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# ANALYSIS OF AZANAPHTHALENES AND THEIR ENZYME OXIDATION PRODUCTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, INFRARED SPECTROSCOPY AND MASS SPECTROMETRY

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## SUMMARY

Adsorption and reversed-phase high-performance liquid chromatography (HPLC) have been successfully used to separate metabolites from the parent heterocycles (isoquinoline, 3-methylisoquinoline, phthalazine, quinazoline, quinoxaline and cinnoline). Retention data are reported. The metabolites, hydroxyazanaphthalenes, which arise as a result of aldehyde oxidase catalysed oxidation, could be extracted in $\mu$g quantities from incubation mixtures by *n*-butanol. Complete identification of the oxidation products was achieved by collecting fractions corresponding to each compound from the HPLC eluate and subjecting these to infrared and mass-spectral analysis.

## INTRODUCTION

Aldehyde oxidase (EC 1.2.3.1), an enzyme present in the 100,000 $\times$ *g* supernatant fraction prepared from liver homogenates, catalyses the oxidation of a number of nitrogen-containing heterocycles[1], including the drug quinine[2], at a significantly fast rate. As part of a general study[3] of the aldehyde oxidase-catalysed oxidation of the basic nuclei contained in mono- and diazanaphthalenes, physical methods for the separation and identification of the oxidation products were examined. The compounds employed in the present study were isoquinoline, phthalazine, quinazoline, quinoxaline and cinnoline. This report describes the combined application of high-performance liquid chromatography (HPLC), infrared (IR) and mass-spectrometry techniques to the isolation and identification of the metabolites that arise as a result of the enzymic oxidation of these compounds.

Although the parent heterocycles can be chromatographed directly by gas–liquid chromatography (GLC), this method is unsatisfactory for the oxidation products, owing to their low volatility. The O-trimethylsilyl derivatives of the hydroxyquinolines can be separated by GLC[4], but analysis of the enzymic incubation mixtures containing such oxidation products by HPLC is more efficient for a number of reasons: (*a*) It is difficult to achieve complete separation of the various silylated hydroxy-

quinolines by GLC on a single column. (*b*) *n*-Butanol is the most convenient solvent for extraction of the hydroxylated heterocycles, and other contaminants are extracted from the incubation mixtures which interfere with the separation of the metabolites. (*c*) The use of HPLC enables the eluate to be collected and used for further characterisation of the separated compounds by UV, IR and mass-spectroscopic techniques.

An HPLC system for the separation of hydroxyquinolines using a reversed-phase partition column has been reported[5], and this system has been adapted for more general use in the present study. Hydroxyquinolines have also been separated by TLC on silica gel, with methanol–chloroform as solvent[6]; this system also has been successfully transferred to HPLC with an adsorption column and methanol–chloroform as eluting solvent.

## EXPERIMENTAL

### *Materials*

Isoquinoline, 1-hydroxyisoquinoline, 3-methylisoquinoline, quinazoline, 4-hydroxyquinazoline, 2,4-dihydroxyquinazoline, quinoxaline, 2-hydroxyquinoxaline, 2,3-dihydroxyquinoxaline, phthalazine and cinnoline were purchased from Aldrich (Milwaukee, Wisc., U.S.A.). 3-Hydroxyisoquinoline[7,8], 1-hydroxy-3-methylisoquinoline[9], 1-hydroxyphthalazine[10], 2-hydroxyquinazoline[11] and 4-hydroxycinnoline[12] were synthesised in this laboratory according to published procedures.

The solvents used for HPLC were of reagent grade, except chloroform, which was redistilled AnalaR grade.

### *Extraction and isolation of oxidation products*

The parent heterocycles were incubated with a 10,000 $\times g$ or 100,000 $\times g$ supernatant fraction prepared from rabbit liver. Each incubation mixture was extracted with four 15-ml portions of *n*-butanol, the combined extracts were evaporated to dryness, and the residue was dissolved in 1 ml of *n*-butanol and retained for analysis.

### *Thin-layer chromatography*

Aliquots (10–20 $\mu$l) of the butanol extract, together with the pertinent authentic azanaphthalenes, were applied to thin layers of silica gel G containing a fluorescent indicator for irradiation at 254 nm. The retention values were measured after the plates had been developed in 5% methanol–chloroform, and the spots were detected by native fluorescence (365 nm) or fluorescence quenching (254 nm). Information gained in this manner was used to determine suitable conditions for separation by HPLC.

### *High-pressure liquid chromatography*

All HPLC work was performed with the use of Waters Chromatograph M-6000 pumps, fitted with a gradient-elution programmer (M-660) and a Cecil 212 variable-wavelength UV monitor. Retention volumes of the heterocyclic derivatives were determine dby using both reverse-phase and adsorption systems. The reversed-phase system consisted of a stainless-steel column (6 ft. $\times$ $\frac{1}{8}$ in. O.D.) packed with

$C_{18}$ Corasil (37–50 $\mu$m), a bonded non-polar stationary phase; either methanol–water or isopropanol–water was used as mobile phase. The adsorption system consisted of a stainless-steel column (10 × ¼ in. O.D.) packed with Bondapak Corasil (10 $\mu$m) as stationary phase and methanol–water as eluting solvent. The flow-rates for both systems were varied from 0.5 ml to 2 ml/min (see Table I). A wavelength of 254 nm was generally used for detection of the peaks.

After the retention volume in a particular solvent system had been ascertained, the eluate (2–3 ml) corresponding to that peak was collected and evaporated to dryness; the residue was dissolved in 10–20 $\mu$l of methanol and used for IR and mass-spectral studies.

*IR spectroscopy*

The IR absorption spectra were determined on a Unicam SP 200 or a Perkin-Elmer 297 instrument fitted with a potassium bromide beam condenser and attenuator. Micro-discs were prepared from the residues collected from HPLC, and the spectra of the metabolites were compared with those of reference compounds treated in a similar manner.

*Mass spectrometry*

Mass spectra were determined on an AEI Model MS 902 mass spectrometer. The samples collected from HPLC were also subjected to mass-spectral analysis with direct-probe insertion.

## RESULTS AND DISCUSSION

### *HPLC*

In order to determine suitable operating conditions, the incubation extracts were initially chromatographed with the use of a linear gradient-elution programme and different combinations of eluting solvents and stationary phases. Complete resolution was sometimes achieved on a single column, but for most of the incubation mixtures it was necessary to use more than one system for complete separation of each of the oxidation products. The retention data for the heterocyclic reference compounds and the enzymic oxidation products are presented in Table I.

The adsorption system was the most convenient one to use when the eluate was being collected, because relatively low temperatures were needed for evaporation of the solvent. Problems were encountered with aqueous solvent systems owing to the tendency of the basic compounds to steam-distil during the evaporation process. However, this could be overcome by the addition of two drops of 2 *N* hydrochloric acid to form the salt of the base, which remained in the vial during evaporation of the solvent.

The reaction of nitrogen heterocycles with aldehyde oxidase normally results in hydroxylation at a position adjacent to a ring nitrogen. As most of the hydroxy-azanaphthalenes had to be synthesised in the laboratory, only compounds with the hydroxyl group in the heterocyclic ring were used as reference compounds in the first instance.

Incubation of isoquinoline with aldehyde oxidase produced a single oxidation product, which was separated easily on the silica column with 2.5% methanol–chloro-

TABLE I

RETENTION DATA FOR AZANAPHTHALENES IN REVERSED-PHASE AND ADSORPTION SYSTEMS

Chromatographic conditions: (i) Reversed-phase system; gradient elution (0–10% methanol–water in 10 min) at 2 ml/min. (ii) Adsorption system; eluent 2.5% methanol–chloroform at 1 ml/min. (iii) Adsorption system; eluent 1% methanol–chloroform at 1 ml/min. (iv) Adsorption system; gradient elution (0–10% methanol–chloroform in 40 min) at 1 ml/min. (v) Reversed-phase system; gradient elution (0–10% isopropanol–water in 20 min) at 1 ml/min. (vi) Reversed-phase system; gradient elution (0–2.5% isopropanol–water in 20 min) at 1.5 ml/min. (vii) Adsorption system; eluent 1% methanol–chloroform at 0.5 ml/min.

| *Compound* | *Retention volume (ml) and conditions* | |
|---|---|---|
| Isoquinoline | — * (i) | 8.5 (iii) |
| 1-Hydroxyisoquinoline | 40.0 (i) | 9.8 (ii) |
| 3-Hydroxyisoquinoline | 36.0 (i) | — **(ii) |
| Oxidation product of isoquinoline | 40.8 (i) | 9.8 (ii) |
| 3-Methylisoquinoline | 7.8 (iii) | |
| 1-Hydroxy-3-methylisoquinoline | 10.0 (iii) | |
| Oxidation product of 3-methylisoquinoline | 10.0 (iii) | |
| Phthalazine | 18.8 (iv) | |
| 1-Hydroxyphthalazine | 14.0 (iv) | |
| 1,4-Dihydroxyphthalazine | — (iv) | |
| Oxidation product of phthalazine | 14.0 (iv) | |
| Quinoxaline | 28.4 (v) | |
| 2-Hydroxyquinoxaline | 21.4 (v) | |
| 2,3-Dihydroxyquinoxaline | 8.0 (v) | |
| Oxidation product A of quinoxaline | 21.4 (v) | |
| Oxidation product B of quinoxaline | 8.0 (v) | |
| Quinazoline | 8.5 (ii) | 63.0 (vi) |
| 4-Hydroxyquinazoline | 14.0 (ii) | 30.6 (vi) |
| 2-Hydroxyquinazoline | 16.4 (ii) | — * (vi) |
| 2,4-Dihydroxyquinazoline | 10.0 (ii) | — * (vi) |
| Oxidation product C of quinazoline | 13.4 (ii) | 30.9 (vi) |
| Oxidation product D of quinazoline | 9.9 (ii) | — * (vi) |
| Cinnoline | 30.4 (v) | 4.1 (vii) |
| 4-Hydroxycinnoline | 19.2 (v) | 13.9 (vii) |
| 3-Hydroxycinnoline | 19.6 (v) | 14.2 (vii) |
| Oxidation product of cinnoline | 19.4 (v) | 13.9 (vii) |

* No peak observed under time of elution.
** Not chromatographed in this system.

form as eluting solvent and could be seen to be 1-hydroxyisoquinoline. 3-Methylisoquinoline and phthalazine were both found to be excellent substrates for aldehyde oxidase and thus produced considerable amounts of metabolites during the incubation period (1 h). Separation of these products was achieved easily by using an adsorption system (0–10% methanol–chloroform in 40 min for phthalazine incubations, and 1% methanol–chloroform for 3-methylisoquinoline incubations). As expected, the more polar hydroxy derivatives of isoquinoline and 3-methylisoquinoline were retained on the column for longer than the parent compounds, although 1-hydroxyphthalazine was eluted from the column before phthalazine itself. This is in agreement with TLC data and is probably due to the solubility of these compounds in organic solvents.

Oxidation of quinoxaline with aldehyde oxidase resulted in the formation of

two products, separation of which could not be accomplished using an adsorption system. Complete resolution was obtained by using a reversed-phase system, as shown in Fig. 1. Quinoxaline was not oxidised as rapidly as either phthalazine or 3-methylisoquinoline, so that the quantities of oxidation products detected in the incubation mixtures were less than those in the reference solutions.

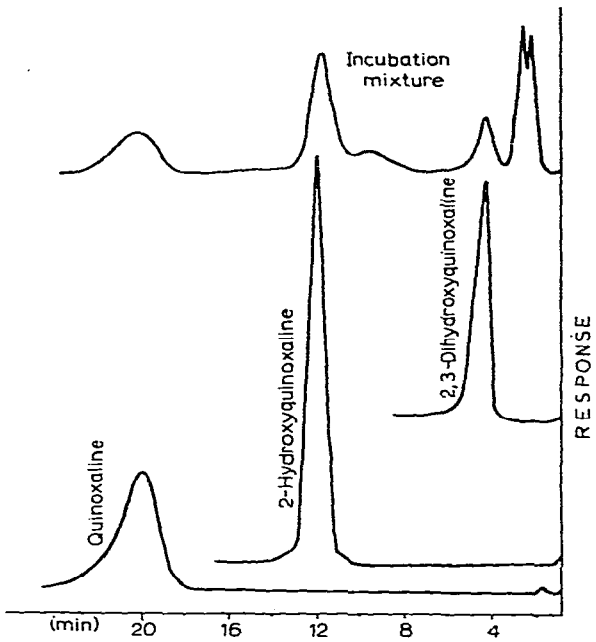



Fig. 1. HPLC traces of quinoxaline, 2-hydroxyquinoxaline, 2,3-dihydroxyquinoxaline and an incubation mixture of quinoxaline. Adsorption system: eluent 0–10% isopropanol–water in 20 min (flow-rate 1 ml/min).

Quinazoline also produced two metabolites as a result of aldehyde oxidase-catalysed oxidation; in this instance, it was necessary to use two systems in order to clearly resolve and collect a sample of each.

The oxidation product of cinnoline appeared as single peak on HPLC, well separated from the parent compound, but with retention characteristics similar to those of both 3-hydroxy- and 4-hydroxy-cinnoline. Unfortunately, as the retention volumes of both these compounds were almost identical in both reversed-phase and adsorption systems, it was not possible to determine which isomer had been produced. However, when submitted to TLC[13], the $R_F$ value and the purple fluorescence at 365 nm of the spot due to the oxidation product indicated that it was, in fact, 4-hydroxycinnoline (3-hydroxycinnoline fluoresces yellow at 365 nm). This was rather surprising, as it was expected that hydroxylation would occur at the 3-position, adjacent to a nitrogen atom.

*IR spectroscopy*

The eluate corresponding to each peak in the incubation mixture was collected and used to prepare micro-discs for IR analysis. The spectrum of each incubation product was compared with that of an authentic specimen of the reference

compound, which had been treated in an identical manner. The IR spectra of these azanaphthalenes are characterised by three main sets of bands in the region 1400–1700 $cm^{-1}$, *i.e.*, isoquinoline exhibits bands at 1600, 1560 and 1650 $cm^{-1}$ and the spectrum of quinazoline shows bands at 1478–1510, 1570 and 1620 $cm^{-1}$. The hydroxy derivatives of the heterocycles show OH bands around 3500 $cm^{-1}$ when substitution occurs in the benzene ring; however, when substitution occurs adjacent to a ring nitrogen atom, this band is absent from the IR spectrum, being replaced by a characteristic band around 1600–1650 due to the lactam tautomer. This is illustrated in Fig. 2, which shows the tautomeric forms of 1- and 3-hydroxyisoquinolines. Also in Fig. 2 are the IR spectra of these compounds compared with that of the oxidation product of isoquinoline.

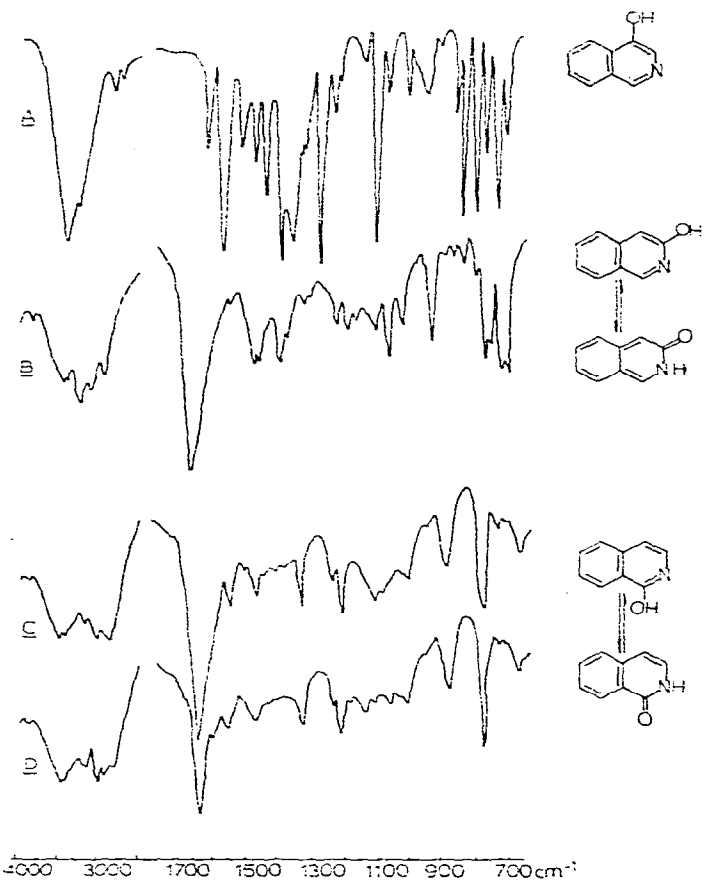



Fig. 2. IR spectra of hydroxyisoquinolines: A, 4-Hydroxyisoquinoline; B, 3-hydroxyisoquinoline; C, 1-hydroxyisoquinoline; and D, metabolite of isoquinoline.

All the oxidation products of the incubations exhibited C=O bands around 1690 $cm^{-1}$, indicating that each compound was in the lactam form, *i.e.*, substituted either *ortho* or *para* to a ring nitrogen.

*Mass spectra*

A further consequence of the amide structure of the oxidation products is the loss of a CO fragment in their mass spectra, a competing reaction with the usual fragmentation pattern observed with the parent compounds. Isoquinoline and quinoxaline fragment by the loss of one or two molecules of HCN, whereas phthalazine can also lose a molecule of $N_2$. The dominant pathway in the fragmentation of cinnoline is the loss of $N_2$ followed by a $C_2H_2$ fragment. The relative contributions of the above pathways lead to characteristic splitting patterns of the hydroxylation products, and the variations that occur can serve as useful diagnostic aids when identical molecular peaks are obtained from two or more possible products. This is illustrated by the results of the isoquinoline incubations (see Fig. 3). The fragmentation pattern of the metabolite is compared with those of 1-hydroxy- and 3-hydroxyisoquinoline: all three show a molecular-ion peak at *m/e* 145. The most abundant fragment in the spectrum of 3-hydroxyisoquinoline is at *m/e* 117 (M—CO), whereas both 1-hydroxyisoquinoline and the metabolite fragment by initial loss of HCN to give an ion peak at *m/e* 118. This is probably due to the direct attachment of the carbonyl-carbon to the benzene ring in the metabolite, thus hindering the fission of the bond that is the key step to produce the M—CO ion[14].

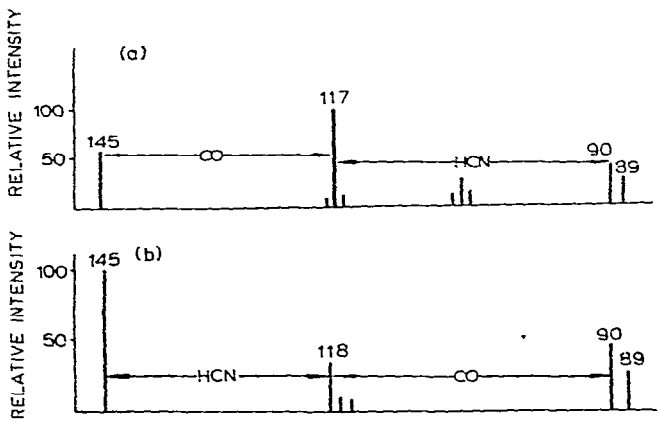



Fig. 3. Mass spectra of 3-hydroxyisoquinoline (a) and 1-hydroxyisoquinoline and metabolite (b).

Thus, it would appear from mass-spectral and other data that 1-hydroxyisoquinoline is produced as a result of aldehyde oxidase-catalysed oxidation of isoquinoline.

The mass-spectral data for the oxidation products and the corresponding reference compounds are presented in Table II. The mass spectrum of the metabolite of 8-methylisoquinoline incubations was identical to that of 1-hydroxy-3-methylisoquinoline. The spectra showed a molecular-ion peak at *m/e* 159 and a strong [M— (H + CO)] ion at *m/e* 130. This fragmentation is probably hindered in the same way as that of 1-hydroxyisoquinoline, because an ion at *m/e* 118 ($M-CH_3CN$) is also present; this ion is not apparent in the spectrum of the isomeric 3-hydroxy-1-methylisoquinoline. Both phthalazine and quinoxaline have planes of symmetry, thus, for each, only one position of substitution in the heterocyclic ring is possible, i.e., 1-hydroxyphthalazine and 2-hydroxyquinoxaline. These compounds were duly

TABLE II

MASS-SPECTRAL DATA FOR THE AZANAPHTHALENES

The *m/e* values are shown, with the relative abundances in parentheses.

| *Compound* | $M^+$ | *Other abundant ions* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 1-Hydroxyisoquinoline | 145 | 118 | 117 | 116 | 90 | 89 | | | |
| | (100) | (30) | (6) | (5) | (45) | (25) | | | |
| 3-Hydroxyisoquinoline | 145 | 118 | 117 | 116 | 105 | 104 | 103 | 90 | 89 |
| | (89) | (17) | (100) | (17) | (11) | (28) | (11) | (50) | (42) |
| Metabolite | 145 | 118 | 117 | 116 | 90 | 89 | | | |
| | (100) | (30) | (6) | (6) | (45) | (25) | | | |
| 1-Hydroxy-3-methylisoquinoline | 159 | 128 | 129 | 130 | 118 | 117 | 116 | 115 | 103 |
| | (100) | (8) | (15) | (65) | (17) | (3) | (8) | (12) | (12) |
| Metabolite | 159 | 128 | 129 | 130 | 118 | 117 | 116 | 115 | 103 |
| | (100) | (7) | (13) | (65) | (17) | (2) | (7) | (12) | (12) |
| 1-Hydroxyphthalazine | 146 | 128 | 118 | 117 | 91 | 90 | 89 | 65 | 64 |
| | (100) | (7) | (14) | (8) | (11) | (14) | (42) | (8) | (18) |
| Metabolite | 146 | 128 | 118 | 117 | 91 | 90 | 89 | 65 | 64 |
| | (100) | (8) | (15) | (8) | (12) | (14) | (42) | (9) | (19) |
| 2-Hydroxyquinoxaline | 146 | 119 | 118 | 92 | 91 | 64 | 63 | | |
| | (100) | (4) | (64) | (3) | (27) | (18) | (18) | | |
| Metabolite | 146 | 119 | 118 | 92 | 91 | 64 | 63 | | |
| | (100) | (4) | (64) | (3) | (27) | (18) | (18) | | |
| 2,3-Dihydroxyquinoxaline | 162 | 135 | 134 | 133 | 106 | 105 | 98 | 97 | 95 |
| | (100) | (5) | (35) | (5) | (27) | (14) | (10) | (11) | (12) |
| Metabolite | 162 | 135 | 134 | 133 | 106 | 105 | 98 | 97 | 95 |
| | (100) | (5) | (35) | (5) | (27) | (14) | (10) | (11) | (12) |
| 2-Hydroxyquinazoline | 146 | 145 | 119 | 118 | 117 | 92 | 91 | 90 | |
| | (100) | (14) | (11) | (74) | (7) | (5) | (19) | (13) | |
| 4-Hydroxyquinazoline | 146 | 145 | 119 | 118 | 117 | 92 | 91 | 90 | |
| | (100) | (25) | (17) | (23) | (7) | (14) | (14) | (16) | |
| Metabolite | 146 | 145 | 119 | 118 | 117 | 92 | 91 | 90 | |
| | (100) | (25) | (17) | (25) | (9) | (12) | (12) | (15) | |
| 2,4-Dihydroxyquinazoline | 162 | 120 | 119 | 93 | 92 | 91 | 90 | 64 | 63 |
| | (100) | (6) | (84) | (4) | (60) | (10) | (7) | (17) | (13) |
| Metabolite | 162 | 120 | 119 | 93 | 92 | 91 | 90 | 64 | 63 |
| | (100) | (6) | (85) | (4) | (60) | (10) | (7) | (17) | (13) |
| 3-Hydroxycinnoline | 146 | 119 | 118 | 91 | 90 | 89 | 65 | 64 | 63 |
| | (100) | (5) | (43) | (10) | (30) | (34) | (6) | (22) | (10) |
| 4-Hydroxycinnoline | 146 | 119 | 118 | 92 | 91 | 90 | 65 | 64 | 63 |
| | (100) | (50) | (5) | (58) | (25) | (14) | (7) | (29) | (27) |
| Metabolite | 146 | 119 | 118 | 92 | 91 | 90 | 65 | 64 | 63 |
| | (100) | (52) | (5) | (50) | (24) | (12) | (4) | (30) | (29) |

identified as metabolites of the parent heterocycles, along with 2,3-dihydroxyquinoxaline from quinoxaline (molecular-ion peak at *m/e* 162).

Incubation of quinazoline produced two metabolites, one of which was easily identified as 2,4-dihydroxyquinazoline (molecular-ion peak at *m/e* 162). The second product was a monohydroxyquinazoline, but showed a mass spectrum identical to that of 4-hydroxyquinazoline. Whereas both 4-hydroxy- and 2-hydroxyquinazoline fragment by the initial loss of a neutral CO molecule to give an ion peak at *m/e* 118, 4-hydroxyquinazoline can also lose two successive molecules of HCN, giving

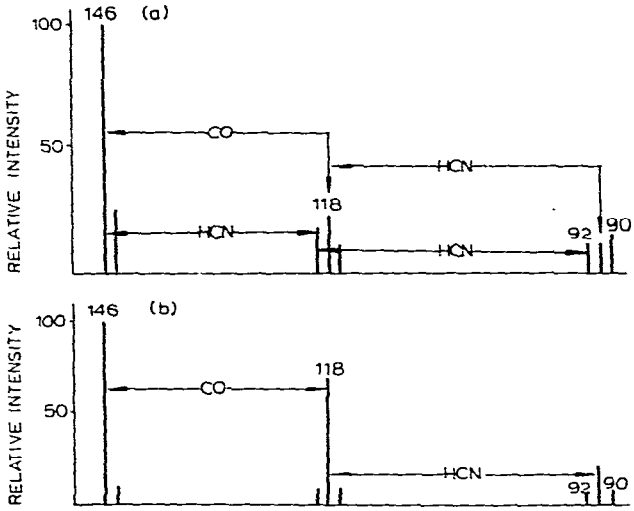
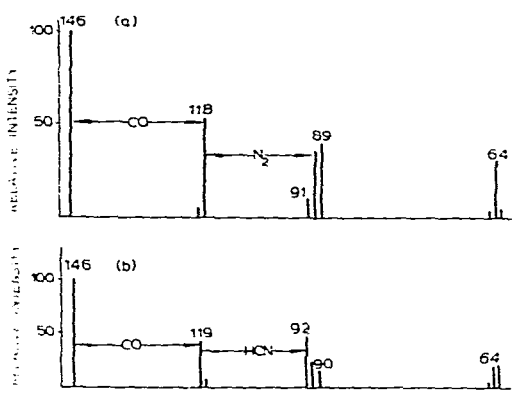



Fig. 4. Mass spectra of 4-hydroxyquinazoline and metabolite (a) and 2-hydroxyquinazoline (b).

ions at *m/e* 119 and 92 (ref. 15). The ratios between peaks at *m/e* 119 and 118 and at *m/e* 92 and 91 thus differ in these spectra (Fig. 4).

The expected product resulting from the incubation of cinnoline with aldehyde oxidase was 3-hydroxycinnoline, *i.e.*, where substitution occurs adjacent to a nitrogen atom. However, the chromatographic and IR data did not confirm this assumption. The mass spectrum shown in Fig. 5 confirms the conclusion that the product was, in fact, 4-hydroxycinnoline. 3-Hydroxycinnoline fragments by the loss of an even-numbered fragment (CO) to yield an ion peak at *m/e* 118, followed by successive loss of $N_2$ and H to produce an ion peak a t*m/e* 89. In contrast, 4-hydroxycinnoline loses two molecules of HCN to give ion fragments at *m/e* 118 and 92: only then is a molecule of CO lost to form an ion peak at *m/e* 64 (ref. 16). The metabolite shows a fragmentation pathway identical with that of 4-hydroxycinnoline.




ig. 5. Mass spectra of 3-hydroxycinnoline (a) and 4-hydroxycinnoline and metabolite (b)

## CONCLUSIONS

The hydroxyazanaphthalenes formed as result of the action of aldehyde oxidase on the unsubstituted heterocycles do not show phenolic properties and cannot be identified by TLC with the use of the usual spray reagents for the identification of phenols. A successful method of separating the metabolites from the parent heterocycles has been devised by combining two HPLC systems (adsorption and reversed-phase); this permits resolution of the metabolites to be carried out. This method has also been combined with IR and mass-spectral analysis to achieve absolute identification of each oxidation product. It is thus possible to separate and identify cyclic amides of this type after extraction from biological fluids even when only $\mu$g quantities are available.

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