

# Properties of Vinorine Synthase – the *Rauwolfia* Enzyme Involved in the Formation of the Ajmaline Skeleton

Artur Pfitzner, Leo Polz, and Joachim Stöckigt

Institut für Pharmazeutische Biologie der Ludwig Maximilians Universität München, Karlstr. 29, D-8000 München 2, Bundesrepublik Deutschland

Z. Naturforsch. **41c**, 103–114 (1986); received July 3, 1985

Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday

*Rauwolfia serpentina* Benth., Cell Suspension Cultures, Vinorine Synthase, Ajmaline Skeleton

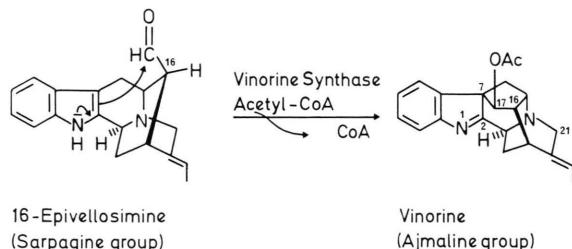
Vinorine synthase, a key enzyme in the formation of the *Rauwolfia* alkaloid ajmaline and its derivatives, has been isolated from *Rauwolfia serpentina* cell suspension cultures. The new enzyme has been 160-fold purified and characterized in detail. The synthase catalyses a single step in the biosynthesis of ajmalan alkaloids by the acetyl-coenzyme A and 16-epi-vellosimine dependent formation of vinorine, which shows the basic ajmaline skeleton. Besides a characteristic substrate specificity the major properties of the enzyme are a relatively high pH optimum (pH 8.5), a temperature optimum of 35 °C, an isoelectric point of pH 4.4, and a relative molecular weight of  $31000 \pm 8\%$ . The apparent  $K_m$  values for 16-epi-vellosimine and acetyl-coenzyme A were  $19.4 \mu\text{M}$  and  $64 \mu\text{M}$  resp. for the formation of vinorine. Kinetic data of the catalysed reaction indicate that 17-deacetylvinorine is not involved as a free intermediate during the vinorine biosynthesis. Vinorine was proved by *in vivo* feeding experiments to be the biosynthetic precursor of the alkaloids in the final stages of the ajmaline pathway, vomilenine (21-hydroxyvinorine), 17-O-acetylnorajmaline and 17-O-acetylajmaline.

## Introduction

Vinorine is one of the structurally simplest indole alkaloids of the ajmaline class and is relatively widely distributed in *Rauwolfia* species. The alkaloid has been isolated from *R. balansae* spp. *balansae* Boiteau [1], *R. balansae* spp. *schumanniana* var. *basicola* Boiteau [1], *R. perakensis* King. Gamble [2], *R. serpentina* Benth. (plant cell culture) [3], *R. sevenetii* Boiteau [1], *R. spathulata* Boiteau [1] and *R. verticillata* Chevalier (plant and cell culture) [4].

In a preliminary communication we described recently the cell-free synthesis of vinorine by a pre-purified enzyme extract obtained from cell suspension cultures of *R. serpentina* Benth. [5]. This new *Rauwolfia* enzyme, which catalyses the ring closure of 16-epi-vellosimine by an acetyl-coenzyme A dependent reaction leading to vinorine, was named vinorine synthase (Scheme 1). It is this enzyme which links biosynthetically sarpagine with ajmaline alkaloids in *R. serpentina* and therefore occupies the key position in the biosynthesis of the latter com-

pounds. Meanwhile the synthase has been partially purified and characterized. In this paper we report in detail on the purification of the enzyme, its properties and the kinetic data of the catalysed reaction and we discuss the biogenetic significance of vinorine.



Scheme 1. Enzymatic formation of vinorine from 16-epi-vellosimine.

## Results

### Isolation and purification of vinorine synthase

From 0.4 kg cultivated *R. serpentina* cells vinorine synthase was extracted with 0.1 M potassium phosphate buffer. A five-step purification procedure

Reprint requests to Dr. J. Stöckigt.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen  
0341-0382/86/0100-0103 \$ 01.30/0

Table. Partial purification of vinorine synthase from 0.4 kg *R. serpentina* cells.

Purification step	Total volume [ml]	Total protein [mg]	Total activity [nkat]	Specific activity [nkat/mg]	Yield [%]	Purification [-fold]
1. Supernatant of the crude extract	1160	1740	—	—	—	—
2. Ammonium sulphate fractionation	57	410.4	6.35	0.015	76	1.0
3. DEAE-Cellulose chromatography	76	36.5	8.32	0.23	100	15.3
4. AcA 54 chromatography	52	11.5	7.9	0.69	95	46
5. Hydroxylapatite chromatography	80	8	6.62	0.83	80	55.3
6. Phenylsepharose chromatography	76	2	4.73	2.4	57	160

comprising ammonium sulphate precipitation, DEAE cellulose, AcA 54, hydroxylapatite and phenylsepharose chromatography resulted finally in a 160-fold enrichment of the enzyme activity. The overall recovery of enzyme during this procedure was 57%. The data for the enzyme purification are summarized in the Table. To monitor the individual purification steps a standard assay for vinorine synthase activity was developed based on the enzymatic generation of the substrate 16-epi-vellosimine from polyneuridine aldehyde by the known polyneuridine aldehyde esterase [6] and adding [<sup>3</sup>H-acetyl]acetyl-coenzyme A. Quantitation of vinorine formation could be achieved by extraction of the enzyme incubation mixture with ethyl acetate and determination of [<sup>3</sup>H]vinorine by scintillation counting of the organic phase. Acetylajmalan esterase which interferes with the assay by hydrolyzing the formed vinorine

could be quantitatively removed from the enzyme mixture by ion exchange chromatography on DEAE DE 52 cellulose. The separation profile of this esterase from vinorine synthase is depicted in Fig. 1. The esterase was monitored by counting [<sup>3</sup>H]acetic acid liberated from [<sup>3</sup>H]acetyltetraphyllicine which could be synthesized from tetraphyllicine by acetylation with [<sup>3</sup>H]acetic anhydride.

Purification of the synthase preparation from DEAE chromatography on a hydroxylapatite column removed a further *Rauwolfia* enzyme, polyneuridine aldehyde esterase. Vinorine synthase is not bound to hydroxylapatite. The final 160-fold enrichment of the synthase was achieved on a phenylsepharose column by elution with decreasing KCl concentration (0.5–0 M) and simultaneous increase of ethylene glycol (0–50%) as shown in Fig. 2.

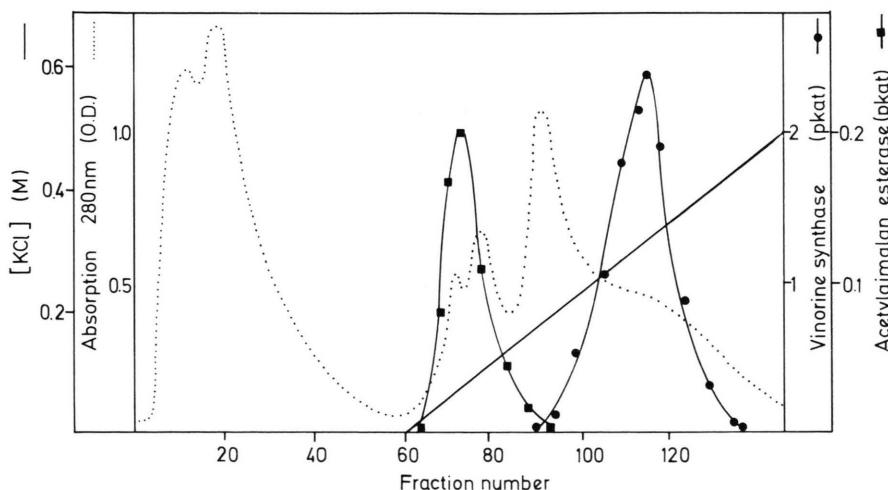


Fig. 1. Separation of vinorine synthase and acetylajmalan esterase on a DEAE DE 52 column.

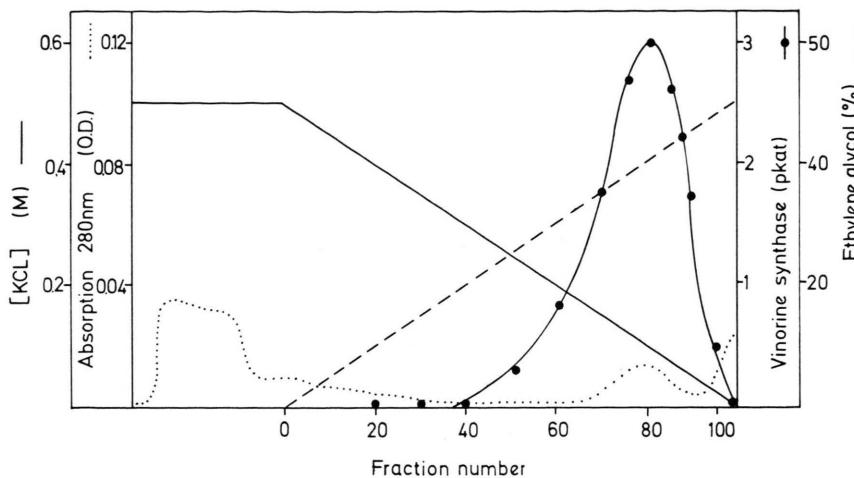


Fig. 2. Elution profile of vinorine synthase on phenyl-sepharose.

#### Properties of vinorine synthase

The vinorine synthase reaction was absolutely dependent on protein and gave a typical saturation curve and normal Michaelis-Menten kinetics for 16-epi-vellosimine (Fig. 3) and acetyl-coenzyme A (Fig. 4). For both substrates double-reciprocal plots of enzyme activity and substrate concentration showed the linear relationship (Figs. 3, 4). The apparent  $K_m$  value for 16-epi-vellosimine was 19.4  $\mu\text{M}$

( $V_{\max} = 5 \text{ pkat}$ ) and for acetyl-coenzyme A 64  $\mu\text{M}$  ( $V_{\max} = 4.4 \text{ pkat}$ ).

Vinorine synthase showed a clear pH optimum at pH 8.5 when enzyme activity was determined under standard conditions (see experimental part) in different buffer systems (Fig. 5). The enzyme tolerates a broad pH range from pH 5 to pH 10 and has a temperature optimum at 35 °C (Fig. 6). The isoelectric point of the enzyme was pH 4.4 as measured by the

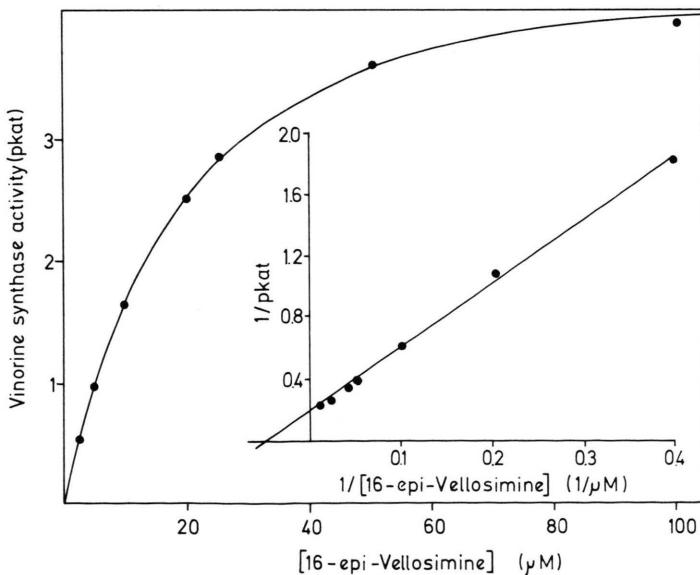


Fig. 3. The effect of the concentration of 16-epi-vellosimine on the reaction rate of vinorine synthase. Standard incubation mixture,  $K_m = 19.4 \mu\text{M}$ ,  $V_{\max} = 5 \text{ pkat}$ .

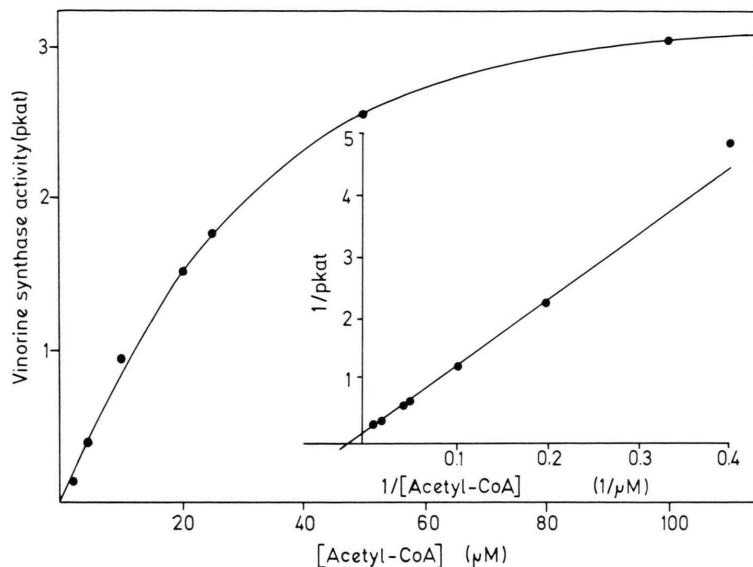


Fig. 4. The effect of the concentration of acetyl-coenzyme A on the reaction rate of vinorine synthase. Standard incubation mixture,  $K_m = 64 \mu M$ ,  $V_{max} = 4.4 \text{ pkat}$ .

chromatofocusing method [7]. Determination of the relative molecular weight of vinorine synthase by gel-chromatography on a calibrated AcA 54 gave a value

of  $31000 \pm 8\%$  Dalton assuming a globular shape of the enzyme.

#### Substrate specificity of the synthase

Substrate acceptance of the enzyme was examined using a variety of sarpagine type alkaloids bearing a

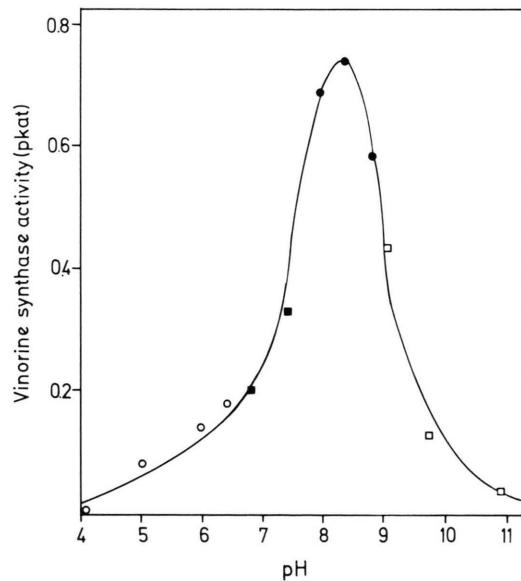


Fig. 5. The effect of pH on vinorine synthase activity. Enzyme activity was measured in presence of 0.1 M citrate-potassium phosphate buffer (○—○), 0.1 M potassium phosphate buffer (■—■), 0.1 M borate buffer (●—●) and 0.1 M glycine-NaOH buffer (□—□).

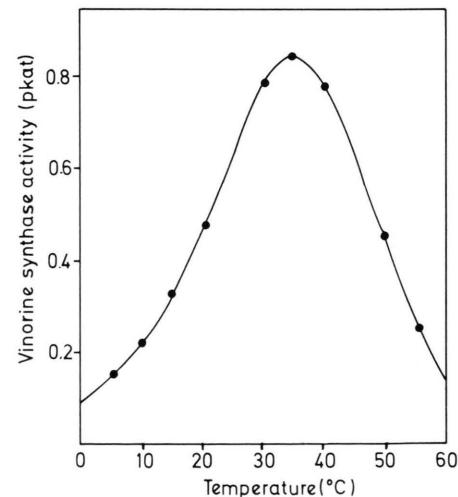


Fig. 6. Influence of temperature on the reaction rate of vinorine synthase.

C-16-aldehyde group and [ $^3\text{H}$ ]acetyl-coenzyme A as co-substrate. None of the alkaloids with a C-16 (R) stereochemistry *e.g.* vellosimine act as a substrate for the ring closure to ajmaline alkaloids. Of six alkaloids exhibiting an *endo* aldehyde group (C-16, S) only two were cyclized by the enzyme, 16-*epi*-vellosimine and its 11-methoxy derivative (gardneral). For the latter compound a  $K_m$  value of 39  $\mu\text{M}$  ( $V_{\max} = 4.3 \text{ pkat}$ ) was determined. Alkaloids of the same type, but  $\text{N}_a$ -methylated (21-deoxyajmal A, methylgardneral) or bearing a carbomethoxy group in addition to the aldehyde function at C-16 (polyneuridine aldehyde, voachalotine aldehyde), were inactive in respect to the catalytic activity of the synthase (Scheme 2) when substrate concentrations were used between 2.5 and 100  $\mu\text{M}$ , acetyl-CoA and 5 pkat enzyme. Co-substrates other than acetyl-CoA, such as malonyl-CoA, benzoyl-CoA, coumaroyl-CoA or oleoyl-CoA were also not accepted nor did they show an inhibition of enzymatic vinorine formation.

The enzymatic ring closure of 11-methoxy-16-*epi*-vellosimine (gardneral) gave a new indolenine alkaloid, 11-methoxyvinorine, as demonstrated by its mass spectral data (UV, MS,  $^1\text{H}$ -NMR) as found for the chemically synthesized alkaloid from acetyltetraphyllicine or for vinorine isolated from *R. serpentina* cell cultures. Optical rotation values of the three vinorine preparations also were identical,  $[\alpha]_D^{20} = 30^\circ \pm 3^\circ$ .

The enzymatic synthesis of vinorine was not inhibited by the pathway endproducts sarpagine and ajmaline in standard incubations containing up to 1 mM concentrations of both alkaloids.

#### Investigation of the mechanism of vinorine formation

Because of the relative instability of 16-*epi*-vellosimine these experiments were carried out with gardneral as substrate. When under standard incubations (5 pkat vinorine synthase) the catalytic activity of the enzyme was investigated dependent on gardneral in presence of different concentrations of the co-substrate acetyl-CoA (Fig. 7) or *vice versa* (Fig. 8), the double reciprocal plots gave a set of curves intersecting the abscissa in the *same* point as observed for enzyme kinetics where a ternary enzyme-substrate-cosubstrate complex is formed.

Moreover experiments were carried out with the endproduct coenzyme A to measure the inhibition of

the enzyme reaction. Vinorine synthase activity was determined in incubations containing 0, 0.5 and 2 mM CoASH in the presence of 0.1 mM acetyl-CoA and different concentrations of gardneral (Fig. 9) or in the presence of 0.1 mM gardneral and different acetyl-CoA concentrations (Fig. 10). In both cases the set of curves intersects the ordinate in the same

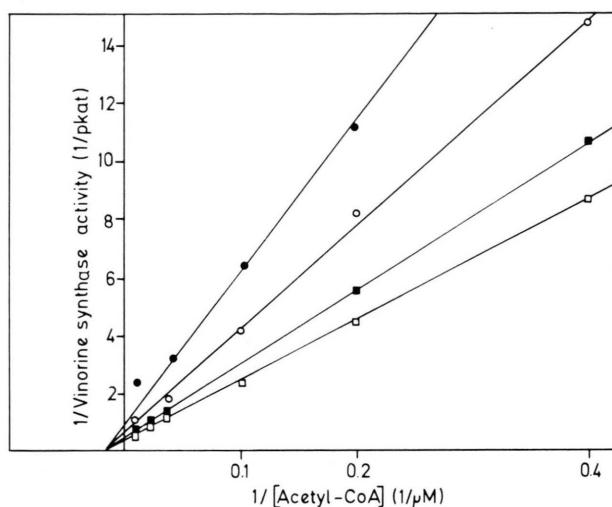


Fig. 7. Reciprocal plot of the vinorine synthase activity dependent on the concentration of gardneral in presence of 10  $\mu\text{M}$  (●—●), 25  $\mu\text{M}$  (○—○), 50  $\mu\text{M}$  (■—■) and 100  $\mu\text{M}$  (□—□) acetyl-coenzyme A.

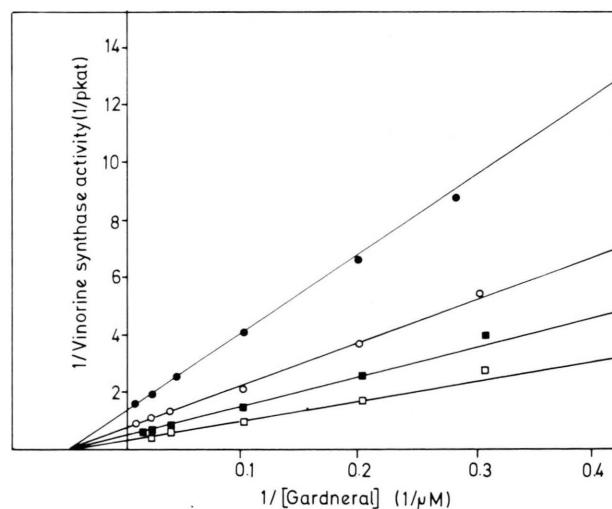


Fig. 8. Reciprocal plot of vinorine synthase activity dependent on the acetyl-coenzyme A concentration in the presence of 0.5  $\mu\text{M}$  (●—●), 10  $\mu\text{M}$  (○—○), 25  $\mu\text{M}$  (■—■) and 50  $\mu\text{M}$  (□—□) gardneral.

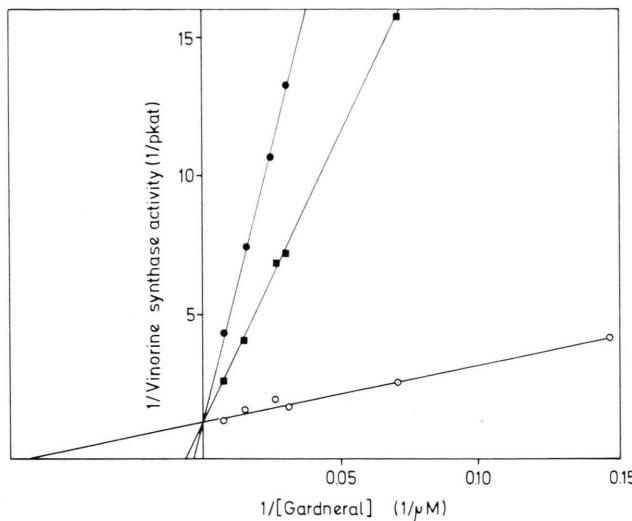


Fig. 9. Reciprocal plot of the activity of vinorine synthase dependent on the concentration of gardneral without coenzyme A (○—○), in presence of 0.5 mM (■—■) and 2 mM (●—●) CoA.

point, demonstrating for both substrates a competitive inhibition due to the presence of CoASH.

#### Feeding experiments with $[^3\text{H}]$ -acetyl- and $[^{14}\text{C}]$ -acetylvinorine

*In vivo* feeding was conducted with 17-O-acetyl labelled vinorine which could be easily synthesized from polyneuridine aldehyde, the corresponding polyneuridine aldehyde esterase, vinorine synthase

and  $[^3\text{H}]$ acetyl-CoA or  $[^{14}\text{C}]$ acetyl-CoA without isolation of the intermediate 16-epi-velliosimine. The specific activity of  $[^3\text{H}]$ -acetylvinorine was 31  $\mu\text{Ci}/\mu\text{mol}$  and 9.2  $\mu\text{Ci}/\mu\text{mol}$  for  $[^{14}\text{C}]$ -acetylvinorine. After a feeding time of 24 h to a cell suspension culture of *R. serpentina*  $[^3\text{H}]$ -acetylvinorine showed incorporation into vomilenine (2%), 17-O-acetylajmaline (1%) and 17-O-acetylajmaline (9%).  $[^{14}\text{C}]$ -acetylvinorine, which had been fed for 2.5 days, was exclusively incorporated into vomilenine (18%).

#### Discussion

Monoterpene indole alkaloids of the ajmaline class are widespread in the genus *Rauvolfia* [8]. So far 53 different alkaloids of this group have been isolated from some plant species [9]. Among them is the pharmacologically important compound ajmaline. The typical structural feature of all these compounds is the characteristic ajmalan bond between C-7 and C-17.

During the last three years we have detected several enzymes involved in the biosynthesis of ajmaline and the biosynthetically related sarpagine alkaloids by using cell suspension cultures of *R. serpentina*. One of these enzymes, which catalyses the formation of the above mentioned bond and therefore the key step in the biosynthesis of the ajmaline skeleton, was named vinorine synthase (EC 4.1.2.-). The properties of this enzyme and the biogenetic significance of the enzyme product vinorine are described in this paper.

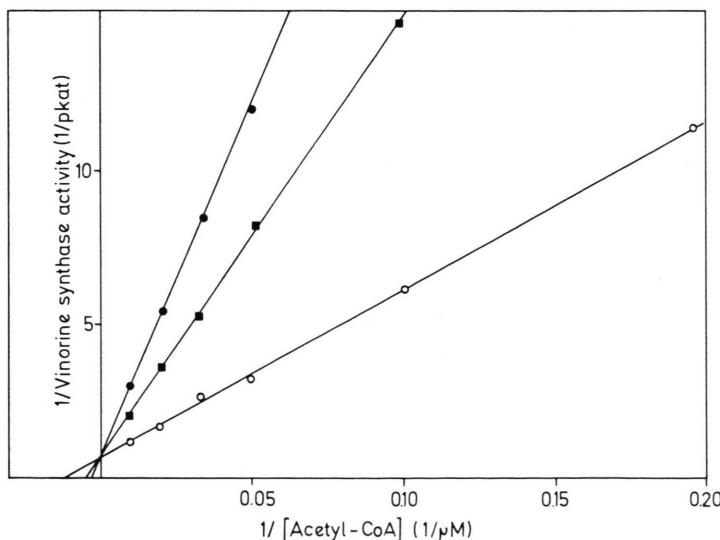


Fig. 10. Reciprocal plot of the vinorine synthase activity against the acetyl-coenzyme A concentration without coenzyme A (○—○), in presence of 0.5 mM (■—■) and 2 mM (●—●) CoA.

Cultivated cells of *Rauwolfia serpentina* which synthesize 15 major alkaloids of the ajmaline and sarpagine biosynthetic pathway are an efficient source for many of the assumed alkaloidal biosynthetic intermediates [3]. Four of the ajmaline type compounds exhibit a 17-O-acetyl group e.g. the indolenine alkaloids vinorine or vomilenine. These acetylated alkaloids were previously also detected in *Rauwolfia* plants [10, 11] and coupled with the observation that 17-OH indolenines are unstable [12] we assumed acetyl-coenzyme A to be directly involved in the formation of the C-7, C-17 bond of vinorine. Moreover, the recent isolation of polyneuridine aldehyde esterase allowed the synthesis of the new alkaloid 16-epi-vellosimine [6] which has the indispensable steric requirement (16-S configuration) for the generation of that bond. Indeed, incubation of a crude enzyme mixture, 16-epi-vellosimine and acetyl-CoA yielded traces of vinorine identified by TLC, UV, MS and <sup>1</sup>H-NMR [5]. Incubation experiments with denatured protein clearly established this formation to be enzyme catalyzed. By a five step purification procedure including ammonium sulphate precipitation, DEAE, AcA 54, hydroxylapatite and phenylsepharose chromatography monitored by an enzyme assay with [<sup>3</sup>H]acetyl-CoA, vinorine synthase could be enriched 160-fold with an acceptable yield of 57%.

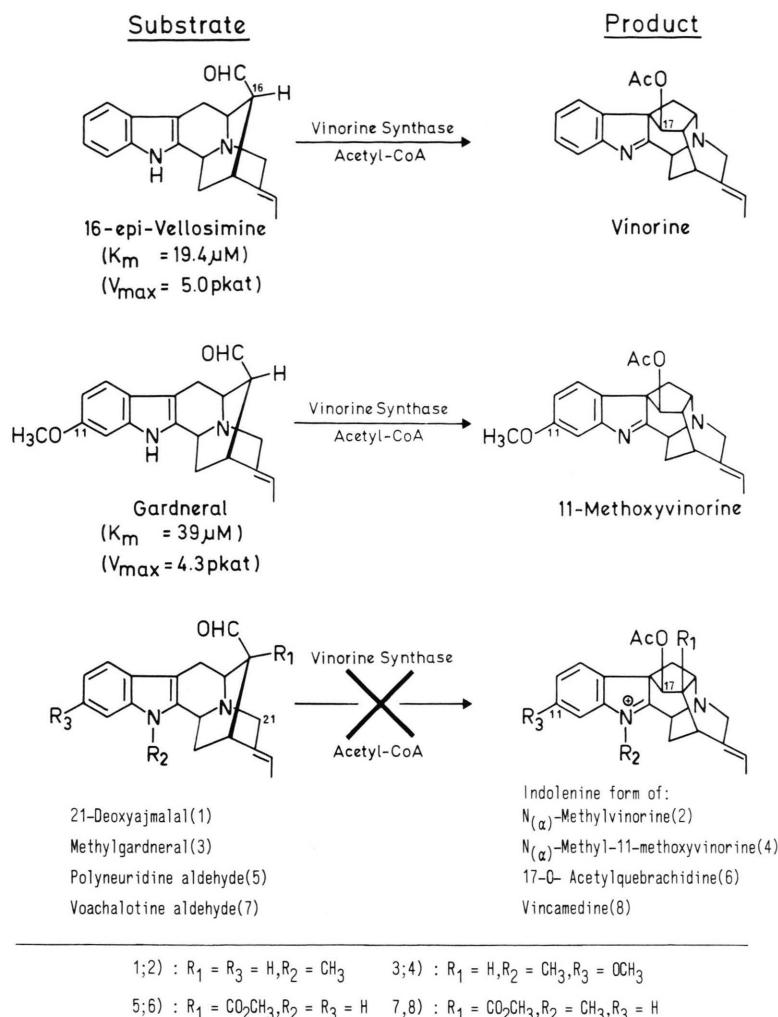
Acetylajmalan esterase, an enzyme hydrolyzing the 17-O-acetyl group of vinorine and obviously involved in the late stages of ajmaline biosynthesis, could be completely separated from vinorine synthase by the first purification step using ion exchange chromatography. After this step vinorine is stable in the presence of the remaining enzymes which would explain the isolation of only trace amounts of the alkaloid from incubation mixtures containing crude enzyme preparations with acetylajmalan esterase activity. Chromatography on hydroxylapatite then removed polyneuridine aldehyde esterase from vinorine synthase quantitatively and allowed a detailed investigation of the substrate specificity of the synthase including also polyneuridine aldehyde.

As illustrated in Scheme 2 enzyme activity was tested with six alkaloids having the required C-17 stereochemistry (*endo* configuration of the aldehyde group) for the ring closure reaction. Exclusively two alkaloids were accepted by the enzyme, 16-epi-vellosimine and its 11-methoxy derivative (gardneral). The former alkaloid is clearly preferred by the en-

zyme as demonstrated by the low  $K_m$  value (19.4  $\mu\text{M}$ ) whereas gardneral gave a  $K_m$  of 39  $\mu\text{M}$ . Gardneral was transformed into 11-methoxyvinorine which so far has not been detected as a natural compound. This result indicates that the synthase might be not strongly selective for aryl substituted alkaloids and that substitution at the aromatic ring might be an earlier process in the biosynthesis of appropriate ajmaline type compounds. The corresponding  $N_a$ -methyl compounds, 21-deoxyajmalal and methylgardneral, do not act as progenitors of their corresponding indolenine alkaloids, a fact which supports our previous conclusion, that  $N_a$ -methylation is a late step in ajmaline biosynthesis [13]. Similarly, polyneuridine aldehyde and its  $N_a$ -methyl congener voachalotine aldehyde, both of which still carry the carbomethoxy group at C<sub>16</sub>, were not cyclized by the synthase (Scheme 2). In complete agreement, the expected indolenines are not known to occur naturally, but their 1,2-hydrogenated derivatives quebrachidine and vincamedine have been isolated from *Aspidosperma quebracho blanco* Schlecht [14], *Cabucala* species [15, 16], *Alstonia spectabilis* R. Br. [17], *Vinca libanotica* Zucc. [18] and from *V. major* L. [19], *V. difformis* Pourret [20] resp.

These results demonstrate a high substrate specificity of vinorine synthase and indicate that in the above mentioned plant species a different enzyme must be responsible for the cyclization reaction in the biosynthesis of alkaloids of the quebrachidine group. When 16-epi-vellosimine was incubated with vinorine synthase in the presence of malonyl-, benzoyl-, coumaroyl- or oleoyl-CoA instead of acetyl-CoA, ajmaline type alkaloids were not synthesized, which again supports the specificity of the enzyme. In addition to that, the activated esters did not show an inhibitory effect on vinorine formation.

In a plausible sense, the enzymatic vinorine synthesis could involve 17-hydroxyvinorine as a biosynthetic intermediate [9]. 17-hydroxyindolenines, however, have not been detected in plants or cell cultures. They also were not isolated during the chemical synthesis of related alkaloids from sarpagan aldehydes by acid catalysis [12]. These compounds can however be trapped as derivatives by reduction of the 1,2-double bond or by acetylation of the 17-hydroxy group. All our attempts to intercept the cell-free vinorine or 11-methoxyvinorine formation in the absence of the co-substrate acetyl-CoA by reduction with sodium borohydride at physiological pH or by



Scheme 2. Substrate specificity of vinorine synthase.

acetylation of freeze dried incubation mixtures gave no evidence for the intermediacy of 17-OH-indolenines. Therefore kinetic experiments were conducted in order to obtain an insight into the enzyme mechanism. Such investigations would confirm whether both substrate and co-substrate are simultaneously bound at the enzyme by formation of a ternary complex or whether they successively react with the protein. Because of the instability of 16-*epi*-vellosimine the experiments were carried out with the more stable gardneral as substrate. When under standard incubation conditions the velocity of the enzyme reaction was measured at different substrate and co-substrate concentrations resp., the resulting graphs coincided at one point (Figs. 7, 8). These data are usually obtained for enzyme reactions involving a

ternary complex between enzyme, substrate and co-substrate. The results obtained support the conclusion that a simultaneous binding of gardneral and acetyl-CoA is a prerequisite for the enzymatic formation of the characteristic ajmaline C-7, C-17 bond. This suggestion was further substantiated by inhibition studies of the cyclization reaction. Since the alkaloid 11-methoxyvinorine is fairly poorly soluble in water an inhibition of the enzymatic reaction could only be tested with CoA, the endproduct of the cosubstrate. As demonstrated in Fig. 9 and Fig. 10, CoA clearly inhibited the vinorine synthase activity. For both substrates a competitive inhibition was observed which is consistent with a enzyme mechanism of a two-substrate reaction. Obviously a mechanism is involved where both substrates are independently

bound at the active site of the enzyme, similar to that reported for the hexokinase reaction [21].

From the discussed kinetic data and with the results of the negative trapping experiments for the assumed hydroxyindolenine compound we propose that 17-OH-vinorine is not an isolatable, free intermediate of vinorine biosynthesis, nor does a second enzyme activity take part in the biochemical process. Our further aim is the NMR-spectroscopic investigation of this particular enzyme reaction to get a further proof of our conclusion.

Vinorine is structurally closely related to vomilenine. Therefore it was interesting to clarify the biogenetic significance of the product of the vinorine synthase reaction for latter alkaloids in the ajmaline pathway *e.g.* for vomilenine, which only differs from vinorine by a hydroxy group at C-21. Until now a hydroxylation at this carbon by isolated enzymes had not been achieved and we therefore decided to clarify this point by *in vivo* experiments. As outlined in the experimental part, [<sup>3</sup>H]- or [<sup>14</sup>C]vinorine was fed to *R. serpentina* cultures and its metabolism was investigated under different culture conditions. When [<sup>3</sup>H-acetyl]vinorine was applied to cells growing in Linsmaier and Skoog medium incorporation rates were observed into vomilenine and alkaloids which appear later in the pathway – acetylajmaline and acetylajmaline – to an extent of 2%, 1% and 9%. Feeding of cells cultivated under conditions where vomilenine formation is enhanced by a factor of about 8 showed 18% incorporation into vomilenine. These results unambiguously demonstrate that vinorine is an excellent biosynthetic precursor for these alkaloids. Apparently vinorine is hydroxylated first at C-21 leading to vomilenine, which is then metabolized to ajmaline as already demonstrated with a crude cell-free system [13].

Vinorine synthase therefore occupies a central position in the biosynthesis of *Rauwolfia* alkaloids belonging to the ajmaline group.

## Materials and Methods

### Cell cultures

Plant cell suspension cultures of *Rauwolfia serpentina* were grown in 1-l Erlenmeyer flasks at 25 °C on a gyratory shaker (100 rpm). Cultures were maintained for 9 days in 250 ml Linsmaier and Skoog medium [22], harvested by suction filtration and stored at –26 °C.

### TLC chromatography

Purification and chromatographic identification of alkaloids were carried out on Sil gel thinlayer plates (0.25 mm, Macherey and Nagel) employing the following solvent systems: A) ethyl acetate/ether/*n*-hexane = 2:2:1, B) petroleum ether/acetone/diethylamine = 7:2:1, C) chloroform/methanol/ammonia = 9:1:0.01.

### Spectroscopic methods

UV spectra were measured with a Perkin Elmer 551 S spectrophotometer using methanol as solvent. Mass spectra were recorded on a Quadrupol instrument (Finnigan MAT 44 S) in EI mode (70 eV). Optical rotation was measured in chloroform with a Perkin Elmer polarimeter 241.

### Quantitation of radioactivity

Radioactivity was determined by scintillation counting with 5 ml Rotiszint (Roth Company) on a Berthold BF 5000 scintillation counter.

### Determination of the molecular weight of vinorine synthase

The relative molecular weight of vinorine synthase was determined by gel chromatography using AcA 54 obtained from LKB (Bromma, Sweden). 5 ml of the enzyme solution containing 4% glycerol were applied to AcA 54 column (2.3 × 92 cm) which was calibrated with known enzymes; bovine serum albumin ( $M_r$  68000), ovalbumin ( $M_r$  45000), trypsin inhibitor ( $M_r$  21500) and cytochrome C ( $M_r$  12500). The synthase was eluted with 50 mM potassium phosphate buffer (pH 7, 10 mM  $\beta$ -mercaptoethanol) at a flow rate of 20 ml/h. The enzyme was eluted at 220 ml buffer corresponding to a relative molecular weight of 31000 ± 8%.

### Determination of the isoelectric point of the synthase

The isoelectric point of vinorine synthase was determined by the chromatofocusing method developed by Sluyterman [7]. Of the enzyme 20 ml (25 mM imidazol-HCl buffer, pH 7.5, 10 mM  $\beta$ -mercaptoethanol) were subjected to a 0.27 × 17 cm column containing with the mentioned buffer equilibrated polybuffer exchanger (PBE 94).

The synthase was eluted with polybuffer 74-HCl (pH 4.0, 1:8 diluted, flow rate of 13 ml/h). Vinorine

synthase was eluted at pH 4.4 corresponding to its isoelectric point.

#### Enzyme quantitation

Protein was determined by the method of Bradford [23] using a calibration curve obtained with bovine serum albumin.

#### Assay for vinorine synthase

Since the substrate of the synthase, 16-epi-vellkosimine, is highly unstable, it was generated from polyneuridine aldehyde by polyneuridine aldehyde esterase, and vinorine synthase was determined by standard incubations as follows: 10 nmol polyneuridine aldehyde were incubated in presence of 40 pkat polyneuridine aldehyde esterase (65-fold enriched), 10 nmol [ $^3$ H-acetyl]acetyl-coenzym A (25 nCi), the enzyme solution containing vinorine synthase in a total volume of 0.2 ml borate buffer (0.1 M, pH 8.5) for 30 min at 30 °C. The incubation mixture was extracted with 0.4 ml ethyl acetate and 0.3 ml of the organic phase was counted for radioactive vinorine.

#### Assay for acetylajmalan esterase

[ $^3$ H-acetyl]acetyltetraphyllicine (5.4 nmol, spec. act. 17  $\mu$ Ci/ $\mu$ mol) was incubated in a total volume of 0.5 ml potassium phosphate buffer (0.1 M, pH 7) with the corresponding protein fraction for 30 min at 30 °C. A suspension of 0.2 ml charcoal (50 g/l) was added and the mixture centrifuged. The radioactive formed [ $^3$ H]acetic acid was counted for activity using 0.5 ml of the supernatant solution.

#### Isolation and purification of vinorine synthase

For the protein isolation 0.4 kg of frozen cultivated *R. serpentina* cells were stirred at 0 °C in 0.8 l potassium phosphate buffer (0.1 M, pH 7.5, 10 mM  $\beta$ -mercaptoethanol) for 30 min. The mixture was filtered through cheese cloth and the filtrate centrifuged at 20000  $\times$  g for 20 min. To the supernatant solution ammonium sulphate was added to yield a final concentration of 70% during 30 min.

After centrifugation for 15 min at 48000  $\times$  g, the precipitated protein was dissolved in 50 ml of the above mentioned buffer and dialyzed for 12 h against 20 mM borate buffer (pH 8, 10 mM  $\beta$ -mercaptoethanol).

#### 1. Purification step

The above protein solution was applied to a DEAE DE 52 column (2  $\times$  25 cm) which was equilibrated with borate buffer just mentioned. The column was washed with 67 ml of the same buffer and then eluted with a linear gradient from 0–0.5 M KCl at a flow rate of 50 ml/h. 180 fractions (each 4.4 ml) were collected and tested for vinorine synthase and acetylajmalan esterase. Fraction 103–120 contained the synthase (spec. act. 0.23 nkat/mg, 15.3-fold enrichment) and were combined and concentrated to 12 ml by ultra filtration. Acetylajmalan esterase was localized in fractions 65–92.

#### 2. Purification step

The concentrated enzyme solution was applied to gel chromatography (AcA 54 column, 2.3  $\times$  92 cm). Elution of the protein was carried out with 50 mM potassium phosphate buffer (pH 7, 10 mM  $\beta$ -mercaptoethanol) at a flow rate of 20 ml/h. Combined fractions containing vinorine synthase gave a total volume of 52 ml (spec. act. 0.69 nkat/mg corresponding to a 46-fold purification of the enzyme).

#### 3. Purification step

The enzyme then was chromatographed on hydroxylapatite (column 1.5  $\times$  9 cm) using the buffer mentioned above. 40 fractions (each 3 ml) were collected, fractions 6–26 exhibited the synthase activity (spec. act. 0.83 nkat/mg, 55.3-fold purification). Polyneuridine aldehyde esterase was retained on the column.

#### 4. Purification step

Finally the synthase was purified on phenylsepharose (column 1  $\times$  11 cm, equilibrated with 0.5 M KCl) after the enzyme solution had been brought to a concentration of 0.5 M KCl by adding solid KCl. The column was eluted with a decreasing salt gradient (0.5–0 M KCl) under simultaneous linear increase of ethylene glycol concentration (0–5%). Two hundred fractions of 2 ml were collected. The synthase was found in fractions 70–90 with a spec. act. of 2.4 nkat/mg protein which corresponds to a 160-fold enrichment of vinorine synthase. The total yield in this 4 step purification procedure was 57%.

### Enzymatic formation of vinorine

Polyneuridine aldehyde (5.25 mg, 15  $\mu$ mol) was dissolved in 50 ml potassium phosphate buffer (0.1 M, pH 7.5), 10 nkat polyneuridine aldehyde esterase (65-fold enriched), acetyl-coenzyme A (12 mg, 15  $\mu$ mol) and 20 ml of partially purified vinorine synthase isolated from an AcA 54 chromatography were added. The mixture was incubated for 60 min at 30 °C. The reaction mixture was extracted with ethyl acetate, the organic solvent evaporated and the residue purified by TLC with solvent system A. Pure vinorine resulted in a yield of 31% (1.5 mg),  $[\alpha]_D^{20} = 30 \pm 3$  °C ( $c = 0.12$  % CHCl<sub>3</sub>).

### Chemical synthesis of vinorine

Vinorine was synthesized from acetyltetraphyllicine as described by Kiang *et al.* [24]. Acetyltetraphyllicine (5.6 mg, 16  $\mu$ mol) was dissolved in 1 ml benzene and an excess (4-fold) of Pb(OAc)<sub>4</sub> was added with stirring. After 60 min the reaction mixture was extracted twice with H<sub>2</sub>O (5 ml). The organic solvent was evaporated and the residue chromatographed by TLC employing solvent system B. Vinorine was isolated in a yield of 15%.  $[\alpha]_D^{20} = 30 \pm 3$  °C ( $c = 0.05$  %, CHCl<sub>3</sub>).

### Isolation of vinorine from cultivated cells

Vinorine was isolated from *R. serpentina* cells as recently reported [3]. UV, MS and <sup>1</sup>H-NMR data of all vinorine preparations were identical and in full agreement with published values. MS: 334 (M<sup>+</sup>, 39), 333 (M<sup>+</sup>-H, 20), 291 (63), 275 (35), 182 (33), 169 (74), 168 (100) m/z.

### Enzymatic synthesis of 11-methoxyvinorine

As described for the enzyme catalysed formation of vinorine, 11-methoxyvinorine was synthesized starting with vinorine synthase, gardneral (11-methoxy-16-epi-vellosimine) and acetyl-coenzyme A. The formed alkaloid was purified by TLC using solvent system A. UV: 228, 268 nm, MS: 364 (M<sup>+</sup>, 25), 363 (M<sup>+</sup>-H, 10), 321 (59), 305 (21), 212 (24), 198 (100), 183 (12), 155 (12) m/z.

### Synthesis of voachalotine aldehyde

For the synthesis of voachalotine aldehyde the corresponding alcohol (voachalotine) was oxidized by a

procedure of Corey and Kim [25]. N-Chlorosuccinimide (80 mg, 0.6 mmol) was stirred for 60 min in 2 ml dry toluene, the unsolved imide was removed by filtration and to the filtrate dimethylsulfide (0.82 mmol) was added at 0 °C. The mixture was then adjusted to -25 °C and with stirring voachalotine (3 mg, 8.2  $\mu$ mol) was added. After 2 hrs the reaction was terminated with 100  $\mu$ l triethylamine, 2 ml H<sub>2</sub>O and 2 ml diethylether. The organic layer was evaporated and the remaining material chromatographically purified using solvent system C. Voachalotine aldehyde was obtained in a yield of 86%, UV: 225, 280 nm; MS: 364 (M<sup>+</sup>, 30), 349 (10), 335 (22), 305 (70), 263 (80), 183 (100), 167 (38) m/z.

### Synthesis of [<sup>3</sup>H-acetyl]17-O-acetyltetraphyllicine

Tetraphyllicine (15.4 mg, 50  $\mu$ mol) was dissolved in dry pyridine. [<sup>3</sup>H]-Acetic anhydride (25 mCi, 50  $\mu$ mol) was added under vacuum (0.1 Torr). After 16 hrs the reaction mixture was evaporated under vacuum and the residue was purified by TLC in solvent system C. [<sup>3</sup>H]Acetyltetraphyllicine (yield 3%) was isolated showing a specific activity of 457.8  $\mu$ Ci/ $\mu$ mol.

### Synthesis of [<sup>3</sup>H-acetyl]vinorine

[<sup>3</sup>H-acetyl]vinorine was accordingly synthesized from [<sup>3</sup>H-acetyl]tetraphyllicine (2.1 mg, 34  $\mu$ Ci/ $\mu$ mol) and Pb(OAc)<sub>4</sub> (24  $\mu$ mol). After working up of the reaction mixture and purification by TLC [<sup>3</sup>H-acetyl]vinorine was obtained in a yield of 10% (specific activity: 31  $\mu$ Ci/ $\mu$ mol).

### Enzymatic synthesis of [<sup>14</sup>C-acetyl]vinorine

In an total volume of 4 ml Tris/HCl buffer (50 mM, pH 8.0) polyneuridine aldehyde (1.5  $\mu$ mol) was incubated for 90 min at 30 °C in the presence of polyneuridine aldehyde esterase, vinorine synthase and 5  $\mu$ Ci [<sup>14</sup>C]acetyl-CoA (0.55  $\mu$ mol). The incubation mixture was twice extracted with 2 ml ethyl acetate and the organic layer chromatographed on TLC in solvent system A. Elution of the vinorine containing band yielded 0.12  $\mu$ mol (yield 22%) of the [<sup>14</sup>C]alkaloid (specific activity 9.2  $\mu$ Ci/ $\mu$ mol).

### In vivo feeding experiment of [<sup>3</sup>H-acetyl]vinorine

To a cell suspension culture (70 ml) of *R. serpentina* grown for 7 days in Linsmaier and Skoog

medium 15  $\mu$ Ci [ $^3$ H-acetyl]vinorine (0.5  $\mu$ mol) were added. After 24 hrs cells were harvested, frozen with liquid nitrogen and extracted with 75 ml ethyl acetate for 24 hrs. The organic phase was separated, evaporated and the residue purified by TLC in solvent system B and C. *Rauwolfia* alkaloids isolated were vomilenine, 17-O-acetylnorajmaline and 17-O-acetylajmaline showing incorporation rates of 2%, 1% and 9% respectively.

#### *In vivo feeding experiment of [ $^{14}$ C-acetyl]vinorine*

A cell suspension culture of *R. serpentina* (30 ml) grown for 7 days in an alkaloid production medium [26] was fed with 1.0  $\mu$ Ci [ $^{14}$ C-acetyl]vinorine (0.11  $\mu$ mol) for 2.5 days and worked up as described above. The incorporation rate of [ $^{14}$ C-acetyl]vinorine into vomilenine was determined to be 18%. Incorporation

into 17-O-acetylnorajmaline and 17-O-acetylajmaline could not be detected. The identity of the labelled alkaloid (specific activity 8.8 nCi/ $\mu$ mol) was proved by TLC (5 different solvent systems) and chemical conversion into perakine [27].

#### *Acknowledgements*

Our thanks are due to Prof. S. Sakai, Institute of Organic Chemistry, Chiba University, Japan, Dr. Kehrbach and Dr. Zeugner, Kali Chemie, Hannover, for gifts of reference alkaloids. We also thank Prof. W. E. Court, Baildon, England, for helpful suggestions and for checking the English version of the manuscript. The Deutsche Forschungsgemeinschaft, Bonn, is acknowledged for a mass spectrometer (Finnigan MAT 44 S) and for financial support (SFB 145).

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