METABOLIC N^1-OXIDATION OF 9-BENZYLADENINE AND ISOMERIC 9-(NITROBENZYL)ADENINES BY HAMSTER HEPATIC MICROSOMES

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SUMMARY

The metabolism of 9-benzyladenine (BA) and isomeric 9-(nitrobenzyl)adenines (NBAs), i.e. 9-(2-nitrobenzyl)adenine (2NBA), 9-(3-nitrobenzyl)adenine (3NBA) and 9-(4-nitrobenzyl)adenine (4NBA), was investigated with normal hamster hepatic microsomes. Using HPLC, TLC, UV and MS techniques, it was established that metabolic N^1-oxidation occurred for all BA and NBAs. The N^1-oxidative rate was in the order 2NBA>BA>3NBA and 4NBA. The results suggest that the introduction of a nitro group may interfere with the binding between substrates and related N^1-oxidase(s). This may be the reason for the lower N^1-oxidative rates of 3NBA and 4NBA. It is also suggested that 2NBA may possess the most favourable conformation and binding characteristics for the enzymes among the substrates studied as it was metabolised even faster than BA.

KEY WORDS

isomeric 9-(nitrobenzyl)adenines, 9-benzyladenine, metabolism, N^1-oxidation, hepatic microsome, hamster

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INTRODUCTION

Since the finding of adenine in 1885, a great number of adenine derivatives have been isolated from natural sources or prepared by chemical methods. Many of them have important physiological or pharmacological activities as the components of biological molecules or as drugs. Adenine exists widely in organisms as an important component of nucleic acids. Adenosine is also found to be a ubiquitous chemical messenger or "local hormone" involved in regulation of many physiological functions /1/. In addition, adenine occurs as the component of a number of important coenzyme systems such as nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide (NAD), flavine adenine dinucleotide (FAD) and coenzyme A /2/. In recent years, a large number of new derivatives of adenine and adenosine have been synthesised and are currently under development for potential use in cancer and viral infection /3/, renal failure /4/ and Parkinson's and Huntingdon's diseases /5/.

As an important class of amino azaheterocyclic compounds, the metabolism of adenine and its derivatives is an interesting subject. Although it was previously reported that both metabolic N^1-oxidation (with normal rat hepatic microsomes) and N^6-hydroxylation (using 3-methylcholanthrene or isosafrole induced rat hepatic microsomes) of adenine were found /6,7/, no other case of N-hydroxylation has been reported for other adenine derivatives. With different animal hepatic microsomal preparations, it was previously found by Lam *et al.* /8-10/ that metabolic N^1-oxidation occurred for some 9-alkyladenines (9AAs), such as 9-benzyladenine, 9-benzhydryladenine and 9-(4-nitrobenzyl)adenine. But they failed to find the N^1-oxide of adenine and certain other 9AAs, such as methyladenine, adenosine, ATP, ADP and 9-trityladenine, or the N^6-hydroxylamine as metabolites of all the 9AAs studied. The hepatic microsomal N^1-oxidase activity amongst rodents was found to be in the order hamster > mouse > rabbit > rat > guinea-pig. The most favourable conformations of some 9AAs were also established using computer graphic modelling techniques. The possible relationship between N^1-oxidation of adenines and their stereo-structures as well as possible controlling factors for N^1-oxidation have been discussed by Lam *et al.* and Gorrod *et al.* /8-11/.

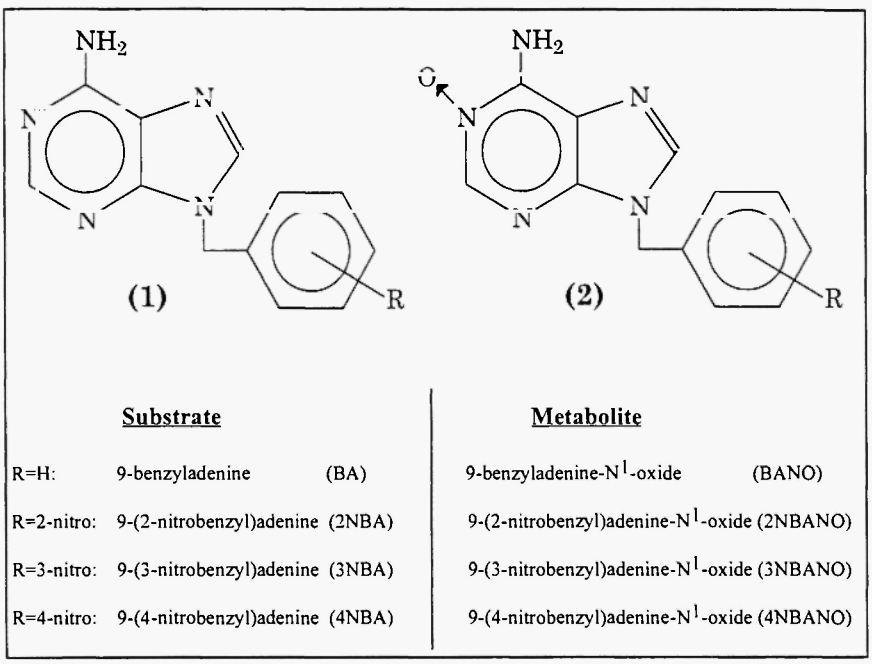
From the above studies, it seems N^1-oxidation occurs as the main metabolic pathway for only some 9AAs. In addition to other factors, the variation of stereo-structure of 9AAs due to the nature of the 9-

substituted groups may be an important factor affecting N^1-oxidation. Based on the above information and as a continuation of previous investigations, we have studied and compared the metabolism of 9-benzyladenine (BA) and isomeric 9-(nitrobenzyl)adenines (NBAs) by normal hamster hepatic microsomes.

MATERIALS AND METHODS

Materials

The structures and abbreviations of the 9AAs studied and their potential metabolic N^1-oxides are indicated in Figure 1. All substrates and potential metabolites were synthesized in our laboratories and the synthetic methods as well as the data obtained for characterization will be separately reported elsewhere. N,N-Dimethylformamide (DMF, HPLC grade) was produced by Aldrich Chemical Co. (Gillingham, Dorset, UK); glucose-6-phosphate (G-6-P) and nicotinamide adenine



	Substrate		Metabolite	
R=H:	9-benzyladenine	(BA)	9-benzyladenine-N^1-oxide	(BANO)
R=2-nitro:	9-(2-nitrobenzyl)adenine	(2NBA)	9-(2-nitrobenzyl)adenine-N^1-oxide	(2NBANO)
R=3-nitro:	9-(3-nitrobenzyl)adenine	(3NBA)	9-(3-nitrobenzyl)adenine-N^1-oxide	(3NBANO)
R=4-nitro:	9-(4-nitrobenzyl)adenine	(4NBA)	9-(4-nitrobenzyl)adenine-N^1-oxide	(4NBANO)

Fig. 1: Structures and abbreviations of 9-alkyladenines (9AAs) and their N^1-oxides.

dinucleotide phosphate (NADP) were obtained from Sigma Chemical Co. (Poole, Dorset, UK); and glucose-6-phosphate dehydrogenase (suspension grade II) was the product of Boehringer Mannheim Corporation (Lewes, East Sussex, UK). Other chemicals and solvents used were either analytical grade or HPLC grade, supplied by commercial sources.

Preparation of hepatic microsomes

Syrian hamsters (male, 80-100 g) were used throughout this investigation and were supplied by King's College animal facilities. The hepatic microsomes were prepared using the procedure of Schenkman and Cinti /12/ as modified by Lam *et al.* /8/, based on sedimentation of the microsomal fraction of liver homogenate with calcium ions. The prepared microsomes were finally resuspended in phosphate buffer (0.2 M, pH 7.4, containing 20% glycerol) equivalent to 0.5 g fresh liver tissue per ml suspension and stored at -80°C until required. The microsomal protein content, cytochrome P-450 content, cytochrome b_5 content and NADP-cytochrome P-450 reductase activity were determined using standard methods as described by Gibson *et al.* /13/.

Incubations

Incubations were carried out in 10 ml non-stoppered glass blood-testing tubes for 30 min at 37°C with a SS40-D shaking bath (Grant Instruments (Cambridge) Ltd., Cambridge, UK). The incubation system consisted of phosphate buffer (1.0 ml, 0.2 M, pH 7.4), NADP (1 μmol), glucose-6-phosphate (5 μmol), glucose-6-phosphate dehydrogenase (0.5 unit), which was preincubated at 37°C for 5 min before addition of magnesium chloride (10 μmol) and microsomal suspension (0.5 ml) with a final volume of 1.5 ml. BA and NBAs were separately dissolved in DMF to the required concentrations (10-30 μmol/ml) and 10-30 μl was added to each incubate for metabolism studies. When incubation was complete, the metabolic reactions were terminated either by placing the incubation tubes in an ice bath or by adding trichloroacetic acid solution (200 μl/incubate, 25% w/v in H_2O) to the incubates. Metabolites present in incubates were further analysed using various techniques (see below).

Solid phase extraction (SPE) AND HPLC methods

Incubates were extracted with an established off-line SPE procedure and analysed with an HPLC method as previously described /14/. After the metabolism was terminated by adding trichloroacetic acid solution, internal standard solution (4NBANO for BA and BANO; BA for others; 100 µl/incubate in MeOH) was added to the incubates, which were mixed and centrifuged at 3500 rpm for 10 min. The supernatants were obtained and diluted with H_2O (1.5 ml) for further extraction.

The SPE procedure utilised ISOLUTE C18 columns (non-end-capped) with 500 mg sorbent mass and 6 ml reservoir volume (Jones-Chromatography, Mid Glamorgan, UK). Briefly, the SPE procedure was as follows: (a) the SPE columns were wetted with MeOH (2 ml) followed by H_2O (1 ml); (b) the columns were conditioned with phosphate buffer (4 ml, 0.1 M, pH 7.4); (c) the samples (incubates or the standards) were applied to the columns; (d) interfering substances were washed off the columns with phosphate buffer (4 ml, 0.1 M, pH 7.4, containing 15% MeOH); (e) the columns were washed again with H_2O (2 ml) to remove salts (the remaining H_2O was removed by blowing air through the columns for one minute with a small air pump); and finally, (f) the analytes were desorbed and eluted from the columns with MeOH (3 ml). The eluents were collected and evaporated to dryness with a Vortex-Evaporator (HAAK-Buchler Instrument Inc., Saddle Brook, USA) under reduced pressure. The residues were dissolved in MeOH (300 µl) for HPLC analysis.

The reversed phase HPLC method used a Spherisorb 5 ODS (2) column (25 x 0.46 cm ID; particle size 5 µm; Phenomenex, Cheshire, UK). The apparatus consisted of a M-45 solvent delivery system (Waters, Milford, USA), a SpectroMonitor 3100 Variable Wavelength UV Detector (LDC/Milton Roy, Riviera Beach, USA), a CI-4100 Computing Integrator (LDC/Milton Roy, Co. Clare, Ireland) and a Rapiscan SA6508 diode array detector (a high speed, multiple wavelength UV/VIS detector; HPLC Technology Ltd., Macclesfield, UK). The isocratic mobile phase comprised H_2O:MeOH:N,N-diethylamine (65:35:0.5, v/v/v), adjusted to pH 6.8 with H_3PO_4 (80% aq.). The samples were injected into a 20 µl valved loop and chromatographed with the mobile phase at a flow rate of 1.0 ml/min. The analytes were monitored at 233 nm with the UV detector or scanned within 200-350 nm wavelength range with the diode array detector. The N^1-oxides

formed during incubation were identified by comparison with the corresponding authentic N^1-oxides according to their retention times, UV spectra and 3-dimensional chromatograms (absorbance-time-wavelength) characteristics. For quantitative analysis of metabolites, the concentrations of the N^1-oxides present in incubates were determined by using peak height ratios of N^1-oxides to internal standards according to constructed calibration curves.

Detection using thin layer chromatography (TLC)

The incubates were extracted with the above SPE procedure, except that the internal standards were not added and the phosphate buffer used for washing out interfering substances did not contain MeOH. The extracts were analysed by TLC and compared to authentic compounds. TLC analysis was carried out on pre-coated silica gel 60 F_{254} analytical plates (0.2 mm, 20 x 20 cm TLC aluminium sheet; Merck Chemical Co., Darmstadt, Germany). The solvent was DMF: chloroform:ammonia solution (SG 0.88) (100:100:3 v/v/v). The plates were examined under a UV lamp after development.

Analysis of 4NBANO by mass spectrometry (MS)

For further confirmation of metabolic N^1-oxidation of 9AAs studied, forty separate incubates with 4NBA as substrate were extracted using the SPE procedure without the addition of internal standard. The eluents were combined and evaporated to dryness. The residue was dissolved in MeOH and applied to silica gel plates; the plates were run in the solvent system chloroform:EtOH:ammonia (20:80:1 v/v/v); the band of silica gel containing 4NBANO formed was collected and extracted with MeOH; the extract was evaporated and the residue analysed by EI-MS with a Kratos MS 890 spectrometer. The MS analysis was performed by the Mass Spectrometry Service, Department of Chemistry, King's College London.

UV spectrum of 4NBANO

The above purified 4NBANO sample, which was formed during incubation, was also scanned in HCl (0.1 M) with a UVIKON 860 Spectrophotometer (Kontron Instrument, Zurich, Switzerland) and the spectrum compared with that of authentic 4NBANO.

RESULTS AND DISCUSSION

Biochemical analysis of microsomes

To evaluate the quality of prepared hamster hepatic microsomes, the content of microsomal protein, cytochrome P-450 and b_5, and the activities of NADP-cytochrome P-450 reductase were determined. Typical results are: (1) microsomal protein content: 15.4 mg/g fresh liver tissue; (2) cytochrome P-450 content: 1.03 nmol/mg protein; (3) cytochrome b_5 content: 0.42 nmol/mg protein; (4) activity of NADPH-cytochrome P-450 reductase: 0.15 μmol cytochrome reduced/min/mg protein. All data were in the normal range and confirmed that the procedure for microsome preparation is reliable and the prepared microsomes were appropriate for metabolism studies.

TLC detection of metabolites

Figure 2 shows the representation of TLC of incubates derived from different 9AA substrates. The results show that the N^1-oxidation of every substrate probably occurred as there was an area present on the chromatograms with the same R_f value as the corresponding authentic N^1-oxide for each incubate. In addition, like certain other 9AAs studied by Lam *et al.* /8-10/, it seems that N^9-dealkylation of 9AAs also occurred during incubation because in each case there was a zone corresponding to authentic adenine. From Figure 2, another metabolite with a higher R_f value than 4NBANO could also be recognised in extracts from incubates of NBAs. It may be the same minor metabolite found in the 3-dimensional HPLC chromatogram of the NBA incubates (see below).

HPLC chromatographic and UV spectral characterization of metabolic N^1-oxides

Figure 3 shows the HPLC chromatograms of authentic substrates and N^1-oxides as well as the extracts from the corresponding incubates. It is obvious that the developed reversed-phase HPLC method gave complete separation of N^1-oxides from their parent substrates and endogenous substances in microsomes as well as from the incubation co-factors. The results showed that there was a peak in each incubate with the same retention time as the corresponding authentic

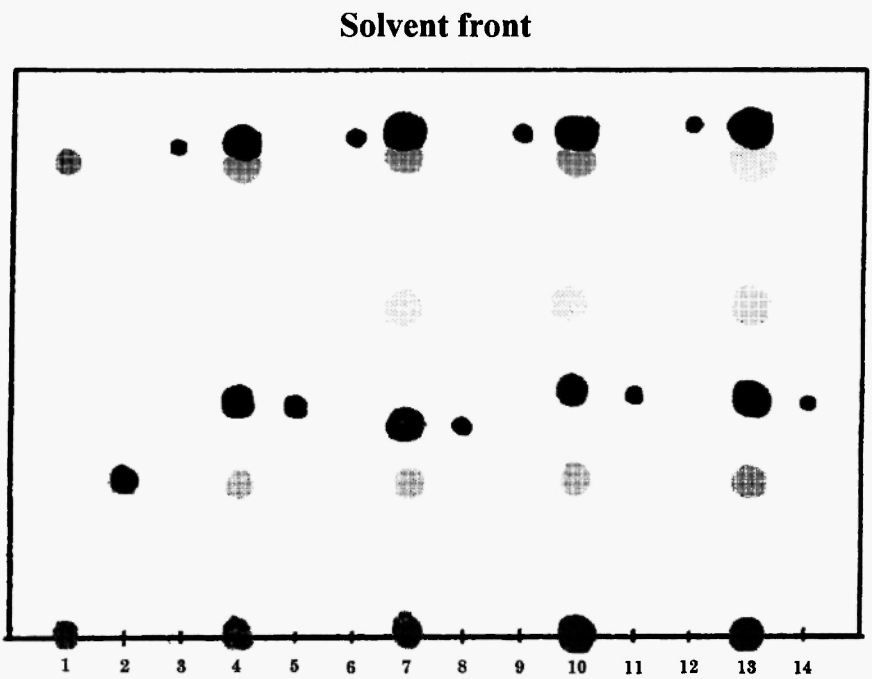


Fig. 2: TLC diagram of extractions of incubates with BA or NBAs as substrate and the related authentic compounds. 1: Control incubate without substrate, 2: adenine, 3: BA, 4: BA incubate, 5: BANO, 6: 2NBA, 7: 2NBA incubate, 8: 2NBANO, 9: 3NBA, 10: 3NBA incubate, 11: 3NBANO, 12: 4NBA, 13: 4NBA incubate, 14: 4NBANO.

N^1-oxide. This strongly implies that the metabolic formation of N^1-oxides from all the studied 9AAs by hamster hepatic microsomes occurred. For further identification of the postulated metabolic N^1-oxides, the UV spectra of these peaks and the authentic N^1-oxides were obtained with the diode array detector (shown in Figure 4) and compared. The spectra provide credable evidence for the identity of the metabolites as the authentic N^1-oxides and the corresponding metabolites possess the same UV spectra. Moreover, comparison of 3-dimensional chromatograms (absorbance-time-wavelength) of metabolic N^1-oxides with authentic N^1-oxides also confirmed their identity (Figure 5). It was apparent that the chromatograms of the metabolically formed N^1-oxides and the corresponding authentic N^1-oxides have the same 3-dimensional shape.

Besides the N^1-oxides and adenine (the N^9-dealkylated metabolite), another minor metabolite, which was found by TLC in the incubates of

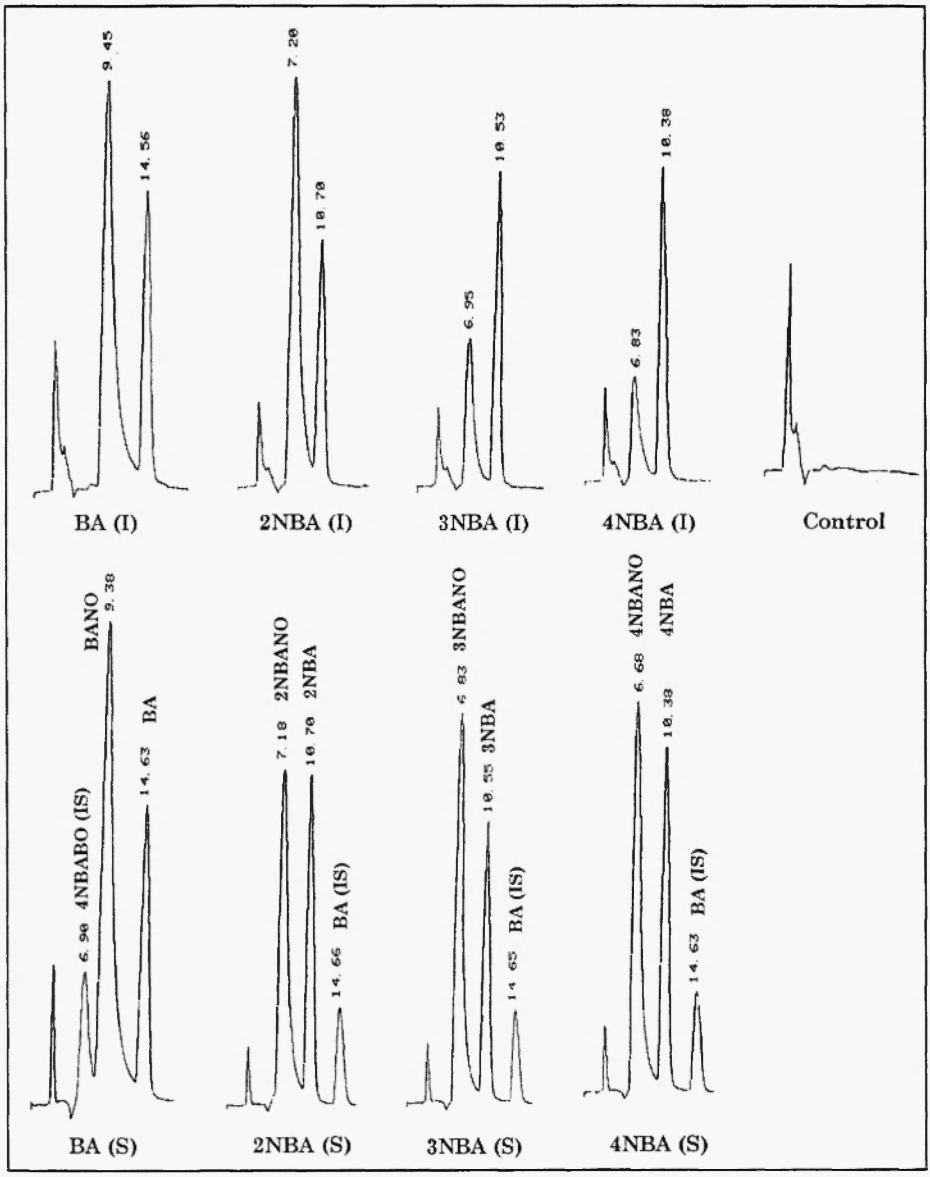


Fig. 3: HPLC chromatograms of (I) hamster hepatic microsomal incubates of BA or NBAs; (S) standards containing authentic BA, NBAs and their N^1-oxides.

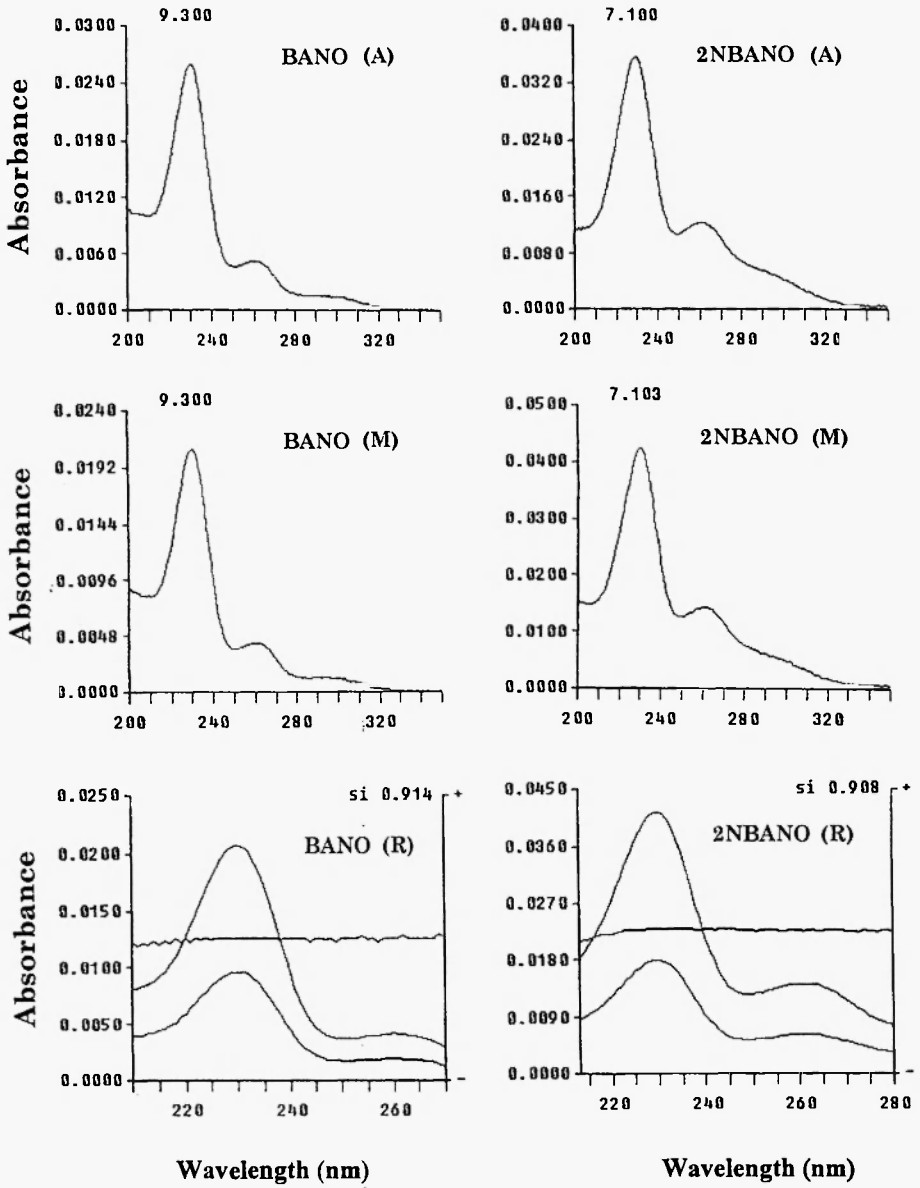


Fig. 4: UV spectra of (A) authentic N^1-oxides and (M) metabolically formed N^1-oxides obtained with a HPLC diode array detector. (R) The ratio of UV spectra of same peak taken at different time slices.

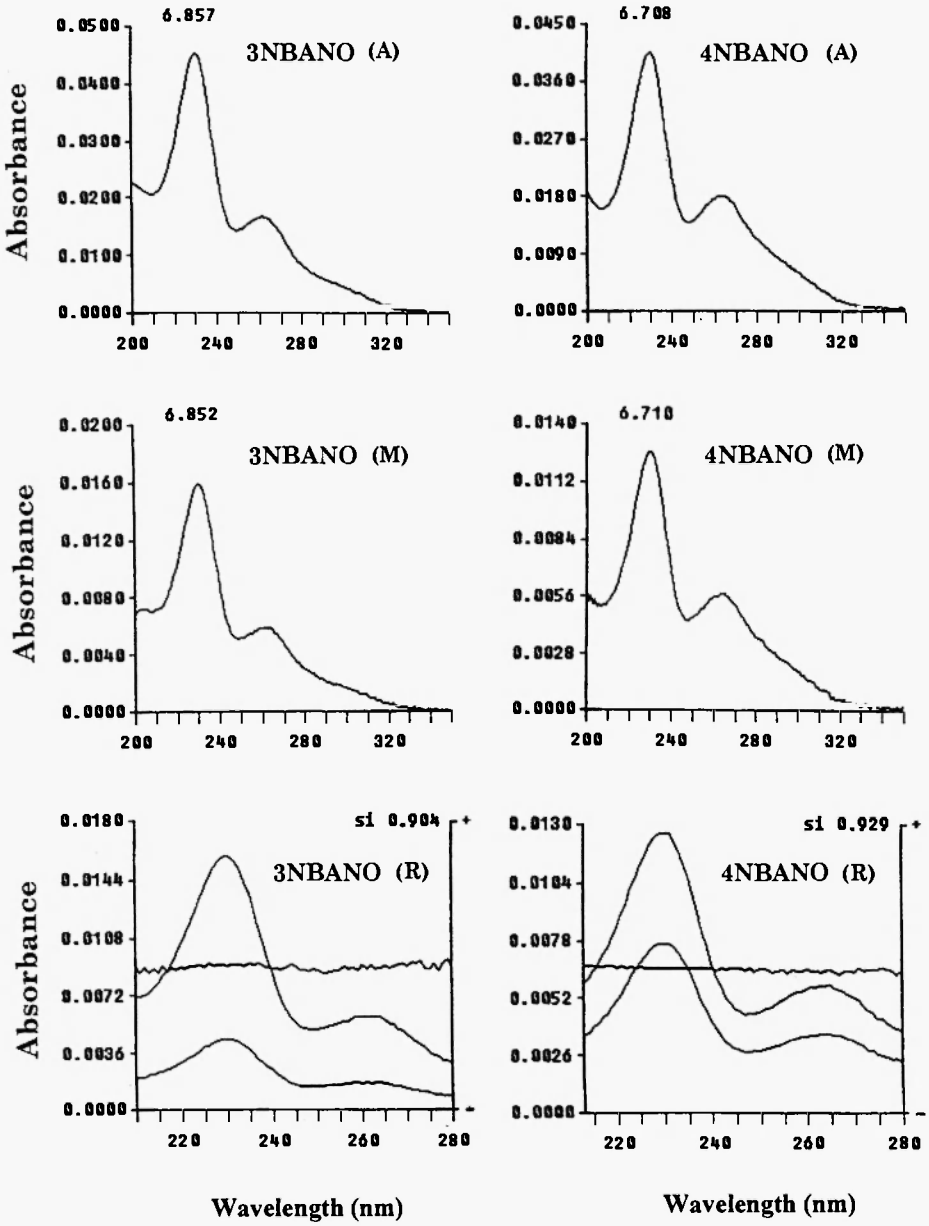


Fig. 4 continued

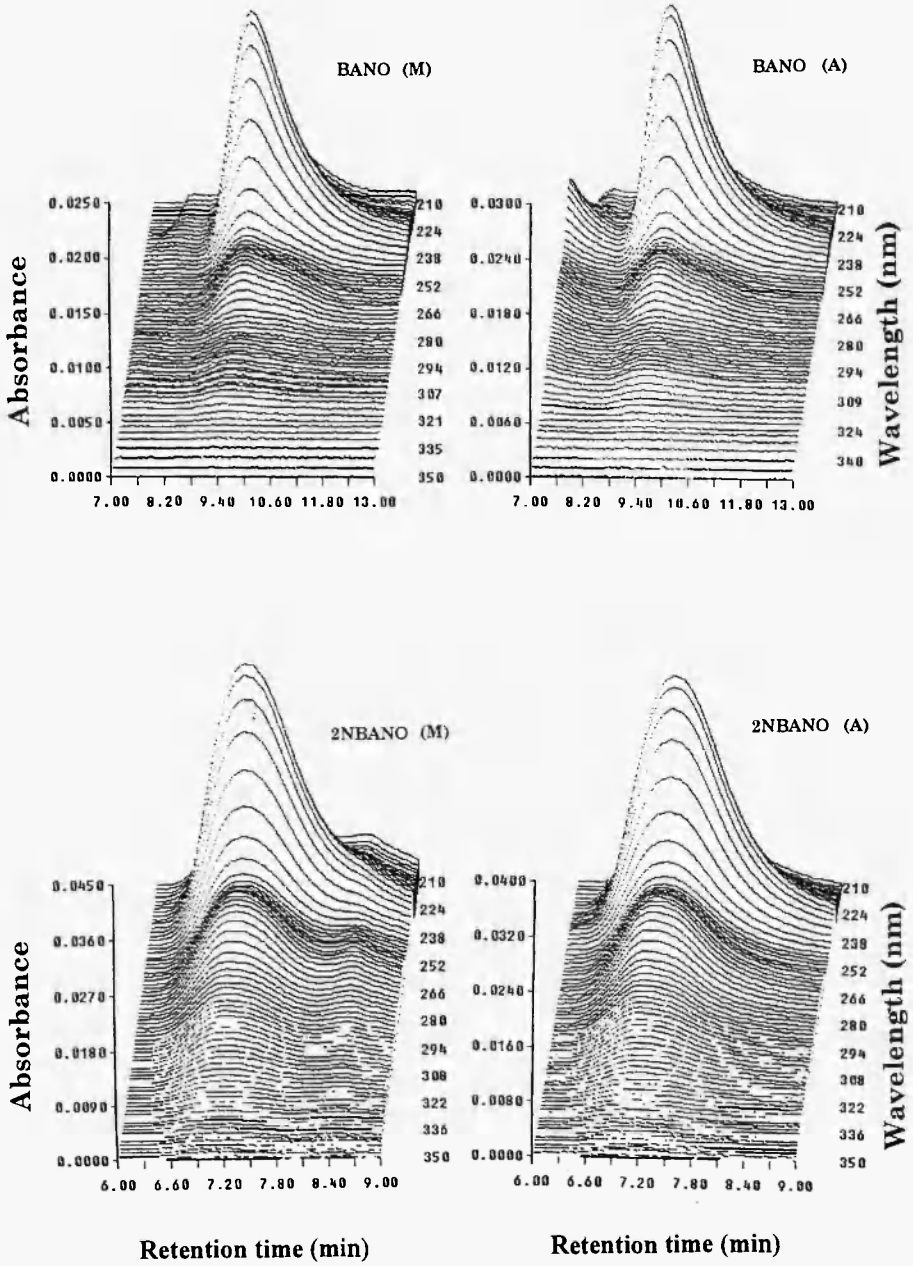


Fig. 5: Three-dimensional chromatograms (absorbance-time-wavelength) of (M) metabolically formed and (A) authentic N^1-oxides, obtained with a HPLC diode array detector.

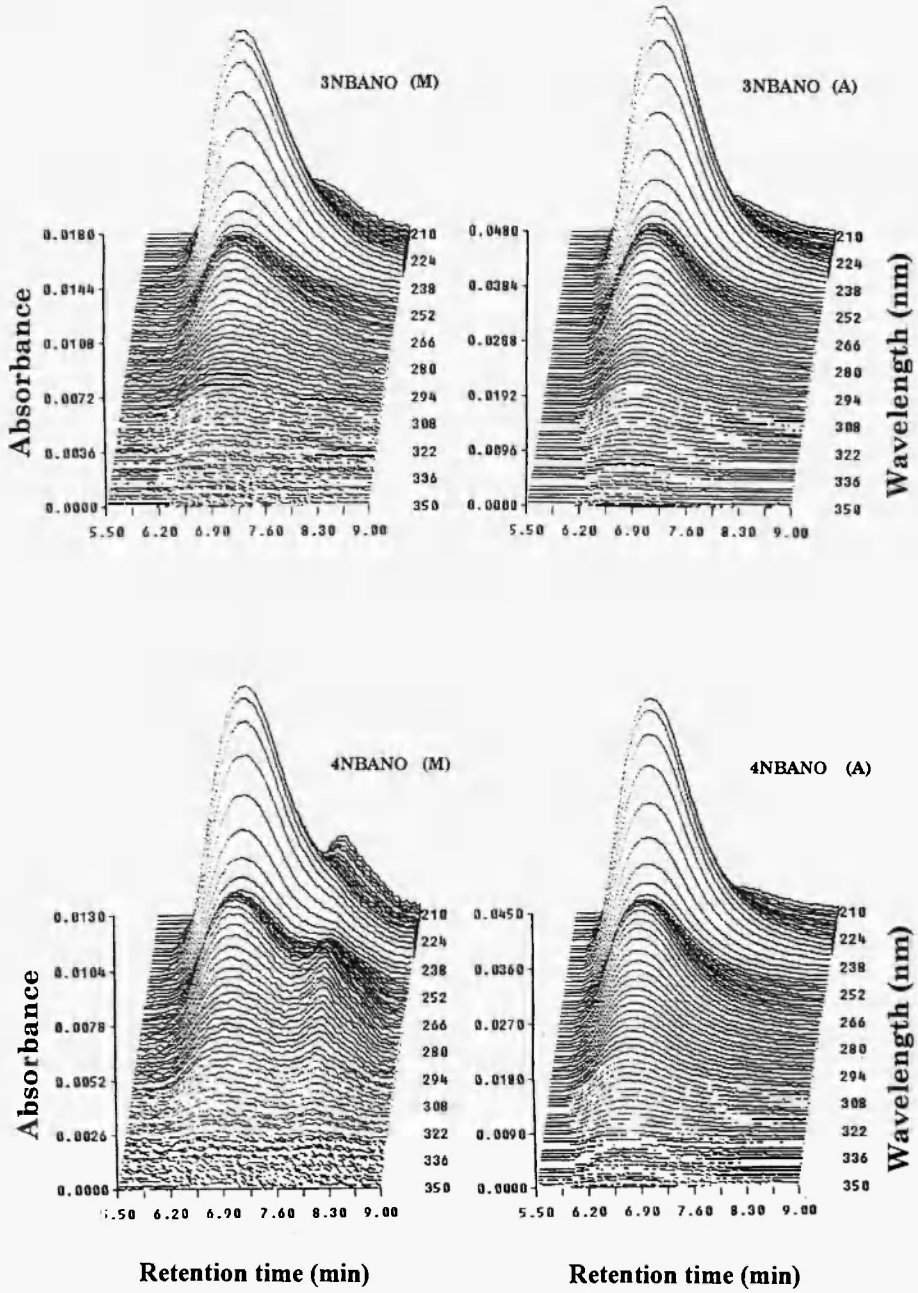


Fig. 5 continued

each NBA, but not BA, was also detectable from the 3-dimensional chromatograms with a retention time about 8 min (Figure 5). Their UV spectra are different from that of the N^1-oxides, i.e. not showing a peak at 233 nm, which is possessed by all N^1-oxides. This means that this minor metabolite does not markedly affect the HPLC analysis of N^1-oxides. However, it was not clear whether these metabolites found in each NBA incubate were the same or isomers (i.e. nitrobenzaldehydes). Further characterization of these metabolites is required before the mechanism of their metabolic formation is understood.

The purity of the chromatographic peak of each metabolic N^1-oxide was checked by comparing the ratio of UV spectra obtained at different time slices of this peak. It was shown that the ratio of UV spectra of every N^1-oxide peak formed a horizontal line ($r>0.9$) (Figure 4). This means the ratio at any recorded wavelength is approximately the same, indicating that the chromatographic peak consists of only one material, i.e. N^1-oxide. However, it should be noted that other potential polar metabolites of 9AAs, e.g. the N^9-dealkylation metabolite, adenine, cannot be observed under the chromatographic conditions employed. The SPE procedure may also not be suitable for the extraction of those very polar metabolites.

UV spectrum of 4NBANO

As additional proof of metabolic N^1-oxidation of the 9AAs studied, some metabolically formed 4NBANO was further purified and analysed by UV scanning (Figure 6). The results show that both metabolically formed 4NBANO and authentic 4NBANO have the same UV spectra.

MS analysis of 4NBANO

Figure 7 shows the MS spectra of both metabolically formed and authentic 4NBANO. The molecular ion peaks of both samples have the same m/z value (286), and the fragmentation profiles of both samples were also similar. This provides additional evidence to confirm that N^1-oxidation is the main metabolic pathway, not only for BA /8-10/ but also for 4NBA.

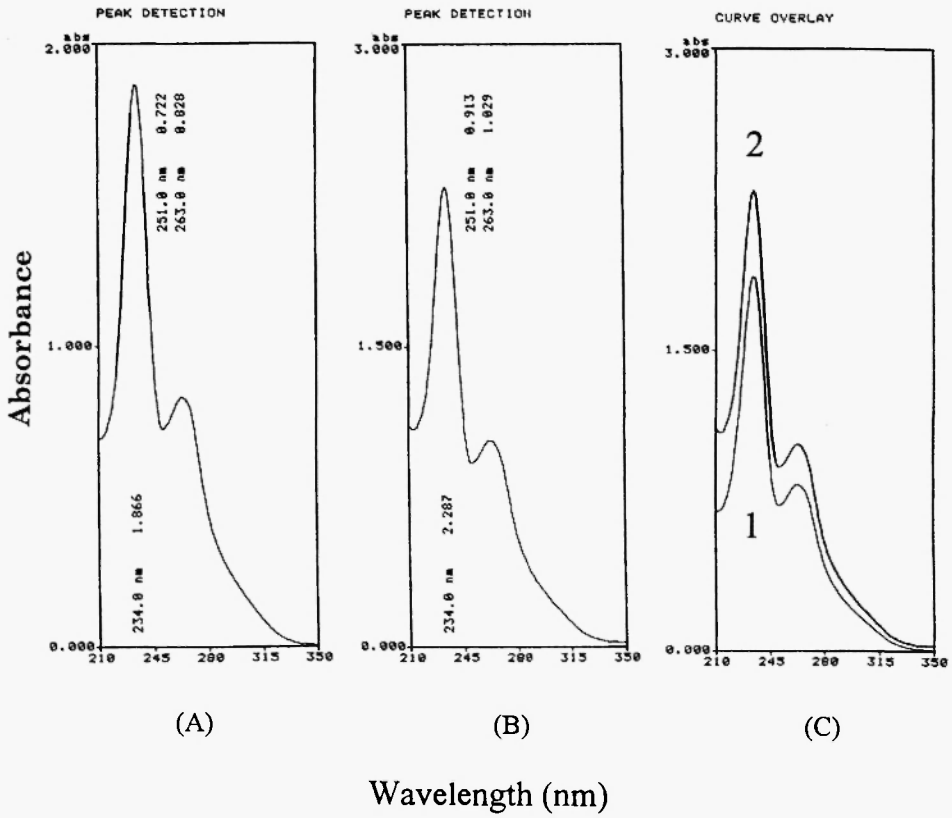


Fig. 6: UV spectra of (A) authentic 4NBANO, (B) purified metabolically formed 4NBANO and (C) the overlay of both spectra: 1 = (A); 2 = (B). Solvent: 0.1 M HCl.

Comparison of N^1-oxidation of BA and NBAs

To evaluate the possible effects of substitution of BA with a nitro group, as well as its substituted position, on N^1-oxidation of NBAs, the metabolic rates of BA and NBAs were compared using different substrate concentrations (see Figure 8). In addition, the amount of N^1-oxides formed during incubation and the amount of unmetabolised substrates present after incubation were also simultaneously determined. The total amount of unknown metabolites (such as the dealkylation product adenine) was obtained by subtracting the amount of formed N^1-oxides plus unmetabolised substrates remaining from the amount of added substrates (Figure 9).

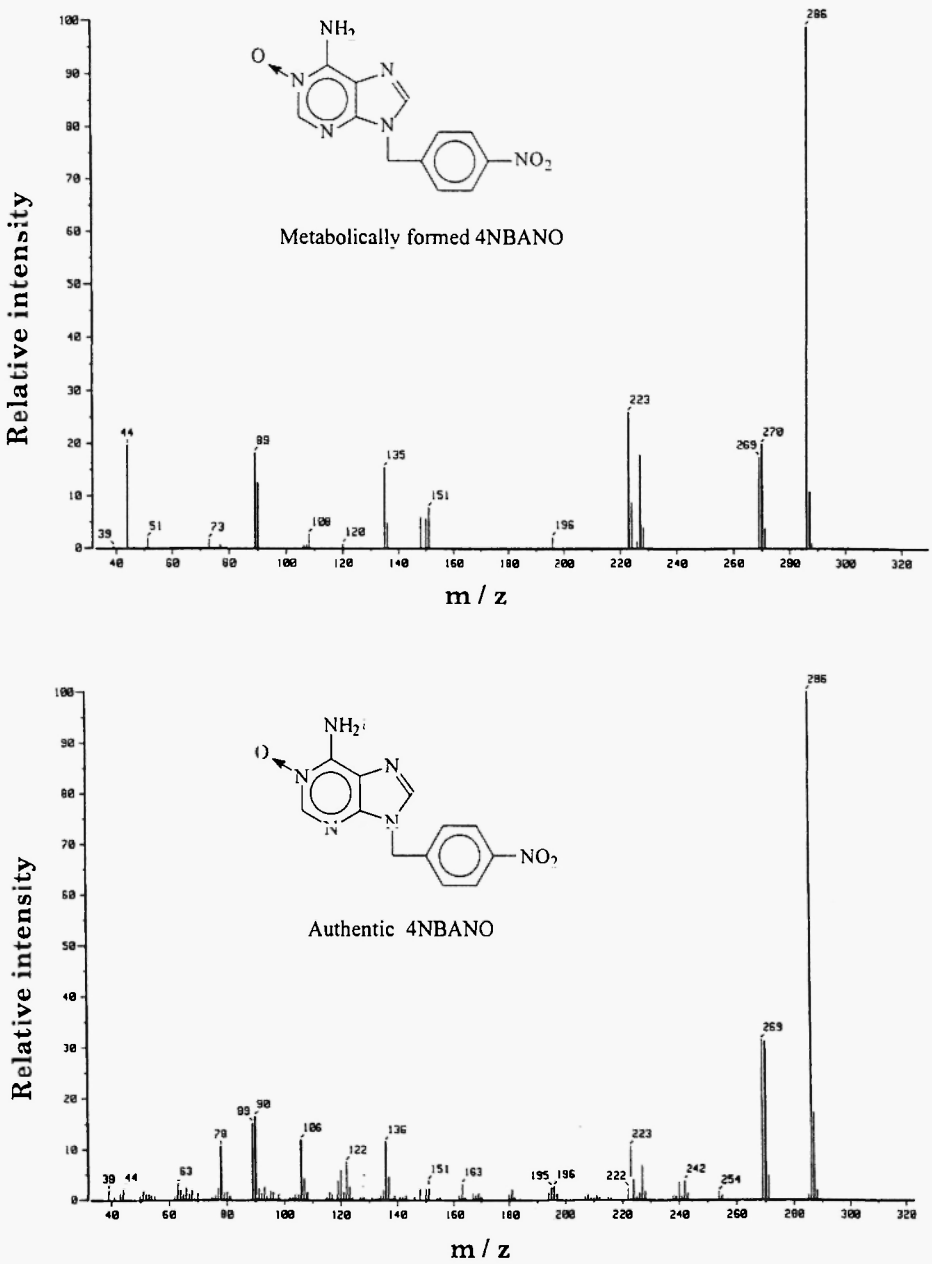


Fig. 7: Mass spectra of metabolically formed and authentic 4NBANO.

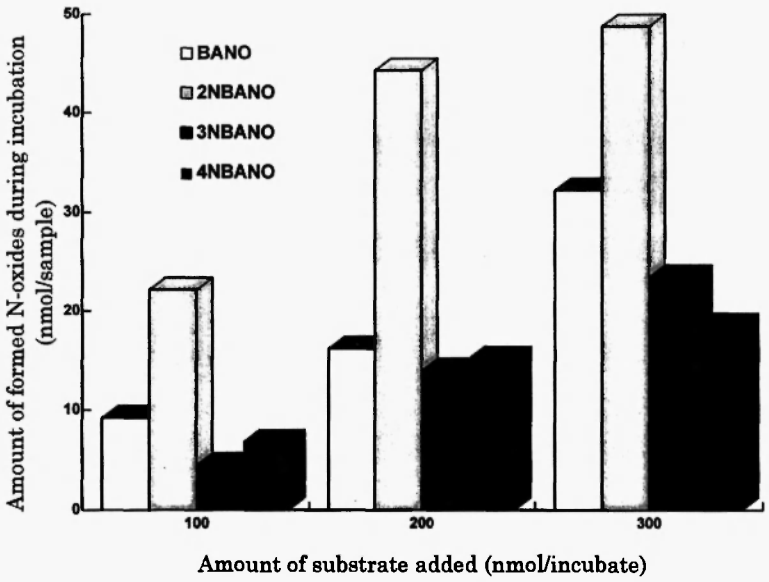


Fig. 8: Comparison of the N^1-oxidation rates of BA and NBAs with different substrate concentration (n = 3).

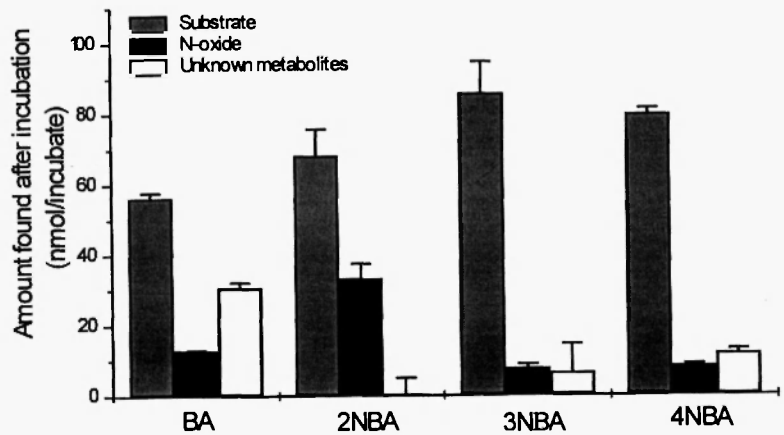


Fig. 9: The metabolism of BA and NBAs by hamster hepatic microsomes (n=3).

The results indicate that: (a) N^1-oxidation was a main pathway of BA and NBA, but not the only one; (b) the order of total metabolic rate was BA>2NBA>4NBA and 3NBA; (c) the order of N^1-oxidation rate was 2NBA>BA>4NBA and 3NBA; (d) the metabolic rate order for the other unknown metabolic pathways was BA>4NBA and

3NBA>2NBA. The results clearly show that introduction of a nitro group into the phenyl ring diminishes the total metabolism compared to BA, which may be due to an effect on lipophilicity as nitro substitution is known to increase the lipophilicity of certain substrates, e.g. aniline, whereas it decreases others, e.g. benzene and toluene.

As the N^1-oxidation rates of 3NBA and 4NBA are slower than that of BA, it can reasonably be deduced that the introduced nitro group affected the binding of substrate to N^1-oxidase(s) of microsomes, compared with BA. It was interesting that 2NBA was metabolised to the N^1-oxide faster than BA, 3NBA and 4NBA, but was the poorest substrate to yield other uncharacterised metabolites among the substrates studied. This phenomenon implies that any negative effect of the nitro group on N^1-oxidation was completely overturned by some other factor in the case of the 2-isomer. Lam *et al.* /9/ indicated that BA had a *trans* conformation with the phenyl ring close to the 8-H proton, hence exposing the N^1-nitrogen atom for oxidation. It is possible that introduction of a nitro group at the 2-position of the phenyl ring may limit its rotation around the two single bonds connecting it to the adenine nitrogen, thereby leading 2NBA to possess a more rigid and more favourable conformation to the N^1-oxidase(s), like 9-phenyladenine /10/, but least favourable for those enzymes responsible for other metabolic pathways. When the nitro group is at either the 3- or 4-position, its distance to the adenine moiety becomes longer and possibly less able to maintain a favourable conformation. However, this deduction needs to be further confirmed and a detailed kinetic analysis of NBAs and certain other 2-substituted benzyladenines is in progress.

CONCLUSION

After BA or NBAs were incubated with hamster hepatic microsomes, the incubates were analysed by HPLC, TLC, UV and MS techniques. According to the data obtained, it was concluded that N^1-oxidation is one of the major metabolic pathways for NBAs by hamster hepatic microsomes, in agreement with those susceptible 9AAs investigated earlier /8-10/. The introduction of a nitro group into the phenyl ring of BA did not prevent the metabolic N^1-oxidation of NBAs *in vitro*. However, BA and the isomeric NBAs were metabolised at different rates, indicating that the nitro group exhibits effects on the

N^1-oxidation of NBAs. It was interesting that the metabolic rates of 3NBA and 4NBA were slower than that of BA, while 2NBA was metabolised faster than BA. Whether the substitution position of the nitro group affects the affinity of the substrate with the N^1-oxidase(s), or whether the isomers are metabolised by different isoenzymes is not clear at present. Further investigations are ongoing which it is hoped will aid our understanding of the processes controlling N^1-oxidation of amino azaheterocycles.

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