ROTHAMSTED RESEARCH

Report for 1974 - Part 1

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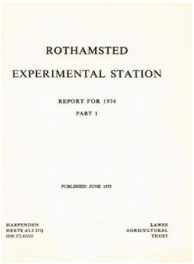

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B. J. Miflin

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Introduction

This year has seen the initiation of several new projects. These deal with plant–fungal relationships, intermediary nitrogen metabolism, factors affecting protein synthesis in developing seeds, and studies with tissue cultures. Few results are as yet available; much time has been spent in planning objectives, establishing methods and doing preliminary experiments.

Many of the results reported below are preliminary, but significant progress has been achieved in certain areas, notably in the discovery of an alternative route of nitrogen assimilation in higher plants.

Host plant pathogen relationships

Modification of potato viruses by o-quinones. The reaction of Potato Virus X (PVX) with enzymically generated *o*-quinones is likely to occur in infected senescing leaves and to produce a modified but infective form of the virus. We have continued to investigate the chemistry of the reaction, and have started to examine other strains of the virus to see if the ability to retain infectivity after modification is related to the ease with which these strains survive and spread in the field.

There are over 1000 protein subunits per particle of PVX, and each contains approximately 11 lysine residues. Previous evidence suggests that one or, at the most, two lysine amino groups per subunit react with chlorogenoquinone. It has now been shown that of the 11 amino groups one reacts more readily than the others with trinitrobenzene sulphonic acid and, contrary to an earlier impression, only one residue reacts with pyridoxal 5′-phosphate (PLP). It is not clear whether it is the same amino group that is most reactive with these three reagents and most accessible to the proteolytic enzyme trypsin. Competition experiments suggest that quinone and PLP react with the same residue; thus, bound quinone more than halves the amount of PLP that reacts with the virus. Moreover, PLP-treated PVX is not split by trypsin, suggesting that the modified amino group is the one recognised by the enzyme. However, other facts, such as the susceptibility of quinone-treated PVX to trypsin, argue that the quinone has reacted with a different amino group. Attempts are being made to resolve this question by

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examining ('mapping') the peptides in tryptic digests of virus modified by the different reagents.

A strain of PVX (KE/WS2) which affects different varieties of potato than does X^N, and which is rarely isolated from the field, also reacts with chlorogenoquinone. It is not inactivated, although its UV spectrum, its fluorescence and its electrophoretic mobility are altered. Attempts are being made to detect its formation in infected leaves. (Ireland, Jewer and Pierpoint)

Plant–fungal relationships. There is evidence that the pathogenicity of different strains of some fungi is correlated with the production of cell wall degrading enzymes. Isolates of *Gaeumannomyces graminis* are being tested to see whether a similar correlation holds for the take-all fungus. The fungus has been grown in liquid culture under various conditions and the extracellular activity of the enzymes polygalacturonase and cellulase determined by viscometric methods. An isolate of *G. graminis* var. *tritici* excreted polygalacturonase in response to the presence of pectin in the growth medium, whereas an isolate of var. *avenae* has so far only produced traces of activity under all conditions tested. An isolate of *Phialophora radicicola*, which may be the imperfect state of a variety of *G. graminis*, produced polygalacturonase as it started to grow, whether or not pectin was present. However, the activity diminished rapidly, particularly in shaken cultures, and at the period of incubation when the var. *tritici* isolate had maximal activity there was none detectable in the culture fluid of the *Phialophora* isolate. A range of other isolates is now being tested.

The polygalacturonase from var. *tritici* has an optimum pH near 5·5 with pectic acid as substrate and is a very stable enzyme.

Preliminary experiments suggest that none of the isolates so far tested excretes significant amounts of an enzyme that will reduce the viscosity of carboxymethyl cellulose solutions (i.e. cellulase).

Parallel studies on the composition of the cell walls of wheat roots have been initiated. Analysis has been made of soluble polysaccharide of week-old coleoptiles and roots; fructosan represented half of this polysaccharide in the coleoptiles but was absent from the roots. (Festenstein and Holden)

Nitrogen metabolism of crop plants

Intermediary nitrogen metabolism. Between applying nitrogen fertiliser to the soil and harvesting the protein in cereal and legume seeds, a range of complex biochemical transformations involving nitrogen occur in the crop plant. The cost of nitrogen fertilisers and the cost of importing protein feeding stuffs for animals require that these processes occur efficiently. The department is concerned in increasing the understanding of these transformations with the eventual aim of developing crop plants and agronomic techniques that maximise nitrogen utilisation.

Entry of nitrogen into organic combinations. The manner in which inorganic nitrogen is incorporated into the organic form as amino acids, prior to movement through the plant and incorporation into proteins, is of primary importance. This route has generally been considered to be via glutamate dehydrogenase, despite many reservations about the suitability of this reaction (see Paper 12). We have now shown that plants have an alternative pathway available in both leaves and roots in which ammonia is incorporated into glutamine via the action of glutamine synthetase, an enzyme showing a much higher affinity for ammonia than glutamate dehydrogenase. Reaction:

$$\text{L-glutamate} + NH_3 + ATP \xrightarrow{Mg^{2+}} \text{L-glutamine} + ADP + Pi$$

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The amido nitrogen thus formed is then transferred by the enzyme glutamine-α-oxoglutarate amino transferase (GOGAT) to α-oxoglutarate:

$$\text{L-glutamine} + \alpha\text{-oxoglutarate} \xrightarrow{(2H)} 2\ \text{L-glutamate}$$

One of the molecules of glutamate produced is recycled to accept another molecule of ammonia, and the second is available for transamination to form other amino acids. GOGAT activity in chloroplasts is unique in that it utilises reduced ferredoxin rather than reduced pyridine nucleotides as the electron donor. The enzyme is also present in roots, where it is also located in the plastids. The enzyme from both sources is specific for glutamine as the amido nitrogen donor and α-oxoglutarate as acceptor. This mode of nitrogen assimilation has the advantage of operating at and maintaining low ammonia concentrations, and it is irreversible. Nitrate is the major form of nitrogen present in the soil. Three of the key enzymes involved in its utilisation, nitrite reductase, glutamine synthetase and GOGAT, are all present in the plastids which suggests that the plastids are important in the synthesis of organic nitrogen. Other workers have found glutamine synthetase and GOGAT in the bacteroids of root nodules in addition to their presence in the root, and it is therefore probable that this pathway is of primary importance in the assimilation of the products of nitrogen fixation.

Route of amides in nitrogen mobility. Glutamine plays an essential role in the transport and transformation of nitrogen compounds in plants. Besides the formation of glutamate, the amido nitrogen of glutamine can be transferred to a wide range of compounds including asparagine (see below). Glutamine and asparagine are the major nitrogen constituents of the xylem fluid of most plants. They are efficient transporters in that they carry two nitrogens per molecule. On arrival in the developing apex or seed, the amido nitrogen must be transferred to keto acids to form amino acids for protein synthesis. Although we have described a glutamine amido nitrogen transferase, as yet little is known about the transfer of the amido nitrogen of asparagine. We are continuing to search for possible enzymic mechanisms.

Asparagine appears to act as a secondary nitrogen store at times of nitrogen excess and of carbohydrate deficiency, and this tends to delay or prevent the build up of high concentrations of ammonia. The mechanism of the synthesis of asparagine has been studied, and a glutamine-dependent asparagine synthetase from lupin seedlings has been partially purified and characterised. This enzyme carries out the following reaction:

$$\text{L-aspartate} + \text{L-glutamine} + \text{ATP} \xrightarrow{Mg^{2+}} \text{L-asparagine} + \text{L-glutamate} + \text{AMP} + PP_i$$

The enzyme is also able to utilise ammonia as the nitrogen donor, although the K_m for glutamine is much lower, indicating that glutamine is the probable physiological substrate. In crude plant extracts the synthesis of asparagine is inhibited by α-oxoglutarate, due to the competition for aspartate as a substrate for aspartate transaminase. Possibly, when the plant has a plentiful supply of carbon, α-keto acids will prevent the formation of asparagine *in vivo*. However, at times of low carbohydrate and high nitrogen levels the keto acids will be converted to amino acids and the glutamine amido nitrogen transferred to asparagine.

Enzymes of amino acid biosynthesis. Work has been concentrated on the synthesis of the aspartate family of amino acids. Attempts to purify and characterise aspartate kinase (the first key enzyme of the pathway) from barley and peas, using the same techniques as developed for maize (Cheshire & Miflin, *Phytochemistry*, in the press) have not yet proved successful, due to very low levels in the tissues. Further attempts will be made using affinity chromatography to improve the isolation of enzymes present in only small amounts. Dihydrodipicolinate synthase activity has been demonstrated in crude

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extracts of a number of plants, and is particularly active in extracts of field bean seedlings. This enzyme, like aspartate kinase, is subject to feedback control by lysine. Diaminopimelate decarboxylase, which catalyses the final step in lysine biosynthesis in plants, is also present and reasonably active. These enzymic studies, together with labelling experiments from other laboratories (Møller, B. L., *Plant Physiology* (1974) **54**, 638) confirm that plants, as well as bacteria, utilise the diaminopimelate pathway of lysine biosynthesis. This is in contrast to fungi, most of which use the α-aminoadipic pathway. Enzymes of the fungal pathway have also been studied with particular attention to saccharopine dehydrogenase, the enzyme responsible for the last reaction in the pathway.

$$\text{saccharopine} + \text{NAD}^+ \rightleftharpoons \text{L-lysine} + \alpha\text{-oxoglutarate} + \text{NADH} + \text{H}^+$$

This enzyme has been extracted and partly purified from the mycelium of the rice blast fungus (*Pyricularia oryzae*). The enzyme reaction is reversible and has two distinct pH optima, 6·8 when assayed in the saccharopine forming direction, and 9·7 for the opposite direction. It is highly specific with respect to substrates and co-enzymes. Substrate analogues inhibit the reaction.

Giovanelli and co-workers (Datko, Giovanelli & Mudd, *Journal of Biological Chemistry* (1973), **249**, 1139) suggested that the pathway to methionine branches from that to threonine and isoleucine after the production of *O*-phosphohomoserine. In methionine synthesis phosphohomoserine condenses with cysteine to form cystathionine, a precursor of methionine. Alternatively phosphohomoserine is cleaved to yield threonine. Studies have been initiated on the formation and utilisation of phosphohomoserine in plants, and attempts were made to synthesise it enzymically from ATP and homoserine, using homoserine kinase prepared from baker's yeast. Initially there was difficulty in reproducing the published methods, probably due to the nature of the supplies of yeast available. However, by rupturing the cells with liquid nitrogen and maintaining the pH at 7·5 the yield of homoserine kinase was improved; such preparations were stable for at least five months. Inclusion of small amounts of NH_4^+ in the reaction mixture increased the formation of phosphohomoserine to >75%. ^{14}C studies show all the radioactivity to be coincident with *O*-phosphohomoserine on TLC plates. Attempts were made to establish the conditions required for affinity chromatography of homoserine kinase from yeast and plant sources. Threonine was coupled to aminopropyl silica gel, using a glutaraldehyde spacer. This complex completely removes all homoserine kinase activity from the yeast preparations, but so far the activity has not been recovered.

Chemical syntheses of *O*-phosphohomoserine have been attempted. The best results were obtained by reacting the anhydrous copper chelate of homoserine with phosphorus oxychloride, removing excess reagent by evaporation at low pressure, followed by hydrolysis with saturated sodium bicarbonate solution. Yields were never better than 25% of theoretical.

Non-protein amino acids in plants. Plants produce many other amino acids than those incorporated into proteins. These amino acids are of interest because, first they are often analogues of the protein amino acids and can be used in probing the biochemical reactions of the latter; secondly they can be used as selection agents for isolating desired mutants (see below); and thirdly the unusual amino acids produced in plants are often toxic to other species of plants, micro-organisms and animals and possibly act as a natural defence mechanism.

The glutamine analogues, albizziine, azaserine and methionine sulphoximine, have been used in studies on the amido-nitrogen transfer reactions mentioned above. Albizziine has been shown to inhibit asparagine synthetase from lupins. However, the enzyme isolated from *Acacia farnesiana* seeds, which contain the analogue in high

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concentrations, is far less susceptible. GOGAT is also inhibited by the analogues and is most sensitive to azaserine, followed by albizziine and methionine sulphoximine.

Recent studies by workers at the Food Research Institute (Mbadiwe & Synge), and in Belgium (Dardenne) (personal communications) have shown that the tropical legume *Pentaclethra macrophylla*, found widely in Zaire, contained a novel amino acid 3(R)-[1′-(S)-amino-carboxymethyl]2-pyrrolidone-5(S)-carboxylic acid. This compound can be split in acid to give 2,5-diamino-3-carboxyadipic acid, a structural analogue of α-amino-adipic acid, which is a key intermediate in the fungal biosynthesis of lysine (see above). In attempts to obtain new analogues, Shewry, who represented Rothamsted on the Zaire River Expedition (supported by the Scientific Exploration Society), has collected a range of seeds and pods from members of the Leguminosae family growing adjacent to the Zaire River. The Upper Shaba region was particularly suitable due to the presence of the Miombo woodland dominated by tree legumes, especially of the genera *Brachystegia*, *Julbernardia* and *Isoberlinia*. These are all members of the subfamily Caesalpinoideae and have not been previously investigated biochemically to any extent.

Recent work in animals has suggested that pyrrolidone-5-carboxylic acid plays an important role in the transport of amino acids. This compound has consistently been reported in plant extracts but is generally considered as an artifact of isolation. Preliminary evidence, suggesting that it might be a normal plant metabolite, has now been obtained: an enzyme capable of catalysing the ATP-dependent hydrolysis of pyrrolidone carboxylic acid to glutamate has been found in field bean seedling extracts. (Fowden, Hill, Lea, Mazelis, Miflin, Pratt, Shewry, Wade and Wallsgrove)

Protein synthesis in cereal seeds. Experiments to establish a range of suitable analytical techniques have been carried out. These have included taking samples of the xylem contents of developing wheat plants; adding amino acids to the xylem stream of developing ears on the plant; feeding radioactive tracers to excised ears; extracting, hydrolysing and analysing the amino acid content of seed proteins; and extracting and analysing the nature of the proteins formed during seed development.

Extraction of protein from whole milled wheat grain. A technique has been established for extracting protein from the grain of individual ears of wheat. Milled grain was shaken with 0·01N-NaOH, at 4°C, for 1 h (20 ml NaOH/0·5 g sample). Of the total N 8% remained insoluble, 82% was precipitable from the NaOH extract by trichloroacetic acid, leaving a final 10% in solution. This was the optimum concentration of NaOH for protein extraction. Lower concentrations left more N insoluble and higher ones increased protein hydrolysis. The addition of detergents did not increase extraction.

The amino acid composition of the NaOH insoluble residue and the TCA-precipitable protein differed considerably. In particular the amount of lysine (as compared to the other amino acids) in the residue was double that found in the extracted protein; however, this is not necessarily nutritionally available.

Comparison of hydrolysis methods for cereal grains. To minimise the formation of humin and the destruction of certain amino acids, samples with a large carbohydrate content are usually prepared for amino acid analysis in a large volume of mineral acid, 1 : 500 being a commonly used ratio. A comparison of hydrolysis methods and the ratio of sample to acid was made to find which offered most advantages.

Different amounts of milled wheat grain (var. Kleiber), ranging from 10 to 500 mg, were treated with redistilled 6N-HCl (sample to acid ratio ranging from 1 : 100 to 1 : 1000) and hydrolysed by refluxing in air, refluxing under nitrogen, heating in a sealed evacuated tube, or heating in a tube filled with nitrogen following evacuation.

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Serine, threonine and lysine seemed unaffected either by the sample to acid ratio or by the method of hydrolysis. The maximum amount of cystine and methionine was found in evacuated tube hydrolysates (at all sample to acid ratios), followed by those refluxed under nitrogen (provided the acid was boiled under nitrogen before adding the sample to remove dissolved air); results from nitrogen-filled tubes were somewhat lower. Destruction of *S*-containing amino acids occurred in air hydrolysates, but surprisingly methionine was more affected than cystine. In evacuated tube hydrolysates methionine and its sulphoxide (in about equal amounts) represent only one-half to two-thirds of the original methionine.

Choice of hydrolysis conditions depends on which amino acid(s) are of most interest. A nitrogen-filled tube should be satisfactory for all except the *S*-amino acids. For the latter, an evacuated tube is still recommended.

Factors affecting protein synthesis in developing seeds. Studies are in progress on the possible effects on protein composition of altering the flow of amino acids to the developing grain by feeding various combinations and contents of amino acids into the top internode of growing wheat plants by means of wicks. Single wheat ears have taken up 4–8 mg nitrogen, as different amino acids, which is equivalent to 10–20% of the final nitrogen content of the grain. The protein derived from ears fed in this manner is now being worked up for analysis.

In collaboration with Talibudeen (Chemistry Department), we have sampled the xylem contents and the seed protein of wheat plants grown in the greenhouse with different levels, and times of application, of N and K fertilisers. This is part of a larger project to investigate the effects of environmental conditions on the quantity and quality of seed protein in cereals, in part supported by the Home Grown Cereals Authority. Some 600 samples of Cappelle wheat and Julia barley, with widely different protein contents, have been taken from Broadbalk and other field experiments and selected samples are being analysed.

In a related project the interaction of sulphur and nitrogen fertilisation on the amino acid composition of wheat proteins has been analysed. Previously the Chemistry Department (see Bolton, *Rothamsted Report for 1973*, Part 1, 51) determined the ratio of straw to grain, and the total N and total S content in each, of wheat (var. Kleiber) grown with differing amounts of N and S fertiliser. Grain from plants receiving both N and S, at all levels, had an S : N ratio of about 0·066, while the ratio in grain from plants receiving only N decreased to 0·032 as applied N increased. Our interest was in the effect of N and S fertilisers on the amino acid composition of whole grain.

Gliadin, one of the storage proteins of wheat, contains more glutamic acid and proline and less lysine than other protein fractions making up the endosperm. Its synthesis is known to be stimulated by applying N fertiliser at the appropriate growing stage. When the seed plants given no sulphur were analysed large amounts of glutamic acid and proline and less lysine were found, as expected, in the plants receiving most nitrogen. However, they also contained around 50% less cystine and methionine than the plants receiving the least nitrogen. Adding S fertiliser at high levels of N restored the methionine and cystine contents, but caused no significant increase at low levels of N.

The number of sulphydryl groups and disulphide bonds present in flour protein is an important factor in bread making. There is evidence that the composition and properties of gluten from S-deficient wheat are different from those of wheat grown with adequate S fertiliser. Because of the large amounts of N fertiliser currently used to increase protein yields in the grain it would now seem necessary to ensure that adequate S is also available.

Samples of barley mutants have been obtained from Risø, and studies of protein and

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amino acid metabolism in the developing grain, particularly in the high lysine mutant 1508, are in progress. (Byers, Kirkman, Lea, Miflin, Pratt and Shewry)

Tissue culture studies

Establishment of cultures. Sterile cultures growing on defined media have been initiated from barley (*Hordeum vulgare*, vars. Julia, Hiproly and Risø 1508), pea (*Pisum sativum*, var. Meteor), field bean (*Vicia faba*, var. Minor), and carrot (*Daucus carota*).

Selection of mutant lines. In bacterial systems strains of bacteria have been selected on the basis of their resistance to analogues of the protein amino acids. On analysis these are often, but not necessarily, found to overproduce the protein amino acid. Strains isolated in this way have been used for the commercial production of amino acids. Similar selection experiments have been initiated using carrot cells, and these will be extended to the other tissue cultures mentioned above. So far lines resistant to the two lysine analogues, *S*-2-aminoethyl-L-cysteine (AEC) and α-aminocaprylic acid (ACA) have been selected. Five cell lines of carrot have been isolated which are resistant to 0·5 mM-AEC. A further 40 resistant lines have been produced after treating carrot cells with 0·15% solutions of the mutagen ethyl methane sulphonate. Two carrot cell lines resistant to ACA have also been isolated. The basis of the resistance in these cell lines is being investigated by amino acid analysis and growth experiments, but so far no evidence of resistance due to the over production of lysine has been obtained. (Bright, Miflin, Pallet and Restall)

Chlorophyll degradation studies

Recent work by McKinney and co-workers (*Agricultural and Food Chemistry* (1973), **21**, 279–281) has suggested that when chlorophyll breaks down during leaf senescence the porphyrin ring may split into fragments with the phytyl side-chain remaining attached to one of them. With a view to investigating this, methods are being developed for determining phytol.

It seemed possible that magnesium might likewise remain attached to one of the fragments of the ring, but work done in collaboration with V. Cosimini showed no evidence for this. The ether-soluble magnesium of fresh leaves of several species corresponded exactly with the chlorophyll content of the fraction as determined spectrophotometrically. When the green pigment disappeared the magnesium and chlorophyll were lost from the ether fraction at the same rate, indicating conversion of magnesium from an organic to an inorganic form. (Holden)

Staff and visiting workers

New staff appointed to the department during the year were S. W. J. Bright (previously at the University of Cambridge), M. A. Kirkman (previously at the Norwegian Agricultural Research Centre), and P. R. Shewry (previously at Westfield College, London). Professor M. Mazelis, from the Department of Food Science and Technology, University of California, Davis, is spending one year in the laboratory. H. M. Davies and R. J. Ireland joined the department as Ph.D. students, supported by the ARC and Potato Marketing Board respectively. During the year P. Jewer, K. E. Pallet and C. Restall worked as sandwich students. Monica Newman replaced Victoria Green as secretary.

B. J. Miflin was invited to give a paper at the 'International Workshop on Genetic Improvement of Seed Proteins' in Washington, USA, in March 1974. P. J. Lea, M. Mazelis and B. J. Miflin gave invited papers at the Phytochemical Society International Symposium on 'Nitrogenous compounds of current interest in plant biochemistry' at the University College of Swansea in September 1974.

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Publications

General Papers

1 Lea, P. J. (1975) Asparagine metabolism in higher plants. *Phytochemistry* **14**, 855.

2 Mazelis, M. (1975) The enzymatic cleavage of the C–S bond of substituted cysteines in higher plants. *Phytochemistry* **14**, 855.

3 Miflin, B. J. (1975) Amino acid biosynthesis in chloroplasts. *Phytochemistry* **14**, 855.

Research Papers

4 Bright, S. W. J. & (Northcote, D. H.) (1974) Protoplast regeneration from normal and bromodeoxyuridine-resistant sycamore callus. *Journal of Cell Science* **16**, 445–463.

5 Byers, M., Nowakowski, T. Z. & Bolton, J. (1974) Effect of replacing potassium by sodium on growth and on inorganic and organic composition of Italian ryegrass. *Journal of the Science of Food and Agriculture* **25**, 271–283.

6 Fowden, L. & (Norris, R. D.) (1974) Cold-lability of prolyl-tRNA synthetase from higher plants. *Phytochemistry* **13**, 1677–1687.

7 Fowden, L., (Wray, J. L. & Bruce, R. E.) (1974) Development of aminoacyl tRNA synthetases in cultured *Nicotiana tabacum* cells. *Phytochemistry* **13**, 697–701.

8 Holden, M. (1974) Chlorophyll degradation products in leaf protein preparations. *Journal of the Science of Food and Agriculture* **25**, 1427–1432.

9 Jervis, L. & Pettit, N. M. (1974) Purification of ribonuclease T_1 on porous glass affinity adsorbents. *Journal of Chromatography* **97**, 33–38.

10 Kirkman, M. A. (1974) Comparative determination of protein amino acids in plant materials by automated cation exchange and gas liquid chromatography of the amino acid N-heptafluorobutyryl, n-propyl esters. *Journal of Chromatography* **97**, 175–191.

11 Lea, P. J. & Miflin, B. J. (1974) Alternative route for nitrogen assimilation in higher plants. *Nature, London* **251**, 614–616.

12 Miflin, B. J. (1974) The location of nitrate reductase and other enzymes related to amino acid biosynthesis in the plastids of roots and leaves. *Plant Physiology* **54**, 550–555.

13 Miflin, B. J. & (Beevers, H.) (1974) Isolation of intact plastids from a range of plant tissues. *Plant Physiology* **53**, 870–874.

14 Pierpoint, W. S. (1974) Chemical modification of the lysine–amino groups of potato virus X. *Journal of General Virology* **25**, 303–312.

15 Shewry, P. R. & (Stobart, A. K.) (1974) Effect of gibberellic acid on sterol production in *Corylus avellana* seeds. *Phytochemistry* **13**, 347–355.

16 Shewry, P. R., (Pinfield, N. J. & Stobart, A. K.) (1974) Effect of gibberellic acid on mevalonate activation in germinating *Corylus avellana* seeds. *Phytochemistry* **13**, 341–346.

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