitude of the bathochromic shift in the absorption maximum of the dicationic spectrum of such *N*-alkylated porphyrins was related to the size of the substituent on the tetrapyrrole nitrogen (De Matteis & Cantoni, 1979; De Matteis & Gibbs, 1980). The bathochromic shift of the diethylnitrosamine-induced green pigment was compatible with that which might be expected from an ethyl substituent. However, evidence from h.p.l.c. (Fig. 1) suggested the pigment to be more polar, with a retention time the same as that of *N*-hydroxyethylprotoporphyrin IX. The identity of this green pigment as *N*-hydroxyethylprotoporphyrin IX was supported by mass-spectral data and the ability to acetylate the pigment with acetic anhydride. More indirect evidence came from the weak porphyrinogenic effect of diethylnitrosamine in mice. *N*-Ethylprotoporphyrin IX is an inhibitor of protohaem ferro-lyase (De Matteis *et al.*,

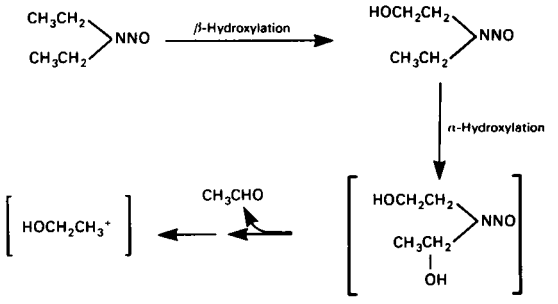

Fig. 8. *Suggested pathway for metabolic activation of diethylnitrosamine to β-hydroxyethylcarbonium ion*

1981). Its formation in the liver at the concentrations indicated by the present results would be expected to cause greatly elevated porphyrin levels in the liver. In contrast, *N*-hydroxyethylprotoporphyrin IX has no inhibitory effect on protohaem ferro-lyase at least *in vitro* (De Matteis *et al.*, 1980).

The expected *N*-ethylprotoporphyrin IX did not accumulate in the livers of mice given diethylnitrosamine. When *N*-ethylprotoporphyrin IX was produced in the livers of mice *in vivo*, e.g., by the administration of 3,5-diethoxycarbonyl-4-ethyl-1,4-dihydro-2,6-dimethylpyridine (De Matteis *et al.*, 1981) or when *N*-ethylprotoporphyrin IX was incubated with microsomes *in vitro*, it was not transformed enzymically to the corresponding *N*-hydroxyethyl derivative. *N*-Hydroxyethylprotoporphyrin IX may be the primary reaction product between the active metabolites of diethylnitrosamine and hepatic haem. Similar results have been obtained with Fischer rats (I.N.H. White, unpublished work).

The nature of the enzyme system responsible

A major metabolic pathway for the metabolism of the dialkylnitrosamines is generally accepted to involve a cytochrome *P*-450-mediated oxidative dealkylation, releasing an aldehyde and forming an alkylcarbonium ion (reviewed by Magee & Barnes, 1967; Michejda *et al.*, 1981).

The present results indicate that an additional pathway may exist for the activation of diethylnitrosamine. Mice exposed to ethylene form the same *N*-hydroxyethylprotoporphyrin IX product in their livers as animals exposed to diethylnitrosamine. Ethylvinylnitrosamine has been suggested as a metabolite of diethylnitrosamine (Althof *et al.*, 1977). Although there is no evidence to support this pathway, the possibility that ethylene may be a metabolite of diethylnitrosamine cannot be excluded. Alternatively, metabolic oxidation of the β-carbon atom has been implicated in the activation of longer-chain nitrosamines (reviewed by Michejda *et al.*, 1981; Loeppky *et al.*, 1981). β-Hydroxylation of one ethyl substituent of diethylnitrosamine followed by α-hydroxylation of the other would yield a β-hydroxyethyl carbonium ion (Fig. 8). β-Hydroxylation of diethylnitrosamine to di-(hydroxyethyl)nitrosamine has been demonstrated *in vivo* (Blattman *et al.*, 1974). This latter compound given chronically to rats is carcinogenic (Lijinsky *et al.*, 1980).

To our knowledge there are no reports of hydroxyethylation of other cellular macromolecules, e.g. DNA, after the administration of diethylnitrosamine. The expected greater chemical instability of the hydroxyethylcarbonium ion relative to an ethylcarbonium ion may prevent alkylation of cellular DNA. The role of such an activation pathway in the cytotoxic or genotoxic effects of diethylnitrosamine remains to be assessed.

We thank Janet Cox for technical assistance and John Lamb for running the mass spectra.

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