Enzymatic Biosynthesis of Vomilenine, a Key Intermediate of the Ajmaline Pathway, Catalyzed by a Novel Cytochrome P450-Dependent Enzyme from Plant Cell Cultures of *Rauwolfia serpentina*

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Microsomal preparations from *Rauwolfia serpentina* Benth. cell suspension cultures catalyze a key step in the biosynthesis of ajmaline – the enzymatic hydroxylation of the indole alkaloid vinorine at the allylic C-21 resulting in vomilenine. Vomilenine is an important branch-point intermediate, leading not only to ajmaline but also to several side reactions of the biosynthetic pathway to ajmaline. The investigation of the taxonomical distribution of the enzyme indicated that vinorine hydroxylase is exclusively present in ajmaline-producing plant cells. The novel enzyme is strictly dependent on $NADPH_2$ and O_2 and can be inhibited by typical cytochrome P450 inhibitors such as cytochrome *c*, ketoconazole and carbon monoxide (the effect of CO is reversible with light of 450 nm). This suggests that vinorine hydroxylase is a cytochrome P450-dependent monooxygenase. A pH optimum of 8.3 and a temperature optimum of 40 °C were found. The K_m value was 3 µM for $NADPH_2$ and 26 µM for vinorine. The optimum enzyme activity could be determined at day 4 after inoculation of the cell cultures in AP I medium. Vinorine hydroxylase could be stored with 20% sucrose at −28 °C without significant loss of activity.

Introduction

Cell suspension cultures of the Indian medicinal plant *Rauwolfia serpentina* (L.) Benth. are an excellent system for the investigation of the biosynthesis of *Rauwolfia* alkaloids at the enzymatic level, especially those of the ajmalan group. Based on our present knowledge, at least 10 different enzymes are involved in the straightforward *in vitro* transformation of tryptamine and secologanin into the antiarrhythmic alkaloid ajmaline. This target alkaloid exhibiting nine chiral carbon centres is expected to be biosynthesized by highly complex reactions. In addition about the same number of enzymes seem to catalyze several side reactions of the biosynthetic pathway to ajmaline (Stöckigt and Schübel, 1988; Stöckigt *et al.*, 1992). The most efficient of these side paths is the glucosylation of the pathway intermediate vomilenine, which is dependent on the recently described UDPG: vomilenine 21-β-D-glucosyltransferase (Ruyter and Stöckigt, 1991). Vomilenine also accumulates as one of the major alka-

loids of *Rauwolfia* cell suspensions and occupies therefore an important branch-point leading to ajmaline, which is the end-product of the pathway, or to its glucoside raucaffricine or through the aldehyde perakine to its alcohol raucaffrinoline (Scheme 1) (Stöckigt, 1986).

Because of this central function of vomilenine in the alkaloid metabolism in *Rauwolfia* cell suspensions, its biosynthesis is of special interest. *In vivo* feeding of its labelled 21-deoxy derivative vinorine to *Rauwolfia* cells resulted in a remarkable incorporation of 18% into vomilenine. This observation suggested vinorine as the immediate biogenetic precursor of vomilenine (Pfitzner *et al.*, 1986; Polz, 1989). Apparently vinorine is directly hydroxylated at the allylic C-21 leading to vomilenine, which is then channelled into various biosynthetic sequences.

The involvement of cytochrome P450-dependent enzymes in hydroxylation reactions in plant secondary metabolism has often been described (Hagman *et al.*, 1983; Petersen *et al.*, 1988; Clemens *et al.*, 1993). In alkaloid biosynthesis we find few examples (Rüffer and Zenk, 1987), only different reaction types such as the formation of a methylenedioxy bridge (Bauer and Zenk, 1991) or

Scheme 1. Biosynthesis of vomilenine and its central function in the metabolism of alkaloids of the Indian medicinal plant *Rauwolfia serpentina*.

an oxidative phenol coupling (Gerardy and Zenk, 1993) have been investigated.

In this paper we describe the isolation and characterization of a cytochrome P 450-dependent monooxygenase generating the hydroxylation of the indole alkaloid vomilenine from vinorine.

Results and Discussion

During our investigation of soluble enzymes from *Rauwolfia* cell suspension cultures catalyzing steps of the biosynthesis of the antiarrhythmic alkaloid ajmaline, we were never able to observe the synthesis of vomilenine, which for phytochemical and biochemical reasons should occupy a central role as an ajmaline precursor. After the

detection of the vinorine synthase (Pfitzner and Stöckigt, 1983; Pfitzner *et al.*, 1986) we concentrated on the search for the next enzyme, which in a formal sense would hydroxylate the product of vinorine synthase at carbon 21 during the formation of vomilenine. The presumptive substrate vinorine was separated easily by HPLC on a reverse phase column from the enzyme product vomilenine and thus made it possible to screen a number of enzyme preparations. In particular membrane-bound protein fractions were tested using different methods to isolate microsomal protein (Diesperger *et al.*, 1974; Britsch *et al.*, 1981; Hakamatsuka *et al.*, 1991) from *R. serpentina* cells grown for different times. The microsomal fractions of the chosen standard method (Diesperger

et al., 1974), when incubated with vinorine and NADPH$_2$, indicated by HPLC analysis the cell-free formation of vomilenine.

After scaling-up such an incubation 20-fold, GC-MS analysis of the acetylated assay mixture showed the enzymatic formation of vomilenine unequivocally by the characteristic fragmentation pattern of its acetylated derivative (Falkenhagen *et al.*, 1993).

Because the cell-free conversion was not very high yielding, we screened for the highest enzyme activity during the whole period of cell growth (Fig. 1). In contrast to most of the enzymes of ajmaline biosynthesis, which express a maximum of activity between day 9 and 13, the vinorine hydroxylase here detected exhibited its optimum 4 days after cell inoculation. In fact, at this time the highest amounts of microsomal protein were extractable from the fresh cells. The total enzyme activity was, however, still relatively low (max. 0.1 nkat/l medium). For a further optimization of hydroxylase isolation, its activity in *Rauwolfia* cells grown in different nutrition media was measured at day 4. As demonstrated in Fig. 2, cells grown in the AP I medium exhibited good hydroxylase activity (1.3 nkat/l after optimization of all other parameters). For standard enzyme isolation, however, cells from LS medium (Linsmaier and Skoog, 1965) which we routinely use for cell cultivation were efficient enough to isolate the hydroxylase for further characterization.

Fig. 2. Hydroxylase activity of *Rauwolfia* cells grown in different nutrition media under standard conditions as described. Cells for activity tests were harvested on day 4.

The microsomal bound enzyme showed quite good stability when stored at −28 °C in buffer with 20% sucrose. Even after one year of storage 70% of the original activity was retained. A major property of this enzyme, a pH optimum at pH 8.3, was determined. This is relatively high compared to that of most of the other enzymes of ajmaline biosynthesis. One exception is that of vinorine synthase, the enzyme immediately preceeding the hydroxylase and exhibiting nearly the same pH optimum at pH 8.5. Whether both enzymes are located in the same compartment and are acting under these *in vivo* optimum pH conditions is still unknown. The temperature optimum of the reaction was 40 °C.

The enzyme reaction was clearly dependent on NADPH$_2$ (and oxygen) which could not be replaced by NADH$_2$, indicating a remarkable coenzyme specificity. In fact many examples have been described for plant monooxygenases preferring NADPH$_2$ rather than NADH$_2$ (West, 1980; Rüffer and Zenk, 1987; Kochs and Grisebach, 1987; Schmidt and Stöckigt, in preparation). The appropriate Michaelis-Menten kinetic (Fig. 3) showed a K_m value of NADPH$_2$ of 3 µM demonstrating a very high affinity of the enzyme towards the cofactor. In addition, vinorine served as an excellent substrate and its Menten kinetics (Fig. 4) revealed also a low K_m value of 26 µM, which suggested that vinorine was in fact the natural substrate of the isolated hydroxylase. From these K_m
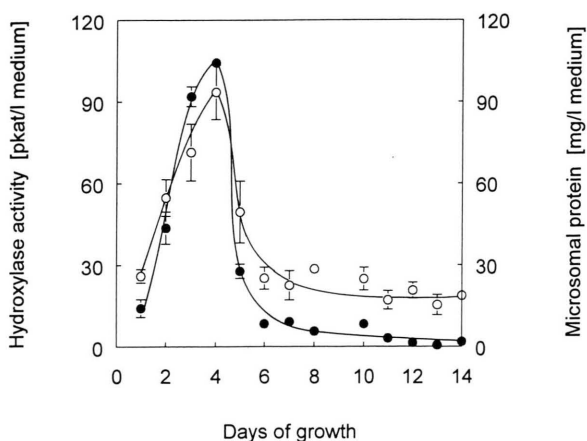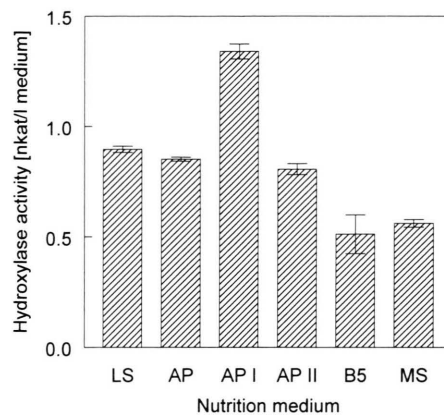

Fig. 1. Changes in enzyme activity and content of microsomal protein of *R. serpentina* cell cultures during a cultivation period of 14 days (●, enzyme activity; ○, protein content/l medium).

values it might be quite possible that a change of concentration of both the substrate and the co-enzyme can markedly influence *in vivo* the rate of vinorine hydroxylation and might therefore be a point of regulation of the alkaloid biosynthesis after the vinorine formation has taken place.

The necessity of $NADPH_2$ and oxygen for the hydroxylase pointed to a cytochrome P450-dependent reaction. In order to verify this assump-

tion a number of inhibition studies were performed. A typical competitive inhibitor of this class of enzyme is the oxidized cofactor $NADP^+$ (Madyastha and Coscia, 1979). When we measured the hydroxylase activity of different $NADPH_2/NADP^+$ ratios (Fig. 5), it became evident that the ratio has a distinct influence with increasing inhibition if the concentration of the oxidized cofactor increases. A Lineweaver-Burk plot for three different $NADPH_2$ concentrations proved the competitive nature of the inhibition by $NADP^+$ (inhibitory constant of 1.5 mm).

Moreover, cytochrome *c* interfering with the electron transfer from the cytochrome P450 reductase to the final oxidase (Fujita *et al.*, 1982; Takemori *et al.*, 1993) should also inhibit the vinorine hydroxylase. When 10 μm cytochrome *c* were included in the assay a 76% inhibition was recorded for the enzyme. Complete inhibition was reached at 20 μm cytochrome *c*, which again was a significant indication of the cytochrome P450 character of the hydroxylase. The action of a series of typical inhibitors (Coulson *et al.*, 1984; Graebe, 1984) of this class of enzymes, as illustrated in Table I, was in excellent agreement with the assumption that the hydroxylase belongs to cytochrome P450 monooxygenases. In fact at a concentration of 100 μm all tested compounds gave a significant inhibition. Ketoconazole and the

Fig. 3 The effect of $NADPH_2$ concentration on vinorine hydroxylase activity (standard incubation mixture, K_m = 3 μM, V_{max} = 0.99 nkat). The insert shows the double-reciprocal plot.
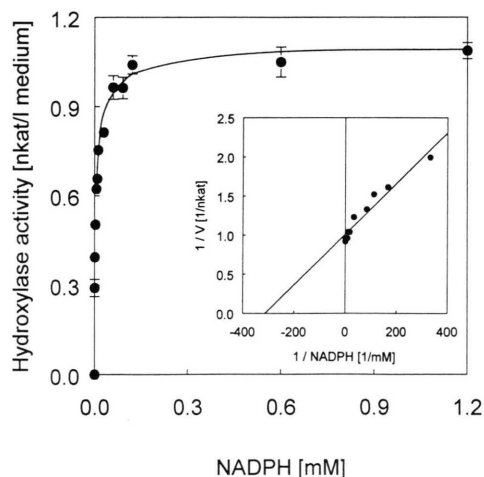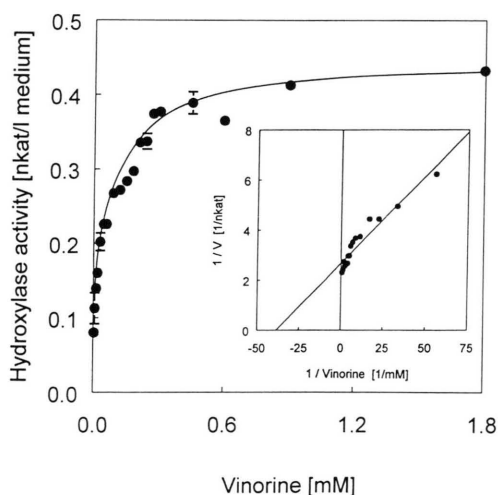

Fig. 4. The effect of vinorine concentration on vinorine hydroxylase activity (standard incubation mixture, K_m = 26 μM, V_{max} = 0.38 nkat). The insert shows the double-reciprocal plot.
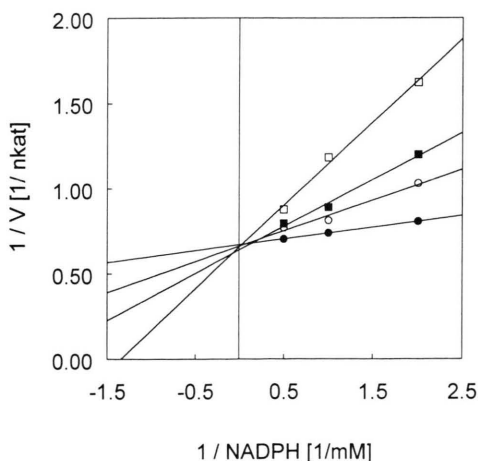

Fig. 5. Competitive inhibition of vinorine hydroxylase by $NADP^+$ (Lineweaver-Burk plot; ●, without $NADP^+$; ○, 0.6 mm $NADP^+$; ■, 1.2 mm $NADP^+$; □, 2.4 mm $NADP^+$; K_i = 1.5 mm).

Table I. Influence of typical cytochrome P450 inhibitors on vinorine hydroxylation (standard incubation mixture; 100% enzyme activity = 1.22 nkat).

Inhibitors	Concentration [mM]	Inhibition [%]
Cytochrome c	0.02	100
	0.01	76
Ketoconazole	1.0	52
	0.1	38
Metyrapone	1.0	44
	0.1	29
Tetcyclacis	0.5	46
	0.1	23
Ancymidole	1.0	40
	0.1	11
LAB 150978	1.0	62
	0.1	34
BAS 110 W	1.0	45
	0.1	24
BAS 111 W	1.0	72
	0.1	22

triazole growth regulators LAB 150978 and BAS 110 W (Jung *et al.*, 1986) were the strongest inhibitors besides metyrapone.

The inhibition of the hydroxylase could additionally be achieved by carbon monoxide. In particular the reversal of the inhibition by light with a maximum at 450 nm is regarded as one of the most reliable proofs for a cytochrome P450-containing enzyme (Estabrook *et al.*, 1963; West, 1980). As demonstrated in Table II, the hydroxylase activity is reduced by 70% if the gas mixture is changed from N_2/O_2 to CO/O_2 and the reaction is carried out in absence of light. Under the same conditions light at 450 nm but not at 700 nm clearly reversed this inhibition effect. The system for the gas experiments was evaluated by

Table II. Effect of light and carbon monoxide on vinorine hydroxylase activity in microsomes of *R. serpentina* cell cultures (standard incubation mixture in a special apparatus for varying illumination and different gas mixtures; 100% enzyme activity = 0.37 nkat).

Gas mixture	Illumination	Vinorine hydroxylase activity [%]
N_2/O_2 9:1	light	100
N_2/O_2 9:1	dark	103
CO/O_2 9:1	dark	30
CO/O_2 9:1	light	75
CO/O_2 9:1	blue (450 nm)	67
CO/O_2 9:1	red (700 nm)	38

the known cytochrome P450-dependent hydroxylation of aniline (Estabrook *et al.*, 1972) which showed the same inhibition characteristics when tested with guinea-pig liver microsomes.

The CO-difference spectrum (Fig. 6) exhibited absorptions at 420 and 450 nm indicating the presence of cytochrome P450 and cytochrome P420 which is its inactive form that may occur during preparation of the microsomes. From the absorption at 450 nm the content of cytochrome P450 enzymes in *R. serpentina* microsomes could be calculated as 113 pmol/mg protein (Omura and Sato, 1964; Estabrook and Werringloer, 1978). This amount agrees with other authors who found between 5 pmol and 400 pmol/mg protein in plant microsomal fractions (Rich and Bendall, 1975; Petersen *et al.*, 1988; Bauer and Zenk, 1991). All these data indicate that the vinorine-converting enzyme is without doubt a novel cytochrome P450-dependent monooxygenase.

We have previously found that the enzymes of the ajmaline biosynthetic pathway have a very distinct taxonomic distribution. Conclusively these enzymes are obviously highly specific which is supported by their pronounced substrate specificity and the limited occurrence of the appropriate alkaloids. We are now investigating the substrate specificity of vinorine hydroxylase with various structurally interesting alkaloids and the high
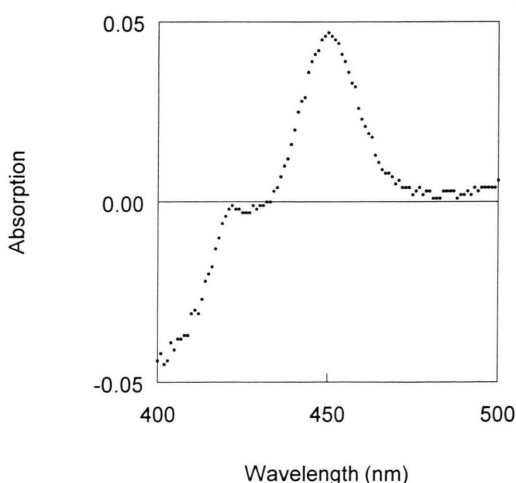

Fig. 6. Carbon monoxide difference spectrum of *R. serpentina* microsomes. The sample was bubbled with CO and treated with sodium dithionite as well as the reference cuvette as described by Omura and Sato (1964). Cytochrome P450 content: 113 pmol/mg protein.

specificity seems also to be true for the formation and accumulation of vomilenine (these data will be presented elsewhere). A broad screening program was carried out during recent years and clearly indicated that vomilenine is a typical *Rauwolfia* alkaloid not detected in other genera of the Apocynaceae family. This finding is in complete agreement with chemotaxonomic investigations also including other plant families *e.g.* Loganiaceae or Rubiaceae (Kisakürek *et al.*, 1993). In this context it was necessary to know whether the enzyme here described is randomly distributed or has a more limited distribution. Therefore we have analyzed 9 cell culture systems of 6 different plant families (Table III). It turned out that exclusively in one culture – *R. serpentina* – hydroxylase activity could be measured, and this is the only one of the tested cell systems synthesizing vinorine and vomilenine resp. Therefore it appears to be clear that the occurrence of both the substrate vinorine and the enzyme vinorine hydroxylase are strongly correlated and that the hydroxylase is an extraordinarily specific enzyme.

Table III. Taxonomic distribution of vinorine hydroxylase in various cell suspension cultures. Standard growth conditions and harvest on day 4 (* no detectable activity in HPLC, detection limit <1% rel. act.; 100% activity = 1.11 nkat).

Cell culture	Family	Vinorine hydroxylase [rel. act. %]
Rauwolfia serpentina Benth. *ex* Kurz	Apocynaceae	100
Catharanthus roseus G. Don.	Apocynaceae	*
Nicotiana tabacum L.	Solanaceae	*
Solanum marginatum L.	Solanaceae	*
Lycopersicon esculentum Mill.	Solanaceae	*
Agrostis tenuis Sibth.	Poaceae	*
Viola calaminaria Lej.	Violaceae	*
Ophiorrhiza pumila Champ.	Rubiaceae	*
Lonicera morrowii A. Gray	Caprifoliaceae	*
Liver microsomes	guinea-pig	*

Materials and Methods

Biochemicals

NADPH$_2$ (98% purity), NADP$^+$ (98%) and NADH$_2$ (98%) were purchased from Boehringer (Mannheim). Cytochrome *c*, ketoconazole and metyrapone were obtained from Sigma (München). Ancymidole was from Fluka (Neu-Ulm).

Tetracyclacis, LAB 150978, BAS 110W and BAS 111W were kindly provided by BASF (Ludwigshafen). All other commercially available materials were of highest purity. Vinorine and vomilenine were isolated from *R. serpentina* hairy root cultures in our laboratory (Falkenhagen *et al.*, 1993).

Cell cultures

Cell suspension cultures of *Rauwolfia serpentina* were grown as recently described (Ruyter and Stöckigt, 1991) under constant illumination on gyratory shakers in 1 l Erlenmeyer flasks containing LS medium (Linsmaier and Skoog, 1965) (400 ml total volume). For the investigations concerning the optimal nutrition medium for hydroxylase activity we also used AP, AP I, AP II, B 5 and MS medium in the same way (Murashige and Skoog, 1962; Gamborg *et al.*, 1968; Zenk *et al.*, 1977; Schübel *et al.*, 1989). Cultures used for enzyme preparation were harvested by suction filtration after cultivation for 4 days. The cell material was filtered, frozen with liquid N$_2$ and stored at −25 °C until further operation.

Guinea-pig liver

Guinea-pig liver was excised and frozen with liquid N$_2$ and then used for enzyme isolation.

Enzyme preparations

The deep frozen cell material of *R. serpentina* and the guinea-pig liver were pulverized in presence of liquid nitrogen, thawed in buffer A (liver in buffer B) and filtered through 4 layers of cheese-cloth. This crude extract was centrifuged at 13,000×*g* for 20 min. From the supernatant the microsomes of *R. serpentina* were prepared by Mg^{2+} precipitation as described earlier (Diesperger *et al.*, 1974). For preparation of liver microsomes the supernatant was centrifuged at 100,000×*g*, washed with buffer B and re-centrifuged at 100,000×*g* each for 1 h. The microsomal pellets were diluted with buffer A, or with buffer B resp., homogenized with a Potter homogenisator and stored at −25 °C. All operations were carried out at 4 °C. Protein determination was carried out according to Bradford (Bradford, 1976).

The following buffers were used: buffer A: 0.1 M Tris-HCl, pH 8.0, 10 mM KCl, 20% sucrose and 10 mM β-mercaptoethanol; buffer B: 0.1 M Tris-HCl, pH 8.0, 10 mM KCl and 20% sucrose; buffer C: 0.1 M citrate-phosphate buffer from pH 3–7, 10 mM KCl and 20% sucrose. Buffer C was used in experiments to determine the pH optimum of the hydroxylase.

HPLC assay for vinorine hydroxylase

Vinorine (0.3 mM), NADPH (1.2 mM), and microsomal protein (1 mg) were incubated in buffer B (total volume 0.5 ml) for 60 min at 35 °C with shaking in open vials. The reaction was terminated by addition of 500 μl MeOH. The mixture was subsequently centrifuged and a 40 μl aliquot of the supernatant was used for HPLC analysis.

HPLC column: Merck LiChrosolv RP select B, 4×125 mm, gradient 68% 0.01 M $(NH_4)_2CO_3$: 32% MeCN for 6 min, 40% $(NH_4)_2CO_3$: 60% MeCN for 6 min and re-equilibration of the column for 8 min. The alkaloids were detected at 260 nm. Retention time of vinorine and vomilenine were 11 min and 4.8 min respectively.

Photometrical assay for aniline hydroxylase

The assay for aniline hydroxylase was performed as previously reported (Nishigori and Iwatsuru, 1982).

Product identification

The analytical assay was scaled up 20 times. In an open 100 ml Erlenmeyer flask 20 mg of microsomal protein, 1 mg vinorine, 10 mg $NADPH_2$ and buffer B up to 10 ml were incubated while shaking at 35 °C for 1 h. The appropriate control experiment was performed without substrate. To stop the reaction the incubation mixture was extracted twice with 5 ml EtOAc. EtOAc was evaporated and the residue acetylated overnight with 250 μl pyridine/acetic anhydride (1:1 vol.) at room temperature.

After removal of the solvents 10 μl MeOH were added and an aliquot of 2 μl was used for GC-MS analysis. 21-O-acetyl-vomilenine could be clearly detected with a retention time of 3.18 min (vinorine 2.36 min). The detection limit was 0.5 μg. The fragmentation pattern of acetylated vomilenine was identical to that of an authentic sample. For GC-MS experiments a Finnigan MAT 44 S quadrupole instrument coupled with a DB 1 column was used with the following time program: initial temperature 280 °C, 10 °C/min up to 320 °C final temperature.

Inhibition experiments with controlled gas atmosphere and illumination

Three assay mixtures at a time could be incubated in a glass apparatus with three side vessels, each closed with a septum. The apparatus had a stopcock for vacuum and one for gas supply. After the standard assay mixtures without $NADPH_2$ were introduced, the apparatus was evacuated 3 times for 3 s and each time refilled with the required gas mixture. Subsequently the reaction was started with the injection of $NADPH_2$ through the septum of each side vessel.

The gas mixture was provided by displacement of water in graduated cylinders.

The whole apparatus was placed in a box impervious to light, but with a hole in the bottom. The filters (450 nm and 700 nm) fitted exactly the opening to provide the vessels with the red and blue illumination. In the case of working with normal light (halogen spot light, 20 W) or darkness the hole was left open or closed tightly. The reaction temperature was held constant at 30–35 °C by a ventilator. The reaction was stopped by the addition of 500 μl MeOH through each septum.

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