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METABOLIC ACTIVATION OF OLEFINS

CONVERSION OF 1-OCTENE TO A PUTATIVE REACTIVE INTERMEDIATE 1-OCTEN-3-ONE: AN ALTERNATIVE PATHWAY TO EPOXIDATION

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Abstract—1. The enzymic activation of a model olefin oct-1-ene was studied in rat liver microsomal systems *in vitro*.

2. An active metabolite was trapped using *N*-acetylcysteine and identified by means of capillary GLC/mass spectrometry and 360 MH_z ^1H NMR as S-3-oxo-octyl-*N*-acetylcysteine. A two step pathway for the formation of this adduct was proposed involving first the production of oct-1-en-3-ol by NADPH dependent mixed function oxidases and secondly a NADP or NAD linked oxidation, independent of cytochrome P-450, to yield the putative reactive intermediate oct-1-en-3-one.

3. Under physiological conditions, oct-1-en-3-one, prepared chemically, reacted non-enzymically with *N*-acetylcysteine with a t_i of about 6 sec.

4. Enzymes catalysing the NADP-dependent oxidation of octen-3-ol were present in microsomal preparations from a number of organs apart from the liver, those from adrenal and intestinal epithelia showing the next highest levels of activity. Unlike the activation of octene, rates of hepatic activation of octen-3-ol were not induced by pretreatment of rats with phenobarbitone or 3-methylcholanthrene.

5. Using 1-octene as the substrate, comparisons were made of alternative routes of hepatic metabolism activation. Relative to the rate of formation of the 3-oxo intermediate trapped with *N*-acetylcysteine, epoxidation of octene and subsequent hydrolysis to octane-1,2-diol was over 40 times more rapid. The rate of formation of a presumptive oxirane precursor trapped with the haem of cytochrome P-450 as *N*-(2-hydroxyoctyl)protoporphyrin IX was about 17-fold lower.

Olefinic compounds are generally considered to undergo oxidation by the cytochrome P-450 dependent mixed function oxidases to epoxides, the electrophilic species responsible for covalent binding to DNA and to proteins [1–3]. Following incubation of 1-octene with rat liver preparations *in vitro*, the 1,2 epoxide does not accumulate but is rapidly hydrolysed to octane-1,2-diol [4]. In addition to these reactions, after metabolic activation, many terminally substituted olefinic compounds can alkylate the haem of cytochrome P-450 [5, 6]. In the case of octene, the adduct has been identified as *N*-(2-hydroxyoctyl)protoporphyrin IX [7]. Octene epoxide is not involved in this reaction. Results suggest that abstraction of electrons from the olefinic π bond yields an acyclic carbocation as the reactive species [7].

We have recently demonstrated that certain terminally substituted acetylenes such as 1-octyne can undergo metabolic activation by an alternative cytochrome P-450 mediated pathway giving rise to oct-1-yn-3-one as the putative reactive species [8]. In this paper evidence is presented for an analogous 3-oxo pathway in the metabolic activation of octene. A comparison is made between this and the more classical epoxidative routes of metabolism.

MATERIALS AND METHODS

Chemicals

Oct-1-ene (97% pure) oct-1-en-3-ol, hex-1-en-3-ol and octane-1,2-diol were from the Aldrich Chemical Co. Ltd. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NAD, NADP and *N*-acetyl-L-cysteine were from the Sigma Chemical Co. Oct-1-en-3-one was prepared by the CrO_3 oxidation of oct-1-en-3-ol as described by Tursh *et al.* [9]. Capillary GLC showed the final product to be >98% purity. Octane-1,2-oxide was synthesised from 1-octene according to the procedure of Ortiz de Montellano *et al.* [7]. $^{65}ZnCl_2$ (sp. radioactivity 490 Ci/mol) was from the Radiochemical Centre, Amersham.

Preparation of S-*3-oxo-octyl*-L-N-*acetylcysteine*

To an ice-cold solution of L-*N*-acetylcysteine (0.82 g, 5 mmol) in 10 ml 0.1 M Tris/NaOH buffer, pH 8.0 was added octen-3-one (0.62 g, 5 mmol) in methanol (10 ml) with stirring. After 5 min at RT, the mixture was adjusted to pH 1.5 with c.HCl and extracted (×3) into diethyl ether (20 ml). The combined either extracts were washed with water, dried (anhyd.Na_2SO_4) and concentrated to yield 0.8 g (72%) of crude product. This was recrystallised from diethyl ether light petroleum (b.p. 40–60°) 1:4 v/v) to give *S*-3-oxo-octyl-*N*-acetylcysteine (m.p. 86°). ^1H NMR (60 MH_z, Perkin Elmer R12B, tetramethylsilane internal standard) (ppm), [^2H]chloroform; 9.28 (1H,s,—CO_2H); 6.88, (1H,d,—N*H*, J = $6H_z$); 4.80 (1H,m,—C*H*COO); 3.05 (2H,d,—SCH_2,J = $6H_z$); 2.75 (4H,m,—S(CH_2)$_2$); 2.42 (2H,t,—CH_2CO); 2.10 (3H,s,—CH_3CO); 1.38 (6H,m,(—CH_2)$_3$; 0.90 (3H,t,—CH_3(CH$_2$)$_2$).

Found C,54.0%; H,8.2%; N,4.9%; S,11.1%. Calc. for C_{13} H_{23} N O_4 S: C,54.0%; H,8.0%; N,4.8%, S,11.1%. Mass spectral studies (CI or EI) failed to show a molecular ion. However, following derivatization using established procedures to form the methyl ester, chemical ionization mass spectrometry gave a protonated molecular ion m/z 304 (21.8% of the base peak) and a fragmentation pattern consistent with the proposed structure.

S-3-oxo-hexyl-*N*-acetylcysteine (m.p. 88°), used as an internal standard was prepared from hex-1-en-3-one and L-*N*-acetylcysteine in an analogous manner. Found: C,50.4%; H,7.4%, N,5.3%; S,12.1%. Calc. for C_{11} H_{19} N O_4 S: C,50.6%; H,7.3%; N,5.4%; S,12.3%. Chemical ionisation mass spectrometry of this compound derivatized as the methyl ester gave a protonated molecular ion m/z 276 (29.5% of base peak). Data following mass spectrometry fragmentation and 60 MH_z NMR (not shown) were consistent with the structure.

Animals

Male Fischer F334/N rats (150–170 g) were used. In some instances, hepatic mixed function oxidase activities were induced by pretreatment of the animals with: phenobarbitone, 0.1% (w/v) in the drinking water for 7 days; 3-methylcholanthrene 20 mg/kg (20 mg/ml in trioctanoyl glycerol) intraperitoneally once a day for 3 days; 2-(*p*-chlorophenoxy)-2-methyl propionic acid (Clofibrate) 200 mg/kg, i.p. (100 mg/ml in trioctanoin) once daily for 4 days. Animals were killed 24 hr after the last dose. In one experiment, phenobarbitone pretreated rats were given 1-octene, 100 mg/kg, i.p. (100 mg/ml in trioctanoyl glycerol) and killed 2 hr after dosing.

Preparation of microsomal fractions

Washed microsomes from liver or from other organs were prepared using the procedures previously described for liver [10]. The final microsomal pellet was made up in 0.25 M phosphate buffer pH 7.4 containing 30% v/v glycerol. Intestinal microsomes were prepared from the duodenal segment. The lumen was perfused with ice-cold saline and the tissue sectioned longitudinally. Epithelial cells were lightly scraped off using a microscope slide and homogenised in 0.25 M sucrose in the usual manner. Protein concentrations were determined using the Lowry procedure [11] with a bovine serum albumin standard.

Microsomal conversion of 1-octene to octane-1,2-diol

Reaction mixtures of 2 ml volume were in 0.1 M potassium phosphate buffer pH 7.4 containing: $MgCl_2$ (2 mM), EDTA (0.5 mM); NADP (0.5 mM); glucose-6-phosphate (5 mM); glucose-6-phosphate dehydrogenase, 2 units and microsomal fraction (3–4 mg of protein). Reactions were started with octene (10 μl in dimethyl sulphoxide) to give a final concentration of 1 mM. After incubation at 37° in a shaking water bath reactions were stopped by taking 0.2 ml of the mixture and adding it to 40 μl 0.1 M NaOH in tubes on ice. After the addition of internal standard (octane-1,8-diol 40 nmoles), the mixture was extracted with *n*-pentane (200 μl) and the pentane extracts discarded. The tubes were then extracted (×2) with ethyl acetate (0.2 ml). The combined ethyl acetate phases were cautiously concentrated to dryness at room temperature. Following the addition of ethyl acetate (0.2 ml), the reaction products were derivatized with heptafluorobutyric anhydride (10 μl) by heating in a sealed vial for 15 min at 110°. The derivatized products were made up in ethyl acetate (1 ml), washed with 0.1 M phosphate buffer, pH 7.4 (0.2 ml) and subjected to capillary GLC using electron capture as described below. Recovery of authentic octane-1,2-diol (25 nmoles) added to microsomal incubation mixtures was 75.7 ± 1.9% (mean ± S.E., 4 experiments). The sensitivity was adequate to measure down to levels below 0.5 nmoles/ml. In some experiments, octane-1,2-oxide and octane-1,2-diol were estimated in ethyl acetate extracts without derivatization using capillary GLC with flame ionization detection. Such procedures permitted the estimation of concentrations down to about 10 nmoles/ml.

Formation of S-3-oxo-octyl-N-acetylcysteine by microsomal systems

(a) *From 1-octene.* Reaction mixtures of 2 ml were as described above except they also contained *N*-acetylcysteine (10 mM). Reactions were stopped after various times with an equal volume of ice-cold 1 M HCl. Following the addition of *S*-3-oxo-hexyl-*N*-acetylcysteine internal standard (2 nmoles) and precipitation of protein (3000 *g* for 20 min), the supernatant was extracted with diethyl ether (3 × 2 ml). The combined ether extracts were dried (anhyd-Na_2SO_4) and concentrated to dryness. The residue was dissolved in methanol (0.2 ml) and derivatized with diazomethane in ether (0.1 ml). After 15 min at RT, the solvent was removed under N_2 at 30° and the residue taken up in cyclohexane (1 ml). This was washed with water (0.2 ml) and the cyclohexane phase concentrated to dryness under N_2. The derivatized products were dissolved in ethyl acetate and subjected to capillary GLC/mass spectrometry with selective ion monitoring as described below. The extraction efficiency of authentic *S*-3-oxo-octyl-*N*-acetylcysteine (25 nmoles) added to microsomal incubation mixtures was 45.2 ± 0.6% (mean ± S.E., 4 determinations). Sensitivity by GLC (nitrogen/phosphorus detector) was adequate to measure down to levels below 5 nmoles/ml. For greater sensitivity using GLC/mass spectrometry with selective ion monitoring levels of *S*-3-oxo-octyl-*N*-acetylcysteine methyl ester below 0.1 nmoles/ml could be detected.

(b) *From 1-octen-3-ol.* Reaction mixtures were as described in (a) except octen-3-ol (1 mM) replaced octene and the NADPH generating system was omitted. Cofactors were NADP or NAD (2 mM). In some instances flasks were purged with N_2 or CO/O_2 8:2 (v/v) (250 ml/min) for 5 min prior to and during the course of the reaction. Work up procedures were the same as described above except that quantitation of the reaction products was achieved using capillary GLC in conjunction with a nitrogen phosphorus detector as described below.

Gas chromatograph

Gas chromatography was carried out using a Carlo Erba Fractovap 4160 instrument equipped with a flame ionization, nitrogen-phosphorus (NPD-40) or a ^{63}Ni electron capture (HT-25) detection system.

For the resolution and detection of derivatized octene-*N*-acetylcysteine adducts using gas chromatography with nitrogen phosphorus detection, a SE 52 glass capillary column (20 m × 0.3 mm) was employed. The column was operated at 100° for 3 min then programmed at 30°/min to 280°. The He carrier gas flow rate was 2 ml/min and the N_2 detector purge flow rate was 20 ml/min. Samples were injected by using a split injection system (10:1 split ratio).

In some initial experiments underivatized octane-1,2-diol and octene epoxide were estimated using GLC with an OV-1701 coated glass column (20 m × 0.3 mm) and a flame ionization detector. The initial column temperature of 60° was held for 3 min then increased to 280° at 30°/min. Due to the limited sensitivity of this procedure, further work used derivatized samples in conjunction with an electron capture detector. Quantitation of octane-1,2-diol as the heptafluorobutyryl derivative was made using a CP wax 57 fused silica capillary column (20 m × 0.25 mm). This was maintained at 80° for 1 min then programmed at 5°/min to 110° using He as carrier gas at a flow rate of 2 ml/min. The electron capture detector was operated at 300° in a constant current mode with a pulse width of 5 μsec. Nitrogen was employed as purge gas at a flow rate of 40 ml/min. Quantitation of octane-1,2-diol was made by reference to a standard calibration curve constructed with each batch of samples analysed. The standard curve was obtained from the analysis of blank microsomal incubation mixtures to which had been added 40 nmoles of the internal standard (octane-1,8-diol) and various amounts of octane-1,2-diol (2–100 nmoles).

Gas chromatography—mass spectrometry

The mass spectrometer used was a 70-70 VG Analytical double focussing instrument interfaced to a Carlo Erba HRGC 5160 Mega Series Gas chromatograph. The gas chromatographic separation for the analysis of derivatized octene-*N*-acetylcysteine by selective ion monitoring was made using a SE 52 fused silica capillary (20 m × 0.3 mm) coated with SE 52 or OV 1701 stationary phase. Samples were introduced to the column with a falling needle solid injector. The temperature programme for the column was 100° for 1 min followed by a 50°/min temperature rise to 280°. The mass spectrometer was operated with a source temperature of 220° and an accelerating voltage of 4 kv. For electron impact (EI) operation the electron energy was 70 eV and the trap current 200 μA; for chemical ionisation (CI) the electron energy was 50 eV and the emission current 500 μA. Iso-butane was used as reagent gas for CI.

Mass spectra were recorded at a scan speed of 1 sec/decade and processed by a VG2035 Data System. Selective ion detection at 0.05 sec per mass channel was carried out using the ion at m/z 266 in the spectrum of tris-(trifluoromethyl)-*S*-triazine (Fluorochem Ltd., Glossop, U.K.) as the lock mass.

Quantitation of derivatized *S*-3-oxo-octyl-*N*-acetylcysteine in microsomal extracts was carried out by reference to calibration curves determined from the analysis of mixtures of *S*-3-oxo-octyl-*N*-acetylcysteine and the internal standard *S*-3-oxo-hexyl-*N*-acetylcysteine added to samples of blank microsomal mixtures. The ions monitored in EI represent $(M—CH_3CONH_2)^+$: m/z 244 for *S*-3-oxo-octyl-*N*-acetylcysteine and m/z 216 for *S*-3-oxo-hexyl-*N*-acetylcysteine methyl esters.

NMR

The 360 MH_z NMR spectra were obtained with a Bruker model WH 360 instrument in the Fourier transform mode using a 2.5 mm microprobe. The octene-*N*-acetylcysteine methyl ester adduct purified by HPLC [8] was dissolved in C^2HCl_3.

Estimation of N-*(2-hydroxyoctyl)protoporphyrin IX and its reaction with* $^{65}ZnCl_2$

Reaction mixtures containing 1-octene (1 mM) and a NADPH generating system as described above were incubated for various lengths of time with rat liver microsomal preparations. Reactions were terminated by the addition of 40 ml ice-cold 5% H_2SO_4 in methanol. Extraction of the esterified products and their separation by silica gel HPLC was as described previously [12]. Extraction of 2-hydroxyoctyl-protoporphyrin IX from the livers of rats and its purification was carried out using the procedures of White *et al.* [13]. The green pigment adduct (30 nmoles) in $CHCl_3$/methanol (2:1 v/v) was mixed with $^{65}ZnCl_2$ (2 μmoles in 10 μl water). Incorporation of the zinc into the protoporphyrin IX ring was followed spectrophotometrically by observing the bathochromic shift in the Soret band from 413 to 426 nm. When the reaction was complete (15 min at RT in the dark), the zinc complex was extracted into $CHCl_3$, washed with water and subjected to TLC essentially as described elsewhere [12]. The absorbance of the purified zinc complex at 427 nm was determined and then its radioactivity, the latter in a Packard (model 5330) auto gamma counter.

Non-enzymic reaction of 1-octen-3-one with N-*acetylcysteine*

This was carried out at 37° in 0.1 M phosphate buffer pH 7.4 in the presence of a 10-fold molar excess of N-acetylcysteine using described procedures [10], the concentrations of 1-octen-3-one and *S*-3-oxo-octyl-*N*-acetylcysteine being determined by reverse phase HPLC using the procedures described previously [8].

RESULTS

Microsomal conversion of 1-octene to octane-1,2-diol

Initial GLC estimations using underivatized ethyl acetate extracts of microsomal incubation mixtures containing 1-octene and a NADPH generating system showed that octane-1,2-oxide did not accumulate but was rapidly hydrolysed to octane-1,2-diol. After an initial lag period of 1–2 min, time courses

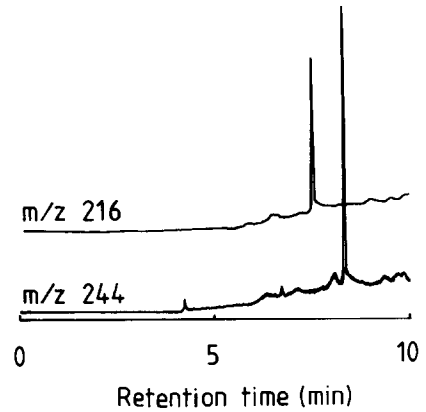

Fig. 1. Capillary GLC/mass spectrometry trace using selective ion monitoring of a derivatized microsomal extract following incubation with octene, *N*-acetylcysteine and a NADPH generating system. Rat liver microsomes (1–2 mg protein) were incubated with octene (1 mM), *N*-acetylcysteine (10 mM) and a NADPH generating system for 5 min at 37°. After stopping the reaction (1 M-HCl) and the addition of internal standard (*S*-3-oxo-hexyl-*N*-acetylcysteine, 2 nmoles), ether extracts of the mixture were derivatized with diazomethane and subjected to GLC/mass spectrometry. Selective ion monitoring was carried out for fragments m/z 216 (upper trace) and 224 (lower trace) representing $(M—CH_3CONH_2)^+$ for internal standard and *S*-3-oxo-octyl-*N*-acetylcysteine methyl esters respectively.

for the formation of octane-1,2-diol were linear up to 8 min. Over this period the rate of diol formation was 7.5 ± 0.8 nmol/min/mg microsomal protein (mean ± S.E., 4 experiments). No octane-1,2-diol formation could be detected if octene or the NADPH generating system were omitted from the reaction mixtures.

Formation of N-*(2-hydroxyoctyl)protoporphyrin IX)*

N-(2-hydroxyoctyl)protoporphyrin IX was extracted and purified from the livers of rats given 1-octene as described in the Methods section. Assuming a 1:1 incorporation of ^{65}Zn into *N*-(2-hydroxyoctyl)protoporphyrin IX dimethyl ester, the metal free adduct was calculated to have an extinction coefficient in $CHCl_3$ at 413 nm of 21000 l/mol. Recoveries of this product (50 pmoles) when added to microsomal incubation mixtures and processed in the usual way was 64 ± 2.4% (mean ± S.E., 4 experiments). The initial rate of accumulation of 2-hydroxyoctyl-protoporphyrin IX with time in microsomal mixtures incubated with 1-octene and a NADPH generating system was 32 ± 4 pmol/min/mg protein (mean ± S.E., 4 experiments). Although there was no lag period, linearity of the reaction time course was maintained for only 6–8 min. No formation of this adduct could be detected when 1-octene was omitted from the incubation mixtures.

Identification and quantitation of S-*3-oxo-octyl*-N-*acetylcysteine in microsomal mixtures incubated with 1-octene,* N-*acetylcysteine and a NADPH generating system*

Electron impact mass spectrometry of authentic *S*-3-oxo-octyl-*N*-acetylcysteine methyl ester did not yield a detectable molecular ion but gave an abundant peak m/z 244 $(M—CH_3CONH_2)^+$. GLC/mass spectrometry with selective ion monitoring for this fragment was used on the derivatized ether extracts from microsomes incubated with octene, *N*-acetylcysteine and a NADPH generating system. Figure 1 shows a typical selective ion monitoring trace from such an experiment. A peak, retention time 8.3 min, coincident with authentic *S*-3-oxo-octyl-*N*-acetylcysteine methyl ester was seen. This peak was absent

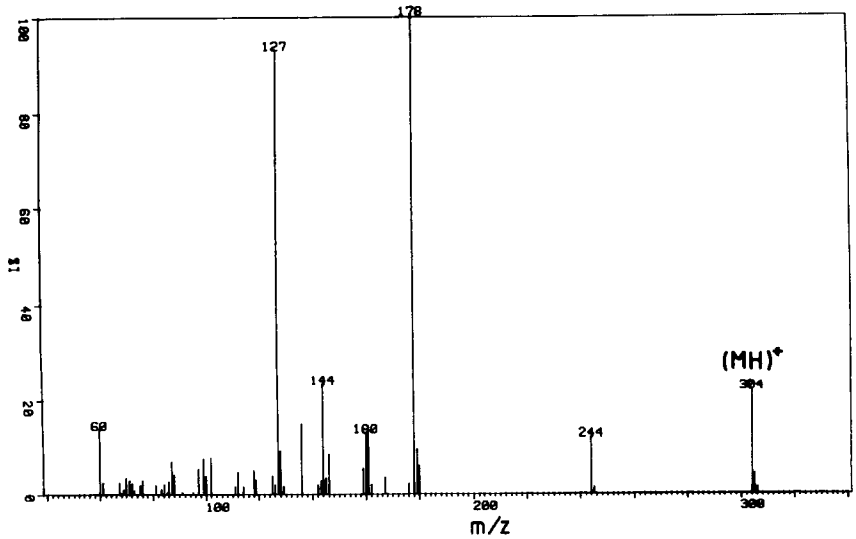

Fig. 2. Mass spectrum (CI) of a derivatized octene-*N*-acetylcysteine adduct extracted from microsomal mixtures following incubation with 1-octene, *N*-acetylcysteine and a NADPH generating system. Conditions were similar to those described in the legend to Fig. 1 except that no internal standard was added. The derivatized extract purified by HPLC was subjected to capillary GLC and the mass spectra of the single major component retention time 8.3 min was recorded.

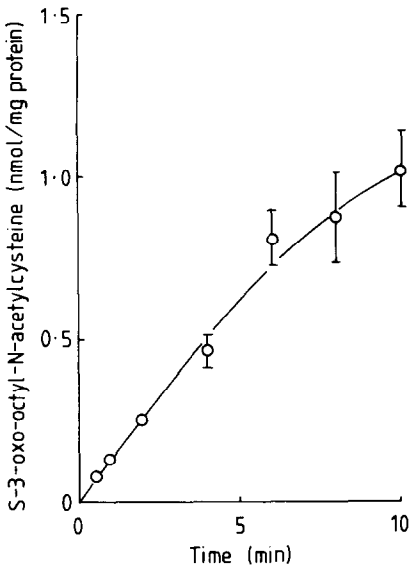

Fig. 3. Effects of time of incubation on the formation of *S*-3-oxo-octyl-*N*-acetylcysteine from 1-octene and *N*-acetylcysteine. Reaction conditions and assay procedures were the same as described in the legend for Fig. 1 except incubation times were varied as indicated. Results represent the mean ± S.E. for 4 experiments.

at zero time or after 10 min incubation with NADPH if either octene or *N*-acetylcysteine were omitted from the reaction mixtures. The methyl ester adduct isolated from such microsomal incubates was purified using HPLC and subjected to GLC-chemical ionization mass spectrometry. A protonated molecular ion m/z 304 was seen together with fragments m/z 244 $(M - CH_3CONH_2)^+$ and m/z 178 $(MH - CH_2 = CHCO(CH_2)_4CH_3)^+$. Additional fragmentation peaks m/z 127 and 144 represented cleavage of the molecule about the thioether bond, results consistent with the proposed structure (Fig. 2).

Further structural evidence was gained from 360 MH_z ^1H NMR of the methyl ester dissolved in ^2H $CHCl_3$ assisted by homonuclear decoupling experiments and comparison with the 60 MH_z NMR spectra of authentic *S*-3-oxo-octyl-*N*-acetylcysteine. The assignments for the proton resonances are (ppm, relative number of protons, multiplicity, coupling): 6.41 (1H,s,—N*H*); 4.82 (1H,m,—C*H*COO); 3.78 (3H,s,—CH_3COO); 3.02 (2H,d,—CH_2S,J = 7H_z); 2.75 (2H,m,—CH_2S); 2.68 (2H,m,—CH_2CO); 2.06 (3H,s,—CH_3CO); 1.6 (m,—$(CH_2)_3CH_2$) resonance partially obscurred by residual water peak; 1.28 (6H,m—$(CH_2)_3$); 0.86 (3H,t,$CH_3(CH_2)_3$—, J = 7H_z).

Figure 3 shows the time course for the formation of *S*-3-oxo-octyl-*N*-acetylcysteine in microsomal mixtures incubated with octene,*N*-acetylcysteine and a NADPH generating system. Initial reaction rates became non-linear after 4–5 min incubation. Over a 4 min incubation period, a linear relationship was found between the amount of adduct formed and the microsomal protein concentration over a range of 0.5–2 mg protein/2 ml reaction mixture.

Chemical reactivity of oct-1-en-3-one and N*-acetylcysteine in phosphate buffer, pH 7.4*

Octen-3-one reacted very rapidly with a 10-fold molar excess of *N*-acetylcysteine in phosphate buffer, pH 7.4 at 37° with a $t_{\frac{1}{2}}$ of about 6 sec to give *S*-3-oxo-octyl-*N*-acetylcysteine (Fig. 4). Under similar conditions, octen-3-one did not react with haem or protoporphyrin IX. Oct-1-en-3-ol had no reaction with *N*-acetylcysteine in this system. However, when oct-1-en-3-ol was incubated with rat liver microsomes, *N*-acetylcysteine and the oxidised coenzyme: either NAD or NADP, an adduct could be extracted which when derivatized as the methyl ester had the same retention time following capillary GLC and gave the same molecular ion and fragmentation

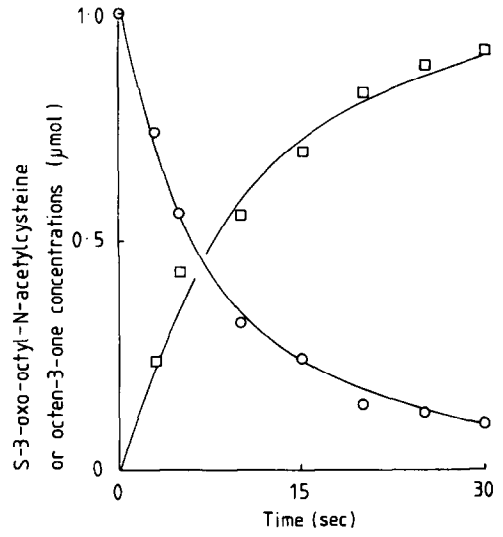

Fig. 4. Effects of incubation time on the non-enzymic formation of *S*-3-oxo-octyl-*N*-acetylcysteine from octen-3-one and *N*-acetylcysteine. Reaction mixtures (2 ml) at pH 7.4 and 37° contained 1-octen-3-one (2 μmoles) and N-acetylcysteine (20 μmoles). Reactions were terminated by the addition of 1 M HCl and an aliquot of the mixture immediately subjected to reverse phase HPLC. Results represent the mean of 2 experiments: ○, concentration of octen-3-one; □, concentration of *S*-3-oxo-octyl,*N*-acetylcysteine.

Table 1. Formation of *S*-3-oxo-octyl-*N*-acetylcysteine in microsomal mixtures from various tissues incubated with octen-3-ol, *N*-acetylcysteine and NADP

Tissue	*S*-3-oxo-octyl-*N*-acetylcysteine formed (nmol/min/mg protein)
Liver	9.7 ± 2.1
Adrenal	7.5 ± 1.2
Intestine	2.5 ± 0.8
Lung	1.3 ± 0.2
Kidney	1.0 ± 0.2
Spleen	0.8 ± 0.1
Brain	0.5 ± 0.2

Microsomal preparations (1–2 mg protein) were incubated for 4 min at 37° with octen-3-ol (1 mM), *N*-acetylcysteine (10 mM) and NADP. Following the addition of internal standard, reaction products were extracted into ether, derivatized as the methyl esters and estimated by capillary GLC as described in the Methods section. Results represent the mean ± S.E. of 4 experiments.

Table 2. Effects of pretreating rats with mixed function oxidase inducers on the formation of *S*-3-oxo-octyl-*N*-acetylcysteine in liver microsomal systems incubated with either octene or octen-3-ol

Pretreatment	Substrate	
	1-octene	1-octen-3-ol
	S-3-oxo-octyl-*N*-acetylcysteine formed (nmol/min/mg protein)	
Controls	0.13 ± 0.004	10.9 ± 0.5
Phenobarbitone	0.39 ± 0.007	9.9 ± 0.9
3-Methylcholanthrene	0.22 ± 0.02	12.6 ± 1.2
Clofibrate	0.16 ± 0.01	10.5 ± 0.5

Liver microsomes (1–2 mg protein) were incubated for 4 min at 37° with *N*-acetylcysteine (10 mM) and either 1-octene (1 mM) and a NADPH generating system or octen-3-ol (1 mM) and NADP (2 mM). Work up procedures were the same as described in the legend for Table 1. With 1-octene products were assayed using GLC/mass spectrometry with single ion monitoring. Results represent the mean ± S.E. for 4 experiments.

pattern following CI mass spectrometry as *S*-3-oxo-octyl-*N*-acetylcysteine. Reaction rates were linear with time up to 10 min incubation and were more rapid when NAD rather than NADP was used as the cofactor (15.3 ± 2.1 and 8.5 ± 0.5 nmol/min/mg protein respectively, mean ± S.E., 4 experiments). Initial rates (NADP as cofactor) were not significantly reduced if reactions were carried out anaerobically under N_2 or in the presence of CO/O_2(9:1) (9.4 ± 0.5 and 8.9 ± 0.5 nmol/min/mg protein respectively). The enzyme involved was principally located in the microsomal fraction although liver cytosol did show some activity (2.5 ± 0.3 nmol/min/mg protein). Table 1 shows that microsomal fractions prepared from other organs were also able to catalyse this reaction, adrenal microsomal preparations being especially active in this respect.

Comparison between the rates of microsomal activation of oct-1-ene and oct-1-en-3-ol: the effects of pretreatment of rats with mixed function oxidase inducers

Table 2 shows a comparison of the effects of pretreatment of rats with phenobarbitone, 3-methylcholanthrene or clofibrate on the subsequent microsomal catalysed formation of *S*-3-oxo-octyl-*N*-acetylcysteine from either 1-octene or octen-3-ol. The rate of formation of the active metabolite from octen-3-ol, trapped with *N*-acetylcysteine, was not affected by such pretreatment procedures whereas both phenobarbitone and to a lesser extent 3-methylcholanthrene induced the activation of octene. Pretreatment of rats with clofibrate did not greatly affect rates of activation of either octene or octen-3-ol.

DISCUSSION

Activation of 1-octene via a 3-oxo-intermediate

The results described in this paper show that the model olefin,1-octene, when incubated with rat liver microsomes in the presence of NADPH, undergoes metabolic activation to an intermediate which was trapped with *N*-acetylcysteine, as *S*-3-oxo-octyl-*N*-acetylcysteine. This pathway was induced by pretreatment of rats with phenobarbitone and to a lesser extent, 3-methylcholanthrene. Clofibrate was also investigated since treatment of rats with this compound results in an increased hydroxylation of certain fatty acids [14]. However, such pretreatment procedures did not induce the formation of *S*-3-oxo-octyl-*N*-acetylcysteine from octene.

The evidence suggests *S*-3-oxo-octyl-*N*-acetylcysteine arises as a result of a two stage activation sequence (Fig. 5). Octene is first metabolised by mixed function oxidases to oct-1-en-3-ol. This is followed by further NAD or NADP-dependent oxi-

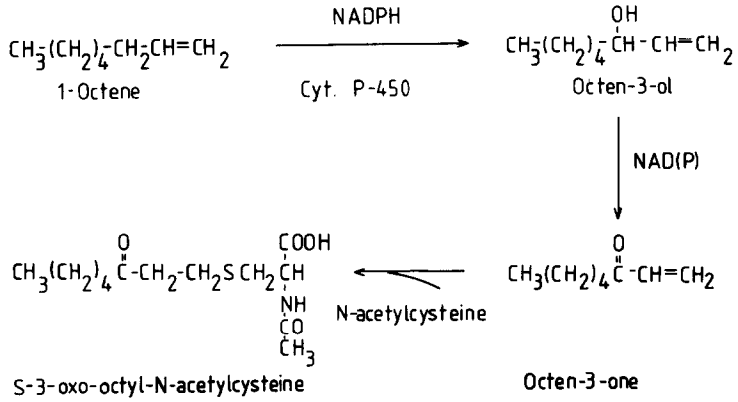

Fig. 5. Proposed route for the activation of 1-octene via a 3-oxo activated metabolite.

dation by a cytochrome P-450 independent enzyme system to the putative reactive intermediate oct-1-en-3-one. Since at least 20-fold more active metabolite is trapped with *N*-acetylcysteine using octen-3-ol as the substrate than with octene (Table 2), it is suggested that in the activation of octene, the first step in the pathway, is rate limiting. These findings show activation of olefins may proceed by a route analogous to that proposed for acetylenic substituted compounds [8].

Hydroxylation of allylic compounds at a position α to the carbon–carbon double bond has been described for safrole and related structures [15]. In such instances, some activation may occur via the 1′-oxo-safrole intermediate [16], although the evidence from experiments carried out *in vivo* suggest that the sulphuric acid ester of 1′-hydroxysafrole is the ultimate carcinogenic metabolite [17]. It is not known the extent to which octen-3-ol may be a substrate for the cytosolic sulphotransferases.

Octen-3-ol occurs naturally as a flavour constituent in some foods such as mushrooms [18] certain cheeses [19] and shrimps [20]. As the present results show, it can be activated by the 3-oxo pathway in microsomal preparations from many organs apart from the liver. It would be of some interest therefore to assess the extent to which this could occur following its administration *in vivo*.

Comparison of metabolic activation of 1-octene by the 3-oxo pathway in relation to other routes

Relative to the rate of formation of *S*-3-oxo-octyl-*N*-acetylcysteine, via the 3-oxo pathway, the results show the classical route of activation of octene via the 1,2 epoxide followed by hydrolysis to octane-1,2-diol [4] to be about 40 times higher. In contrast, trapping an active metabolite of octene with the haem of cytochrome P-450 to yield *N*-(2-hydroxyoctyl)protoporphyrin IX gave rates about 17 times lower. If such *N*-alkylated porphyrins are formed from an oxirane precursor [7] it is somewhat surprising that the rate of accumulation of *N*-(2-hydroxyethyl)protoporphyrin IX should be over 600-fold lower than that of octane-1,2-diol. Studies with 2-alkyl-2-isopropylacetamide suggest during the course of activation of the alkylic function via an epoxide intermediate, about 200 moles of substrate are consumed for every mole of cytochrome P-450 destroyed [21, 22].

Many epoxides prepared chemically are carcinogenic or mutagenic (reviewed in ref. 23). However, in the case of octene, the epoxide formed in the liver *in vivo* will be a substrate for both epoxide hydrolase and the glutathione transferases [24]. Thus 1-octene administered to rats *in vivo* as a single dose (200 mg/kg, intraperitoneally) is not acutely hepatotoxic (H. E. Driver and I. N. H. White unpublished results). The possible mutagenic or carcinogenic effects of 3-oxo-activated olefins are being investigated.

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