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Report for 1978 - Part 1

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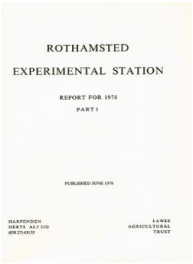

Biochemistry Department

B. J. Miflin

B. J. Miflin (1979) *Biochemistry Department ;* Report For 1978 - Part 1, pp 19 - 33 **- DOI: https://doi.org/10.23637/ERADOC-1-135**

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Introduction

During the year the ARC received funds to initiate a programme on the genetic manipulation of crop plants. The aim of the overall programme, which is spread between four institutes, is to develop techniques that will enable genetic information to be readily transferred between crop plants beyond the restrictions of normal plant breeding. The Department has been asked to take responsibility for about one-third of the programme. This work will be in two areas; one concerned with studies on the isolation, identification and cloning of plant nuclear genes coding for storage-protein synthesis in cereals and the second concerned with the development of techniques which will allow the regeneration of crop plants (particularly barley, wheat, potatoes, and brassicas) from protoplasts. The first area is one in which we are already involved and will mean an expansion of our existing effort on cereal storage proteins in order to accelerate and widen the programme. The second area is one which will be attached to our mutant selection programme because mutual benefits should accrue. Thus protoplasts that can regenerate are a more efficient selection system than hand-dissected embyros and conversely the biochemical mutants already identified are of great potential in selecting the products of fusion between protoplasts of different genetic origin. However, the most serious limitation in the use of protoplasts in selection or genetic manipulation programmes is that regeneration of whole plants in a reliable and reproducible manner is chiefly restricted to tobacco and related species.

The rest of the Department's work has continued on the same programmes as in previous years and is reported below.

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Host/plant pathogen relationships

Virus studies. The aims of this work are to identify the nature and function of novel proteins that are formed in plant leaves in which the spread of invading virus is restricted; and to describe some of the surface features of potato virus X (PVX) that affect its behaviour in senescing infected leaves.

Virus-induced plant proteins associated with resistance. Infection of the leaves of *Nicotiana tabacum* cv. Xanthi-nc with tobacco mosaic virus (TMV) is followed by the appearance of necrotic local lesions at the sites of infection and the virus is restricted to a narrow zone around the lesions. The localisation of the virus is a result of the development of resistance to infection and correlates with the appearance of a number of novel proteins which are 'coded' for by the plant. A procedure for isolating and purifying to homogeneity one of these proteins, b_1, was described previously. This procedure has now been modified to separate and purify a further two proteins, b_2 and b_3, which can be distinguished by their different mobilities during polyacrylamide gel electrophoresis (PAGE) at pH 8·9. The three purified proteins were found to co-migrate as a single band on sodium dodecylsulphate-PAGE at pH 7·2 demonstrating that each consists of a single polypeptide chain of mol. wt. $1{\cdot}5 \times 10^4$. The molecular weights obtained for these proteins by analytical ultracentrifugation were essentially the same as by gel filtration. The electrophoretic and sedimentation behaviour of these three proteins suggested that they may be charge isomers. This was confirmed by analysing the mobility of these proteins in gels containing different amounts of acrylamide using a Hedrick-Smith plot.

Similar proteins have been described in extracts of TMV-infected leaves of the Samsun NN cultivar of tobacco. The purification procedure developed for the b-proteins of Xanthi-nc was also found to purify these proteins. The Samsun NN proteins resemble the b_1, b_2 and b_3 proteins very closely in mol. wt. as determined by SDS-PAGE and ultracentrifugation, and in their behaviour on PAGE and gel filtration. Only the Samsun NN protein corresponding to the b_2 protein was obtained in sufficient amount to determine its amino acid composition and peptide map. The preliminary results indicate that this Samsun NN protein is very similar to the b_1 protein from Xanthi-nc. (Antoniw, Norbury and Pierpoint)

Potato virus X, its structure and modifications. Most strains of PVX contain only one type of protein sub-unit. This can be degraded, while still attached to the virus particle, by the proteolytic enzymes of leaf extracts. An exceptional form of the virus, an isolate X^4, was previously reported by us to contain two types of subunit which could be separated on SDS-PAGE. We now believe that the strain as previously cultured, was a mixture of two separate strains growing in almost equal amounts in leaves of Xanthi-nc tobacco. When purified by repeated passage through single lesions, the virus contains only one type of protein subunit which is unusually resistant to digestion by brief exposure to trypsin. However, it is still uncertain that it is a single, stable strain because some of its properties, such as the amount of chlorogenoquinone that it will bind, vary from one preparation to another.

The disposition of protein subunits in the virus particle has been examined for this and for the X^N strain of PVX by cross-linking them with reagents such as dimethyl suberimidate which cross-link suitably located ϵ-amino groups. At pH 8·5 about half the subunits in a virus preparation are converted to polymers up to octamers, and further treatment with the suberimidate does not increase the extent of polymerisation. At pH 9·5 more subunits (75%) are polymerised and larger polymers, containing at least 12 subunits, are formed. The results so far are best explained if the protein subunits form a close-packed

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array without grouping, and with only a few of the total number of ϵ-NH_2 groups conveniently juxtaposed to form crosslinks between adjacent subunits. Protein subunits isolated from virus particles are more readily crosslinked than are the subunits of intact particles: reaction with other reagents has previously suggested that more of the —NH_2 groups of separated subunits are available for reaction than are those in the intact virus.

A blue form of PVX, PVX-Q_2, is produced when virus is exposed under alkaline conditions to chlorogenic acid which is undergoing enzymic oxidation. Chromatically, PVX-Q_2 resembles the blue and blue-green proteins which have been extracted from leaf material by alkali and described in the literature on a number of occasions. PVX-Q_2 contains two molecules of chlorogenic acid bound per protein subunit, and, judging from its colour reactions contains a quinone group probably conjugated to phenolic OH. The chromophore is probably attached to the lysine ϵ-NH_2 of the proteins, because similar blue colours can be made by reacting chlorogenic acid with lysine which is attached to an inert carbohydrate by its α-amino group. Attempts to extract and isolate the chromophore so that it can be identified have not been successful. (Pierpoint, and Strowman, with Carpenter, Plant Pathology Department)

Fungi of the *Gaeumannomyces-Phialophora* complex

Comparison of isolates. The behaviour of isolates of *Phialophora radicicola* when grown on agar plates containing either roots or leaves of oat seedlings was compared with that previously observed with isolates of the three varieties of *Gaeumannomyces graminis*. *P.r.* var. *radicicola* behaved like *G.g.* var. *tritici* in not growing on either root- or leaf-agar; *P.r.* var. *graminicola* was similar to *G.g.* var. *graminis* in having the growth inhibited on root-agar but growing well on leaf-agar. It has been suggested that *P.r.* var. *radicicola* is the imperfect state of *G.g.* var. *graminis* but this test seems to indicate a clear distinction between them.

Cell-wall degrading enzymes. Several isolates of each of the various types in the complex were grown in liquid media with and without carboxymethylcellulose present. Culture fluids from both *G. graminis* var. *avenae* and *G.g.* var. *graminis* were found to have lower carboxymethylcellulase (CMCase) activity than those from the least active of the isolates of *G.g.* var. *tritici*. *P.r.* var. *radicicola* had higher CMCase activity which was in the same range as found for *G.g.* var. *tritici*. Isolates of *P.r.* var. *graminicola* all had low CMCase activity; these had earlier been found to have very low pectin-degrading activity. Thus the least pathogenic type in the complex seems unable, under the conditions used, to produce more than traces of cell-wall degrading enzymes. (Holden and Ashby)

Nitrogen metabolism

Intermediary nitrogen metabolism

Ammonia assimilation and reassimilation. It has been widely established that the enzymes of nitrite reduction and ammonia assimilation are present in the chloroplast and that the chloroplast can carry out the light-dependent synthesis of glutamate from nitrite and 2-oxoglutarate. What has not been clear is whether all of the enzymes were present in the chloroplast or if other parts of the leaf cell also possessed the capability to reduce nitrite and assimilate ammonia. To answer this question a method of isolating a large proportion of intact organelles from leaf cells has been developed. Pea leaf protoplasts are first produced by digesting pieces of leaf with 1·5% (w/v) cellulysin for 3·5 h. The isolated and purified protoplasts are then ruptured in various buffers appropriate to the enzyme under study and the organelles separated by sucrose density gradient centrifugation. This technique allows the recovery of 80% of the total chlorophyll of the cell

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in intact chloroplasts. It was found that 81 and 80% of the nitrite reductase and glutamate synthase respectively were also recovered in the intact chloroplast whereas only 46% of the glutamine synthetase and less than 7% of the nitrate reductase co-sediment with the chloroplasts. There was no evidence that either of these enzymes was associated with any of the other organelles or with any membrane fraction. The conclusion is therefore that in a pea leaf cell all of the nitrite reductase and glutamate synthase are in the chloroplast, nitrate reductase is in the cytoplasm and that glutamine synthetase is present both in the cytoplasm and the chloroplast.

Studies on the assimilation of ammonia produced from N_2 fixation in *Phaseolus vulgaris* root nodules have continued (in collaboration with Day and Roughley, Soil Microbiology Department). The pattern of development of the activities of nitrogenase, glutamine synthetase, glutamate synthase and glutamate dehydrogenase during nodule development on plants grown under different regimes of nitrate nutrition has been studied through a second season with similar results to last year. The glutamate synthase of the nodule has been considerably purified and its properties studied. Sub-cellular localisation studies with nodule tissue show the enzyme is outside the bacteroid and in the root plastids. However, its properties seem to differ considerably from those of the pea root enzyme described previously, in particular the K_m for 2-oxoglutarate is considerably lower. Further studies on the mechanism of the glutamate synthase reaction, the sub-cellular localisation of the other enzymes and the possible control mechanisms of nitrogen fixation in nodules are in progress.

Besides the primary assimilation of ammonia from that produced by nitrate reduction, nitrogen fixation or ammonium uptake there is now evidence that in certain organs the plant needs to reassimilate considerable quantities of ammonia that have been released from organic combination. During the past year we have been studying two of these systems: those concerned with photorespiration in leaves and with asparagine metabolism in developing seeds.

Leaves of temperate crop plants photorespire at rates of about 80 μmol CO_2 h^{-1} g^{-1} fresh weight (Keys *et al.*, *Journal of Experimental Botany* (1977) **28,** 525–533, Thomas *et al. Ibid.* **29,** 1161–1168). This CO_2 is considered to arise from the decarboxylation of glycine during the conversion of two molecules of glycine to one molecule of serine, CO_2 and ammonia. The rate of ammonia release in photorespiration is thus also likely to be of the order of 80 μmol h^{-1} g^{-1} fresh weight, a value that far exceeds the rate of primary assimilation of ammonia. This release of ammonia would involve the leaf in the rapid loss of all its organic N unless the ammonia is reassimilated and co-operative work with Keys, Bird and Cornelius of the Botany Department was undertaken to study the problem. (See also the Botany Department Report.) The results obtained with isolated leaf mitochondria, which were decarboxylating and deaminating glycine, showed that addition of purified glutamine synthetase led to reassimilation of the ammonia. Such re-assimilation was not obtained when all the reactants necessary for the reductive amination of 2-oxoglutarate by the glutamate dehydrogenase present in the mitochondria, were added. Studies with ^{15}N-glycine fed to intact, detached wheat leaves in the presence and absence of methionine sulphoximine (an inhibitor of glutamine synthetase) confirmed that reassimilation *in vivo* also occurs via glutamine synthetase. A photorespiratory nitrogen cycle has thus been proposed, based on these results and our knowledge of the location of glutamine synthetase and glutamate synthase (reported above), in which the ammonia released from glycine is reassimilated by cytoplasmic glutamine synthetase into glutamine. Glutamine and 2-oxoglutarate move into the chloroplast where, in the presence of ferredoxin-dependent glutamate synthase, two molecules of glutamate are formed. One of these molecules becomes the acceptor for a further released ammonia and the other provides a transamination equivalent for the conversion of glyoxylate to

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glycine. The operation of the cycle would minimise the energy required in the cytoplasm since the major energy-demanding step is directly coupled to the light-dependent reduction of ferredoxin.

In previous years we have reported our investigations on the pathway of asparagine breakdown. Although an asparaginase (which hydrolyses asparagine to aspartate and ammonia) has been isolated from the maturing seeds of *Lupinus polyphyllus* and a few other lupin species it could not be detected in other legumes. We have now isolated an asparaginase from developing pea seeds and discovered that the reason for our previous failure to detect activity is that, unlike the *L. polyphyllus* enzyme, it is completely dependent on the presence of K^+ ions (although Na^+ or Rb^+ can substitute to some extent). The K^+-dependent asparaginase was also found in the developing seeds of a wide range of other plants. The time course of development of the activity in the maturing pea seed has been followed. Asparaginase activity developed initially in the testa of the seed, maximum amounts being present 13 days after flowering. The activity in the cotyledon developed later reaching a peak 21 days after flowering. From the maximum rates of activity observed it was calculated that there was sufficient enzyme activity present to enable the cotyledon to derive all its protein N from asparagine – which isolated cotyledons can do in culture. Therefore, depending on the level of asparagine in the transport stream to the cotyledon, up to half of the N in a cotyledon could be released as ammonia and have to be reassimilated. Assays of glutamine synthetase and glutamate dehydrogenase show that both enzymes are present in the cotyledon during its development although the peak amounts of activity are seen later than that of asparaginase. This may explain the build up of ammonia observed in legume seeds during the early stages of seed development to amounts sufficient for the high K_m of glutamate dehydrogenase not to be a barrier to ammonia reassimilation via that enzyme. However, studies with methionine sulphoximine have suggested that the ammonia from asparagine is reassimilated via glutamine synthetase.

Sites of nitrate reduction and ammonia assimilation. Last year we reported on the composition of the reduced N fraction of the xylem exudates of spring wheat plants grown in the greenhouse. Further analysis have been made of these exudates and some collected over the growing season from field grown Cappelle wheat supplied with different amounts of N fertiliser (with Page and Talibudeen, Soils and Plant Nutrition Department) in order to compare the concentrations of nitrate and reduced N. It has been generally assumed that this ratio reflects the relative amount of incoming nitrate that is reduced in the root compared to that transported to the shoot for reduction in the leaves.

N assimilation in the shoots occurs in the chloroplasts at the expense of light energy and is thus less likely to compete with carbohydrate accumulation than reduction in the root.

However, the results have shown, both in the field and in the greenhouse, that most of the N in the xylem sap is in the reduced form. In the earliest field samplings (4 May, Zadoks' decimal growth stage 30) from plants given 200 kg NO_3^-—N ha^{-1} the ratio of NO_3^-—N to reduced N was 0·13 and this dropped to 0·01 at the latest sampling of the same treatment. The ratios on those plots given less N were generally lower. When uptake of N was assessed through the growing season there was no evidence that it ceased at anthesis and evidence was obtained for continued uptake and transport of N from the roots to the shoot for a major part of the ear-filling period.

Biosynthesis of amino acids. Studies have continued on the enzymology of the synthesis of lysine, threonine and methionine. Previous work has shown that there are several methods for the control of aspartate kinase (the first enzyme in the pathway) including the presence in carrot tissue cultures of two isoenzymes, one sensitive to lysine

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and one to threonine. We have continued to study the aspartate kinase of peas which other workers have shown to be inhibited by threonine when the enzyme is isolated from cotyledons at early stages of germination. We have confirmed this but have found that the enzyme from young leaves is inhibited by both lysine (65%) and by threonine (25%). These inhibitions are separate and additive and are the first indication of a lysine-sensitive aspartate kinase in legumes. The enzyme has also been isolated from developing cotyledons and found to be inhibited only by lysine. The two forms have not been physically separated but it seems likely that there are two isoenzymes present, the proportions of which change according to the physiological state of the plant. As in carrots, the lysine-sensitive activity predominates in rapidly dividing tissues whilst the threonine-sensitive enzyme is found in older tissues.

Studies on the distribution of amino acid biosynthetic enzymes in leaves have shown that several of them are associated with chloroplasts. Although we cannot yet say whether the enzymes studied are present solely in the chloroplast their levels are sufficient for the chloroplast to be a major site of amino acid biosynthesis. Isolated chloroplasts have been fed ^{14}C aspartic acid under conditions in which they are carrying out light-dependent protein synthesis. These chloroplasts catalyse a light-dependent incorporation of ^{14}C into homoserine, lysine, threonine and leucine plus isoleucine present in the soluble pool and into lysine and threonine in protein. Lysine inhibited its own synthesis as well as that of threonine whereas threonine inhibited its own synthesis but not that of lysine. Although both amino acids partially inhibit homoserine synthesis when added separately at 2 mM, together they synergistically completely prevent incorporation of ^{14}C from aspartate into homoserine. These results are thus in good agreement with experiments on isolated aspartate kinase and homoserine dehydrogenase.

The enzymes responsible for lysine synthesis in fungi have been further studied. The final two steps in this pathway, which differs from that of higher plants, involve the conversion of 2-aminoadipic semialdehyde and glutamate to lysine and 2-oxoglutarate via the free intermediate saccharopine. The enzymes have been studied working in the opposite direction but little is known about their activity and properties in the synthetic direction. The enzymes catalysing these two steps have been extracted from *Pyricularia oryzae* and purified and separated by chromatography on Sephadex G-100. Using these preparations and enzymatically synthesised ^{3}H-saccharopine it has been demonstrated unequivocably that the second enzyme is responsible for lysine synthesis as well as breakdown. The properties of this enzyme are now being determined. (Awonaike, Kirkman, Lea, Miflin, Mills, Sodek, Thomson and Wallsgrove)

Protein synthesis in cereal seeds. We are interested in the way in which a cereal seed lays down its protein store and in determining the factors that affect the quantity and quality of that protein. To do this, we are attempting to (1) identify and characterise the major individual protein components of the seed, (2) locate their sites of synthesis and deposition, (3) identify the nuclear DNA and its transcribed mRNA responsible for the synthesis of storage proteins, (4) understand the factors, both genetic and agronomic, that alter the relative distribution of individual proteins in the grain, and (5) relate the properties of the individual proteins to their importance in the utilisation of cereals.

The characterisation of cereal seed proteins. The protein fractions of cereal seeds were classified by Osborne at the beginning of the century into four groups, depending on their extractability by different solvents, and were given the general names albumin (water-soluble), globulin (salt-soluble), prolamin (alcohol-soluble), and glutelin (dilute acid- or alkali-soluble). The different fractions have specific names in different plants: the prolamin of barley is termed hordein and of wheat, gliadin. We have previously reported on

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techniques for the characterisation of hordein from barley; work has continued along similar lines to characterise gliadins from wheat. The effect of protein-lipid interactions on the extractability of N from wheat has received little attention although most workers defat the milled seed or flour before extracting the various protein groups. Ethanol-ether-water (EEW) mixtures or butan-1-ol, either dry or water-saturated, are the most commonly used solvents. Defatting as a routine procedure appears to have arisen because of the better-textured gliadins obtained on drying. Those extracted from un-defatted material are much less easy to handle because of the associated lipid.

There are few quantitative studies on the effect of whole or partial removal of lipid on the subsequent extractability of the proteins. One report says that less N is extracted in the gliadin fraction when defatted wheat is used, and that some of the higher mol. wt. polypeptides found in extracts from undefatted wheat are absent, or present in much smaller amounts. We found that from 2·5–5·0% of the N from whole milled grain was removed by EEW (v/v 2:2:1), twice as much being extracted from one variety (Sappo) than from the other two tested (Flinor and Hobbit). Water-saturated butan-1-ol removed about 2% of the total N from Hobbit, but dry butan-1-ol only 1%.

Quantitatively the most interesting effect produced by defatting wheat was to decrease the amount of N extracted by 0·5M–NaCl at the optimum temperature of 4°C (i.e. the non-protein N, albumin and globulin fractions) from around 35 to 25%. Subsequent treatment with gliadin solvents at the optimum temperature of 60°C extracted about 5% more N than when undefatted wheat was used. This would appear to contradict the results quoted above, but the extraction solvent and conditions were different. The polypeptides in these gliadin fractions from defatted wheat are currently being compared with those extracted from whole milled wheat using SDS-PAGE.

Studies in many other centres have produced suggestive evidence that the baking quality of wheat flours is associated with their content of aggregated high mol. wt. protein. However, these studies have not carefully defined this fraction and it is even uncertain whether it consists of gliadins or glutelins. We have, with the support of the HGCA, initiated a programme to more carefully define this fraction in bio- and physico-chemical terms and to determine which properties are most important for baking quality.

Synthesis and deposition of storage proteins. Cereal seeds have protein bodies which contain the storage proteins. The origin of these organelles is a subject for debate and further information is required in order to understand the process of protein deposition. The relationship of the protein bodies of cereal seeds to those found in other seeds (e.g. legumes) is also not clear. We have therefore studied the development of protein bodies during the maturation of seeds of barley, wheat, maize and pea. Developing seeds were homogenised in sucrose-containing buffers in either the presence or absence of Mg^{++} which respectively leads to the retention or loss of ribosomes on the endoplasmic reticulum (e.r.) and the homogenate subjected to density gradient centrifugation. The distribution of the e.r., as measured by the activity of the marker enzyme NAD(P)H-cytochrome c reductase, and of the storage protein, monitored by SDS-PAGE, has been followed. In barley the main e.r. peak has a density (ρ) of 1·21 when the ribosomes are attached and of 1·19 in the absence of Mg^{++}; there is a secondary peak of activity associated with the protein bodies at $\rho = 1{\cdot}26$. The e.r. from maize is less dense (+ Mg^{++} $\rho = 1{\cdot}15$, − Mg^{++} $\rho = 1{\cdot}10$) but a much greater percentage of the total cytochrome c reductase activity is associated with the protein bodies (ρ 1·25). The e.r. in wheat has densities intermediate between barley and maize and has least e.r. associated with the protein bodies. In peas the density of the e.r. peaks are 1·13 and 1·21 (−Mg^{++} and + Mg^{++} respectively) but none of the e.r. marker enzymes are associated with the protein bodies ($\rho = 1{\cdot}28$). The increased density of the e.r. in barley as compared with

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maize is considered to be due to attachment of storage proteins, a conclusion supported by SDS-PAGE analysis of the e.r. bands.

Proteinase-k, which degrades proteins but does not pass through organelle membranes, has been used to determine whether the protein bodies are surrounded by a semi-permeable membrane. The enzyme completely degraded the protein bodies of barley and wheat but left untouched those of maize and pea. This suggests that the protein bodies in the latter two species are completely surrounded by a semi-permeable membrane, a conclusion supported by electron microscope studies. It is probable that the membrane surrounding the maize protein bodies is that of the endoplasmic reticulum but that in peas is of different origin.

In vitro *protein synthesis.* We have previously found that the polysomes derived from the rough e.r. of developing barley seeds contain the mRNA for several of the hordein polypeptides. The mRNA has been extracted from the polysomes and contaminating protein by treatment with SDS and affinity chromatography on oligo dT-cellulose. This technique selects all those messenger RNAs that have polyadenylate (poly A) sequences attached. The poly A rich fraction has then been translated in a wheat-germ protein-synthesising system and shown to code for the production of polypeptides that are soluble in 50% (v/v) propan-1-ol + 2% 2-mercaptoethanol, precipitated by salt and have similar electrophoretic mobility to authentic hordeins. The characteristics of the wheat germ system have been studied to optimise the translation of the hordein mRNAs. The poly A RNA fraction contains a range of molecules of different sizes around a peak of 18S. Samples of these poly A fractions have been reverse transcribed and double-stranded DNA copies made by Dr. R. Thompson and Dr. R. Flavell at the Plant Breeding Institute, Cambridge.

Genetic analysis of barley storage proteins. The alcohol-soluble storage protein fraction of barley grain (termed hordein) can be separated into three groups of polypeptides ('A', 'B' and 'C') which differ in electrophoretic mobility and molecular weight. Of these the low mol. wt. 'A' hordein has a constant polypeptide composition in all varieties, comprises only 1–2% of the total fraction and is probably not a storage protein. In contrast the 'B' and 'C' groups show considerable varietal differences, in the number, molecular weights and isoelectric points of the component polypeptides. Analysis of the hordein fractions from a large number of varieties suggests that the 'A' and 'B' groups are under the control of closely linked loci. Previous work (Oram *et al.*, *Hereditas* (1975), **80**, 53–58) has shown that the genes controlling the 'B' group of polypeptides are closely linked to the Mla locus on chromosome 5. We have analysed by one-dimensional SDS-PAGE the hordein of F_2 seed of a cross between two varieties with contrasting 'B' and 'C' hordein patterns (Goldfoil and Nielsson Ehle No. 2). The results showed that the 'B' and 'C' groups of polypeptides were controlled by two separate loci 10–16 recombination units apart. Using one-dimensional analysis it is difficult to detect recombination within the two loci. We have therefore used two-dimensional analysis of either single F_2 seed or homozygous doubled monoploid lines produced by the *Hordeum bulbosum* technique. This has confirmed the linkage of the 'B' and 'C' loci but so far failed to show any recombination with them. Our present conclusion is thus that the storage proteins are both coded for by genes on chromosome 5 which are grouped in two complex loci.

The protein fractions of high-lysine barleys. We have investigated the protein fractions of a number of high-lysine barley lines. These include Hiproly and two high-lysine lines (Sv 73608, Sv 76457) derived at Svalof from crosses between Hiproly and the normal varieties Mona and Birgitta. We have also studied mutant high-lysine lines produced at

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Risø, Denmark (Risø mutants 1508 and 56 in the varieties Bomi and Carlsberg II respectively), New Delhi (Notch mutants 1 and 2 in NP 113) and Italy (*lys* mutants 95 and 449 in Perga). The results are summarised in terms of relative amounts because some of the mutations cause drastic reductions in seed size.

In all the high-lysine lines there is a decrease in the relative amount of the lysine-poor hordein fraction and increases in other more lysine-rich fractions. The depression in hordein synthesis is greatest in the lines with larger increase in lysine and least in those with the smallest increase. For example, Risø 1508 has 45–50% more lysine but only 30–35% of the hordein of the parent variety Bomi while the Hiproly-derived lines have 17–20% more lysine than the normal lines Mona and Birgitta and approximately 20% less hordein.

The high-lysine lines differ also in the relative amounts of salt-soluble protein, glutelin protein and non-protein N fractions (all of which are lysine-rich). In Risø 1508, Notch 1 and Notch 2 there are increases in the relative amounts of all three fractions while in *lys* 449 and 95 the effects are mainly on the glutelin and non-protein N fractions with little effect on salt-soluble proteins. In Hiproly and the derived lines there is little effect on non-protein N.

Separation of the salt-soluble protein fractions by iso-electric focusing in the pH range 3·5–10 showed that patterns of *lys* 95, *lys* 449 and Risø 56 were similar to those of the parental varieties while the Notch mutants and Risø 1508 differed. Analysis of endosperms and embryos of Risø 1508 and Bomi showed that the differences were due to changes in endosperm proteins. The polypeptide patterns of the fractions from Sv 73608 and Sv 76457 had some bands derived from Hiproly and others from the normal-lysine parents (Mona and Birgitta).

The reduced and pyridylethylated hordein fractions were separated by two-dimensional isoelectric focusing (pH range 5–9)/SDS-PAGE. In Sv 73608 and Sv 76457 the hordein pattern was similar to Mona while the fractions from the Notch and *lys* mutants showed only minor differences to those from the normal-lysine parental lines. In Risø 1508 and Risø 56, however, the polypeptide patterns were considerably different from the parents. In Risø 1508 the characteristic 'C' hordein pattern present in Bomi was completely absent, although other polypeptides in the same molecular weight range were present. Several major 'B' hordein polypeptides were also absent and the general characteristics of those remaining were altered, with a tendency to form multiple spots. The Risø 1508 hordein also contained relatively large amounts of the low molecular weight 'A' hordein, resulting in an increase in the lysine content of the total fraction from less than 1% to about 2·5%. In Risø 56 there was an increase in the relative amount of 'C' compared to 'B' hordein. Although the polypeptide pattern of the 'C' hordein was not affected, changes did occur in the relative polypeptide composition of the 'B' hordein. This mutation did not affect the relative amount of the 'A' hordein.

The conclusion is that although all the high-lysine lines had decreased levels of hordein there are considerable differences in the effects of the mutations on the amounts and polypeptide composition of the other protein fractions as well as differences in their effects on the polypeptide composition of hordein. This is in agreement with genetic studies at Risø and elsewhere which show that the high-lysine mutations map at different places spread over several chromosomes. Thus it appears that there are a number of mutational events that can occur at different places in the genome that all lead to a depression of hordein synthesis; the exact nature of the changes in the biochemistry of the seed directly caused by the mutations remains unknown.

Immunology of barley hordeins. Hordein fractions separated in various ways were tested against the two active antisera described last year; from rabbits injected with Risø

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1508 hordein, rabbit 74 was injected with firstly a mixture of B polypeptides of about $5{\cdot}1 \times 10^4$ mol. wt. and subsequently total hordein and rabbit 93 with 'A' hordein.

Individual components of Risø 1508 hordein separated by SDS-PAGE were tested by slicing the gels and placing individual slices in Ouchterlony wells along with 0·1% SDS-phosphate buffered saline (PBS) to react with 74 antiserum. Both the major 'B' the polypeptide bands gave strong precipitation lines, the $5{\cdot}1 \times 10^4$ mol. wt. fraction giving stronger reaction. SDS eluates of 'A' hordein and of the two 'B' horde in fractions from both Bomi and Risø 1508 from SDS-PAGE gels were also tested by immuno-diffusion against 74 and 93 antisera. Antiserum 74 gave strong reactions with Bomi 'B' hordeins suggesting that although the 1508 mutation alters the nature of the polypeptides formed as judged by two-dimensional electrophoretic analysis (see above) they still contain antigenic determinants in common with the normal hordein. Antiserum 93 (anti-'A' bands) also reacted to some extent with 'B' hordein but not as strongly. It remains to be determined whether the antibodies to 'A' and 'B' hordeins can be separated, or whether there will always be some cross reaction.

The various fractions obtained by sucrose density gradient centrifugation of cell-free homogenates of developing barley endosperm were tested for their antigenicity. Insoluble pellets produced by centrifuging the endoplasmic reticulum and protein body bands were taken up in 0·1% SDS-PBS and tested against sera 74 and 93. Both fractions gave positive results with 74 but not 93. These results appear to confirm the conclusions made above about the distribution of hordein in the cellular organelles. However, a positive result was also obtained with the supernatant from the homogenate; SDS-PAGE analysis of the immunoprecipitate formed showed the absence of 'A' and 'B' hordein bands but the presence of some high molecular weight material. This suggests that the antiserum 74 also reacts with some non-hordein proteins and requires further purification.

The nutritional status of the plant and the composition of seed proteins. We are interested in the effect of N and S fertiliser treatments on the proteins of wheat and barley because it is known that variation in these nutrients leads to changes in the amino acid composition of the grain, particularly in lysine, cysteine and methionine. All of these amino acids are important in non-ruminant animal nutrition and there is considerable suggestive evidence of a link between S-S bond formation (from two cysteines) and baking quality. In our studies on S nutrition we have used pot-grown plants in which S limits yield and field-grown material showing no evidence of a S limitation. Seed produced in these experiments is being assessed for its baking characteristics.

S-deficient wheat has a much decreased S-amino acid content compared with that of wheat grown with adequate amounts of both N and S, but it is not known if, or how, S-deficiency affects the polypeptide composition of the seed proteins. To obtain grain with a low S content (and the comparable control grain grown with adequate S), wheat cv Sappo was grown on vermiculite using nutrient solutions. N was supplied at two levels; half the plants in each treatment received just enough S to maintain vegetative growth whilst the remainder were given a much larger amount. Average grain yields per plant reflected the treatments. Supplying sufficient S doubled the yield of grain at both levels of N (from 1·43–3·50 and from 2·66–5·32 g per plant at the lower and higher levels of N respectively). The 1000 grain weight from plants receiving high N but little S was considerably lower at 35·4 than from those getting the other treatments (41·1–43·6), in agreement with previous results. The proteins formed in the different seeds are in the process of analysis.

It was previously reported that a negative correlation exists between the S-amino acid content of wheat seed protein and % N content of the grain. Using X-ray fluorescence techniques to measure S we have shown that the ratio of S/N is an alternative indicator

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of S-amino acid content of the protein, and is more easily measured than the methionine and cysteine content. Analysis of a wide range of field grown samples of wheat has given a negative relationship between S/N ratio and % N but suggested that the slope of the relationship is displaced to higher S/N values in seeds from plants which have received single superphosphate (containing S) as compared with triple superphosphate (no S). This observation has been checked in a pot experiment and a replicated field experiment is in progress.

A similar negative correlation of S/N ratio with % N has also been measured in barley. The whole seed and the extracted protein fractions have been analysed for methionine and cysteine to establish the reason for the decreased proportion of S. The results show that the effect is not due simply to an increasing proportion of hordein with increasing N fertiliser since this fraction is not particularly deficient in S-amino acids. When Bomi and its hordein-deficient mutant 1508 (see above) were grown with different levels of N there was no difference in the negative relationship of S/N and % N between the two genetic lines. Similarly, analyses of a wide range of normal and low-hordein barley varieties have failed to show that the low-hordein mutants differed from the normals. (Burgess, Byers, Faulks, Field, Festenstein, Hill, Kirkman, Leggatt, Matthews, Miflin, Parmar, Pearton, Pratt, Shewry and Smith)

Selection of biochemical mutants

For the last few years we have been selecting for biochemical mutants of crop plants which have lost the capacity to regulate the synthesis of certain amino acids, notably lysine and methionine, and consequently produce extra amounts. Initially carrot tissue cultures were used but these have been abandoned in favour of excised embryos of mutated barley. Two selection systems have been used: resistance to S-aminoethyl-cysteine (AEC, an analogue of lysine) and resistance to lysine plus threonine (which inhibits methionine synthesis).

This year's experiment (with Small Plots staff of the Field Experiments Section) on mutagenesis of barley with sodium azide has produced the highest rates of mutation which we have so far obtained, with one plot producing more than 7% of progeny seed with some form of chlorophyll mutation. This quite high rate of mutation should allow more efficient selection of desirable mutations in our biochemical screens.

One AEC-resistant mutant R906 has been extensively characterised. The regulation of lysine biosynthesis by lysine is unchanged in the mutant as is the soluble lysine content of young plantlets. The seeds have the same amino acid and protein content as the parent variety. Purified ^{3}H-AEC has been prepared and used in studies of uptake. Both long (6 day) and short (0·5–5 h) term experiments show that far less AEC is taken up by R906 than its normal parent. We attribute the resistance to a modified root permease system which either fails to take up the inhibitor or transports out what has been taken up. This conclusion is consistent with the genetic analysis of the mutation which has shown it to be due to a single, recessive nuclear gene. Another mutant R2501 has been selected this year which is resistant to lysine and threonine. Methods are being developed to assay the enzymes of the aspartate pathway from single plants to examine the changes in this mutant.

Under the genetic manipulation programme we are expanding this work and are selecting for other resistance mutations. We also intend to increase the range of plants used in the selection programme by taking advantage of regenerating protoplast systems that will be developed in the Department. (Bright, Featherstone, Hill, Kueh, Lea, Leggatt, Miflin, Norbury and Petzing)

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Methodology

Amino acid analyser. The TSM amino acid analyser has continued to be used with one ion-exchange column. The dual wavelength measurement facilities (570 nm and 410 nm) have been changed and both colorimeter channels are now operated at 410 nm but with different electronic sensitivities. This has greatly reduced the need to duplicate amino acid analyses at different sample loadings and now, for most analyses, all amino acids can be measured from a single run. The change to measuring the ninhydrin colour at 410 nm has resulted in little or no loss in sensitivity for all amino acids, including proline, when compared with measurement at 570 nm and 440 nm. Recent problems in the loss of resolution of amino acids have been traced to microbial growth on the ion exchange resin which resisted the operating temperature of 60° and periodic washing with 0·2 M-NaOH solution. Pentachlorophenol and other bactericides are now being incorporated routinely in the buffers used with this instrument.

An AA-1 amino acid analyser was acquired at the end of October from the Soils and Plant Nutrition Department. This has now been adapted for peptide analysis and in particular for peptide 'fingerprinting'. The peptides are separated on a sulphonated polystyrene ion-exchange column in a similar way to amino acid mixtures. The eluate is split into two streams, one stream being hydrolysed with NaOH and the other left unchanged, before reaction with ninhydrin. This system not only indicates the relative time of elution of the peptide but also gives a measure of both the free and the total $-NH_2$ groups in the peptide. This system has now been set up and is being evaluated. (Hill, Leggatt and Smith)

Staff and Visitors

Outside support. The Department gratefully acknowledges the financial support for personnel and materials that have been provided by various organisations including the Home-Grown Cereals Authority, Ciba-Geigy Ltd., Shell Research Ltd. with the SRC, NATO and the EEC. We also thank all those people who have co-operated with us both inside the Station and in many organisations outside.

Visitors. Besides increasing numbers of day visitors the Department was pleased to welcome Dr. C. M. Wilson from the University of Illinois, USA, who is spending 10 months with us, and also Dr. J. S. Wall from Northern Regional Laboratory, Peoria, USA, Mr. R. Altmann from the Justus-Liebig University, Giessen, Germany, Dr. A. Tallberg from Svalof, Sweden, Dr. V. Esanu from the Institute of Virology, Romania, and Dr. L. C. van Loon and Miss C. E. Ritter both of Wageningen, Netherlands, who spent shorter periods learning or exchanging techniques.

Visits abroad. S. W. J. Bright attended the 4th International Tissue Culture Conference in Calgary, Canada, to present a poster demonstration and then visited and gave seminars in several laboratories in the USA.

P. J. Lea gave an invited paper at an International Conference on the Biological Applications of Solar Energy and taught in an associated workshop at the University of Madurai, India.

W. S. Pierpoint gave an invited paper at a meeting of the Group Polyphenols at Nancy, France.

B. J. Miflin and P. R. Shewry gave an invited paper at the FAO/IAEA Seed Protein Conference in Munich and both visited laboratories in Italy. P. R. Shewry also visited and gave seminars at two laboratories in France. B. J. Miflin gave an invited paper at an International Sugar Cane Conference in Nigeria. He also visited Professor J. E. Fox in

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Kansas, USA, to carry out research work and went to various other American Universities to deliver seminars.

Staff. During the year Susan Veck, Maureen Leggatt and Helen Pratt resigned and were replaced by Gillian Watson, Saroj Parmar and Audrey Faulks. Jane Pearton, Joseph Kueh from Nottingham University, and Stephanie Petzing from the Insecticides and Fungicides Department, joined the Department as part of the genetic manipulation team. J. M. Field from St. Andrews University and D. Parkin from Sheffield University joined the Department as post-doctoral fellows supported by outside funds.

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