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EFFECTS OF PYRAZOLE ON NITROSODIMETHYLAMINE DEMETHYLASE AND OTHER MICROSOMAL XENOBIOTIC METABOLISING ACTIVITIES

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Abstract—Pyrazole administered to immature rats at one day or on four successive days prior to sacrifice increased a microsomal NDMAD with apparent K_m 0.04 mM. Aniline hydroxylase activity was also increased by these treatments. Ethoxycoumarin deethylase and amino pyrine demethylase activities were not altered when animals were treated with pyrazole one day prior to sacrifice but were reduced to below control activity when animals were treated for four successive days. All microsomal mono-oxygenases were decreased when animals received a single administration of pyrazole four days prior to sacrifice and the cytochrome P-450 content of these microsomes was reduced by up to 50%. When microsomes from untreated animals or animals treated for four successive days were incubated with pyrazole in the presence of NADPH, cytochrome P-450 content decreased in a time dependent process to a limiting value. The effect was dependent on pyrazole concentration and saturable. These results suggest that pyrazole induces a cytochrome P-450 isoenzyme with high affinity for NDMA but also acts as a suicide inhibitor of the cytochrome.

Nitrosamines are a class of potent carcinogens which occur widely as environmental pollutants and whose biological effects result from metabolic activation [1–3]. Studies of the metabolism of nitrosodimethylamine, NDMA*, and other nitrosamines suggest that such activation proceeds via hydroxylation to give an α-hydroxymethyl (or α-hydroxyalkyl) nitrosamine [1, 4]. This undergoes a spontaneous fragmentation to give formaldehyde (or appropriate aldehyde) and diazonium ion. The diazonium ion can in turn react with cellular nucleophiles and this may result in tumourigenesis. Much evidence indicates that the initial hydroxylation reaction is catalysed by a cytochrome P-450 containing microsomal mono-oxygenase termed nitrosodimethylamine demethylase, NDMAD [5–10]. Despite this, alternative pathways have also been invoked. Such suggestions have in part resulted from the finding that NDMAD activity is differentially affected by inducers of cytochrome P-450 when compared to other mono-oxygenase activities and is differentially affected *in vitro* by inhibitors and activators of mono-oxygenases [11–13]. The stoichiometry of reaction products would also appear to deviate from that predicted by the α-hydroxylation pathway [4, 14]. Resolution of this controversy may be assisted by isolation of enzymes involved and such an objective is being pursued in this laboratory.

As a working hypothesis it is assumed that metabolic activation via α-hydroxylation plays a significant role and is catalysed by a cytochrome P-450 isozyme [5, 16] with high affinity for its substrate. Steady state kinetic analysis indicates the presence of several NDMAD enzymes in microsomal membranes which give rise to multiline inverse velocity substrate plots [9, 11, 17, 18]. One component of these activities has an apparent K_m of 0.06 mM and may be of significance to activation of nitrosamines *in vivo*. This low K_m enzyme can be induced by pyrazole [9], alcohol administration [17] or fasting [18]. These treatments result in the appearance of a protein band on SDS-polyacrylamide gel electrophoretograms with M_r approximately 52,000. Induction of this band and NDMAD activity were blocked by $CoCl_2$ indicating the involvement of haemoprotein synthesis. The use of pyrazole as a means of enriching microsomal NDMAD prior to enzyme purification would appear to be promising. However, the large inductive effect was only obtained by a regime of four daily successive administrations of the agent [9, 10]. Such treatment has an acute toxicological effect on the young rats used in these experiments. The animals fail to gain weight and eventually weight is lost ([9]; J. A. Craft unpublished observation). In addition while NDMAD activity is greatly enhanced by pyrazole, total cytochrome P-450 shows only a modest induction and other mono-oxygenase activities are depressed [9, 10]. Such observations are themselves of some interest since pyrazole can inhibit dimethylhydrazine-induced tumourogenesis [19] but produces microsomes which promote NDMA induced mutagenesis [20]. This study was undertaken to extend the studies on pyrazole as an inducer of NDMAD and to seek an explanation of the anomalous effects it has on cytochrome P-450 mediated reactions.

* Abbreviations used: NDMA, nitrosodimethylamine; NDMAD, nitrosodimethylamine demethylase; SDS, sodium dodecyl sulphate.

MATERIALS AND METHODS

Materials

Chemicals were obtained from the sources indicated: aminopyrine, cytochrome *c* (Horse heart Type 11A), ethoxycoumarin, glucose-6-phosphate Na, glucose-6-phosphate dehydrogenase, from yeast Type VII, NADP, Na, NADPH, Na_4, NADH, Na_2, NDMA and pyrazole from Sigma Chemical Co. (Poole, Dorset, U.K.); 7-hydroxycoumarin from Aldrich Chemical Co. (Gillingham, Dorset, U.K.); 7(*n*) 3H styrene oxide from Amersham International (Bucks., U.K.).

Methods

Animals and microsomal preparation. Male Wistar rats were 50–55 g at the start of each experiment and were divided into groups consisting of at least four animals. Pyrazole, dissolved in 0.9% saline, was administered i.p. 200 mg/kg [9] as detailed below and animals were permitted food and water *ad lib.* For each experiment there were four groups of animals which were (a) untreated, or (b) received a single administration of pyrazole one day prior to sacrifice, or (c) received a single administration of pyrazole on four successive days prior to sacrifice, or (d) received a single administration of pyrazole four days prior to sacrifice. All animals were sacrified on the fifth day. Salt washed microsomes were prepared from pooled livers of each group as described [21] and were resuspended in 0.2 M potassium phosphate/1.0 mM EDTA, pH 7.0 at approximately 20 mg/ml. Mono-oxygenase assays other than NDMAD were performed on the day of microsomal preparation. NDMAD and all other determinations were performed on samples which had been stored at −20° for not longer than 1 week.

Enzyme assays. NDMAD was determined by the rate of formaldehyde formed, essentially as described by Tu and Yang [18]. The incubations were carried out in tightly stoppered tubes and all manipulations were carried out in a well ventilated fume hood. Plastic tubes and cuvettes were used throughout to facilitate disposal of NDMA contaminated equipment by incineration. Incubation was carried out at 37° in mixtures containing 70 mM Tris HCl/0.07 mM EDTA/14 mM $MgCl_2$/215 mM KCl, pH 7.4 at 37°. An NADPH regenerating system consisting of 0.4 mM NADP, 4 mM glucose-6-phosphate and 4 units/ml glucose-6-phosphate dehydrogenase was also added. NDMA was varied over a concentration range of 0.02–40.0 mM. After a 2 min preincubation, reaction was initiated by addition of a microsomal suspension containing 0.76–1.0 mg of protein. Blanks contained all reagents other than NDMA. No HCHO was formed from NDMA when the NADPH regenerating system was omitted. Reaction was terminated after 30 min as described [18] by the addition of Ba $(OH)_2$ and $ZnSO_4$ and HCHO determined in an aliquot of the supernatant by a modification of [18] of the procedure of Nash [22]. Quantitation of HCHO by standard was unaffected by the inclusion of NDMA or pyrazole with the Nash reagents. Under the assay conditions, HCHO production was linear with incubation time and microsomal protein. Data thus obtained were initially analysed by double reciprical plots (1/v against 1/(NDMA)). When these plots were linear the kinetic parameters K_m and V_{max} were obtained by linear regression analysis. However, many data sets yielded plots which were curved, concave downwards (see Results Section). It was assumed that such phenomena result from the simultaneous action of two NDMAD enzymes (low K_m form and high K_m form) [9, 11, 17, 18]. The kinetic parameters of both enzyme forms were obtained by the method of Osmundsen [23, 24]. This method uses an iterative, non linear regression to obtain the best fit of the data to the equation:

$$v_0 = \frac{\text{VML.S}}{\text{KML} + \text{S}} + \frac{\text{VMH.S}}{\text{KMH} + \text{S}}$$

where VML and VMH are V_{max} associated with the low K_m and high K_m enzyme forms respectively.

Table 1. Effect of pyrazole on the apparent kinetic parameters of microsomal NDMAD activity

Pyrazole treatment	KML (mM)	VML (nmoles/min/mg)	KMH (mM)	VMH (nmoles/min/mg)
Untreated	0.023 (0.01–0.05)	2.16 (0.54–2.16)	1.95 (0.74–1.95)	0.50 (0.50–1.99)
One, one day prior	0.038 (0.028–0.056)	7.40 (4.93–9.26)	— —	— —
On four successive days prior	0.046 (0.046–0.085)	10.6 (9.34–10.6)	— —	— —
One, four days prior	0.029 (0.01–0.05)	1.49 (0.32–1.54)	2.50 (1.38–9.4)	0.29 (0.29–0.63)

Animals were untreated, received a single administration of pyrazole (200 mg/kg) one day prior to sacrifice, received a daily administration of pyrazole on four successive days prior to sacrifice on the fifth, or received a single administration of pyrazole four days prior to sacrifice on the fifth day. NDMAD activity was determined as described in the Methods over a substrate concentration range of 0.02–40.0 mM. Data obtained with microsomes from untreated animals and animals receiving a single administration four days prior to sacrifice were analysed by the method of Osmundsen (see Methods) to give values for KML and KMH (K_m values for low K_m enzyme and high K_m enzyme) and VML and VMH (V_{max} for these enzymes). Data obtained with microsomes from animals receiving a single administration one day prior to sacrifice or on four successive days was analysed by plotting $1/v$ against 1/(NDMAD) over the range 0.02–5 mM. Values of K_m and V_{max} were obtained by linear regression analysis. The results shown were obtained in a single experiment and the range of values found over five exeriments are shown in parenthesis.

KML and KMH are the K_m values associated with these enzymes. The data were processed by the DEC-2050 computer of Glasgow College of Technology Computer Centre using a program compiled in Fortran IV.

Aminopyrine demethylase activity was determined as for NDMAD but with 5 mM aminopyrine replacing NDMA and reaction was terminated after 5 min. Aniline hydroxylase was determined by the method of Schenkman *et al.* [25]. Pyrazole did not affect colour development by this method. Ethoxycoumarin-7-*O*-deethylase was determined by the method of Greenlee and Poland [26]. Epoxide hydrolase was determined by the rate of ^{3}H-styrene oxide hydrolysis as previously described [21].

NADPH-cytochrome *c*-reductase [27] and NADH-cytochrome *c*-reductase [28] were measured at ambient temperature and are expressed as μmoles cytochrome *c* reduced/min per mg protein. Cytochrome P-450 was determined by CO difference spectroscopy [29] and cytochrome *b*5 by difference spectroscopy of oxidised vs NADH reduced microsomes [30].

Incubation of microsomes with pyrazole in vitro. The method was similar to that of Ortiz de Montellano and Mathews [31]. Microsomes (1 mg/ml) were incubated at 37° with pyrazole in a mixture containing 0.1 M potassium phosphate/150 mM KCl/5 mM $MgCl_2$/2.5 mM EDTA, pH 7.4 and an NADPH regenerating system as used for NDMAD. Mixtures were preincubated for 3 min and reaction initiated by addition of the NADPH regenerating system. Aliquots were removed at various times and the cytochrome P-450 content determined. Protein was determined by the method of Lowry [32] using bovine serum albumin as standard.

RESULTS

Effects of pyrazole on microsomal NDMAD activity

Rats were treated with pyrazole following various protocols and NDMAD determined as a function of substrate concentration. The apparent kinetic parameters obtained are shown in Table 1. Microsomes from untreated animals displayed NDMAD activity which was not saturated by substrate at concentrations as high as 40 mM. Over a NDMA concentration range of 0.02–40 mM, double reciprical plots ($1/v_0$ against 1/(NDMA)) gave continuous curves which were concave downwards. When analysed by the method of Osmundsen [23, 24] the data provided a good fit to a two enzyme model with values of 0.023 mM and 1.95 mM for the low K_m and high K_m forms respectively. When animals were given pyrazole one day or for four successive days prior to sacrifice, microsomal NDMAD activity was increased. Treatment of the animals with pyrazole under these protocols caused an alteration of the steady state kinetics. Thus double reciprocal plots over a range 0.02–5.0 mM NDMA display only a single component corresponding to the lower K_m site found in untreated animals. At high substrate concentrations (10–40 mM), a progressive decrease of maximal NDMAD activity was observed. For this reason these data points were not used in the determination of kinetic parameters for these microsomes. When animals received a single administration of pyrazole and were sacrificed four days later, NDMAD was consistently lower than in untreated animals. The microsomes obtained from these animals displayed NDMAD with kinetic properties similar to untreated animals. Thus saturation was not found at the highest substrate concentration employed and double reciprical plots were curved, concave downwards. When these data were analysed by the method of Osmundsen K_m values for the low K_m form and high K_m form of enzyme were obtained which were similar to those obtained for untreated animals. The equivalent V_{max} figures were by comparison reduced.

Effect of pyrazole on other mono-oxygenase activities

The effects of pyrazole administration on aniline hydroxylase, ethoxycoumarin-*o*-deethylase and aminopyrine demethylase have also been investigated and results are shown in Table 2. When animals received a single administration of pyrazole one day prior to sacrifice aniline hydroxylase activity was

Table 2. Effect of pyrazole on microsomal mono-oxygenase activities

Pyrazole treatment	Enzyme activity (nmoles/min/mg)		
	Aniline hydroxylase	Ethoxycoumarin-*o*-deethylase	Aminopyrine demethylase
Untreated	0.71 ± 0.01 (0.55–0.71)	0.59 ± 0.01 (0.59–1.36)	10.15 ± 0.31 (8.04–10.15)
One, 1 day prior	1.72 ± 0.11* (1.30–2.19)	0.70 ± 0.01 (0.65–1.39)	8.51 ± 0.14 (8.37–8.51)
On 4 successive days prior	1.95 ± 0.06* (1.93–1.95)	0.12 ± 0.03* (0.12–0.50)	6.07 ± 0.02* (5.88–6.09)
One, 4 days prior	0.41 ± 0.07* (0.17–0.41)	0.23 ± 0.02* (0.20–0.50)	5.88 ± 0.10* (3.78–6.07)

Animals were administered pyrazole as indicated and microsomal mono-oxygenase activities determined by the methods quoted. The results are taken from a single experiment and show means ± S.D. of triplicate determinations. Similar results were obtained in two other experiments using different microsomal preparations, and the range of mean values obtained in all experiments is shown in parenthesis.

* $P < 0.01$.

Table 3. Effect of pyrazole on microsomal components

Pyrazole treatment	Cytochrome P450 (nmoles/mg)	NADPH-cyt c-reductase (μmoles/min/min)	Cytochrome b5 (nmoles/mg)	NADH-cyt c-reductase (μmoles/min/mg)	Epoxide hydrolase (nmolcs/min/mg)
Untreated	0.73 ± 0.01 (0.70–0.96)	0.053 ± 0.001 (0.053–0.093)	0.41 ± 0.01 (0.41–0.43)	0.506 ± 0.028 (0.506–0.720)	8.62 ± 0.55 (8.62–9.73)
One day prior	1.02 ± 0.03* (1.02–1.46)	0.044 ± 0.001* (0.040–0.091)	0.37 ± 0.01 (0.37–0.40)	0.275 ± 0.007* (0.251–0.30)	7.82 ± 0.33 (7.82–8.46)
On 4 successive days	1.23 ± 0.02* (1.23–1.34)	0.044 ± 0.003* (0.044–0.070)	0.32 ± 0.02* (0.32–0.34)	0.241 ± 0.011* (0.241–1.288)	12.67 ± 0.13* (11.21–13.10)
One, 4 days prior	0.39 ± 0.01* (0.39–0.68)	0.053 ± 0.003 (0.031–0.101)	0.33 ± 0.02* (0.30–0.35)	0.484 ± 0.013 (0.480–0.520)	12.41 ± 0.51* (12.13–14.49)

Animals were treated with pyrazole as indicated and microsomal components were determined by the methods quoted. The results are taken from a single experiment and show the mean ± S.D. for triplicate determinations. Similar results were obtained in two other experiments using different microsomal preparations and the range of values found in all experiments are shown in parenthesis.

* $P < 0.01$.

increased by more than twofold when compared to the activity of untreated animals. The one day treatment had very little effect on ethoxycoumarin deethylase and aminopyrine demethylase activities. When microsomes were obtained from animals receiving four successive, daily administrations aniline hydroxylase activity was still induced but ethoxycoumarin deethylase and aminopyrine demethylase activities were significantly less than found for untreated animals. When microsomes were obtained from animals receiving a single administration of pyrazole four days prior to sacrifice all three mono-oxygenase activities were less than found for untreated animals.

Effects of pyrazole on microsomal cytochromes, cytochrome reductases and epoxide hydrolase

The effects of pyrazole administration on microsomal cytochrome P-450, NADPH–cytochrome *c*-reductase, cytochrome *b*5, NADH–cytochrome *c*-reductase and epoxide hydrolase are shown in Table 3. Pyrazole administered for one day or for four successive days cause only a small increase in cytochrome P-450. However, when animals received a single administration of pyrazole four days prior to sacrifice the microsomal cytochrome P-450 content was reduced to approximately 50% of that of microsomes from untreated animals. None of the pyrazole treatments caused a marked change in NADPH–cytochrome *c*-reductase activity. When pyrazole was administered once, one day prior to sacrifice or for four successive days prior to sacrifice a small but consistent decrease in cytochrome *b*5 was observed. In the experiment shown in Table 3 a decrease of 20% was found but in all other experiments amounted to a decrease of 10%. The microsomes from animals receiving this type of pyrazole treatment showed significant decreases (approximately 50%) in the activity of NADH–cytochrome *c*-reductase.

Epoxide hydrolase activity of microsomes prepared from animals treated for four successive days or once four days prior to sacrifice was found to be higher than for untreated animals. It is possible that this effect results from residual pyrazole since the isomer imidazole (J. A. Craft, unpublished results) and imidazole derivatives [33] activate this enzyme *in vitro*. However, pyrazole in the range 5.0–25.0 mM did not alter epoxide hydrolase activity of microsomes from untreated animals (data not shown).

Effect of pyrazole in vitro *on microsomal cytochrome P-450*

Since certain pyrazole administrations can lead to decreased microsomal cytochrome P-450 and microsomal mono-oxygenases, it was considered that pyrazole may cause destruction of the cytochrome. To assess this hypothesis microsomes from untreated animals and from animals treated with pyrazole for four successive days were incubated with pyrazole in the presence and absence of an NADPH regenerating system. The results are shown in Fig. 1. The cytochrome P-450 content of both microsomal preparations was relatively stable to incubation in the presence of pyrazole alone or of the NADPH regenerating system alone. However, with both pyra-

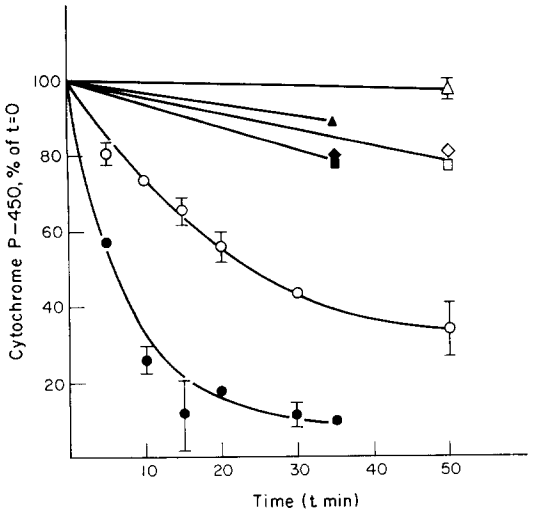

Fig. 1. Effect of pyrazole *in vitro* on microsomal cytochrome P-450. Microsomes obtained from untreated animals, open symbols, or animals treated with pyrazole on four successive days prior to sacrifice, closed symbols, were incubated as described in the Methods. Incubations contained pyrazole (10 mM) alone (△ ▲), NADPH regenerating system alone (□ ■) or combination of both (○ ●). The combination of imidazole (10 mM) and the NADPH regenerating system is also shown (◇ ◆). Results are expressed as a percentage of the cytochrome P-450 remaining compared to the content without incubation. Initial values were: untreated, 0.76 nmoles/mg; treated, 1.20 nmoles/mg. Results show the means of duplicate determination.

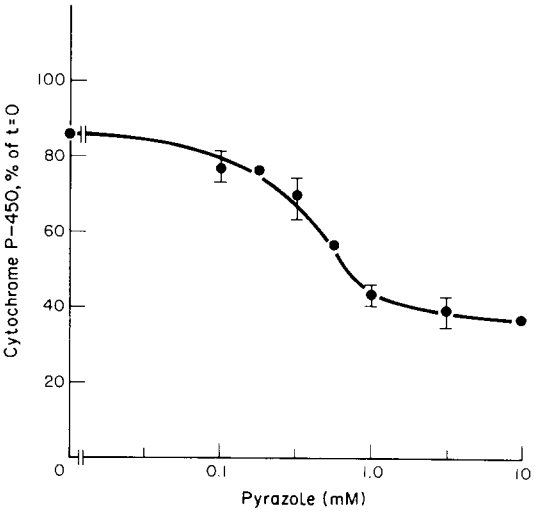

Fig. 2. Effect of pyrazole concentration on the destruction *in vitro* of cytochrome P-450. Microsomes from animals treated for four successive days with pyrazole were incubated with pyrazole and an NADPH-regenerating system for 10 min and cytochrome P-450 determined. Results are expressed as a percentage cytochrome P-450 remaining compared to the content without incubation. This was 1.00 nmoles/mg protein.

zole and the NADPH regenerating system were included, the cytochrome P-450 content decreased and approached a limiting value. The rate of disappearance of the cytochrome appeared to be faster in microsomes from animals receiving four pyrazole administrations than in microsomes from untreated animals. Further a higher percentage of total cytochrome P-450 was labile to this treatment when microsomes were obtained from treated animals (~90%) than from untreated animals (~60%). Imidazole caused no decrease in the content of cytochrome P-450. When data for the pyrazole mediated cytochrome P-450 decay is plotted on a logarithmic basis straight lines were obtained indicating pseudo first order kinetics. The effects of pyrazole *in vitro* were found to be concentration dependent and saturable (Fig. 2) with 50% maximum effect at 0.6 mM. Pyrazole had no effect on cytochrome *b*5 or the activities of NADH–cytochrome *c*-reductase or NADPH–cytochrome *c*-reductase when incubated with microsomes from untreated animals (data not shown).

DISCUSSION

Steady state kinetic analysis of NDMAD using microsomes from untreated rats indicates the presence of at least two enzymes (Table 1). As found by others [9, 11, 17, 18], double reciprocal plots give curves, concave downwards. The kinetic parameters K_m and V_{max} for these two enzymes have been derived by dividing the double reciprocal plots into two pseudo linear portions [9, 11, 17, 18]. The values obtained from the intercepts of the $1/v_0$ axis (V_{max}) and the 1/NDMA axis (K_m) of the two segments cannot give true values of these parameters [24, 34]. In the study reported here the values have been derived by an iterative, non linear regression analysis which obtains the best fit of the data to the equation generated by summing each component [23, 24]. By comparing these values to those which would have been obtained by the simple analysis, the latter method yields values which are in reasonable agreement for the low K_m enzyme but are in error for the high K_m enzyme. (KMH is under-estimated and VMH is over-estimated).

The two enzyme components present in microsomes from untreated animals are presumed to be distinct cytochrome P-450 isozymes [9, 15] but could be accounted for by enzymic components other than cytochrome P-450 [4, 11–13]. As found by others [9] the low K_m form of the enzyme (K_m = 0.04 mM) is induced by pyrazole when given to animals on four successive days (Table 1). Following this treatment the high K_m enzyme form is no longer apparent but instead high substrate concentrations cause submaximal velocity possible due to substrate inhibition [35]. The induction of the low K_m enzyme and the altered kinetic properties of NDMAD occur as early as 24 hr after pyrazole administration confirming the assumption of others [10] that the early increase of NDMAD activity reflects induction of an enzyme with high affinity for NDMA. That the induction results from the synthesis of a cytochrome P-450 rather than increased NADPH–cytochrome P-450-reductase is indicated by a modest increase of cytochrome P-450 but unaltered NADPH–cytochrome *c*-reductase (Table 3). The induced cytochrome P-450 would appear to have high specificity for its

substrates since one day treatment causes a threefold increase of NDMAD but does not alter mono-oxygenase activity towards aminopyrine or ethoxycoumarin (Table 2). These compounds are prototype substrates for the major phenobarbital induced isozyme P-450b [36] and major 3-methyl-cholanthrene induced isozyme, P-450c, [37, 38] respectively. Aniline hydroxylase activity was increased by pyrazole administration. Cytochrome P-450 isoenzymes inducible by ethanol in the rabbit [39] and rat [40] have high specificity for aniline. The low K_m NDMAD is also induced by ethanol [17].

The decline of aminopyrine demethylase and ethoxycoumarin deethylase after four successive daily administrations of pyrazole are similar to declines in benzphetamine demethylase [9] and aryl hydrocarbon hydroxylase [10, 20] which occur after this treatment. Surprisingly the mono-oxygenase activities towards all substrates tested, including NDMA and aniline were reduced in animals which received a single administration of pyrazole four days prior to sacrifice (Tables 1 and 2). The decrease of activities correlates with decreased cytochrome P-450 but not of NADPH–cytochrome *c*-reductase (Table 3). These observations suggest that the action of pyrazole may not be restricted to induction of cytochrome P-450 isozyme but may also be responsible for the destruction of cytochrome P-450. It is now well established that a wide range of compounds, including some heterocyclics, act as suicide inactivators of cytochrome P-450 [41] by the formation of haem adducts with activated metabolites. Evidence that pyrazole is such a suicide inactivator was provided by the finding that incubation of microsomes *in vitro* with pyrazole resulted in a decrease of cytochrome P-450 by a mechanism which was dependent on the presence of NADPH. The process was time dependent, followed pseudo first-order kinetics and was saturable, all necessary properties for a suicide inactivation event [42–44]. The chemical basis for inactivation remains a matter for speculation but may be elucidated by isolation and characterisation of a putative chemically modified haem.

The effects of pyrazole on other microsomal constituents appear to be mediated by mechanisms distinct from those operating on mono-oxygenases. Thus, the *in vivo* treatments leading to decreases in cytochrome *b*5 do not correlate with those leading to decreased cytochrome P-450 (Table 3). Further pyrazole does not affect cytochrome *b*5 content *in vitro*. Incubation of microsomes with pyrazole also had no effect on either NADPH–cytochrome *c*-reductase or NADH–cytochrome *c*-reductase. It is not understood why pyrazole administration at one day or for four successive days should cause a decrease in the activity of NADH–cytochrome *c*-reductase. Lastly, pyrazole *in vivo* but not *in vitro* increased the activity of epoxide hydrolase. Again this increase did not correlate with alterations of mono-oxygenase activities since it was not apparent by one day but was after four successive administrations or after one administration four days previously.

The effects of pyrazole on microsomal constituents are thus complex and no satisfactory regime for induction of NDMAD for protein purification is immediately apparent. The results also suggest that caution should be adopted in the formulation of mechanisms to explain the antitumourigenic activity of pyrazole [19] and in the use of microsomes from pyrazole treated animals for mutagenesis testing [20].

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