ROTHAMSTED RESEARCH

Report for 1973 - Part1

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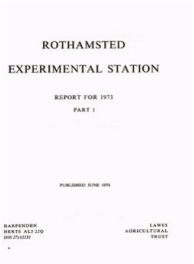

Biochemistry Department

B. J. Miflin

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BIOCHEMISTRY DEPARTMENT

B. J. MIFLIN

This year marked the retirement of the founding head of the department N. W. Pirie. The large leaf-protein extraction plant has been transferred to Reading but, happily, Pirie is continuing to work on protein extraction as a temporary worker in the Chemistry Department. Work on leaf protein extraction in this department has now ceased. However, this year has involved tidying up various aspects of the leaf protein work and these are reported.

Although a change in leadership inevitably results in a reappraisal of the aims of the department, it is not envisaged that any swift and dramatic changes will take place. In the past the department has been concerned, amongst other things, with the problems of the content and quality of protein in plant products and with the biochemical relationship between crop plants and their pathogens. These problems are of continuing importance and we shall still devote a major part of our effort to aspects of them.

The approach to the first problem will be different from that used in the past. We aim to gather information on the enzymology of amino acid biosynthesis in crop plants. We wish to establish how the synthesis of nutritionally essential amino acids is limited by internal regulatory mechanisms and what can be done to override these. We also hope to investigate the flow of nitrogen into the storage proteins of the seed and the means by which this flow is regulated.

Since its inception the department has been involved in work on plant viruses and we shall continue our studies of the biochemistry of plant–virus relationships. It is hoped to expand these in the future. In addition we have started work on biochemical aspects of plant–fungus interactions.

Studies on leaf protein

Chlorophyll breakdown products in leaf protein preparations. Previous work has shown that leaf proteins have no adverse effects when fed to humans and animals. Recently, however, Lohrey, Tapper and Hove (to be published in the *British Journal of Nutrition*) found that rats fed with lucerne leaf protein and exposed to natural daylight developed skin lesions of varying severity up to the sloughing of ears and tails. This photosensitisation was not observed in rats fed on ryegrass (*Lolium perenne*) leaf protein and Lohrey *et al.* suggested that pheophorbide *a* and related pigments were the agents responsible. From our earlier work it seemed likely that the difference between the two species was caused by the difference in chlorophyllase activity. The chlorophyll breakdown products in proteins from a range of species and the chlorophyllase activity of the leaves used were investigated further.

When the juice from lucerne, wheat, rye (*Secale cereale*), sugar-beet and spinach-beet leaves is coagulated by heating rapidly at 80°C the chlorophyll in the extracts is mostly converted to chlorophyllide and a small amount of pheophorbide. The chlorophyllide is further converted to pheophorbide on washing the preparation at pH 4. These species are rich in chlorophyllase and some chlorophyllide was actually formed during maceration. Potato and red clover leaves have low chlorophyllase activity and the juice browned rapidly; about half the chlorophyll was converted into pheophytin during coagulation but little or no pheophorbide was formed. Marrow and ryegrass leaves also have low chlorophyllase activity; the chlorophyll remained practically intact on coagulating the

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protein. These results confirm that pheophorbide formation is related to high chlorophyllase activity.

Chlorophyllase activity and chlorophyllide formation is largely prevented by increasing the coagulation temperature to about 100°C and it was suggested to Hove and his colleagues that they should compare the photosensitising effect of lucerne leaf proteins made at 80° and 100°C. They have recently reported (personal communication) that preparations made at 100°C do not, in fact, cause photosensitisation. It seems, therefore, that a small modification in the processing, which is desirable from several points of view, removes possible adverse effects due to the presence of photosensitising pigments.

Carotene in leaf protein preparations. Leaf protein is a useful source of β-carotene but there was a distinct possibility that during processing much of it might have been converted into other stereoisomers which are less effective as vitamin A precursors. This was checked and it was found that artefactual stereoisomers account for less than 15% of the total carotene in the preparations examined.

The relationships between protein and pigments in leaves. Samples were taken from selected plots on Broadbalk to obtain leaves with different total nitrogen and therefore different protein contents. Wheat leaf samples were taken from section 0 (last fallowed in 1951) and section 5 (last fallowed in 1972); potato leaf samples were taken from section 2. In samples from section 5, as the total N (expressed as % DM) went up from 2·29 to 4·57% with increasing nitrogen fertiliser so the xanthophyll content increased from 0·79 to 1·25 g kg^{-1} and carotene from 0·29 to 0·48 g kg^{-1}. With samples from section 0 the carotenoid difference was much larger whereas the nitrogen increase was smaller. The protein N content was about 87% of the total N for all the plots. Thus, although both the carotenoid and protein N content of the leaves vary in the same manner in response to nitrogen fertilisation, there is no absolute correlation between their relative values. Potato leaves showed less variation than wheat with fertiliser treatment; total N increased by only 20% and the carotenoid levels increased by 47%. (Holden)

Carbohydrates in leaf extracts. The carbohydrate content of the leaf protein preparation and of the residual liquor produced during the extraction of leaf protein from crops has been analysed. The liquor contains the soluble sugars, fructose, glucose and sucrose, and the polysaccharide fraction. This latter fraction is about one-quarter of the total carbohydrate and, for most crops, consists chiefly of fructosans which could be hydrolysed with 0·01*N* H_2SO_4 at 100°C. The residual material, remaining after the hydrolysis products were removed by dialysis, was further analysed. It contained some N and on treatment with 2*N* H_2SO_4 arabinose, galactose, glucose and xylose were released.

The protein fraction was usually free of the soluble sugars found in the liquor but hydrolysis with 2*N* H_2SO_4 yielded galactose, arabinose and xylose, with a little glucose. Pronase treatment of the protein released a polysaccharide fraction which gave the same sugars on hydrolysis. The non-fructosan liquor polysaccharide (the residual material mentioned above) and a polysaccharide preparation obtained from a Pronase digest of red clover leaf protein have been chromatographed on Sephadex G-75: each gave two fractions one of which was eluted in the region of the void volume.

Hydrolysis with dilute acid (0·2*N* H_2SO_4) of the non-fructosan liquid polysaccharide and also of the polysaccharide chain released by Pronase treatment showed that three-quarters of the total pentose was liberated. Arabinose was the main sugar identified. Glycopeptides containing arabinose and galactose, but yielding only arabinose under similar conditions of dilute acid hydrolysis, have been found in primary cell walls. It is

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probable that the polysaccharides in the liquor and protein fractions described here are related to these cell wall components. (Festenstein)

Nutritional quality of leaf proteins. Although all unfractionated leaf proteins have a similar amino acid composition they vary widely in nutritional quality; there can be differences between preparations made from the same species as well as between preparations made from different species. The need for fast processing during extraction to minimise enzymic oxidation of phenols, polymerisation of quinones and similar reactions, has been recognised for some time, as has the use of additives to inhibit such reactions (Arkcoll, *Rothamsted Report for 1970*, Part 1, 116). Dr. M. Fafunso (University of Ibadan, Nigeria) while working at IBVL, Wageningen, has investigated the effects of pre-treating lucerne foliage on the extraction and properties of leaf protein. Protein, extracted from leaves treated with polyvinylpyrrolidone (PVP) (2% of leaf wet weight) at varying pH, and with and without steam treatment before pulping, was sent to Rothamsted for amino acid analysis and *in vitro* digestibility tests.

However, all preparations, irrespective of PVP and/or steam treatment had a similar amino acid profile, which resembled that of normally-prepared unfractionated lucerne protein. From 65–75% of protein N was released as trichloroacetic acid-soluble N from all preparations after a 24-hour digestion (at 60°C) with thioglycollic acid-activated papain: this is within the usual range for unfractionated leaf protein. Thus these treatments do not appear to confer any advantage. (Byers)

Modification of potato viruses by *o*-quinones

During the enzymic oxidation of leaf phenols *o*-quinones (e.g. chlorogenoquinone) are formed. These are extremely reactive and combine with the *N*-terminal groups and those on the side chains of the lysyl residues in viral proteins. Studies in this department and elsewhere have shown that the infectivity of some viruses, modified in this way, is diminished. However, although potato virus X (PVX) reacts with *o*-quinones, the product retains its infectivity suggesting that the virus has reactive groups which are not essential for infectivity.

It was not known how many of the lysyl residues are modified by treatment with chlorogenoquinone and so this has been investigated. When successively more of the 11 lysyl residues are titrated with pyridoxal phosphate or trinitrobenzene sulphonic acid only two to three residues can be modified without affecting the infectivity of the virus. However, these reagents convert the lysine-NH_2 into acidic or much less basic groups; it seemed possible, therefore, that more lysine residues could be modified, without loss of infectivity, by reagents such as imidoesters which retain the positive charge.

Three imidoesters were prepared and tested on PVX but only methyl picolinimidate reacted with the virus under suitably mild conditions. The amount bound was estimated from the changes in u.v. absorption after correction for scattered light. As expected, the modified virus had a similar surface charge to PVX, judged by its electrophoretic mobility, and a decreased tryptophan-fluorescence. Although its infectivity was not completely abolished by extensive modification, it was substantially decreased (>50%) by modification of three or more of the lysyl residues. Inactivation by methyl picolinimidate therefore resembles that by the other reagents and suggests that inactivation is not primarily due to altering the electrical charge of the viral protein.

These results suggest that only one or two of the lysine groups in each subunit of PVX can be modified without substantially decreasing infectivity, and that infective quinone-modified virus (PVX-Q) therefore contains only one or two phenolic residues. This conclusion is consistent with experiments in which the reaction of PVX and PVX-Q

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with pyridoxal phosphate were compared; the phenolic residues do not apparently affect the rate at which up to four lysine residues are modified by this reagent.

Recent work has shown that in contrast to other viruses, modification of potato virus Y by reaction with enzymically-generated chlorogenoquinone increases its infectivity several fold. The spectrum of the re-isolated virus suggests that it has been modified. It is not yet known if the modified virus has enhanced activity, or if it is less susceptible to any denaturation or aggregation that occurs with unmodified virus in the test conditions. (Pierpoint)

Studies on amino acids and amines

Free amino acids in different parts of the pea plant. In connection with the work on synthetic diets for aphids (Griffiths and Greenway, Insecticides and Fungicides Department) various parts of the pea plant were analysed for their non-protein amino acid content. It had been noted that *Acyrthosiphon pisum* (Harris) settled preferentially on the upper leaves and stipules of pea plants, *Aphis fabae* (Scop.) on the veins and tendrils of the middle leaves and *Myzus persicae* (Sulz.) on the lower leaves. Amino acids are important in aphid phagostimulation and it was hoped that information about the free amino acid composition of, and relative amounts in, these different parts of the plant might assist in the improvement of such diets.

Extracts were made from plants at the early flowering stage by exposing the appropriate part of the plant to chloroform vapour and expressing the (chlorophyll-free) extract. There were large amounts of 4-aminobut-1-anoic acid, serine and homoserine in all extracts, more homoserine being consistently found in the middle leaves. However, the most unexpected result was the huge amount of proline present in the upper leaves, accounting for some 20% of the total identifiable amino acids. The accumulation of proline in the non-protein fraction of leaves is often associated with severe water stress (Barnett, N. M. & Naylor, A. W. *Plant Physiology* (1966) **41**, 1222; Singh, T. N. *et al. Australian Journal of Biological Science* (1973) **26**, 46) but although these plants were grown during June in a glasshouse they had been regularly watered and appeared normal.

Following this observation pea plants were grown in a constant environment (28°C); some were watered *ad lib.* and the remainder received minimal watering. More proline was found in the upper leaves of the stressed than in those of well-watered plants (20 and 1% of the total identifiable amino acids respectively) confirming its accumulation in plants receiving insufficient moisture. The deliberately stressed plants looked shrivelled, unlike those grown in the glasshouse, and it is felt that other factors, possibly high temperature and light intensity may be involved in proline accumulation.

So far, diets based on the free amino acid composition of the upper and lower leaves have given poor survival and growth rates with *M. persicae* and *A. pisum*. This may be because the extracts do not resemble the composition of the plant fluid upon which the aphids are feeding, and further work may depend on success in obtaining samples of 'stylet sap'. (Byers, with Greenway, Insecticides and Fungicides Department)

Oxidation of complexes of amino acids with transition metals catalysed by peroxidase. It has already been shown that some peroxidase-catalysed reactions can cause oxidation of amino acids. Amino acids complex with transition metals; some of these complexes have distinct absorption spectra. The effect of peroxidase and peroxidase-coupled reactions on these complexes has been examined spectrophotometrically. Peroxidase only affected the complexes of tryptophan with Co^{2+} and histidine with Co^{2+}, Fe^{2+} and Mn^{2+}. There was a slow rise in the absorption at 360 nm of the spectrum of the tryptophan-Co^{2+}

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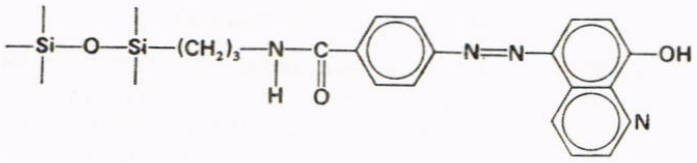
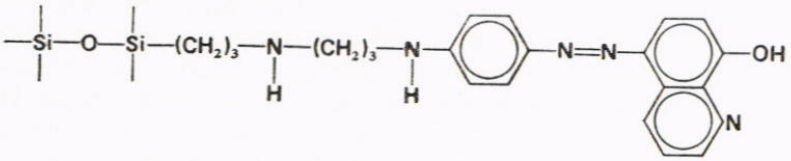
complex and at 340 nm and 360 nm respectively with histidine-Mn^{2+} or -Fe^{2+}. There was rapid formation of a new absorption band at 380 nm in the histidine-Co^{2+} spectrum similar to that formed more slowly when the oxygenated *bis*-cobalto-dihistidine complex ages in air. This suggests that a valency change of the cobalt might be involved but the products of the reaction were not identified.

Affinity chromatography of diamine oxidase. Last year it was reported that the 8-hydroxyquinoline derivative of silica gel



(HOQ I)

could be used to remove selectively transition elements from aqueous solutions but did not selectively adsorb metalloproteins. Another 8-hydroxyquinoline derivative of silica gel has now been prepared with increased chain length



(HOQ II)

which adsorbs the copper-containing diamine oxidase from pea seedlings; the adsorbed enzyme remained active. All attempts to desorb this active enzyme, including changing the pH, eluting with the substrate putrescine or with Zn^{2+} and some other metal ions, and changing the salt concentration, have been unsuccessful. An inactive protein was recovered when this gel containing adsorbed diamine oxidase was treated with solutions of Cu^{2+} ions or with *N* HCl. The precursors of the HOQ II derivative of silica gel and some of the intermediate by-products produced during its formation have been tried as adsorbents of the diamine oxidase. With the exception of the diazonium salt formed immediately before the coupling of the 8-hydroxy-quinoline, all showed some absorption in neutral solution which could be reversed by changing the pH. The diazonium salt, as expected, also coupled to the enzyme and this could not be reversed by changing the pH, eluting with *N* HCl or with Cu^{2+} ions, although the protein could be removed by reduction with aqueous sodium dithionite. The binding of the diamine oxidase to the modified silica gel is therefore considered to be due to its reaction with the HOQ II derivative probably *via* the copper of the enzyme. If this is so, the fact that the diamine oxidase retains enzymic activity when bound suggests that, although this copper is essential for the activity of the enzyme, it is probably not at the active catalytic site but may be involved in maintaining the essential tertiary structure of the protein. (Hill)

Staff and visiting workers

Bill (N. W.) Pirie retired as head of the department on 31 March. He was appointed by Sir John Russell in 1940 as Virus Physiologist, with the understanding that this was to be interpreted as Biochemist. He became head when the Biochemistry Department was founded in 1947. Before coming to Rothamsted he had worked in the Biochemistry

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Department at Cambridge and had started to collaborate with F. C. Bawden on problems concerned with plant viruses. This work led to their election to the Royal Society in 1949 and continued until Bawden's death in 1972. Bill gave the Leeuwenhoek Lecture to the Royal Society in 1963 and was awarded the Copley Medal in 1971. He was elected a Fellow of the New York Academy of Sciences in 1963.

He has devoted much time and effort to advocating the use of green leaves as a source of protein for human feeding. His arguments, no less than the machinery he has designed, form the basis of the pilot-scale protein plants now operating in several developing countries. In addition to his research papers he has commented cogently and lucidly in many articles and reviews on subjects ranging from World Nutrition to Scientific Semantics and the Origin of Life. He spent a year in 1946–47 in the USA at the Worcester Foundation for Experimental Biology working on the biochemistry of animal contraception. A two-year grant from the Potato Marketing Board enables him, now attached to the Chemistry Department, to continue protein extraction studies.

Margaret Holden was acting Head until the arrival of B. J. Miflin in September. D. B. Arkcoll resigned early in the year. During the year Helen Pratt and R. M. Wallsgrove were appointed and P. Lea, M. Wade and E. Major came as temporary workers.

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