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Metabolic Activation of Acetylenic Substituents to Derivatives in the Rat Causing the Loss of Hepatic Cytochrome *P*-450 and Haem

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1. A number of acetylenic-substituted steroidal and non-steroidal compounds, including 2,2-dipropargylacetamide, pregna-2,4-dien-20-yno[2,3-*d*]isoxazol-17-ol (Danazol) and acetylene gas, when administered to rats *in vivo* brought about a decrease in the concentrations of hepatic microsomal cytochrome *P*-450 and haem. Abnormal haem-breakdown products, 'green pigments', and porphyrins accumulated in the livers of these animals. 2. For loss of microsomal cytochrome *P*-450 to occur *in vitro*, metabolic activation of the acetylenic substituent was necessary. The enzyme system responsible required NADPH and air, and was induced by pretreatment of rats with phenobarbitone; these are characteristics typical of the microsomal mixed-function oxidases. 3. When rats were dosed with 17α-ethynyl-17β-hydroxyandrost-4-en-3-one (ethynyltestosterone, 1 mmol/kg) the pattern of green pigments extracted from the liver 4 h after dosing and separated by t.l.c. was quite different from that in rats given 17β-hydroxy-17α-vinylandrost-4-en-3-one (vinyltestosterone), suggesting that reduction of the unsaturated triple bond to a double bond is not normally part of the metabolic activation pathway of the acetylenic substituent. 4. The green pigments extracted from the livers of rats 4 h after the administration of the acetylenic-substituted compounds (1 mmol/kg) when separated by silica-gel t.l.c. had variable R_F values. The number and distribution of green pigments was characteristic for each compound examined. There was little correlation between the total loss of hepatic microsomal haem and the apparent intensity of the green pigments seen on the thin-layer chromatograms. 5. After incubation of [^{14}C]acetylene *in vitro* with microsomal preparations from phenobarbitone-pretreated rats and a NADPH-generating system, no significant covalent binding to microsomal protein was detected over a 30 min incubation period, although under similar conditions there was a significant loss of cytochrome *P*-450.

Acetylenic-substituted (–C≡C–) contraceptive steroids such as 17β-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one (norethindrone, Fig. 1, compound I) can be metabolically activated by mixed-function oxidase type of enzymes in the livers of rats to derivatives capable of causing a loss of hepatic microsomal cytochrome *P*-450 (White & Muller-Eberhard, 1977). Abnormal haem-breakdown products, 'green pigments', accompany this loss of cytochrome *P*-450. Green pigments are not formed in the liver after the administration of naturally occurring steroids, such as oestradiol, progesterone or the synthetic ethyl-substituted analogue of norethindrone, the anabolic steroid 17α-ethyl-17β-hydroxy-19-nor-androst-4-en-3-one (norethandrolone). Metabolic activation of norethindrone also leads to the formation of derivatives which covalently bind to liver macromolecules (Bolt & Kappus, 1974; Kappus & Remmer, 1975). It has been suggested that this involves the formation of a 4β,5β-epoxy intermediate (Cook *et al.*, 1974; Kappus & Remmer, 1975).

Our earlier results (White & Muller-Eberhard, 1977) suggested that only steroids with an acetylenic substituent were capable of giving rise to green pigments in the liver. The properties of these abnormal haem-breakdown products appeared similar in a number of respects to green pigments isolated from the livers of rats given allyl (H_2C=CH–CH_2–)-substituted compounds (De Matteis, 1971; De Matteis & Unseld, 1976). We wished to establish if the metabolic activation of acetylenic substituents to derivatives capable of causing a loss of liver cytochrome *P*-450 and the formation of green pigments in the liver of rats was a general phenomenon. A number of acetylenic-substituted steroid and non-steroidal compounds such as a possible male contraceptive, pregna-2,4-dien-20-yno[2,3,*d*]isoxazol-17-ol (Danazol, Fig. 1, compound II), 2,2-dipropargylacetamide (Fig. 1, compound III) and acetylene gas (Fig. 1, compound

IV) have been examined in this respect. The possibility that metabolic activation of acetylene might give rise to derivatives capable of covalently binding to microsomal protein has also been studied *in vitro*. The results are presented in this paper.

Experimental

Chemicals

Norethindrone acetate, bovine serum albumin, protoporphyrin IX, $NADP^+$, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K. 17α-Ethyl-17β-hydroxyandrostan-3-one (5αH), 17α-ethynyl-17α-hydroxyandrost-4-en-3-one (ethynyltestosterone) and 17β-hydroxy-17α-vinylandrost-4-en-3-one (vinyltestosterone) were from Steraloids Ltd., Croydon, Surrey, U.K. Danazol was a gift from Sterling-Winthrop Research Institute, Renesselaer, NY 12144, U.S.A. The purity of the steroids was ascertained by t.l.c. Each steroid migrated as a single spot on silica-gel plates (Silica gel 60; layer thickness 0.25mm; E. Merck, Darmstadt, Germany). A chloroform/acetone (19:1, v/v) solvent system was used and the spots were detected with a concentrated H_2SO_4 spray (Wald, 1965). 2,2-Dipropargylacetamide was a gift from Professor C. Rimington, Department of Chemical Pathology, Kings College Hospital Medical School, London SE5 8RK, U.K. This was purified by boiling an aqueous solution with activated charcoal (Norit AL activated, Hopkin and Williams, Chadwell Heath, Essex, U.K.), filtering and recovering the white crystals on cooling. These were dried *in vacuo* over P_2O_5 (m.p. 130.5°C). Acetylene (99.5% purity) was from British Oxygen Ltd., London SW19, U.K. [U-^{14}C]Acetylene (119mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. Trioctanoin was from Eastman Organic Chemicals, Kirby, Liverpool, U.K. All remaining chemicals were from BDH Ltd., Poole, Dorset, U.K. unless mentioned otherwise.

Treatment of animals

Male Wistar rats (140–150g) were used. These were fed M.R.C. 41B pellets and had free access to water. Phenobarbitone sodium was given as a 0.1% (w/v) solution in the drinking water for 7 days followed by normal tap water for 24h before being dosed. Steroids and dipropargylacetamide were dissolved in trioctanoin and given intraperitoneally in a volume of 0.3–0.5ml at the doses indicated, between 09:00 and 10:00h. Ethynyltestosterone was only poorly soluble at the required concentration in trioctanoin and was administered as a suspension. Control animals received trioctanoin only. Rats were exposed to acetylene in glass-fibre chambers as described by Magos *et al.* (1970). Air flow was 5 litres/min and acetylene 250ml/min. Free access to drinking water was allowed but food was with-held for the duration of the exposure. Commercial acetylene showed an absorption band at 1750cm^{-1} in the i.r. spectrum, probably due to acetone contamination (C=O stretching). To remove this and other impurities the gas was passed through wash bottles connected in series containing: 10% (w/v) sodium metabisulphite; water; 1M-I_2 in 10% (w/v) KI; 10% (w/v) sodium thiosulphate; 10% (w/v) KOH and 0.5M-potassium phosphate buffer, pH 7.4, as described by Conn *et al.* (1939). After passing through a cold trap (solid CO_2) the gas was dried through a column of Drierite (anhydrous $CaSO_4$; Hopkin and Williams). The i.r. spectrum of the purified gas was similar to that described by Pierson *et al.* (1956). The concentration of acetylene in the exposure chambers was monitored continuously from its absorption at 3300cm^{-1} in a 5cm gas-flow cell. Control rats were placed in similar chambers through which only air was passed.

Preparation of liver microsomal fractions

Determination of cytochrome b_5, P-450 and haem. The livers of rats killed by decapitation were perfused *in situ* with ice-cold 0.14M-NaCl via the hepatic vein for 60s before being removed, rinsed in ice-cold 1.15% KCl and blotted dry. Homogenates (10%, w/v) were prepared in 0.25M-sucrose. After centrifugation at 10000*g* for 20min, 4vol. of 12.5mM-sucrose in 8mM-$CaCl_2$ (Ca^{2+}/sucrose) was added to the supernatant (Kamath & Rubin, 1972). The microsomal fraction was precipitated by centrifugation at 10000*g* for 10min, washed in Ca^{2+}/sucrose and was resuspended in 0.25M-sucrose so that 1ml of microsomal suspension was equivalent to 1g of liver wet wt. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. The microsomal fractions were used on the day of preparation. Cytochrome b_5 was determined from the NADH reduced versus oxidized difference spectrum by using an absorption coefficient of 185 litre·$mmol^{-1}$·cm^{-1} (Omura & Sato, 1964*a*). Cytochrome *P*-450 was determined from the CO–dithionite reduced versus dithionite reduced difference spectrum by using an absorption coefficient of 91 litre·$mmol^{-1}$·cm^{-1} (Omura & Sato, 1964*b*).

Haem was determined as the pyridine haemochromogen by diluting 0.5ml of the microsomal suspension with 5.5ml of pyridine/0.3M-NaOH (1:1.8, v/v). Immediately after mixing, the haem concentration was estimated from the difference spectra between the sodium dithionite-reduced versus potassium ferricyanide (final concn. 10μM)-oxidized sample, by using an absorption coefficient ($A_{557-575}$) of 32.4 litre·$mmol^{-1}$·cm^{-1} (Omura & Sato, 1964*a*). The potassium ferrocyanide solution was prepared on the day of use.

Loss of cytochrome P-450 in vitro. Solutions of water-insoluble steroids were prepared at a nominal concentration of 1.5mM by ultrasonic dispersion in 1.15% KCl (Dawe Soniprobe; Dawe Instruments Ltd., London W.3, U.K.; 6A for 3min). Dipropargylacetamide (1.5mM) was dissolved in 1.15% KCl. Reaction mixtures of 6ml final volume contained: steroid, 3.0μmol; $NADP^+$, 2.6μmol; glucose 6-phosphate, 32.8μmol; glucose 6-phosphate dehydrogenase, 2i.u.; EDTA, 10μmol; $MgCl_2$, 20μmol; potassium phosphate buffer, pH7.4, 400μmol. After equilibration at 37°C, the reaction was started with 1.0ml of microsomal suspension. After incubation at 37°C in a shaking water bath (100 strokes/min) in air for various times as indicated, the flasks were placed on an ice/salt mixture and the cytochrome *P*-450 content was determined as described above. Control mixtures contained no steroid. In some experiments, where indicated, $NADP^+$ was omitted. Where acetylene was the substrate, flasks containing all constituents except $NADP^+$ were fitted with Teflon-sleeved rubber stoppers and a purified acetylene/oxygen mixture (4:1, v/v) passed through for 5min at a rate of 125ml/min. During this time the flasks were shaken (100 strokes/min) at 37°C. After 5min, the flow of gas was stopped and the reaction started by injecting $NADP^+$.

Experiments with [U-^{14}C]acetylene

Incubation conditions were identical with those described above except that after gassing the flasks with unlabelled acetylene, the interconnecting tubes were clamped and 0.5ml (1μCi) of [^{14}C]-acetylene was injected into each flask. The reaction was started by injecting $NADP^+$. At the end of the incubation period, the reaction was stopped with 6ml of ice-cold 15% (w/v) trichloroacetic acid. The precipitate was recovered by centrifugation at 3000*g* for 5min at 0–4°C. The precipitate was then washed successively with 6ml portions of the following reagents to remove non-covalently bound radioactivity: ice-cold 15% (w/v) trichloroacetic acid (three times); 7% (w/v) trichloroacetic acid (three times); ethanol (once); 7% (w/v) trichloroacetic acid at 50°C for 10min (once); ethanol at 80°C for 10min (once); ethanol/diethyl ether (1:1, v/v) (once); diethyl ether (once). The final pellets were dried *in vacuo*, weighed and a known weight was dissolved in 1ml of Soluene tissue solubilizer (Packard Instrument Co., Caversham, Berks., U.K.). Instagel scintillator (Packard Instrument Co; 10ml) was added and the samples were counted for radioactivity in a Phillips liquid-scintillation counter. Conversion of c.p.m. into d.p.m. was computed by means of an external channel-ratio method.

Liver porphyrins

To 0.5ml of a 10% (w/v) liver homogenate in 0.25M-sucrose was added 4.5ml of ice-cold 1M-$HClO_4$/methanol (1:1, v/v) as described by Abbritti & De Matteis (1971). After mixing and centrifuging (3000*g* for 20min), the porphyrin content of the supernatant was determined in a Perkin–Elmer MPF 3 fluorescent spectrophotometer (excitation wavelength 402nm, emission wavelength 654nm) with a protoporphyrin IX standard.

Isolation of green pigments from the liver

The isolation procedure was similar to that described previously (White & Muller-Eberhard, 1977). Portions of liver were homogenized in ice-cold 5% H_2SO_4 in methanol (20ml/g wet wt. of liver). After 17h at room temperature in the dark, the mixtures were filtered. To the filtrate in a separating funnel was added dichloromethane (20%, v/v), followed by ice-cold 2M-NaCl (1:1, v/v). The combined red–brown dichloromethane phases from two extractions were washed with a small volume of 0.5% (w/v) $NaHCO_3$ and water, and dried over anhydrous Na_2SO_4; the product was then concentrated in a stream of N_2 at 37°C. Separation of the dimethylhaem from green pigments was achieved on silica-gel

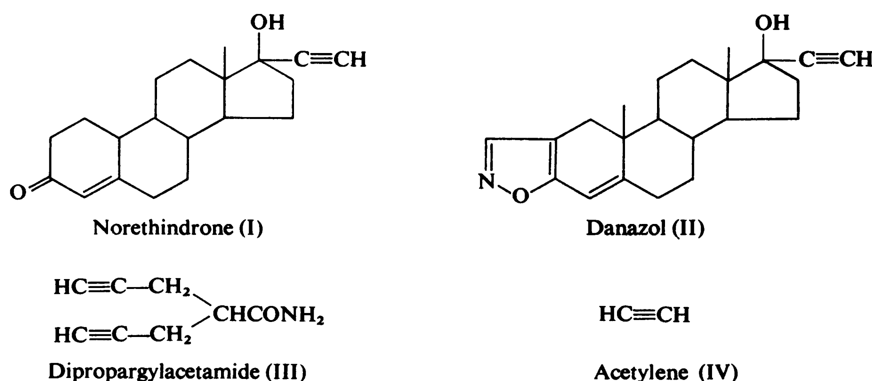

Fig. 1. *Chemical structures of some acetylenic-substituted compounds*

thin-layer plates (Silica gel 60, layer thickness 0.25mm) in a chloroform/kerosine/methanol (20:5:3, by vol.) solvent system. Kerosine (boiling range 190–250°C) was from Hopkin and Williams.

Results

Effects of dipropargylacetamide on hepatic microsomal cytochrome P-450 in vivo

We first examined the effects of the non-steroidal acetylenic-substituted acetamide, dipropargylacetamide (Fig. 1, compound III), on hepatic microsomal cytochrome *P*-450. Fig. 2 shows that in non-pretreated rats administration of this compound (100mg/kg) caused first a small decrease in the concentration of cytochrome *P*-450 with time,

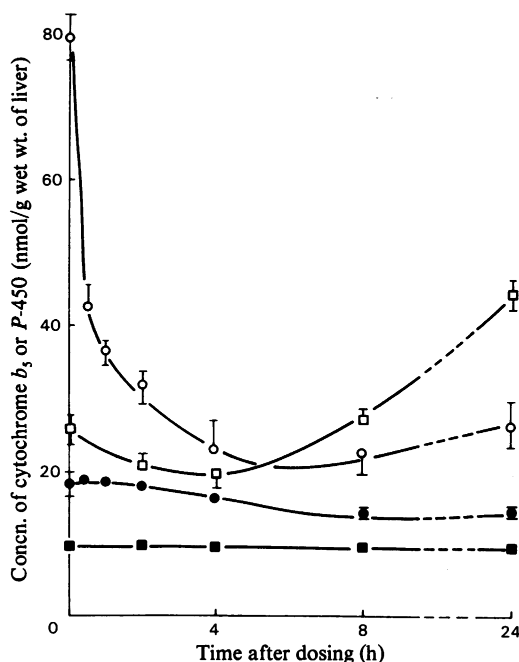

Fig. 2. *Effect of a single dose of dipropargylacetamide (100mg/kg) on the loss of hepatic cytochrome b_5 or P-450 with time in non-pretreated and phenobarbitone-pretreated rats*

Phenobarbitone-pretreated animals received 0.1% phenobarbitone in the drinking water for 7 days, followed by tap water for 24h, before being dosed. At various times after the administration of dipropargylacetamide, animals were killed and cytochrome *P*-450 (open symbols) and cytochrome b_5 (closed symbols) content in the liver microsomal fraction was determined as described in the Experimental section. Each point represents the mean (±S.E.) for four rats. □, ■, Control rats. ○, ●, Phenobarbitone-pretreated rats.

reaching a minimum at 4h, followed by a marked inductive effect at 24h after dosing. Hepatic microsomal cytochrome b_5 concentrations were not significantly affected. When rats were pretreated with phenobarbitone to induce the activity of the microsomal mixed-function oxidase enzymes, the same dose of dipropargylacetamide caused a much more marked and prolonged fall in cytochrome *P*-450 concentration. There was also a small time-dependent decrease in the concentration of cytochrome b_5. The yield of total microsomal protein decreased from 40±6mg/g of liver wet wt. (mean±S.E., four rats) at the time of dosing to 30±6mg/g liver wet wt. ($P<0.01$) in animals killed 8h after dosing. There was a small (though not significant) rise in the liver weight (expressed as a percentage of the body weight) over this time period.

Fig. 3 shows the effects in phenobarbitone-pretreated rats of different doses of dipropargylacetamide on the loss of hepatic microsomal cytochromes b_5 and *P*-450, measured 4h after dosing. There was a corresponding decrease in the total microsomal haem concentration, but the correlation between the total haem concentration and the sum of the molar concentrations of cytochromes b_5 and *P*-450 was generally poor. The reason for these discrepancies is not known. The loss of cytochrome *P*-450 in these experiments was not accompanied by the appearance of a cytochrome *P*-420 component.

Effects on some other acetylenic-substituted compounds in liver cytochrome P-450 in vivo

Using phenobarbitone-pretreated rats to obtain induced hepatic drug-metabolizing enzymic activities, we investigated the effects of administering a number of other steroidal and non-steroidal acetylenic compounds (1mmol/kg). Table 1 shows there to be a general effect on these derivatives in causing the loss of hepatic microsomal cytochrome *P*-450 and haem. Cytochrome b_5 was not usually affected. In the case of acetylene gas, rats were exposed for 18h to a concentration of 5% as described in the Experimental section. Although at high concentrations acetylene has a narcotic effect, at the concentrations used in these experiments the behaviour of the animals did not appear to be affected. As with the other acetylenic compounds, however, acetylene gas caused a significant decrease in hepatic cytochrome *P*-450 and haem concentrations.

Absorption of water-insoluble steroids from the site of administration and their availability to the hepatic parenchymal cell may be important in determining their relative efficacy. In preliminary experiments we found norethindrone, which had a low solubility in the trioctanoin vehicle, much less effective in causing loss of cytochrome *P*-450 *in vivo* than the more soluble norethindrone acetate. We

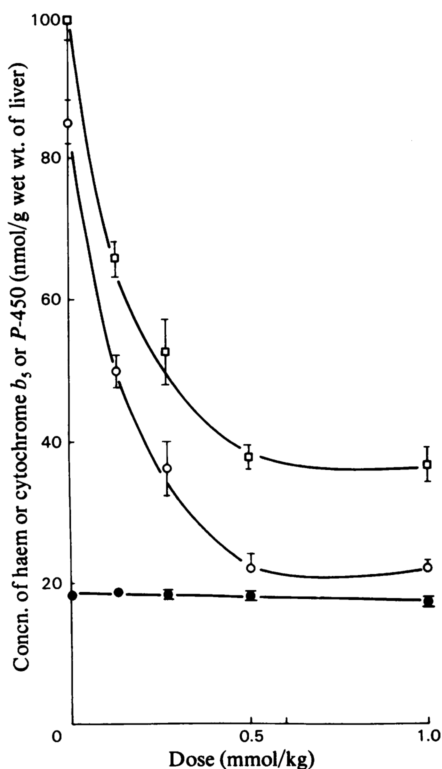

Fig. 3. *Effects of different doses of dipropargylacetamide on the loss of hepatic cytochrome b_5, P-450 and total microsomal haem in phenobarbitone-pretreated rats*

Phenobarbitone-pretreated animals received 0.1% phenobarbitone in the drinking water for 7 days, followed by tap water for 24h, before being dosed with dipropargylacetamide at the concentrations shown. The animals were killed 4h after dosing, and the microsomal cytochrome b_5, cytochrome P-450 and haem content determined as described in the Experimental section. Each point represents the mean (±S.E.) for four animals. □, Haem; ○, cytochrome P-450; ●, cytochrome b_5.

suggest that the apparent low activity of ethynyltestosterone in this respect, relative to norethindrone (Table 1) is due to the extremely low solubility of the former compound.

All of the acetylenic-substituted compounds also caused an increase in the concentration of porphyrins in the liver. This might be expected as a consequence of loss of liver haem, in view of the central role haem plays in regulating the rate-limiting enzyme of haem synthesis, 5-aminolaevulinate synthase (Granick, 1966). There was not, however, always a direct relationship between the loss of liver haem and the concentrations of porphyrins in the liver. This was particularly evident in rats exposed to acetylene gas (Table 1), where there was only a small loss of microsomal haem but a large increase in the concentration of porphyrins in the liver. The reason for this apparent discrepancy is not known.

Formation of abnormal green pigments in the liver

The loss of liver haem and cytochrome P-450 in the livers of rats dosed with the acetylenic-substituted compounds was always accompanied by the appearance of abnormal green pigments in the liver. These were extracted as their methyl esters and separated from dimethylhaem by t.l.c. (see the Experimental section). Except for norethindrone and ethynyloestradiol, which gave a similar pattern of green pigments, the remaining compounds tested, somewhat unexpectedly, gave different patterns of green

Table 1. *A comparison between the effects of some acetylenic-substituted compounds on various liver parameters in vivo*

Rats were given 0.1% phenobarbitone in the drinking water for 7 days, followed by tap water for 24h. The acetylenic-substituted compounds (except acetylene) were dissolved in trioctanoin and given intraperitoneally at a dose of 1mmol/kg. Controls received trioctanoin only. Animals were killed 4h after dosing. With acetylene, rats were exposed for 18h to 5% acetylene as described in the Experimental section. Results represent the means (±S.E.) for four experiments. Probability of significance between control and treated animals: $*P<0.05$; $**P<0.01$; $***P<0.001$.

	Liver wt. (% of body wt.)	Liver porphyrins (nmol/g wet wt.)	Total microsomal haem (nmol/g wet wt. of liver)	Microsomal cytochrome P-450 (nmol/g wet wt. of liver)	Microsomal cytochrome b_5 (nmol/g wet wt. of liver)
Controls	4.85±0.22	1.68±0.06	109.0±3.5	95.6±4.3	18.3±1.0
Ethynyltestosterone	4.92±0.16	2.27±0.07**	102.1±3.9	86.5±2.0	18.7±0.8
17α-Ethynyl-17β-hydroxy-androstan-3-one	4.93±0.18	2.35±0.15**	78.2±4.4***	81.9±1.1***	18.6±0.2
Acetylene	4.53±0.25	10.83±2.58**	76.6±4.3***	64.7±2.8***	19.7±0.3
Danazol	4.88±0.24	3.63±1.65**	68.3±2.7***	53.9±5.7***	18.4±2.2
Norethindrone acetate	4.68±0.27	4.01±0.90*	58.1±3.8***	37.2±2.5***	19.6±1.4
2,2-Dipropargylacetamide	5.03±0.17	4.27±0.88*	46.5±1.4***	20.9±2.6***	17.2±1.4

pigments on t.l.c. The numbers of green pigments were characteristic for a given compound, but they had variable R_F values, possibly owing to lipid contamination of the samples.

All the green pigments fluoresced red under short-wavelength u.v. light. They were not seen in control animals dosed only with trioctanoin. Animals dosed with dipropargylacetamide showed a large loss of liver haem 4h after dosing (Table 1), but at the same time there was only a poor formation of green pigments. There was generally little correlation between overall haem loss and the intensity of the green bands seen on t.l.c.

In addition to the acetylenic-substituted compounds, the vinyl (>C=C<)-substituted analogue of ethynyltestosterone, vinyltestosterone, was tested. When injected into phenobarbitone-pretreated rats (1 mmol/kg) this compound caused a substantial decrease in hepatic cytochrome *P*-450 concentrations to 39.0±1.1% (mean±S.E., three animals) of control values. Green pigments were also seen in the methylated liver extracts (Fig. 4). Their pattern, however, was quite different from that given by ethynyltestosterone. As far as we are aware, the production of green pigments in the livers of rats dosed with vinyl-substituted compounds has not previously been reported. However, vinyl derivatives such as vinyl chloride and 2,2,2-trifluoroethylvinyl ether (fluorexene) can undergo metabolic conversion into derivatives which cause the loss of liver haem and cytochrome *P*-450 (Guengerich & Strickland, 1977; Marsh *et al.*, 1977).

Effects of acetylenic-substituted compounds on cytochrome P-450 in vitro

To study further the mechanism of action of the acetylenic-substituted compounds, the effects of incubating some of these with rat liver microsomal preparations and a NADPH-generating system *in vitro* were examined. Fig. 5 compares the loss of cytochrome *P*-450 caused by dipropargylacetamide, Danazol and acetylene gas with time, with microsomal preparations from phenobarbitone-pretreated rats. Results are expressed relative to control mixtures containing no acetylenic derivative to compensate for destruction of cytochrome *P*-450 caused by NADPH-catalysed lipid peroxidation (De Matteis & Sparks, 1973). The loss of cytochrome *P*-450 was not accompanied by a shift in the absorption maxima or in the formation of a cytochrome *P*-420 component. NADPH (20 μmol) could effectively replace $NADP^+$, glucose 6-phosphate and glucose 6-phosphate dehydrogenase. No destruction of cytochrome *P*-450 occurred if NADH was substituted for NADPH.

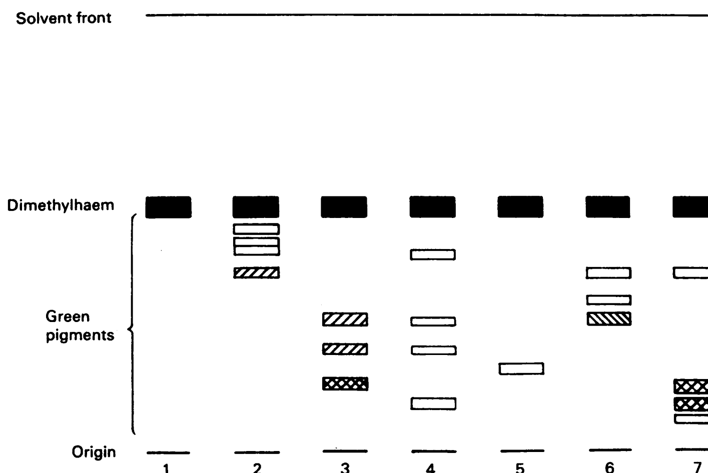

Fig. 4. *Schematic representation of the major green pigments extracted from the livers of rats dosed with various acetylenic-substituted compounds*

Rats were pretreated with phenobarbitone (0.1%, w/v) in the drinking water, followed by tap water for 24h, before being dosed with the acetylenic-substituted compounds (1 mmol/kg). The animals were killed 4h after injection and 5g portions of the liver homogenized in ice-cold 5% (v/v) H_2SO_4 in methanol. The methylated derivatives were extracted into dichloromethane, concentrated and run on silica-gel thin-layer plates as described in the Experimental section. Columns represent rats dosed with: 1, trioctanoin (controls); 2, 17α-ethynyl-17β-hydroxyandrostan-3-one; 3, acetylene (animals exposed to 5% acetylene in air for 18h before being killed); 4, Danazol; 5, dipropargylacetamide; 6, ethynyltestosterone; 7, vinyltestosterone. Cross-hatching represents the relative intensity of green colour: □, light; ■, dark.

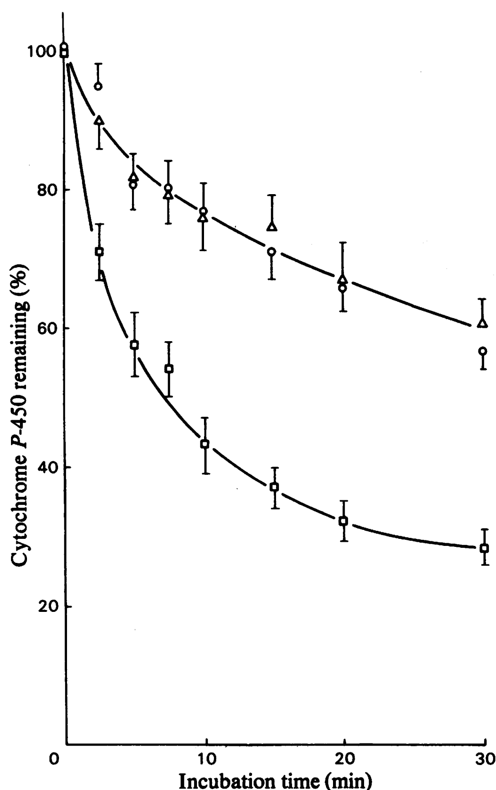

Fig. 5. *Decreased cytochrome P-450 in vitro after incubation of phenobarbitone-pretreated rat liver microsomal fractions with dipropargylacetamide, Danazol or acetylene*

Results are expressed as the percentage of cytochrome *P*-450 remaining relative to control incubation mixtures containing a NADPH-generating system, but no acetylenic-substituted compound. Points represent the mean (±S.E.) of four determinations. Details of the incubation mixture are given in the Experimental section. Flasks were shaken in air, or where acetylene was the substrate, in acetylene/oxygen (4:1, v/v) for the times indicated. They were then placed on an ice/salt mixture, and the cytochrome *P*-450 content was determined from the CO–dithionite-reduced versus dithionite-reduced difference spectrum. △, Acetylene; ○, Danazol; □, dipropargylacetamide.

Fig. 6 shows the effects of substrate concentration on the loss of cytochrome *P*-450 caused by dipropargylacetamide or Danazol in microsomal preparations from phenobarbitone-pretreated rats. Dipropargylacetamide remained in solution over the concentration range used in these experiments, whereas Danazol proved to be highly water-insoluble. The apparent inhibition of enzyme-mediated destruction of cytochrome *P*-450 at Danazol concentrations greater than 1 mM may have been due to physical

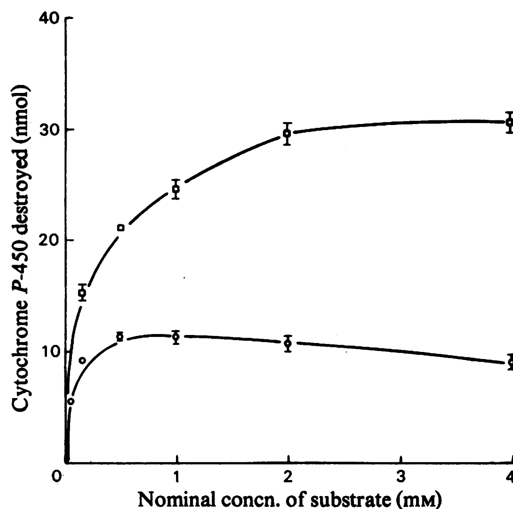

Fig. 6. *Effects of dipropargylacetamide and nominal Danazol concentrations on the loss of cytochrome P-450 in vitro*

Points represent the mean (±S.E.) for four experiments. Incubation mixtures of 6 ml final volume were as described in the Experimental section. Flasks were shaken for 15 min (100 strokes/min) in air at 37°C, then placed on a salt/ice mixture and the cytochrome *P*-450 content was determined. The microsomal fraction used was from phenobarbitone-pretreated rats. Incubation mixtures initially contained 50 nmol of cytochrome *P*-450. □, Dipropargylacetamide; ○, Danazol.

effects of steroid granules on the microsomal membranes.

Absence of covalent binding by active metabolites of acetylene

We wished to establish if, in addition to causing a loss of cytochrome *P*-450, active metabolites of the acetylenic group could also be involved in covalent binding to microsomal protein. Since our experiments had established that acetylene gas was effective in causing a loss of cytochrome *P*-450 *in vitro* (Fig. 5), we repeated the procedure using microsomal preparations from phenobarbitone-pretreated rats and acetylene gas containing a [U-^{14}C]acetylene tracer. After various incubation times, one part of the incubation mixture was used to determine total radioactivity in the aqueous phase, another part was added to trichloroacetic acid. The protein precipitates were thoroughly washed to remove non-covalently bound radioactivity (see the Experimental section). After 30 min incubation, about 20% of the radioactivity injected into the gas phase as [^{14}C]acetylene was present in the aqueous phase. The amount of covalently bound radioactivity showed a slight

increase with time of incubation, but still was not significantly greater than the background after 30 min incubation. No attempt was made to measure possible binding of radioactive label to green pigments as procedures for the isolation of these products from microsomal incubation mixtures *in vitro* have not yet been developed.

Discussion

Our results show for the first time that metabolic activation of the acetylenic (–C≡C–) substituent to derivatives capable of causing a loss of hepatic cytochrome *P*-450 and haem is a general phenomenon.

The present findings show that for loss of cytochrome *P*-450 to occur, unsaturation of neither the steroid ring A, cf. 17α-ethynyl-17β-hydroxyandrostan-3-one (Table 1) nor the steroid nucleus itself is required. The enzyme responsible for the metabolic activation of the acetylenic substituent has a requirement for NADPH and O_2, and is inhibited by CO (White & Muller-Eberhard, 1977), properties typical of the microsomal mixed-function oxidases. The nature of the activation step(s), however, remains unclear.

The loss of microsomal cytochrome *P*-450 caused by dipropargylacetamide represents destruction of this cytochrome and not modification of the spectral characteristics of the dithionite reduced versus reduced *P*-450–CO complex as observed with some water-insoluble compounds (Franklin, 1972). This is substantiated by the marked loss of total microsomal haem which occurs at the same time as a decrease in cytochrome *P*-450 concentrations in the livers of rats dosed with this compound (Fig. 3).

In control rats given dipropargylacetamide, the initial loss of cytochrome *P*-450 followed by induction (Fig. 2) was very similar to the action on the liver produced after the administration of the allyl (H_2C=CH–CH_2–)-substituted 2-allyl-2-isopropylacetamide (De Matteis, 1971). For both compounds, pretreatment of rats with phenobarbitone markedly enhances the loss of hepatic cytochrome *P*-450 and haem. The loss of haem in both instances is also accompanied by the appearance of abnormal haem-breakdown products (green pigments) in the liver. Although green pigments have only been reported as being formed as a result of haem destruction by 2-allyl-2-isopropylacetamide, a number of other compounds containing unsaturated double bonds, such as vinyl chloride, fluorexene and secobarbital, when administered to rats, also bring about destruction of liver haem. It has been suggested that similar reaction mechanisms involving the metabolic formation of active metabolites from the unsaturated double bond may be involved (Guengerich & Strickland, 1977). In order to investigate further the mechanism of activation of acetylenic groups, we compared the destruction of cytochrome *P*-450 and the appearance of green pigments after the administration of ethynyltestosterone and its vinyl (>C=C<) analogue, vinyltestosterone, in rats. Both compounds caused a loss of cytochrome *P*-450, but the patterns of green pigments produced by the two were quite dissimilar (Fig. 4). This suggests that reduction of the unsaturated triple bond to a double bond is not the normal metabolic activation route of acetylenic substituents; it does not exclude the possibility, however, that this metabolic pathway is a minor route.

The results with radioactively labelled acetylene suggest that the acetylenic substituent was not involved in covalent binding to protein, but does not exclude the possibility that these derivatives bind to lipid or to haem or to haem-degradation products. The loss of cytochrome *P*-450 haem caused by acetylene suggests that either the active metabolites have a very short half-life, so they are only able to attack nucleophiles at their site of formation, i.e. cytochrome *P*-450 haem, or that a free-radical mechanism is involved in the metabolic activation step.

It is concluded from these studies that for steroids, such as ethynyloestradiol or norethindrone, metabolic activation in rat liver can occur at two sites. The acetylenic substituent gives rise to derivatives capable of breaking down liver haem and the unsaturated ring gives rise to derivatives capable of covalently binding to liver protein (Cook *et al.*, 1974).

In view of the current widespread use of acetylene, it is of interest that this compound can cause loss of haem and a marked accumulation of porphyrins in the liver of rats (exposed for 18 h to 5% acetylene, Table 1). Human occupational exposure to acetylene is limited because of the explosive hazard in air at concentrations greater than 3%. In the past, however, acetylene was used as an anaesthetic up to concentrations of 40% (Seevers & Waters, 1938). Studies at that time showed that exposure of a variety of animal species to high concentrations of this gas caused no damage to the hepatic parenchymal cells (Franken & Miklos, 1933).

The mechanism of activation of acetylene and other acetylenic-substituted compounds and the nature of the active metabolite remains unclear. Our results suggest that the active species is either highly unstable or remains in the lipid environment of the endoplasmic reticulum, unable to react with nucleophiles at locations remote from its site of formation.

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