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THE MECHANISM OF INHIBITION BY 2,2′-PYRIDYLISATOGEN TOSYLATE OF NADPH-LINKED ENZYME ACTIVITIES IN MICROSOMES ISOLATED FROM RAT LIVER

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Abstract—Microsomal preparations isolated from rat liver were used to study the action of 2.2′-pyridylisatogen tosylate (PIT) on aniline hydroxylation, cytochrome *c* reduction and NADPH oxidation. PIT was found to inhibit both the NADPH-dependent (5–100 μM, PIT) and the NADPH-independent (0.05–2.5 mM, PIT) hydroxylation of aniline, but had no significant effect on either the NADPH-dependent oxidation of hexobarbital, or the NADPH-independent hydrolysis of glucose-6-phosphatase. PIT was also found to inhibit cytochrome *c* reductase competitively (K_i = 35 μM) and to stimulate NADPH oxidation (ED_{50} = 6.5 μM) PIT and aniline were both found to bind to the microsomal haemoprotein cytochrome P-450 and produce Type II spectral changes. It is proposed that PITs ability to bind to the haemoprotein and its ability to accept electrons from the microsomal NADPH–cytochrome *c* reductase system leads to the inhibition of aniline hydroxylase activity.

Interest in the heterocyclic molecule, 2,2′-pyridylisatogen has centred around its ability to act as an antagonist of the inhibitory actions of ATP on smooth muscle [1–7]. One factor that complicates the use of 2,2′-pyridylisatogen as an ATP-receptor antagonist is the direct muscle relaxant action of the compound [8, 9]. This effect may be due to an inhibitory action of isatogens on mitochondrial oxidative phosphorylation [10–12]. In support of this suggestion, the results of a structure–activity study showed that the smooth muscle relaxant activity wihin a series of isatogens was related to inhibition of ADP-stimulated respiration in tightly-coupled mitochondria [3].

A further aspect to the action of isatogens was discovered when 2-phenylisatogen was found to accept electrons from the NADPH dehydrogenase region of the mitochondrial respiratory chain [13]. Although NADH was oxidized at a faster rate in the presence of 2-phenylisatogen, there was no concomitant increase in ATP synthesis, since the respiratory chain was bypassed. The observation that NADPH oxidation was stimulated markedly in a post-mitochondrial fraction of rat liver, whereas mitochondrial NADPH oxidation was unaffected [14] has led us to examine this effect in greater detail. The present paper, therefore, describes the results of experiments designed to investigate the mode of action of the ATP-receptor antagonist, 2,2′-pyridylisatogen tosylate (Fig. 1) on microsomal enzyme activities.

MATERIALS AND METHODS

Microsomes. Microsomes were prepared from the livers of male Wistar rats by the method of Remmer *et al.* [15], and suspended in 0.1 M phosphate buffer, pH 7.4, to give a final protein concentration of 20 mg/ml.

Enzyme activities. The rate of cytochrome *c* reduction was determined at 550 nm using a Pye Unicam SP1800 spectrophotometer [16]. Aniline hydroxylase activity was determined by the method of Mazel [17]. The hydroperoxide-dependent hydroxylation of aniline was determined by the method of Imai *et al.* [18] which is based on coupling the product *p*-aminophenol to phenol in an alkaline medium, to give a blue compound having a maximal absorbance at 630 nm. Hexobarbital oxidase activity was measured by the rate of disappearance of hexobarbital from an incubation mixture, according to the method of Cooper and Brodie [19]. Aqueous extractions were measured at 245 and 280 nm using a Pye Unicam SP8-100 U.V. spectrophotometer. NADPH oxidation was measured at 340 nm using an SP-1800 spectrophotometer [20]. Glucose-6-phosphatase activity was measured by following the release of inorganic phosphate according to the method of Fiske and Subbarow [21]. The specific conditions employed in the measurement of the above reactions are given in the legends to the appropriate figures and tables.

Difference binding spectra. Difference binding spectra were obtained using the method of Schenkman *et al.* [22].

Protein. Protein was determined by the biuret method [23] after solubilization of the microsomal pellet with sodium deoxycholate (0.16% w/v); bovine serum albumin was used as standard.

Chemicals. 2,2′-Pyridylisatogen was prepared by the method of Ruggli and Cuenin [24], as modified by Robertson [25]. The tosylate salt was prepared by treating the isatogen with *p*-toluene sulphonic acid

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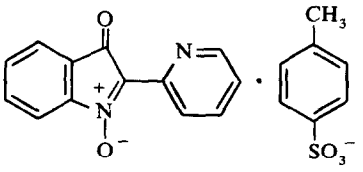

Fig. 1. Structure of 2,2′-pyridylisatogen tosylate (PIT).

in ether 2,2′-pyridylisatogen was added to reaction media as a solution in dimethylformamide (DMF); controls carried out with equivalent amounts of DMF showed that the solvent had no effect on the reactions under consideration. Analytical grade chemicals and biochemicals were purchased from British Drug Houses Ltd (Poole, U.K.) and Sigma Chemical Co. (St. Louis, MO).

Statistics. Where appropriate, results are expressed as the mean ± S.E.M.

RESULTS

Aniline hydroxylation. In the presence of PIT (80 μM) marked inhibition (95%) of aniline hydroxylase activity was observed. Using a Lineweaver and Burk double reciprocal plot the inhibition was found to be of the non-competitive type (increasing concentrations of aniline failed to overcome the inhibition), with a K_m value of 0.57 mM being obtained. In the presence of PIT (80 μM) the V_{max} value for the control reaction was reduced from 0.13 to 0.01 μmole *p*-aminophenol produced min^{-1} mg of $protein^{-1}$. The inhibition was found to be concentration-dependent; increasing the concentration of PIT caused a concomitant decrease in the rate of aniline hydroxylation (Fig. 2), with an IC_{50} value (concentration required to cause a 50% reduction in enzyme activity) of 23.6 ± 2.9 μM (N = 5) being obtained. These findings were further confirmed using a Dixon Plot [26] with a K_i value of 17.5 μM being obtained (Fig. 3).

For the NADPH-independent hydroxylation of aniline, cumene hydroperoxide (CHP) was used to replace NADPH, molecular oxygen and the flavoenzyme requirements of the microsomal cytochrome P-450 system. In this system CHP directly oxidizes cytochrome P-450 to the active hydroxylating species [27]. In the presence of PIT (2.5 mM) the rate of *p*-aminophenol production fell from a control level of 0.18 ± 0.02 to 0.09 ± 0.01 μmole min^{-1} mg of $protein^{-1}$ (both N = 5); however, concentrations of PIT below 2.5 mM failed to produce a significant inhibition (Table 1). Replacement of PIT with known inhibitors and stimulators of the CHP dependent reaction produced the characteristic responses. Low concentrations of either cytochrome *c* (10 μM) or haematin (10 μM) produced marked inhibition while pyrogallol (100 μM), glutathione (1 mM) and ascorbate (1 mM) were all found to stimulate the production of *p*-aminophenol (Table 1).

Hexobarbital oxidase

In contrast to its action on aniline metabolism PIT (0.1–2.5 mM) was found to have no significant ($P > 0.05$) effect on the oxidation of the Type I substrate hexobarbital.

Cytochrome c reduction. The microsomal reduction of cytochrome *c* (NADPH present) by the

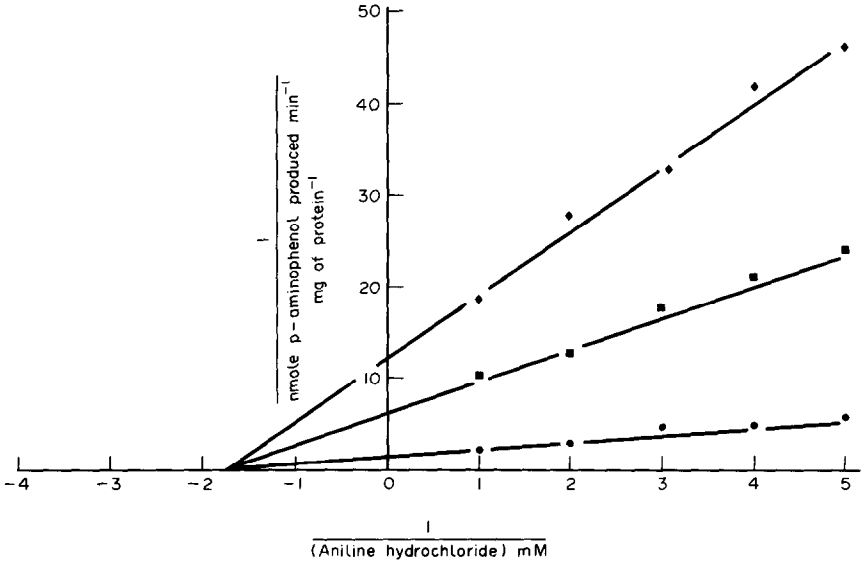

Fig. 2. Effect of 2,2′-pyridylisatogen tosylate on aniline hydroxylase activity in rat hepatic microsomes. Each reaction mixture contained 1.6 μmoles NADPH 20 μmoles nicotinamide, 30 μoles $MgCl_2$, 16 μmoles glucose-6-phosphate, 0.4 unit glucose-6-phosphate dehydrogenase, 300 μmoles potassium phosphate buffer, pH 7.4 and rat hepatic microsomes (2 mg protein). At zero time 10 μmole aniline hydrochloride was added. After 20 min incubation the reaction was stopped by the addition of 2 ml 20% (w/v) trichloroacetic acid and the amount of *p*-aminophenol produced was estimated by the method of Mazel [17]. The incubation temperature was 37° and the final volume 3 ml. When present 60 or 240 nmoles 2,2′-pyridylisatogen tosylate was added 2 min prior to the addition of substrate. Values are the mean of five different experiments: ◆——◆, control; ■——■, 20 μM PIT; ●——●, 80 μM PIT.

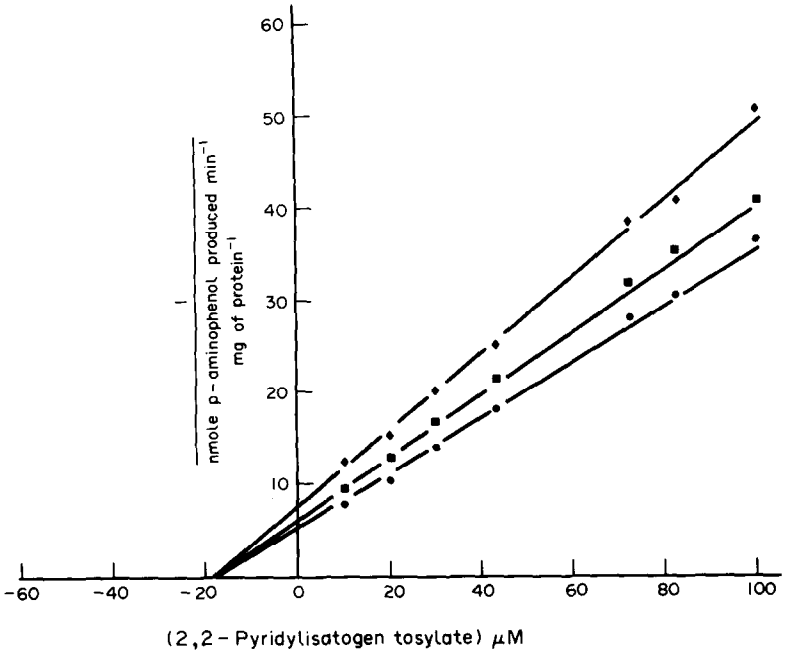

Fig. 3. A Dixon plot showing the inhibition of aniline hydroxylase activity associated with rat hepatic microsomes by PIT. Reaction conditions are described in the legend to Fig. 2. The reaction was initiated by the addition of substrate: ◆——◆, 0.5 mM; ■——■, 1.0 mM; ●——●, 2.0 mM. The incubation temperature was 37° and the final volume 3 ml. When present 30–300 nmoles 2,2'-pyridylisatogen tosylate was added 2 min prior to the addition of substrate. Valves are the means of five different experiments.

enzyme NADPH cytochrome *c* reductase (EC 1.6.2.3.) was found to be inhibited in a concentration-dependent manner by PIT giving an IC_{50} value of 16.5 ± 1.7 μM (N = 5). The inhibition was found to be competitive (Fig. 4) with a K_i value of 35 μM PIT, being obtained.

NADPH oxidation. Low concentrations of PIT (1–40 μM) were found to cause a marked concentration-dependent stimulation of microsomal NADPH oxidation, with a maximum stimulation of 471% (N = 5) occurring at a PIT concentration of 18 μM (Table 2). The ED_{50} value (concentration required to cause a 50% increase) for the stimulatory effect was found to be 6.5 ± 1.5 μM (N = 5).

Glucose-6-phosphatase. The effect of PIT on glucose-6-phosphatase (EC 3.1.3.9), a microsomal

Table 1. The effect of PIT on the hydroperoxide dependent hydroxylation of aniline associated with rat hepatic microsomes

Compound	Concentration (mM)	Rate (nmole *p*-aminophenol produced min^{-1} mg of $protein^{-1}$)
Control		181 ± 22
PIT	0.05*	185 ± 25
PIT	0.25*	161 ± 13
PIT	2.50	88 ± 13
Cytochrome *c*	0.01	94 ± 8
Hematein	0.01	99 ± 13
Pyrogallol	0.10	290 ± 24
Glutathione	1.00	230 ± 21
Ascorbate	1.00	213 ± 15

Each reaction mixture contained 170 μmoles Tris–HCl buffer, pH 7.5. 0.5 mmole aniline hydrochloride and rat hepatic microsomes (2 mg of protein). The reaction was initiated by the addition of 3 μmoles cumene hydroperoxide following a 2 min pre-incubation. The temperature was 37° and the final volume 2 ml. When present PIT and the other components were added in the concentrations stated in the table prior to the addition of hydroperoxide. The results are the means of five different experiments ± S.E.M.

* Not significantly different from the control ($P > 0.05$).

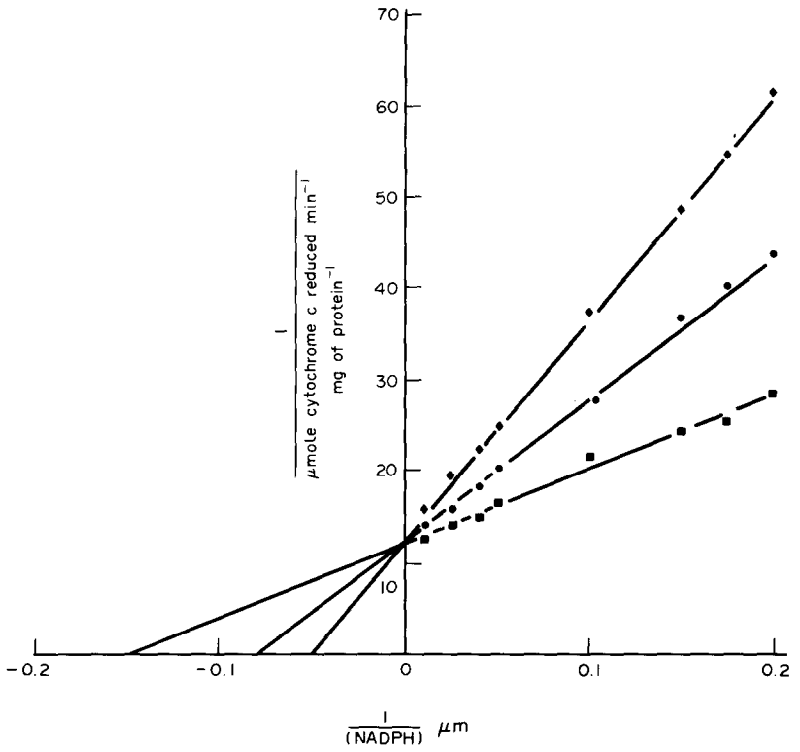

Fig. 4. The effect of 2,2'-pyridylisatogen tosylate on NADPH-cytochrome *c* reductase activity in rat hepatic microsomes. The reaction medium contained 76 μmoles potassium phosphate buffer, pH 7.6, 10 μmoles nicotinamide and rat hepatic microsomes (2 mg of protein). The reaction was initiated by the addition of 150 nmoles NADPH to the experimental cuvette. The reaction temperature was 37° and the final volume 3 ml. Reduction of 2 μmoles cytochrome *c* was measured according to the method of Masters *et al.* [16] by observing the increase in optical density at 550 nm using a Pye Unicam SP 1800, u.v. spectrophotometer. When present 30 or 60 nmoles 2,2'-pyridylisatogen tosylate was added to test and blank cuvettes 2 min prior to the addition of NADPH (■——■) control, (◆——◆) 10 μM PIT and (●——●) 20 μM PIT. The valves are the means of five different experiments.

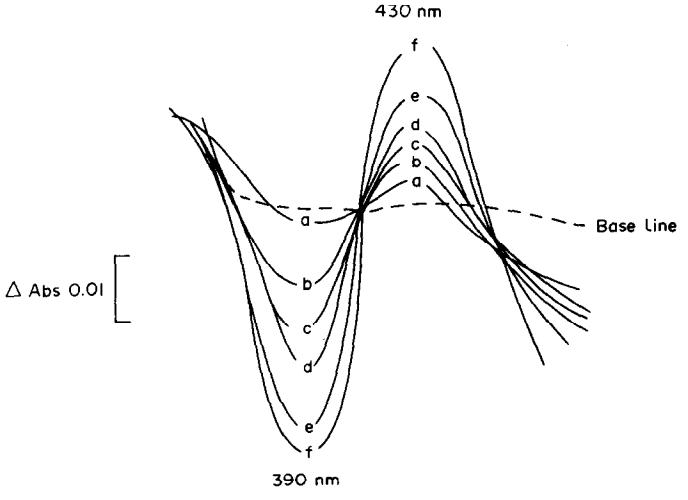

Fig. 5. Difference spectra of 2,2'-pyridylisatogen tosylate obtained in the presence of rat hepatic microsomes. Each cuvette contained microsomes (2 mg protein) suspended in 0.1 M potassium phosphate buffer, pH 7.4. The experimental cuvette received (a) 1.0 mM, (b) 1.5 mM, (c) 2.0 mM, (d) 2.5 mM, (e) 3.0 mM, (f) 4.0 mM PIT; blank cuvette received equal volumes of solvent (DMF)/The temperature was 37°, final volume 3.0 ml and the difference spectra were obtained using a Pye Unicam SP 1800 u.v. spectrophotometer based on the method of Schenkman *et al.* [22]. ΔAbs represents absorbance change.

Table 2. The effects of 2,2'-pyridylisatogen tosylate on NADPH oxidation in rat hepatic microsomes

PIT (mM)	Rate (nmole NADPH oxidized min^{-1} mg of $protein^{-1}$)
Control	24 ± 9
4	46 ± 11
6	70 ± 10
8	81 ± 13
10	110 ± 12
12	124 ± 16
18	137 ± 15
30	137 ± 15
40	135 ± 12

The reaction medium contained 300 μmoles potassium phosphate buffer, pH 7.4 and rat hepatic microsomes (2 mg of protein). The reaction was initiated by the addition of 150 nmoles NADPH to the experimental cuvette, reaction temperature was 37° and the final volume 3.0 ml. NADPH oxidation was measured spectrophotometrically at 340 nm using a Pye Unicam SP 8-100, u.v. spectrophotometer according to the method of Chvapil *et al.* [18]. When present, 12–120 nmoles 2,2'-pyridylisatogen tosylate was added 2 min prior to the addition of NADPH. Values are the mean ± S.E.M. of five different experiments.

enzyme which does not require NADPH as a cofactor, was also studied. In the presence of PIT (1 mM) the K_m and V_{max} values of 6.3 mM and 0.17 μmole of inorganic phosphate liberated min^{-1} mg of $protein^{-1}$ respectively were found to remain unchanged for the enzyme-catalysed hydrolysis of glucose-6-phosphate.

Substrate binding. The addition of PIT to microsomal suspensions (2 mg of protein) produced a characteristic Type II difference spectrum [22] exhibiting a peak at 430 nm and a trough at 390 nm (Fig. 5). The spectral changes were found to be concentration-dependent and a K_s (spectral dissociation constant) value of 2.2 mM for PIT was obtained. Similar changes were observed when aniline repeated PIT (peak at 426 nm and trough at 390 nm), a K_s value for aniline of 0.36 mM was obtained.

DISCUSSION

The endoplasmic reticulum of liver cells contains a mono-oxygenase enzyme system which catalyses the biotransformations of steroids, fatty acids, drugs, carcinogens and other xenobiotics [28]. In this enzyme system the reducing equivalents from NADPH are transferred through the flavoprotein, NADPH cytochrome *c* reductase, to the haemoprotein, cytochrome P-450, which in turn catalyses the oxygenation of various substrates [29]. It therefore appears that hepatic mono-oxygenase activities can be controlled by either the regulation of electron flow through the microsomal electron transport chain from NADPH to the cytochrome P-450 substrate complex [28], or by altering the ability of the haemoprotein to bind substrates [22].

The results obtained in the present study show that the heterocyclic compound 2,2'-pyridylisatogen tosylate has the ability to bind to microsomal cytochrome P-450, to inhibit the NADPH-dependent as well as the NADPH-independent hydroxylation of aniline hydrochloride, to inhibit the NADPH-dependent reduction of cytochrome *c*, and to stimulate the oxidation of NADPH.

Aniline was found to produce a typical Type II spectral change due to the binding of its amino group to the haem iron, resulting in cytochrome P-450 being changed from the high to low spin state [30]. The formation of the enzyme–substrate complex with cytochrome P-450 is the triggering event for the mono-oxygenation process. Only the enzyme-substrate complex seems to be reduced at a rate fast enough to ensure the observed hydroxylation rate. It is not clear whether a conformational change induced by the addition of substrate and/or an increase in the redox potential is the actual determinant for the initiation of the reduction to the ferrous cytochrome [31]. The ability of PIT to produce a similar spectral change indicates that the heterocyclic compound has the capacity to interfere with substrate binding and thus prevent the reduction process. This is further supported by PIT's inability to alter the oxidation of the Type I compound hexobarbital, a compound which is known to bind to the protein component of the cytochrome P-450 complex and not the haem iron [30].

In addition to the potential importance of binding interactions, the observation that the heterocyclic compound is a potent promotor of microsomal NADPH-oxidation may further explain its ability to inhibit both aniline hydroxylation and cytochrome *c* reduction. It was observed that the rate of NADPH-oxidation increased in the presence of PIT. Over the same concentration range of PIT, the NADPH-dependent microsomal enzyme activities were inhibited instead of the expected concomitant increase, thus indicating that the electrons generated by the oxidation of NADPH were not being transferred to either cytochrome *c* or cytochrome P-450.

When the NADPH-independent hydroxylation of aniline was studied, PIT was also observed to have a direct effect, however, the concentrations require to produce inhibition were significantly ($P < 0.05$) higher than those required to inhibit the NADPH-dependent reactions. Studies on mitochondrial oxidations have shown that 2-phenylisatogen, a compound closely related to PIT, can act as an electron acceptor in the NADH dehydrogenase region of the respiratory chain [13]. It is concluded that the primary action of PIT on the microsomal NADPH-dependent reactions, cytochrome *c* reduction and aniline hydroxylation is inhibition, resulting from its ability to accept electrons from the microsomal NADPH oxidase system.

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