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The Metabolic Oxidation of the Ethynyl Group in 4-Ethynylbiphenyl *in vitro*

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1. The metabolism of 4-ethynylbiphenyl has been studied *in vitro* with subcellular fractions of normal and induced rat liver, and rat intestinal microflora (caecal contents). 2. Oxidation of the ethynyl group occurred in washed microsomal preparations, but not significantly in soluble fractions. Oxidation was NADPH-dependent, was inhibited by CO and stimulated by pretreatment with phenobarbitone or 3-methylcholanthrene. 3. Oxidation of the ethynyl group by a microsomal fraction preceded aromatic hydroxylation and no metabolites containing the intact ethynyl group were detected. 4. The major metabolite in liver fractions was biphenyl-4-ylacetic acid. This was the only product produced by a modified Udenfriend system. 5. Metabolism of 4-ethynylbiphenyl by rat caecal contents under anaerobic conditions produced very small amounts of 4-vinylbiphenyl. 6. In a modified Ames test with *Salmonella typhimurium* TA98, 4-ethynylbiphenyl gave a weak positive result that was doubled after 'activation' with an induced rat S9 fraction.

Metabolism studies of 4-ethynylbiphenyl in rat and rabbit (Wade, 1978; Wade *et al.*, 1979) have shown that unlike other acetylene compounds, oxidation of the ethynyl group occurred readily to produce biphenyl-4-ylacetic acid and 4-hydroxybiphenyl-4-ylacetic acid, the latter being excreted as the major product in both species. No metabolites containing an intact ethynyl group were detected. The work reported in the present paper was performed to identify the subcellular site of oxidation of the ethynyl group and to elucidate the mechanism of this reaction. Possible metabolism of the acetylenic bond by gut microflora was also investigated, as it is known that reduction of acetylene to ethylene is catalysed by some anaerobic bacteria (Dilworth, 1966; Schollhorn & Burns, 1966). In addition the possible occurrence of reactive and potentially toxic intermediates in the metabolism of 4-ethynylbiphenyl was investigated by using an Ames mutagenicity test.

Experimental

Radiochemical

4-[^{14}C]Ethynylbiphenyl (sp. radioactivity 0.33 Ci/mol) was synthesized (Wade, 1978). The chemical and radiochemical purity, by t.l.c. chromatography [silica gel G, light petroleum (b.p. 60–80°C)/toluene (19:1, v/v)] and radioscanning, were >99.0%.

Animals

Male Wistar albino rats (140–160 g) were used for all studies. Intestinal contents were collected from adult rats that had been resident in the Departmental Animal Unit for at least 7 days.

Scintillation counting

Aqueous and non-aqueous samples were dissolved in 0.5 ml of water and 4 ml of a Synperonic NXP/toluene/2,5-diphenyloxazole scintillant (Wood *et al.*, 1975). Areas from silica-gel thin-layer chromatograms were counted for radioactivity in a similar system but with the addition of 4% thixotropic gel (Packard, Caversham, Berks., U.K.).

Mass spectrometry

Electron-impact mass spectra were recorded on an A.E.I. MS12 mass spectrometer. Spectra were obtained by direct insertion of samples at an accelerating voltage of 8 kV and an ionizing voltage of 70 eV.

Chromatography

T.l.c. was performed on 0.25 mm layer silica-gel G.F.254 plates or on aluminium-backed plates coated with silica gel G.F.254 (Merck, Darmstadt,

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Germany, supplied by Schiecher and Schull, London). Solvent systems used were (A) toluene/methanol/ethyl acetate/acetic acid (90:10:17:3, by vol.) and (B) chloroform/methanol (1:1, v/v).

Incubations with caecal contents

The incubations of [^{14}C]ethynylbiphenyl *in vitro* with samples of caecal contents were performed by the method of Scheline (1966). The contents of a single caecum were suspended in 10ml of 0.1M-sodium/potassium phosphate buffer, pH 7.4 (previously degassed under vacuum), and the supernatant fraction was left for 5 min and then taken for incubation. The medium for incubations comprised 0.5% (w/v) each of D-glucose, yeast extract and proteose-peptone in 0.1M-phosphate buffer, pH 7.4. Portions of this medium (10ml) were placed in 25-ml Thunberg tubes and the contents were sterilized by autoclaving at 110°C for 15 min (103.5 kPa). When cooled, test material and caecal extract (1.0ml) were added to the tubes, which were immediately evacuated to remove any dissolved or gaseous O_2 and then N_2 was admitted from an N_2-filled bladder. This evacuation and flushing procedure was repeated three times to remove as much O_2 as possible.

[^{14}C]Ethynylbiphenyl, in combination with pure unlabelled material (1–10mg containing 0.5–5.0μCi of ^{14}C) dissolved in 10–50μl of dimethylformamide, was added to the caecal mixture and incubated at 37°C for 48–72h in N_2 as described above. Incubations were performed (*a*) with untreated caecal extract, (*b*) with boiled caecal extract and (*c*) with caecal extract to which antibiotics (neomycin and tetracycline) had been added. The incubation mixtures were extracted with 5 × 20ml of ethyl acetate and the extracts were combined, evaporated under reduced pressure and the residues redissolved in ethyl acetate (100–500μl) for analysis by t.l.c. in solvent systems (A) and (B). Radioactivity was determined by radioscanning and autoradiography.

A larger scale preparation of the metabolite formed from 4-ethynylbiphenyl was made by using the caecal contents of five rats. The concentrated ethyl acetate was purified by column chromatography [silica-gel 80–230 mesh, 20cm × 2.0cm column, with light petroleum (b.p. 60–80°C)/toluene (19:1, v/v) as eluting solvent], and by preparative t.l.c. About 60μg of the metabolite was isolated and purified for mass spectrometry.

Studies using Udenfriend's system

The incubation system used was a modification of that described by Udenfriend *et al.* (1954). The solutions were made up with 0.1M-phosphate buffer (pH 5.5) to give the final concentrations: ferrous sulphate (10mM), EDTA (30mM) and ascorbic acid (50mM). [^{14}C]Ethynylbiphenyl (500μg, 1.1μCi) was added in dimethylformamide (100μl) to 10ml portions of the incubation medium to give a final concentration of 0.3mM. Incubation was carried out at 35°C in air for 10h; control incubations contained buffer alone. After incubation the mixtures were extracted with ethyl acetate (4 × 20ml) and the combined radioactive extracts were concentrated and investigated by t.l.c. in solvent systems (A) and (B).

Studies with rat liver preparations

The metabolism of 4-ethynylbiphenyl was studied using a washed microsomal fraction (100000$g_{av.}$ pellet), a 10000$g_{av.}$ supernatant fraction and a 100000$g_{av.}$ supernatant fraction (soluble fraction) from rat liver. These preparations were made using livers from male Wistar albino rats (140–160g) that were (*a*) saline-treated, (*b*) pretreated with phenobarbitone (70mg/kg body wt. intraperitoneally injected twice daily for 4 days) and (*c*) pretreated with 3-methylcholanthrene (20mg/kg body wt. intraperitoneally injected daily for 3 days). Rats in all groups were killed at 20–22h after the last injection.

Rats were killed by cervical dislocation and the livers were quickly excised into ice-cold KCl solution (1.15%, w/v). A 25% homogenate was prepared by scissor chopping and homogenizing in ice-cold KCl (1.15%, w/v) with a motor-driven Potter–Elvehjem glass/Teflon homogenizer (four complete slow strokes). The homogenate was centrifuged at 10000$g_{av.}$ (MSE High-Speed 18 centrifuge) for 20min, the supernatant fraction decanted and used for metabolism studies. A microsomal fraction was prepared by centrifugation of the 10000$g_{av.}$ supernatant fraction at 100000$g_{av.}$ (Beckman L5-65 ultracentrifuge) for 60min. The pellet was resuspended in KCl (1.15%, w/v) by using a Potter–Elvehjem homogenizer (hand-held, three strokes) and recentrifuged at 100000$g_{av.}$ for 60min. The thrice-washed microsomal pellet was finally resuspended in phosphate buffer (0.1M, pH 7.4) to give a concentration equivalent to 1g of liver/ml.

Portions of the 10000$g_{av.}$ supernatant fraction (2.0ml, equivalent to 0.5g of liver) were incubated with [^{14}C]ethynylbiphenyl (0.5μCi, 0.63mM) in 10μl dimethylformamide, in the presence of isocitrate (10μmol), $NADP^+$ (1.0μmol), isocitrate dehydrogenase (2.0 units, 0.2mg) and $MgSO_4$ (20μmol) at 37°C in a shaking water bath in air (10-ml unstoppered flasks) for periods of 1 min to 1h. One unit of enzyme activity is the amount required to convert 1μmol of substrate/min.

Portions of the 100000$g_{av.}$ supernatant fraction (2.0ml, equivalent to 0.5g of liver) were incubated with [^{14}C]ethynylbiphenyl (as described above) in the presence of glucose 6-phosphate (12μmol), $NADP^+$ (1.0μmol), glucose 6-phosphate dehydrogenase (50 units, 0.25mg) and $MgCl_2$ (20μmol).

Portions of microsomal suspensions (2.0ml, equivalent to 4mg of microsomal protein) were incubated with [^{14}C]ethynylbiphenyl (as described above) in the presence of isocitrate (10μmol), isocitrate dehydrogenase (2.0 units, 0.2mg), $MgSO_4$ (20μmol) and $NADP^+$ (1.0μmol). In all cases the reaction was started by addition of [^{14}C]ethynylbiphenyl after pre-incubation of microsomal fraction and cofactors for 2min at 37°C; final incubations were carried out for time periods of 1 to 60min. The reaction was stopped by the addition of 1ml of ice-cold acetone followed by freezing at −60°C in a methanol bath.

Extraction of metabolites

The frozen incubation mixtures were freeze-dried and extracted with ethyl acetate/methanol (1:1, v/v). Freeze-drying was complete within 6h and preliminary checks were performed to investigate possible loss of radioactivity due to sublimation at the very-low vapour pressures obtained. No significant loss of radioactivity was observed after 6h (<1.0%) but samples left under vacuum overnight lost between 1 and 5% of radioactivity.

The organic extracts were dried under a stream of N_2 and redissolved in 600μl of the same solvent. Portions (50μl) were subjected to t.l.c. as previously described.

Mutagenicity studies (Ames test) in vitro

The methods used were as described by Ames *et al.* (1975), with the exception that the concentrations of histidine were decreased to obtain a lower rate of spontaneous reversion.

As 4-ethynylbiphenyl was insoluble in the medium, it was added in dimethylsulphoxide at a level of 400μg/plate. The bacteria used was *Salmonella typhimurium* TA98. For metabolic activation, an S9 fraction (9000g supernatant fraction) obtained from the livers of rats pretreated with Aroclor 1254 was used. Benzopyrene (2μg) and nitrofluorene (1μg) were used as known positive standards.

Results

Metabolism by rat liver fractions

(*a*) *10000gav. supernatant fraction.* T.l.c. of extracts of incubation mixtures showed that several metabolites were produced. The identity of the major metabolite was confirmed as biphenyl-4-ylacetic acid by its R_F value in solvents (A) and (B), co-chromatography with authentic material and by mass spectrometry (Wade *et al.*, 1979). Other metabolites accounted for less than 10% of the total metabolite radioactivity and apart from 4-hydroxybiphenyl-4-ylacetic acid were not present in large enough quantities to be identified.

(*b*) *100000$g_{av.}$ supernatant fraction (soluble fraction).* Biphenyl-4-ylacetic acid was the only metabolite detected in these studies and was only produced in trace amounts (<1.0% normal microsomal metabolism) in incubations of longer than 40min duration.

(*c*) *100000$g_{av.}$ pellet (microsomal fraction).* With a normal microsomal fraction, and with a microsomal fraction from phenobarbitone-induced and 3-methylcholanthrene-induced rats, the major metabolite was biphenyl-4-ylacetic acid. The formation of this metabolite was O_2- and NADPH-dependent and inhibited by CO. As with the 10000$g_{av.}$ supernatant fraction other metabolites accounted for less than 10% of total metabolite radioactivity.

The rate of metabolism of 4-ethynylbiphenyl was significantly increased by both phenobarbitone and

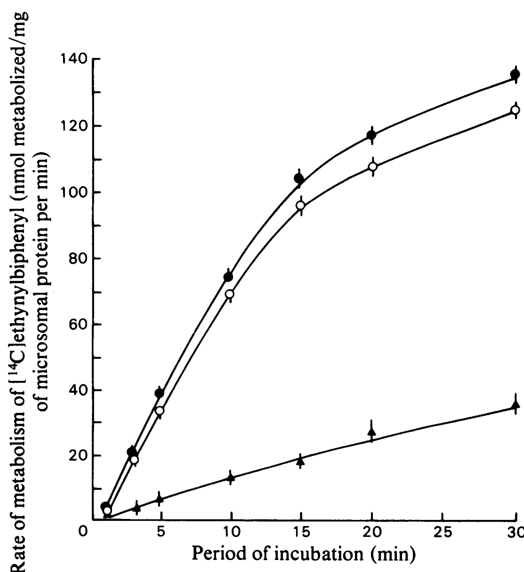

Fig. 1. *Rate of production of 4-biphenyl-4-ylacetic acid in untreated and in induced rat liver microsomal fraction*

The metabolism of [^{14}C]ethynylbiphenyl by microsomal preparations of rat liver (untreated and induced) was determined at various time intervals by extraction of radioactivity from incubation mixtures and determination of the radioactivity in the major metabolite, 4-biphenyl-4-ylacetic acid, by scintillation counting of t.l.c. plate scrapings. Results are expressed as nmol of 4-biphenyl-4-ylacetic acid produced/mg of microsomal protein per min, and are means ± S.E.M. for four male rats; ▲, untreated rats; ●, induced with 3-methylcholanthrene (20mg/kg body wt. intraperitoneally daily for 4 days); ○, induced with phenobarbitone (70mg/kg body wt. intraperitoneally twice daily for 4 days).

3-methylcholanthrene treatments. The rate of production of biphenyl-4-ylacetic acid was linear over 10 min with the induced microsomal fraction and over 20 min with the normal microsomal fraction. During the linear phase the rate of production of biphenyl-4-ylacetic acid (nmol produced/mg of microsomal protein per min) was increased approx. 6-fold by both inducing agents (Fig. 1).

Metabolism by intestinal microflora

Only one metabolite of 4-ethynylbiphenyl was isolated from the incubations with caecal contents and this in very small amounts (10–15 μg/incubation). Mass spectrometry showed that this compound was identical with 4-vinylbiphenyl, *m/e* 180 (Fig. 2). This compound was not formed in the presence of boiled caecal contents.

Studies with Udenfriend's system

Incubation of 4-ethynylbiphenyl with Udenfriend's system resulted in the production of biphenyl-4-ylacetic acid (identity confirmed by co-chromatography and mass spectrometry). No other products were detected.

Ames mutagenicity test

The results of this study are shown in Table 1. Mutagenicity was observed both without and with 'activation' by a 9000 $g_{av.}$ supernatant fraction. The reversion rate was very low, although doubled by activation.

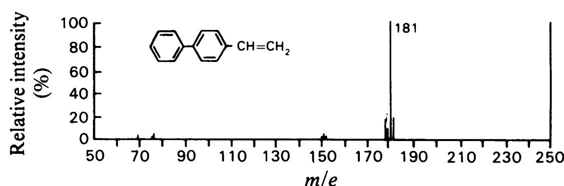

Fig. 2. *Mass spectrum of the metabolite produced by rat caecal contents when incubated with 4-ethynylbiphenyl*

Table 1. *Results of the Ames mutagenicity test*
Abbreviations used: −S9, without induced 9000 *g* rat liver supernatant fraction; +S9, with induced 9000 *g* rat liver supernatant. Results are duplicates of each plate.

	Number of revertant TA98/plate	
	−S9 mix	+S9 mix
(*a*) Control (dimethylsulphoxide)	16, 31	13, 15
(*b*) 4-Ethynylbiphenyl (400 μg)	48, 47	84, 98
(*c*) Benzopyrene (2 μg)	—	107, 108
(*d*) Nitrofluorene (1 μg)	175, 150	—

Discussion

Incubation of [^{14}C]ethynylbiphenyl with caecal contents produced only one metabolite, 4-vinylbiphenyl, in which the acetylene group of the original material had been reduced by a two-electron transfer to the alkene analogue. Reduction of acetylene to ethylene is known to occur in some bacteria, and the reaction is used as a method of assaying nitrogen fixation in plants (Dilworth, 1966; Schollhorn & Burns, 1966). The nitrogen molecule is very similar to that of acetylene, being linear and possessing a triple bond of similar dimensions. It is noteworthy that acetylene has been shown to competitively inhibit nitrogen fixation (Dilworth, 1966). Although no work appears to have been performed on the metabolism of acetylene and acetylenic compounds by gastrointestinal micro-organisms, it seems reasonable to suppose that similar reductions could occur. Reduction of acetylene occurs under highly anaerobic conditions and is strongly inhibited by O_2. It is probable that in aerobic organisms that exhibit the reduction, the process occurs in an O_2-free microenvironment (Brock, 1974). By using deuterated water Dilworth (1966) showed that the two-electron transfer did not involve the acetylide ion, $HC{\equiv}C^{-\cdot}$, as an intermediate, for the ethylene produced was not tri-deuterated.

There are several species of bacteria within the gastrointestinal tract that possess reductase activity (*Klebsiella* spp. and *Clostridium* spp.), which may have been responsible for the reduction of 4-ethynylbiphenyl (Drasar *et al.*, 1970). It is difficult to estimate the importance of this reduction *in vivo*, for although only very small amounts of the vinyl compound were produced *in vitro* this may have been due to oxygen contamination. Neither 4-vinylbiphenyl nor its likely metabolites, biphenyl-4-ylglycol and biphenyl-4-ylmandelic acid, were isolated from the faeces of rats dosed with 4-ethynylbiphenyl *in vivo*, except for small amounts of biphenyl-4-ylmandelic acid. The contribution of the gut microflora to the metabolism of 4-ethynylbiphenyl seems, therefore, to be small, although the hydrolysis of the conjugated biliary metabolites was significant (Wade, 1978).

The mechanism of the metabolism of the ethynyl group remains unproven. However, the simple addition of water suggested by El Masry *et al.* (1958) for the metabolism of phenylacetylene seems untenable for ethynylbiphenyl. The metabolism of ethynylbiphenyl is catalysed by the microsomal mono-oxygenase system, is induced by phenobarbitone and 3-methylcholanthrene and inhibited by CO. Metabolism therefore proceeds by oxygen-

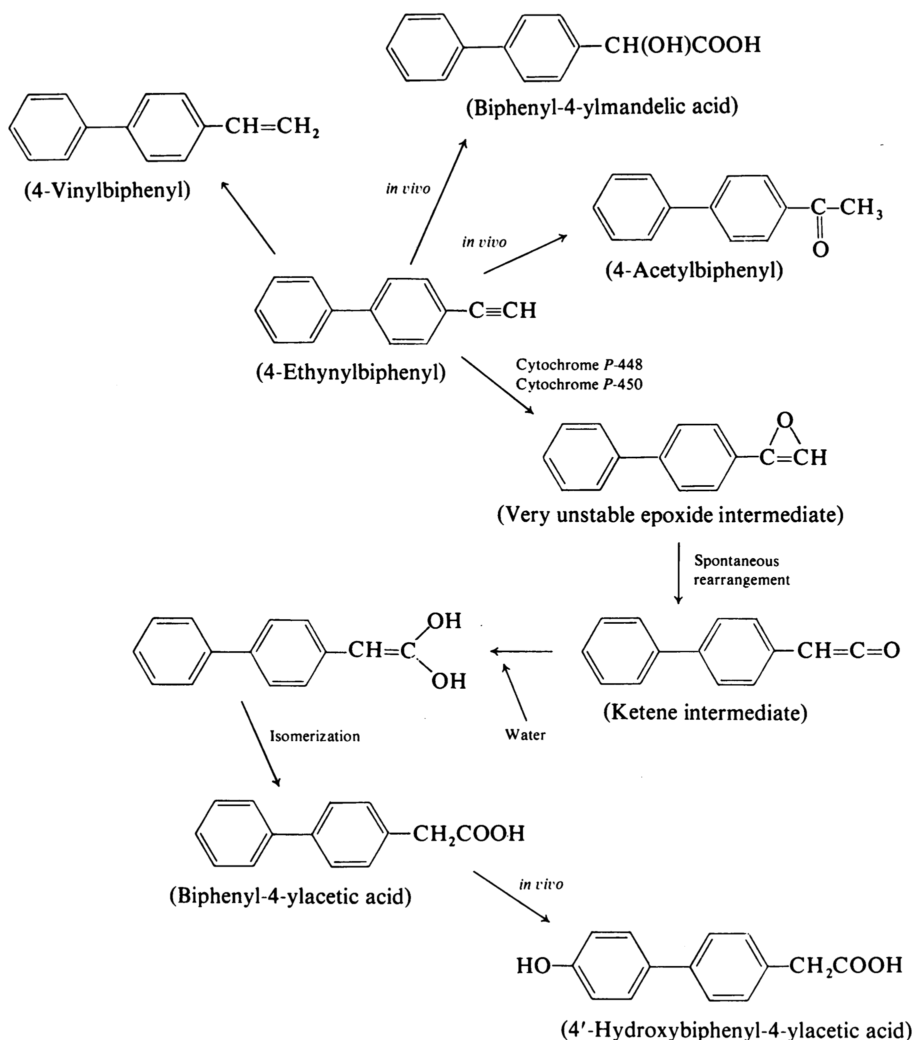

Fig. 3. *Metabolites of 4-ethynylbiphenyl in vitro and in vivo and a possible mechanism for the oxidation of the triple bond*
Observations *in vivo* are taken from Wade *et al.* (1979)

ation, probably by addition of oxygen across the carbon≡carbon triple bond, and it is interesting that this is apparently equally well catalysed by either cytochrome *P*-450 or cytochrome *P*-448 (Fig. 1). The formation of epoxides in the metabolic oxidation of double bonds is well established (Daly *et al.*, 1972) and a similar process seems likely to occur with the ethynyl group. The stability of the epoxide formed, however, would be much less than for the corresponding alkenes and would probably rearrange immediately to the ketene. The very reactive ketene thus produced would rapidly hydrolyse to produce the corresponding acid (Fig. 3). Such a mechanism involving unstable or reactive intermediates would explain the failure to isolate intermediate metabolites both *in vivo* and *in vitro*.

The mechanism of phenylacetylene oxidation suggested by El Masry *et al.* (1958) involved the asymmetrical addition of water (contrary to the Markovnikov principle). This would in the case of 4-ethynylbiphenyl produce an aldehyde intermediate. To produce the acid derivative this aldehyde would have to be oxidized, a reaction that is associated with the enzymes of the soluble fraction of liver homogenates such as aldehyde oxidase, xanthine oxidase and alcohol dehydrogenase. The mechanism suggested by El Masry and his colleagues seem untenable for 4-ethynylbiphenyl as

biphenyl-4-ylacetic acid was formed readily by washed microsomal fraction. The symmetrical addition of water to 4-ethynylbiphenyl would result in the formation of a ketone, the oxidation of which again requires the enzymes of the soluble fraction. The oxidation products of such a ketone intermediate, as well as the ketone itself, were isolated in trace amounts *in vivo* (Wade *et al.*, 1979), suggesting that this might represent a minor route of metabolism. Support for the epoxide mechanism is found in the work of McDonald & Schwab (1964), who studied the peroxidation of acetylenes. These workers showed that phenylacetylene was oxidized to phenylacetic acid by trifluoroperacetic acid and produced evidence for the formation of a ketene intermediate, although neither this nor the oxirene precursor were stable enough to be isolated. It is interesting that in the present study Udenfriend's system produced biphenyl-4-ylacetic acid from 4-ethynylbiphenyl with no apparent intermediates.

The evidence from the present work suggests that the route of metabolism for the acetylenic group in 4-ethynylbiphenyl is by liver microsomal monooxygenase oxidation *in vitro*, and it is probable that the same mechanism is responsible for the formation of biphenyl-4-ylacetic acid, and 4-hydroxybiphenyl-4-ylacetic acid *in vivo*. However, the discovery of very small amounts of biphenyl-4-ylacetic acid and 4-acetylbiphenyl *in vivo* suggests that a minor pathway involving direct hydration of the acetylenic bond also occurs. Clearly the structure of the compound containing the acetylenic bond is important in determining the route of metabolism, for example there is evidence that some acetylenic compounds characteristically undergo hydroxylation α to the triple bond. Examples are *N*-[5-(*N*-pyrrolidinyl)pent-3-ynyl]succinimide (Lindeke *et al.*, 1978) and pargyline (*N*-methyl-*N*-prop-2-ynylbenzenemethanamine; Diehl *et al.*, 1976). Hydroxylation in the first example was microsomal, inducible with phenobarbitone, and inhibited by compound SKF 525A, CO and nicotinamide; metabolism before phenobarbitone induction was minimal, and it was concluded that the reaction was mediated by an inducible cytochrome *P*-450 system (Lindeke *et al.*, 1978).

Many aromatic and olefinic compounds are converted by epoxidation into intermediate arene and alkene oxides by mammalian mono-oxygenases. Due to their highly electrophilic reactivity, such oxirenes can bind to proteins, RNA, and DNA, which is associated with mutagenic and/or carcinogenic properties. Their effectiveness depends on several factors, such as the stability of the epoxide towards spontaneous (or enzymic) degradation and on its electrophilic reactivity (Daly *et al.*, 1972; Oesch, 1973). The possible occurrence of highly reactive and short-lived oxirene and ketene intermediates in the metabolism of 4-ethynylbiphenyl is supported by the findings of White (1978), who showed that some acetylenes apparently degrade cytochrome *P*-450 haem after metabolic activation but do not bind to other proteins.

The results of the Ames test showed that 4-ethynylbiphenyl was mutagenic in the bacterial system used, both with and without 'activation' by a $9000\,g_{av.}$ rat liver supernatant fraction. Although activation doubled the reversion rate, the amount observed was still low, about 8 revertants/10μg of 4-ethynylbiphenyl (0.007 revertants/nmol). Short-term tests such as the Ames test would need to be supported by carcinogenicity and teratogenicity studies *in vivo* before any conclusions could be made regarding the potential mutagenicity of 4-ethynylbiphenyl.

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