Biochemical Pharmacology, Vol. 24, pp. 621–626. Pergamon Press, 1975. Printed in Great Britain.

OXIDATION OF *N*-NITROSOPIPERIDINE IN THE UDENFRIEND MODEL SYSTEM AND ITS METABOLISM BY RAT-LIVER MICROSOMES

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(*Received* 14 *June* 1974; *accepted* 12 *August* 1974)

Abstract—The major product isolated from the oxidation of the carcinogen, *N*-nitrosopiperidine, by the ascorbic acid model system of Udenfriend *et al.* has been identified as *N*-nitroso-4-piperidone both by its spectral properties and by comparison with authentic material. On incubation of *N*-nitrosopiperidine with rat-liver microsomes in the presence of the appropriate co-factors, *N*-nitroso-4-hydroxypiperidine was the major metabolite. This was identified by comparison of its mass spectrum and chromatographic properties with those of *N*-nitroso-4-hydroxypiperidine obtained by nitrosation of 4-hydroxypiperidine. The significance of these products with respect to the mechanism of action of the parent carcinogen, *N*-nitrosopiperidine, is discussed.

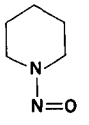
N-Nitrosopiperidine (I) is a potent carcinogen causing tumours of the oesophagus, pharynx and nasal cavity in the rat when administered intravenously and subcutaneously [1–3] and of the liver and oesophagus when fed in the drinking water [4, 5]. In the hamster, it causes tumours of the lung when administered subcutaneously [6].

In common with other nitrosamines, this compound probably requires metabolic activation before acting as a carcinogen, but the mechanism by which this occurs is not fully understood. There is much evidence [7] for the release *in vivo* of formaldehyde following the administration of dimethylnitrosamine and this has led to suggestions [7] that the metabolism of dialkylnitrosamines usually involves an initial α-hydroxylation followed by rapid decomposition of the product to give an alkyl carbonium ion. More recently, however, Kruger [8] and Okada and Suzuki [9], have suggested that β-hydroxylation of long chain alkyl nitrosamines may also be an important metabolic activating process, and Kolar [10] has discussed a possible mechanism whereby di-*n*-butylnitrosamine might be activated by ω-hydroxylation of one of the alkyl groups.

In an attempt to cast further light on the mechanism of action of nitrosamines, we have examined the oxidation of *N*-nitrosopiperidine using, in the first instance, the Udenfriend model system, since the breakdown of dialkylnitrosamines in this system had been reported by Preussman [11], although no products were isolated or identified. The results obtained were compared with those obtained when the same compound was metabolised by rat-liver microsomes.

The postulated mechanism for metabolic activation of dialkylnitrosamines led us to expect the formation of α-oxidised products. Since authentic *N*-nitroso-2-piperidone (II), though not *N*-nitroso-2-hydroxypiperidine, was available, we also investigated the stability of this compound in both oxidative systems.

A preliminary account of some of these results has already been given†.



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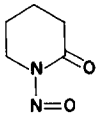

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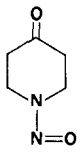

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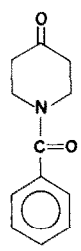

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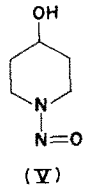

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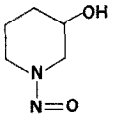

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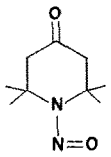

(VII)

METHODS

Synthesis of compounds

(*a*) N-*Nitrosopiperidine* (I). This compound was prepared by nitrosation of piperidine with sodium nitrite and dilute acid as described by Lee and Lijinsky [12].

(*b*) N-*Nitroso*-2-*piperidone* (II). This compound, prepared by a modification of the method described by White [13], was kindly supplied by Dr. C. N. Berry.

(*c*) N-*Nitroso*-4-*piperidone* (III). *N*-Benzoyl-4-piperidone (IV, 10 g, 0·049 moles) was hydrolysed by refluxing in 25% hydrochloric acid (25 ml) for 1 hr. The benzoic acid was filtered from the cooled reaction mixture leaving 4-piperidone hydrochloride in solution. The pH of this solution was adjusted to 2·85 by the addition of concentrated aqueous sodium hydroxide. Nitrosation was accomplished by slow addition, with stirring, of potassium nitrite (4·86 g, 10 per cent in excess of the theoretical equivalent) to the hydrochloride solution at room temperature. After 2·5 hr, thin-layer chromatography (t.l.c.) indicated that almost complete conversion into *N*-nitroso-4-piperidone had occurred. The reaction solution was extracted with dichloromethane (5 × 50 ml). The combined extracts were washed, dried and concentrated. The resulting oily residue was purified by two successive vacuum sublimations (50° 1–2 torr) to give a pale yellow crystalline solid m.p. 63·5–64·5°. (Found: C, 46·85; H, 6·1; N, 21·6%. $C_5H_8N_2O_2$ requires C, 46·9; H, 6·25; N, 21·85%).

(*d*) N-*Nitroso*-4-*hydroxypiperidine* (V). (i) To an ice-cooled, stirred solution of 4-hydroxypiperidine (Aldrich Chemical Co., 1·1 g) in 25% w/v aqueous H_2SO_4 (3·6 g) was added a solution of $NaNO_2$ (1·7 g) in water (3 ml) during 1 hr. After a further 1 hr at room temperature, the mixture was extracted with chloroform (2 × 10 ml) and the combined extracts were washed with 40% aqueous K_2CO_3. The dried (K_2CO_3) organic phase was concentrated and applied to a column (15 × 2 cm) of silicic acid (25 g) which was eluted with chloroform (10-ml fractions). The required product (0·8 g) appeared in fractions 32–95. Distillation (b.p. 135° 0·2 torr) afforded the pure product as a yellow oil. (Found: C, 45·6; H, 8·0; N, 21·2%. $C_5H_{10}N_2O_2$ requires C, 46·15; H, 7·75; N, 21·55%) u.v. data (ethanol): λ_{max} 351 (ϵ = 86·40) and 236 nm (ϵ = 7830). i.r. data (film): 3390, 2945, 1425, 1370, 1280, 1160, 1070, 1020, 985, 955 cm^{-1}.

(ii) A solution of *N*-nitroso-4-piperidone in water was treated with solid sodium borohydride. T.l.c. of the reaction solution after 30 min showed complete conversion into *N*-nitroso-4-hydroxypiperidine.

(*e*) N-*Nitroso*-3-*hydroxypiperidine* (VI). Nitrosation of 3-hydroxypiperidine (Aldrich Chemical Co.) was conducted as for the 4-hydroxy analogue [see (d) (i) above]. Distillation (b.p. 120° 0·2 torr) of the product (0·5 g) obtained after column chromatography gave a yellow oil, which solidified on cooling to 0°. (Found: C, 45·75; H, 8·1; N, 21·05%). u.v. data (ethanol): λ_{max} 350·5 (ϵ = 88·20) and 238 nm (ϵ = 7630). i.r. data (film): 3390, 2940, 1450, 1365, 1270, 1175, 1080, 995, 960 cm^{-1}.

Udenfriend oxidation system [14, 15]. A number of variations of this system were tried in order to maximise the yield of oxidised product. Variations in the amount of ascorbic acid, pH, temperature and time did not appear to have much influence on the yield. The following was a typical experimental protocol.

Ascorbic acid (8·24 g), $FeSO_4.7H_2O$ (1·208 g) and EDTA (disodium salt, 8·06 g) were dissolved in 0·1 M potassium dihydrogen orthophosphate (100 ml) and 0·1 M disodium hydrogen orthophosphate (700 ml). The pH was raised to 6·7 by the addition of 4 N sodium hydroxide (8·75 ml). The solution was transferred to a three-necked flask fitted with a mechanical stirrer, condenser and sintered glass oxygen inlet tube and the flask was placed in a water bath at 60°. *N*-Nitrosopiperidine (1 ml) was added to the reaction mixture. A blank reaction was also set up omitting the nitrosopiperidine. After passage of oxygen for 48 hr, the reaction solution was extracted with dichloromethane (4 × 60 ml), the extracts were combined, washed, dried over $CaSO_4$ and concentrated by rotary evaporation. The products extracted were then examined by t.l.c.

Microsomal oxidation system

Preparation of microsomes. The livers from male Wistar rats (1 g/liter sodium phenobarbital in the drinking water for 7 days) were homogenised to give a 25% suspension in 0·1 M Tris buffer pH 7·4. This suspension was centrifuged at 10,000 *g* for 20 min and the resulting supernatant was centrifuged at 105,000 *g* for 1 hr. The microsomal pellet thus obtained was resuspended in buffer and recentrifuged at 105,000 *g* for 1 hr. The supernatant fluid was decanted and the pellet was resuspended in buffer to give microsomes equivalent to 0·33 g liver per ml (8 mg protein per ml).

Incubations were carried out at 37° for 1 hr under air in stoppered 25 ml conical flasks. Each flask contained 3 ml of microsomal suspension, 2·75 μmoles of $NADP^+$, 55 μmoles of glucose-6-phosphate, 50 μmoles of $MgCl_2.6H_2O$ and 3·5 units of glucose-6-phosphate

dehydrogenase, in a total volume of 10 ml, buffered at pH 7·4 with 0·1 M Tris–HCl. Substrate (*N*-nitrosopiperidine, 20 mg or *N*-nitroso-2-piperidone, 5 mg) was added in the Tris–HCl buffer. To maximise metabolism, microsomes and cofactors were added in three portions at 0, 20 and 40 min. At the end of the incubation period, protein was co-precipitated with barium sulphate by the method of Somogyi [16] and removed by centrifugation at 1200 *g* for 10 min. The supernatant was extracted (3 ×) with an equal volume of dichloromethane. The combined extracts were concentrated to a small volume at 30°/10 torr and examined by t.l.c.

The extent of oxidative metabolism under the conditions described was determined as follows. Parallel incubations of *N*-nitrosopiperidine with microsomes and cofactors and of microsomes and cofactors alone (control) were carried out. Immediately prior to precipitation of the proteins, the control incubation was divided into two portions and *N*-nitrosopiperidine in buffer was added to one portion and buffer alone to the other. All three suspensions were extracted as described above, except that the dry dichloromethane extract was concentrated to a residue, which was taken up in redistilled ethanol. The absorbance of each extract was measured at 349 nm and that due to the extract from microsomes and cofactors alone was subtracted from the absorbance of those containing *N*-nitrosopiperidine (metabolised and unmetabolised). In addition, the ratio of major metabolite to starting material on thin-layer chromatograms was estimated by comparing the absorbance (at 254 nm) of components of the metabolism extract with known amounts of synthetic standards spotted onto the t.l.c. plate.

Thin-layer chromatography. Examination of products from the Udenfriend oxidation system was carried out on 0·4 mm layers of silicic acid (Merck Kieselgel GF 254). The plates were developed in light petroleum (b.p. 40–60°)–acetone (4:1, v/v). Preparative separation of products from this system was carried out on 1 mm layers using the same solvent for development. Three consecutive developments were needed to achieve a good separation. The major product was recovered by removing the relevant band and eluting it with dichloromethane. Evaporation of the dichloromethane yielded the crude product.

Products from the microsomal metabolism were separated on 0·25 mm layers of silicic acid (Merck, Kieselgel GF 254). Each plate was developed twice using the same solvent as above. Silicic acid in the appropriate u.v.-absorbing areas was removed and the products were eluted [17] with chloroform for mass spectrometric investigation.

Infra-red spectra. I.r. spectra were determined in spectroscopically pure chloroform solutions on a Perkin-Elmer 700 spectrometer, or, for synthetic *N*-nitroso-3 and 4-hydroxypiperidines as thin films, using a Perkin-Elmer 257 spectrometer.

Ultra-violet spectra. U.v. spectra were determined in aqueous solution on a Unicam SP 1800 spectrometer, or, for synthetic *N*-nitroso-3 and 4-hydroxypiperidines, in ethanol solution using a Unicam SP 800 spectrometer.

Nuclear magnetic resonance spectra. NMR spectra were determined in deuteriochloroform on a Varian HA 100 spectrometer.

Mass spectra. These spectra were determined with an ionising voltage of 70 eV and source temperature of 100°. The mass spectrum of the major product from Udenfriend oxidation was determined with an AEI MS-9 spectrometer. Products from the microsomal metabolism were examined on an AEI MS-12 spectrometer.

RESULTS

Udenfriend oxidation system. Oxidation of *N*-nitrosopiperidine with molecular oxygen in the presence of ascorbic acid, Fe^{2+} and EDTA afforded 4 products of R_f 0·11, 0·19, 0·30 and 0·37 [t.l.c., light petroleum (b.p. 40–60°)–acetone (4:1, v/v)]. The percentage conversion of nitrosopiperidine (R_f 0·48) was low (*ca* 10%). None of these products was formed in the blank reaction (where *N*-nitrosopiperidine was omitted from the system) nor did any of them have the same R_f value as the marker compound *N*-nitroso-2-piperidone (R_f 0·31).

The major product (R_f 0·19) was isolated as a crude material by preparative t.l.c. It was purified by vacuum sublimation (52–60° 1–2 torr) to yield a pale yellow crystalline solid, m.p. 63–64°. (Found: C, 46·9; H, 6·1; N, 21·85%. $C_5H_8N_2O_2$ requires: C, 46·9; H, 6·25; N, 21·85%). The i.r. spectrum in chloroform solution showed a strong absorption at 1720 cm^{-1} suggesting the presence of a carbonyl group. A doublet at 1460 and 1435 cm^{-1} associated with an absorption at 1360 cm^{-1} indicated the N—N=O group. Other absorption bands appeared at 1310, 1290, 1145, 1028 and 907 cm^{-1}. The u.v. spectrum in water gave a major absorption at 235 nm with smaller absorption peaks at 342 and 418 nm. The NMR spectrum showed two triplets at 5·42 and 6·06τ and a quintuplet at 7·38τ, integrating for 2, 2 and 4 protons, respectively. Spin decoupling resolved the quintuplet into two overlapping triplets, the triplet at lower-field being coupled to the triplet at 5·42τ while that at the higher field was coupled to the triplet at 6·06τ. The low field triplets at 5·42 and 6·06τ are characteristic of *N*-nitroso-compounds [18] having α-methylene groups *anti* and *syn* to the nitroso function, coupled to two adjacent equivalent hydrogens. The pair of overlapping triplets corresponding to two sets of two protons have chemical shifts consistent with their being α to a keto-function, the difference in chemical shifts arising from their *syn* and *anti* conformation with respect to the β nitroso-function. Taken with the analytical and infra-red data, the NMR spectrum is consistent with a cyclic nitrosamine structure in which the two hydrogens at the 4-position in nitrosopiperidine have been replaced by a carbonyl oxygen. Exchange reactions of this compound carried

out in deuterium oxide illustrated the lability of the protons both α and β to the nitroso group.

The mass spectrum of this compound showed a parent ion at 128 agreeing with the formula $C_5H_8N_2O_2$ suggested by the analytical data. This formula was confirmed by accurate mass measurement (calculated: 128·0586; found, 128·0572). Principal ions in the mass spectrum were: *m/e* 128 (M^+, base peak), 111 ([M − OH]$^+$, 25% of base peak, metastable peak *m** *m/e* 96·3), 99 (10%), 98 ([M^+ − NO], 3%), 97 (8%), 84 (3%), 69 (5%), 56 (79%).

Interpretation of the analytical and spectral data suggested that the major product isolated from the Udenfriend oxidation of *N*-nitrosopiperidine was *N*-nitroso-4-piperidone. This was confirmed by a structure-determining synthesis of this compound from *N*-benzoyl-4-piperidone. The *N*-nitroso-4-piperidone thus obtained had the same spectral characteristics (see above) as the major product from the Udenfriend oxidation. T.l.c. and a mixed melting point determination confirmed the identification of the major oxidation product as *N*-nitroso-4-piperidone.

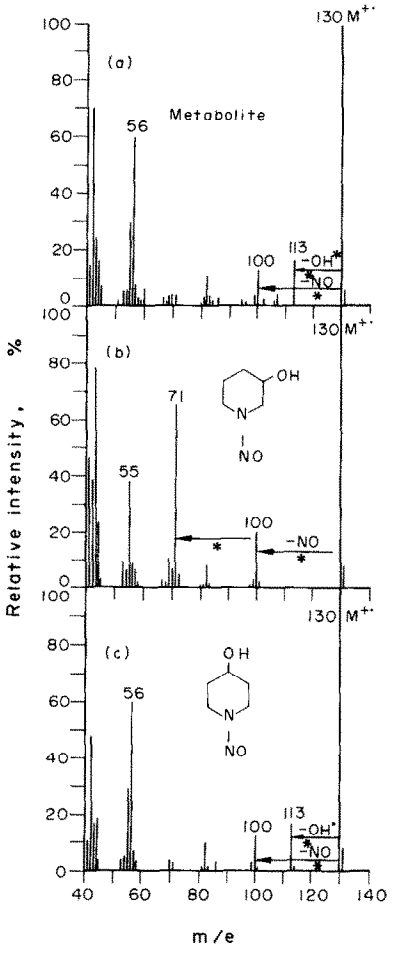

Fig. 1. Mass spectra: (a) the major microsomal metabolite of *N*-nitrosopiperidine; (b) synthetic *N*-nitroso-3-hydroxypiperidine; (c) synthetic *N*-nitroso-4-hydroxypiperidine. An asterisk (*) indicates that a metastable peak is present corresponding to the appropriate transition.

Addition of *N*-nitroso-2-piperidone to the Udenfriend system (under the same conditions as those used in the oxidation of *N*-nitrosopiperidine) allowed a check to be made on the stability of this compound in this system. T.l.c. showed no trace of *N*-nitroso-2-piperidone in the concentrated dichloromethane extract of the reaction mixture indicating that any *N*-nitroso-2-piperidone formed in this system would not be detected.

Microsomal oxidation system. T.l.c. on silicic acid in light petroleum (b.p. 40–60°)–acetone (4:1) of the dichloromethane extract, prepared as described above, showed that three principal mobile products had been formed from *N*-nitrosopiperidine (R_f 0·76). These products had R_f values of 0·04, 0·24 and 0·31, the major product being that of R_f 0·24.

However, incubation of *N*-nitroso-2-piperidone with microsomes and cofactors resulted in rapid and complete hydrolysis, yielding one principal product (R_f 0·04) in the dichloromethane extract. Total metabolism, estimated by comparison of the absorbance of the dichloromethane-extractable material, was a maximum of 9 per cent. The ratio of *N*-nitrosopiperidine to the major product on t.l.c. (that of R_f 0·24) was approximately 150:1.

Comparison of the R_f values of the products from both experiments showed that nitroso-2-piperidone decomposes or is metabolised to give a product with the same mobility on t.l.c. as one of the products obtained from the microsomal metabolism of *N*-nitrosopiperidine (i.e. that of R_f 0·04). Thin-layer chromatographic comparison with other marker compounds, i.e. *N*-nitroso-2- and 4-piperidones and *N*-nitroso-4-hydroxypiperidine (obtained by reduction of *N*-nitroso-4-piperidone with borohydride) showed that the major metabolite from *N*-nitrosopiperidine had the same R_f value (0·24) as *N*-nitroso-4-hydroxypiperidine. One metabolic product additional to those described above and present only in trace amounts appeared to have the same R_f value (0·36) as *N*-nitroso-4-piperidone but there was insufficient of this compound for its mass spectrum to be determined. In keeping with the observed instability of *N*-nitroso-2-piperidone under the conditions of metabolism there was no trace of a metabolite corresponding to this compound (R_f 0·54).

Mass spectrometry showed (Fig. 1a) that the major metabolite had a molecular weight (130) consistent with the insertion of one oxygen atom into the original nitrosopiperidine molecule, and that it showed the losses of 17 mass units (OH·) and 30 mass units (NO) (characteristic of *N*-nitrosopiperidine [19]). A hydroxy derivative would have the appropriate molecular weight, and the three possible *N*-nitroso derivatives, those of 2-, 3- and 4-hydroxypiperidine were considered. Of these, only *N*-nitroso-3-hydroxypiperidine

(an intermediate in the synthesis of 1-amino-3-hydroxypiperidine) had been previously described [20] but no physical or analytical data were presented. *N*-Nitroso-4-hydroxypiperidine was prepared from 4-hydroxypiperidine by conventional nitrosation, but this route could not be applied to the 2-hydroxy derivative, since 2-hydroxypiperidine is unknown. An alternative route to *N*-nitroso-4-hydroxypiperidine was the reduction, with sodium borohydride in water or methanol, of *N*-nitroso-4-piperidone [see Methods (d) (ii)]. However, although *N*-nitroso-2-piperidone was stable in methanol for at least 1 hr at room temperature, the addition of sodium borohydride resulted in the immediate loss of the u.v. absorption associated with the *N*-nitroso group. It was concluded that *N*-nitroso-2-hydroxypiperidine was unstable, and was probably therefore unlikely to survive the conditions of incubation and extraction used in the metabolism of *N*-nitrosopiperidine.

The mass spectrum (Fig. 1a) of the major metabolite was virtually identical with that of synthetic *N*-nitroso-4-hydroxypiperidine (Fig. 1c) and differed from that (Fig. 1b) of the analogous 3-hydroxy derivative which was also slightly less mobile on t.l.c. in light petroleum–acetone (4:1) than the 4-hydroxy derivative. The mass spectra of the two hydroxy derivatives differed markedly in the relative intensitites of the signals (*m/e* 113) ascribed to the loss of OH· from the molecular ion, though the reason for this is unknown.

N-Nitroso-4-hydroxypiperidine was therefore the sole metabolite isolated and identified following the metabolism of nitrosopiperidine by rat liver microsomes.

DISCUSSION

Oxidation of *N*-nitrosopiperidine both in the Udenfriend system and by rat-liver microsomes gave major products identified as those resulting from oxidation at the 4-position, namely *N*-nitroso-4-piperidone and *N*-nitroso-4-hydroxypiperidine.

Although the Udenfriend system is now believed to be an unsatisfactory biological model since it does not exhibit the NIH shift, it does resemble the microsomal system in producing an electrophilic oxidising species [15]. It is not surprising, therefore, that in both systems, oxidation takes place remote from the ring nitrogen which is thought to bear a partial positive charge [18, 21].

A precedent for the biological oxidation of a piperidine derivative at the 4-position exists. The organism *Sporotrichum sulphurescens* found in corn-steep liquor oxidises *N*-benzoylpiperidine to *N*-benzoyl-4-hydroxypiperidine [22].

The relevance of metabolism at the 4-position to the carcinogenicity of *N*-nitrosopiperidine remains an unanswered question at present. Other work, however, has shown that γ-keto nitrosamines [e.g. 4-methyl-4-(methylnitrosamino)-pentan-2-one] are mildly carcinogenic on oral administration to rats [2] and there

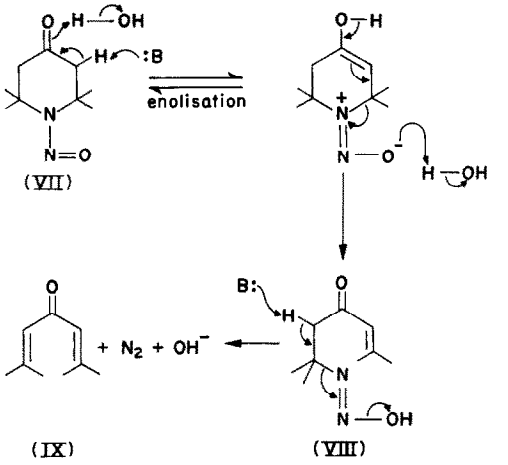

Fig. 2. Postulated base catalysed decomposition of *N*-nitrosotriacetonamine.

is a precedent for the hydrolytic cleavage of *N*-nitroso-4-piperidone under mildly basic conditions. Thus, *N*-nitrosotriacetonamine (VII) decomposes in aqueous or alcoholic solution in the presence of a catalytic amount of base to yield 2,6-dimethyl-4-keto-hepta-2,5-diene (phorone, IX) [23] presumably via a primary alkyldiazonium hydroxide (VIII) (see Fig. 2). Either (VIII) or the α,β-unsaturated ketone (IX) will effect alkylation of suitable nucleophilic species [24, 25].

The present results for either the Udenfriend or microsomal system do not exclude concurrent oxidation at the 2-position (i.e. α-oxidation) of *N*-nitroso piperidine. The resultant *N*-nitroso-2-hydroxypiperidine is unknown but is expected to be very unstable and we have demonstrated that the alternative *N*-nitroso-2-piperidone, if formed, would not survive the conditions of our oxidative experiments. Since *N*-nitroso-4-hydroxypiperidine accounted for only 10 per cent of the metabolites formed by liver microsomes, it is probable that most of the 90 per cent of the metabolites not accounted for had undergone decomposition and lost the u.v.-absorption due to the nitroso group. Experiments are in progress to effect oxidation under conditions where *N*-nitroso-2-piperidone would survive.

The carcinogenicity of the *N*-nitroso-2- and 4-piperidones is at present under test but following the identification of *N*-nitroso-4-hydroxypiperidine as the major isolable metabolite from rat-liver microsomes, there is an obvious need to test the carcinogenicity of this compound so that its relevance to the carcinogenicity of the parent *N*-nitrosopiperidine might be assessed.

Acknowledgements—This investigation was supported by a grant to B. C. Challis from the Cancer Research Campaign and by grants to the Chester Beatty Research Institute (Institute of Cancer Research, Royal Cancer Hospital) from the Medical Research Council (Grant no. G973/786/K and 973/787/K). The A.E.I. MS-12 mass spectrometer was purchased

on a special grant (G969/189/C) from the Medical Research Council. The work was carried out during the tenure (by P.J.C.) of the Ward Blenkinsop Fellowship and a fellowship (awarded to M.J.) by the Ludwig Institute for Cancer Research. We thank Professor A. B. Foster and Dr. T. A. Connors for their interest and Mr. M. H. Baker and Mr. L. J. Griggs for technical assistance.

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