ROTHAMSTED RESEARCH

Report for 1970 - Part1

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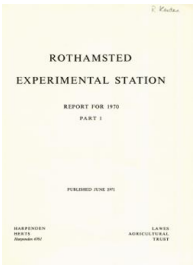

Biochemistry Department

N. W. Pirie

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BIOCHEMISTRY DEPARTMENT

N. W. PIRIE

Large-scale work on the extraction of leaf protein started in 1940 as a war-time project, but was discontinued when 'lease-lend' food began to arrive. It was resumed in 1947 with a grant from the Agricultural Research Council, in cooperation with the Grassland Research Station, then at Drayton. The state of the work was reviewed in the *Rothamsted Report for 1952* and equipment was installed here soon after. For the next 10 years we were mainly concerned with the basic principles on which efficient pulping and pressing depended and could devote little attention to agricultural and other factors that influence the quantity and quality of the end product. As the design of equipment, and our skill in handling it, have improved, we have been able to give more attention to these factors, with the result that, as recorded in recent *Rothamsted Reports*, we usually get about 2 tons of extracted protein from a hectare in a year, with a nutritional value better than that of any of the seed proteins, and acceptable by people who are familiar with it.

Official support for the idea of using leaf protein as a human food waned and our work could not have continued without support from the Rockefeller Foundation and the Wolfson Foundation. With the passage of time, the idea has become more familiar and accumulating evidence on yield and quality is overcoming the initial prejudices against it. There is now work connected with the bulk production of leaf protein in 12 countries and it is being planned in others. In some countries, notably India, Sweden and U.S.A. several different institutes are involved. Commercial interest develops surprisingly slowly, perhaps because of the essential simplicity of the process—no important step in it seems to be patentable, but is now growing. In Sweden the main commercial interest is in making equipment, in Hungary and U.S.A. it is producing animal feed, in Britain it is in the economical way in which the fibre residue, from which part of the protein has been extracted, can be dried. From the outset we have stressed that this is a process yielding three products—protein, fibre and 'whey'—and that each must be used. Failure to realise this has led to some fallacious economic argument.

In spite of this world-wide interest there is still work to be done at Rothamsted. Perfecting machinery can perhaps be left to industry now that the principles are established. Our studies of the most suitable crops and how best to grow them must be looked on as models, because relevant work must be, and is being, done in countries with an actual protein shortage, but we also need to produce protein to supply to institutes starting work on its presentation. Our main research is now on the factors that influence the quality of what is made. As the article on quinones in Part 2 of this *Report* shows, we have a fund of knowledge on one aspect of deterioration. We have also accumulated much experience on the separation and handling of leaf protein, and this needs to be systematised. More information about other components of the leaf that can react with the protein during or after extraction is an essential prelude to the rational search for better species, and for the optimum stage of growth at which they should be harvested.

Research on leaf protein is only a small part of the work of the department, but attention is focused on it in this introduction because it now seems to be gaining recognition as a practical part of agricultural research.

Enzymes

Acyl methionine deacylase. Concentrated preparations of *o*-diphenol oxidase from tobacco leaves unexpectedly liberated methionine from N-acetyl methionine. Such

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deacylase activity is common in both animals and bacteria but not in plants. An interesting possibility is that the enzyme responsible is a methionine deacylase primarily involved in protein synthesis.

Protein chains being synthesised by bacterial systems *in vitro* start with an N-formyl methionine residue. As the chain grows, the formyl group is removed by a specific deacylase; usually the methionine is also removed leaving the N-terminal amino acid characteristic of the protein. Protein synthesis in the chloroplasts and mitochondria of plant cells is thought to resemble that of bacteria in being initiated by formyl methionine, whereas synthesis in the cytoplasm does not. The functioning and the intracellular distribution of leaf deacylases would therefore be expected to provide information on the different protein synthesising systems of the leaf: their specificity might help explain the frequent occurrence of N-terminal acetyl groups in the proteins of plant viruses.

The deacylase can be measured by reacting the amino group of liberated methionine with 2 : 4 : 6 trinitrobenzene sulphonic acid. Using this assay, a procedure for partially purifying the enzyme was devised. The enzyme is extracted from an acetone powder of leaves, precipitated with ammonium sulphate and passed through a Sephadex column. During the chromatography it separates sufficiently from the *o*-diphenol oxidase to establish that it is a different enzyme. We hope to make preparations sufficiently pure and concentrated to determine whether its specificity is that expected for an enzyme involved in protein synthesis, and whether its other properties resemble those of the specific bacterial deacylase. (Pierpoint)

Tobacco leaf ribonucleases. The phenol-stable and unstable RNase fractions from tobacco leaf are both active against the same substrates, and are inhibited by the same reagents, although to different degrees. The phenol-stable enzyme has a pH optimum of 5·7 and the unstable a pH optimum of 5·1. Under optimal conditions, using as a substrate large molecular weight ribonucleic acid (RNA), the phenol-stable enzyme has a Km of 0·2 g/l, and the unstable a Km of 0·08 g/l. Both enzymes are endo-nucleases and give the same hydrolysis products when acting on the RNA. The phenol-stable enzyme is unaffected by heating at 100° in neutral solution, whereas the unstable one loses about 30% of its activity. Treating either enzyme with dilute diethyl pyrocarbonate (DEP) irreversibly inhibits the RNase activity. This fact might be useful in studying RNase-sensitive components of healthy or virus-infected leaves and in elucidating the role of nuclease in inactivating RNA of tobacco mosaic virus (TMV) in phenol extracts of leaves.

The similarity in properties suggested that the phenol-stable enzyme might be a modified form of the unstable enzyme produced during the extraction. A new purification procedure was therefore devised that omitted treating the plant extracts with phenol. Leaf extracts were desalted on a Sephadex G50 column followed by ion-exchange chromatography of the RNase-containing fractions on CM-Sephadex using a citrate, pH and concentration gradient. This yielded two RNase fractions. One, the major fraction, has a pH optimum of 5·1 and is destroyed by phenol. The other has a pH optimum of 5·6–5·8 and loses more than 90% of its activity when treated with phenol, a greater loss than was expected from the amount of phenol-stable RNase present in crude leaf extracts. Seemingly crude leaf extracts contain some component(s) protecting the enzyme from the denaturing effect of phenol and largely removed during the purification procedure, so increasing the sensitivity of the purified enzyme to phenol.

The phenol-stable enzyme is certainly not a modified form of the unstable one (its molecular weight is 17 500 as opposed to 22 000 for the phenol unstable enzyme), but seems to gain its stability to phenol by interacting with other components when crude extracts are treated with phenol. The extent to which substances in this molecular weight

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range penetrate dialysis membranes depends, among other things, on the extent to which the membrane is, or has been, stretched. Using atmospheric pressure to drive ultrafiltration, both enzymes can appear in the diffusate and there is an element of uncertainty in any conclusions drawn from the results of dialysis. (Jervis)

Distribution of some copper-containing enzymes in clover seedlings. Visible symptoms of copper-deficiency did not develop in red clover (*Trifolium pratense* cv. Dorset Marlgrass) grown in nutrient solutions without copper, but extracts from these differed greatly from those of plants grown with copper (0·061 ppm) in their ability to catalyse the oxidation of ascorbate, putrescine and caffeic acid. These compounds are substrates for the copper-containing enzymes: ascorbate oxidase, diamine oxidase and o-diphenol oxidase respectively. Because heating to 100°C and sodium diethyldithiocarbamate, but not ethylenediaminetetra-acetate (EDTA), were inhibitory, it is assumed that these enzymes in the extracts were catalysing the oxidations.

Two days after germination, plants grown with and without copper had similar copper contents and enzyme activity. By the ninth day, the copper contents were still similar, but the hypocotyls and cotyledons of the plants grown with copper had twice as much ascorbate oxidase activity as those without.

By the fifteenth day, there was 18% less copper in the roots of plants grown without copper than in those with, but the copper content of other parts of the two groups of plants was similar. Ascorbate oxidase activity of the plants with copper was still double that of the others. Six-week-old plants grown with and without copper contained 15 and 4 ppm copper respectively in the leaves and 25 and 4 ppm in the roots; the plants without copper had only 20% of the ascorbate oxidase activity of the plants with copper.

Diamine oxidase also occurred in 2-day-old seedlings, 95% of the total activity in the radicle, whether grown with or without copper. The diamine oxidase activity in roots of 9-day-old seedlings grown without copper was half and in the cotyledons only a sixth of that in plants with copper, whereas the hypocotyls and leaves of both groups contained similar amounts. The total diamine oxidase activity of 15-day-old plants differed little from that of 9-day-old plants, although the enzyme was distributed differently; the roots, hypocotyls, cotyledons and leaves of plants without copper had between 10% and 40% of the activity of those given copper. Diamine oxidase activity was only just detectable in 6-week-old plants grown without copper and in leaves and roots was only 5% and 12% of that of plants given copper.

o-Diphenol oxidase was not detected in 2-day-old seedlings nor in the roots of older plants. Small amounts of this enzyme occurred in the cotyledons of 9- and 15-day-old plants, but copper had only a slight effect on the amount. However, at 9 days, 15 days and 6 weeks respectively after germination, plants grown without copper had only 9%, 20% and 20% as much enzyme in their leaves as plants given copper. Assuming that the diamine oxidase, ascorbate oxidase, and o-diphenol oxidase in these extracts contain amounts of copper per unit of enzyme activity similar to those in the enzymes purified from other sources, their copper content accounts for 1–5% of the total copper in the plants given copper and up to 2·5% in the plants grown without copper.

Distribution of copper in clover roots. Last year we reported that about half of the total copper in clover roots could be extracted with phosphate–citrate buffer at pH 7. Ultrafiltration and gel-chromatography showed that about 60% of this soluble copper is present as complexes with molecular weights less than 10 000. These complexes have no ascorbate oxidase, diamine oxidase or *o*-diphenol oxidase activity and their ratio of copper to nitrogen is five times as much as in the original extract.

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Modification of cytochrome-*c* by anionic detergents. The modification of cytochrome-*c* by anionic detergents is reversible. When mixtures of cytochrome-c and sodium dodecyl sulphate (SDS) with or without NNN′N′-tetramethyl-p-phenylene diamine (TMPD) were passed through Sephadex G-25 columns, cytochrome-*c* (indistinguishable from untreated cytochrome-*c*) was recovered in the eluate. It did not possess the Soret band at 396 nm reported last year as being characteristic of the detergent-treated cytochrome-*c*, and only catalysed oxidation of ascorbate in the presence of TMPD when SDS or another suitable anionic detergent was added. Hydrogen peroxide, one of the end products of the oxidation of ascorbate, is formed in amounts stoichiometric both with the oxygen uptake and the disappearance of ascorbate. TMPD solutions alone slowly absorb oxygen to give the stable free radical, Wurster's blue, and hydrogen peroxide. Ascorbate, cytochrome-*c*, and SDS-treated cytochrome-c in the absence of ascorbate, all failed to affect the rate of this reaction.

These results suggest that this oxidation of ascorbate catalysed by cytochrome-*c* differs from that catalysed by ascorbate oxidase, which does not form hydrogen peroxide. The modified cytochrome-*c* may catalyse the initial oxidation of TMPD with the formation of hydrogen peroxide but the oxidised TMPD may inhibit oxidation. Ascorbate reduces oxidised TMPD to TMPD, so removing the inhibitor and allowing the reaction to go rapidly until all the ascorbate is oxidised. (Hill)

Pigments

Chlorophyll bleaching. EDTA inhibits chlorophyll breakdown in detached leaves in the dark and speeds bleaching in the light (*Rothamsted Report for 1969*, Part 1, 136). The suggestion that formaldehyde might be formed when a suspension of leaves in EDTA is illuminated, and that this might bleach chlorophyll was disproved. A sensitive test for formaldehyde failed to detect it unequivocally, and experiments with leaves floated on formaldehyde solutions showed that much larger amounts than could be present were needed to bleach chlorophyll.

The extent of bleaching depended on the concentration of EDTA and the period of illumination. Bleaching stopped when leaves, floating in EDTA solution, were transferred from light to dark, indicating that it is a photochemical process. Illumination in an atmosphere of nitrogen diminished the EDTA bleaching but did not completely prevent it. Chlorophyll *a* was lost faster than chlorophyll *b* during bleaching on EDTA, whereas in normal senescence the ratio of *a* to *b* alters only slightly. Carotenoids were also broken down during illumination of leaves on EDTA. Pheophytins were not formed but a 'changed' chlorophyll *a* was sometimes found. Leaves in the dark for 10 days on 0·05*M* EDTA, pH 7·3, still looked fresh and green and had retained 90% of the chlorophyll, whereas on water all the chlorophyll had disappeared after 7 days and the leaves had started to go mouldy. The effective range of EDTA concentrations for inhibiting chlorophyll breakdown in the dark was the same as for promoting bleaching in the light.

During illumination on EDTA for about 20 hours the amount of TCA-soluble nitrogen in the leaves increased and N leached into the surrounding solution. There was no change in the TCA-soluble N of leaves on water. In leaves in the dark for several days the reverse occurred: soluble N increased much more in the leaves floated in water than in the leaves on EDTA.

A report that EDTA prevents chlorophyll breaking down in boiled illuminated leaves and in solvent extracts was not confirmed.

To see whether the effects of EDTA are specific, a range of compounds, including chelators of the same type as EDTA, chelating agents of other types, salts of inorganic

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and organic acids, surface active agents and herbicides, were tested at various concentrations. Many materials promoted chlorophyll bleaching in the light and inhibited its breakdown in the dark, with the effects of individual substances depending on their concentration. Even concentrated salts had little effect whereas other compounds, such as the bipyridylium herbicides, diquat and paraquat, and the non-ionic detergent Triton X-100, were active in very small amounts. (Holden)

Betacyanins. Extracts of *Amaranthus caudatus* and *Atriplex hortensis cupreata* leaves are reddish purple after precipitating the protein with TCA. The colour (λ_{max}540 nm) was discharged by alkali and re-appeared on acidification—like the purple colour (λ_{max}570–580 nm) in extracts of sycamore leaves (*Rothamsted Report for 1969*, Part 1, 136). The leaves of both these plants are coloured initially so that the extract is coloured before acidification; with sycamore the colour appears only after acidification. The pigments from the coloured leaves, the betacyanins, are related to betanin, which occurs in beetroot. The nature of the compounds in the sycamore extracts is not known. (Festenstein with King, Pedology Department)

Allagochrome. A green pigment that reddened on acidification was noticed in several plant extracts by Kozlowski (*Nature* (1950), **165**, 495), and named allagochrome by Habermann (*Plant Physiol.* (1963), **38**, 381). More information about the conditions in which it is formed arises incidentally from observations on the varied types of tobacco-leaf extract made while studying TMV infection. The pigment is easily distinguished from chlorophyll or its green derivatives, because it remains in the water layer during extraction with phenol, it reddens on acidification, and has a different absorption maximum. Extracts made by boiling, or by pulping in the presence of enough phenol to saturate the extract, are not initially green though some of them go green at the surface on standing for several days. Leaf pulp to which phenol is added after intervals of 5–30 minutes gives green extracts; greenness increases as the interval is prolonged, is intensified in the presence of citrate, slightly diminished by phosphate and greatly diminished by EDTA. Little or no colour forms in extracts from the lower four or five leaves of tobacco plants 1–1·5 m tall, but it invariably forms in extracts from leaves above this. It is retained during dialysis in 'Visking' tubes.

These observations are all compatible with the suggestion that it is a complex formed by oxidation of a chlorogenic acid complex. It is probably related to the green pigment that appears when the water in which young potato tubers have been boiled is left standing. (Pirie)

Viruses

The nucleic acid content of leaf extracts containing TMV. Differences in the infectivity of extracts made in different ways (but all disrupting TMV to yield infective nucleic acid) from infected tobacco plants, are in part the consequence of differences in the extent to which ribonuclease is being extracted and remains active in the extracts. It is still not certain that this enzyme action is the main reason for the loss of infectivity when extracts are stored. Several other components of leaf extracts e.g. aldehydes, nicotine and spermine, inactivate nucleic acid but not at the dilutions or conditions in which they occur in the leaf—e.g. in the presence of leaf salts. Various *ad hoc* assumptions must be made if inactivation by these substances is being invoked. Differences in the extent to which nucleic acid is being extracted from the fibrous part of the leaf are a more important reason for differences in infectivity.

Measurements of acid-precipitable phosphorus and of the UV absorption of partly

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purified leaf extracts show that there is more nucleic acid in extracts that have been allowed a brief period of autolysis before adding the phenol used to disrupt TMV. But much of this nucleic acid is not derived from TMV. Extracts from infected and uninfected plants to which purified TMV labelled with 32P had been added before or during the extraction were therefore studied: the amount of 32P in these extracts need not be proportional to the total amount of nucleic acid in them, but all the 32P would be derived from TMV nucleic acid. We already know (*Rothamsted Report for 1958*) that, when labelled TMV is used as inoculum, much of the 32P becomes firmly combined with the leaf fibre, but the amount that combines, the rapidity of the process and the firmness of the attachment was unexpected. When pulped for a minute in the presence of phenol, 10 g (fresh weight) combines with 80% of the 32P in 14 mg of TMV and it is only slowly released on extraction at 0° with neutral citrate, or EDTA, or N acid or alkali. There is less fixation when citrate, oxalate or commercial nucleic acid are present during pulping, or when the phenol is added after pulping. All these variants on the extraction process also increase the infectivity of the extracts, but the magnitude of the increase differs with different batches of plants, for as yet unexplained reasons. The increases are invariably greater in lower than in upper leaves, but the state of nutrition or illumination of the leaves during the few days before harvest has no invariable effect. Fortunately, the behaviour of a batch of leaves is not greatly affected by freezing, so stored material with known behaviour, can be used.

Insofar as conclusions can be drawn from infectivity measurements alone, infected leaves behave similarly to uninfected leaves to which purified TMV has been added. (Pirie with Bawden)

TMV extraction in presence of diethyl pyrocarbonate. As much infective TMV is extracted from infected tobacco leaves when they are ground with DEP as without, but the virus tends to aggregate and lose its infectivity. This suggests, rather surprisingly, that DEP combines with the unreactive amino groups of TMV and possibly polymerises the particles, as it does RN-ase. The streaming birefringence of purified TMV is intensified by DEP but electronmicrograms did not show any linking of the particles. The treatment slowly inactivates and three-quarters of the infectivity was lost after standing in 10% DEP for four days. (Jervis and Pierpoint)

Oxygen and TMV formation. Discs punched from TMV-inoculated leaves illuminated for 48 hours will synthesise TMV in the light, but not in the dark, when atmospheric CO_2 and O_2 are greatly diminished. With more rigorous efforts to remove CO_2 and O_2, there was little synthesis even in the light. Photosynthesis in these conditions would continue to produce a little O_2: this can be inhibited by adding 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). When the fluid on which the discs were floated contained 10 μM DCMU there was no synthesis of TMV. This suggests that a little O_2 is necessary for synthesis.

The interpretation of these results is complicated by the fact that the virus-forming mechanism of infected discs is damaged by even 8 hour anaerobiosis so that they subsequently form less TMV in the air and light than discs not kept anaerobic. (Olsson)

Large-scale preparation of leaf protein

Yields. The best species selected so far are winter wheat and rye, cocksfoot, mustard, and fodder radish all of which can synthesise extractable protein at more than 8 kg/ha/day. These have been grown either alone or in sequence and given various treatments to

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make the maximum use of our growing season. Annual yields of between 1600 and 2000 kg/ha of extracted protein can be expected most years from cocksfoot or from winter cereals followed by successive fodder crops, but not in unusually dry years. Protein yields have sometimes been nearly doubled by irrigation even when dry matter yields were little affected. An improved cultivar of fodder radish yielded more than 800 kg/ha in seven weeks when irrigated, and two crops grown in sequence produced 2000 kg/ha.

Of new species and varieties of grasses tried, Westerwolds, S24 and S22 ryegrasses all outyielded cocksfoot in their establishment year.

Protein quality. The nutritional quality of leaf protein differs between species and between batches prepared from the same species. This is not accounted for by the very small differences in amino acid composition. *In vitro* digestibility tests suggest that faster processing gives better products. Any delay after pulping before coagulating allows the enzymic oxidation of phenols to orthoquinones, which react with thiol and amino groups and probably make cysteine and lysine less available. The quinones also polymerise to form brown pigments that complex with the protein and make it less digestible *in vitro*. Consequently delays in coagulating the protein and in separating the liquor lead to poor products with small nitrogen contents and many phenolic and other contaminants. Digestibilities are improved by adding reducing agents, sodium hydroxide or a detergent during pulping, probably by inhibiting phenolase activity and the hydrogen bonding of polymerised quinones to protein.

Flavour also deteriorates with delays in processing. A bitter flavour develops enzymically in lucerne juice before coagulation. The protein curd from most other species is sweet and pleasant immediately after heating, then less acceptable 'tea and leaf' flavours develop. Rapid processing gives bland and uniform products from various species. Later changes in flavour, caused by oxidation of the lipid fraction, can be prevented by careful preservation and storage. (Arkcoll)

Fractionation and amino acid composition of protein in leaf extracts. Extracts from the leaves of lupin (*Lupinus albus*) and chinese cabbage (*Brassica chinensis*) were fractionated by centrifuging and by controlled heating (cf. barley leaf extracts, *Rothamsted Report for 1969*, 132). With all three species, but especially barley, the chlorophyll-containing fraction sediments less readily from extracts of old than of young leaves. The amount of protein-N remaining after removing this fraction is the same in young and old leaves of one species, but differs between species.

The composition of protein extracted from different species is similar and uninfluenced by leaf age. However, statistical analysis shows small but consistent differences between the contents of aspartic acid, alanine and methionine in preparations from the three species. Protein from barley had most methionine and protein from lupin least. There were also differences, unrelated to species, between the lysine contents of different preparations from the same extract; preparations coagulated by heat had 10–15% less lysine than those precipitated by acid. Heat damage to protein in food is usually associated with the Maillard reaction between the ϵ-amino group of lysine and aldehyde groups: this reaction probably occurs in heat-coagulated extracts rich in reducing sugars and in easily-oxidised unsaturated fatty acids. These condensation products are usually thought to be acid labile, but some resist acid hydrolysis; the apparently smaller lysine content of heat-coagulated leaf protein may reflect its incomplete liberation during hydrolysis.

Similar differences in amino acid composition to those reported last year with some

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fractionated barley proteins were found between chloroplastic (both sedimented and heat-coagulated) and cytoplasmic fractions from lupin and chinese cabbage. Both fractions from any one species contain the same amounts of aspartic acid, proline, alanine, valine and methionine, and differ only slightly in their contents of threonine, serine, glutamic acid, iso-leucine, tyrosine, phenyl-alanine and arginine, but there is less leucine and substantially more histidine and lysine in the cytoplasmic than in the chloroplastic fraction. The availability of the essential amino acids, rather than their absolute amounts, often determines the nutritive value of a protein. This is especially so with lysine and methionine. Comparison with the FAO reference protein, and rat feeding experiments, show methionine to be the first limiting amino acid in unfractionated leaf protein. Cytoplasmic protein, with a methionine content similar to the unfractionated protein, has a nutritive value approaching that of casein, so unavailability probably explains the poorer performance of the unfractionated preparations. Cytoplasmic and unfractionated protein contain enough lysine, but it may be marginal in some chloroplastic proteins. (Byers)

Carotenoids in leaves and leaf protein. Carotene is important as a precursor of vitamin A, and lutein is added to chicken feeds to increase yolk and skin pigmentation. Carotenoids are concentrated during the preparation of leaf protein, so they were studied in some detail and methods developed whereby carotene and xanthophylls in fractions from leaves could be measured rapidly.

Laboratory tests showed little or no loss of carotene when leaves were boiled, frozen or ground. Little carotene or xanthophyll was lost when ground leaves were kept overnight at pH 7 but much was lost at pH 4·5; there was no loss when leaves were boiled before grinding.

In bulk preparations of leaf protein, carotene is stable during the extraction of the juice and the coagulation of the protein. Carotene remaining in the fibre after protein extraction is quickly destroyed; this gives yet another reason for separating extract and fibre quickly. Carotene in leaf protein survived freeze-drying and most samples prepared in this way contained about 1·5 mg of β-carotene and 3·0 mg of xanthophyll per gram. Thus 2–3 g of leaf protein can supply a person with the daily requirement of vitamin A.

Autoxidation during storage can cause substantial losses, which are increased by hot-air drying. Drying, storing cold, and keeping the protein in darkness and away from oxygen prevent these losses. Oxidation of carotene was especially rapid in the light, and this action may be catalysed by the chlorophyll present. Xanthophylls were more stable than carotene in stored leaf protein. (Arckoll and Holden)

Carbohydrates in leaf extracts. The 'whey' remaining after heating extracts from cocksfoot, fat hen, fodder radish, lucerne, mixed grasses, mustard, red clover, rye, ryegrass and wheat contained on average 1% of carbohydrate and 2% of dry matter; 75% of the total carbohydrate was soluble in 80% ethanol.

The separation of the sugars by thin-layer chromatography on silica gel impregnated with monosodium phosphate was improved by using triple instead of single development: the developing solvent (acetone–water–chloroform–methanol, 8 : 0·5 : 1 : 1) was allowed to run the full length of the plate, then evaporated and the development repeated. This is qualitatively satisfactory; work proceeds on a quantitative adaptation of the method. All the liquors contained fructose and glucose, and most contained sucrose; arabinose and galactose were not detected. Xylose did not exceed 10% of the reducing sugars in any of the liquors. All the crops studied contained the same sugars, only the proportions differed.

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Hydrolysates of the polysaccharide fraction (insoluble in 80% ethanol) contained fructose, arabinose, glucose, galactose and xylose. Fructose was the major sugar in ryegrass and other crops rich in fructosan; lucerne contained less and mustard very little.

Preparations of protein from cocksfoot, lucerne, mustard, ryegrass and wheat contained about 5% carbohydrate. The chief sugars, identified by chromatography, were galactose, arabinose and xylose, with only small amounts of glucose and fructose. The composition thus differed qualitatively from that of the polysaccharide in the liquor, so the carbohydrate associated with heat-precipitated leaf protein is probably combined with the protein, rather than co-precipitated with it from the liquor. (Festenstein)

Machinery. Unless large amounts of water are added during the first extraction, only half the protein in a crop is extracted. Half of what remains comes out in a second extract provided it is made quickly but there are losses when the pulp or fibre are allowed to stand. The prototype International Biological Program (IBP) pulper, now obsolete and replaced by the stainless steel pulper mentioned last year, was modified by chamfering its beaters at 45° and, in conjunction with the small belt press mentioned last year, makes first and second extraction a continuous process. Unevenness in the feed to the pulper wastes power. We therefore fitted an auger feed to the IBP pulper and it worked so well that a similar feed is being made for the large pulper. (Davys and Pirie)

Cooperation with other organisations. IBP pulpers and presses for agronomic work were sent to the Department of Agriculture in Reading University, to Lysoform Industrias Quimicas in Brazil, and to the Department of Scientific and Industrial Research in Pakistan. The Royal Society sent an IBP pulper to Gaveston Hall School in Sussex. Small belt presses have been (or are being) sent to Pakistan, the Department of Agriculture in Eire and the Indian Statistical Institute in Calcutta. We designed, and supervised manufacture of, a 2 ton per hour (the largest yet made) pulper and belt press combination for Lysoform. Because of the voltage difference between Britain and Brazil the pulper cannot be properly tested here, but the press works admirably. We have an enquiry for a similar unit from the Tea Research Institute of Ceylon.

From time to time since 1951 (e.g. ARC circular 89/1951) the advantages of using the process of leaf protein extraction to cheapen grass drying have been stressed. This suggestion may now be acted on. The National Research Development Corporation is discussing the possibilities of cooperative work with grass driers and engineers. This is a project that deserves encouragement because the protein, fibre and 'whey' are more valuable and easily handled when separate than when mixed in a crop. The value of the 'whey' as a medium for growing microorganisms is being studied at the National College of Food Technology (Weybridge) and Miss M. Mehta spent a few days familiarising herself with the production process.

Reaction of *o*-quinones with other components of leaf extracts

The formation of *o*-quinones in biological material, and their effects on processes of agricultural importance, are summarised in Part 2 of this report.

The study of the o-quinones formed by the enzymic oxidation of polyphenols in leaf extracts was extended to their reactions with methionine and carnation ringspot virus (CRV). These reactions were expected to explain part of the 'unavailability' of the methionine in leaf proteins, and some difficulties encountered in the extraction of CRV.

Chlorogenoquinone and caffeoquinone, the quinones formed from chlorogenic acid and caffeic acid respectively, react with methionine and methionine sulphoxide, absorbing

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oxygen and giving coloured products. They seem not to react with N-acetyl methionine, and chromatographic examination of the reaction mixture detected no trace of adducts formed between acetyl methionine and quinone, or of the sulphone or sulphoxide formed by oxidation of acetyl methionine. It did reveal traces of an S-containing, ninhydrin-reacting substance, probably methionine, but this was formed from acetyl methionine and leaf extract even in the absence of quinones. Thus only the amino group of methionine seems to react with leaf *o*-quinones, and only the methionine that forms the N-terminus of leaf proteins is likely to be rendered 'unavailable' by this reaction. However, judging by the frequency with which methionine forms the N-terminus of bacterial proteins, this might involve as much as a third of total leaf-protein methionine.

Preliminary experiments have not borne out the suggestion that CRV reacts with, and is inactivated by, *o*-quinones. Thus enzymically-generated chlorogenoquinone does not appreciably decrease the infectivity of extracts of CRV-infected leaves of *Nicotiana clevelandii*. Moreover, the spectrum of CRV separated from solutions with and without quinones is the same, and there is no indication of protein-bound quinones. (Pierpoint)

Free amino acids and amines in leaf extracts. Work continued on the kind and amount of free amino acids and amines in extracts of ryegrass grown under different conditions of mineral nutrition. (Byers with Nowakowski, Chemistry Department)

Serological identification of *Ophiobolus graminis*. (Olsson, *see* Hornby and Govier, Plant Pathology Department, p. 134.)

Travel

N. W. Pirie spent two months at the beginning of the year in the Indian Statistical Institute (Calcutta) establishing research there on leaf protein extraction. In November he organised a 'Technical Group' meeting of the International Biological Program at Coimbatore on leaf protein, attended by 30 participants, nine from outside India. A book is being compiled from the papers.

M. Holden and W. S. Pierpoint attended the 8th International Biochemical Congress in Switzerland.

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