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REVIEW

THE USE OF AMINO ACID ANALOGUES IN STUDIES ON PLANT METABOLISM

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INTRODUCTION

Since many metabolic reactions require amino acids as substrates [1,2] and nearly all cellular reactions depend on proteins (and thus ultimately on a supply of amino acids), the replacement of an amino acid by an amino acid analogue may lead to diverse metabolic effects.

An effective analogue usually possesses similar stereochemical and charge properties to one or more of the amino acids found in proteins [3], the structures of some analogues are illustrated in Fig. 1. Although the growth-inhibitory effect of amino acid analogues has been extensively reviewed [4,5], the potential use of these compounds in biological studies has been little discussed especially with regard to plant systems [6]. Since many higher plants are able to synthesise amino acid analogues which are toxic to some organisms [7,8], it is of interest to ascertain the extent to which the enzymes of such plants are altered in order to minimise 'analogue poisoning'.

Problems involved in the use of amino acid analogues

Amino acid analogues usually only inhibit the growth of an organism when the endogenous pools of free protein amino acids in the cells are sufficiently low to ensure competition between the analogues and the amino acids which they antagonise [9]. However, a number of analogues, which will be discussed later, have the ability to bind to enzymes irreversibly and are able to exert their toxic effect at low concentrations.

The response following the administration of an analogue to higher organisms is sometimes difficult to interpret because different control mechanisms may operate in various tissues. Many tissues are able to detoxify certain analogues [10], e.g. azetidine-2-carboxylic acid (A-2-C) (**1**) inhibits the development of *Phaseolus aureus* seedlings but has little effect on *Agrobacterium* sp which degrade the analogue to α-hydroxy-γ-aminobutyric acid [11].

Similar problems may be encountered when testing the substrate specificity of individual enzymes *in vitro*. The presence of enzymes such as deaminases, hydrolases and deacetylases in impure enzyme preparations may lead to errors in the estimation of the effectiveness of the analogue as a substrate for a particular reaction. Enzymatic processes within the cell may also catalyse the synthesis of an amino acid analogue from a non-toxic precursor. After treatment of tomato plants with 3-amino-1,2,4-triazole, the histidine analogue, β-3-amino-1,2,4-triazol-1-ylalanine (**2**) is formed [12,13].

The ability of certain analogues to complex with pyridoxal phosphate (a cofactor for many cellular reactions) may also confuse the interpretation of their specific effects *in vivo*. Mimosine chelates pyridoxal phosphate and may thus inhibit mammalian transaminases and decarboxylases. The corresponding plant enzymes, however are not affected in this manner since the cofactor is firmly bound to the enzyme surface [4]. Canaline, a breakdown product of canavanine in *Canavalia ensiformis* [14], and inhibitor of ornithine-α-oxoglutarate transaminase, also inhibits other transaminase reactions not utilising ornithine as a substrate [15] by non-enzymic oxime formation with pyridoxal phosphate.

Growth

The growth inhibitory effect of an analogue is usually competitively reversed by the inclusion of a specific protein amino acid in the growth medium e.g. the growth inhibition of *Avena* roots by A-2-C is reversed by proline [16]. When more than one amino acid is capable of reversing such growth inhibition, it is usually structurally related to the others: isoleucine, leucine and norleucine are able to reverse the *O*-methylthreonine-mediated inhibition of cell multiplication and chlorophyll formation in *Euglena gracilis* [17]. Some compounds may act as analogues of two unrelated amino acids: methionine sulphoximine (**3**) inhibition of the growth of *Chlorella* is reversed by methionine [18], whilst glutamine reverses the inhibitory action of this analogue on glutamine synthetase [19]. Substances unrelated to amino acids may also reverse the growth inhibitory action of analogues: the ethionine-induced inhibition of the elongation of *Avena* coleoptiles is reversed by purines [20].

The type of growth inhibition caused by an amino acid analogue depends on the organism concerned.

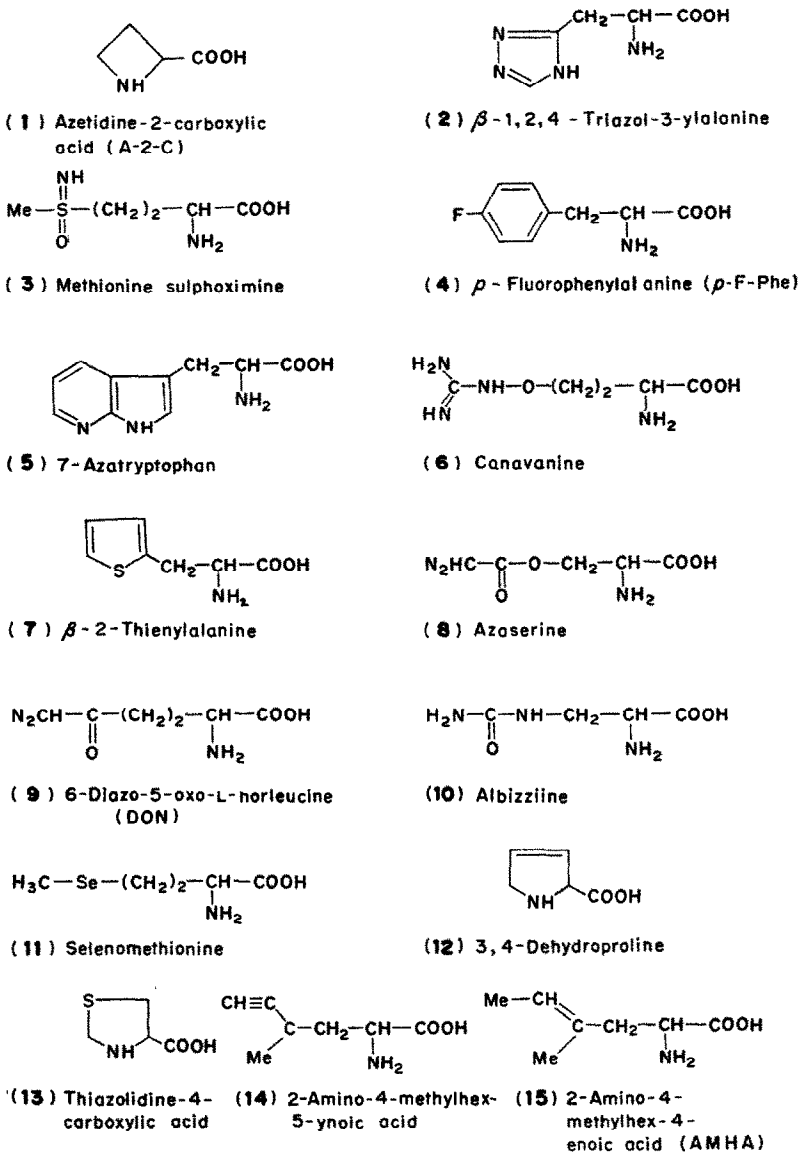

Fig. 1. Structure of a number of amino acid analogues.

Methionine and threonine antagonise the growth inhibitory effect of *O*-methyl-threonine in *E. coli* [21], although in higher plants and *Euglena* this inhibition is reversed by isoleucine [17].

Cell structure

Analogues may be of value in correlating biochemical functions with morphological changes in the cell. Aeration of beetroot discs causes a rapid synthesis of endoplasmic reticulum followed after 50 hr by the synthesis of crystalline bodies in the cisternae. Aeration in the presence of *p*-fluorophenylalanine (*p*-F-Phe) (4) increases the amount of crystalloid protein formed at the expense of the endoplasmic reticulum because non-functional protein is overproduced from the surplus amino acids [22,23].

Development

Although both *p*-F-Phe and ethionine inhibit the elongation of pea seedling root tips [24], they have no effect on the growth of excised embryonic axes from bean plants [25]. *p*-F-Phe stimulates both the increase in fresh weight of embryonic bean axes and the elongation of *Avena* coleoptiles [25,26]. This stimulatory effect has been attributed to the ability of *p*-F-Phe to inhibit phenylalanine ammonia lyase, an enzyme catalysing the formation of *trans*-cinnamic acid from phenylalanine. Since *trans*-cinnamic acid is a potent inhibitor of coleoptile elongation a decrease in its intracellular concentration causes a relief of the natural growth inhibition [26,27]. Many amino acid analogues inhibit the auxin-induced elongation of plant coleoptiles [28–30]. Although it has been suggested that *p*-F-Phe inhibits auxin-stimulated coleoptile growth by inhibiting the synthesis of proteins required for the auxin effect, there is no decisive evidence for this [31].

The formation of inactive proteins by incorporation of analogues into their polypeptide chains (see below) has led to an assessment of the importance of newly-formed proteins at a number of developmental stages

in the life cycle of a plant. The time taken for *Begonia* tubers to enter into the dormant state is increased by *p*-F-Phe and ethionine. This suggests that the production of specific inhibitors required for the shutdown of the biosynthetic machinery of the plant requires the synthesis of new proteins [32]. In contrast *p*-F-Phe has no effect on the elongation of *Avena* coleoptiles induced by red light, thus suggesting that the biochemical factors responsible for this growth stimulation are already present in the young coleoptile [26]. The same analogue also inhibits the induction of flowering in cocklebur (*Xanthium pennsylvanium*) by interfering with processes occurring in the inductive dark period, but has no effect on vegetative development [33]. Flower and frond production in *Lemna gibba* is inhibited by ethionine although low concentrations of the analogue stimulated the former [34].

The synthesis of chlorophyl *a* and phycocyanin is inhibited by ethionine and *p*-F-Phe when dark grown *Cyanidium caldarium* is exposed to the light, thus indicating that protein synthesis is a prerequisite of pigment synthesis. The enzymic degradation of chlorophyll and protein in excised segments of the mature leaf of *Avena sativa* is inhibited by ornithine and 2,4-diaminobutyric acid. It is possible that the inhibition of enzymes which break down chlorophyll, by ornithine is a mechanism by which the degradation of chlorophyll in the dark is prevented [35].

An increase in the frequency of heterocyst formation is specifically stimulated in the alga *Anabaena catenula* by low concentrations of 7-azatryptophan (**5**). It is possible that the analogue prevents the formation of a natural inhibitor of heterocyst development [36].

Cell division

There is little information regarding the effect of analogues on cell division in plants. In *Chlorella vulgaris* cell division is uncoupled from growth by the addition of selenomethionine to the culture medium [37]. Although the cells continue to increase in size and protein content and exhibit increased oxygen uptake, cell division is prevented. In the rapidly dividing and growing cells of certain legumes canavanine (**6**) appears to interfere with arginine utilisation in the initiation of DNA replication [38]. However in most cases, where the inhibition of cell division by analogues has been studied, it is not certain whether the effect is due to the production of an analogue-containing protein associated with DNA replication or the production of faulty proteins involved in the cell division process itself.

Although anomalous mitoses and meioses have been observed in ethionine-treated animal cells [39], similar observations have yet to be reported in detail for plant tissues, despite the apparent suitability of such material for studying the effects of analogues on chromosome patterns.

Organelles

Enzymes from the cytoplasm and cell organelles which catalyse the same overall reaction, may exhibit a different substrate specificity for analogues. The cytoplasmic arginyl-*t*RNA synthetase from *Phaseolus vulgaris* is inhibited by a concentration of canavanine which still stimulates ATP–PP_i exchange catalysed by the chloroplast enzyme [40]. Protein synthesis on chloroplast ribosomes of *Euglena gracilis* is inhibited by ethionine to a greater extent than on cytoplasmic ribosomes [41]. By the extension of such experiments in which the enzymes from one subcellular compartment are selectively inhibited by an analogue, the role of organelles in the overall metabolism of the cell may be elucidated.

It is possible to study the transport of some metabolites across the nuclear membrane by placing microelectrodes and micropipettes in the cytoplasmic and nuclear compartments of large cells. The effect of amino acid analogues on such a system has been studied using animal cells [42] but has as yet received little attention in plants.

Incorporation of amino acid analogues into proteins

One of the chief mechanisms by which an amino acid analogue may exert its toxic effect is through incorporation into proteins at the sites normally occupied by the amino acid which it mimics. This replacement often leads to the loss or impairment of enzyme activity. The inhibitory action of analogues on the synthesis of 'induced' enzymes in higher plants has been extensively studied. However, care must be taken in interpreting the results because of the possibility that secondary inhibitory reactions of the analogues may be responsible for the decreased synthesis of specific proteins.

Ethionine, A-2-C, *p*-F-Phe and *trans*-4-hydroxyproline inhibit the increase of invertase and acid phosphatase activity in excised pea root segments [43], whilst the *de novo* synthesis of α-amylase in barley endosperm is inhibited by ethionine and norleucine [44].

The level of extractable nitrate reductase rapidly increases in nitrate starved plants fed with nitrate or in molybdenum deficient plants fed with molybdenum in the presence of nitrate. A-2-C inhibits the nitrate 'induced' rise in activity by 86% and the molybdenum induced rise by 54% [45], indicating that a different type of protein synthesis is involved in the two 'induction' reactions. *p*-F-Phe was also shown to completely inhibit the formation of nitrate reductase in barley roots, whilst a non-inducible enzyme, alkaline phosphatase was not effected [46]. No effect on the rate of respiration of the tissue was detected. However, nitrate reductase exhibits a rapid turnover rate and it is not possible to ascertain if the increase in levels of activity are due to changes in the rate of *de novo* synthesis.

By using density labelling techniques with D_2O, Shepard and Thurman [47] were able to show that the ammonia induced increase in glutamate dehydrogenase levels in *Lemna gibba* was due to *de novo* synthesis. *p*-F-Phe and A-2-C prevented this increase in enzyme activity, but as both compounds were found to inhibit respiration in *Lemna*, the authors were rightly sceptical about their precise mode of action.

The rise in level of alcohol dehydrogenase in germinating pea cotyledons is not suppressed by *p*-F-Phe, suggesting that this enzyme is already preformed in the dormant seed. However, the normal decrease in level after the fifth day does not take place in the presence of *p*-F-Phe, indicating that a protein inhibitor is probably synthesised at this time [48]. The wall bound invertase activity increases three-fold when *Convulvulus* callus tissue is transferred to solid medium. This increase is enhanced by the addition of the phenylalanine analogue β-thienylalanine (**7**) [49]. It has been suggested that the analogue is incorporated into a rapidly turning over inhibitor or

repressor molecule [50], thus allowing the apparent level of invertase activity to rise.

The specificity of protein synthesis is controlled by aminoacyl-*t*RNA synthetases, which catalyse a two step reaction:

(a) amino acid + ATP $\overset{Mg^{2+}}{\rightleftharpoons}$ aminoacyl – AMP + PP_i

(b) aminoacyl – AMP + *t*RNA $\rightleftharpoons$ aminoacyl-*t*RNA + AMP

Providing an analogue is able to participate in both these reactions there is usually no further restriction of its ability to become incorporated into protein [51]. The transfer of each of the protein amino acids to its cognate *t*RNA molecule is catalysed by a specific synthetase. These enzymes exhibit a remarkable diversity in their properties, even in closely related species, and their mechanism of action has been studied in higher plants by the use of analogues [52]. In several instances a range of amino acid analogues has been used to investigate the conformational features of the active site of these enzymes [53].

The extent and rate at which an analogue is able to replace an individual amino acid in protein depends on the discriminatory ability of the aminoacyl-*t*RNA synthetases, the level of endogenous amino acid pools and the efficiency of transport of the analogue into the cells. *p*-F-Phe is incorporated into protein of the Cocklebur plant at half the rate of phenylalanine [33], in contrast to the situation for rabbit reticulocytes, where the rate is the same for both compounds [54]. The substitutions most likely to impair the activity of an enzyme are those involving groups at the active site or those which cause a change in the three-dimensional structure of the protein sufficient to alter the shape and size of the active centre [55]. The effect of analogue substitution on enzyme chemistry is an important field for the understanding of structure-function relationships in proteins [6], but has as yet received little attention using purified plant proteins.

Inhibition of protein synthesis

Amino acid analogues may inhibit protein synthesis by five different processes (a) mimicing the normal protein amino acid in both reactions catalysed by aminoacyl-*t*RNA synthetases, hence forming inactive proteins associated with protein synthesis itself; (b) inhibition of one of the two reactions catalysed by synthetases, thereby reducing the transfer of the normal protein amino acid to *t*RNA [56]; (c) acting as a false feedback inhibitor of amino acid biosynthesis (see below), thus depleting the cell of the normal protein amino acid [57]; (d) inhibition of amino acid transport through the cell membrane resulting in a fall in the intracellular concentration of amino acids [58]; (e) depletion of the ATP and GTP levels in the cell [6].

Little work has been reported concerning these problems using plant material. In bacteria and animals some analogues inhibit the synthesis and assembly of ribosomes because they cause sub-methylation of RNA components [59] or become incorporated into ribosomal proteins [60]. 3-Amino-1,2,4-triazole prevents the formation of normal green chloroplasts, possibly by preventing 70S ribosome formation, and thus inhibits the synthesis of the large subunit of fraction 1 protein.

Nucleic acid metabolism

Several amino acids serve as N- and C-donors in the biosynthesis of the purine and pyrimidine precursors of nucleic acids. The amide N of glutamine is utilised in three steps in the biosynthesis of both purines and pyrimidines, whilst aspartic acid is utilised in the second step of pyrimidine synthesis and in several reactions of the purine biosynthetic pathway. The glutamine analogues azaserine (**8**) [62], 6-diazo-5-oxonorleucine (DON) (**9**) [62] and albizziine (**10**) [63], and the aspartic acid analogues hadacidin [64] and alanosine [65], have been used to elucidate these pathways in animals and bacteria but as yet little is known of their action in plants. The occurrence of high concentrations of albizziine (**10**) in a number of species of *Albizzia* [66] and *Acacia* [67] could indicate that the enzymes of purine and pyrimidine biosynthesis in these plants are resistant to the inhibitory actions of this analogue.

Nucleic acid metabolism may be affected by the action of analogues on specific enzymes: *p*-F-Phe inhibits the increase of RNA'ase activity observed in *Avena* leaves during senescence [68]. Canavanine (**6**) inhibits RNA and then DNA synthesis in *Glycine max*; although some of the inhibition may be due to competition with arginine for protein synthesis, the analogue may also have a direct action on RNA synthesis [38].

DNA and several species of RNA contain a proportion of structurally important methylated bases. In animals and microorganisms analogues of methionine have been used to investigate the mechanism and extent of methylation of such bases. Some possible actions of selenomethionine (**11**) in biological systems have been discussed by Shrift [69]. Treatment of sugar beet discs with ethionine results in a decrease in the aminoacylation of leucine to specific *t*RNA [70], thus indicating that the ability of the *t*RNA to be acylated by an amino acid may in some instances be correlated with the degree of methylation of the *t*RNA molecule [71]. However, the general validity of this assumption remains to be tested.

Amino acid biosynthesis

The synthesis of many amino acids in bacteria is regulated by enzyme repression or inhibition [72]. No unequivocal evidence has been produced for the repression of amino acid biosynthetic enzymes in higher plants [73]. Although feedback inhibition (the end product of a pathway binding at an allosteric site of the enzyme catalysing the first step in the sequence and inhibiting its activity) has been reported [1,73]. Such a system is believed to prevent organisms synthesising compounds which require a large amount of energy for their production, in excess of their immediate requirements. It is interesting however that higher plants have an 'overflow' mechanism by which products of a biosynthetic pathway may be broken down by a separate series of reactions [74].

Several amino acid analogues are able to mimic the action of their naturally occurring counterparts by acting as false feedback inhibitors. In this manner an analogue may starve the cell of a particular amino acid and subsequently inhibit cell growth [4,6,75,76].

In *E. coli* anthranilate synthetase, the first enzyme specific to tryptophan biosynthesis, is feedback inhibited by tryptophan, 7-azatryptophan (**5**) and 5-methyltryptophan [4]. Although three of the four remaining enzymes of

bacterial tryptophan biosynthesis are specifically inhibited by tryptophan analogues, in carrot and tobacco tissue culture cells the same specificity of the different steps was not found [77]. A wide range of tryptophan analogues were found to inhibit the growth of tissue culture cells, but the effect could be reversed by indole or anthranilate. Only the enzyme anthranilate synthetase was shown to be inhibited by the analogues [77].

The synthesis of proline has been extensively studied in maize by Oaks and her colleagues [78]. In maize roots exogenously added proline inhibits the formation of proline from ^{14}C-acetate. Azetidine-2-carboxylic acid and 4-hydroxyproline also inhibit the formation of proline. A-2-C was found to greatly reduce the amount of newly synthesised proline incorporated into protein, probably due to its ability to interfere with the attachment of proline to *t*RNA [79]. Hydroxyproline, on the other hand, which has little effect on the formation of prolyl-*t*RNA, was found to be a greater inhibitor of the formation of proline in the soluble fraction. Thus it would appear that in the case of feedback inhibition hydroxyproline is more able to mimic the action of proline than A-2-C.

Acetohydroxyacid synthetase, the first enzyme unique to leucine, isoleucine and valine biosynthesis, has been partially purified from barley [80]. The enzyme is inhibited by leucine and valine and cooperative inhibition was demonstrated in the presence of both amino acids or their analogues. Further studies with analogues of leucine and valine suggested that there were two distinct binding sites on the enzyme where feedback inhibition could take place. [80].

Cell wall synthesis

Plant cell walls contain a number of amino acid residues in various types of linkage. The most important of these is *trans*-4-hydroxyproline linked to arabinose oligosaccharides [81]. The increase in protein-bound hydroxyproline in the cell walls observed after the maximum growth rate of seedlings may be a contributing factor in the cessation of cell elongation. Hydroxyproline is formed by the hydroxylation of protein-bound proline prior to its transportation to the cell wall [82]. A number of proline analogues inhibit the extension growth of pea roots in culture [24] and inhibit amino acid incorporation into protein [43]. However, both *cis* and *trans*-hydroxyproline promote the extension of root segments [83], although neither affect the incorporation of proline into protein [84]. Both isomers probably inhibit the hydroxylation of protein-bound proline thus preventing the formation of the extensive cross linkages in the wall [81] and allowing further cell extension [84]. In contrast, hydroxyproline inhibits the growth of wheat coleoptiles [29] and the auxin-induced elongation of oat coleoptiles [30]. Auxin causes a decrease in wall bound peroxidase, which is prevented by hydroxyproline, a known constituent of peroxidases [81]. It has been suggested amongst several other hypotheses, that auxin can control its own activity by regulating the levels of peroxidase activity, and this could be prevented by hydroxyproline [85].

Amino acid transport

Although amino acid analogues have been of value in distinguishing between multiple membrane transport systems in microorganisms and mammalian cells, little is known about the mechanism by which amino acids are transported into the cells of higher plants. The uptake of an amino acid into a plant cell may be affected by its rate of metabolism after entry. To overcome this problem Shtarkshall *et al.* [86] used the synthetic amino acid α-aminoisobutyric acid, which was not metabolised, to investigate amino acid transport in barley leaves.

Glutamine, but not glutamic acid, is able to support the growth of *Chlorella* due to the inability of the organism to transport the dicarboxylic amino acid across the cell membrane. However the γ-methyl substituted ester of glutamic acid is transported, although the γ-ethyl ester is too bulky to bind to the permease system [87].

Cell suspension cultures of sugar cane have a specific arginine transport system which is poorly inhibited by lysine or canavanine (**6**). However, arginine inhibits the uptake of lysine, and to a lesser extent canavanine, indicating that the uptake of lysine and arginine involve separate transport systems [88]. The uptake of phenylalanine and tyrosine by young seedlings of *Caesalpinia tinctoria* and *Cucumis melo* has been studied using a number of analogues of these amino acids. At least two types of transport systems (with different kinetics) have been postulated for these aromatic amino acids [89]. The uptake of phenylalanine in synchronous cultures of *Chlorella fusca* is inhibited by *p*-F-Phe [90].

Amino acid analogues also affect the uptake of monovalent ions by plant cells. Aerated beet tissue is able to develop a mechanism for absorbing K^+, Na^+ and Cl^-; *p*-F-Phe causes a shortening of the time required for the development of Na^+ and K^+ uptake capabilities but completely prevents the development of Cl^- uptake [23]. Chloride uptake is also inhibited in pea root segments by a number of analogues [43]. The transport of ^{36}Cl and ^{86}Rb in maize and barley roots has been very carefully studied using *p*-F-Phe [46]. The analogue inhibits ion transport across the root without reducing the initial uptake. There was no prevention of movement into the stele, but the site of action apparently lay between the xylem parenchyma and xylem vessels. The authors suggested that a specific carrier protein with a short effective life was involved [46].

Enzyme studies

The substrate specificity and mechanism of action of individual enzymes has been studied in detail with amino acid analogues. The ability of analogues to bind to individual enzymes varies markedly according to the organism concerned. For example in *Brassica* sp [91] *S*-alkyl-L-cysteine lyase has a restricted substrate specificity compared with the same enzyme from *Albizzia lophanta* [92]. An extensively purified enzyme has recently been isolated from *Acacia farnesiana* which is specific for the $-S-CH_2-CHNH_2-COOH$ group, but can carry out the β-elimination reaction on a wide range of substituents [93]. Proline dehydrogenase extracted from wheat germ, peanuts or pumpkins [94,95] is able to utilize 3,4-dehydroproline (**12**) and thiazolidine-4-carboxylic acid (**13**) at similar rates but A-2-C is not a substrate for this enzyme although it is a good substrate for prolyl-*t*RNA synthetase [53,96–98]. Conversely, albizziine (**10**) which is firmly bound to the active site of a number of glutamine-requiring enzymes [62] has little effect on glutaminyl-*t*RNA synthetase [99]. These observations reflect basic differences in the mechanism of substrate binding of amino acids. Glutamate dehydrogenase exhibits a limited substrate specificity; although

threo-γ-fluoroglutamate is a substrate for the beef liver enzyme [100], the slightly more bulky analogue *threo*-γ-hydroxy-glutamate is a poor substrate for the plant enzyme (Lea, unpublished observations).

A comparison of the substrate specificity of a given enzyme isolated from two different fractions of the plant cell may reveal whether or not they are the same protein. The soluble ornithine transaminase isolated from *Cucurbita maxima* cotyledons is inhibited to a greater extent by canavanine (**6**) than is the particulate enzyme [101].

Separate reactions apparently catalysed by the same enzyme can be distinguished by observing the action of analogues on the two reactions. The oxidation of NAD(P)H has been detected with impure enzyme preparations from sycamore cells in the presence of α-oxoglutarate and either asparagine or glutamine. Fowler *et al.* [102] suggested that the asparagine and glutamine dependent reactions are catalysed by the same or similar enzymes. However, in pea roots the glutamamine-dependent reaction is inhibited by azaserine (**8**) and albizziine (**10**) (two analogues known to inhibit the transfer of the amide nitrogen group to an acceptor molecule) [62,63]; no inhibition was detected for the asparagine dependent reaction [103]. Later studies showed that the latter reaction was due to the presence of contaminating aspartate, which was rapidly transaminated to oxaloacetate the substrate of malate dehydrogenase [103].

It has long been assumed that the major point of entry of ammonia into amino acids in plants is via glutamate and the enzyme glutamate dehydrogenase (Reaction (i)) [104].

$$NH_3 + \alpha\text{-oxoglutarate} + NADPH + H^+ \rightleftharpoons \text{Glutamate} + NAD^+ \quad \text{(i)}$$

^{15}N-labelled feeding experiments in blue–green algae [105], green algae [106] and higher plants [107] all suggest that there is an initial synthesis of the amide nitrogen of glutamine (Reaction (ii)) followed by a rapid synthesis of glutamate.

$$\text{Glutamate} + NH_3 + ATP \rightarrow \text{Glutamine} + ADP + P_i \quad \text{(ii)}$$

The enzyme involved in the transfer of the amide nitrogen group to α-oxoglutarate, GOGAT (glutamine(amide):α-oxoglutarate amino-transferase) (Reaction iii), has now been isolated from a wide range of plant sources [103,108–110].

$$\text{Glutamine} + \alpha\text{-oxoglutarate} + 2H \rightarrow 2\,\text{Glutamate} \quad \text{(iii)}$$

The reducing power may be provided from ferredoxin or coenzymes. The enzyme glutamate dehydrogenase is not inhibited by either methionine sulphoximine (an inhibitor of glutamine synthetase) or azaserine and DON (inhibitors of GOGAT) (Lea and Miflin unpublished results).

However the addition of methionine sulphoximine (**3**) causes a build up of ammonia is blue–green algae [105] and in leaves [111], suggesting that glutamine is probably the first product of ammonia assimilation. In *Chlorella* DON causes the level of glutamine and α-oxoglutarate to rise and the level of glutamate to fall [112], suggesting that glutamate is formed from glutamine and α-oxoglutarate and not by the direct amination of α-oxoglutarate. Thus the action of analogues can be used as a third line of *in vivo* evidence, along with ^{15}N labelling data and enzymological studies, that the major route of ammonia assimilation in a variety of plants is through reactions (ii) and (iii) and not by the long established reaction (i).

The stereochemical and charge requirements of an enzyme reaction may often be determined by using amino acid analogues as inhibitors or substrates. Computer analysis of analogue binding constants with the enzyme may be correlated with their three dimensional structure and the geometry of the active site of the enzyme determined [113]. Additional information concerning reaction mechanisms and the nature of groups near the active centres of enzymes may be deduced from the degree of protection which analogues provide against reagents which inhibit specific amino acid residues in the protein [114].

A good example of the use of analogues as probes for the active sites of enzymes has been described for the prolyl-*t*RNA synthetase from *Phaseolus aureus* and *Delonix regia*. The thermodynamic constants obtained by using analogues of proline to protect the enzyme against thermal denaturation in the presence or absence of ATP, suggest that ATP is probably bound to the synthetase prior to the amino acid substrate [79] and that the nucleotide triphosphate causes a conformational change in the enzyme that modifies one of the two proline binding sites (Norris, unpublished results). The protection afforded by several proline analogues against methylene blue mediated photoinactivation and *p*-chloromercuribenzoate inhibition of prolyl-*t*RNA synthetase activity and lack of protection observed by proline analogues lacking a carboxyl groups lends additional support to the hypothesis that histidine and cysteine residues are in close proximity to that part of the proline binding site which is important for aminoacylation [114].

The occurrence of amino acid analogues in phytotoxins produced by plant parasites

The mode of action of some bacterial and fungal toxins may involve interference with the amino acid metabolism of the host plant [115]. The toxic effects of tabtoxin (a β-lactam derivative of threonine) [116] is reversed by glutamine and to a lesser extent by methionine [117]. Methionine sulphoximine (**3**) has similar properties to tabtoxin, giving the characteristic yellow lesions of wildfire disease caused by *Pseudomonas tobaci*. Leaves treated with either tabtoxin or methionine sulphoximine accumulate ammonia; it would thus seem probable that the toxin is exerting its effect by preventing glutamine formation, the primary step in ammonia assimilation [108].

N-Amino substituted derivatives of aspartic acid from *Fusarium oxysporum* [118] and *Aspergillus flavus-oryzae* [119] cause necrosis and severe wilting in certain plants but their mode of action is not known [120]. A close examination of their structures suggests they could well act as analogues of aspartic acid, asparagine, glutamic acid, glutamine or even α-aminoadipic acid.

Tentoxin, a cyclic peptide containing dehydrophenylalanine, causes irreversible chlorosis in a number of seedlings [121]. The toxin apparently interferes with chlorophyll synthesis but also causes the formation of deformed chloroplasts which accumulate starch [122,123].

Strains of *Rhizobium japonicum* which are involved in nitrogen fixation in soybean root nodules synthesise 2-amino-4-(2-amino-3-hydroxypropyl)-*trans*-but-3-enoic acid which induces chlorosis in new leaf growth [124]. The toxin inhibits the enzyme β-cystathionase which

converts cystathionine to homocysteine and thus blocks methionine formation.

Helminthosporum carbonum produces a cyclic peptide containing α-amino-2,3-dehydro-3-methyl pentanoic acid [125], which may be considered as dehydroisoleucine. The toxin inhibits the seedling growth of maize and increases carbon dioxide fixation in the dark [126] and mineral uptake [127]. It is possible that dehydroisoleucine is incorporated into protein in place of isoleucine and alters the properties of enzymes involved in transport across cell membranes.

Pseudomonas phaseolica produces a peptide toxin which causes chlorotic halos in the leaves of bean plants [128]; two amino acids released on hydrolysis remain to be identified. Infected plants have been shown to accumulate ornithine, due to the inhibition of ornithine carbamoyltransferase. The toxic action could also be alleviated by citrulline and arginine, suggesting that the symptoms were probably caused by a prevention of arginine synthesis.

It is of considerable interest that a number of phytotoxins contain potential amino acid analogues either free or bound in small peptides. An understanding of their mode of action is essential, before steps can be taken to prevent their toxic effects.

Amino acid analogues as fungicides, insecticides and herbicides

Apart from a few non-protein amino acids, e.g. γ-methylene glutamine and canavanine, which may act as temporary nitrogen stores, no precise role for these compounds has been suggested. Although it is possible that plants which produce amino acid analogues have an advantage during germination (when the highest levels of analogues are normally found) by preventing fungal and insecticide attack and inhibiting the growth of surrounding plants.

A simple test for the potential use of an analogue is to float wheat leaves previously innoculated with rust on solutions at various concentrations. Canavanine (**6**) at 10 ppm, *p*-F-Phe (**4**) at 200 ppm and ethionine at 100 ppm were found to completely inhibit rust development without apparent phytotoxicity [129]. Two acetylenic amino isolated from *Euphoria longan* inhibited spore germination of a wide variety of fungi. The compounds also protect cucumber from infection by mildew (*Erysiphe cichoracearum*) and broad bean from rust (*Uromyces fabae*). However 2-amino-4-methylhex-5-ynoic acid (**14**) was shown to have considerable phytotoxic action on the two plants at concentrations of 100 μg/ml [8].

Ethionine has been shown to lower the incidence of potato common scab, after the spraying of a 0·2% solution on the leaves [130]. During the early stages of germination of *Acacia farnesiana* there is a strong mercaptan odour at the base of the hypocotyl. It has been suggested that the *S*-alkyl-L-cysteine derivatives, which are broken down at this time by a β-elimination reaction, are used to provide volatile sulphur compounds which prevent fungal attack at the root-stem junction [93].

In Central America, legume seeds are attacked by a variety of bruchid species; certain legume species however are conspicuously free from attack by the southern armyworm *Prodenia eridania* [131]. The seeds were found to contain a number of non-protein amino acids of which canavanine and β-hydroxy-γ-methylglutamic acid were repellent to the larvae, whilst 5-hydroxytryptophan was toxic at low levels but repellent at the high levels found in the seeds of *Griffonia simplicifolia* [132]. The high level of mortality produced by a diet of *Albizzia julibrissin* was attributed to the combined action of albizziine and *S*-(β-carboxymethyl)-cysteine. The resistance to attack of *Mucuna* seeds is probably due to high concentrations of L-3,4-dihydroxyphenylalanine. This compound inhibits tyrosinase, an enzyme required for cuticular hardening [133]. Canavanine inhibits the formation of pupae from larvae of the boll weevil [134], and at a similar stage in the silkworm [135].

The synthetic compound amitrole (3-amino-1,2,4-triazole) has been used extensively as a herbicide; administration causes severe bleaching, chloroplast disruption and inhibition of carotenoid synthesis [136,137]. In bacteria the compound inhibits histidine biosynthesis at the level of the enzyme imidazole glycerol phosphate dehydratase [138], although in plants its mechanism of action seems more complicated [13].

Amino acid analogues are thus a class of compounds which have great potential as selective inhibitors of fungal and insect pathogens of plants. Chemical modification of natural plant products could lead to important advances in this field.

Resistance and adaption to analogues

Resistance. If plants that produce amino acid analogues are to carry out efficient metabolism, they must develop mechanisms to prevent the toxic action of their products. The classic example of such a mechanism is the alteration of the substrate specificity of prolyl-*t*RNA synthetase in plants which produce A-2-C [96,97]. The active site of the enzyme is altered in such a manner that the analogue is not efficiently bound, thus preventing A-2-C from interfering with proline incorporation into protein [53,79,98,114].

2-Amino-4-methylhex-4-enoic acid (**15**) (AMHA) which occurs in high concentrations in the seeds of *Aesculus californica* [139], is a potent analogue of phenylalanine which acts as a substrate for phenylalanyl-*t*RNA isolated from non-producer species of *Aesculus* with a comparable K_m to that of phenylalanine [140]. The enzyme isolated from the producer species catalyses the formation of AMHA-adenylate at a rate of thirty-seven times less than the rate of formation of the phenylalanine-adenylate, and thus AMHA does not seriously inhibit the incorporation of phenylalanine into protein.

Hemerocallis fulva produces *threo*-γ-hydroxyglutamic acid [141] and *Caesalpinia bonduc* seeds contain *erythro*-γ-methylglutamic acid [142]. Both these substituted glutamic acids serve as substrates of glutamyl-*t*RNA synthetase from *Phaseolus aureus*, but the *Hemerocallis fulva* enzyme is unable to utilise any *threo*-substituted derivatives and the *Caesalpinia bonduc* enzyme fails to use any *erythro*-substituted derivatives as substrates. Thus the active sites of glutamyl-*t*RNA synthetases have been altered in such a way as to prevent incorporation of the endogenous analogue into protein [143].

Little information is available concerning possible alterations of other enzymes in analogue-producing plants, especially with regard to the enzymes of amino acid metabolism. Such experiments are urgently required especially in view of the finding that specific aminoacyl-*t*RNA synthetases in some analogue-producing plants appear not to discriminate against their own toxic

Table 1. Plant aminoacyl *t*RNA synthetases which have been studied for their ability to utilize amino acid analogues as substrates

Amino acid	Plant source	Reference
Proline	*Phaseolus aureus* *Polygonatum multiflorum*	96, 97
	Phaseolus aureus *Delonix regia* and other plant sources	53, 79, 98, 114
Phenylalanine	*Phaseolus aureus* *Leucaena leucocephala*	144
	Aesculus sp.	140
	Delonix regia *Caesalpinia tinctoria*	145
Tyrosine	*Phaseolus aureus*	144
	Delonix regia *Caesalpinia tinctoria*	145
Leucine	*Aesculus* sp.	146
Valine	*Aesculus* sp.	146
Arginine	*Phaseolus vulgaris* *Canavalia ensiformis*	147
Lysine	*Canavalia ensiformis*	147
Glutamate	*Phaseolus aureus* *Caesalpinia bonduc* *Hemerocallis fulva*	143
Aspartate	*Phaseolus aureus*	99
Glutamine	*Phaseolus aureus* *Albizziia julibrissin*	99
Asparagine	*Phaseolus aureus* *Vicia sativa*	99

products [99]; a full list of the substrate specificities of plant aminoacyl-*t*RNA synthetases is given in Table 1.

Canavanine inhibited root elongation in *Glycine max*, *Phaseolus aureus* and *Zea mays*, no such action was however detected in the producer species *Canavalia ensiformis* [148]. The authors concluded that the inhibitory action was more than just a direct competitive action with arginine for protein synthesis. Lathyrine and homoarginine inhibited pollen tube growth in a number of plant species, but promoted the growth in the producer species *Lathyrus niger* [149].

Adaption

The selection of bacterial mutants which have developed a mechanism of adaption is usually performed by growing them in the presence of the analogue. Such techniques can be readily used for fungi and algae but until recently could not be used as a mechanism for selection in higher plants, although Widholm has been able to screen 100 000 wheat seedlings, and found five that were resistant to the action of 20 ppm 5-methyltryptophan [150]. With the improvement in methods of growing plants in tissue culture [151] or as protoplast suspensions [152], similar techniques can now be used.

An important mechanism of resistance occurs when the pathway involved in the synthesis of an amino acid is no longer inhibited by the end product. The amino acid thus builds up to much higher levels than normal and is able to compete with the applied analogue. Carrot cells resistant to 5-methyltryptophan have been isolated by Widholm [153], the tryptophan levels were increased from 81 μM in the wild type to 2170 μM in the mutant line. 5-Methyltryptophan normally exerts its toxic action by acting as a false feedback inhibitor of anthranilate synthetase [77], thus starving the cell of tryptophan. The anthranilate synthetase isolated from the mutant cells required much higher concentrations of either tryptophan or 5-methyltryptophan before inhibition occurred [153].

The ability to regenerate whole plants from tissue culture cells, in admittedly only a few species at the present time, allows the possibility of a new style of breeding procedure for economically important crop plants which are resistant to an analogue, and may over-produce one amino acid. Resistance to wildfire disease in tobacco, caused by a bacterial pathogen [117] (see above), has been selected for using methionine sulphoximine, (3) a compound which has a similar mode of action to the natural toxin. Leaves of the regenerated plants did not show the characteristic 'halos' produced by the toxin [154].

The major nutritionally limiting amino acid in cereals is lysine and in legumes is methionine. Both amino acids are synthesized from aspartate [1], and their synthesis is subject to control, possibly by the enzyme aspartokinase [75,155]. The feasibility therefore arises of selection of mutant lines resistant to analogues of lysine and methionine which over-produce these amino acids. Mutants of *Chlorella* resistant to ethionine had seven fold higher levels of free methionine and cysteine. Exogenous methionine had no action on methionine production in the mutant, but prevented synthesis in the wild type [156].

Chaleff and Carlson [157], by mutating rice cells with ethyl methanesulphanate, have selected three lines resistant to *S*-(β-aminoethyl)-cysteine with higher levels of lysine in their free amino acid pools. Similar mutants formed by selecting with α-aminocaprylic acid and *S*-(β-aminoethyl)-cysteine for lysine overproducers, and selenomethionine for methionine overproducers, are being investigated at Rothamsted. Mutants resistant to α-aminocaprylate and 1-6, diaminohexane have higher levels of lysine and other amino acids present in their soluble amino acid pool [158].

Not all mechanisms of resistance, however, are due to the overproduction of the natural amino acid. Mutants of *Chlorella vulgaris*, which are resistant to methionine sulphoximine, have a permease system in which the uptake of the analogue is only 10% of the wild type [158]. Similar alterations in the permeases of carrot tissue culture cells resistant to 5-methyltryptophan have been detected [18]. Occasionally a mutation against an analogue may affect the permeation of more than one amino acid. Resistance to 4-methyltryptophan in *Neurospora crassa* also induces resistance to ethionine and impairs the uptake of leucine and α-aminobutyric acid, thus indicating that the mutation acts via a general permease system [160]. Mutants of *Saccharomyces cerevisiae* are resistant to ethionine as they are able to rapidly break down *S*-adenosylethionine and thus prevent ethylation reactions taking place [161]. In another species of yeast a mutant is able to degrade ethionine to methionine [162].

CONCLUSIONS

Although the effect of analogues on *in vivo* systems may be difficult to interpret due to their action on more than one metabolic pathway, the increasing availability and use of new 'custom built' analogues may provide the means to study various cellular reactions with a greater specificity than hitherto possible.

In higher plant systems, analogues have been extensively used to determine the specificity (and in some cases the mechanism) of individual enzyme reactions, especially those involved in protein synthesis. However, detailed studies concerning how the effect of analogue substitution into the polypeptide chain of a protein causes a modification of its enzymic properties has yet to be attempted. From an extension of the few experiments attempted so far concerning the effect of analogues on cell structure, development, cell wall synthesis, cell permeability and amino acid biosynthesis in higher plants, it may be expected that, given the great variety of analogues, many problems involving the specificity of these cellular reactions may be elucidated. An attempt to use analogues to elucidate differences between the cytoplasmic and organelle-specific enzymes and the interaction between these compartments of the cell would seem to be a feasible proposition.

The mechanism of resistance and adaptation of plants toward amino acid analogues has not been extensively studied and it is by no means certain that changes in the specificity of aminoacyl-*t*RNA synthetases are always involved. Studies on the permease, amino acid biosynthetic and nucleic acid biosynthetic pathways and especially the isolation of altered DNA sequences responsible for resistance are required before a complete picture of the mechanism of resistance may be constructed.

Recent experiments indicate that certain analogues may be used as herbicides, fungicides or insecticides. These results encourage the view that certain analogues may be of agricultural importance in the future.

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REFERENCES

1. Miflin, B. J. (1973) in *Biosynthesis and its Control in Plants* (Milborrow, B. V. ed.), p. 49. Academic Press, New York.
2. Bryan, J. K. (1976) in *Plant Biochemistry* (Bonner, J. and Varner, J. E. eds.), 2nd edition (in press). Academic Press, New York.
3. Richmond, M. H. (1962) *Bacteriol. Rev.* **26,** 398.
4. Fowden, L., Lewis, D. and Tristram, H. (1967) in *Advances in Enzymology* (Nord, F. F. ed.), Vol. 29. p. 89. Academic Press, New York.
5. Nass, G., Poralla, K. and Zahner, H. (1971) *Naturwissenschaften* **58,** 603.
6. Norris, R. D. and Lea, P. J. (1976) *Sci. Prog.* **65,** 6.
7. Fowden, L. (1970) in *Progress in Phytochemistry* (Rheinhold, L. and Liwschitz, Y. eds.), Vol. 2. p. 203. Wiley, London.
8. Fowden, L. (1973) in *Biosynthesis and its Control in Plants* (Milborrow, B. V. ed.) p. 323. Academic Press, London.
9. Leisinger, T., Haas, D. and Hegarty, M. P. (1972) *Biochim. Biophys. Acta* **262,** 214.
10. Dolan, G. and Godin, C. (1966) *Biochemistry* **5,** 922.
11. Dunnill, P. M. and Fowden, L. (1965) *Phytochemistry* **4,** 445.
12. Massini, P. (1963) *Acta Botan. Neerl.* **12,** 64.
13. Frisch, D. M., Dunnill, P. M., Smith, A. and Fowden, L. (1967) *Phytochemistry* **6,** 921.
14. Whiteside, J. A. and Thurman, D. A. (1971) *Planta* **98,** 279.
15. Rahiala, E.-L. (1973) *Acta. Chem. Scand.* **27,** 3861.
16. Vaughan., D. and Cusens, E. (1973) *Planta* **112,** 243.
17. Gray, R. A. and Hendlin, D. (1962) *Plant Physiol.* **37,** 223.
18. Meins, F. and Abrams, M. L. (1972) *Biochim. Biophys. Acta* **266,** 307.
19. Tate, S. S. and Meister, A. (1973) *The Enzymes of Glutazmine Metabolism* (Pruisner, S. and Stadtman, E. R. eds.) p. 77. Academic Press, New York.
20. Norris, W. E. (Jr). and Mangum, H. E. (1970) *Physiol. Plantarum* **23,** 131.
21. Neale, S. and Tristram, H. (1963) *J. Bacteriol.* **85,** 1241.
22. van Stevenick, R. F. M. and van Stevenick, M. E. (1971) *Protoplasma* **73,** 107.
23. van Stevenick, M. E. (1972) *Physiol. Plantarum* **27,** 407.
24. Vaughan, D. and Cusens, E. (1970) *Biochem. J.* **120,** 18p.
25. Walton, D. C. (1966) *Plant Physiol.* **41,** 1549.
26. Hopkins, W. G. and Bonell, K. F. (1969) *Plant Physiol.* **44,** 281.
27. Camm, E. L. and Towers, G. H. N. (1973) *Phytochemistry* **12,** 961.
28. Bonner, J. (1949) *Am. J. Botany* **36,** 323.
29. Schrank, A. R. (1956) *Arch. Biochem. Biophys.* **61,** 348.
30. Cleland, R. (1963) *Nature* **200,** 908.
31. Nooden, L. D. and Thimann, K. V. (1966) *Plant Physiol.* **41,** 157.
32. Esashi, Y. and Leopold., A. C. (1969) *Plant Physiol.* **44,** 1200.
33. Miller, J. and Ross, C. (1966) *Plant Physiol.* **41,** 185.
34. Umemura, K. and Oota, Y. (1965) *Plant Cell Physiol.* **6,** 73.
35. von Abrams, G. J. (1974) *Z. Pflanzenphysiol.* **72,** 410.
36. Mitchison, G. J. and Wilcox, M. (1973) *Nature, New Biol.* **246,** 229.
37. Shrift, A. (1959) *Plant Physiol.* **34,** 505.
38. Weaks, T. E. and Hunt, G. E. (1973) *Physiol. Plantarum* **29,** 421.
39. Herbert, S. and Fournier, E. (1970) *Compt. Rend. Soc. Biol.* **164,** 238.
40. Burkard, G., Guillemaut, P. and Weil, J. H. (1970) *Biochim. Biophys. Acta* **224,** 184.
41. Aaronson, S., Ellenbogen, B. B., Yellen, L. K. and Hutner, S. H. (1967) *Biochem. Biophys. Res. Commun.* **27,** 535.
42. Kohen, E., Siebert, G. and Kohen, C. (1971) *Hoppe-Seylers Z. Physiol. Chem.* **352,** 927.
43. Vaughan, D., DeKock, P. C. and Cusens, E. (1974) *Physiol. Plantarum* **30,** 255.
44. Yomo, H. and Iinuma, I. (1964) *Agr. Biol. Chem.* **28,** 273.
45. Hewitt, E. J. and Smith, T. A. (1974) *Plant Mineral Nutrition*, English University Press, London.
46. Schaefer, N., Wildes, R. A. and Pitman, M. G. (1975) *Aust. J. Plant Physiol.* **2,** 61.
47. Shepard, D. V. and Thurman, D. A. (1973) *Phytochemistry* **12,** 1937.
48. Susuki, Y. and Kyuwa, K. (1972) *Physiol. Plantarum* **27,** 121.
49. Klis, F. M. and Hak, A. (1972) *Physiol. Plantarum* **26,** 364.
50. Tomkins, G. M., Gelehrter, T. D., Granner, D., Martin, D., Samuels, H. and Thompson, B. (1969) *Science N.Y.* **166,** 1474.
51. Chapville, F., Lipmann, F., Ehrenstein, G. von., Weisblum, B., Ray, W. J. and Benzer, S. (1962) *Proc. Natl. Acad. Sci. U.S.* **48,** 1086.
52. Lea, P. J. and Norris, R. D. (1972) *Phytochemistry* **11** 2897.
53. Norris, R. D. and Fowden, L. (1973) *Phytochemistry* **12** 2829.
54. Arnstein, H. R. V. and Richmond, M. H. (1964) *Biochem. J.* **91,** 340.
55. Schlesinger, S. (1968) *J. Biol. Chem.* **243,** 3877.

56. Horowitz, N. H., Fling, M., Feldman, H. M., Pall, M. L. and Froener, S. C. (1970) *Dev. Biol.* **21,** 147.
57. Ezekial, D. H. (1965) *Biochim. Biophys. Acta* **95,** 54.
58. Finerman, G. A. M., Downing, S. and Rosenberg, L. E. (1967) *Biochim. Biophys. Acta.* **135,** 1008.
59. Beaud, G. and Hayes, D. H. (1971) *European J. Biochem.* **18,** 323.
60. Oravec, M. and Sourkes, T. L. (1970) *Biochemistry* **9,** 4458.
61. Bartels, P. G., Matsuda, K., Siegel, A. and Weier, T. E. (1967) *Plant Physiol.* **42,** 736.
62. Buchanan, J. M. (1973) in *The Enzymes of Glutamine Metabolism* (Prusiner, S. and Stadtman, E. R. eds.) p. 387. Academic Press, New York.
63. Horowitz, B. and Meister, A. (1973) in *The Enzymes of Glutamine Metabolism* (Prusiner, S. and Stadtman, E. R. eds.) p. 573. Academic Press, New York.
64. Demain, A. L. (1966) *Nature* **212,** 93.
65. Gale, G. R. and Schmidt, G. B. (1968) *Biochem. Pharmacol.* **17,** 363.
66. Gmelin, R., Strauss, G. and Hasenmaier, G. (1958) *Z. Naturforsch.* **13b,** 252.
67. Seneviratne, A. S. and Fowden, L. (1968) *Phytochemistry* **7,** 1039.
68. Bagi, G. and Farkas, G. L. (1966) *Acta. Biochem. Biophys. Acad. Sci. Hung.* **1,** 329.
69. Shrift, A. (1969) *Ann. Rev. Plant Physiol.* **20,** 475.
70. Stone, B. P., Whitty, C. D. and Cherry, J. H. (1970) *Plant Physiol.* **45,** 636.
71. Pegg, A. E. (1969) *Biochim. Biophys. Acta* **177,** 361.
72. Umbarger, H. E. (1969) *Ann. Rev. Biochem.* **38,** 323.
73. Miflin, B. J. and Cheshire, R. M. (1976) in *International Workshop on Genetic Improvement of Seed Proteins.* National Research Council of U.S.A. (in press).
74. Sodek, L. and Wilson, C. M. (1970) *Arch. Biochem. Biophys.* **140,** 29.
75. Bryan, P. A., Cawley, R. D., Brunner, C. E. and Bryan, J. K. (1970) *Biochem. Biophys. Res. Commun.* **41,** 1211.
76. Martin, R. G. (1963) *J. Biol. Chem.* **238,** 257.
77. Widholm, J. M. (1972) *Biochim. Biophys. Acta.* **261,** 44.
78. Oaks, A., Mitchell, D. J., Barnard, R. A. and Johnson, F. J. (1970) *Can. J. Botany* **48,** 2249.
79. Norris, R. D. and Fowden, L. (1973) *Phytochemistry* **12,** 2109.
80. Miflin, B. J. (1971) *Arch. Biochem. Biophys.* **146,** 542.
81. Lamport, D. T. A. (1970) *Ann. Rev. Plant Physiol.* **21,** 235.
82. Holleman, J. (1967) *Proc. Natl. Acad. Sci. U.S.* **57,** 50.
83. Vaughan, D. (1973) *Planta* **115,** 135.
84. Cleland, R. and Olson, A. C. (1968) *Biochemistry* **7,** 1745.
85. Whitmore, F. W. (1971) *Plant Physiol.* **47,** 169.
86. Shtarkshall, R. A., Reinhold, L. and Harel, H. (1970). *J. Exp. Botany* **21,** 915.
87. Lynch, H. V. and Gilmor, R. G. (1966) *Biochim. Biophys. Acta.* **115,** 253.
88. Pederson, A. G. and Knubsen, G. (1974) *Physiol. Plantarum* **32,** 294.
89. Watson, R. and Fowden, L. (1975) *Phytochemistry* **14,** 1181.
90. Maretski, A. and Thom, M. (1970) *Biochemistry* **9,** 2731.
91. Mazelis, M., Beimer, N. and Crevelling, R. K (1967) *Arch. Biochem. Biophys.* **120,** 371.
92. Schwimmer, S. and Kjaer, A. (1960) *Biochem. Biophys. Acta* **42,** 316.
93. Mazelis, M. and Crevelling, R. K. (1975) *Biochem. J.* **147,** 485.
94. Mazelis, M. and Crevelling, R. K. (1974) *Phytochemistry* **13,** 559.
95. Rena, A. B. and Splittstoester, W. E. (1974) *Physiol. Plantarum* **32,** 127.
96. Peterson, P. J. and Fowden, L. (1963) *Nature* **200,** 148.
97. Peterson, P. J. and Fowden, L. (1965) *Biochem. J.* **97,** 112.
98. Norris, R. D. and Fowden, L. (1972) *Phytochemistry* **11,** 2921.
99. Lea, P. J. and Fowden, L. (1973) *Phytochemistry* **12,** 1903.
100. Unkeles, J. C. and Goldman, P.. (1971) *Mol. Pharmacol.* **7,** 293.
101. Splittstoester, W. E. and Fowden, L. (1973) *Phytochemistry* **12,** 785.
102. Fowler, M. W., Jessup, W. and Sarkissian, L. S. (1974) *FEBS Letters* **46,** 346.
103. Miflin, B. J. and Lea, P. J. (1975) *Biochem. J.* **149,** 403.
104. Lea, P. J. and Thurman, D. A. (1972) *J. Exp. Botany* **23,** 440.
105. Stewart, W. D. P. (1975) *Biochem. Biophys. Res. Commun.* **65,** 846.
106. Baker, J. E. and Thompson, J. F. (1961) *Plant Physiol.* **36,** 208.
107. Lewis, O. A. M. and Pate, J. S. (1973) *J. Exp. Botany* **24,** 596.
108. Lea, P. J. and Miflin, B. J. (1974) *Nature* **251,** 616.
109. Lea, P. J. and Miflin, B. J. (1975) *Biochem. Soc. Trans.* **3,** 381.
110. Lea, P. J. and Miflin, B. J. (1975) *Biochem. Biophys. Res. Commun.* **64,** 856.
111. Braun, A. C. (1955) *Phytopathology* **45,** 659.
112. van der Meulen, P. Y. and Bassham, J. A. (1959) *J. Am. Chem. Soc.* **81,** 2233.
113. Gass, J. D. and Meister, A. (1970) *Biochemistry* **9,** 1380.
114. Norris, R. D. and Fowden, L. (1974) *Phytochemistry* **13,** 1677.
115. Strobel, G. A. (1974) *Ann. Rev. Plant. Physiol.* **25,** 541.
116. Stewart, W. W. (1971) *Nature* **229,** 174.
117. Sinden, S. L. and Durbin, R. D. (1968) *Nature* **219,** 379.
118. Hardegger, E., Liechti, P., Jackman, L. M., Boller, A. and Plattner, P. A. (1963) *Helv. Chim. Acta* **46,** 60.
119. Haenni, A. L., Barbier, M. and Lederer, E. (1965) *Helv. Chim. Acta* **48,** 729.
120. Bousquet, J. F., Vegh, I., Pouteau-thouenot, M. and Barbier, M. (1971) *Ann. Phytopathol.* **3,** 407.
121. Koncewicz, M., Mathiaparanum, P., Uchytil, T. F., Sparapano, L., Tam, J. Rich, D. H. and Durbin, R. D. (1973) *Biochem. Biophys. Res. Commun.* **53,** 653.
122. Halloin, J. M., Dezoeten, G. A., Gaard, G. and Walker, J. C. (1969) *Plant Physiol.* **45,** 310.
123. Arntzen, C. J. (1972) *Biochim. Biophys. Acta* 283, 539.
124. Owens, L. D. and Thompson, J. F. (1972) *J. Chem. Soc. Chem. Commun.* 714.
125. Pringle, R. B. (1971) *Plant Physiol.* **48,** 756.
126. Kuo, M. S., Yoder, O. C. and Scheffer, R. P. (1970) *Phytopathol. Z.* **57,** 29.
127. Yoder, O. C. and Scheffer, R. P. (1973) *Plant Physiol.* **52,** 513.
128. Patil, S. S., Tamm, L. Q. and Sakai, W. S. (1972) *Plant Physiol.* **49,** 803.
129. Samborski, D. J. and Forsyth, F. R. (1960) *Can. J. Botany* **38,** 467.
130. McIntosh, A. H. (1973) *Ann. Appl. Biol.* **73,** 189.
131. Janzen, D. H. (1969) *Evolution* **23,** 1.
132. Rehr, S. S., Bell, E. A., Janzen, D. H. and Feeny, P. P. (1973) *Biochem. Syst.* **1,** 63.
133. Rehr, S. S., Janzen, D. H. and Feeny, P. P. (1973) *Science, N.Y.* **181,** 81.
134. Vanderzant, E. S. and Chremos, J. H. (1971) *Am. Ent. Soc. Am.* **64,** 480.
135. Isogai, A., Murakoshi, S., Suzuki, A. and Tamura, S. (1973) *J. Agr. Chem. Soc. Japan* **47,** 449.
136. Castlefrance, P. and Bisalputra, T. (1967) *Am. J. Botany* **52,** 222.
137. Burns, E. R., Buchanan, G. A. and Carter, M. C. (1971) *Plant Physiol.* **47,** 144.
138. Hilton, J. L. (1969) *J. Agr. Food Chem.* **17,** 182.
139. Fowden, L. and Smith, A. (1968) *Phytochemistry* **7,** 809.
140. Anderson, J. W. and Fowden, L. (1970) *Biochem. J.* **119,** 677.

141. Fowden, L. and Steward, F. C. (1957) *Ann. Bot. N.S.* **21,** 53.
142. Watson, R. and Fowden, L. (1973) *Phytochemistry* **12,** 617.
143. Lea, P. J. and Fowden, L. (1972) *Phytochemistry* **11,** 2129.
144. Smith, I. K. and Fowden, L. (1968) *Phytochemistry* **7,** 1065.
145. Norris, R. D., Watson, R. and Fowden, L. (1975) *Phytochemistry* **14,** 393.
146. Anderson, J. W. and Fowden, L. (1970) *Biochem. J.* **119,** 691.
147. Fowden, L. and Frankton, J. B. (1968) *Phytochemistry* **7,** 1077.
148. Weaks, T. E. and Hunt, G. E. (1974) *Bot. Gaz.* **135,** 45.
149. Simola, L. K. (1967) *Planta* **77,** 287.
150. Singh, M. and Widholm, J. M. (1975) *Crop Sci.* **15,** 79.
151. Street, H. E. (1973) in *Plant Tissue and Cell Culture,* Blackwell, Oxford.
152. Carlson, P. S. (1973) *Proc. Natl. Acad. Sci. U.S.* **70,** 598.
153. Widholm, J. M. (1972) *Biochim. Biophys. Acta* **279,** 48.
154. Carlson, P. S. (1973) *Science, N.Y.* **180,** 1366.
155. Furuhashi, K. and Yatazawa, M. (1970) *Plant Cell. Physiol.* **11,** 569.
156. Sloger, M. and Owens, L. D. (1974) *Plant Physiol.* **53,** 469.
157. Chalef, R. S. and Carlson, P. S. (1975) in *Modification of the Information Content of Plant Cells* (Markham R., Davies, D. R., Hopwood, D. A. and Horne, R. W. eds.) p. 197, North-Holland, Amsterdam.
158. Miflin, B. J., Pallett, K. and Restall, C. unpublished results.
159. Widholm, J. M. (1974) *Plant Sci. Letters* **3,** 323.
160. Lester, G. (1966) *J. Bacteriol.* **91,** 677.
161. Cherest, H., Talbot, G. and deRobichon-Szulmajster, H. (1968) *Biochem. Biophys. Res. Commun.* **32,** 723.
162. Spence, K. D., Parks, L. W. and Shapiro, S. K. (1967) *J. Bacteriol.* **94,** 1531.