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Metabolic activation of acetylenes

Covalent binding of [1,2-^{14}C]octyne to protein, DNA and haem *in vitro* and the protective effects of certain thiol compounds

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1. [1,2-^{14}C]Oct-1-yne was used to investigate metabolic activation of the ethynyl substituent *in vitro*. Activation of octyne by liver microsomal cytochrome *P*-450-dependent enzymes gave intermediate(s) that bound covalently to protein, DNA and to haem. 2. The time course and extent of covalent binding of octyne to haem and to protein were similar. However, two different activating mechanisms are probably involved. Whereas covalent binding to protein or to DNA was inhibited by nucleophiles such as *N*-acetylcysteine, that to haem was little affected. 3. When *N*-acetylcysteine was included in the reaction mixtures, two major octyne-*N*-acetylcysteine adducts were isolated and purified by high-pressure liquid chromatography. G.l.c.–mass spectrometry and n.m.r. suggest that these are the *cis–trans* isomers of *S*-3-oxo-oct-1-enyl-*N*-acetylcysteine. 4. Oct-1-yn-3-one reacted non-enzymically with *N*-acetylcysteine at pH 7.4 and 37°C with a $t_{\frac{1}{2}}$ of about 6 s also to yield *S*-3-oxo-oct-1-enyl-*N*-acetylcysteine. The same product was formed when microsomal fractions were incubated with oct-1-yn-3-ol, *N*-acetylcysteine and NAD(P)$^+$. Octyn-3-one did not appear to react with haem or protoporphyrin IX. 5. A mechanism for the metabolic activation of oct-1-yne is proposed, consisting in (*a*) microsomal hydroxylation of the carbon atom α to the acetylenic bond and (*b*) oxidation to yield octyn-3-one as the reactive species.

Ethynyl-substituted contraceptive steroids such as norethindrone are metabolically activated in the liver to derivatives that cause the destruction of cytochrome *P*-450 and the formation of abnormal green pigments (White & Muller-Eberhard, 1977). Such green pigments represent a covalent adduct between the steroid and the protoporphyrin IX ring of haem (Ortiz de Montellano *et al.*, 1979). The possibility that reactive metabolities of the ethynyl substituent may also bind to other cellular macromolecules has not been explored.

Because of the potential difficulties in distinguishing between different sites of metabolic activation in the steroidal structure (see, e.g., Bolt, 1977), a model acetylene, oct-1-yne, was chosen for the present study. Oct-1-yne causes the loss of hepatic cytochrome *P*-450 *in vivo* and *in vitro* (White, 1982), and has been employed to study the regiospecificity of haem alkylation (Kunze *et al.*, 1983). [1,2-^{14}C]Oct-1-yne was used to investigate the extent, if any, that active metabolites of the ethynyl substituent might bind covalently to protein or to DNA *in vitro*.

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

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Materials and methods

Chemicals

Oct-1-yne (99% purity) and oct-1-yn-3-ol (97% purity) were from Aldrich Chemical Co., Gillingham, Dorset, U.K. Octyn-3-one was prepared by the CrO_3 oxidation of octyn-3-ol (Nobuhara, 1969). Capillary g.l.c. showed the final product to be of > 99% purity. L-[^{35}S]Cysteine hydrochloride

(specific radioactivity 32mCi/mmol) was from Amersham International, Amersham, Bucks., U.K. NAD^+, $NADP^+$, glucose 6-phosphate, *N*-acetylcysteine, haemin chloride, protoporphyrin IX and glucose-6-phosphate dehydrogenase were from Sigma Chemical Co., Poole, Dorset, U.K.

Synthesis of [1,2-^{14}C]oct-1-yne

The radioactive synthesis of [1,2-^{14}C]oct-1-yne (specific radioactivity 28mCi/mmol; 27% radiochemical yield) was carried out at the Services des Molecules Marquées, C.E.N.-Saclay, France, by adapting the procedure of Beckmann *et al.* (1975).

In a high-vacuum system, [U-^{14}C]acetylene (1.5mmol, 42mCi) was frozen in a 100ml two-necked flask containing tetrahydrofuran (10ml). The flask was cooled at −10°C with stirring while n-butyl-lithium in hexane (1ml, 1.6mmol) was added dropwise. The mixture was stirred for 1h at about −1°C, and then 1-bromohexane (0.2ml, 1.5mmol) in hexamethylphosphoramide (2ml) was added dropwise. The mixture was stirred for 30min at room temperature, then decomposed by ice/water. The mixture was extracted with diethyl ether, and the combined ether extracts were then washed with water and dried. The product (29mCi) was carefully concentrated to about 4ml, then 1ml of this concentrate was purified by preparative g.l.c. (C.E.N.-Saclay apparatus, silicone SE-30 10%, 100°C) to afford 2.7mCi of [1,2-^{14}C]oct-1-yne of chemical and radiochemical purity >98%, checked by g.l.c.

Conversion of [^{35}S]cysteine into N-acetyl-[^{35}S]cysteine

To [^{35}S]cysteine hydrochloride (5μmol, 50μCi) in 50mM-sodium phosphate buffer, pH 7.4, (0.1ml) was added acetic anhydride/1-methylimidazole (5:1, v/v) (0.1ml). After 5min at room temperature, 3M-NH_3 was added and the mixture left to stand for 15min under N_2. It was then freeze-dried. Silica-gel t.l.c. (silica gel 60, F_{254}, layer thickness 0.25mm; E Merck, Darmstadt, Germany) of the product dissolved in water (0.1ml) with the solvent system butan-1-ol/acetic acid/water (3:3:4, by vol.) showed under u.v. light a single component, R_F 0.53, which was ninhydrin-negative [0.25% ninhydrin in acetone/lutidine (9:1, v/v)] but gave a yellow colour when sprayed with 5,5′-dithiobis-(2-nitrobenzoic acid) (0.5% in 50mM-phosphate buffer, pH 7.4). This R_F value corresponded to that of authentic *N*-acetylcysteine. The plate monitored in a Packard radiochromatogram scanner (model 7200) showed the product to be >98% radiochemical purity. It was used without further purification.

Preparation of S-3-oxo-oct-1-enyl-L-N-acetylcysteine

To an ice-cold solution of L-*N*-acetylcysteine (0.82g, 5mmol) in 10ml of 0.1M-Tris buffer (adjusted to pH 8.0 with 2M-NaOH) was added octyn-3-one (0.63g, 5mmol) in methanol with stirring. After 5min at room temperature, the mixing was adjusted to pH 1.5 (with conc. HCl), and 200ml of ice-cold water was added. The white precipitate was washed with water and dried to yield 0.9g (74%) of crude product. Recrystallization from diethyl ether/light petroleum (b.p. 40–60°C) (1:4, v/v) gave *S*-3-oxo-oct-1-enyl-*N*-acetylcysteine, m.p. 131°C, ^{1}H-n.m.r. (60Hz, Perkin–Elmer model R12B instrument, tetramethylsilane internal standard) δ (p.p.m.) ([^{2}H]chloroform) 9.4 (1H, s, $-CO_2H$), 7.9–6.1 (2H, q, $CH{=}CH$), 7.2–7.0 (1H, s, NH), 5.1–4.7 (1H, broad, s, $CH{-}CO_2$), 3.6–3.1 (2H, d, $-S{-}CH_2$), 2.7–2.1 (2H, t, $-CH_2{-}CO$), 2.2–1.9 (3H, s, CH_3CO-), 1.8–1.1 (6H, broad, s, $-[CH_2]_3-$), 1.1–0.8 (3H, t, $CH_3[CH_2]_4-$) (Found: C, 54.1; H, 7.4; N, 4.8; S, 11.4. Calc. for $C_{13}H_{21}NO_4S$ C, 54.3; H, 7.4; N, 4.9; S, 11.2%). Mass-spectral studies (chemical ionization or electron impact) did not yield a molecular ion. However, formation of derivatives of the product by using established procedures to form the methyloxime methyl ester (see below) gave a product with a molecular ion by high-resolution mass spectrometry $m/z = 330.1604$ (2.5% of the base peak), (calc. for $C_{15}H_{26}N_2O_4S = 330.1613$), and a fragmentation pattern consistent with the proposed structure.

Animals

Male Fischer F-344N rats (150–170g) were used. In some instances, hepatic mixed-function oxidase activities were induced by pretreatment of the animals with either phenobarbitone (0.1%) in the drinking water for 7 days or by dosing with 3-methylcholanthrene (dissolved in glycerol trioctanoate; 20mg/ml) (20mg/kg) intraperitoneally once a day for 3 days. The rats were killed 24h after the last dose.

Preparation of liver microsomal fractions

Washed liver microsomal fractions were prepared as described previously (White, 1978). The final pellet was made up in 0.25M-sucrose so that 1ml was equivalent to 0.5g wet wt. of liver. Protein concentrations were determined by the method of Lowry *et al.* (1951), with a bovine serum albumin standard. Microsomal haem was determined as the pyridine haemochromogen (White, 1978).

Covalent binding of [^{14}C]octyne to microsomal protein haem or to DNA: reaction conditions

Microsomal incubation mixtures of 2ml volume

in stoppered tubes contained potassium phosphate buffer, pH 7.4 (0.1 M), $MgCl_2$ (2 mM), EDTA (0.5 mM), $NADP^+$ (0.5 mM), glucose 6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (2 units) and microsomal fraction (3–4 mg of protein). Reactions were started by the addition of [^{14}C]octyne (2 μCi in 10 μl of dimethyl sulphoxide) to give a final concentration of 1 mM. In some instances $NADP^+$ and the NADPH-generating system were omitted or replaced by NADPH (1 mM) and/or NADH (1 mM). In other experiments tubes were gassed with N_2 or CO/O_2 (4:1) at a flow rate of 100 ml/min for 5 min before and during the time of reaction. Reaction mixtures were incubated at 37°C in a shaking water bath in the dark for 2 min, except where indicated.

(*a*) *Covalent binding to protein*. Reactions were stopped with ice-cold 1 M-$HClO_4$/methanol (1:1, v/v) (2 ml). This was used in preference to trichloroacetic acid to facilitate the removal of octyne–haem adducts (green pigments) (I. N. H. White, unpublished work). After centrifugation (3000 *g* for 15 min at 4°C), the protein precipitates were exhaustively extracted with organic solvents as described by Sun & Dent (1980). The extracted pellets were dissolved in 1 M-NaOH and assayed for protein content (Lowry *et al.*, 1951) by using a bovine serum albumin standard (Sigma Chemical Co.) and for ^{14}C radioactivity in a Searle model 6880 liquid-scintillation counter after addition of scintillant (Beckman Readysolv CP).

(*b*) *Covalent binding to DNA*. Reaction conditions were the same except that reaction mixtures contained in addition 1 mg of calf thymus DNA (Sigma Chemical Co.). Reactions were stopped with 0.6 M-NaCl/1.6% (w/v) sodium dodecyl sulphate (2 ml). The work-up procedures and extraction of DNA with organic solvents were as described by Loosemore *et al.* (1981).

(*c*) *Covalent binding to haem*. Reactions were terminated with 40 ml of ice-cold conc. H_2SO_4/methanol (1:19, v/v). Extraction of the esterified products and separation by h.p.l.c. were as described by White (1981), except the isocratic solvent used for silica-gel h.p.l.c. was changed to cyclohexane/chloroform/methanol (6:2:1, by vol.) containing 0.2% (v/v) acetic acid.

Determination of octyne metabolites by reverse-phase h.p.l.c.: metabolic profile

Incubation mixtures (2 ml) were as described above. Reactions were stopped, generally after 5 min incubation at 37°C, with ice-cold acetonitrile (2 ml), and the tubes were shaken and placed on ice. A 0.5 ml portion of the mixture was spun in an Eppendorf centrifuge (10000 *g* for 2 min), and 0.05 ml of the clear supernatant was injected directly on to a reverse-phase h.p.l.c. column (25 cm × 0.47 cm) (Macherey Nagel, Nucleosil 5C_{18}). A concave gradient (G8, model 660 Solvent Programmer; Waters Associates) with a solvent system of methanol/water (3:2, v/v) to methanol over 15 min was used. Both solvents contained 0.02% (v/v) H_3PO_4. Flow rate was 1.5 ml/min, and the detector wavelength 206 nm. Peak areas were computed by using a Pye–Unicam DP88 integrator. Fractions (0.3 min) were collected from the detector outlet, and, after the addition of scintillant (Beckman Readysolv CP), assayed for radioactivity in a Searle model 6880 liquid-scintillation counter.

Time course for the formation of N-acetylcysteine adducts from microsomal fractions incubated with octyn-3-ol and N-acetylcysteine

Incubation mixtures of 2 ml were as described above, except that octyne was replaced by octyn-3-ol (1 mM) and the NADPH-generating system by either $NADP^+$ (1 mM) or NAD^+ (1 mM). Reactions were stopped after various times with ice-cold 1 M-HCl (2 ml), and, after centrifugation (10000 *g* for 10 min), the supernatant (1.5 ml) was extracted with diethyl ether (3 × 2 ml). The combined ether extracts were washed with water, dried (over anhydrous Na_2SO_4) and concentrated to dryness. The residue was dissolved in ethanol (0.1 ml), and diazomethane in diethyl ether (0.1 ml) was added. After 15 min diethyl ether (1 ml) and water (0.5 ml) were added, and the esterified products were extracted into the ether phase (3 × 1 ml). The combination ether extracts were dried, concentrated to dryness under N_2 at 37°C and subjected to reverse-phase h.p.l.c. as described above. The peak areas of the esterified octyne-*N*-acetylcysteine adducts (retention times 6.3 and 7.0 min) were determined. For confirmation of the identity of the reaction products, in some experiments, the ether extracts were treated with methoxamine hydrochloride before treatment with diazomethane. Purification by silica-gel and analysis by h.p.l.c. and g.l.c./mass spectrometry were as described below.

Non-enzymic reaction of octyn-3-one with N-acetylcysteine

Reaction mixtures of 1 ml volume at 37°C contained 0.1 M-potassium phosphate buffer, pH 7.4, and *N*-acetylcysteine (10 μmol). Reactions were started by the addition of octyn-3-one (or in some experiments octyn-2-ol or octyne) in methanol (1 μmol, 10 μl), and effectively stopped with ice-cold 1 M-HCl (1 ml) (see the Results section). A sample (50 μl) was immediately subjected to reverse-phase h.p.l.c. as described above, and the

peak areas of octyn-3-one (retention time 7.0min) and the octyn-3-one–*N*-acetylcysteine adducts (retention times 4.7 and 4.9min) were determined.

In other experiments, *N*-acetylcysteine was replaced by haem or protoporphyrin IX (50nmol) in dimethyl sulphoxide (0.1ml) or rat liver microsomal fraction (0.1ml, 1.5–2mg of protein). Reactions were carried out either at pH7.4 or at pH10.0, in the latter instance in a 0.1M-$NaHCO_3$/NaOH buffer. Reactions were terminated with 40ml of ice-cold conc. H_2SO_4/methanol (1:19, v/v) and processed as described above (covalent binding to haem). Esterified extracts were separated by silica-gel t.l.c. developed in a chloroform/kerosene/methanol (60:15:9, by vol.) solvent system (White, 1978).

Isolation of octyne–N-acetylcysteine adduct from microsomal incubation mixtures

Silica-gel h.p.l.c. Microsomal incubation mixtures (2ml) as described above contained octyne (1mM), *N*-acetylcysteine (10mM), an NADPH-generating system and hepatic microsomal fraction from phenobarbitone-pretreated rats. In some instances unlabelled octyne was used or in conjunction with *N*-acetyl[^{35}S]cysteine. Reactions were stopped after 5min incubation at 37°C with an equal volume of 1M-HCl, and the reaction products were extracted into diethyl ether and esterified as described above. In other experiments, before esterification, extracts were dissolved in dry pyridine (0.1ml) containing methoxamine hydrochloride (1mg/ml) (Eastman Kodak, Kirby, Liverpool, U.K.). Tubes were heated at 80°C for 30min under N_2. After cooling to 37°C, pyridine was removed under N_2. H.p.l.c. was carried out on a silica column (0.25cm × 0.47cm) (Macherey Nagel Nucleosil 50:5) and an isocratic hexane/ethanol (9:1, v/v) solvent system at a flow rate of 1.5ml/min. The detector was set at 212nm. Fractions (0.3min) were collected from the detector outlet and assayed for radioactivity, or, as appropriate, pooled and concentrated to dryness under N_2.

G.l.c./mass spectrometry

A Carlo Erba gas chromatograph equipped with a flame ionization detector was used for g.l.c. analyses. Separations were carried out on either a glass or fused-silica capillary column (20m × 0.3mm) coated with the stationary phase SE52. The column was operated at 100°C for 3min, then programmed at 30°C/min to 280°C. The flow of He carrier gas was 2ml/min. Samples were injected by using a split-injection system (10:1 split ratio). Mass-spectrometric analyses were carried out on a 70-70 VG Analytical mass spectrometer, linked with a VG 2035 Data System. Electron-impact analysis was carried out with an ionization energy of 70eV, an accelerating voltage of 4kV, and a source temperature of 200°C. Samples were admitted to the source by the gas chromatograph/mass spectrometer interface and held at 300°C, and spectra were acquired at 1s/decade. G.l.c. separations for mass spectrometry were made on a fused-silica capillary column (20m × 0.3mm), coated with SE52 stationary phase and housed in a Pye–Unicam series 204 gas chromatograph. Samples were injected by using a falling-needle solid injector at 250°C. He was used as carrier gas at a flow rate of 2ml/min. The temperature programme for the column was 100°C for 15s followed by a 32°C/min temperature rise to 250°C.

N.m.r.

The 360MHz n.m.r. spectra were obtained with a Bruker model WH360 instrument in the Fourier-transform mode by using a 2.5mm microprobe. The octyne–*N*-acetylcysteine adduct (0.3μmol), converted into the methyl ester and purified by h.p.l.c., was dissolved in C^2HCl_3.

Results

Metabolic activation of octyne to derivatives involved in covalent binding to microsomal protein

Fig. 1 shows that, when [1,2-^{14}C]oct-1-yne was incubated with rat liver microsomal fraction and an NADPH-generating system, radioactive label became covalently bound to microsomal protein, although reaction rates rapidly became non-linear with time. Covalent binding (expressed as pmol of octyne bound/min per mg of microsomal protein) was increased if microsomal fraction from phenobarbitone-pretreated animals was used and decreased with microsomal fraction from 3-methylcholanthrene-pretreated rats. There was a linear relationship between covalent binding and microsomal protein concentration over a range of 2–8mg of protein/2ml of reaction mixture (results not shown). Studies on the cofactor requirements showed (Table 1) that maximal binding occurred in the presence of NADPH and NADH, but the principal cofactor requirement was for NADPH. Binding was much lower when incubations were carried out in the absence of O_2, in the presence of CO or with compound SKF 525A or metyrapone.

Covalent binding of [^{14}C]octyne to DNA

When calf thymus DNA was included in the microsomal activating system with [^{14}C]octyne, radioactive label also became covalently bound to this macromolecule in a time-dependent manner (Fig. 2). DNA was particularly effective in this respect, since the amount of binding (pmol/min per mg of DNA) was 8-fold greater than to

Table 1. *Covalent binding of [1,2-^{14}C]octyne to microsomal protein: cofactor requirements and effect of inhibitors*
Reaction mixtures (2ml) contained microsomal fraction from phenobarbitone-pretreated rats (3–4mg of protein), [^{14}C]octyne (1mM) and cofactors as indicated. Tubes were incubated for 2min at 37°C in a shaking water bath. Reactions were stopped with ice cold 1M-$HClO_4$/methanol (1:1, v/v) (2ml). Protein precipitates were recovered by centrifugation and exhaustively extracted with organic solvents as described in the Materials and methods section. Results represent the means±S.E.M. for four experiments.

Incubation conditions	Covalent binding (pmol/min per mg of protein)
(*a*) Cofactor requirements	
Complete incubation mixture contains:	
NADPH+NADH	59.6±2.9
NADPH only	48.2±1.9
NADH only	15.9±0.4
No cofactors	6.4±1.3
NADPH: reaction carried out under N_2	10.3±0.5
NADPH: reaction carried out under CO/O_2 (4:1)	19.0±0.4
(*b*) Effects of inhibitors	
Complete incubation mixture in air contains NADPH	
+Semicarbazide (1mM)	49.3±1.7
+Compound SKF 525A (1mM)	15.4±1.5
+Metyrapone (1mM)	13.6±0.3

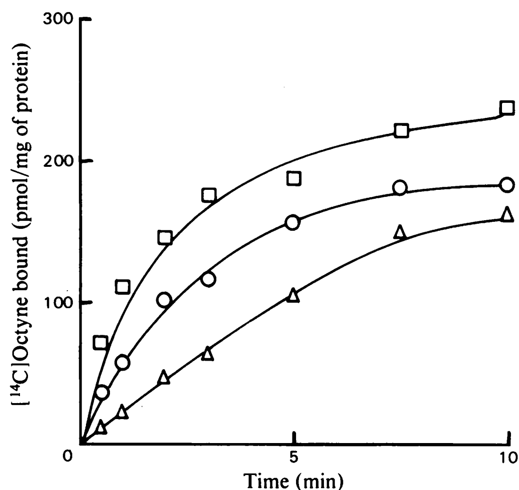


Fig. 1. *Effects of time of incubation on covalent binding of [1,2-^{14}C]octyne to microsomal protein*
Mixtures (2ml) contained rat liver microsomal fraction (3–4mg of protein), octyne and an NADPH-generating system. These were incubated at 37°C in a shaking water bath for the times shown. Reactions were stopped with ice-cold 1M-$HClO_4$/methanol (1:1, v/v) (2ml). After centrifugation and exhaustive extraction with organic solvents as described by Sun & Dent (1980), the protein content and ^{14}C radioactivity of the protein precipitates were determined. ○, Control rat liver microsomal fraction; △, microsomal fraction from rats pretreated with 3-methylcholanthrene; □, microsomal fraction from phenobarbitone-pretreated rats. Results represent the means for two experiments.

microsomal protein. In order to obtain information on the likely stability of the active octyne metabolite, in one experiment DNA was separated from [^{14}C]octyne and the microsomal activating system by a Visking cellulose dialysis membrane by using the apparatus described by Neal *et al.* (1979). No covalent binding to the DNA (or to the dialysis membrane) could be detected over a 30min incubation period, suggesting a very short half-life for such active metabolites.

Covalent binding of [^{14}C]octyne to cytochrome P-450 haem

In the present study, two green pigments (retention times 6.2 and 6.8min) were separated from haem by h.p.l.c. after incubation of octyne with rat liver microsomal fraction and an NADPH-generating system (Fig. 3). When [^{14}C]octyne was used as the substrate, radioactive label became associated with these components (Fig. 3*b*). Fig. 4 shows that covalent binding of octyne to haem increased in a non-linear time-dependent manner. The extent of covalent binding was of a similar order of magnitude as that to protein (Fig. 1).

Protective effects of thiol compounds against covalent binding of octyne to microsomal protein

In order to make comparisons between different thiol compounds, short (2min) incubation times were used on the near-linear part of the time-versus-covalent-binding progress curve (Fig. 1). Various thiol compounds shown in Table 2 were used. All inhibited covalent binding of octyne to microsomal protein, although the concentration required to bring about a 50% decrease (I_{50} values) varied greatly. Reduced glutathione was outstandingly effective in this respect, an action dependent on the complete molecule, since either cysteinyl-

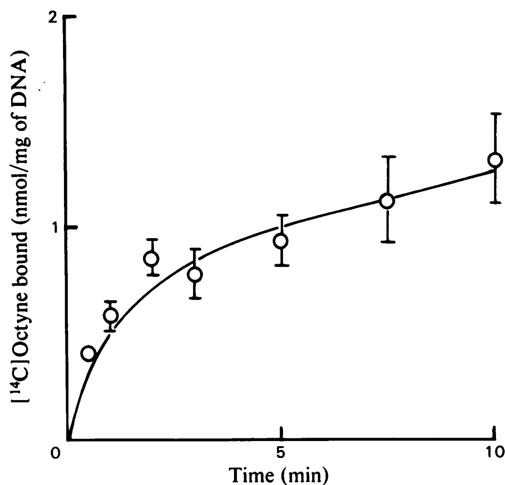


Fig. 2. *Effects of time of incubation on covalent binding of [1,2-^{14}C]octyne to DNA*

Reaction conditions were similar to those described in the legend to Fig. 1 except that reaction mixtures contained in addition 1 mg of calf thymus DNA. At the end of the incubation reactions were stopped with 0.6M-NaCl/1.6% sodium dodecyl sulphate (2ml). DNA was extracted from the incubation mixture and washed with organic solvents as described in the Materials and methods section. Results represent the means ± S.E.M. for four experiments with microsomal fraction from phenobarbitone-pretreated rats.

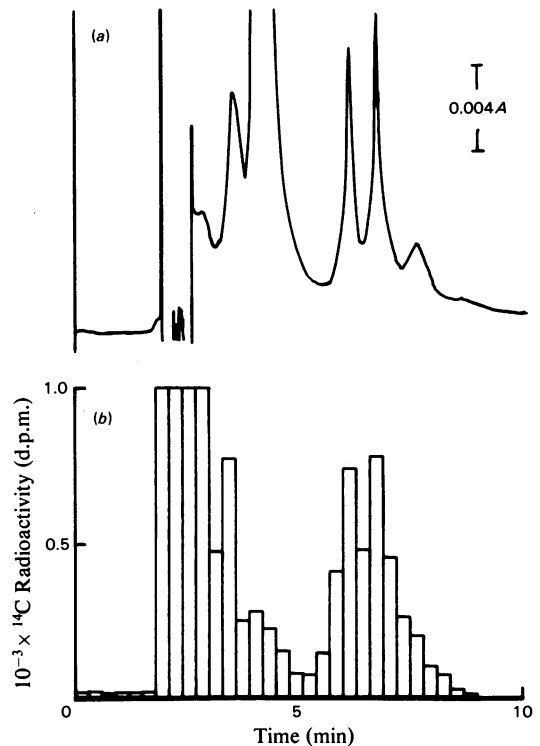


Fig. 3. *H.p.l.c. elution profile of green pigments as a result of incubating [^{14}C]octyne with a liver microsomal system*

[^{14}C]Octyne was incubated with microsomal fraction from a phenobarbitone-pretreated rat and an NADPH-generating system for 2min at 37°C. The reaction was stopped with ice-cold conc. H_2SO_4/methanol (1:19, v/v) (40ml). After 18h in the dark at 4°C, the esterified products were extracted into chloroform, washed, dried and subjected to silica-gel h.p.l.c. as described in the Materials and methods section. (*a*) Relative absorbance at 417nm. In (*b*) fractions (0.3min) were collected at the detector outlet and assayed for ^{14}C radioactivity.

glycine or cysteine was an order of magnitude less effective. Microsomal glutathione transferase may participate in this reaction. The protective action was not solely dependent on the extent of ionization of the thiol group of these compounds, since, as Table 2 shows, there was little correlation between the pK_a of the thiol group and the effect on covalent binding. No decrease in the extent of covalent binding occurred when glycine (up to concentrations of 20mM) was included in the reaction mixtures.

Protective action of thiol compounds against covalent binding of octyne to DNA or to haem

The effects of glutathione, cysteine or cysteamine at concentrations that decreased covalent binding of octyne to microsomal protein relative to controls by 50% were investigated to see if a similar protection would be afforded against binding to DNA or to haem. Table 3 indicates that these compounds protected against binding to DNA, but had little or no protective action against the formation of green pigment.

Metabolic fate of octyne: reverse-phase h.p.l.c. of metabolites

Three major radiolabelled metabolites (retention times 2.2, 3.2 and 5.0min) were separated by reverse-phase h.p.l.c. when [^{14}C]octyne was incubated with rat liver microsomal fraction and an NADPH-generating system (results not shown). No conversion of octyne (retention time 15.0min) into octanoic acid (retention time 10.3min) could be detected, although for certain other terminally substituted acetylenic compounds conversion into the corresponding acid is a major metabolic pathway (Wade *et al.*, 1979; El Masri *et al.*, 1958). The addition of the thiol compounds shown in Table 2 at their respective I_{50} concentrations did not decrease the radioactivity in the metabolite peaks, but selectively increased certain compo-

Table 2. *Covalent binding of [1,2-^{14}C]Octyne to microsomal protein: inhibition of binding by certain thiol compounds*
Reaction conditions were similar to those described in the legend to Table 1. Incubation mixtures (2ml) contained microsomal fraction from phenobarbitone-pretreated rats (3–4mg of protein), octyne (1mM), an NADPH-generating system and thiol compound at four concentrations in the range shown below. From a plot of the percentage of inhibition of covalent binding (relative to controls having no thiol compound added) versus the logarithm of the concentration of nucleophile added, the concentration required to cause a 50% decrease in covalent binding (I_{50}) was obtained. Results represent the means ± S.E.M. for four experiments.

Compound	Concentration range used (mM)	pK_a of thiol group	Concentration required to decrease covalent binding by 50% I_{50} (mM)
Reduced glutathione	0.001–0.1	9.12*	0.035 ± 0.01
Cysteamine	0.1–10	9.58*	1.21 ± 0.23
Cysteine	0.1–10	8.5†	2.33 ± 0.16
Cysteinylglycine	0.1–10	9.55*	2.05 ± 0.35
N-Acetylcysteine	1.0–30	9.7†	11.9 ± 1.7

* Data from Dawson *et al.* (1972).
† Data from Torchinsky (1981).

Table 3. *Protective effects of certain thiol compounds against the covalent binding of [^{14}C]octyne to DNA or to haem*
Reaction mixtures (2ml) were similar in composition to those described in the legend to Table 1 and contained microsomal fraction from phenobarbitone-pretreated rats (3–4mg of protein). For experiments looking at covalent binding to DNA, incubation mixtures also contained 1mg of calf thymus DNA. Reaction mixtures contained one of the thiol compounds at the I_{50} concentrations shown below. After incubation at 37°C for 2min, either DNA or haem was extracted from the mixtures and purified as described in the Materials and methods section. Results represent the means ± S.E.M. for four experiments and are expressed as the percentages of covalent binding to haem or to DNA that occurred in the absence of added thiol compounds.

Compound	Concentration (mM)	Covalent binding (% of controls)	
		To DNA	To haem
Reduced glutathione	0.035	54.5 ± 6.2	98.3 ± 3.0
Cysteamine	1.21	40.8 ± 4.4	105 ± 1.8
Cysteine	2.3	49.1 ± 2.1	89.0 ± 2.2

nents, e.g. the inclusion of *N*-acetylcysteine caused an increase in the radioactive label in the component eluted at 4.8min. *N*-Acetylcysteine was used in subsequent experiments, since initial studies with cysteine gave products that were unstable during subsequent work-up procedures.

Identity of a reactive octyne metabolite trapped with N-acetylcysteine

The reaction products from incubations with octyne, *N*-acetylcysteine and microsomal fraction from phenobarbitone pretreated rats were extracted into diethyl ether, treated with diazomethane and the derivatives subjected to silica-gel h.p.l.c. with a hexane/ethanol solvent system as described in the Materials and methods section. Two components (retention times 9.8 and 11.8min) were observed. The larger of these (retention time 9.8min) was rechromatographed and subjected to n.m.r. (see below). These components were absent if octyne or *N*-acetylcysteine was omitted from the reaction mixtures. The components whose derivatives were made in this way were not sufficiently stable for g.l.c./mass spectrometry. Treatment of the microsomal extracts with methoxamine hydrochloride followed by diazomethane caused the retention time of these components after h.p.l.c. to decrease to 7.3 and 7.8min (Fig. 5). Inclusion of either [^{14}C]octyne or *N*-acetyl[^{35}S]cysteine in the incubation mixtures led to radioactivity being associated with these components. Their retention times on h.p.l.c. were the same as that of the derivatives of the *cis–trans* isomers of authentic *S*-3-oxo-octenyl-*N*-acetylcysteine. Capillary g.l.c. of the h.p.l.c. purified peaks showed them to have retention times of 10.0 and 9.9min, the same as the authentic standard, and g.l.c./mass spectrometry showed both peaks to have similar fragmentation patterns with the same molecular ion ($m/z = 330$), consistent with the *S*-3-oxo-octenyl-*N*-acetylcysteine structure. The most abundant fragmentation peaks, m/z 186, 154 and 144, represented cleavage of the molecule about the thioether bond. In addition peaks at m/z 299 ($M^+ - CH_3O$) and m/z 240 ($M^+ - CH_3O - CH_3CONH_2$) were consistent with the proposed structure.

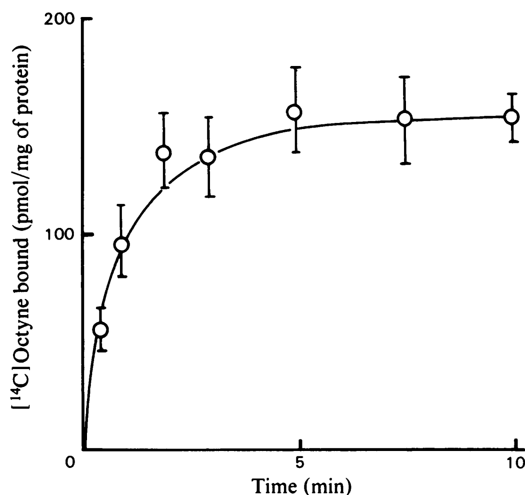


Fig. 4. *Effects of time and incubation on covalent binding of [14C]octyne to haem: formation of green pigments*
Reaction conditions were the same as those described in the legend to Fig. 1. Microsomal fraction was from phenobarbitone-pretreated rats. At the end of the incubation period, reactions were stopped with conc. H_2SO_4/methanol (1:19, v/v) (40ml). The esterified haem was separated from the haem–octyne adducts by h.p.l.c. as shown in Fig. 3, and the radioactivity in the green-pigment peaks was determined. Results represent the means ± S.E.M. for four experiments, expressed relative to the amount of microsomal protein (3–4mg) in the incubation mixtures as the haem concentration (initially 2.6 ± 0.26nmol/mg of protein) decreased with time owing to destruction of cytochrome *P*-450 by the active metabolites of octyne.

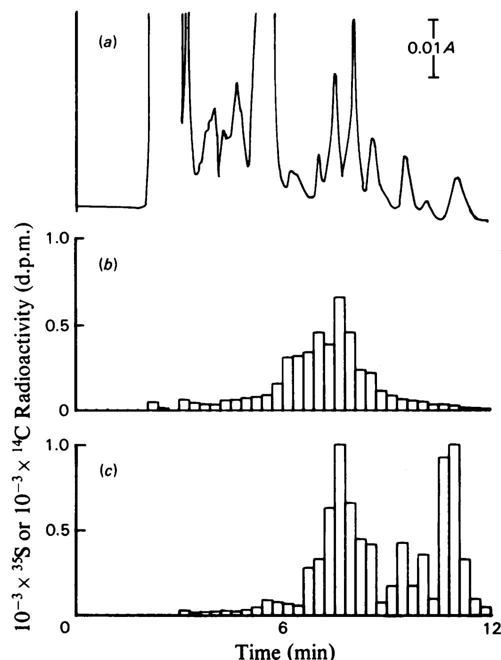


Fig. 5. *Silica-gel h.p.l.c. elution profile of reactive octyne metabolite trapped as the N-acetylcysteine adduct*
Reaction conditions were similar to those described in the legend to Fig. 1 except that mixtures contained 10mM-*N*-acetylcysteine. Incubations were for 5min at 37°C with microsomal fraction from a phenobarbitone-pretreated rat. The reaction was stopped with ice-cold 1M-HCl (2ml). After centrifugation, the reaction products were extracted into diethyl ether, treated with (i) methoxamine hydrochloride and (ii) diazomethane and subjected to h.p.l.c. with a hexane/ethanol (9:1, v/v) solvent system. (*a*) Relative absorbance from the u.v. detector at 212nm. Ordinates in (*b*) and (*c*) represent ^{14}C and ^{35}S radioactivity in fractions collected from the detector outlet. In (*b*) the microsomal reaction was conducted in the presence of [^{14}C]octyne and unlabelled *N*-acetylcysteine; (*c*) shows the result of a similar experiment with *N*-acetyl[^{35}S]cysteine and unlabelled octyne.

^1H n.m.r.

Confirmatory evidence for the structure of the octyne–*N*-acetylcysteine adduct was obtained from the 360MHz ^1H-n.m.r. spectrum of the h.p.l.c.-purified methyl ester (retention time 9.8min) dissolved in [^2H]chloroform, assisted by homonuclear decoupling experiments and comparisons with the 60MHz n.m.r. of authentic *S*-3-oxo-octenyl-*N*-acetylcysteine. The assignments of the proton resonances are as follows (p.p.m., relative number of protons, multiplicity, coupling): 6.93 (1H, d, $J = 9.6$Hz, =C*H*–S–), 6.30 (2H, d, $J = 9.6$Hz, =C*H*–CO–), 4.89 (1H, m, –C*H*OOCH$_3$), 3.79 (3H, s, –OOCH$_3$), 3.28 (2H, m, –C*H*$_2$S–), 2.48 (2H, t, $J = 7$Hz, –C*H*$_2$CO–), 2.03 (3H, s, C*H*$_3$CO–), 1.62 (2H, t, –C*H*$_2$CH$_2$CO–), 1.32 (4H, m, CH$_3$[C*H*$_2$]$_2$–), 0.84 (3H, t, $J = 7$Hz, C*H*$_3$[CH$_2$]$_2$–). There was a coupling of 7.1Hz from the –CH of the amino acid to the –NH proton, which is hidden under one of the vinyl proton resonances $\delta = 6.30$p.p.m. The small size of the coupling constant ($J = 9.6$Hz) of the vinyl protons suggests that in this component the double bond is in the *cis* position.

Reaction of octyn-3-one with N-acetylcysteine under physiological conditions

Octyn-3-one reacted rapidly with a 10-fold molar excess of *N*-acetylcysteine non-enzymically at pH7.4 and 37°C with a $t_{\frac{1}{2}}$ of about 6s to produce a product that had h.p.l.c., g.l.c. and mass-spectroscopic properties identical with those of authentic *S*-3-oxo-octenyl-*N*-acetylcysteine (Fig. 6). No reaction of octyn-3-ol or octyne with *N*-acetylcysteine occurred over a 10min incubation period. The reaction of octyn-3-one with *N*-acetylcysteine was

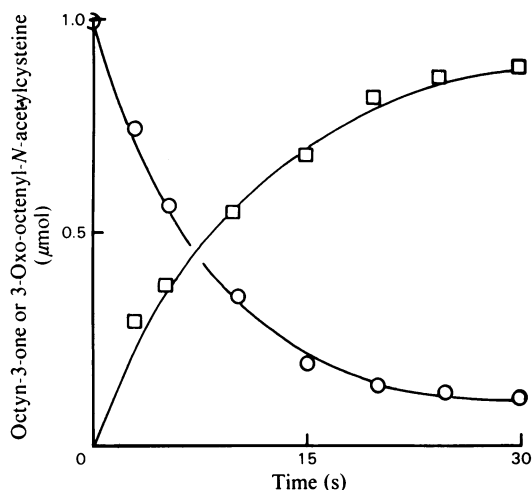


Fig. 6. *Effects of incubation time on the non-enzymic formation of S-3-oxo-octenyl-N-acetylcysteine from octyn-3-one and N-acetylcysteine*

Reaction mixtures (1 ml) at pH 7.4 and 37°C contained octyn-3-one (1 μmol) and *N*-acetylcysteine (10 μmol). Reactions were terminated by the addition of 1 M-HCl (1 ml), and 50 μl of the incubation mixture was immediately subjected to reverse-phase h.p.l.c. Results represent the mean of two experiments. ○, Concentration of octyn-3-one (μmol); □, concentration of *S*-3-oxo-octenyl-*N*-acetylcysteine (μmol).

pH-dependent; at pH 1.5, the half-life of the octyn-3-one was greater than 60 min. At pH 7.4 or at pH 10.0 there was no detectable reaction of octyn-3-one with haem or with protoporphyrin IX and no evidence of green-pigment formation.

Metabolic activation of octyn-3-ol: trapping of active metabolite with N-acetylcysteine

When octyn-3-ol was incubated with control rat liver microsomal fraction, NAD^+ or $NADP^+$ and *N*-acetylcysteine, the adduct isolated had the same retention times on h.p.l.c. and on g.l.c. and the same fragmentation pattern on mass spectrometry as had *S*-3-oxo-octenyl-*N*-acetylcysteine. The formation of this adduct was linear with time over a 10 min incubation period. Recovery of authentic *S*-3-oxo-octenyl-*N*-acetylcysteine added to microsomal incubation mixture was $87.7 \pm 0.7\%$ (mean $\pm$ S.E.M. for four experiments). Initial reaction rates were greater with NAD^+ as the cofactor (19.5 nmol/min per mg of protein) than with $NADP^+$ (6.3 nmol/min per mg of protein). Increasing the concentration of the latter to 5 mM did not increase the initial rate of formation of the adduct.

The initial rate of formation of *S*-3-oxo-octenyl-*N*-acetylcysteine from oct-1-yne when incubated with control rat liver microsomal fraction, *N*-acetylcysteine and NADPH was 0.54 ± 0.12 nmol/min per mg of protein (mean $\pm$ S.E.M. for four experiments). Inclusion of NAD^+ in the reaction mixtures did not significantly increase the initial rate of formation of the adduct, suggesting that conversion of octyne into octyn-3-ol may be the rate-limiting step in this mechanism.

Discussion

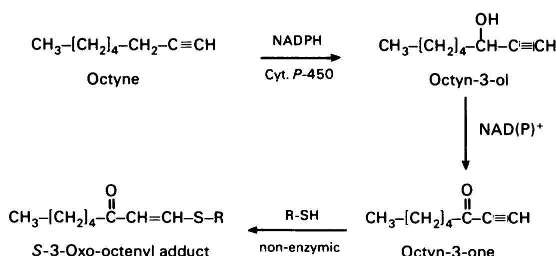
Characterization of the enzyme system responsible for the metabolic activation of octyne

The results presented in this paper show octyne to undergo metabolic activation by an enzyme system having characteristics typical of the cytochrome *P*-450-dependent mixed-function oxidases (Table 1) to derivatives that covalently bind to haem, to protein or to DNA (Figs. 1 and 2). Although the time course for covalent binding of the activated octyne to haem was similar to that to protein (Fig. 4), indicating that the same forms of cytochrome *P*-450 may be catalysing both reactions, the evidence suggests that two distinct activating mechanisms may be involved.

Identity of the activated octyne metabolite

Activation of acetylenes classically involves the formation of an oxirene intermediate (Wade *et al.*, 1979; Ortiz de Montellano & Kunze, 1980*b*). The work of Kunze *et al.* (1983) demonstrated that the octyne–haem adduct contained the saturated 2-oxo-octyl substituent. Our results show an octyne metabolite trapped with *N*-acetylcysteine to be different, containing the unsaturated 3-oxo-octenyl substituent. Analysis of microsomal extracts from incubation mixtures containing octyne and *N*-acetylcysteine by g.l.c./mass spectrometry showed only trace amounts of the saturated *S*-2-oxo-octyl-*N*-acetylcysteine (I. N. H. White & P. B. Farmer, unpublished work). A mechanism for the formation of the 3-oxo-octenyl adduct with *N*-acetylcysteine involving an oxirene reactive intermediate seemed unlikely. The possibility of an alternative activating mechanism for octyne was investigated.

Octyn-3-one reacted rapidly non-enzymically with *N*-acetylcysteine under physiological conditions to form a product that was the same as that formed when microsomal fraction was incubated with octyne, *N*-acetylcysteine and an NADPH-generating system. The evidence suggests that microsomal fraction also possesses an $NAD(P)^+$-dependent enzyme(s) capable of oxidizing octyn-3-ol to octyn-3-one. It is therefore proposed that, with respect to covalent binding to protein or to DNA, octyne undergoes the two-stage activating mechanism shown in Scheme 1. This involves a rate-limiting 3-hydroxylation of octyne followed by oxidation of the octyn-3-ol to the active metabolite,



Scheme 1. *Proposed pathway for the metabolic activation of octyne*

octyn-3-one. A non-enzymic Michael addition of the octyn-3-one then occurs with a thiol group to form the *S*-oxo-octenyl derivative.

Implications for the metabolic activation of other ethynyl-substituted compounds

Such an activation mechanism as the one proposed clearly cannot occur with acetylene, known to bind covalently to haem (White, 1978; Ortiz de Montellano & Kunze, 1980*a*), or in the case of contraceptive 17α-ethynyl steroids, where C-17 is fully substituted. The involvement of the ethynyl substituent in the possible covalent binding to protein or to DNA remains to be elucidated.

Earlier evidence for the occurrence of microsomal hydroxylation at a carbon atom α to the acetylenic bond comes from the work of Lindeke *et al.* (1978) with the oxotremorine analogue *N*-(5-pyrrolidinopent-3-ynyl)succinimide and from the studies made by Sacher *et al.* (1968) with certain propargyl-substituted insecticide synergists.

An activation mechanism such as the one proposed in the present paper could have a more general application to compounds containing a terminal carbon–carbon double bond. Oct-1-en-3-one reacts rapidly and non-enzymically under physiological conditions with *N*-acetylcysteine (I. N. H. White, unpublished work). In a similar manner the allenic substituted compound safrole is metabolically converted via the 1′-hydroxy derivative into 1′-oxo-safrole, which can react non-enzymically with the thiol group of reduced glutathione (Fennell, 1983).

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