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ROTHAMSTED RESEARCH

Report for 1969 - Part 1

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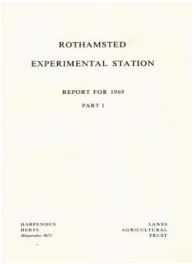
ROTHAMSTED
EXPERIMENTAL STATION
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PART 1

Biochemistry Department

N. W. Pirie

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BIOCHEMISTRY DEPARTMENT

N. W. PIRIE

Lawes and Gilbert established biochemistry at Rothamsted with pioneer research on the interconversion of fat and carbohydrate in pigs, and on the dependence of nitrogen excretion on protein intake rather than on the amount of work that was being done. After their deaths, biochemical work diminished; when it became active again it was at first mainly directed towards a study of plant viruses and phenomena connected with their extraction and purification. Then other aspects of plant metabolism preponderated but our interest is again veering towards viruses and we are trying to interpret some of the phenomena encountered in the study of infection in terms of the known metabolic behaviour of the leaf.

The 'age' of a leaf is difficult to define because physiological age depends not only on the time that has elapsed since germination, but also on environmental temperature, nutrition and other aspects of husbandry. In tall plants, such as tobacco, the newly formed leaves towards the top of an old plant obviously differ in shape and surface texture from leaves on young plants. Metabolic differences would be expected between these leaves and those further down the plant. Striking differences in the behaviour of tobacco mosaic virus (TMV) in superficially somewhat similar leaves are less expected. Extracts, made in the presence of phenol, from the middle leaves of an infected tobacco plant are infective, whereas similar extracts from the lower leaves are not. When phenol is withheld until after the leaves have been pulped, there is little or no difference in the behaviour of leaves from different positions on the plant. Uninfected leaves to which purified TMV has been added exhibit all these phenomena. We are exploring the possibility that this phenomenon can be explained by differences in the state of ribonuclease in leaves of differing age.

Leaf age also affects the multiplication of viruses and the concentration of leaf components that react with them. Thus the synthesis of TMV is less dependent on illumination in older leaves and the effect of an inhibitor of photosynthesis is also less than in younger leaves. As tobacco leaves age, their polyphenol-oxidase activity increases and so does the concentration of metals such as copper and iron that are often associated with oxidations. In the *Rothamsted Reports for 1960–63* difficulties encountered in purifying cucumber mosaic virus were interpreted as the consequence of reactions with the products of oxidation of chlorogenic acid; means to prevent this were described. The protein units in TMV contain groups that can react with the products of oxidation of chlorogenic acid but the intact virus seems not to react with them. The manner in which these groups become reactive as TMV is disrupted sheds some light on the chemical architecture of this very stable virus. For a virus to be able to accumulate to the concentrations attained by TMV it must not only find sites and substrates at which and from which it can be synthesised, but also avoid destruction by the enzymes of the host and the formation of inactive complexes with other host components.

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Work on the conditions in which chlorophyll bleaches in leaves continues, but work on the properties of chlorophyllase ended. The use of diamine oxidase to measure small quantities of copper is being studied. Evidence is presented that the oxidation of linoleic acid in leaves is an enzyme reaction and not, as had been claimed, caused by haematins: conversely the action of cytochrome oxidase can be simulated by anionic detergents.

Improvements in extraction equipment, and increasing skill in using it, prompted us to reconsider early harsh judgements on some crops as potential sources of leaf protein. In this dry year, cocksfoot yielded 1·8 tons (dry weight) of extracted protein per hectare; the wheat : mustard succession, which yielded more than 2 tons in 1968, yielded less this year.

Viruses

The infectivity of extracts from TMV-infected plants. The realisation that part of the ribonuclease in tobacco leaves diffuses through dialysis membranes and is not inactivated by phenol (*Rothamsted Report for 1968*, Part 1, 114) established a framework on which to hang attempts to explain the great differences in infectivity between extracts made in different ways from infected leaves. It is still not certain that ribonuclease is the only, or even the main agent involved but the conditions necessary for getting repeatable results have now been defined. Extracts made with phenol : water mixtures from the upper and middle leaves from infected plants have similar infectivities, regardless of the sequence of events during the extraction. However, when the lower leaves are pulped with phenol and water, the final dialysed extract is much less infective than an extract made by adding phenol to already pulped leaves, as mentioned in the *Rothamsted Report for 1960*, 112. The new development is that, by using lower leaves only, it is easy to get 100 or even 1000-fold differences in infectivity. The phenomenon is also shown by infected leaves that have been stored at −15°C and by the lower leaves of uninfected plants to which purified TMV is added. Diffusates from extracts made by pulping infected or uninfected plants in the presence of phenol inactivate TMV nucleic acid more strongly than extracts made by pulping first and then adding phenol; it is reasonable to suspect that this is a ribonuclease action, but the nature of the change which prevents injurious amounts of ribonuclease from appearing in the second type of extract is still obscure. This work seems relevant to the behaviour of TMV *in vivo* because the diffusible form of ribonuclease is perhaps the one naturally present in cytoplasm or organelles and it may be being protected from 'autolytic' changes by the presence of phenol. (Pirie, in collaboration with Bawden)

TMV multiplication in the light and dark. Light affects the susceptibility of plants to infection and more TMV is formed in inoculated tobacco plants that are kept in the light than in those in the dark. To investigate more closely how light affects virus multiplication, discs from infected tobacco leaves were used. In all experiments discs were kept for 48 hours in the light to get infection well established.

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Plants 5 or 6 weeks old have two prominent leaves. The lower is rounder, thicker and less veined than the upper. The upper leaf, at both ages, formed less virus in the dark than in the light in experiments lasting 24–72 hours. This was also true of the lower leaves of 5-week-old plants but illumination made no difference to the amount of virus formed in the lower leaves of 6-week-old plants.

Virus formation is presumably affected by changes in illumination because of changes in the concentration of the intermediate substances (possibly ATP) formed during photosynthesis. Last year the effects of suppressing photosynthesis by keeping illuminated leaves in an atmosphere free from CO_2 were described. The suppression was incomplete because CO_2 is being formed within the leaf by photorespiration. It has now been found that discs kept under a stream of nitrogen are still able to synthesise some TMV provided that they are illuminated. This suggests that either some photosynthesis occurs even under these conditions, or that a 'cyclic' photophosphorylation, without CO_2 fixation, supports some virus synthesis.

Photosynthesis can be completely inhibited by the herbicide 3-(3 : 4 dichlorophenyl)-1,1-dimethyl urea (diuron or DCMU), which was used to analyse further the difference between the synthesis of virus in the light or dark. As would be expected, DCMU decreases the amount of virus formed aerobically in the light so that it equals the amount produced in the dark; in discs from lower leaves, where the same amount of virus is formed in the light or dark, it has no effect. Preliminary experiments suggest that DCMU completely inhibits the synthesis of virus that occurs in illuminated discs kept under nitrogen. This implies that there is some photosynthesis in these anaerobic conditions, possibly using CO_2 liberated in the leaves by decarboxylation reactions, and the effect of light is not simply to produce ATP by the 'cyclic' photophosphorylation process.

The formation of intermediates can also be influenced by 2 : 4 dinitrophenol (DNP), which uncouples repiration from oxidative phosphorylation. At 10^{-4} M it can double respiration and depress virus formation to half or less in the dark, but has much less effect on virus formation in the light. Although this effect of light has not been explained, light is known to diminish the effect of DNP on other metabolic processes.

TMV multiplication and leaf respiration. Virus synthesis would be expected to deplete tobacco leaf discs of respirable substrates more in the dark than in the light. This would explain why infected leaf discs respire less after incubation in the dark than in the light (*Rothamsted Report for 1968*, Part 1, 117). Some respirable substrates, e.g. reducing sugars, decrease during incubation of the discs in the dark, but these decreases also occur in uninfected discs and there is no evidence that they are accelerated by infection.

Infected discs continuously illuminated respire 10–20% faster than uninfected discs between 24 and 100 hours after inoculation, when virus is being formed fastest. They then respire less than uninfected ones. These results, expressed on a dry weight basis, disagree with earlier reports on

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the effect of TMV on the respiration of intact leaves, and resemble closely the effects of other viruses on leaf respiration. (Olsson)

Reaction of o-quinones with viruses. The o-quinones, formed in leaf extracts by the enzymic oxidation of polyphenols, react with leaf proteins and modify their properties. Previous studies of the *in vitro* reactions of chlorogenoquinone, the quinone derived from chlorogenic acid, were extended to its reactions with plant viruses, especially tobacco mosaic virus. Although its stability and ease of purification argue against TMV reacting with leaf components, each of its protein sub-units contains three groups, two lysine ϵ-amino and one cysteine thiol, which would be expected,under appropriate conditions, to react with quinones.

TMV was extracted from tobacco leaves anaerobically, to prevent the formation of quinones. Its absorption spectrum, electrophoretic mobility and its NH_2 content, all properties that would be affected by combination with quinone, were not measurably different from those of TMV prepared aerobically. Moreover, when the purified virus was exposed to a large excess of chlorogenoquinone, none of these properties was affected; nor was there a loss of virus assessed either by infectivity or by measuring the amount eluted characteristically from a column of Sepharose. When care was taken to remove contaminating bacteria, TMV did not affect either the amount of oxygen absorbed in the oxidation of chlorogenic acid or the colour of the products. This result, also arguing against any reaction with chlorogenoquinone, was obtained at different pHs, and also with the U_2 strain of TMV, which has a terminal amino group.

The amino and thiol groups of TMV protein depolymerised by dilute alkali also seem inaccessible to quinones. After treatment with a ten-fold excess of chlorogenoquinone, the protein could still polymerise in the appropriate conditions of pH and ionic strength. Moreover, the resulting polymerised rods, when separated from the quinone condensation products, had the absorption spectra of chemically unmodified TMV protein. There is, however, a little evidence that TMV protein completely denatured by heating in a salt free solution, reacts with chlorogenoquinone; when it is precipitated with salt and washed well with buffer, it still retains some brown phenolic material. Additional evidence is needed to confirm that this is chemically bonded to the protein and not just physically absorbed.

The small reactivity of the amino and thiol groups of TMV with chlorogenoquinone is shown with the synthetic reagent 2 : 4 : 6 trinitrobenzene sulphonic acid (TNBS); at neutral pHs and colder than 40°C, there is little or no reaction. Reaction occurs only in conditions where TMV denatures, and to obtain what is probably maximal reaction, the virus must be preincubated at 40°C, at pH 9 and in the presence of sodium dodecylsulphate. In these conditions the colour produced from both the ordinary strain and from U_2 can be accounted for by their known amino acid composition.

The small reactivity of the amino and thiol groups of TMV towards o-quinones and TNBS contrasts greatly with the behaviour of bovine serum albumin. This difference supports the suggestion that, in native TMV, these groups are either 'buried' in hydrophobic regions of the

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protein, or protected by some complexing with the side chains of the amino acids. This arrangement undoubtedly contributes to the stability of TMV in leaf extracts, and helps explain the ease with which it can be isolated. (Pierpoint)

Enzymes

Leaf lipoxidase. The assumption that the peroxidation of linoleic acid by plant extracts was catalysed by lipoxidase was challenged by Blain, Patterson and Pearce (*J. Sci. Fd Agric.* (1968) **19**, 713), who suggested that, except in seeds, it is caused by haematin compounds. The leaves of etiolated wheat seedlings cv. Cappelle, which are particularly active in peroxidising linoleic acid, were studied in detail to determine whether haematins or a lipoxidase are responsible. For some experiments green wheat leaves were used and also extracts of other plants that have moderate or large fatty-acid peroxidising activity. Leaf extracts, but not haematin compounds (cytochrome *c*, haemin, haemoglobin), peroxidised linoleic acid that was free from hydroperoxides. Boiled leaf extracts had no peroxidising activity whereas solutions of haematin compounds lost little activity on boiling. The pH activity curve for leaf extracts differed from that of haemoglobin. Leaf extracts and soya lipoxidase were more sensitive than haematins to ethanol in the reaction mixture. More haematin was needed for peroxidation of linoleic acid under the test conditions than the amount present in most plant extracts. Linoleic acid peroxidising activity is widely distributed in leaves (*Rothamsted Report for 1966*, 101) and all the evidence suggests that it is caused by a lipoxidase-type enzyme. (Holden)

Ribonuclease. Most of the ribonuclease activity that withstands treatment with phenol diffuses from intact tobacco leaves that have been robbed of their osmotic control by freezing or exposure to water saturated with phenol. Also most of the activity in these extracts diffuses through cellophane. During purification by extraction into the phenol layer of a phenol : water system, precipitation with ammonium sulphate, removal of contaminants with diethylaminoethyl cellulose, and fractionation on Sephadex, diffusibility is lost and the activity is finally completely retained during dialysis. The obvious interpretation, that a very small 'native' enzyme is aggregating during purification, is borne out by comparisons between crude and purified preparations during gel-filtration on Sephadex. On a dry weight basis the best preparations are about 1000 times as active as the original phenol-saturated diffusate when activity is measured by the inactivation of TMV : RNA. On an E_{280} basis, the best preparations are about 200 times as active as the original extract in their ability to hydrolyse highly polymerised yeast RNA *in vitro*. Though stable to heat and phenol, purified preparations are sensitive to atmospheric oxidation, especially when dry, and activity is lost on boiling in acid solution.

The sap from lower leaves has twice the ribonuclease activity of sap from upper leaves. About one fifth of the total activity is not destroyed or coagulated by phenol whatever the age of the leaf. The differences between upper and lower leaves reported in the section on viruses cannot

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therefore be explained simply by differences in their content of stable ribonuclease. The activities of extracts made by adding phenol to leaves pre-treated in different ways are being compared. No large differences in such properties as pH optimum and inhibitability by metal ions have yet been found between the phenol-stable and -unstable enzyme. Phenol-stable enzyme occurs in the leaves of some other plants. (Jervis and Pirie)

Copper determination with copper-free diamine oxidase. The specific reactivation of copper-free diamine oxidase by Cu^{2+} ions was adapted to measure nanogram amounts of copper. The reactivation of the copper-free enzyme at pH 7 in phosphate buffers is inhibited by EDTA although this chelating agent does not inhibit the reactivated enzyme. Solutions containing less than 10 ng Cu^{2+} are incubated with copper-free pea-seedling diamine oxidase for 15 minutes; further reactivation is prevented by adding Na_2 EDTA pH 7, and the activity of the reactivated enzyme determined polarographically, manometrically or, more usually, spectrophotometrically. The method gives linear calibration curves from 0 to 10 ng Cu^{2+} and is 100 to 1000 times more sensitive than the colorimetric methods usually used to determine copper. This enzymic method is not significantly affected by the ions common in acid digests of plant materials, although large amounts of ammonia interfere.

Modification of cytochrome *c* by anionic wetting agents. Cytochrome oxidase activity is often determined polarographically or manometrically by coupling the oxidation of cytochrome *c*, to that of ascorbate, using N : N : N′ : N′-tetramethyl-p-phenylenediamine (TMPD) as the intermediate electron carrier. In this system cytochrome *c* and TMPD are alternately oxidised and reduced and need only be present in small amounts in the reaction mixtures. In the presence of small amounts of sodium dodecyl sulphate (SDS) or other anionic detergents (e.g. sodium dioctyl sulphosuccinate) and in the absence of cytochrome oxidase, cytochrome *c* will catalyse the aerobic oxidation of ascorbate, provided TMPD is present as the electron carrier. Oxygen is only taken up in this 'non-enzymic' system when all of the four components (ascorbate, cytochrome *c*, TMPD, or anionic detergent) are present. Non-ionic detergents, e.g. Triton X-100, Triton X-315, and Tween 20, cannot replace SDS but the cationic detergent, cetyl methyl ammonium bromide, competitively inhibits the action of SDS. Spectrophotometric changes in the Soret band of cytochrome *c* induced by SDS suggest that this detergent changes the cytochrome *c* structure, and that these changes may be responsible for this new catalytic activity of cytochrome *c*. (Hill)

Large-scale protein preparations

Fractionation of protein in leaf extracts. The chloroplasts and their fragments in extracts from young barley leaves sediment readily at 1600 g. Sedimentation becomes more difficult as the plants age, but all the pigmented protein sediments at 50 000 g leaving 25–30% of the total protein in the extract still in solution. When precipitated with trichloroacetic acid

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(TCA), this 'cytoplasmic' protein contains little ash, carbohydrate or lipid and is more than 95% digestible by papain.

There are small but definite differences between the amino acid compositions of whole extract barley protein, the chloroplastic sediment and the 'cytoplasmic' fraction. The percentage of tyrosine, lysine, histidine and arginine increase, and serine, glutamic acid, glycine, alanine and leucine decrease, as the precipitated protein becomes increasingly free from chloroplasts. These differences are too small to account for the nutritional differences between 'chloroplastic' and 'cytoplasmic' fractions. It is the availability, rather than the total amount of each amino acid, which determines the nutritive value and digestibility of a product. Therefore, in collaboration with Dr. J. E. Ford, NIRD, the 'availability' of several amino acids was determined microbiologically. In Table 1 the 'available' is expressed as a percentage of the 'total' for six essential amino acids in four barley leaf fractions.

TABLE 1

'Available' amino acids expressed as percentage of 'total' in some preparations made from barley leaf extracts

	TCA-ppt of whole extract	1600 g sediment	TCA-ppt of 1600 g supernatant fluid	TCA-ppt of 50 000 g supernatant fluid
Methionine	93	37	87	100
Leucine	63	28	75	90
Iso-leucine	72	31	74	86
Histidine	45	23	43	80
Valine	89	40	93	—
Arginine	71	35	76	86

Properties of unfractionated preparations. Freeze-dried preparations, made at Rothamsted and selected to cover a wide range of plant species and processing conditions, contained from 50–65% protein and were from 71–76% digestible. Except for lysine, the percentages of all other amino acids in the 9 preparations analysed so far were similar. Some clue to the variation in lysine content (which ranges from 6·2 to 7·3 g per 100 g total amino acids) comes from analyses on two wheat proteins. These were made from the same batch of juice but one preparation (A) was precipitated immediately, separated and washed within 10 minutes, whereas the other (B) was precipitated after standing for 2 hours, then allowed to stand 12 hours in its own liquor before finishing the isolation. Preparation (B) contained about 10% less lysine than (A), indicating that quick processing may be advantageous. Also, whereas about 30% of the methionine of (B) appeared as methionine sulphoxide (metO), only 18% did in (A). These amounts of metO have not been found in hydrolysates of laboratory-prepared, TCA-precipitated, proteins; metO is therefore probably formed during processing rather than hydrolysis. If this is correct, it could have important nutritional implications as the nutritive value of metO is uncertain. 'Available' methionine results, expressed as a percentage of the total are inconsistent. Cocksfoot grass protein, with the smallest lysine and greatest metO content, has least available methio-

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nine (68%) but there is only a small difference between the slow and quick processed wheat proteins (88% and 94% respectively). Whole extract barley protein also had about 90% available methionine. Seven whole-leaf proteins made at the Central Food Technological Institute, Mysore, whose nutritional value had already been measured there, were analysed for their amino acid content and their *in vitro* digestibility with papain. The amino acid composition fell within the range found for preparations made at Rothamsted, except for methionine. This was smallest in samples with a large carbohydrate content and could well be the result of degradation during hydrolysis. Available methionine, estimated microbiologically, ranged from 36–78% of the total methionine and was most in those preparations with the greatest *in vitro* digestibilities. (Byers)

Yields and new species. Because of the dry summer we did not equal last year's production record of 2 tons of extracted protein per hectare, from a cereal followed by mustard or fodder radish, but Cocksfoot grass gave 1·8 tons. With irrigation and more experience 2 tons should be easily achieved. Hitherto we considered cereals and broad-leaved plants better sources of leaf protein than grasses, because the grasses tend to be tougher and were not disintegrated effectively by the original types of pulper. Recent improvements in design have made pulping more efficient and in some circumstances, grasses can now be satisfactory protein sources.

Protein is poorly extracted from some soft and easily pulped leaves (*Rothamsted Report for 1966*, 105). Experience with the extraction of viruses from plants such as strawberry and raspberry suggests that the protein may not extract because polyphenols and other tanning agents are liberated and precipitate the protein as soon as a leaf is macerated. Neutralised commercial tannic acid precipitates most of the protein from extracts of wheat and cocksfoot. Measurements of the tannin content of Spanish trefoil (*Desmodium uncinatum*) show that it contains enough to account for the poor extraction from what would otherwise be an attractive source of protein in the tropics. We have often considered the possibility of using perennial crops such as coppiced trees. Protein extracts satisfactorily from elder (*Sambucus nigra*) but not from sycamore (*Acer pseudoplatanus*), which contains much tannin. (Arkcoll and Festenstein)

Appearance and quality. Many traditional foods are considered attractive in spite of, or because of, oxidative and other changes undergone during processing. A novelty such as leaf protein is considered more attractive when these changes are avoided. Delay between pulping and pressing, pressing and coagulation, coagulation and filtering, or filtering and washing, all promote changes that lead to dark-coloured products with a pronounced 'leafy' flavour. These changes are easily avoided on the laboratory-scale, and it will be easy to organise rapid separation in continuous commercial production, but it is more difficult with the equipment we have and on the intermediate scale on which we work. Therefore, for the time being, we have to be reconciled to making products that are less attractive than they could be.

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The moist protein press-cake is at pH 4 and keeps at room temperature for several days. It will keep for longer when there is 2% acetic acid in the water remaining in the cake but moulds ultimately grow. The flavour can be interestingly modified by seeding with *Aspergillus oryzae*, and it should be possible to introduce a useful amount of vitamin B_{12} by seeding with *Streptomyces*.

New and improved equipment. Laboratory-scale extraction units of the type designed for the International Biological Program were sent to Nigeria, Ceylon, Eire, India (Calcutta), New Zealand and Sweden (2), in addition to the countries named last year. A stainless steel IBP pulper was made to minimise the introduction of iron into the product. This will be used to study the stability of carotene in extracted leaf protein because, if carotene could be preserved, 3 or 4 grams of protein from some leaves would supply the human day-dose of Vitamin A. Though it was designed as an agronomic tool, the IBP pulper is so satisfactory that a continuous belt-press was made to match it. The unit can handle 100 kg of leaf an hour and supersedes the old 'village unit'.

The good performance of the IBP pulper led us to reconsider the basic features of the large-scale pulper and to realise that it still retains some unnecessary or unsuitable features of the Christy and Norris 'coir sifter' that was installed in 1948. Total reconstruction is not feasible but the rotor was rebuilt on what we hope is an improved plan.

A full size version of the IBP pulper was designed that should be cheaper than the present large-scale pulper and have the same capacity, but there are no present plans to build it. (Davys and Pirie)

Pigments

Chlorophyll bleaching systems in leaves

Bleaching in solutions of surface active agents. Chlorophyll is bleached enzymically in leaf discs or chopped leaves suspended in aqueous acetone in the dark (*Rothamsted Report for 1966*, 101). Assuming that the role of the acetone is to loosen substrates and bring them into closer contact with enzymes, some surface-active compounds, were tested as substitutes for the solvent. Triton X-100 and Tween-20 (non-ionic), CTAB (cationic) at pH 6·5 and dimethylsulphoxide did not have the same effect as acetone. The anionic detergents, Manoxol OT (sodium dioctylsulphosuccinate) and sodium lauryl sulphate, caused some bleaching. However, although the pH did not fall below 6·2, these detergents caused enough pheophytin to be formed to account for the loss of absorption of pigment-containing extracts.

Some factors affecting chlorophyll breakdown in detached leaves. Growth substances often delay leaf senescence and the disappearance of chlorophyll in detached leaves. Peptone delayed the breakdown of chlorophyll in the dark in leaf discs from eight species of *Rumex* (Reynolds, *Nature, Lond.* (1969) **233**, 505). *Rumex* responds to gibberellins and it was of interest to

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test whether peptone affects other species that respond to these and also species that respond to kinins. Discs from *Rumex obtusifolius* lost their chlorophyll slowly, even at 37°C, and bacterial contamination, especially with peptone present, complicated some of the results. However, peptone (1%) clearly delayed chlorophyll breakdown, not only in *Rumex*, but also in dandelion and nasturtium, which are also gibberellin sensitive. With some kinin-sensitive leaves, such as oat and brussels sprout, peptone retarded the disappearance of chlorophyll but not with others, such as wheat and barley.

Kotaka and Krueger (*Pl. Physiol.* (1969) **44**, 809) stated that EDTA protected chlorophyll in barley leaves from breakdown in the dark, but speeded bleaching in the light. The effect was confirmed with wheat and bean leaves. EDTA is unstable when illuminated in the presence of flavin coenzymes and the bleaching might be caused by a breakdown product such as formaldehyde. Bleaching was also accelerated when the leaves were floated on acetate solutions in the light but not on citrate solutions of the same pH values. Neither buffer affected chlorophyll breakdown in the dark.

Earlier experiments showed an effect of glycollate on chlorophyll bleaching in barley leaf extracts. Discs of bean leaves and chopped wheat leaves floated on sodium glycollate bleached rapidly in the light, but not in the dark.

Studies on leaf pigments

(a) Some plants of *Epipactis sessilifolia* (violet helleborine) that seemed chlorophyll-free were examined. They contained only 0·2% of the chlorophyll present in normal green plants of this species, 1% of the carotene, 5% of the lutein and traces of violaxanthin and neoxanthin. Normal helleborine plants depend partly on a mycorrhizal association for their nutrition. The aberrant plants were only about half the size of the green ones and seem to have become entirely saprophytic.

(b) Large amounts of xanthophylls were needed for continuing the study of the relationship between these pigments and growth substances, and a new method was developed to remove the chlorophylls before separating the individual xanthophylls by chromatography on cellulose. Leaves were ground with half their weight of polyethylene powder in 70% aqueous acetone and filtered. The clear filtrate was yellow, as the chlorophylls were adsorbed on the polyethylene, which was removed with the leaf debris. This method is quicker than saponifying with methanolic potash and the pigments are not exposed to alkali which may be one of the factors responsible for the frequent appearance of violaxanthin degradation products. (Holden)

(c) The fluids after precipitating protein from extracts of sycamore (*Acer pseudoplatanus*) maple (*Acer campestre*) and Spanish trefoil (*Desmodium uncinatum*) with TCA are purple. The colour is discharged by alkali but reappears more weakly on acidifying. The pigment seems to be formed enzymically because it is not present in extracts made by grinding the leaves directly in 80% ethanol or 5% TCA. The absorption maximum at

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570–580 nm suggests that it is not related to the anthocyanins. (Festenstein and Smith, in collaboration with King)

(d) Oestrogenic isoflavones have caused reproductive disturbances in animals fed on the clovers. A method developed for determining the individual isoflavones depends on extracting them with solvents, then passing an ammoniacal solution through a Sephadex column and measuring the ultra-violet absorption of successive fractions. Washed leaf protein from red clover (*Trifolium pratense*) contains 5–10% of the isoflavone present in the original leaf and the fibre residue contains 15–20%. (Festenstein and Glencross, in collaboration with King)

Metals

Distribution of copper in clover seedlings. The amount of copper, expressed as a percentage of the dry matter, in the roots of clover seedlings increases up to 6 weeks after germination. Thereafter, though the total amount of copper per plant increases, the percentage does not. The leaves, petioles, crowns and roots of 3-month-old plants contain respectively 37, 31, 12 and 20% of the dry matter and 35, 6, 10 and 48% of the copper.

The possibility was considered that part of the large amounts of copper and manganese present in the roots of clover seedlings could be adsorbed on the surface. Roots washed with 0·01 *M* Na_2 EDTA or 0·1 *N* hydrochloric acid for 30 minutes released respectively 8% and 35% of the copper. This suggests that little is adsorbed at the root surface: the hydrochloric acid probably changes the structure of the epidermis and allows increased diffusion of copper from the internal parts of the root. This conclusion is supported by a histological examination of roots. Roots of 3-month-old clover plants were irrigated with a 1% solution of diethyldithiocarbamate (DIECA). This forms a yellow, insoluble copper complex but little or no yellow colour developed in the root caps, epidermal cells or root hairs. The apical meristem was stained yellow, and the colour was less intense the further cells were from the meristem; little staining was seen in cells more than 2–3 mm from the meristem. Root nodules also developed a yellow stain but there was little staining in the cells adjacent to the nodules. These histological results suggest that much of the copper in roots is concentrated at the sites of active metabolism and attempts are now being made to determine the copper concentration in these areas.

When clover roots were ground with phosphate-citrate buffer at pH 7 and centrifuged at $10^4 \times g$ for 30 minutes, 49% of the initial copper, 87% of the manganese and 53% of the nitrogen remained in the supernatant phase. The supernatant phase was separated into two fractions on Sephadex G-100 columns. The first fraction was almost colourless and contained 15% of the initial copper, 16% of the manganese and only 7% of the nitrogen present in the original homogenate. The second fraction was yellow and contained 41% of the original copper, 74% of the managanese and 47% of the nitrogen. Attempts are now being made to characterise the first fraction further because it has a much larger Cu : N and Mn : N ratio than either the original homogenate or the second fraction.

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The copper-free apoenzyme of pea-seedling diamine oxidase (Diamine : Oxygen oxidoreductase (deaminating), E.C. 1.4.3.6) is specifically reactivated by Cu^{2+} ions. Homogenates of clover roots prepared with phosphate buffers would not reactive the copper-free diamine oxidase. These homogenates did not inhibit the holo-pea-seedling diamine oxidase, suggesting that the copper in the homogenates was not present as free Cu^{2+} ions. (Hill)

Staff and visiting workers

M. F. Bacon resigned and L. Jervis was appointed. Dr. G. M. Wallace (Faculty of Food Science and Biotechnology, Massey University, Palmerston North, New Zealand) and Dr. R. M. Allison (Plant Chemistry Division, D.S.I.R., Christchurch, New Zealand) spent several months here learning the use of the IBP extraction unit bought by the New Zealand D.S.I.R., and studying the separation of lipids from leaf protein. Dr. F. B. de Jorge, from Lysoform (São Paulo, Brazil), which is considering leaf protein production, came for a month to study the different types of equipment we have and advise his company. We had shorter visits from Dr. O. L. Oke (Ibadan) and Mr. C. P. Gnacadja (Dahomey).

N. W. Pirie attended the meeting of the committee on the IBP of the Commission on Operational Programs of the International Union of Nutritional Sciences in Belgrade and the symposium on 'World Food Supply' organised in conjunction with the International Botanical Congress in Seattle.

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