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THE OXIDATION OF MONODANSYLDIAMINES BY PEA SEEDLING DIAMINE OXIDASE

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Key Word Index—*Pisum sativum*; Leguminosae; pea; diamine oxidase; monodansyldiamines; monodansylcadaverine; lysine; dansylation.

Abstract—In the oxidation of a homologous series of monodansyldiamines by pea seedling diamine oxidase, monodansylcadaverine was the best substrate. Monodansyldiaminohexane was oxidized at 74% of the rate with monodansylcadaverine, and monodansylputrescine and monodansyldiaminopropane were oxidized only very slowly. The optimum pH for the oxidation of monodansylcadaverine was 8.5, and the K_m 2.4×10^{-4} M. Under optimum conditions, putrescine was oxidized eleven times faster than monodansylcadaverine. Oxidation of monodansylcadaverine by diamine oxidase, and the exhaustive dansylation of lysine in equivalent amounts ultimately showed equal fluorescence in the dansyl-5-aminovaleraldehyde formed, indicating stoichiometric conversion to this product in both reactions.

INTRODUCTION

Monodansylcadaverine has been used as a fluorescent probe in the study of protein structure, since it can be substituted on the amide group of glutamine in place of $-NH_2$. This reaction is mediated by the enzyme, transglutaminase, which appears to be confined to animals [1]. Monodansylcadaverine is a strong competitive inhibitor of transglutaminase with lysine as substituent, and this property enables it to prevent endocytotic uptake of the epidermal growth factor by a mouse cell culture [2]. Monodansyldiamines will also prevent cross-linking of fibrin, a reaction which requires the formation of amide bonds between lysine and glutamic acid residues [3]. In a homologous series of monodansyldiamines, monodansylcadaverine was the most effective inhibitor for this cross-linking [3,4]. Monodansylcadaverine will also bind non-covalently to membranes [5].

The structure of monodansylcadaverine suggested that it might act as a substrate for diamine oxidase (DAO); moreover, its fluorescence indicates that it might form the basis of a sensitive method for the detection and estimation of DAO. The oxidation of monodansylcadaverine and of related compounds by pea seedling DAO was studied in the present work.

In a method developed for determining lysine using exhaustive dansylation, the final product is dansyl-5-aminovaleraldehyde (dans-5-AV) [6]. This is also the expected product of the oxidation of monodansylcadaverine by pea seedling DAO. It was of interest to compare the stoichiometry of the dans-5-AV formed by these two quite different mechanisms (Scheme 1).

RESULTS AND DISCUSSION

pH *optimum*

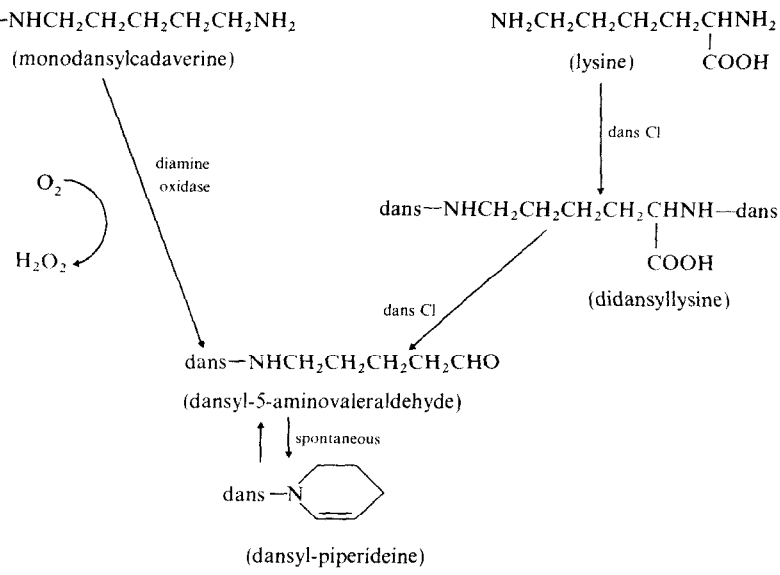
Using Tris, borate, phosphate and carbonate buffers, the DAO activity measured by the peroxidase/guaiacol assay was investigated over the pH range 7–10 (Fig. 1). The optimum pH for the oxidation of monodansylcadaverine was 8.5 with half-maximal activity at pH 7.6 and 9.5. Activity in Tris was *ca* 50% greater than in other buffers. Oxidation of di- and polyamines and benzylamine by pea seedling DAO in Tris is similarly faster than in other buffers [7–9]. The optimum pH for activity with benzylamine [8], tryptamine [10], 2-phenylethylamine [11] and 2,4-dichlorophenoxyethylamine [12] is 8–9, while activity with unsubstituted diamines is usually best at pH 6–7.5 [9,11]. Monodansylcadaverine appears to behave in this respect like the monoamines.

Michaelis constant

In Tris buffers at pH 8.5 (optimum), the K_m for monodansylcadaverine was 2.4×10^{-4} M, and at pH 7.5 the K_m was 6.7×10^{-4} M. For putrescine, the K_m at pH 7.5 was 4×10^{-5} M [9].

Activity of the DAO with monodansylcadaverine and other amines

The relative activity of substrates at a final concentration of 1 mM in pH 7.5 and 8.5 Tris buffer measured by the peroxidase/guaiacol assay is shown in Table 1. Under optimum conditions of pH the rate of oxidation of putrescine is *ca* eleven times greater than the rate for monodansylcadaverine. Cadaverine was oxidized at only 75% of the rate with putrescine. In previous work, using a manometric assay in pH 7 phosphate buffer, cadaverine was found to be oxidized faster than putrescine [13]. This is consistent with the work of Kenten and Mann [11] who showed that oxidation of cadaverine has a pH optimum of *ca* 6.1. Amongst the monodansyldiamines tested, greatest activity was obtained with monodansylcadaverine as substrate (Table 2). By comparison, activity with monodansyldiaminopropane or monodansyldiaminobutane (monodansylputrescine) as substrate was very slow. Although it appears that the



(The position of the double bond is not known.)

Scheme 1.

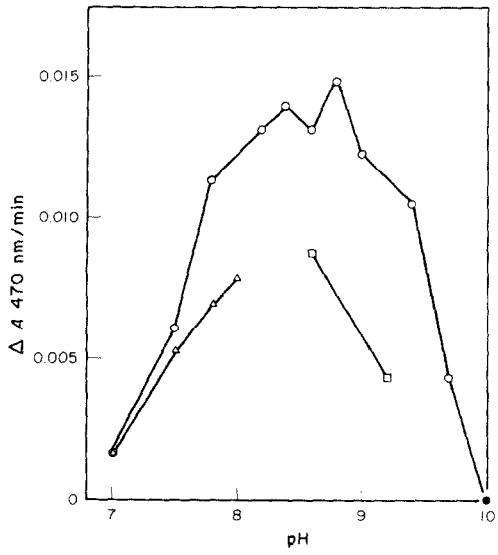


Fig. 1. Effect of pH on the oxidation of monodansylcadaverine measured by the peroxidase/guaiacol method. Results are expressed as the change in A 470 nm/min. Buffers (0.1 M) Tris ○———○; phosphate △———△; borate □———□; Tris and carbonate (separate estimations) ●———●.

Table 1. Relative rate of oxidation of monodansylcadaverine and other amines by pea seedling diamine oxidase in Tris buffer (0.1 M) normalized with respect to the rate with monodansylcadaverine at pH 7.5

	Putrescine	Cadaverine	Monodansylcadaverine
pH 7.5	20	15	1
pH 8.5	15.4 (8.8)	9.8 (5.6)	1.75 (1)

The ratio in brackets for pH 8.5 is normalized with respect to the rate with monodansylcadaverine at that pH.

Table 2. Relative activity of diamine oxidase with a homologous series of monodansyldiamines as substrates

	Volume of DAO (μl)	Relative activity corrected for enzyme concentration
Monodansyldiaminopropane	100	0.5
Monodansyldiaminobutane (monodansylputrescine)	100	1.9
Monodansyldiaminopentane (monodansylcadaverine)	10	100
Monodansyldiaminohexane	10	74

All estimations were made in Tris buffer (0.1 M, pH 8.5) using the peroxidase/guaiacol assay method.

DAO is more active with the free diamines than with their monodansyl derivatives, it is possible that monodansylcadaverine could be used as the substrate in a very sensitive assay for DAO, since dansylated amines may be detected and measured by fluorescence in very small amounts (10^{-11}–10^{-12} mol) [14]. The product may be separated from the substrate by extraction into toluene.

Stoichiometry in the peroxidase/guaiacol DAO assay

The increment of absorbance obtained on adding equivalent amounts of amine substrate was established for various amines (Table 3). Previous work [9] had indicated good agreement in the increment of absorbance for putrescine and spermidine. In the present study, putrescine, monodansylcadaverine, monodansyldiaminohexane and benzylamine showed good agreement, though 2-phenylethylamine and histamine gave a relatively small increment of absorbance. The products of oxidation of the 2-phenylethylamine and histamine are also good substrates for peroxidase [15], and might be further oxidized by the peroxidase in preference to the guaiacol, leading to a reduction in chromogen formation. It is not easy to explain the increased absorbance increment with cadaverine as substrate. It is possible that the Δ′-piperideine formed by oxidation of the cadaverine condenses with the quinones produced on peroxidative oxidation of guaiacol to yield a substance with an increased molar absorbance. In other work, out of ten amines used as substrates for DAO, stoichiometry for O_2 uptake and NH_3 formation with cadaverine in the presence of peroxidase was found to be anomalously low [15]. In the present work, good agreement was found for the stoichiometry of putrescine, cadaverine, benzylamine and monodansylcadaverine in the oxygen electrode assay.

Stoichiometry of dans-5-AV formation

In the exhaustive dansylation of lysine, the final product is dans-5-AV [6], the same as the expected product of oxidation of monodansylcadaverine by pea seedling DAO (Scheme 1). Experiments were therefore designed to compare the fluorescence of the dans-5-AV produced by equivalent amounts of lysine and monodansylcadaverine in the two systems.

Initially, the fluorescence of dans-5-AV produced by monodansylcadaverine oxidation was only 40–60% of the fluorescence of the dans-5-AV produced by exhaustive lysine dansylation in two experiments (Table 4). However, on resampling the toluene layer, TLC showed that the fluorescence increased progressively during 7–9 days' storage at 4°, until it was nearly 100% of the expected value. In principle, this result was repeated in both experiments. This phenomenon was independent of the pH during extraction, since a similar effect was observed on extraction at pH 2 and 10.

The explanation for this progressive increase in the fluorescence may lie in the initial enzyme-mediated production of an isomer of dansylpiperideine in which the position of the double bond differs from that in the final product, and from the position in the product of lysine dansylation. The bond may change position progressively in the toluene, although a similar change does not appear to take place on the TLC plate. Although the mass spectrum indicates that the dans-5-AV is not cyclized during TLC [6], cyclization and polymerization in the aqueous phase may explain the initial loss of fluorescence. An alternative and perhaps more plausible explanation may be found in the formation of an unstable complex with the protein present in the enzyme incubate. However, much less protein was used in experiment 2 (Table 4) although the results were similar for the two experiments. Even so, in the oxidation of tryptamine by pea seedling DAO, a non-specific binding of indoleacetaldehyde was thought to be the cause of a failure to extract stoichiometric quantities of indoleacetaldehyde from the reaction mixture into toluene [16]. If a stable Schiff base is formed between enzyme and substrate, up to 1 mol of amine may be bound to 1 mol of enzyme [16, 17]. Since the specific activity of the pure enzyme is 830 nkat/mg and as the MW is 96 000 [18], *ca* 0.26 nmol of DAO was added to each incubate. The amount of product which might be bound cannot therefore be only associated with the active site.

Despite the anomalous behaviour in the recovery experiment, only one product could be found on TLC of the DAO preparation, corresponding in R_f precisely with the dans-5-AV formed from lysine. Moreover, the ultimate fluorescence of the dans-5-AV produced by the two reactions suggests that both reactions are stoichiometric.

Table 3. Relative stoichiometry of amine oxidation by pea seedling DAO in pH 8.5, 0.1 M Tris buffer

Amine	Peroxidase/ guaiacol assay	Oxygen electrode
Putrescine	100	100
Cadaverine	123	99
2-Phenylethylamine	75	ND
Histamine	42	ND
Benzylamine	90	110
Monodansylcadaverine	90	96
Monodansylhexylamine	93	ND

Increment of absorbance for the peroxidase/guaiacol assay and increment of voltage for the oxygen electrode obtained with 62.5 nmol substrate. Each value is the mean of at least 2 estimates. The data are expressed relative to the deflection obtained with putrescine (= 100). ND = not determined.

Table 4. Change with time in the relative fluorescence of the dans-5-AV produced by exhaustive lysine dansylation and by the oxidation of monodansylcadaverine with equimolar amounts of substrate

	Time (days)	Lysine dansylation	Monodansylcadaverine oxidation
Experiment 1	0	100	41
	7	99	97
Experiment 2	0	100	58
	3	99	89
	9	98	96

Toluene extracts were stored at 4° in the dark and sampled at intervals for TLC. Fluorescence of the dans-5-AV was measured *in situ*.

EXPERIMENTAL

Chemicals. Monodansylcadaverine was purchased from Sigma. The monodansyl derivatives of diaminopropane, diaminobutane (putrescine), and diaminohexane were synthesized and purified by the method of ref. [3]. All monodansyldiamines were dissolved in equivalent vols of HCl to give neutral solns and washed in toluene or $CHCl_3$ to remove traces of residual didansyldiamines. The concns were established by dansylation of the monodansyldiamine solns and quantitative comparison of the fluorescence of the didansyldiamine spots with the fluorescence of the didansyldiamine spots obtained on dansylation of diamine solns of known concn, using the method of ref. [6]. The vols of the monodansyldiamine solns were then adjusted to provide 25 mM solns. All monodansyldiamine samples were free of unsubstituted diamines, as demonstrated by paper electrophoresis effected using pyridine (10 ml)–HOAc (4 ml) to 1 l. (pH 5.4) for 1 hr at 300 V [4]. Electrophoresis of the final stock solns of the four monodansyldiamines gave green-yellow fluorescent ninhydrin-positive spots with indistinguishable mobilities (*ca* 4 cm/hr). No other spots were visible on separation of these solns. The underivatized amines were well separated from the monodansyldiamines with mobilities ranging from *ca* 10.5 cm/hr for diaminopropane to *ca* 9 cm/hr for diaminohexane. The monodansyldiamines could not be effectively separated by TLC on cellulose (CC41) or Kieselgel G. The didansyldiamines were separated by TLC in cyclohexane–EtOAc (5:4) on Kieselgel G plates (250 μm). Fluorescence was measured by the method given in ref. [6].

Diamine oxidase was purified according to the method of ref. [8]. The fraction obtained after $(NH_4)_2SO_4$ precipitation was used for all experiments, other than experiment 2 in the determination of the stoichiometry of dans-5-AV formation, for which the more purified preparation obtained by EtOH fractionation was used [7]. DAO activity was measured by the peroxidase/guaiacol method [9] or by the oxygen electrode [19]. The stoichiometry of the peroxidase/guaiacol method used for DAO estimation was also studied, by the procedure given in ref. [9]. The pH optimum was investigated using the peroxidase/guaiacol method of assay. Buffers used were Tris (pH 7–9.6), Pi (pH 7–8), borate (pH 8.6–9.2) and carbonate (pH 10).

Stoichiometry of the dans-5-AV product. (1) Oxidation of monodansylcadaverine. The incubate contained 0.8 ml Tris–HCl buffer (0.1 M, pH 8.5 correct at 30°), 0.1 ml catalase (1 mg/ml, Sigma, 2000 units/mg), 1 μmol monodansylcadaverine and 0.1 ml DAO (23 nkat). The soln was saturated with O_2 and incubated at 30°. Samples were taken at 2 hr, when 0.1 ml M HCl was added. The incubate was extracted in 2.5 ml toluene and 10 μl samples were taken for TLC. Conversion was calculated to be complete after 15 min. Dansylation of the residue obtained after oxidation and toluene extraction, followed by TLC in cyclohexane–EtOAc (5:4) revealed no didansylcadaverine, indicating that all of the monodansylcadaverine had been oxidized.

(2) Lysine dansylation. In principle, this was conducted by the method given in ref. [6]. The incubate contained 22 μmol dansyl chloride in Me_2CO, 0.1 ml H_2O with 1 μmol lysine, and 50 mg of $NaHCO_3$. After 16 hr at 30°, 2.3 ml toluene was added and the final extract (2.5 ml of toluene–Me_2CO) was sampled (10 μl) for TLC.

Samples from both incubates were chromatographed in parallel on Kieselgel G plates in cyclohexane–EtOAc (5:4). The R_f of dans-NH_2 was 0.41 and the R_f of dans-5-AV was 0.54.

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REFERENCES

1. Pober, J. S., Iwanij, E., Reich, E. and Stryer, L. (1978) *Biochemistry* **17**, 2163.
2. Haigler, H. T., Maxfield, F. R., Willingham, M. C. and Pastan, I. (1980) *J. Biol. Chem.* **255**, 1239.
3. Nilsson, J. L. G., Stenberg, P., Ljunggren, C., Eriksson, O. and Lunden, R. (1971) *Acta Pharm. Suec.* **8**, 497.
4. Lorand, L., Rule, N. G., Ong, H. H., Furlanetto, R., Jacobsen, A., Downey, J., Öner, N. and Bruner-Lorand, J. (1968) *Biochemistry* **7**, 1214.
5. Pincus, J. H., Chung, S., Chace, N. M. and Gross, M. (1975) *Arch. Biochem.* **169**, 724.
6. Smith, T. A. and Best, G. R. (1980) *J. Sci. Food Agric.* (in press).
7. Smith, T. A. (1979) *Analyt. Biochem.* **92**, 331.
8. McGowan, R. E. and Muir, R. M. (1971) *Plant Physiol.* **47**, 644.
9. Smith, T. A. (1974) *Phytochemistry* **13**, 1075.
10. Clarke, A. J. and Mann, P. J. G. (1957) *Biochem. J.* **65**, 763.
11. Kenten, R. H. and Mann, P. J. G. (1952) *Biochem. J.* **50**, 360.
12. Nash, R. J., Smith, T. A. and Wain, R. L. (1968) *Ann. Appl. Biol.* **61**, 481.
13. Smith, T. A. and Wilshire, G. (1975) *Phytochemistry* **14**, 2341.
14. Seiler, N. (1970) *Methods Biochem. Analysis* **18**, 259.
15. Hill, J. M. (1967) *Biochem. J.* **104**, 1048.
16. Yamasaki, E. F., Swindell, R. and Reed, D. J. (1970) *Biochemistry* **9**, 1206.
17. Suresh, M. R. and Adiga, P. R. (1979) *J. Biosci.* **1**, 109.
18. Hill, J. M. and Mann, P. J. G. (1964) *Biochem. J.* **91**, 171.
19. Smith, T. A. and Bickley, D. A. (1974) *Phytochemistry* **13**, 2437.