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THERMAL ISOMERIZATION OF THE NEUROTOXIN β-*N*-OXALYL-L-α,β-DIAMINOPROPIONIC ACID

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Key Word Index—*Lathyrus sativus*; β-*N*-oxalyl-L-α,β-diaminopropionic acid (β-ODAP); neurotoxin; flow injection; thermal isomerization of β-ODAP; kinetics; α-ODAP.

Abstract—The rate constants and reaction order for the conversion of the neurotoxin β-ODAP to α-ODAP have been determined, after off-line thermal treatment, using the recently developed flow injection system for the neurotoxin based on a glutamate oxidase reactor. The effects of the initial concentration of the toxin, temperature and pH on the kinetics and the equilibrium of the endothermic process were examined. The conversion followed a zero order rate law. The reaction rate at pH 7 increased by about two-fold for every 10° temperature rise. The rate at pH 2 was about 60% higher than at pH 7. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The seeds of the drought-tolerant legume, *Lathyrus sativus* (grass pea, also known as guaya in Ethiopia and khesari in India), are important sources of dietary protein in Ethiopia and the Indian sub-continent. Foods from the lathyrus seeds occasionally become the major means of survival in times of flood- and drought-triggered famine. However, consumption of *L. sativus* as a staple diet for a few months is known to cause human neurolathyrism, an irreversibly crippling disease. The neurotoxin responsible for the disease, identified independently by two Indian groups in 1964, is the non-protein amino acid β-*N*-oxalyl-L-α,β-diaminopropionic acid, β-ODAP, also called β-*N*-oxalyl-amino-L-alanine, BOAA [1, 2].

Bell and O'Donovan [3] reported that β-ODAP slowly equilibrates with its isomer α-ODAP in ethanolic solution, and the inter-conversion is facilitated when heated [3]. The α-isomer is non-toxic [4, 5]. The non-toxicity of the α-isomer and the observation that the β-isomer can be isomerized to the non-toxic form paved the way to explore various processing and cooking methods as a means of thermal detoxification of the legume. Abegaz and co-workers [6] as well as De Bruyn and co-workers [7] monitored the thermal isomerization of β-ODAP by NMR spectroscopy. HPLC was also used to follow the thermal process [8]. However, no report has so far been made on the quantitative aspects of the equilibrium and the kinetics of the $\beta \rightarrow \alpha$ conversion with respect to initial toxin concentration, pH and temperature. Such a study may be useful not only in understanding the thermal isomerization in regard to detoxification, but also in examining the metabolic fate of the neurotoxin after ingestion.

Moges and Johansson recently reported a flow injection-glutamate oxidase (FI-GlOD) reactor system for selective determination of β-ODAP in the presence of α-ODAP [9]. We report some of the kinetic and equilibrium data for the thermal isomerization of β-ODAP, based on the toxin-selective enzymatic assay, using the reported FI-GlOD system.

RESULTS AND DISCUSSION

Optimization of the flow system

The optimum pH, flow rate and reagent concentrations for the determination of β-ODAP, using the glutamate oxidase (GlOD) reactor in the FI system (Fig. 1), were found to be essentially the same as

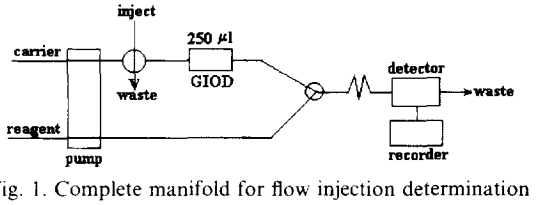

Fig. 1. Complete manifold for flow injection determination of ODAP.

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the reported values [9]. Calibration curves were linear in the range 10–300 μM β-ODAP. The sensitivities of the FI system to β-ODAP, glutamate and hydrogen peroxide were found the same, which shows that the conversion efficiency of the reactor for β-ODAP was 100%. However, the linear region for glutamate and hydrogen peroxide was wider, ranging from 5 μM to the studied limit, 1000 μM.

Equilibrium data

At 80°, the relative response to the toxin was found to be 63% (rsd 1.3% for $n = 5$) at equilibrium. This corresponds to 60% of the original total ODAP out of which is 95% β-ODAP [8]. This is in good agreement with results obtained from the same detection system as reported by Moges and Johansson (62% response or 59% of the original total ODAP) [9]. Hence, this report reconfirms the selectivity of GIOD to the β-form in the presence of both isomers, and the difference accounts for the undetected α-ODAP. At equilibrium, the percentage of β-ODAP at 60° was 68.0% which decreased to 66.5 and 60.0% at 70 and 80°, respectively. The usually reported equilibrium concentrations of the β- and the α-isomers after thermal isomerization, at 55°, are between 60 and 65%, and 40 and 35%, respectively [6, 8].

A linear relationship was found between the equilibration time and initial concentration of β-ODAP (Fig. 2). Earlier workers employed very high concentrations e.g. 3–5 mg β-ODAP in 1–1.5 ml D_2O, i.e. *ca* 15–28 mM, for NMR studies of thermal isomerizations at 55° [6]. Therefore, the high β-ODAP concentration (and the low temperature) that had been applied account for the long equilibration times reported by these workers (*ca* 20–48 hr).

The conversion of β-ODAP to the α-form reached equilibrium after 200, 105 and 60 min at 60, 70 and 80°, respectively. The rate at 90° was about twice as great as at 80°, and, correspondingly, the time to reach equilibrium should be about half (*ca* 30 min) of that at the latter.

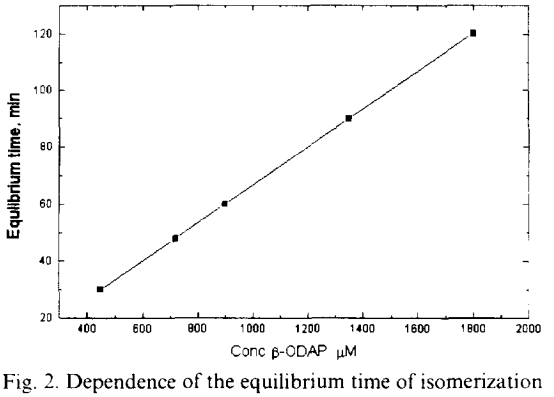


Fig. 2. Dependence of the equilibrium time of isomerization on the initial concentration of β-ODAP.

Kinetic studies

Concentration dependence. During the study, sampling of the heated solution was made 1 min after β-ODAP was added. The residual β-ODAP after the 1 minute interval was taken as the initial concentration. This is to exclude the effect of mixing, to give time for thermal equilibrium and to establish steady state. During this interval, about 10% of β-ODAP was isomerized at 80°, irrespective of the concentration of the toxin initially added to the buffer. The initial rate of isomerization of the compound was found to be independent of its initial concentration. This is reflected in the plot of ln rate versus ln [β-ODAP] which gives a horizontal line and an intercept of ln k_0, $k_0 = 14.5$ μM min^{-1} (2.42×10^{-7} M sec^{-1}, rsd 1.64% for $n = 5$) and is shown in Fig. 3. This strongly suggests that the conversion follows a zero order rate law with respect to β-ODAP. Furthermore, the linear relationship that exists between the equilibrium time and concentration (Fig. 2) further confirms that $\beta \rightarrow \alpha$ conversion follows zero order kinetics, as supported by the conclusion which follows the derivation below.

For a zero order reaction, the integrated form of the rate law is

$$[A]_t - [A]_0 = -k_{app}t \quad (1)$$

At equilibrium, when $t = t_\infty$, eqn (1) may be rewritten as:

$$[A]_\infty - [A]_0 = -k_{app}t_\infty \quad (2)$$

or

$$(m[A]_0 - [A]_0) = -k_{app}t_\infty \quad (3)$$

where at time t_∞, $[A]_\infty$ is $m[A]_0$, and m is the fraction of $[A]_0$ at equilibrium ($m = 0.7$ for β-ODAP at 80°). Rearrangement of eqn (3) yields,

$$[A]_0(m-1) = -k_{app}t_\infty \quad (4)$$

Hence, the time required to reach equilibrium is directly proportional to the initial concentration of $[A]_0$. If the conversion had been first order, the time to reach equilibrium would have been the same irrespective of the initial concentration i.e. in a first order reaction a constant fraction of the substrate concentration should decompose at a given time, e.g. $t_{1/2}$ is independent of the initial concentration. Therefore,

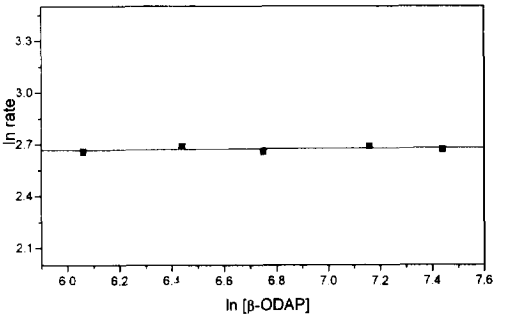


Fig. 3. Plot of ln rate vs ln[β-ODAP].

the long equilibrium times for the isomerization of β-ODAP in the earlier reported experiments can now be explained by the direct dependence of the equilibrium time on concentration that is characteristic of a zero order reaction.

The effect of temperature. The effect of temperature on the rate of isomerization were studied using 1 mM β-ODAP solutions at 60, 70, 80 and 90°. The residual β-ODAP concentration measured after a 1-min heating was again taken as the initial concentration. The observed rate of isomerization at 60° was found to be 3.7 μM min^{-1}, which correspondingly increased to 6.5, 14.5 and 29.4 μM min^{-1} for 70, 80 and 90°. The Arrhenius plot of ln(rate) vs $1/T$ (K) resulted in a straight line with a negative slope (see Fig. 4). Evaluation of the slope shows that for every 10° rise in temperature, the rate of isomerization increases by about two-fold.

From the slope of the line in Fig. 4, the apparent energy of activation was found to be +72.2 kJ mol^{-1}. The positive Gibb's energy of the reaction shows that the reaction is endothermic. This is in agreement with the equilibrium constant which slightly favours the β-form, $[\alpha]/[\beta] < 1$. The endothermicity can also be seen from the increasing equilibrium constant with temperature.

The effect of pH. β-ODAP undergoes three acid dissociation steps similar to that of L-glutamic acid. The compound has apparent pK_a values in the order of 1.95, 2.95 and 9.25 corresponding to the two carboxyl and α-amino functions, respectively [1, 10]. A distribution diagram of the four species of β-ODAP (percentage vs pH) shows that, as expected, mixtures of each form exist at a particular pH [11]. From coupling constants data in NMR spectroscopy and molecular mechanics, De Bruyn and his group [12] determined the proportions of different rotational isomers (rotamers) for the ionic forms of β-ODAP that dominantly occur at pH 0.3, 2.5, 6.9 and 12.4. The orientation of the functional groups that are involved in the isomerization will affect the rate by the existence of rotamers. It can, therefore, be seen that a simple pattern may not be observed between pH and kinetic parameters. At the selected pH (pH 7) for the major study, β-ODAP exists as one with a protonated α-amino group and deprotonated carboxylic groups. At pH 2, β-ODAP is 40% fully protonated while the form with a deprotonated α-carboxyl group (the zwitterion) amounts to 40%.

A study of the rate of conversion at this pH was made to compare with that at pH 7. During the first minute it was found that about 13% of the toxin was converted into the α-form and equilibrium was attained within 35 min. The equilibrium concentration of the toxin was about 57%. The slightly lower concentration of β-ODAP assayed at this pH (57%) is expected to be due to the formation of a small amount of α,β-diaminopropionic acid, resulting from the hydrolysis of the oxalyl compound in acidic conditions [7]. In the thermal process at pH 2, the initial rate was 23.7 μM min^{-1}. It is evident that the isomerization process is faster at pH 2 than at pH 7 (14.5 μM min^{-1}). Abegaz and coworkers [6] suggested that the faster process at the acidic pH might be due to the existence of a more preferred rotamer of the amino acid. Further, they suggested that the mechanism might involve the reaction of a protonated oxalyl carboxyl group. Figure 5 shows concentration of β-ODAP as a function of time for pH 2 and 7.

Mechanism of isomerization. The fact that the initial toxin concentration does not influence the rate of isomerization, but the equilibrium time linearly increases with concentration, confirms zero order kinetics, suggesting a rapid formation of an intermediate (I), whose decomposition to both isomers should be the rate limiting step.

Bell and O'Donovan [3] as well as Abegaz and coworkers [6] suggested the formation of a six-membered cyclic intermediate (DKP, α,β-diketopiperazine) via intramolecular rearrangement. The latter found that higher homologues of β-ODAP ($HOOCONH(CH_2)_nCHNH_2COOH$ where $n = 3$ or 4) did not favour oxalyl transfer to the α-amino group. DKP is a stable compound which can be synthesized. Its conversion to both β- and α-ODAP takes place through a slow hydrolysis reaction. The more recent work by De Bruyn and co-workers [7] experimentally showed that DKP is stable and is not the likely intermediate since no trace of this compound was detected

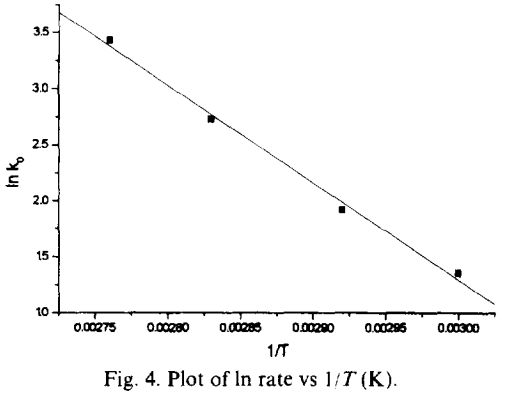


Fig. 4. Plot of ln rate vs $1/T$ (K).

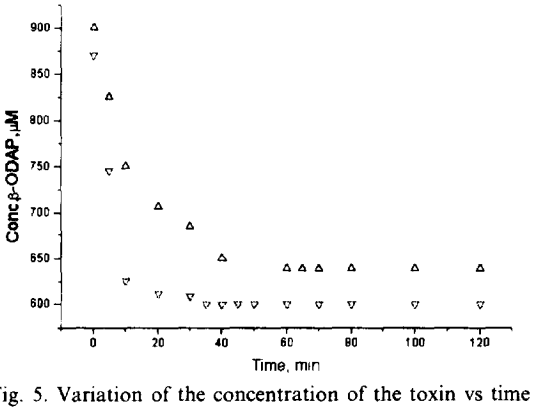
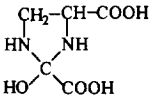


Fig. 5. Variation of the concentration of the toxin vs time during isomerization at pH 2 (∇) and at pH 7 (Δ).



2-Hydroxy-imidazolidine-2,4-dicarboxylic acid

(Intermediate)

$$\beta\text{-ODAPH}^+ + H_2O \overset{fast}{\rightleftharpoons} \beta\text{-ODAP} + H_3O^+$$

$$\beta\text{-ODAP} \overset{slow}{\rightleftharpoons} I$$

$$H_2O + \beta\text{-ODAPH}^+ \longleftarrow I + H_3O^+ \longrightarrow \alpha\text{-ODAPH}^+ + H_2O$$

Fig. 6. Proposed mechanism of the isomerization of ODAP.

during the thermal isomerization of the amino acid. They proposed 2-hydroxy-imidazolidine-2,4-dicarboxylic acid, an unstable five-membered structure (see structure in Fig. 6), as the intermediate. The formation of the five-membered intermediate was presented as purely intra molecular that did not account for any possible involvement of water, acid or base.

At both pH 2 and 7, the α-amino group is protonated ($-NH_3^+$) which renders the lone pair in the nitrogen unavailable for the nucleophilic attack on the oxalyl carbon. Hence water may act as a base catalyst to abstract the proton in $-NH_3^+$. The present experimental results do not reveal that water may have a catalytic role. However, the fact that the conversion rate nearly doubles for every 10° rise in temperature and the high value of the activation energy suggests the occurrence of a chemical reaction that may involve another compound. The proposed participation of water should be a catalytic one, since the final products are α- and β-forms in the reversible process. This report and an early work [6] have shown that the conversion is faster in acidic medium (pH 2 and 2.5) than at neutral or near-neutral pH. This may be explained by the fact that the oxalyl carbon could be protonated which makes it more prone to electrophilic attack by the α-amino group. Hence a mechanism suggested by De Bruyn [7] appears to be reasonable provided it accounts for the possible involvement of water as a base. The mechanism is shown in Fig. 6, with the five-membered cyclic intermediate recently suggested by De Bruyn and coworkers [7].

EXPERIMENTAL

Enzymes and reagents. Glutamate oxidase (GIOD, EC 1.4.3.11, 200 U ml^{-1}, Yamasa Corp., Japan) and horseradish peroxidase (HRP, EC 1.11.1.7, 268 purpurogallin U mg of $solid^{-1}$, L-glutamic acid, L-aspartic acid, β-ODAP monohydrate (all Sigma) and 4-AP (BDH) were used as received. 2,4-Dichlorophenol-6-sulphonate, DCPS, was synthesized from 2,4-dichlorophenol, DCP (Merck 3774), and conc. H_2SO_4 [13] and stored at pH 7 and 4°. H_2O_2 was standardized by permanganate titration.

Immobilization of enzymes. Glutamate oxidase was immobilized by glutaraldehyde covalent coupling on controlled pore glass (CPG-10, particle size 0.12–0.20 mm and pore size 51 nm, Serva). 80 U of the redox enzyme was added to 200 mg of the glutaraldehyde activated CPG. Evaluation of the *A* of the enzyme soln before and after immobilization showed that the coupling yield was 73%. The immobilized enzyme was packed in a 250-μl Plexiglass tube (i.d. 2.0 mm) and, when not in use, stored at 4° in 0.1 M phosphate buffer (NaPi) (pH 7).

The flow injection set up. The two channel FI set-up is a modification of the earlier report in which the present system does not consist of an HRP indicator reactor and glutamate-destroying reactors (see Fig. 1). Samples from the thermal conversion experiments were injected using a 20-μl loop, fixed on a Rheodyne 7125 injection valve. The sample carrier was 0.1 M NaPi at pH 7 (0.3 ml min^{-1}) which passed through the GIOD reactor and merged with the Trinder reagent (0.12 ml min^{-1}) at a confluence point. The H_2O_2 produced from the oxidation of β-ODAP reacted with the reagent for detection as a red quinoneimine dye at 512 nm, using an LKB 2151 UV-Vis flow-through spectrophotometer. The reagent line consisted of HRP (2 mg 100 ml^{-1}), 2.5 mM DCPS, 0.5 mM 4-AP, and 0.5 mM EDTA 0.25 mM DCP in NaPi buffer at pH 7.

Isomerization of β-ODAP. A fixed vol. of 0.1 M NaPi buffer (pH 7) was introduced into a screw-capped test-tube and heated in a water bath thermostated at 80°. 10 mM β-ODAP, in the same buffer, was added to the pre-heated buffer so that the concn of the compound would be 500–2000 μM (final vol. 3 ml). Heating continued after inverting the mixture twice (*ca* 4 sec). A 100-μl sample was withdrawn after 1 min and added to the same buffer, cooled over ice, to stop any further isomerization. More samplings were made every 5 or 10 min. Dilutions to the cooled buffer were made to give 200 μM total ODAP. The un-isomerized β-ODAP was determined by injection of the sample at room temp. into the flow system. At least two determinations were made for each sample. Similar experiments were performed at 60, 70 and 90° for 1 mM standards (pH 7). For comparison, a rate study was also made at 80° for 1 mM toxin prepared from its 10 mM aq. soln in NaPi at pH 2.

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