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ROTHAMSTED RESEARCH

Rothamsted Experimental Station Report for 1984

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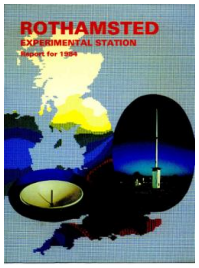

Molecular Sciences Division

B. J. Miflin

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Introduction

Despite the many and various cutbacks in research spending, the area of plant biochemistry has been identified as an important one for research by numerous groups including the Advisory Board for the Research Councils and the Inter-Research Council Committee on Biotechnology. The Department forms a major centre for plant biochemical research within the UK and much of its work relates to objectives defined by

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these committees. We are therefore encouraged to pursue our aims of, firstly, obtaining a detailed understanding of metabolic processes and of the control mechanisms operating at molecular and genetic levels that regulate those processes, and secondly, developing ways of manipulating them to improve crop plants. To do this we are using a blend of traditional biochemical techniques with the increasingly powerful methodology of molecular and cell biology. For the past two years we have concentrated on taking advantage of the reorganization following our merger with part of the Botany Department and it has been a profitable time in terms of results obtained and methodologies mastered. We have found that these have opened up new problems, new opportunities and new applications. We are gratified that the potential of some of these has been recognized by the grant of New Initiative money from both the Plants and Soils and Food Science Committees of the AFRC. These new developments will lead to a reappraisal of our objectives and organization during the coming year. Further encouragement has been gained from the interest shown in our strategic work by private and public representatives of farmers, agrochemical and plant breeding organizations. This has been manifested by the large numbers of visitors to the Department as well as the increasing invitations to members of the Department to speak to audiences outside the normal range of scientific conferences. Selected aspects of the different research areas are reported below.

Ribulose bisphosphate carboxylase

The mechanisms of carboxylation and oxygenation of ribulose bisphosphate catalysed by ribulose bisphosphate carboxylase are being investigated within the AFRC priority programme on photosynthesis. The relative rates of these reactions determines the rates of carbon dioxide fixation and photorespiration in illuminated plants. We seek to increase carboxylation relative to oxygenation in crop species. Natural variation in carboxylases and changes produced by conventional and *in vitro* mutagenesis are of increasing interest in the search for an enzyme with superior properties or with properties that may be interpreted in terms of the mechanisms of catalysis. Studies of the purified enzyme from wheat and other species has continued with significant advances in our understanding of the catalytic mechanism, activation and structure. (Area coordinator: Keys; Burton, Cornelius, Gutteridge, Johnson, Millard, Parry, Schmidt)

Proton NMR studies. Studies using high resolution proton NMR spectroscopy have shown that in the virtual absence of CO_2 and O_2 but in the presence of activated enzyme, exchange of a proton on C-3 of ribulose bisphosphate occurs with $^2H^+$ from ^2H_2O in solution. This is new evidence that the formation of an enediol bisphosphate intermediate occurs prior to reaction with either substrate CO_2 or O_2. It was also observed that during carboxylation, but not during oxygenation, half of the 3-phosphoglycerate molecules formed acquired ^2H at the C-2 position when reaction was in the presence of ^2H_2O. Thus a protonation of the C-2 derived from ribulose bisphosphate by the medium is associated with the formation of the 3-phosphoglycerate containing C from CO_2. (With the National Institute for Medical Research, Mill Hill, London)

Activation of RuBP carboxylase with inorganic phosphate. Ribulose bisphosphate carboxylase has to bind CO_2 as a carbamyl group at lysine residue 201 in the large subunit polypeptide. A divalent metal is also required. We have shown that the amount of catalytic activity per CO_2 bound in the carbamyl group can be varied by the presence of inorganic phosphate. Thus, when only between one and two of the eight active site lysyl residues per molecule of holoenzyme is carbamylated, the addition of 10 mM

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inorganic phosphate increased both oxygenase and carboxylase activities by a factor of four. With between five and six active sites per molecule carbamylated, the addition of inorganic phosphate only doubled activity. We conclude that the potential activity per active site is much higher than was previously proposed and that negative cooperativity between the eight active sites prevents the full potential activity of the enzyme from being exhibited.

Site specific *in vitro* mutagenesis of RuBP carboxylase. During a period of study leave S. Gutteridge with Dr G. H. Lorimer and colleagues of the research laboratories of E. I. Du Pont de Nemours and Company, USA, developed ways of manipulating the structure of RuBP carboxylase using site specific techniques. The aspartyl residue at position 198 in the polypeptide chain of native RuBP carboxylase from *Rhodospirillum rubrum* was changed to a glutamyl residue in a mutated form of the enzyme. This was achieved by substituting a synthetic oligonucleotide for the native sequence in the gene cloned in an expression plasmid constructed by Dr C. R. Somerville and his co-workers (Michigan State University). The carboxylase and oxygenase activities of the mutant carboxylase were decreased by 30% compared to the normal enzyme. EPR spectra of the two enzymes with Mn^{2+} as the divalent metal ion, and in the presence of a transition state analogue to stabilize the binding, showed that residue 198 contributes to the coordination sphere of the metal. Further *in vitro* mutagenesis work in the Department is likely to involve the genes for the cyanobacterial enzyme (which is similar in structure to that of the higher plant). These genes are functionally expressed in *E. coli* albeit presently at a low level.

Photosynthetic carbon and nitrogen metabolism

This research area examines the interaction of photosynthetic carbon and nitrogen metabolism within the leaf. A major aim has been the isolation of mutant plants of barley unable to grow in normal air but able to survive at enhanced CO_2 concentrations. A number of such plants have biochemical lesions in the photorespiratory pathway and offer a means of identifying key steps in photorespiratory metabolism. However, over 40 mutant plants have now been isolated that have no obvious blocks in their ability to assimilate $^{14}CO_2$. The possibility that such mutants are unable to detoxify activated oxygen and hydroxyl radicals is being considered. (Area coordinator: Lea; Hall, Hill, Kendall, Keys, I. K. Smith, Temple, Turner and Wallsgrove)

Characterization of new photorespiratory mutants of barley. A long standing mutant RPr79/2 has now been identified positively as being unable to transport 2-oxoglutarate into the chloroplast. The mutant has very similar properties to that of the ferredoxin dependent glutamate synthase deficient lines RPr82/1 and 82/9 and the leaves contain seven-fold increased concentration of 2-oxoglutarate when exposed to air.

A slow growing plant RPr83/202 has been identified as unable to decarboxylate glycine and thus convert glycine to serine. As yet it has proved impossible to obtain fertile ears from the plant and attempts are being made to promote stem elongation using growth regulators.

One mutant plant (RPr84/51) showed reduced activity of superoxide dismutase after staining on polyacrylamide gels. Quantitative studies confirmed that the leaves of this mutant contained two- to four-fold less superoxide dismutase (expressed per mg protein) than the leaves of the wild type barley.

Glutathione metabolism. The total glutathione content of the leaves of the catalase deficient mutant of barley RPr79/4 increased five- to ten-fold to a maximum of $3\,\mu mol\,g^{-1}$

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fresh weight after growth in air. The increase was accounted for by a rise in oxidized glutathione in both the chloroplast and cytoplasm, the reduced glutathione concentration remained relatively constant. Only 2–3% of applied $^{35}SO_4$ was metabolized to glutathione by wild-type shoots, whilst this value rose to 32% in the leaves of RPr79/4 exposed to air. We have proposed that glutathione synthesis is feedback regulated by the amount of the reduced form but is insensitive to oxidized glutathione (e.g. that produced by H_2O_2).

Chlorophyll *b* mutants. Two barley plants containing very low levels of chlorophyll *b* have been isolated (R.Chl 46 and 47). The chloroplast membranes of both mutants are deficient in the peptides associated with the light-harvesting, chlorophyll *a*/*b* binding complex. The granal stacking of the membranes within the chloroplast of the two mutants is greatly reduced (with Dr Mary L. Parker, PBI).

Glycollate oxidase. The quaternary structure of glycollate oxidase differs according to the plant from which it is extracted. The enzyme from wheat, spinach, barley and pea has an M_r of 160–180 000 (tetramer) whereas from maize and sugarcane the M_r is 290–310 000 (octamer).

Metabolic regulation

The regulation of agriculturally significant pathways can be studied using mutants. Selection and characterization of biochemical mutants particularly in nitrogen metabolism gives a way both to understand metabolism and, in some cases change it in directed ways. (Area coordinator: Bright; de Bry, Franklin, Hill, John, Karp, Kueh, Norbury, Risiott, Steven, Wallsgrove)

Aspartate pathway and branched chain amino acids. Lysine, threonine and methionine are nutritionally important amino acids deriving their carbon skeletons from aspartic acid; cereal mutants accumulating these amino acids should have increased nutritional quality. The R2506 mutant resistant to lysine plus threonine accumulates exceptionally high amounts of soluble threonine in grains as well as lysine to a lesser extent. The R2506 mutation, giving rise to an aspartate kinase isoenzyme III with decreased feedback sensitivity to lysine, is at the same genetic locus as the R3004 mutation and the R1t8 mutation (selected in the group of Prof. M. Jacobs, Brussels). Dihydrodipicolinic acid synthase from normal barley has been characterized; so far no barley mutants have been identified at this enzyme in spite of considerable effort. Ethionine and selenomethionine are being used to select resistant cell lines and plants of *Nicotiana plumbaginifolia* in an effort to find mutants in the enzymes of methionine metabolism. The effects of D and L analogues of methionine are also being studied.

Isoleucine, leucine and valine share four common biosynthetic enzymes and their carbon skeletons are derived from threonine or pyruvate. Two auxotrophic cell lines of *N. plumbaginifolia* requiring isoleucine lack threonine dehydratase as measured by enzyme assay and precursor feeding. The enzyme lesions in other auxotrophic lines requiring threonine, isoleucine plus valine or methionine are being established. Acetolactate synthase in a valine-resistant tobacco mutant has altered feedback sensitivity to valine plus leucine, two other mutants are being investigated.

Nitrate reductase. Six chlorate-resistant barley mutants selected at Rothamsted are being characterized by biochemical and genetic tests. Nitrite reductase and nitrate uptake systems are functional in all mutants as well as various degrees of cytochrome-c reductase

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and other partial activities of nitrate reductase. Four mutants are defective in synthesis or assembly of the molybdenum-containing cofactor. They contain very low or zero nitrate reductase and are unable to grow with nitrate as nitrogen source. In these mutants nitrate is toxic. At least three genetic loci are involved. Provision of unphysiologically high amounts of molybdenum did not correct the growth of plants of R9401 or R11301. However, large amounts of molybdenum supplied *in vitro* restored activity in extracts of R11301, but not in those of R9041 to allow functional enzyme production in the presence of apoprotein from *Neurospora crassa* (with Dr J. L. Wray, University of St Andrews)

Cultivated cereal caryopses. Culture of isolated cereal caryopses would allow the study of metabolic pathways during endosperm development. Cultured wheat (20–30 mg) and barley caryopses (5–30 mg) increased considerably in fresh and dry weight although not to as great an extent as on the plant. The effects of standard growth regulating chemicals on the growth of cultured grains were examined. In preliminary tests, growth of barley caryopses was inhibited by atrazine. (In conjunction with Dr M. Evans, ICI)

Plant cell biology

In this part of the genetic manipulation programme a major aim is to develop efficient systems for the regeneration of important UK crop plants from isolated protoplasts, cells or tissues by *in vitro* culture. Following the introduction or modification of genetic information into individual cells, the systems can be used to produce genetically modified plants that can be characterized to evaluate potential applications to agriculture. Genetic information is being introduced using *Agrobacterium* spp. or their engineered derivatives as vectors, and via protoplast fusion; and changes induced by culture itself are being evaluated. (Area coordinator: M. G. K. Jones; Bains, Bossen, de Both, Bright, Darrell, Fish, Foulger, Karp, Kueh, Maddock, Magan, Ooms, Perret, Pfisterer, Quayle, Risiott, Roberts, Tempelaar, Twell)

Cereals. Studies have centred on analysis of somaclonal variation in regenerated plants, on cereal lectins and on isolation and culture of protoplasts. Field trials (at Nickerson RPB Ltd) on the second progeny generation (R3) of 307 wheat plants regenerated from immature tissues have led to the identification of several lines which show stable differences in height. Similarly, 44 R2 regenerants, which have been characterized cytogenetically (and include aneuploids and plants with structural changes), have been screened in the field. Chromosome mutations involving storage protein loci in regenerants have been analysed. One interesting variant has been identified. Seeds from 600 regenerants have been examined by gel electrophoresis for changes in the gliadin fraction of the storage proteins.

Potato. Improvements in the efficiency of culture of protoplasts of three major cultivars (Maris Piper, King Edward and Désirée) have been made. Structural chromosome aberrations have been identified in protoplast-derived regenerants.

On average, 67% of regenerated cv. Majestic plants had the normal euploid chromosome number (2n=4x=48), an improvement over earlier results (30–50% normal). In continued analysis of somaclonal variants (with Dr N. Evans, Northern Ireland Plant Breeding Station), improved scab scores of Désirée regenerants have been maintained for a third year in replicated field trials and several lines have been incorporated into breeders trials. A white tubered variant passed on this trait to its progeny after selfing, indicating a genetic basis for the change.

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Protoplast fusion. The transfer of a defined gene conferring resistance to the amino acid analogue aminoethylcysteine (Aec) from *Nicotiana sylvestris* into potato by protoplast fusion has been examined in detail following chemical fusion of protoplasts. Plants have been regenerated from heterokaryons following fusion of complete viable protoplasts, or after irradiation of the Aec resistant donor. The analysis so far indicates that with low or zero irradiation doses of the donor *N. sylvestris* protoplasts, elimination of potato chromosomes occurs during culture. The possible reversal of this situation by higher irradiation (100 krad) of the donor is being examined.

The technique of electrofusion of plant protoplasts has been studied in depth, and principles that can be used to direct the production of one-to-one heterokaryons described. The electrofusion approach has been scaled up for mass fusion of protoplasts. Viable cells have been obtained that divided, formed callus and regenerated shoots. The extension of electrical techniques to produce pores through which particles and DNA may be taken up is being examined.

Agrobacterium. The usefulness of *Agrobacterium* as a vector to introduce foreign genes into cells of agronomically important crop plants followed by the regeneration of whole plants has been extended. The crops involved have been chiefly potato and rape but exploratory work has been done with a wide range of species (sugar beet, beans, forage legumes and some cereals). Transformed derivatives have been regenerated routinely using shoot-inducing, root-inducing and disarmed *Agrobacterium* strains for a number of potato cultivars. Field tests of potato plants transformed with natural Ri T-DNA were probably the first in the world to test the stability of expression, under natural conditions, of genes introduced in this way. Variation in expression and tissue specific expression of introduced genes has been studied by steady state RNA analysis of potato plants transformed by Ti T-DNA and Ri T-DNA. Similarly, the first plant derivatives of oilseed rape transformed using *Agrobacterium rhizogenes* have been regenerated.

Disease resistance

Studies on the response of plants to virus infection have continued. They show that part of this response is the production of large amounts of pathogenesis-related (PR) proteins whose appearance is closely associated with restriction of virus spread. The formation of these proteins is also induced by certain chemicals and the effects of various compounds, including interferon, on virus infection has been studied. *In vivo* labelling studies show that at least part of the increase in PR proteins is due to *de novo* synthesis.

Besides completing the description of the response we are also attempting to identify proteins involved in the initial interaction between virus and host. (Area coordinator: Miflin; Antoniw, Burrell, Buxton, Ooms, Pierpoint, with White, Plant Pathology Department)

Interferon. Bawden and Pirie (*Journal of General Microbiology* (1959) **21,** 438–456) observed that crude preparations of chick interferon decreased the infectivity of TMV nucleic acid, and more recently Orchansky, Rubenstein and Sela (*Proceedings of the National Academy of Sciences* (1982) **79,** 2278–2280) claimed that human leukocyte interferon reduced the accumulation of TMV in systematically infected leaf discs of *N. tabacum* cv. Samsun NN. We re-examined these claims using purified human α interferon (sp. activity 10^7–10^8 units mg^{-1} protein) provided by Drs P. J. Cayley and I. M. Kerr at the Imperial Cancer Research Fund Laboratories. We found no significant effect of purified human α interferon at 0·1–1000 units ml^{-1} on either TMV accumulation or infectivity in leaf discs of cv. Samsun. Similarly no effect was observed on TMV

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infectivity when TMV RNA was incubated for one hour at 20°C with human α interferon at 100–10 000 units ml^{-1}. Furthermore injection of 1000 units ml^{-1} human α interferon into leaves of cv. Xanthi-nc caused no significant change in the number of lesions produced by inoculation with TMV. Thus our experiments showed no significant effects of human α interferon on either TMV infectivity or its systemic accumulation in tobacco.

Pathogenesis-related proteins

Immunological studies. Antisera to the protein and TMV were used in F(ab′)2 enzyme linked immunosorbent assays (ELISA) to monitor PR protein and TMV concentrations respectively during the first six days of a systemic TMV infection (cv. Xanthi) and a localized TMV infection (cv. Xanthi-nc).

TMV was detectable in the systemically infected cv. Xanthi two days after inoculation but the largest increase occurred after day 4 and by day 6 had reached the equivalent of 1 mg TMV g^{-1} fresh weight of leaf. There were small amounts of PR proteins in healthy Xanthi leaves which did not change. In the localized infection of Xanthi-nc TMV reached only 1/25th of the amount attained in the systemic infection of cv. Xanthi. A detectable increase in PR protein was found at day 2 but the main increase was from day 3 onwards reaching 20 $\mu g\, g^{-1}$ fresh weight of leaf: a 20 000-fold increase over healthy Xanthi-nc leaves. The largest increase in PR protein occurred after necrotic lesion appearance (two to three days) suggesting that PR proteins are not involved in the initial collapse of the lesion, but significantly PR proteins reached a maximum during the time when growth of the lesion was being restricted.

Other PR proteins in virus-infected leaves. Extracts of hypersensitively responding tobacco leaves contain seven protein species (N–R′) which migrate slowly on gel electrophoresis. They resemble the four well characterized PR proteins (PR-Ia,b,c and PR-II; or b_1, b_2, b_3 and b_4) in a number of respects and are, for instance, induced in flowering senescent plants and are readily extracted at low pHs. They differ in other respects however, and are less evident in callus tissue cultured *in vitro*. Component N may be a 'charge-isomer' of PR-II (b_4) but the electrophoretic behaviour of O–R′ does not suggest a close relationship to PR-I (b_1) and II (b_4). Direct attempts have not yet demonstrated a functional role for these proteins against bacterial or viral pathogens. Crude extracts as well as purified preparations have been tested in collaborating laboratories and found to have no significant agglutinating activity against either *Pseudomonas solanacearum* or *Erwinia amylovora*, and not to restrict the growth and final size of lesions resulting from inoculation with TMV. No evidence could be obtained for an interaction of any PR protein with the coat-protein of TMV: coat-protein bound on to an immobilized matrix so that it retains its immunological properties, does not absorb measurable amounts of any of them from an extract of infected leaves. However the immobilized protein did absorb a number of proteins from extracts of healthy leaves, and the nature and specificity of these interactions is being examined.

Cereal seed proteins

The seed protein group is studying various aspects of the chemistry, immunology, genetics, deposition and regulation of synthesis of cereal seed proteins. The major storage proteins in the seed of barley, rye and wheat are alcohol-soluble prolamins. These determine the poor nutritional quality of the grain and, in the case of wheat, are the major components of gluten which is largely responsible for the baking properties of dough. It is considered that the information provided by this research will facilitate the

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improvement of grain quality, whether by conventional plant breeding, genetic manipulation or, in the case of the baking quality of wheat, by chemical modification of the gluten proteins. Two selected aspects of this work are described below. (Area coordinator: Shewry; Bunce, Burgess, Byers, Festenstein, Fox, Franklin, Henderson, Karp, Makin, Parmar, S. J. Smith, Tatham)

The chromosomal locations and linkage relationships of the structural genes for rye storage proteins. Rye is, to a large extent, intermediate between barley and wheat in its breadmaking properties. We are therefore making a comparative study of the proteins of the three cereals. Previous studies have shown that rye prolamins (secalins) can be classified into four groups which are related to groups of prolamins present in barley and wheat.

Analysis of the seed proteins of lines of wheat with added chromosomes of rye, and of F_2 grain from crosses between different genotypes of rye, have enabled us to designate three structural loci for secalins in cultivated rye *(Secale cereale)*. *Sec 1* is located on the short arm of chromosome 1R and encodes the ω-secalins, and possibly also the M_r 40 000 γ-secalins. *Sec 3,* located on the long arm of the same chromosome (57·4±11·30 cM from *Sec 1*) encodes the high molecular weight (HMW) secalins. Loci encoding related proteins are present in similar positions on the homoeologous (homologous but non-pairing) chromosomes of wheat and barley. A third locus, *Sec 2,* is present on the short arm of chromosome 2R and encodes the M_r 75 000 γ-secalins. In contrast, this locus is found on chromosome 6R, not 2R, in the primitive wild rye *S. montanum*. This is consistent with the reported presence of a translocation between chromosomes 2R and 6R of cultivated rye, and indicates that *Sec 2* may be homologous with the *Gli 2* loci present on the group 6 chromosomes of wheat.

The conformations of cereal prolamins. As part of our studies of the structure and functionality of wheat gluten we have determined the secondary structures of gluten proteins and related proteins of barley using a combination of circular dichroism spectroscopy and computer prediction from amino acid sequences.

The high molecular weight (HMW) gluten subunits, which have been implicated as determinants of baking quality have a domain structure with a long central domain consisting of tandemly and interspersed repeated blocks of six and nine residues. These repeats probably form regularly repetitive β-turns. Short non-repetitive domains at the N- and C-terminals are α-helical, and contain the only cysteine residues so far detected. On the basis of these results we have proposed that the HMW subunits form elastic polymers with a similar structure to the mammalian connective tissue elastin. The β-turns in the repetitive domain form a β-spiral which is intrinsically elastic. The individual subunits are assembled into polymers by disulphide bonds between the cysteine residues in the α-helical N- and C-terminal regions. The HMW subunits, therefore, fulfil the two major structural requirements for an elastomeric material: intrinsically elastic monomers assembled into large polymers by covalent cross-links.

The S-poor ω-gliadins of wheat and 'C' hordein of barley also have a repetitive block structure which results in the presence of regularly repeated β-turns. Although the β-turns have lower predicted probabilities than in the HMW subunits, they appear to be present throughout the polypeptide with no regions of α-helix or β-sheet. These proteins do not, however, contain cysteine, and are unlikely to contribute to an elastic polymer.

The S-rich prolamins have a more complex structure, with a repetitive domain close to the N-terminus. This domain is also rich in β-turns, but these are distributed irregularly and would not be expected to form an elastic β-spiral. Other non-repetitive domains are rich in α-helix. The overall contributions of α-helix, β-sheet and β-turns to the

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conformations of S-rich α-, β- and γ-gliadins are about 33–35%, 8–20% and 30–35% respectively (determined in 70% (v/v) aq. ethanol at 20°C. The α-, β- and γ-gliadins are monomeric with intramolecular disulphide bonds. The polymeric S-rich low molecular subunits of glutenin probably have similar conformations.

Gene isolation and expression

The major activity is in genes expressed in the cereal endosperm and this year two genomic clones have been sequenced and several cDNA clones identified. We have extended our interest outside of the major storage proteins to include, for example, clones containing sequences related to the 'high-lysine' chymotrypsin inhibitors of barley. This work has provided much fundamental information on the nature of cereal seed proteins and their evolution, some of which may be used for applied ends (e.g. explaining their role in breadmaking—see above). In future more emphasis will be placed on the expression of these genes and the manner in which this is controlled at the molecular level. Differential expression of members of the glutamine synthetase multigene family is also being investigated. (Area coordinator: Miflin; Burgess, Burrell, Buxton, Clark, B. G. Forde, J. Forde, Fry, Gebhardt, Gibson, Halford, Heyworth, V. Jones, Kreis, Lewis, Malpica, Oliver, Ooms, Pywell, Saarelainen, Schmutz, Williamson)

Characterization of cereal storage protein genes. Analysis of cDNA and genomic clones containing sequences related to the major storage proteins of the Triticeae has given a generalized picture of the structure of these proteins and their genes. Unlike many eukaryotic genes, the two genomic clones sequenced so far do not contain intervening sequences. The proteins encoded by the genes are made up of a central domain consisting of multiple repeated sequences between regions of non-repetitive sequences at the amino- and carboxy-termini. The extent of the non-repetitive sequences and the nature of the repeats differ between the different groups. The HMW prolamins have about 35 residues in the carboxy-terminal and about 100 residues in the amino-terminal domains with about 45 repeats of a glycine-rich hexapeptide and 15–20 repeats of an interspersed nonapeptide repeat. The S-poor prolamins consist almost entirely of a peptide repeat made up of glutamine, proline and phenylalanine. The S-rich prolamins have a long carboxy terminal region rich in S-amino acids and usually, but not always, a short non-repetitive amino-terminal region. Between these two domains is a series of repeats very similar to the proline-rich repeats in the S-poor prolamins.

Evolution of storage protein genes. Comparison of the sequences of these non-repetitive regions of the cereal prolamins with those of other seed proteins shows the presence of homologous regions. In particular, we have defined three regions (A, B and C) in the sequence of a family of α-amylase and trypsin inhibitors present in cereal seeds which are homologous to sequences in the non-repetitive domain of the carboxy terminals of S-rich prolamins. These regions are also present in seed storage proteins of the two dicotyledons, *Ricinus communis* and *Brassica napus*. Homologous regions are also present in the sequences of HMW prolamins, but in this case A and B are found at the amino- and C is at the carboxy-terminus. The results are interpreted as showing that although the prolamins are a recent evolutionary development their genes may have originated from those of an ancient seed protein family.

Control of expression of glutamine synthetase. Different forms of the enzyme glutamine synthetase are found in different plant organs. We have isolated cDNA clones for one nodule-specific and two root-forms of the enzyme subunit. Although they cross-

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hybridize, each of these clones gives a different pattern of fragments after digestion with restriction endonucleases. We have used these clones to probe for the presence of different GS genes within the genome and for their transcription in different organs. Our results show that GS is encoded by a small multigene family and that different members of this family are expressed in different organs. We are currently isolating and characterizing genomic clones (in association with Dr J. V. Cullimore, University of Warwick) in order to study the way in which this differential expression is achieved.

Glasshouse services

As predicted in the *Rothamsted Report for 1982* the facilities of the Botany and Biochemistry Departments were merged to form an interdivisional facility for the Molecular Sciences and Agronomy and Crop Physiology Divisions. Because of the poor state of the glasshouses on the central site a replacement development was planned together with other users. This project has started during the year and is due to finish in March 1985 to provide 670 m^2 of new glass with computer controlled heating, lighting and ventilation systems in order to provide comprehensive facilities for experimental control and recording of environmental conditions. A data-logging system has also been installed in the Ninnings glasshouse facility.

During the year the glasshouse services section has provided about 20 000 pots and trays of plant material for the two divisions. Development of techniques for growth of cereals under controlled nutrient regimes has also taken place. (Services coordinator: Franklin; Gilroy (PEP), Plumb (PEP), Ward)

Staff and visitors

Outside support and collaboration. The Department gratefully acknowledges the financial support for personnel and materials that have been provided by the Home-Grown Cereals Authority, Shell Research Ltd, the Potato Marketing Board, Directorate DG XII (Education and Science) of the EEC, Sigma Chemical Company, NATO, Limagrain and ICI Plant Protection.

We have also shared SERC/CASE research students with the Universities of St Andrews, Durham and Warwick. We also wish to thank our many collaborators in a variety of institutions throughout the world.

Visitors. During the year the Department was pleased to welcome for extended visits: Miss Margaret Bossen, Mr M. de Both, Dr M. J. Tempelaar, Miss S. de Vries, and Mr J. Wybrandi (The Netherlands), Miss Dipti Thakar (University of Essex), Mr U. Pfisterer (Germany), Dr T. Bryngelsson (Sweden), Dr F. Greene (USA), Dr W. Park (USA), Dr J. Perret (France), Dr A. Deggerdal (Norway), Dr D. Schmutz (Switzerland), and Mr D. de Brouer (Belgium)

Visits abroad

Members of the Department attended the following conferences: EEC Meeting on *Agrobacterium* Versailles, France (G. Ooms), EEC Contractants Meeting, Copenhagen, Denmark (N. Fish, C. Gebhardt, M. G. K. Jones, M. Kreis, G. Ooms, M. Tempelaar), Phytochemical Society Meeting on The Biochemistry of Plant Phenolics, Ghent, Belgium (P. J. Lea, W. S. Pierpoint), Workshop on Photosynthesis and Physiology in the Whole Plant, Braunschweig, Germany (A. J. Keys), AACC Meeting, Minneapolis, USA (B. J. Miflin), Sulphur Institute Symposium on Sulphur in Agriculture in Western Europe, Madrid, Spain (M. Byers), UNEP Training Course on Bioproductivity and Photosynth-

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esis, Fortaleza, Brazil (P. J. Lea), Bioenergy 84, Gothenburg, Sweden (P. J. Lea), ASPP Meeting, Davis, California, USA (P. J. Lea), FEBS Symposium, Moscow, USSR (B. J. Miflin), Gas Enzymology Symposium, Odense, Denmark (A. J. Keys), 2nd International Workshop on Wheat Gluten Proteins, Wageningen, Netherlands (M. Byers, P. R. Shewry, A. S. Tatham), UCLA Symposium on Molecular Biology of Development, Colorado, USA (B. G. Forde), International Symposium on Genetic Manipulation of Crops, Beijing, China (S. W. J. Bright), Planning Conference, International Potato Centre, Lima, Peru (M. G. K. Jones), NATO Conference on Plant Genome Structure and Function, Renesse, The Netherlands (B. J. Miflin, G. Ooms), First Nordic Cell and Tissue Culture Symposium, Svalov, Sweden (B. J. Miflin), INRA-AFRC Workshop on Cereal Seed Proteins, Montpellier, France (B. J. Miflin, P. R. Shewry), Scandinavian Association of Geneticists Meeting, Leangkollen, Norway (B. J. Miflin), and EMBO Course on Biotin Labelling, Paris, France (A. Karp).

Visits were made to the following institutions to further collaboration or for exchange of information: Max Planck Institute, Cologne, W. Germany (S. Maddock), University of Würzburg, W. Germany (M. G. K. Jones, M. J. Tempelaar), Massachusetts General Hospital, Boston, Agrigenetics Inc., Wisconsin, Rockefeller University, New York, Monsanto Inc., USA, Queen's University, Kingston, Canada (B. J. Miflin), State University of Utrecht, The Netherlands; University of Chicago, Michigan State University, USDA Laboratories, University of Illinois, Urbana, University of Nebraska, Lincoln, Connecticut Agricultural Experiment Station, University of Vermont, USA (A. J. Keys), Agricultural University, Wageningen, Netherlands (P. R. Shewry), E. I. du Pont de Nemours, Wilmington, Delaware, USA (A. J. Keys, P. J. Lea, B. J. Miflin), Swedish University of Agriculture, Svalov and Uppsala, University of Lund (B. J. Miflin), Institute of Genetics, Bejing, Institute of Plant Physiology, Shanghai, China National Rice Research Institute, Hangzhou, China (S. W. J. Bright), and Vrije Universiteit, Brussels, Belgium (N. Fish, J. Perret).

Staff

During the year R. Fry, J. Kueh, P. J. Lea, Hilary Lewis, Valerie Magan, Jackie Roberts, Jan Roberts, Patricia Roberts, G. Schmidt left. Eileen Ward transferred on promotion to PEP.

Newcomers to the Department were Joanna Clark (from Edinburgh), Nicola Darrell (from Nottingham), Josephine Dunham, Angela Fox (from Hull), Jane Gibson (from Whitbread Ltd), Val Jones (from Edinburgh), Tricia Makin (from Nematology), Jane Oliver (from University of Warwick), Corinne Quayle (from PEP).

MOLECULAR STRUCTURES DEPARTMENT

Introduction

The report covers a period mainly devoted to writing-up and disbanding. There was one new development, the attempt to make ion-selective cellulose membranes. Several syntheses and crystal structure determinations were completed. In collaboration with the Food Research Institute, Norwich, ^{13}C nuclear magnetic resonance (NMR) measurements have been made on a range of crown ethers and their complexes; detailed calculations have provided explanations for the chemical shifts. The power and limitations of the method for structure elucidation is well illustrated by the results.

Coordination chemistry

Monocyclic ligands. Collaboration with the Food Research Institute in Norwich in the field of solid state ^{13}C nuclear magnetic resonance spectra continued and the range of

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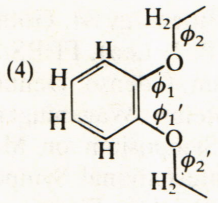

FIG. 1. Fragment of a benzene derivative of a macrocyclic polyether.

compounds examined expanded to include complexes of alkali and alkaline earth metal iodides, thiocyanates and perchlorates with crown ethers containing five and ten ligating O atoms in addition to uncomplexed ligands with five, eight and ten oxygen atoms. Detailed analyses of both aromatic and aliphatic regions were performed to assess the relative importance of the various intramolecular and intermolecular chemical shift contributions. All the compounds contained the partial structure in Fig. 1. The chemical shifts of aromatic carbons are strongly influenced by the torsion angles ϕ_1, ϕ_1' and ϕ_2, ϕ_2' as a result of variation both with the degree of resonance interaction of filled $O_{p\pi}$ orbitals with vacant π acceptor orbitals on the aromatic ring, and with the severity of steric interaction between the α-CH_2 and ortho C-H entities. However, the extraction of conformational information (e.g. ϕ_1, ϕ_2) from the spectrum alone is, in practice, complicated by additional contributions made by intermolecular mechanisms such as (i) π polarization of the aromatic system, (ii) by neighbouring anions, (iii) C—H . . . O interactions to charged or neutral O atoms and (iv) steric effects. In many instances the individual contributions made by these effects could be extracted from the results enabling the testing and subsequent developments of theories of chemical shift effects.

In general intramolecular effects dominated when the degree of asymmetry was large, e.g. $\phi_1 \sim 180°$ $\phi_1' \sim 90°$.

Similar conclusions pertain for the aliphatic region. The spectra for the free ligands could easily be assigned on the basis of *intra*-molecular C—H . . . O (H bond) and C—H . . . H—C (steric) interactions and demonstrated the considerable potential for the extraction of conformational information, while the spectra of complexes in several cases were complicated by ion-pairing and other intermolecular effects. The attempted evaluation of relative chemical shifts by means of simple models of steric and electric field effects revealed the deficiencies in the theoretical models employed.

The sensitivity of the solid state NMR method was well illustrated by the easily observable differences, up to 1 ppm, in spectra of the isomorphous dibenzo-30-crown-10 hydrated complexes of KSCN and RbSCN although the X-ray crystal structures had shown torsion angles and intermolecular distances to differ by less than 15° and 0·01 nm respectively. (Payne, Truter, Wingfield with Dr P. S. Belton, Dr S. F. Tanner and Mr K. M. Wright, Food Research Institute)

Measurements of ^{13}C chemical shifts in solution were interpreted with the greater understanding of π-polarization, steric and resonance effects achieved through the solid state work. In combination with the results of ^{1}H NMR nuclear Overhauser difference spectroscopy (NOE) the studies have provided a detailed insight into the nature of aromatic substituent effects on the conformation of derivatives of benzo-15-crown-5 and benzo-18-crown-6 and their complexes and, as a result, a greater understanding of factors influencing complexing power has emerged.

Substituents on the aromatic ring, e.g. NO_2, MeO, Br, primarily affect the mean C_{ar}–O torsion angle ϕ_1 adopted in the ligand. For example, mesomerically electron-withdrawing substituents at position (4) such as NO_2 and CH_3CO favour a coplanar

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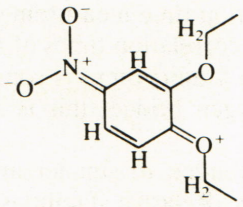


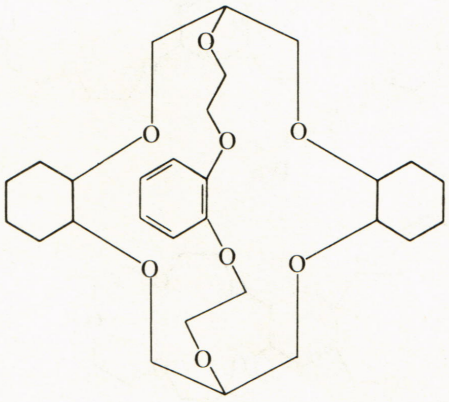
FIG. 2. Fragment of a 4-nitro-substituted benzene derivative of a macrocyclic polyether.

geometry with $\phi_1 \sim 180°$ due to a through-ring resonance contribution of the type shown in Fig. 2. This increases the steric interaction between the α-CH_2 and the ortho C—H. Conversely, mesomerically electron-donating substituents increase the mean value of ϕ_1, probably as a consequence of resonance saturation. The mean ϕ_1 value adopted reflects a compromise between maximum resonance stabilization and minimum steric interactions.

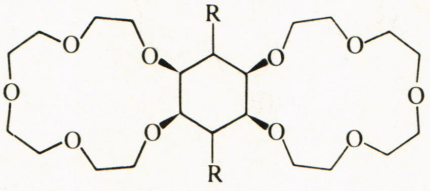
If the substituent is unsymmetrical, e.g. —C(═O)CH_3, the crown ether ring may induce a preferred orientation. For 4-acetylbenzo-15-crown-5 ^{1}H NOE studies showed a two-to-one preference for the conformation with the C═O (not the C—CH_3) bond parallel to the ortho C—H bond. This is probably a consequence of stabilization by electrostatic dipole-dipole interactions.

In the benzo-18-crown-6 series (and to a lesser degree in the benzo-15-crown-5 series) complexation results in increased planarity $\phi_1 \sim 180$ in M^+ (benzo-18-crown-6) X^- (M^+=Na^+, K^+, Rb^+) the enhanced stabilization arising from the optimum geometric arrangement of a lone pair of electrons in oxygen being more important energetically than the increased ortho C—H to α-CH_2 steric interaction. Unlike the free ligands the conformation of the complexes is to a first approximation independent of aromatic substituents in the 4- and 5-positions. The results of ^{1}H NMR competition experiments between unsubstituted and substituted benzo-18-crown-6 indicated a surprisingly small reduction of complexing power on nitro substitution. This can now be understood because the increase in non-bonded interactions on complex formation is less for the substituted than for the unsubstituted molecules.

The greater conformational rigidity of the complexes is shown by the much smaller temperature dependence of their ^{13}C chemical shifts compared with those of the uncomplexed molecules. (Payne)



Ia
Ib



II

FIG. 3. Macrobicyclic polyethers. In Ia the 'open' six-membered rings are benzene in Ib these rings are cyclohexane. In II the R groups are —COOH.

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Bicyclic ligands. ^{13}C NMR relaxation time measurements of the free ligand, Ia (Fig. 3), and its complexes indicate that the correlation times of the carbon atoms are governed by a relatively rigid dibenzo-14-crown-4 unit; there is some conformational mobility of the CH_2—CH_2 chains of the four oxygen bridge, this is significantly reduced on complex formation.

^1H NMR studies have been extended to smaller and larger bicyclic systems and to cations other than alkali metal ions. Relative stabilities have been determined by means of competition studies in mixtures of metal ions. For instance, Ba^{2+} forms a complex with Ia practically isostructural with that of K^+, but considerably less stable, *relative* formation constants for K^+, Ba^{2+} and Rb^+ being *c* 300, 10 and 1 respectively. (Payne)

Use of lanthanide ions as subtle probes for the effect of radius has continued. To avoid the extraneous problems of hydrolysis and hydration associated with the chlorides we made complexes of the nitrates with a range of bicyclic molecules including the isomer Ib. For this isomer having the highest formation constant with alkali metals (Parsons, 1984) a crystalline complex was isolated and shown by crystal structure analysis to consist of two complex cations $[La(NO_3)_2Ib]^+$ and a complex anion $[La(NO_3)_5MeOH]^{2-}$. In one cation the lanthanum is 12-coordinated by the eight oxygen atoms of Ib and by two bidentate nitrate ions, in the other it is 11-coordinated because one nitrate ion is only monodentate. The conformation of the ligand in both cations is similar to that found in its complex with potassium perchlorate (Bandy & Truter, *Acta Crystallographica* (1982) B **38,** 2639–2648) subtle features result in six of the La–O distances being 0·003 nm shorter in the 11- than in the 12-coordinated ligand while, unexpectedly, two are longer. (Hughes, Parsons and Truter, with Dr F. Benetollo, Prof. G. Bombieri and Dr G. de Paoli, Padua, Italy)

After two years of (intermittent) work there has been a breakthrough in the extremely difficult problem of synthesizing compound II. As reported previously (*Rothamsted Report for 1982,* Part 1, 150) the aim was to link the 1 and 4 positions by forming the anhydride, CO—O—CO bridge. This should force the cyclohexano ring to adopt the relatively unstable boat conformation so that the two cyclic ether rings would form a 'clam' compound. The difficulty lay in obtaining the dicarboxyl compound from the benzo bis 15-crown-5 diamide precursor, (IId of Fig. 6 of *Rothamsted Report for 1982,*

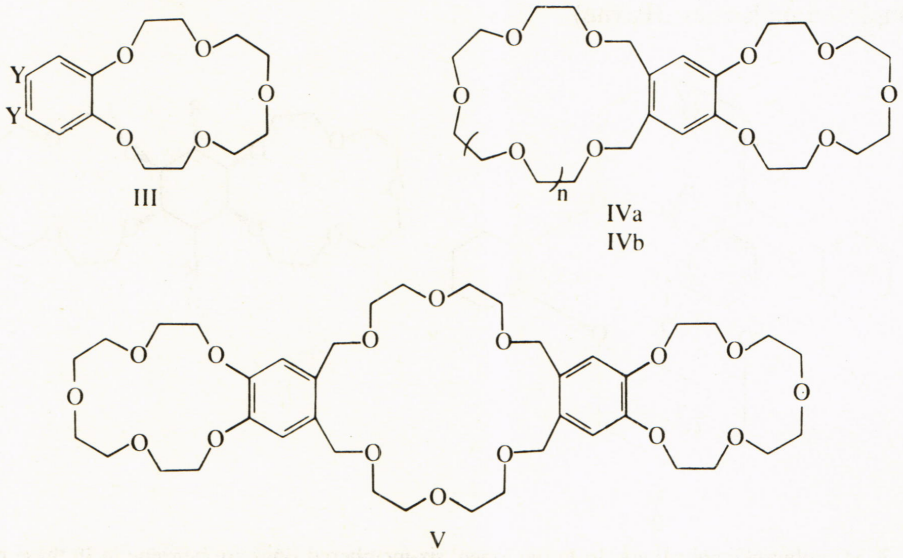

FIG. 4. Precursor monocyclic polyether III and derivatives having polyether rings of different sizes.

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Part 1, 150) because steric hindrance reduces reactivity of the groups. Eventually hydrolysis was achieved, then hydrogenation of the aromatic ring has given a product which should contain two isomers *cis*-anti-*cis* and *cis*-syn-*cis*, the latter is depicted as II. (Parsons)

Crown ethers containing two macrocyclic rings of different sizes have been synthesized from benzo-15-crown-5, III, Y=H through the series Y=Br, $CONH_2$, COOH, COOEt to CH_2OH. Reaction with the necessary polyethyleneglycol ditosylate has given compounds IVa n=1 and IVb n=2 and V, Figure 4.

Compound V has the potential for the central 22-membered ring to fold round bringing the two 15-membered rings in a position to form a clam. Compounds of the type IV provide two ring entities, one having all oxygen atoms separated by two carbon atoms, the other having one pair of oxygen atoms separated by four carbon atoms; the latter arrangement usually reduces the affinity for alkali metal cations. With potassium thiocyanate a complex was isolated for IVa. (Parsons)

The beautiful, clear, colourless crystals of the complex $[K_2(IVa)_2]^{2+}.2SCN^-.3MeOH$ disintegrate when removed from their solvent. After they were hurriedly coated with epoxy-resin, they were subjected to X-ray structure analysis.

The bis-crown ligand adopts a three-plane arrangement; the planes of the two crown rings are folded, in a *trans* configuration, away from the central benzo-group plane. This ligand pairs with a centrosymmetrically related ligand, and two potassium ions are sandwiched within the dimeric unit. Each K-atom is thus surrounded by four oxygen atoms of the 15-crown-5 ring of one ligand molecule and by five oxygen atoms of the 17-crown-5 ring of the other ligand, in approximately a capped cube arrangement. One of the 15-crown-5 ring oxygen atoms is not coordinated; this is not the predicted preference.

The anions and methanol molecules appear to be grouped in hydrogen-bonded dimeric clusters. There are remarkably few intermolecular contacts in this crystal; the methanol molecules can escape easily, and the crystal then rapidly disintegrates. (Hughes)

Polymeric ligands. A procedure was developed to bind crown ethers covalently to cellulose backbones. Homogeneous partial hydrolysis of commercial cellulose, which contains approximately five acetyl groups per cellobiose unit, gave a range of cellulose acetates with one to four acetyl groups per cellobiose unit. Reaction between that having the lowest substitution and increasing ratios of 4-chlorocarbonylbenzo-15-crown-5 yielded a range of cellulose acetate 15-crown-5 benzoates all of which gave tough clear films when cast from 20% solutions in pyridine. A perspex cell was constructed to measure, by conductivity, the permeability of these membranes to different salt solutions in water. Large differences in the rate of diffusion of anions was found, thiocyanates and bromides diffusing much faster than nitrates and sulphates. For the same anion, selectivity between alkali metal cations was rather poor, potassium diffusing about three times as fast as sodium. The infrared spectra of films which had been soaked in aqueous potassium thiocyanate, and appeared swollen, showed conformational changes in the crown ether indicating complexation of the potassium ion. The films returned to normal after being washed in water. They were disintegrated by lead acetate but stable to the salts of sodium, potassium, magnesium, calcium and copper(II). Selectivity might be improved by changing the crown ether moiety and/or including a non-mobile plasticizer. (Parsons)

Collaboration with the Unit of Nitrogen Fixation

Yellow crystals of a compound with formula $Mo(N_2)_3(PPh^nPr_2)_4$ had been obtained in the UNF by reaction of nitrogen with *trans* $Mo(N_2)_2(PPh^nPr_2)_4$. Crystal structure analysis

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confirmed that the compound was, in fact, a *tris* dinitrogen complex, the first to be synthesized. The ligands are octahedrally disposed about the molybdenum in the meridional configuration (i.e. the three N_2 ligands and the metal are approximately coplanar). (Hughes with S. N. Anderson and Dr R. L. Richards)

As part of an investigation in which coordinated dinitrogen was replaced by analogues which are also substrates for nitrogenase, *trans* $ReCl(N_2)(Ph_2PCH_2CH_2PPh_2)_2$ had been treated with an alkyne $PhC{\equiv}CCH_3$, the product being crystals with the expected chemical formula $ReCl(C_9H_8)(Ph_2PCH_2CH_2PPh_2)_2$ solvated with benzene or tetrahydrofuran. Crystal structure determination showed that there had been a rearrangement, the coordinated entity being not an alkyne but an allene $PhCH{=}C{=}CH_2$. The CH_2—Re and C—Re distances 0·231(6) and 0·2087(6) nm respectively and the η^2 allene group occupies one vertex of an octahedron *trans* to the chlorine, while the equatorial 'plane' consists of a shallow tetrahedron of phosphorus atoms. (Hughes with Drs A. J. L. Pombeiro, C. J. Pickett and R. L. Richards)

Staff

Mary R. Truter was reappointed Visiting Professor in University College London and worked there on secondment from 1 October. D. L. Hughes transferred to the Unit of Nitrogen Fixation, D. G. Parsons transferred to the Plant Breeding Institute, J. N. Wingfield was promoted to PSO on appointment to AFRC Headquarters, M. P. Payne took up a Post-doctoral Research Assistantship at Oxford University, P. K. E. Trinder continued his sandwich training at Sandoz in Basel. J. D. Owen left on 1 January to be the AFRCCC Graphics Officer. Joyce Johnson became part-time Personal Secretary in the Nematology Department.

Visitors included Professor H. L. Nigam, Awadhesh Pratap Singh University, India and Professor A. I. Popov, Michigan State University, USA.

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