ROTHAMSTED RESEARCH

Rothamsted Experimental Station Report for 1983

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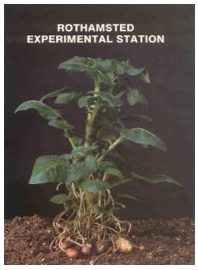

Molecular Sciences Division

B. J. Miflin

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INTRODUCTION

The Molecular Sciences Division was formed with the hope that it would lead to the application of a spectrum of physical, chemical, biomolecular and cellular techniques to problems in plant science of agricultural importance; it was implicit in this hope that the total effect would be synergistic. Unfortunately serious budgetary reductions mean that the Station will not be able to continue research in Molecular Structures. Nevertheless

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we still believe in the validity of the approach which will be prosecuted with the resources remaining.

BIOCHEMISTRY DEPARTMENT

Within the Biochemistry Department the year has seen the consolidation of the scientific reorganization begun in October 1982. As part of this change the Department's research interests in plant growth regulators, and two of its personnel were transferred to the new centre created for this work at Long Ashton Research Station. The Department completed its move out of the Ogg Building into laboratories in the Bawden Building.

Ribulose bisphosphate carboxylase

Our role within the AFRC's priority programme on 'Photosynthesis' is to study the enzymology of the carboxylation and oxygenation of ribulose bisphosphate (RuBP). The relative rates of these two reactions determines the rates of carbon dioxide fixation and photorespiration. We have therefore improved the purity and specific activity of enzyme isolated from wheat to study the mechanism of catalysis of the oxygenase and carboxylase reactions by spectroscopic and stopped flow techniques, and to investigate the three-dimensional structure of the enzyme using electron microscopy, circular dichroism, Electron paramagnetic resonance (EPR), and computer modelling. RuBP carboxylases from different species of plant are being compared especially with respect to affinity for O_2 and activation. A CASE student, supervised jointly with the University of Warwick, is studying the mechanism of assembly of carboxylase polypeptide subunits into active protein. (Area coordinator: A. J. Keys; Boyle, Burton, Cornelius, Gompertz, Gutteridge, Johnson, Millard, Parry, Schmidt)

Activity of ribulose bisphosphate carboxylase. Preparations of RuBP carboxylase with high specific activity are best made from young leaves grown with a plentiful supply of nitrogen fertilizer at high light intensities and harvested in the light immediately prior to extraction. The apparent decline in specific activity that accompanies later steps in the purificiation procedure is due mainly to the removal of endogenous orthophosphate. Further increases in specific activity are thought to be possible and may be related to activation rather than extraction and purification. Contrary to published results, Ca^{2+} can replace Mg^{2+} as the activating cation for RuBP carboxylase. Activation by Ca^{2+}, like that by Mg^{2+}, is increased by the presence of certain effectors, for example inorganic orthophosphate and 6-phosphogluconate. Replacement of Mg^{2+} by Ca^{2+}, unlike replacement with Mn^{2+}, does not alter the relative activity of carboxylase as compared to oxygenase.

The slow activating form of wheat RuBP carboxylase (E_s), contrary to our previous report (Machler, Keys & Cornelius, *Journal of Experimental Botany* (1980) **31,** 7–14), is converted to the rapidly activating form (E_r) by heat in the absence of Mg^{2+} and CO_2. Conversion of E_s to E_r is increased in rate and extent by the addition of certain metabolites. The nature of the two enzyme forms, their conversion one to the other, and their activation by Mg^{2+} and CO_2 are being investigated by circular dichroism. Amino acid sequences of the peptide subunits of various carboxylases have been stored in a microcomputer and used to deduce likely folding patterns. These patterns together with the known changes in circular dichroism spectra will be used to indicate structures near the active site.

Species comparisons. The carboxylases from wheat, tobacco, maize, pea and spinach differ mainly in the extent to which they are converted to an E_s form at 0°C in the

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absence of CO_2 and Mg^{2+}. However, preliminary results show a small but statistically significant decrease in the ratio of carboxylase to oxygenase activities for the enzymes from maize and tobacco compared to those from wheat, pea and spinach.

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. Such plants may have biochemical lesions in the photorespiratory pathway; they offer a means of identifying key steps in photorespiratory metabolism. We are particularly interested in altering the rate of photorespiration in C_3 plants and are currently examining the effects of nitrogen nutrition and of CO_2 and O_2 concentration. The effect on photorespiratory metabolism of a number of potential herbicides, based on the glutamine synthetase inhibitor phosphinothricin, is being examined. (Area coordinator: P. J. Lea; Festenstein, Hall, Hill, Kendall, Keys, Reggiani, I. K. Smith, Temple, Turner and Wallsgrove)

Photorespiratory mutants of barley. Electron microscopical examination of the leaves of the catalase-deficient mutant RPr79/4 (with Dr Mary L. Parker, PBI) shows that the enzyme is absent from the peroxisomes. In leaf tissue exposed to air, the chloroplasts were extensively damaged and contained large osmiophilic droplets.

We have isolated two ferredoxin-dependent glutamate synthase deficient mutants of barley (RPr 82/1 and 82/9); enzyme activity is less than 5% of the wild type in both roots and leaves, but NADH dependent glutamate synthase activity is normal. Immunodiffusion studies indicated that both mutants lack the enzyme protein. After exposure to air the mutant plants accumulated large amounts of glutamine, asparagine and ammonia, but little of other amino acids (e.g. glutamate, glycine and serine).

The mutants, when exposed to air, were unable to metabolize ^{14}C-glutamine, and when fed with $^{14}CO_2$ accumulated relatively more radioactivity in glycollate and sugar phosphates and less in glycine and serine compared with the wild type. After a prolonged period in air, mutant plants accumulated ^{14}C in malate and the total concentration of malate was tenfold higher than normal, This was probably due to the activation of malic enzyme and PEP carboxylase to act as a pH-stat to neutralize the ammonia.

Effect of nitrogen source on photorespiration. Barley and wheat plants grown on nitrate or ammonia had similar rates of photorespiration as measured by two independent methods. In maize there was a slight increase in the compensation point in plants grown on ammonia, but the rate of CO_2 evolution into CO_2-free air remained constant at approximately 2·2% of the rate of CO_2 fixation with either nitrogen source. Similar results were obtained when plants were transferred from a nitrate to an ammonia medium. We are therefore unable to confirm, under our conditions of growth, that the rate of photorespiration in either C_3 or C_4 plants can be altered by nitrogen nutrition.

As part of this study we have detected four isoenzymes of glycollate oxidase in wheat leaves. The two more electropositive forms have a Mr of 160 000 (tetramer), and the two more electronegative forms a Mr of 300 000 (octomer).

Inhibition of ammonia assimilation. Methionine sulphoximine (MSO), phosphinothricin (PPT) and its keto analogue (PPO) all inhibit the rate of photosynthetic CO_2 fixation when fed to leaves of barley in the following order of potency,

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PPT>PPO>MSO. Although PPO is not an inhibitor of glutamine synthetase, it is readily transaminated by glutamate within the plant to yield the active form PPT. (With K. W. Joy, Carleton University, Canada)

Metabolic regulation

This research area uses biochemical mutants to study agriculturally significant pathways in crop plants, particularly barley. The benefits are threefold, (1) they provide increased understanding of plant metabolism, (2) some mutants are altered in beneficial ways and (3) the mutations are valuable as selectable markers. The pathways or enzymes of interest are (a) the aspartate pathway (b) the branched-chain pathways of amino acid biosynthesis (c) nitrate reductase (d) proline synthesis (e) alcohol dehydrogenase. (Area Coordinator: S. W. J. Bright; Arruda, Bottacin, Bright, Creissen, Franklin, Hill, John, Karp, Kueh, Nelson, Norbury, Risiott, S. J. Smith, Steven, Wallsgrove)

Aspartate pathway. Lysine, threonine and methionine are nutritionally important amino acids; cereal mutants accumulating these amino acids in the grain should have improved nutritional quality. The three well-characterized lysine plus threonine-resistant mutants (R2501, R3004, R3202) have been crossed with a fourth (R2506) and with a further mutant selected at the Vrije Universiteit, Brussels. R2506 possesses an aspartate kinase (isoenzyme III) insensitive to feedback inhibition by lysine. The four Rothamsted mutants each have unique aspartate kinase phenotypes as well as characteristic degrees of soluble threonine accumulation. So far the greatest accumulation in seeds causes about a 10% increase in total seed threonine. S-aminoethylcysteine-resistance in two new mutants selected from re-mutagenized R3004 was dominant, unlike that in previous mutants which had an altered permease. These are being analysed as potential lysine accumulators.

Branched chain amino acid synthesis. Auxotrophic mutants of higher plants have recently been produced by other research groups. We have been given auxotrophic cell lines of *Datura innoxia* and *Nicotiana plumbaginifolia* requiring isoleucine (+/− valine) and some valine-resistant mutants of *N. tabacum*. Biochemical analyses of these mutants will provide new information about the synthesis of these amino acids.

Proline. Proline accumulation during water or salt stress in normal barley is greater than the three- to sixfold accumulation in non-stressed, hydroxyproline-resistant mutants and the response of the mutants to stress is little different to their parents. Remutagenesis and selection was used to seek mutants with greater accumulation. The synthesis of proline from glutamate under non-stress conditions is being studied.

Nitrate reductase. Seeds have been obtained from six chlorate-resistant barley plants with low or zero *in vivo* nitrate reductase activity. Mutant R9401 lacks xanthine dehydrogenase, as do three others, so is probably defective in the synthesis of a cofactor common to both enzymes. The mutant gene is not allelic to the *nar-1* and *-2* loci (Kleinhofs *et al.*, *Molecular General Genetics* (1980) **177,** 421). Two other mutants may have lesions at the nitrate reductase structural gene.

Cultured cereal caryopses. Culture of isolated cereal caryopses would allow study of biochemical pathways occurring during grain development in normal and mutant plants. The culture of barley grains of 20 mg fresh wt was repeated with wheat. Barley grains of 5 mg fresh wt will also develop in culture. The storage proteins of cultured

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barley grains are analagous to those in sulphur-starved plants, having more 'C' than 'B' hordein. Manipulation of the proportions of supplied glutamine and cysteine changed the ratio of 'B'/'C' hordeins from 2·3 to 0·37.

Plant cell biology

Plants can potentially be regenerated from single cells either within tissues or from isolated protoplasts. A major aim of this part of the genetic manipulation programme is to regenerate plants of important crop species by *in vitro* culture, so that modified plants can be produced following introduction or modification of genetic information at the single cell level. The crop species of particular interest are wheat, barley, potato and rape. Genetic information is being transferred using *Agrobacterium* as a natural vector and via protoplast fusion. (Area coordinator: M. G. K. Jones; Bains, Bright, Burrell Creissen, Fish, Foulger, Karp, Kueh, Maddock, Magan, Nelson, Ooms, Purdy, Risiott, Roberts, Twell, Wilcox)

Wheat, *Triticale*. Analysis of somaclonal variation in wheat plants regenerated from culture has continued with cytogenetic studies and field trials of regenerated plants (R1) and their progeny (R2). Cytological examination of R1 plants showed that 29% were aneuploid, and many structural chromosome changes, especially interchanges, were observed. Some chromosome mutants obtained are being studied further. Progeny from over 800 R1 plants were evaluated in field trials at Nickerson RPB Ltd. Variation was transmitted to some R2 plants, but was less extensive than in the R1 generation. Five lines have been selected for further breeding trials.

About 400 *Triticale* plants, regenerated from immature embryos, have been transferred to the Plant Breeding Institute Cambridge, for assessment of variation and cytological characteristics.

Potato. Plants have been regenerated from cultured explants of monohaploid (2n=12), dihaploid (2n=24) and tetraploid (2n=48) genotypes. The cytology of regenerants was: monohaploids—doubled in chromosome number: dihaploids—about two-thirds doubled chromosome number; tetraploids—90% euploid. Results for protoplast-derived plants of tetraploid cultivars showed regenerants were on average 50% aneuploid. Field assessment of 257 explant-derived clones (cv. Désirée) has continued (with Dr N. Evans, N.I. Plant Breeding Station). Stable expression of previously observed variation in pigmentation was again apparent. For the second season, a large number of regenerant clones had tubers that were less affected by scab *(Streptomyces scabies)* than those of the controls, but resistance to *Globodera pallida* was not detected. Some of the white skin colour variants of plants (cv. Fortyfold) regenerated from protoplasts, and now in the third tuber generation, regained colour. Thus both stable and unstable changes can occur after passage through a tissue culture cycle.

Protoplast fusion. Techniques required to fuse the protoplasts of crop plants, select heterokaryons and obtain hybrid callus are being developed. Heterokaryons between potato, tobacco and barley protoplasts have been obtained and putative hybrid callus is under selection.

Agrobacterium. Much of the work has focused on isolation and characterization of potato plants transformed by *Agrobacterium*. Transformed plants without roots have been recovered from galls induced by shoot-inducing strains. A transformed tuber formed after grafting on to untransformed root-stock has been grown to a second generation. Potato plants have been regenerated from roots transformed by *A.*

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rhizogenes, and also from cells transformed by avirulent *A. tumefaciens* strains. Auxin and cytokinin levels and transcription of T-DNA have been examined (in conjunction with N. Appleford and J. Lenton now at LARS) in plants without roots. The cytokinin level and TDNA gene transcription decrease when transformed shoots regain a more normal phenotype on grafting. Initial success in transforming rape tissues has been obtained.

Disease resistance

One of the objectives of crop improvement, either by conventional or novel means, is the incorporation of disease resistance traits into crop cultivars. With staff in the Plant Pathology department, we have had a long-term interest in the phenomenon of induced resistance to virus infection in tobacco *(Nicotiana tabacum)* and the relationship of the pathogenesis-related (PR) proteins to this process. These proteins are being studied by immunological and biochemical techniques and attempts are being made to clone the relevant genes. (Area coordinator: B. J. Miflin; Antoniw, Burrell, Buxton, Carr, Ooms, Pierpoint, with Carpenter and White, Plant Pathology Department)

Pathogenesis-related proteins

Immunological studies. An enzyme-linked immunosorbent assay (ELISA) was developed for the PR proteins using $F(ab')_2$ fragments of an antiserum prepared against the PR-1a protein purified from *N. tabacum* cv. Xanthi-nc. The assay can detect as little as 10 pg purified PR-1a and is more reactive for PR-1a than PR-1b and -1c even though they are serologically related. The $F(ab')_2$ ELISA has shown that normal, apparently healthy, plants contain small (610 ± 750 pg g^{-1} fresh wt leaf) amounts of PR-1a, and that there is a significant increase in PR-1a in TMV-infected Xanthi-nc before the necrotic lesions appear although the largest levels of PR-1a are reached after lesion appearance during the time of virus restriction in the infected leaf. (With D. J. Barbara, EMRS)

Combined immunological and electron microscopical techniques have shown that in leaf sections of cv. Xanthi-nc, most of the PR-1a-related proteins are outside the cell membrane and in the cell wall. (With P. Jones, Plant Pathology)

Last year we reported that aspirin treatment of *Solanum demissum* induced a protein serologically related to PR-1a. We have now shown that this is associated with a reduction in the number of lesions caused by PVY.

PR proteins and virus susceptibility in transformed tobacco. *Agrobacterium rhizogenes* transformed plants, regenerated from hairy-root tobacco tissue, were as susceptible to infection with TMV and PVY as untransformed plants. Furthermore equally small amounts of PR proteins were found in both sets of plants. These results imply that the *A. rhizogenes* plasmid can be used to introduce virus-resistance genes into plants without the transformation procedure significantly disturbing the interaction of virus and plant.

Other PR-proteins in virus-infected leaves. Extracts of TMV-infected leaves of Xanthi-nc tobacco contain five major characteristic peaks of PR-proteins which migrate slower on electrophoresis than the four well-recognized PR-proteins. These are resolved into seven components by chromatofocusing of which Q, R and R^1 can be isolated in quantities sufficient to allow their characterization. These three include two of the proteins which are absorbed by chitin, and which were previously suggested to be lectins. However they do not bind onto affinity columns for lectins, they contain no glucosamine or galactosamine, and they have no haemagglutinating activity. Indeed no haemagglutinating activity can be detected in these infected leaves, although the

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literature frequently mentions the presence of lectins in tobacco leaves with other infections. The proteins do not interact with column-bound lectins. Preliminary amino acid analyses reveal no hydroxyproline, but indicate that one of the three (R) has a high content of cysteine.

The procedure used satisfactorily for isolating Q, R and R^1, involves low pHs. Attempts have been made to adapt it to milder conditions so that the isolated proteins may be tested for biological activities such as bacterial agglutination or for restricting the size of lesions induced in leaves by TMV.

Cereal seed proteins

The aim of the work is to relate the properties of cereal seed proteins to the nutritional and technological (malting and baking) quality of the grain. This will provide a sound basis for crop improvement, whether by conventional plant breeding or recombinant DNA approaches.

In barley and wheat the major seed storage proteins are alcohol-soluble prolamins. These are deficient in the essential amino acid lysine and as a consequence the grains are of poor quality for feeding non-ruminant animals. In wheat the prolamins are the main components of gluten, the visco-elastic mass of proteins which is largely responsible for the ability of wheat flour to be baked into leavened bread. Cereal seeds also contain smaller amounts of other storage proteins which have higher nutritional quality and are soluble in water (albumins) or saline (globulins). In oats the globulins, not the prolamins, are the major seed storage proteins.

We are studying the chemistry, genetics, mechanisms of synthesis and deposition and regulation of synthesis of these groups of proteins. Selected projects are described in more detail below. (Area coordinator: P. R. Shewry; Bradberry, Bunce, Burgess, Byers, Festenstein, Galterio, Henderson, Karp, Miflin, Parmar, Rorat, S. J. Smith, Tardani, Tatham)

Characterization of high molecular weight (HMW) gluten polypeptides of wheat. The baking quality of wheat has been related to the amount of high molecular weight gluten aggregates, and to the presence of specific HMW polypeptides which are components of the aggregates. We have purified and characterized a number of HMW polypeptides to determine the structural basis of their aggregation. N-terminal amino acid sequencing (with Dr D. D. Kasarda, USDA Western Regional Research Centre, Albany, California) showed the presence of two cysteine residues in the first 25, although the distance between these differed. The presence of one cysteine residue in the C-terminal region means that these polypeptides could form long head-to-tail polymers, which might be important in the elasticity of gluten.

We also completed our studies of the immunochemical relationships of these polypeptides. This showed that their reaction with an antiserum raised against an HMW subunit was inhibited by low concentrations of urea, which explained our previous results (*Rothamsted Report for 1982*, Part 1, 56) that the antiserum gave a lower reaction with HMW subunits than with other wheat prolamins.

Identification of barley cutivars. Three systems for sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and two extraction methods were used to assess the feasibility of identifying barley varieties on the basis of their patterns of prolamin storage polypeptides. Each system gave reproducible results and improved methods for discriminating between cultivars were obtained. Nevertheless some cultivars had similar patterns and other characters would be required for complete identification.

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Localization and characterization of oat storage proteins. The components of the 7S and 12S globulin storage proteins have been localized in protein bodies isolated from endosperms and embryos using aqueous and non-aqueous media and sucrose density gradient ultracentrifugation. The 7S globulins were located in the embryos and the 12S globulins in the endosperms.

Gene isolation and expression

This area is part of the ARC's priority programme on genetic manipulation. Our primary interest has been in the genes for the storage proteins of the cereals wheat, barley and rye. This year we have obtained much information on the nature of these genes by sequencing cDNA clones. Currently our attention is shifting to the analysis of the corresponding genomic clones. Progress has also been maintained in isolating clones for glutamine synthetase and further projects for gene isolation are under development. (Area coordinator: B. J. Miflin; Burgess, Burrell, Buxton, Cullimore, B. G. Forde, J. Forde, Fry, Gebhardt, Hirel, Kreis, Lara, Lewis, Malpica, Ooms, Pywell, Saarelainen, Williamson)

Characterization of cDNA clones for cereal storage proteins. Clones have been identified which contain sequences related to the 'B1' and 'B3', 'C' and 'D' hordeins of barley, to the 40k and 75k secalins of rye and the high molecular weight (HMW) prolamins of wheat. Many of the clones have been sequenced and the information used to deduce the amino acid sequences of the corresponding proteins. It has proved possible to compare some of the deduced sequences with partial amino acid sequences derived directly from the proteins. In each case there is the expected correspondence thereby confirming the original identification. The results show that large portions of the cereal proteins are built up of repeated sequences. 'C' hordeins largely consist of repeats based on a consensus sequence of proline-glutamine-glutamine-proline-phenylalanine-proline-glutamine-glutamine. The HMW prolamin clone analysed predicts a mixed repeat based on a consensus sequence of X-glycine-glutamine-glycine-glutamine-glutamine with interspersed repeats of glycine-tyrosine-tyrosine-proline-threonine-serine-proline-glutamine-glutamine. 'B' hordeins appear to consist of two domains, one proline-rich and one proline-poor. The latter has only vestiges of repeats but the former contains repeats based on the same consensus as the repeat in 'C' hordein. This suggests either a mixed origin for the 'B' hordein genes or that the 'C' hordein genes were originally derived from part of the 'B' hordein gene.

Cloning of glutamine synthetase. Glutamine synthetase (GS) is responsible for the assimilation of ammonia in plants. In *Phaseolus vulgaris* different forms of the enzyme occur in different organs and subcellular locations. To examine the genetic basis and regulation of these different forms we have isolated a cDNA clone related to GS. This clone has been sequenced and a partial amino acid sequence for the protein deduced; this will be compared with directly determined sequences currently being determined. The cDNA clone has been used (with Dr T. Hall and colleagues, Agrigenetics Corporation, Madison, Wisconsin, USA) to identify clones for GS from genomic library of *P. vulgaris*. Hybridization of the cloned cDNA to electrophoresed DNA restriction fragments and isolated mRNA preparations have given information on the relationships between GS genes and their transcripts in different tissues and species.

Staff and visitors

Outside support and collaboration. The Department gratefully acknowledges the financial support for personnel and materials that have been provided by the

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Home-Grown Cereals Authority, Shell Research Ltd, the Potato Marketing Board, Directorates DG VI (Agriculture) and DG XII (Education and Science) of the EEC, Sigma Chemical Company and NATO. We have also shared SERC/CASE research students with the Universities of St Andrews, Liverpool, Durham, Warwick, and Bath. As well as numerous collaborations within the AFRS we also recognize the value of the collaboration that we have received with members of many academic and industrial organizations in this country and abroad.

Visitors. During the year the Department was pleased to welcome for extended visits: Dr H. Bauwe (DDR), Dr K. Farnden (New Zealand), Dr G. Galterio (Italy), Dr B. Hirel and Dr J. Vidal (France), Dr I. Jonassen (Denmark), Dr D. D. Kasarda (USA), Dr J. M. Malpica (Spain), Dr Z. Miszalski (Poland), Dr T. Rorat (Poland), Dr I. K. Smith (USA), and Dr P. Vaishnav (India).

Visits abroad. Members of the Department attended the following conferences: EEC Workshop, Bergamo, Italy (B. G. Forde, J. Forde, M. Kreis, B. J. Miflin, P. R. Shewry); 3rd International Seed Proteins Symposium, Gatersleben, GDR (P. R. Shewry); 6th International Wheat Genetics Symposium, Kyoto, Japan (P. R. Shewry); ASPP Meeting, Fort Collins, USA (P. R. Shewry); OECD Workshop on Interactions between Carbon Dioxide and Nitrogen Assimilation in Higher Plants, Zurich, Switzerland (A. J. Keys); Sixth International Photosynthesis Conference, Brussels, Belgium (F. Boyle, N. P. Hall, A. J. Keys, P. J. Lea, M. A. J. Parry); ARC/INRA Meeting on Root Metabolism and Physiology, Centre de Recherches de Bordeaux, France (R. M. Wallsgrove); 5th International Symposium on Nitrogen Fixation, Noordwijkerhout, The Netherlands (J. V. Cullimore, C. Gebhardt, B. J. Miflin); EEC Biomolecular Engineering Programme, Louvain-La-Neuve, Belgium (B. G. Forde, C. Gebhardt, M. G. K. Jones, M. Kreis, J. S. H. Kueh, B. J. Miflin, G. Ooms); FAO Workshop on Durum Wheat, Viterbo, Italy (B. J. Miflin); 6th International Protoplast Symposium, Basel (M. G. K. Jones, S. E. Maddock, R. S. Nelson): EEC Workshop on Genetic Resources for Disease Resistance, Antibes, France (G. Ooms); Workshop on Pathogenesis Related Proteins in Plants, Wageningen, Netherlands (J. Antoniw, M. Burrell, J. Carr, W. S. Pierpoint); Gordon Research Conference on Plant Molecular Biology, New Hampshire, USA (J. V. Cullimore, M. Kreis).

Visits were made to the following institutions to further collaboration or exchange of information: University of Umea, Sweden and University of Khartoum, Sudan (P. J. Lea); Botanical Institute, University of Oslo, Norway (S. W. J. Bright); Molecular Biology, Vrije Universiteit, Brussels, Belgium (S. W. J. Bright, B. J. Miflin); E. I. du Pont de Nemours, USA (S. Gutteridge); Agrigenetics Corporation, The Boyce Thompson Institute, Massachusetts General Hospital, Zoecon Corporation, USA; McGill University, Canada; Centro Fijacion de Nitrogeno, Mexico (J. V. Cullimore); INRA, Versailles, France (M. G. K. Jones, S. E. Maddock, R. S. Nelson); University of Wageningen, The Netherlands (G. Ooms); USDA Western Regional Research Centre, Albany, USA (P. R. Shewry).

Staff. During the year A. W. Wheeler retired, Julie Cullimore, S. Rahman and Ann Roberts left and N. Appleford and J. Lenton transferred to LARS. Frances Boyle, J. Carr, G. P. Creissen, G. Holbrook and R. S. Nelson completed their postgraduate studies. Sandra Purdy and Laura Tardani replaced Valerie Magan and Saroj Parmar during their maternity leave. Newcomers to the Department were Bernadette Buxton (from Insecticides & Fungicides), Neil Fish (from University of East Anglia), Hilary

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Lewis (from University of Sheffield), Patricia Roberts (from Plant Pathology), D. Twell (from University of Durham) and M. Williamson (from University of Sheffield).

MOLECULAR STRUCTURES DEPARTMENT

This is probably the last full Report of the Molecular Structures Department. Since its inception in April 1973, the Department has published 112 papers, and nine more (and a patent application) are in press.

The main achievements from our research have been an increased understanding, at the molecular level, of the discrimination by ionophores between cations of the alkali and alkaline earth metals and substituted ammonium ions. We have synthesized many new complexing agents, principally polyethers, which co-ordinate these cations. One particularly important group of such ligands is a series of isomers which shows widely differing (complex-) formation constants; another series of highly selective ligands contains transition metal centres. The latter are of potential interest as models of enzymes activated by sodium or by potassium. Complexation and transportation of the cations has been investigated chemically and by bioassay (initially on rat liver mitochondria, latterly in stomatal aperture studies). As a result, we have, by rational design, developed biologically active compounds, almost as effective when applied exogeneously as the naturally occuring ones, e.g. abscisic acid, in inhibiting stomatal opening.

In addition to studying, by X-ray diffraction methods, many of these ligands and their complexes, we have determined the molecular structures of a variety of crystalline compounds from other laboratories in the Agricultural and Food Research Service. In the past year, in continuing collaboration with the Unit of Nitrogen Fixation, we have studied three tungsten-dinitrogen complexes, and recently the configuration of a natural fungicide was established for Long Ashton Research Station.

Also, in 1983, we have made a promising start on a NATO-funded project, with Italian workers, to study complexes of the lanthanides in which we can monitor the effect of very small changes in cation radius on complex formation. Our continued investigations into the use of transition metal complexes as ligands for the selective binding of alkali metal and related cations has produced encouraging results with exciting prospective application.

Our collaboration with the solid-state NMR spectroscopists at the Food Research Institute has continued, and, from a knowledge of the X-ray crystal structure data of a series of closely related complexes, we are beginning to understand the contributions to splittings of resonances in the solid-state NMR spectra.

Coordination chemistry

Acyclic ligands. We reported (*Rothamsted Report for 1982*, Part 1, 145) that reaction of cobalt acetate with the hydroxy acid HL (HL=OH-*o*-$C_6H_4OCH_2CH_2O$-*o*-$C_6H_4OCH_2COOH$) in alcohol in the presence of potassium bromide leads to the formation of a blue uncharged complex $[Co(KL_2)_2]$ and that this reaction is highly selective for potassium ions. If the reaction is carried out in water no blue colour is formed, but a crystalline white solid is obtained; this is the acid salt, KHL_2, which then reacts with cobalt acetate in ethanol to give $[Co(KL_2)_2]$. This suggests that KHL_2 may be an intermediate in the formation of the cobalt complex. The crystals of KHL_2 diffract poorly and the structure was determined from only 621 diffraction intensities. The L ligands combine in pairs, roughly coplanar, through three hydrogen bonds, two O(phenol)-H . . . O(carbonyl), as in the cobalt complexes, and the third across a centre of symmetry, between carboxylate groups; the O . . . O distance here is 0·245(3) nm

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corresponding to a strong symmetric hydrogen bond. Formation of the cobalt complex requires replacement of the carboxylate hydrogen by cobalt, coupled with dissociation along the sandwich and re-formation with the ligands in a different orientation.

To change the separation of the sandwich layers, other transition metal derivatives have been prepared. The zinc complexes are found to be less selective so that $Zn(ML_2)_2$, (M=K, Rb and NH_4) is readily formed. The crystal structure of the ammonium complex shows the ligand conformation to be very similar to that in $Co(RbL_2)_2$, with one 'odd' torsion angle at −108°C (rather than *ca* 180°C in the unstrained molecule). Four O atoms form a distorted tetrahedron about the central zinc atom with Zn–O=0·1948(3) nm, very similar to the arrangement about cobalt. The L ligands are in parallel pairs about the ammonium ions, but the pairs on opposite sides of the Zn appear to be twisted away, about 20°C, from an overall parallel arrangement. We believe this results from the directionality required by the ammonium ion in forming its hydrogen bonds.

The ruthenium complex $[Ru(CO)_2(PPh_3)_2L_2]$ does not capture alkali metal cations. Its crystal structure shows that, although the L ligands are in the *cis* configuration required for complexation with an alkali metal, they are prevented from forming the necessary cavity by the steric interaction of benzene rings of the *trans* phosphine groups. The conformation of the L ligand is extended, resembling that of the uncomplexed acid, HL, the crystal structure of which has also been determined. Here, as the free HL molecule is not restrained by metal coordination to approximate planarity, its torsion angles are close to those for an unstrained molecule. The molecules connect, through intermolecular hydrogen bonds, in long chains; phenol groups bond to phenol groups and carboxylic acid groups bond to carboxylic acid groups, in contrast to the phenol to carboxy group bonds in the L_2H^- dimers of KHL_2 and the Co and Zn complexes.

To explore the selectivity between sodium and potassium, in particular the absence of complexes of the former, we determined the crystal structure of the salt NaL, found to be $Na_4L_4.C_2H_5OH$ in which the ethanol solvate is disordered. Molecules of formula Na_4L_4 contains clusters of four Na ions in tetrahedral arrangement (Na...Na distances 0·345–0·357 nm), each face of the tetrahedron capped by a triply-bridging carboxylate O atom. Each Na ion is seven-coordinated and bonded to all four L^- ligands in the molecule. The ligand ion shows little sign of strain in the conformation, with normal torsion angles throughout. The ligands are connected in (symmetrical) dimer pairs by hydrogen bonds from phenol to carboxylate groups, but in contrast to that in K^+, Rb^+ or NH_4^+ containing compounds, the dimer is distinctly non-planar since the Na^+ ions cannot coordinate all five O atoms of any one ligand and hence impose conformational restrictions. (Hughes and Wingfield)

Monocyclic ligands. Dynamic averaging of molecular conformations often occurs in solution, giving rise to simplified nuclear magnetic resonance (NMR) spectra. In the solid state, however, these conformations may be frozen out, giving rise to splittings of the NMR resonances. Unfortunately, splittings in the solid state may arise from other factors, such as crystal packing, molecular incongruity, etc. To assess the contributions of these factors we are studying, in collaboration with FRI Norwich, the ^{13}C solid state NMR spectra of a number of compounds of known crystal structure, principally crown ethers and their complexes with alkali and alkaline earth metals.

In the solid state NMR of benzo-15-crown-5, aromatic ^{13}C resonances, which are singlets in solution, show splittings of the order of 1 ppm or less. Much larger splittings, however, are observed in the complexes of the cyclic polyethers, e.g. for dibenzo-30-crown-10.potassium iodide, (I).KI, there are splittings of 8–11 ppm. Such large splittings must be due to intermolecular effects, since the molecules themselves

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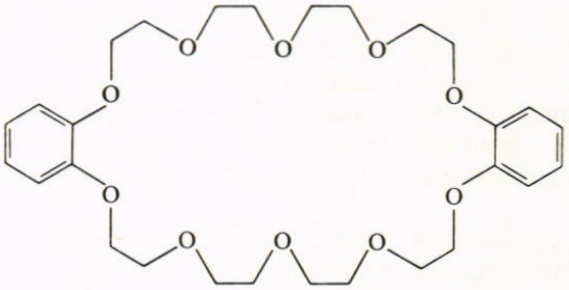
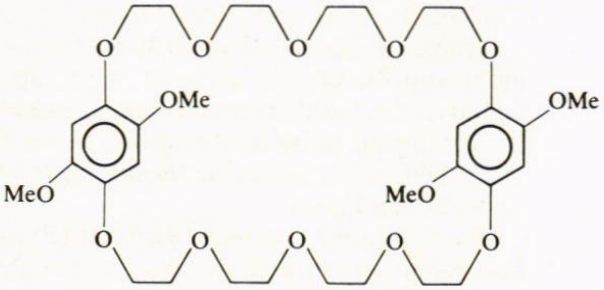
display little intramolecular dissymmetry. Calculations indicate that, although the ring-current effects from neighbouring aromatic rings in the crystal are insufficient to account for the splittings, the different steric effects of packing on the two sides of a benzene ring may be sufficient. For the complexes an additional factor, i.e. the dissymetric packing of the anions with respect to the aromatic rings, is required for the larger splitting. (Payne and Wingfield)



(I)



(II)

To provide a test for anion effects, the dibenzo-30-crown-10 (I) complex of potassium thiocyanate has been prepared; careful work has shown the conditions required to obtain reproducibly the anhydrous form or the monohydrate. In the infrared spectra, the C≡N stretch is at a higher frequency in the hydrate which shows two hydroxy stretching bands. (Wingfield)

The crystal structure of the anhydrous form had, as the original workers (Hašek, Hlavatá and Huml, *Acta Crystallographica* (1980), **B36,** 1782–1785) pointed out, some unsatisfactory features. We have refined it in a different space group, removed the anomalies and shown the complex cation [dibenzo-30-crown-10, K]$^+$ to have the same symmetry and conformation as in the iodide. The anions are in different environments, in the thiocyanate and in the iodide as required for the solid-state NMR study. The crystal structure of the hydrate shows a different conformation of the ligand with the water molecule not coordinated to the cation. (Owen and Truter)

Facile isomerism of *ortho* to *para* quinones to 2,5-dimethoxy-*para*-quinone, has allowed 1,4-dihydroxy-2,5-dimethoxy benzene to be obtained. This has been used to synthesize the tetramethoxy-*para*-cyclophane (II). Reaction of the *para*-cyclophane with sodium thiocyanate in methanol has given a crystalline complex containing two sodium ions for each molecule of the ligand. This is thought to be the first example of a crystalline complex between a Group Ia metal and a *para*-cyclophane. (Parsons)

Crystals of (II) are formed from discrete molecules. Where torsion angles of the C_{benzyl}—$O_{methoxy}$ bonds are ~0°, the bond lengths are significantly shorter than those where torsion angles are large. This is as expected for conjugation of the $O_{methoxy}p$ orbitals, and is observable because of the accuracy of the determination.

The sodium thiocyanate complex has the formula 2(NaSCN.H_2O)(II). Its crystal structure shows two different sodium ions, one coordinated in a distorted pentagonal bipyramid with a thiocyanate N at one apex, and ether oxygen at the other. The other Na^+ is eight coordinated, five O in a plane, two O below and a water O above. (Owen)

Bicyclic ligands. A series of complexes has been made with four representative lanthanide chlorides and four macrobicyclic polyethers (III) with a view to investigating the subtle effect of change of radius on the stoichiometry and structures of complexes. Care was taken to use purified anhydrous lanthanide chlorides and

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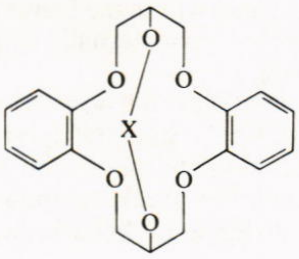

(IIIa) X=$CH_2CH_2OCH_2CH_2$
(IIIb) X=$CH_2CH_2OCH_2CH_2OCH_2CH_2$
(IIIc) X=$CH_2CH_2Oo\text{-}C_6H_4OCH_2CH_2$
(IIId) X=$CH_2CH_2O(CH_2CH_2O)_2CH_2CH_2$

anhydrous solvents. Elemental analyses corresponded to a 1:1 ratio but samples were hygroscopic. Crystallinity required the presence of solvent or water, and it proved very difficult to maintain this even in capillary tubes. One complex, that between $LaCl_3$ and (IIIb) proved sufficiently stable for the crystal structure to be determined. The La atom is in the centre of the polyether molecule, bonded to all eight oxygen atoms of the ligand in a bis-end-capped trigonal prism arrangement; two of the prism faces are also capped, one by a chloride ion, the other by either a chloride ion or a hydroxide ion. This last, disordered, site is only 0·303 nm from a symmetry related site, and this suggests the formation of an O—H . . . Cl hydrogen bond across the centre of symmetry. Partial hydrolysis so that the La/Cl ratio is 1:2·5 (with 0·5 OH^-) is consistent with the elemental analysis.

The La-complex cations pack to leave large channels occupied by disordered water and chloride ions, forming a variety of hydrogen bonding networks through the crystal. Various measurements indicate ratio of La/water of 1:3 to 1:4·5. (Hughes, Parsons and Truter with Prof G. Bombieri and Dr G. de Paoli)

Complexation of the alkali metal cations (Li^+ to Cs^+) with the macrobicyclic polyethers (IIIb) and (IIIc) has been studied in solution using ^1H NMR spectroscopy. Irrespective of the solvent a substantial variation of the complexation induced proton chemical shift changes is observed as a function of cation. Such differences principally reflect changes in the position of the cation within (or in the case of the large cations Rb^+ and Cs^+ on one side of) the eight O atom cavity. Thus for the series of 1:1 complexes formed by Li^+, Na^+, K^+, Rb^+, the progressive movement of the position of the cation away from the basal plane of the four oxygen atoms (to which the Li^+ ion is principally bound) towards those in the link is implied. Coupling constants indicate that the gross conformation of the complexes is similar for all cations.

Nuclear Overhauser Effect (NOE) difference spectroscopy has revealed some subtle changes in conformation between the free and complexed molecules and allowed the calculation of intramolecular distances in specific cases. The NOEs between the *ortho* aromatic protons and the nearest methylene groups are increased on complex formation, indicating an average decrease of about 0·02 nm in the hydrogen-hydrogen distance. (Payne)

Stomatal aperture

Infestations of potato roots by the potato cyst nematode *Globodera rostochiensis* can lead to increase in Ca^{2+} uptake, and hence greater Ca^{2+} concentrations within potato leaves. Ca^{2+} can inhibit stomatal opening. It might, therefore, be expected that the

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presence of increased levels of calcium within the leaves of nematode-infested potatoes could lead to a different response to externally applied calcium compared with nematode-free potatoes (healthy).

Lower epidermal peels of cv. Pentland Dell potato leaves were taken and bathed for 3 h in differing concentrations of Ca^{2+}. Stomatal apertures were then measured. The lack of any difference between stomatal apertures of infested and healthy plants suggests that, although the higher levels of calcium induced by infestation may severely affect the whole plant, there is no apparent behavioural difference in the leaves. The stomata respond normally,

Stomata of control peels of leaves from the nematode-tolerant potato cv. Cara A were found to have reduced apertures (in both healthy and infested plants). This is probably due to the higher levels of abscisic acid known to be present in such tolerant varieties. (Trinder, with Farrokh Fatemy, Nematology Department)

Collaboration with Long Ashton Research Station

Crystals of a naturally-occurring fungicide from infected wood of *Cotoneaster lactea* were supplied by Dr R. S. Burden and Mr M. S. Kemp who had identified the molecule as a dibenzofuran with three methoxy and two hydroxy substituents.

Information from spectroscopic investigation could not distinguish between 20 possible isomers. To obviate the need for each of these to be synthesized, we determined the crystal structure including the location and refinement of all the hydrogen atoms. This established the formula as 2,7-dihydroxy-3,4,6-trimethoxy dibenzofuran. The hydroxy H atoms are close to the molecular plane, and both take part in the H-bonding scheme which forms a three-dimensional network. (Owen)

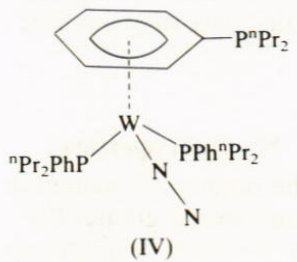
Collaboration with the Unit of Nitrogen Fixation

Three dinitrogen complexes of tungsten were provided by Dr R. L. Richards and their crystal structures determined.

Small, deep crimson-red prisms of a compound, expected to be the bridging dinitrogen complex $[\mu\text{-}N_2\{W(N_2)(PEt_2Ph)_3\}_2]$ (*Rothamsted Report for 1981,* Part 1, 152) were shown to be *trans* $[W(N_2)_2(PEt_2Ph)_4]$ with a disordered tetrahydrofuran of solvation. Steric interaction of the phosphine ligands is relieved by the phosphorus atoms forming a shallow tetrahedron about the tungsten atom. The W—N bonds are 0·1986(6) and 0·1994(6) nm, the N—W—N angle is 179·2(2)°.

Large, bright orange octahedral crystals of $[W(N_2)_2(PMe_2Ph)_4]$ were established as the *cis* isomer. The *trans* phosphorus atoms subtend an angle of 166·3(4)° at the tungsten atom and are bent towards the dinitrogen group having W—N =0·1983(3) nm, while the other dinitrogen group is sandwiched between the two phenyl residues and the W—N length is significantly longer at 0·2015(3) nm.

An air-sensitive compound of formula $W(N_2)$ $(PPh^nPr_2)_3$ was found to have the dinitrogen group and two phosphine groups coordinated to one side of the tungsten



(IV)

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atom and the six atoms of the phenyl group on the third phosphine group on the other to give the 'piano-stool' arrangement, (IV) having W—N 0·1980(8) nm. (Hughes)

Computing

Prime 550. A program BAYES to give better estimates of diffraction intensities with negative nett counts has been written. This uses Bayesian statistics, routines for which were taken from S. French and K. Wilson, *Acta Crystallographica* (1978) **A34,** 517–525. This gives much better E-values for direct methods work, and allows the use of *all* reflections in the least squares refinement.
A program UNIMOL has been written to generate a single connected molecule from atoms which may be in different molecules in the crystal.

VAX 11/750. The molecule drawing program, ORTEP, has been implemented on the VAX, and uses GHOST 80 routines to allow drawing on the Tektronix terminal and the Calcomp 81 flat-bed plotter.

Tektronix 4051. A digitizing pad enables us to measure X-ray diffraction photographs and then the program calculates cell dimensions from these measurements.

PSS gateway. This allows us to access databases at Cambridge and the Edinburgh node of SERCNET, giving quicker response and eliminating character corruption, which we found using the telephone network. R. P. Sharma, AFRCCC, provided us with the recipe for accessing SERCNET. (Owen)

Staff

Mary R. Truter was reappointed Visiting Professor in University College London. In June she lectured in the Universities of Padua and Messina, and presented a poster at the First International Conference on Bioinorganic Chemistry in Florence.

D. L. Hughes presented a paper at the American Chemical Society meeting in Seattle, and attended the 6th West Coast Protein Crystallography Workshop at Asilomar, California in March. With Dr R. L. Richards, he presented a poster at the International Conference on the Chemistry of Chromium, Molybdenum and Tungsten, at Brighton in July.

D. G. Parsons attended the 29th International Union of Pure and Applied Chemistry Congress in Cologne in June and worked in Padua in November. M. P. Payne presented a poster at the 6th International Meeting on NMR Spectroscopy in Edinburgh in July.

Visitors included Professor G. Bombieri (Messina), Dr G. de Paoli (Padua), Professor S. C. Nyburg, University of Toronto and Professor Lydia Vallarino, Virginia Commonwealth University. We are grateful to NATO for support.

PUBLICATIONS

Biochemistry Department

BOOK

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THESES

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