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Preface

Plant amino acid research in retrospect: From Chibnall to Singh

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Introduction

This article's subsidiary title may seem odd initially, but 'From Chibnall to Singh' spans 60 years of plant amino acid research. Chibnall (1939) wrote a book 'Protein Metabolism in the Plant' that summarized most of the information available at that time, whilst the considerably larger volume edited by Singh (1999) and entitled 'Plant Amino Acids: Biochemistry and Biotechnology' brought together just some of our current knowledge of this subject.

As Chibnall prepared his book, he was aware of all 20 primary constituents of plant proteins (19 amino acids, including two amino acid amides, and one imino acid, proline), and all their structures were known. In addition, a very small number of other amino acids (eg citrulline), not components of proteins, were known, and usually were implicated in the synthesis or degradation of the protein amino acids. However, knowledge of the biosynthetic pathways leading to amino acid production in plants was very scarce in 1939, although a few enzymic reactions had been described; these included those catalysed by glutamic dehydrogenase, glutamic decarboxylase and transaminases.

Determination of the amino acid composition of proteins was a tedious and inexact process, and thus only a rudimentary knowledge of plant protein composition (and by inference their nutritive value) existed in 1939. Some amino acid could be assayed by colorimetric procedures of doubtful specificity, whilst others were still estimated by gravimetric precipitation methods.

Early investigators placed great emphasis on the roles of asparagine and glutamine during seed germination when nitrogen is mobilized from seed reserve proteins and made available for new protein synthesis in the developing root and shoot tissues. Legumes (*Vicia*, *Phaseolus* and *Lupinus* spp.) were used most commonly in these studies, which we now realize overemphasized the general importance of asparagine at the expense of glutamine (see later comments concerning the primary pathway of ammonia-N assimilation and reassimilation).

The story from 1939 onwards

Objectives and techniques

Investigations about plant amino acids, like most research, is driven by a mixture of possible goals. These are principally of two types; the first type reflects our continued quest for new fundamental information about the natural world, and the second our desire to understand and manipulate a group of plant constituents that are indispensable for the well-being of humans and livestock. Fundamental questions have involved:

1. the number of amino acids encountered in the plant kingdom, the variety seen in their chemical structures, the modes of their biosynthesis and biodegradation, and whether their distribution across genera and families has chemotaxonomic significance,
2. the molecular biology underlying mechanisms involved in the incorporation of amino acids into plant proteins in the correct amounts and linear sequences, and
3. the characterization of the precursor roles played by selected amino acids in the biogenesis of auxin, chlorophyll, purines and pyrimidines, many alkaloids, glucosinolates and cyanogenetic glucosides.

Practical objectives have focussed on:

1. the opportunities that may exist to modify the ultimate composition of proteins, especially to enhance their processing or nutritional quality, partly by increasing their contents of certain essential amino acids such as lysine, threonine and methionine,
2. the identification of enzymes involved in the biosynthesis of protein amino acids that show degress of plasticity, and can be manipulated in regard to substrate or inhibitor specificity, or through alterations in the number of copies of the genes coding for specific enzymes, and
3. the possible role of certain amino acids, or their simple derivatives, in alleviating abiotic stresses to which plants may be subject.

All these lines of enquiry were facilitated following the development of techniques that permitted increasingly probing, rapid and sensitive separation and assay of amino acids, and proteins and enzymes. The advent first of paper chromatographic techniques, then of ion-exchange chromatography, revolutionized the separation and identification of amino acids in extracts of plant materials; a quantitative audit of the amino acids present in a plant became possible in a few days, rather than months, and the possibilities for recognizing undescribed types of amino acid provided additional excitement. Thin-layer and electrophoretic separation techniques soon augmented these earlier methods, and they in turn were followed by gas chromatography and high pressure liquid chromatography. With suitable choices of column packing materials, proteins and enzymes could often be purified to homogeneity. Developments in spectroscopy in the early 1960s, including ms and nmr techniques, now often allow the unequivocable assignment of chemical structure to a 'new' previously uncharacterized amino acid from a plant source, without recourse to step-wise chemical degradation or synthesis. The development of recombinant DNA techniques and gene cloning has provided quite new insights into amino acid metabolism at the plant cell level, and now underpins many of the practical objectives outlined above.

The assimilation of inorganic nitrogen

Following the description of the reversible reaction catalysed by glutamic dehydrogenase, in which ammonia combines with 2-oxoglutarate to yield glutamic acid in an NADH-requiring step, the concept that this process was the primary pathway whereby plants assimilated inorganic-N became established, and remained in vogue for some 30 years. Gradually the concept was challenged by several types of experimental evidence. Particularly cogent was the fact that studies using ^{15}N-labelled ammonia indicated that ^{15}N entered the amide-N of glutamine earlier and considerably more rapidly than it did the 2-amino group of glutamic acid. These time sequence studies were supported by others using the specific glutamine inhibitor, L-methionine-S-sulphoximine; in the presence of this inhibitor ammonia simply accumulated within the plant cells. The final type of evidence was derived from studies tracing the assimilation of ammonia-N using mutant plants deficient either in glutamine synthetase, when high concentrations of ammonia accumulated, or in glutamate synthase (GOGAT, see Fig. 1), when glutamine accumulated. Miflin and Lea (1976) argued that these lines of evidence represented a convincing case for the assimilation of ammonia occurring via a combined operation of glutamine synthetase (GS) and glutamate synthase (GOGAT, glutamine-2-oxoglutarate amidotransferase) as in the cycle shown in Fig. 1. Subsequently the manner of

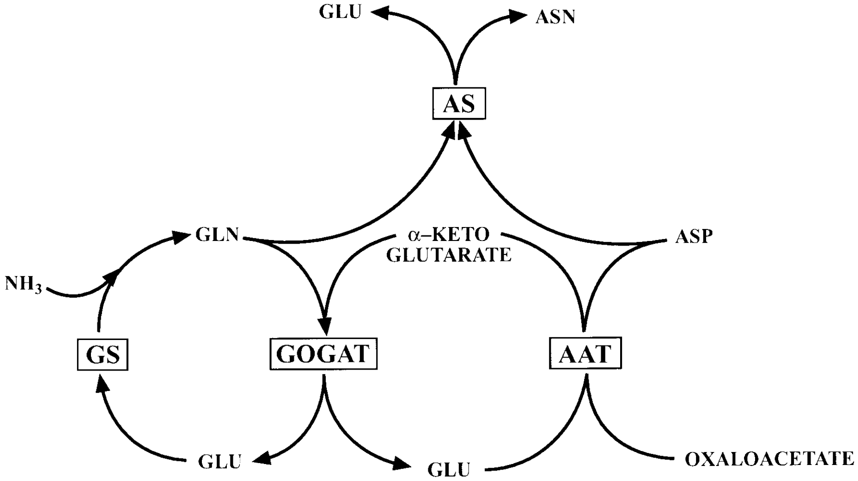

Fig. 1. The pathway of NH_3 assimilation in higher plants, illustrating the roles of glutamine synthetase (*GS*) and glutamate synthase and the relationships with aspartate aminotransferase (*AAT*) and asparagine synthetase (*AS*). After Lea and Ireland in Singh (1999)

asparagine synthesis was clarified; its 2-amino group originates by transfer from glutamic acid to 2-oxaloacetate (catalysed by aspartate aminotransferase) whilst its amide-N is gained by transamidation from glutamine mediated by asparagine synthetase (AS).

The GS-GOGAT sequence of cyclic reactions is now generally accepted as the mode of primary assimilation of inorganic ammonia-N into organic combination. The enzyme sequence is now known to have even greater quantitative importance in relation to its role in the reassimilation of ammonia-N released during photorespiration. In this process, two molecules of glycine give rise to one of serine, and one of molecule of NH_3 is released. Estimates suggest that the rate of reassimilation of NH_3 in this way can be much higher than that associated with the initial assimilation in plant roots.

The exact role of glutamate dehydrogenase in plant metabolism remains somewhat obscure.

Amino acid biosynthetic pathways

As new information accumulated about the steps involved in the biosynthetic pathways of individual amino acids, it became possible to group compounds into biogenetic families as follows:

> Glutamate was the precursor of glutamine, proline and arginine
> Aspartate of asparagine, threonine, methionine and lysine
> Alanine (pyruvate) of serine, glycine, valine and isoleucine
> Shikimate of phenylalanine, tyrosine and tryptophan.

The flow of metabolites along the biosynthetic pathways, which involve some branch points, must be regulated at the cellular level where certain pathway enzymes are subject to strong rate control. End product feedback inhibition of key initial pathway enzymes has been investigated widely; such inhibition can operate similarly on enzymes functioning immediately after a pathway branch point. These regulatory features have been examined further using compounds that are structural analogues of the end products themselves, and thereby mimic as inhibitors the normal end products whilst not fulfilling other cellular functions of the natural amino acid. Such analogues have been useful in screening mutant plant lines, either present in natural populations or after chemical or irradiation mutagenesis, that possess greatly elevated or strikingly reduced levels of key biosynthetic enzymes, or enzymes no longer subject to end product/analogue inhibition. Together these techniques have enhanced understanding of the regulatory mechanisms operating to maintain the cellular concentrations of amino acids required for protein synthesis, and to contribute to the practical objective of increasing biosynthesis of selected amino acids, such as lysine.

The emergence of the non-protein amino acids

In the Introduction, mention was made of amino acids, such as citrulline, that are constituents of plants, but not components of their protein molecules.

Before the advent of chromatographic methods such compounds were few in number, but paper chromatography opened up the possibility of examining the amino acid complements of large numbers of plants speedily and with much greater exactitude than previously. It soon became evident that members of the plant kingdom were far more versatile in synthesizing a range of unusual amino acids than were animals, and as the group of ‘new’ compounds expanded they became known as non-protein amino acids, a term correctly describing their general absence from protein molecules. Recent publications listing these compounds estimate their current number as about 800, a total less than that for the alkaloids or flavonoids, but considerably exceeding that of either the glucosinolates or the cyanogenetic glucosides.

One of the earliest discoveries was 4-methylene-L-glutamine, isolated from peanut (*Arachis hypogaea*) seedlings, where the compound appears to largely displace glutamine as the principal vehicle for N-transport within the plant. Some other non-protein amino acids are illustrated in Fig. 2, these being chosen from among about 50 compounds characterized by the author’s group to illustrate the variety of chemical structure encountered. Non-protein amino acids were the first known examples of the pyrazole moiety or the simple azetidine ring system in natural products. Pyrazolylalanine apparently remains a compound known only in the Cucurbitaceae, whilst azetidine-2-carboxylic acid (A2C) is a common component of liliaceous species yet it is encountered very sporadically in a few members of the unrelated legume family. The cyclopropyl residue occurs in 8 known non-protein amino acids, principally present in members of the related families, Sapindaceae, Hippocastanaceae and Aceraceae. Unsaturated aliphatic amino acids are

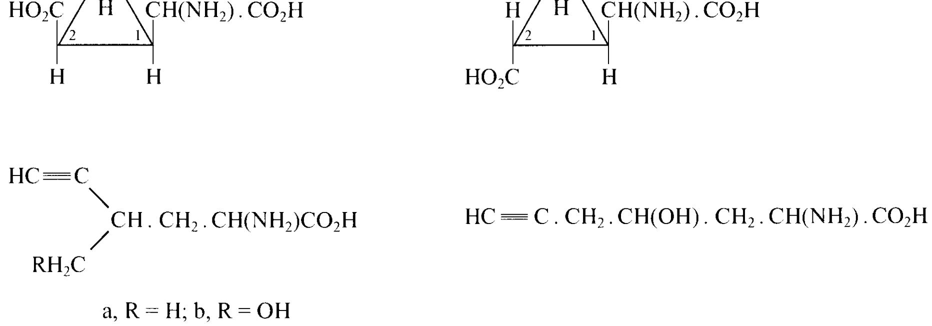
a, R = H; b, R = OH

Fig. 2. Shows some non-protein amino acids chosen to illustrate the variety of chemical structures encountered among members of this group

occasional compounds of plants; three acetylenic ($C \equiv C$ containing) amino acids were isolated from seed of *Euphoria longan* (Sapindaceae), whilst compounds containing an ethylenic ($C=C$) bond are more numerous.

The non-protein amino acids have aroused interest not only by the wealth of structural variety existing, but by the antimetabolite and/or toxic function of some of the compounds. Certain compounds are clearly almost isosteres of protein amino acids, and it is among this group that toxic compounds are found. Examples include A2C in relation to proline, and canavanine in respect of arginine. Both non-protein amino acids can accumulate in very high concentrations in plants producing them, and this posed the intriguing question of how the 'producer' plants avoid the effects of their own toxin. The answer was found in the markedly different substrate specificities encountered in enzymes implicated in amino acid incorporation into protein molecules. The prolyl-tRNA synthetase of a range of plant species that do not produce A2C accepts the analogue molecule as a substrate, and it is incorporated ultimately into protein molecules with disastrous results. In contrast, the same enzyme originating from 'producer' plants shows no affinity whatsoever for the analogue molecule, and protein molecules synthesized remain unaltered (Peterson and Fowden, 1963). This is clearly a case of mutation, occurring early in the evolution of angiosperms, and probably this study was the first demonstrating that an enzyme involved in basic metabolism can exhibit different degrees of substrate/inhibitor specificity dependent upon its origin – a feature later met in the development of herbicide-resistant crops (see later).

The same underlying reason – differential enzyme specificity – allows a bruchid beetle to feed exclusively upon seeds of *Dioclea megocarpa*, which contain up to 8% canavanine. The beetle's arginyl-tRNA synthetase discriminates totally against canavanine, failing to accept the normally-toxic analogue as a substrate and so excludes the toxin from its protein, in contrast to the situation encountered in susceptible species.

The carboxycyclopropylglycines exhibit some structural similarity to glutaminc acid, and Japanese workers have shown that the two natural sterioisomers (of the eight possible steric forms) cause a depolarization of rat spinal motoneurones (Ishida and Shinozaki, 1990).

Modified enzymes and herbicide resistance

Agrochemical industry has devoted major resources to producing cultivars of maize and soya bean that are resistant to wide-spectrum herbicides killing all weed and rogue crop species. Herbicides that have been exploited, such as the sulphonylureas and the imidazolinones, act by strongly inhibiting key enzymes in amino acid biosynthetic pathways. The principal target enzymes have been acetolactate synthase (ALS), catalysing the initial step in branched-chain amino acid synthesis, and 3-enolpyruvyl-shikimate-3-phosphate synthetase (EPSPS) a key enzyme functioning in the synthesis of the aromatic amino acids. Resistance normally takes one of three forms; each involves modification of enzyme levels or affinities.

Type 1 resistance arises when the target enzyme exists in an altered form showing a much diminished affinity in binding the herbicide, ie altered inhibitor specificity.
Type 2 resistance is encountered when plants produce greatly increased amounts of the target enzyme by their possession of a larger number of copies of the coding gene.
Type 3 resistance is found in plants possessing large concentrations of enzymes that bring about metabolic degradation of the herbicide itself.

The natural resistance to these types of herbicide occasionally found in wild species (weeds) is commonly of type 3, whereas resistance displayed by laboratory-generated cultivars normally is of types 1 or 2. For instance the post-emergence herbicide, glyphosate, has the enzyme EPSPS as its target, but strains of *Salmonella* growing in the presence of glyphosate contain a type of EPSPS having little affinity for the herbicide. Subsequent transfer of the bacterial gene coding for EPSPS to crop species have yielded glyphosate-resistant crops of type 1. An example of type 2 resistance is seen in herbicides derived from phosphinothricin, which inhibit glutamine synthetase (GS). Resistant mutants of alfalfa have up to 10× the normal levels of GS, and so the herbicides fail to block the biosynthesis of glutamine to an extent that causes growth inhibition or death of the plants. These studies have further clarified the mechanisms regulating amino acid synthesis, and added a new dimension to global crop production.

Augmenting the essential amino acids in plants

Viewed from the standpoint of human and animal nutrition, a number of important food plants exhibit relative deficiencies in lysine, threonine and methionine. These three essential amino acids share a common initial biosynthetic pathway, in which aspartokinase (AK) catalyses the first reaction converting aspartate to β-aspartyl phosphate. A number of studies, the earliest in the 1970s using barley, examined the kinetics of AK, and concluded that it existed as several distinct isoenzymes each subject to feed-back regulation by one or other of the three amino acid end-products. Mutant lines were isolated containing deregulated AK isoenzymes that allowed a continuous flow of metabolites along the synthetic pathway with the consequent accumulation of threonine, lysine or methionine. Subsequent work using a range of plant species established that lysine biosynthesis was regulated by a second enzyme, dihydrodipicolinate synthase, operating after the pathway branch-point leading specifically to lysine; this enzyme, like AK, can exist in a deregulated form which may determine, more critically, lysine concentrations. At best, the concentrations of the *free* amino acids in mutant lines of crops studied showed increases of: threonine more than ×100, lysine ×20 and methionine ×6. However, these enhanced concentrations are much less significant when judged against total amounts, ie free plus combined (in proteins), and represent increases of only a few percent.

A different approach, using genetic transformation, has been adopted particularly in regard to possible enhancement of the methionine content of crops used prominently in animal feedstuffs. Some seed storage proteins are particularly rich sources of methionine, eg the 2S albumin of the brazil nut and the sunflower seed albumin. Genes coding for these proteins have been introduced into various legume species (lupin, pea, chickpea and *Vicia* spp) when the transformed plants produce seeds whose total soluble protein complement contains up to 5–6% of the new (foreign) proteins, whose very high methionine content results in a possible doubling of seed methionine concentrations. Transfer of the sunflower seed albumin gene may prove to be the more useful approach in view of the allergenic potential of the brazil nut protein. Attempts to augment the total lysine content of seeds by similar gene transfer technology (using genes coding for high-lysine proteins) as yet have not had comparable success.

Later papers in this volume provide more detailed information about these experimental approaches to improve the essential amino acid status of plants.

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