THE PHASE RELATIONSHIPS OF THE OSCILLATIONS OF GLUTATHIONE AND AMINO ACIDS IN THE GERMINATING SEEDS OF *VOANDZEIA SUBTERRANEA*

Considerations pertaining to the wave mechanical characteristics of the γ-glutamyl cycle

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1. Introduction

In the full γ-glutamyl cycle, the binding of amino acid to the transpeptidation complex, to form the diffusion complex, activates the cycle [1]. On the other hand, the binding of glutathione (GSH) to γ-glutamyl cysteine synthetase inhibits the cycle [2]. Since the presence of a product-activated step preceding a product-inhibited step is the basic prerequisite for the intermediates of a metabolic pathway to oscillate [3,4], then the intermediates of the full γ-glutamyl cycle should oscillate. This means that the mechanism of operation of the full γ-glutamyl cycle can be verified by studying the oscillatory characteristics of its intermediates instead of the usual characterization of the enzymes of the cycle. Here, germinating seed was chosen as the experimental material because during seed germination, amino acids are transported from the cotyledons to the embryonic axis [5], so making it possible to study the oscillatory phase relationships of amino acids before and after their translocation via the full γ-glutamyl cycle. Germinating seeds of *Voandzeia subterranea* are also known to contain high activities of γ-glutamyl transpeptidase (γ-GTP) [6].

This study shows that amino acids and GSH oscillate during seed germination. The phase plane plots of GSH against amino acids indicate that a unit of the γ-glutamyl cycle consists of 2 oscillatory motions of the diffusion pathway and 1 of the GSH pathway or vice versa. The pathways of the γ-glutamyl cycle thus have wave-like characteristics.

2. Materials and methods

Healthy seeds of *V. subterranea* (Bambarra groundnut) purchased from a local market were planted on sand as in [7]. They were harvested daily in the evenings, washed with distilled water and decoated. Seeds at the same stage of germination (equal lengths of radicles and/or plumules) were selected each day and stored frozen, but were defrosted before extraction of their contents. The first harvest was 24 h after planting of seeds.

Whole seeds, plumules, radicles, cotyledons or embryonic axis of seeds selected from each day's harvest were weighed and ground with mortar and pestle in 5 times their volume of 75% (v/v) boiling ethanol. The extracts were centrifuged at room temperature at 4000 × *g* for 1 h and the supernatants used for the determination of amino acid nitrogen as in [8]. The supernatants were also assayed for protein as in [9] using bovine serum albumin as standard; no peptide nitrogen was detected in them.

About 10 g whole seeds were ground with pestle and mortar with 10 ml 5% (w/v) trichloroacetic acid. After centrifugation (26 000 × *g* for 30 min) the pellet was extracted with another 10 ml trichloroacetic acid, and after centrifugation (26 000 × *g* for 30 min) the supernatants were combined. The trichloroacetic acid was removed by extraction with ether and the aqueous extract was assayed for total GSH [10].

3. Results and discussion

The oscillations of GSH and amino acids are presented in fig.1. The detection of these oscillations

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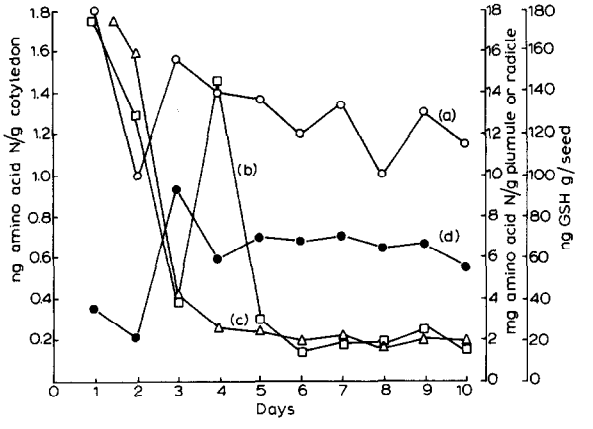


Fig.1. The oscillations of amino acids in : (a) cotyledons; (b) plumules; (c) radicles; and of GSH in (d) whole seeds of *V. subterranea* during germination.

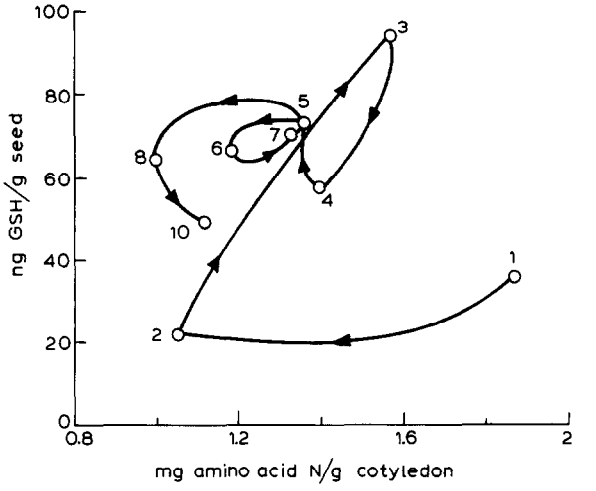


Fig.2. Phase plane plot (data from fig.1) of whole seed GSH against cotyledon amino acids of germinating seeds of *V. subterranea.*

of GSH and amino acid is consistent with the mode of operation of the full γ-glutamyl cycle [1]. To understand the phase relationships of GSH and amino acids, the oscillations of GSH were plotted against those of amino acids to obtain the phase plane plots in fig.2,3. The phase plane plot is the clearest graphic representation of the characteristics of oscillating metabolites [11].

The phase relationship of the GSH in the seed and the amino acids being translocated (fig.2) was obtained by plotting the oscillations of GSH against the cotyledon amino acids, fig.2 has clearly 2 sections. The first is from 1–5 days and consists of clockwise spirals the long axis of which is inclined at 45°. The second section is from 5–10 days and consists of anti-clockwise spirals, the long axis of which is also inclined at 45°. Thus the amino acids being transported and GSH are in phase in the 2 sections. These results are consistent with the postulated mechanism of operation of the full γ-glutamyl cycle which consists of 2 pathways in both of which the amino acids being transported and the GSH are on the same sides of the transpeptidation and diffusion complexes.

The phase relationship of the GSH in the seeds and the translocated amino acids was obtained by plotting the oscillations of GSH against the plumule, radicle or embryonic axis amino acids (fig.3). The phase plane plot consists of 2 sections: the first (1–5 days) being clockwise spirals; the second (5–10 days) anti-clockwise spirals. Fig.3 differs from fig.2 in that in fig.3 the clockwise section is pulled away from the anti-clockwise section so that the clockwise section operates at higher amino acid concentrations (4–18 mg nitrogen/g plumule) while the anti-clockwise section operates at lower amino acid concentrations (<3 mg nitrogen/g plumule). This result is consistent with the mode of operation of the full γ-glutamyl cycle because the diffusion pathway accounts for ~80% of the translocated amino acids [12,13]. On this basis the clockwise section of fig.3 corresponds to the diffusion pathway while the anti-clockwise section corresponds to the GSH pathway.

Fig.3 is also different from fig.2 in that the long axis of the clockwise section of fig.3 is inclined at

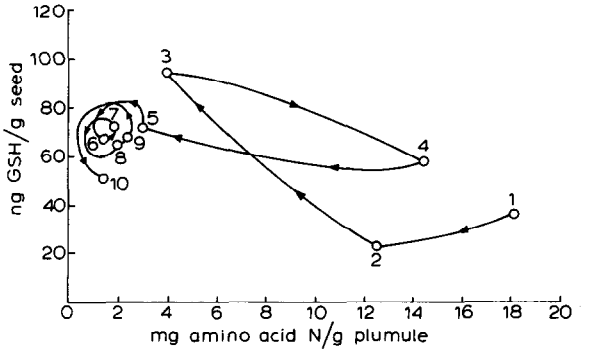


Fig.3. Phase plane plot (data from fig.1) of whole seed GSH against plumule amino acids of germinating seeds of *V. subterranea.*

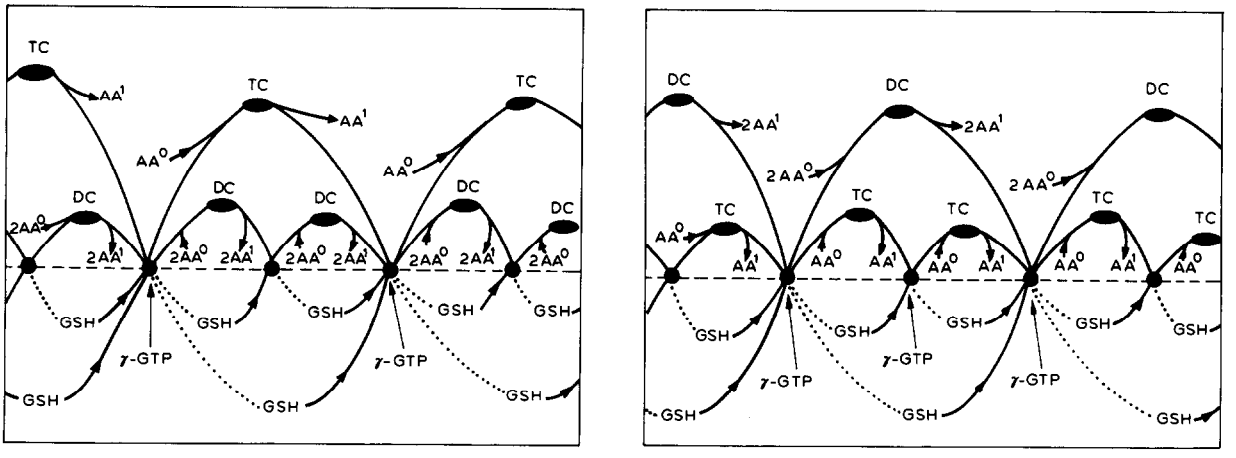
135° while the clockwise section retains its original 45° inclination. Thus, in the clockwise section (diffusion pathway), GSH and the translocated amino acids are out of phase, but in the anti-clockwise section (GSH pathway) GSH and the translocated amino acids are in phase. Fig.2 and 3, therefore, suggest that the members of the basic oscillatory couple are the γ-glutamyl–γ-GTP (γ-glutamyl–enzyme) complex and the diffusion complex. This finding is consistent with the proposed functions of the active sites of γ-GTP during amino acid uptake [13].

The phase relationship of the amino acids being translocated and the amino acids already translocated was obtained by plotting the cotyledons' amino acids against plumule, radicle or embryonic axis amino acids. Data not shown are identical with those in fig.3 and show that the amino acids being translocated and those which have been translocated via the diffusion pathway are out of phase, while those being translocated and those which have been translocated via the GSH pathway are in phase, confirming that the members of the basic oscillatory couple are the γ-glutamyl–enzyme and the diffusion complexes (fig.2,3). The oscillatory characteristics of GSH and amino acids are therefore in complete agreement with the mechanism of operation of the full γ-glutamyl cycle proposed from enzyme kinetic calculations.

Since fig.2 is a plot of the oscillations of GSH against those of amino acids being translocated, the clockwise section is the phase plane plot of the diffusion complex while the anti-clockwise section is the phase plane plot of the transpeptidation complex. The experimental points in the clockwise section are thus the instantaneous 'snapshots' of the diffusion complexes and those in the anti-clockwise section are the instantaneous 'snapshots' of the transpeptidation complexes. Fig.2 has therefore provided the evidence for the physical existence of the diffusion and transpeptidation complexes. So far, only kinetic evidence has been advanced to support the existence of these 2 complexes [1]. The present physical evidence for the existence of these 2 complexes permits the derivation of the oscillatory phase relationships of the diffusion and GSH pathways. Fig.2 shows that the diffusion complexes are shifted 90° against the transpeptidation complexes. Fig.3 shows that the diffusion pathway is shifted 180° against the GSH pathway so that when one pathway is at its maximum the other is at its minimum. The mechanical implication of these phase relationships is that 1 pathway must make 2 oscillatory motions for only one of the other pathways to bring the amino acids being translocated and GSH into phase at the commencement of the next oscillatory cycle of the full γ-glutamyl cycle. The combination

Scheme 1

The oscillatory phase relationship of the diffusion and the GSH pathways of the full γ-glutamyl cycle



(a) A unit of the cycle operating at high concentration of amino acids

(b) A unit of the cycle operating at low concentration of amino acids

Abbreviations: DC, diffusion complex; TC, transpeptidation complex; AA^0, amino acid being translocated; AA^1, amino acid translocated; (...) steps leading to the synthesis of GSH after its cleavage by γ-GTP

of the phase data from fig.2,3 gives the profiles of the wave motions of the 2 pathways of the γ-glutamyl cycle as presented in scheme 1. In scheme 1(a) a unit of the full γ-glutamyl cycle consists of 2 oscillatory motions of the diffusion pathway and one of the GSH pathway, so that for every 4 mol amino acids translocated via the diffusion pathway, 1 mol is translocated via the GSH pathway, to give the theoretical ratio of 4:1 found for the uptake of high concentrations of amino acid via the full γ-glutamyl cycle [13]. In scheme 1(b) a unit of the full γ-glutamyl cycle consists of 2 oscillatory motions of the GSH pathway and 1 of the diffusion pathway, so that 2 mol amino acids are translocated via the GSH pathway for 2 mol translocated via the diffusion pathway, to give the ratio of 1:1 for the translocation of low concentrations of amino acids via the full γ-glutamyl cycle. The full γ-glutamyl cycle therefore has sine wave mechanical characteristics meaning that the biological properties of the cycle can be predicted or verified from wave mechanical calculations. Controversial issues [14] about the mechanism of operation of the full γ-glutamyl cycle such as the turnover rate of GSH, the influence of amino acids on this turnover rate and the mechanism by which the cycle's pathways change from clockwise to anti-clockwise direction can be explained on the basis of the wave characteristics of the full cycle (in preparation).

This is the first demonstration of a functional γ-glutamyl cycle in higher plants. The cycle may operate in most organisms as a general mechanism for the translocation of amino acids. The wave characteristics of the cycle confer on it a high degree of operational efficiency.

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