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Report for 1968 - Part 1

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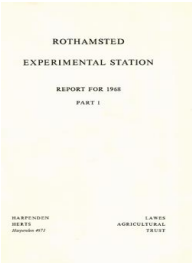

Biochemistry Department

N. W. Pirie

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BIOCHEMISTRY DEPARTMENT

N. W. PIRIE

It used to be assumed that a certain enzyme, when made by one tissue in an organism, would have a definite structure, and that heterogeneity in a purified preparation arose because part of the preparation had been modified during isolation, or because the original tissue was less homogeneous than had been thought. This idealistic assumption—that the cell shares with the biochemist a preference for homogeneity—has been widely questioned during the last decade. Several families of 'isoenzymes' have been described in which enzymes with similar properties are produced by what appear to be homogeneous tissues. When the enzymes are isolated from an excretion, e.g. pancreatic juice, it is reasonable to consider whether this is advantageous: whether a group of similar substrates will be handled more effectively by several enzymes than by one. When there is only one substrate and a tissue has to be extracted to make the enzyme(s), there is increased likelihood that a parent complex is being disintegrated into dissimilar enzymically active fragments. Our inability to prepare homogeneous chlorophyllase preparations may arise in this way, because it is already known that several leaf enzymes appear in extracts as a complex, the so-called 'fraction I protein', which can be dispersed into enzymically active fragments. The position with the leaf ribonucleases is somewhat different because there are many different ways in which ribonucleic acid can be attacked and ribonucleases with characteristically different modes of action are known. An interesting feature of our work on these enzymes is the surprising stability of some of them. During extraction, complexes may be formed as well as being broken down. Quinones produced by the oxidation of polyphenols are outstanding among potential complexing agents. Model experiments on these complexes have now been taken far enough for it to be possible to study the effects of incompletely controlled complex formation on the properties of enzymes and viruses when they are prepared by conventional methods.

Four copper-containing enzymes account for half the copper in the leaves and petioles of clover seedlings, but for only 4% of the copper in the roots. Much remains to be done to ensure that all the enzymes in a tissue are accessible to substrate during the assay, and the activity measurements must be repeated with several different amounts of trace-element in the culture fluid, but the initial steps have been taken in drawing up a trace-element balance sheet.

The lack of official enthusiasm for our work on bulk production of leaf protein has not prevented interest growing in many countries. Ten extraction units either made here or made to our designs are now in use abroad, five more are being manufactured and negotiations have started for a further four.

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Studies relating to leaf enzymes

Chlorophyllase. The yield of chlorophyllase from solvent-treated leaves of sugar beet (*Beta vulgaris*) increases by 60% when 0·4M-sodium chloride is included in the aqueous extraction medium. The yield of enzyme from untreated leaves is similar and also depends on the salt concentration. The tendency of the extracted enzyme to aggregate or associate with other proteins when concentrated is partly reversed by dilution or by adding sodium chloride. Analysis or purification by gel-filtration was therefore done in the presence of 0·5M-sodium chloride. This concentration also minimises interactions between the enzyme and negative groups on the dextran gel.

Because the enzyme is not readily retained by anion-exchangers, material that had been partly purified on carboxymethyl-Sephadex and Sephadex G-100, was purified further on Sephadex, DEAE and QAE at pH 8·9 and ionic strength of 0·006 g ions/l. About one-third of the activity is not retained by the ion-exchangers and most of the remaining activity is eluted by sodium chloride at about 0·03M. The specific activity of both fractions exceeded that of the starting material. Although these results suggest that more than one form of chlorophyllase is present, the possibility that one of the fractions is an artifact of aggregation or association was investigated. Gel-filtration in the same dilute buffer showed that some of the chlorophyllase, and other proteins, are aggregated. Because much of the enzyme was adsorbed by the dextran gel at these ionic strengths, the exact amount of aggregation was difficult to measure; it was at least 15% and may have exceeded 50%. About 10% of high-molecular weight chlorophyllase was present, even when gel-filtration was done in the presence of 0·5M-sodium chloride.

Because of these uncertainties, the properties of the enzyme were studied without purification on anion-exchangers. Solutions containing 0·05 g/l of purified enzyme lost only about 5% of their activity in 20 days at 4° C but intermediate and high molecular weight forms accumulated slowly on storage, even at −18° C. The enzyme was less stable on dilution, and at 2 mg/l lost 56% of its activity at 4° C in 3 days. No activity was lost in 30 minutes at 21° C in 10–55% acetone, and only 25% of the activity was lost in 70% acetone. When heated in aqueous solution for 5 minutes at various temperatures, all activity was lost between 60° and 70° C at pH 6·3.

The ion-exchange properties of the enzyme suggest that its surface groups are mostly basic. Electrophoresis on cellulose acetate indicates that the isoelectric point is at pH 6–7, but the results may be affected by aggregation or by adsorption on the electrophoresis membrane.

Chlorophyll is not soluble in water and is used as a colloidal suspension in water containing acetone in assays for chlorophyllase. Spectral changes in a suspension of chlorophyll *a* in 2% acetone indicate that the physical state of the substrate changes rapidly. The acetone concentration for optimum activity of the enzyme varies considerably, probably because the state of aggregation and accessibility of the substrate depend on the concentration of enzyme, acetone and salts in the test mixture before the substrate is added. In most tests 45% acetone is optimal, but results are not

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always consistent. With 40% of acetone, as usually used, bovine serum albumin increased the conversion to chlorophyllides; sodium chloride at a final concentration greater than 0·1M strongly inhibited the reaction. (Bacon and Holden)

Leaf lipoxidase. Other systems in addition to lipoxidase may be involved in the bleaching of chlorophyll in leaves suspended in aqueous solvents. Some species that have much lipoxidase activity (e.g. *Arctium lappa*) bleach little whereas others with small activity (e.g. *Alliaria petiolata*) bleach strongly. Chlorophyll bleaches well in wheat, barley and rye, but does not bleach in oat seedlings. This failure to bleach was previously correlated with very small lipoxidase activity, but recent assays have not confirmed the exceptionally small activity. Oats contain anti-oxidants, and if these occur in seedling leaves they might prevent chlorophyll being bleached and interfere with lipoxidase determinations. Adding small amounts of the anti-oxidant, nor-dihydroguaiaretic acid, to suspensions of wheat leaves in aqueous acetone inhibited the bleaching.

The lipoxidase activity of cereal seedlings was less stable than some earlier results suggested and the proportion of enzyme associated with the plastid fraction ranged widely depending on the conditions of grinding and extraction.

The change in lipoxidase activity during expansion of the first leaves of *Phaseolus vulgaris* seedlings was followed. The activity per leaf remained almost the same for about two weeks; it therefore fell progressively when expressed on the wet or dry weight basis. (Holden)

Ribonucleases. Leaves of the same species but in different physiological states produced very different numbers of local lesions when rubbed with the same solution of nucleic acid, derived from tobacco mosaic virus (TMV). The infectivity of extracts made from infected leaves in which the virus has been disrupted by phenol is likewise greatly affected by apparently trivial variations in technique. Some well-known constituents of leaf extracts (e.g. Ca^{++}, Mg^{++}, glutathione, ascorbic acid (*Rothamsted Report for 1957* p. 101); chlorogenic acid, hexenal and nicotine (*Rothamsted Report for 1964* p. 123)) abolish the infectivity of TMV nucleic acid, but only when present in unphysiologically great concentrations. Further, infectivity is restored when some of them, e.g. Ca^{++}, are removed by dialysis. Although the possibility was recognised in 1964 that co-action between these various agents, which are usually present simultaneously in a leaf extract, might explain the observed phenomena, we continued to search for more powerful inactivating agents and described (*Rothamsted Report for 1966* p. 109) thermostable fractions that were active at physiological concentrations.

Our early work (Holden & Pirie, *Biochem. J.* (1955), **60**, 39) made us think it inherently probable that there are several ribonucleases in leaf extracts. The predominant enzyme(s) were inactivated on heating to 88° C. This enzyme, or group of enzymes, is inactivated by phenol and so is not present in the extracts of infected leaves that we have been studying. However, there are other ribonucleases, recognised both by their ability to

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abolish the infectivity of TMV nucleic acid and to make yeast nucleic acid soluble in acids, and these are unexpectedly stable. Activity is not destroyed by boiling in neutral solution for some hours, or by exposure to phenol at 100° C for half an hour. The most active preparations are not precipitated by trichloroacetic acid, but are by ammonium sulphate; they give a biuret reaction, and are retained when dialysed. There are puzzling aspects to the last property; much of the activity in crude preparations appears in the diffusate, but is retained to an increasing extent as purification proceeds. This may be the consequence either of aggregation or of passage through the membrane being facilitated by other substances.

Some enzymically active material may be made from leaf ribonuclease(s) during disintegration by phenol, but it is not all made in this way. When tobacco leaf extracts not exposed to phenol are fractionated with ammonium sulphate, the ribonuclease activity of the easily precipitated fractions is destroyed by phenol, but the activity of the later fractions is not. The most active preparations are still inhomogeneous both physically, by the criteria of gel filtration and partition between phenol and water, and enzymically, by the criterion of differences in the extent different fractions are inhibited by such agents as Zn^{++}. These enzymes are academically interesting because of their extreme stability; whether they have a role in causing the anomalies in infectivity of leaf extracts, or influencing the susceptibility of different host plants to infection, remains to be demonstrated. (Pirie in collaboration with Bawden)

Distribution of metallo-enzymes

Copper and copper-containing enzymes in clover seedlings. The copper content of clover seedlings (*Trifolium pratense*) grown in nutrient culture (copper content = 1μM Cu^{2+}, manganese content = 10μM Mn^{2+}) changed only slightly during the first week after germination. Two weeks later the total copper in the seedling had increased by 1·8 times and after six weeks by 20 times. Though the ratio of copper to dry matter in the aerial portions of the plant changed little during the first six weeks of growth, the ratio in the root increased greatly. The copper content of the root of 7-day-old plants was about 8% of the total copper in the plant, in 2-week-old plants it was 20% and in 6-week-old plants 50%; over the same period the dry matter content of the roots remained about one-quarter of that for the whole plant. No precautions were taken to remove copper adsorbed on to the surface of the roots except to wash them in distilled water.

The activities of the copper-containing enzymes, cytochrome oxidase, diamine oxidase, polyphenol oxidase and ascorbate oxidase, were determined in extracts of leaves, cotyledons, roots and petioles of clover seedlings. Only diamine oxidase has been prepared in a pure form from clover seedlings; it contains about 12·3 ng Cu per unit of enzyme activity; the activities of the other enzymes were calculated from those published for preparations purified from other organisms and were assumed to be 42·6 ng Cu per unit of cytochrome oxidase, 1·1 ng Cu per unit of ascorbate oxidase and 9·5 ng Cu per unit of polyphenol oxidase in the conditions employed in these experiments. These four enzymes together accounted for

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43%, 56%, 24%, 4% and 21% of the total copper present in the leaves, petioles, cotyledons, roots and whole plants, respectively, from 6-week-old clover seedlings, and for 16% of the total copper in 2-week-old plants. The relative distribution of these enzymes in leaves, petioles, cotyledons and roots of 6-week-old plants were 33:37:26:4 for cytochrome oxidase, 37:26:20:17 for diamine oxidase, 18:2:1:1 for polyphenol oxidase and 33:37:11:20 for ascorbate oxidation. Polyphenol oxidase and cytochrome oxidase accounted for over 80% of the combined activities of these copper-containing oxidases.

Distribution of manganese in clover seedlings. Clover seedlings contain 5–10 times as much manganese as copper. Six-week-old plants had 75% of their total manganese in the roots, 8% in the cotyledons, 6% in the petioles and 11% in the leaves.

Effect of copper deficiency on copper-containing enzymes in Sitka spruce. The activity of the copper-containing oxidases was determined in apparently healthy (Cu = 4 ppm) and copper-deficient (Cu $<$ 2 ppm) seedlings of Sitka spruce (*Picea sitchensis*). Extracts were prepared in dilute phosphate buffer, pH 7, and centrifuged. Sediments at $10^3 \times$ g, $10^4 \times$ g, and the supernatant liquid were studied. Diamine oxidase was not detected in any fraction; ascorbate was oxidised by all fractions prepared from symptomless plants, but from copper-deficient plants it was oxidised by the supernatant fraction only and then only one-sixth as fast as by the same fraction from the symptomless plant. Polyphenol oxidase was not detected in the copper-deficient plant but was in the $10^3 \times$ g fraction of the healthy one. Cytochrome oxidase was found only in the $10^4 \times$ g precipitate and was twenty times more active from the healthy than from the copper-deficient plant. The symptomless plants used contained relatively little copper, and it is hoped to repeat this work with plants containing more usual amounts (about 10 ppm) and to find how copper concentration affects enzyme activity. (Hill)

Effects of changes in metabolism on the multiplication of tobacco mosaic virus. A chemical method for measuring TMV should be quicker and more precise than infectivity or serological assays, but it is less likely to be specific. Extracts of infected or uninfected leaves were heated to 60° C and shaken with chloroform before precipitation at one-third saturation with ammonium sulphate. The precipitate was dissolved in water and the absorption at 260 nm measured. Absorption was usually negligible by extracts from uninfected tissue. Extraction of virus from young leaves seems to be complete for no more was recovered when the residues were incubated with trypsin or cellulases. The virus solutions gave a single band on electrophoresis.

Virus can be detected by this method after 36 hours in inoculated leaf discs incubated on nutrient solution at 24° C under continuous illumination of 350-ft candles. The multiplication rate increases during the next 100–120 hours, and then decreases. About 200 hours after inoculation the maximum amount of virus (about 300–400 μg) is present in each 1-cm disc.

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A similar amount of virus is formed in undetached leaves of inoculated tobacco plants kept in similar conditions.

More TMV is formed in the light than in the dark. A possible reason is that photosynthesis supplies some substrate necessary for maximum virus synthesis. When photosynthesis is decreased by incubating infected illuminated discs in CO_2-free air, virus synthesis decreases; the addition of 5% (w/v) sucrose to the incubating fluid stimulates synthesis more in the dark than in the light. As much virus is then formed in the dark as in the light. This correlates with the changes in the respiration rate of infected leaf discs. Those incubated in the dark respire less than those incubated in the light, but after floating for three hours on 5% (w/v) sucrose in the light, both sets of discs respire at the same increased rate. Preliminary experiments, using dinitrophenol to uncouple oxidative phosphorylation and electron transport, suggest that virus synthesis is inhibited more in the dark than in the light. This may mean that, in the dark, energy for virus synthesis is supplied from respiratory processes whereas in the light, adenosine triphosphate (ATP) generated photosynthetically is also used. Photosynthesis, therefore, may increase virus synthesis by maintaining respiratory substrates and by supplying ATP formed by photosynthetic phosphorylation. (Olsson)

Reactions of o-quinone with other components of leaf extracts. *o*-Quinones are formed in leaf extracts by the enzymic oxidation of polyphenols, and they combine with and modify the properties of the proteins in the extracts. To learn more about these reactions, chlorogenoquinone was generated from chlorogenic acid by the action of *o*-diphenol oxidase and its reactions with peptides and with bovine serum albumin (BSA) studied. Further evidence was produced that it reacts with an excess of BSA to form an adduct with the single thiol group of the protein. The product is colourless and has no -SH group. Its formation can be represented:

$$\text{BSA-SH} + \text{Quinone} \rightarrow \text{BSA-S-Phenol}$$

A possible alternative reaction that would dimerise the protein,

$$2(\text{BSA-SH}) + \text{Quinone} \rightarrow \text{BSA-S-S-BSA} + \text{Phenol},$$

seems not to occur. Although the quinone reacts avidly with the free -SH of BSA, it does not react with all protein -SH; the free but 'sluggish' thiol groups of ovalbumin, for example, are as unavailable to chlorogenoquinone as they are to many other -SH reagents.

When an excess of chlorogenoquinone reacts with BSA, red protein derivatives are formed. The amino groups of the protein, in addition to the thiol groups, react with quinone, by the reactions:

$$\text{BSA-NH}_2 + \text{Quinone} \rightarrow \text{BSA-NH-Phenol}$$

$$\text{BSA-NH-Phenol} + \text{Quinone} \rightarrow \text{BSA-NH-Quinone} + \text{Phenol}$$

These reactions can be prevented by treating the protein with formaldehyde or succinic anhydride. There is evidence that both the lysine-ε-NH_2 and the aspartate α-NH_2 of the protein react with chlorogenoquinone, although it is the reaction with the ε-NH_2 that is chromogenic. Red products are not

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formed from albumin in which these groups have been specifically guanidinated with O-methyl isourea, whereas they are from albumin in which the terminal amino group is complexed with copper-ions.

The red quinone-modified BSA can be separated from unchanged albumin by chromatographing on DEAE-Sephadex, although much of its colour fades in the process. One preparation (BSA-Q2), prepared by treating the protein with a five-fold excess of chlorogenoquinone generated in four equal lots, proved particularly convenient to separate and study. It sediments like BSA on ultracentrifugation, but moves quite differently on electrophoresis. Its u.v. spectrum has a large shoulder at 320 nm, which is shifted to longer wavelengths by alkali. Preliminary results suggest that it is less easily hydrolysed than is BSA by trypsin.

BSA was chosen because it is readily available in highly purified form, has a known amino acid composition, and has not been exposed to *o*-quinones in its preparation. Experience gained with it, should be useful in studying how plant viruses react with, and are modified by, *o*-quinones. (Pierpoint)

Studies on proteins

Protein in *Septomyxa affinis* spores. Female Wheat Bulb flies feed on the conidiospores of the Hyphomycete *Septomyxa affinis* and Raw, Jones & Gregory, (*Pl. Path.* (1968), **17**, 23) suggested that the spores may supply the protein needed for maturation of the eggs. The total N of the spores was determined (Kjeldahl) and an attempt was made to determine the protein concentration using the biuret method. There are two problems: the protein is insoluble and a yellow pigment is present that interferes with the biuret colour. A preliminary treatment with alkaline ethanol at room temperature removed the pigment and made the protein soluble. However, this may have denatured some of the protein because the biuret colour differed from that given by bovine serum albumin used as standards. Total N was 7% of the dry matter. This suggests the presence of about 40% protein, whereas biuret determinations gave a maximum protein content of only 10%. (Holden)

Leaf protein and its by-products

Equipment. After slight modification, the design of the laboratory-scale pulper and press we made on behalf of the International Biological Program is now settled. Units were sent to Aurangabad and Ibadan and further units are now being made for Eire, New Zealand and Sweden; plans are well advanced to get units made for institutes in some other countries. These units, working on 2-3 kg of crop, give a percentage extraction closely related to that given by our large unit. Less power seems to be consumed in the small pulper per kg of pulp made. If we had the opportunity to redesign the large pulper radically, we would take advantage of this observation and make a scaled-up version of the laboratory pulper in the hope that it also would consume less power.

The belt-press (*Rothamsted Reports for 1963–1965*) does not have a capacity that quite matches the output of the pulper. Because of a probable

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order for a large-scale extraction unit to be operated jointly by the Tea Research Institute and the Institute of Scientific and Industrial Research in Ceylon, we have, in co-operation with the Weedon Engineering Company, redesigned the press with a broader belt and improved drive. This press should have a capacity of 1–2 tons of pulp an hour.

Conditions at the beginning and end of a cycle of freeze-drying are quantitatively very different. At first, especially when the material in the drier is frozen by evaporation of part of the water in it, water vapour is produced rapidly; thereafter, because heat penetrates slowly into the partly dried mass, water vapour is produced slowly. Hitherto we sequestrated the first flush of vapour in a large heat exchanger immersed in a tank of coolant built into one corner of a deep-freeze, and relied on a small commercial freeze-drier during the prolonged second stage. We now sequester the water vapour produced during the second phase in a U mounted in the same coolant tank and, by manipulation of some cocks, can disconnect the large heat exchanger and remove it for thawing out ready for the next cycle. (Davys and Pirie)

Yields. The exceptionally mild spring, and the opportunity that the laboratory-scale extraction equipment gave us for trying several different crop sequences and techniques of husbandry, enabled the yield of extracted protein to reach the two tons per hectare predicted last year. This was obtained with winter wheat followed by two crops of fodder radish, or two of mustard, on land given 22 cwt of 'Nitro-Chalk' during the year. A mixture of wheat and vetch, followed by field beans, gave 1·23 tons of extracted protein per hectare without being given any fertiliser.

Although the amount of leaf protein in a crop is likely to increase until the leaves begin to yellow, extractability diminishes from about the time of flower initiation. There is therefore a maximum on the curve relating the yield of extracted protein per unit area with the date. To prevent flowering, an unvernalised winter wheat (Starke) was sown late in March; it gave excellent yields by producing lush vegetative growth long after winter-sown wheat had flowered. The claim that simazine can increase the amount of protein in a crop prompted an experiment with field beans; it had no effect on the final yield of protein but it slightly increased the amount in young plants.

Crops grown primarily for extraction are likely to be the main source of leaf protein; the argument for diverting farmland from conventional use is that it increases the yield of protein edible by people. However, where possible it is obviously desirable to use by-product leaves. The yield of extracted protein from the waste vines and pods of peas was 350 kg/ha, whereas the yield of protein in the peas that were being harvested was only 250 kg/ha. Potato haulm, taken at the end of August when the tops on the remainder of a field of main-crop were being burnt off, gave 250 kg/ha. Sugar-beet tops can give as much as 500 kg/ha. No promising new species was found during the year, but the improvements in pulping technique have led us to take a renewed interest in some crops previously considered unsatisfactory. Cocksfoot, for example, in spite of a late start, gave 1·1 tons/hectare in three cuts. (Arkcoll and Festenstein)

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Drying. Leaf protein can be preserved by canning, pickling, salting or in deep-freeze. Hitherto we have dismissed air-drying as unsuitable for preparations intended as human food, because the product was hard, gritty and black, although animal-feeding experiments showed that drying was not harmful. Material that had to be dried so that it could be sent to institutes abroad was freeze-dried. Appearance and texture as pleasant as that of a freeze-dried product can be achieved by air drying in two stages. The usual press-cake (65% water) is crumbled and air-dried till the water content is about 20%, when it is finely ground and the drying completed. (Arkcoll)

Digestibility. We now use thioglycollic acid to activate papain in the *in vitro* digestion of various leaf protein preparations because, at digestion pH and temperature, KCN combines with free aldehyde groups to form cyanohydrins which, in contrast to HCN, do not volatilise at the beginning of the digestion. These are TCA-soluble and would introduce inaccuracies when hydrolysis is calculated from the non-protein N fraction of a digest. Bulk preparations of leaf protein are seldom free from reducing sugars: at the concentration of KCN used (0·005M) the errors would be slight, but it is obviously undesirable to have an uncontrolled variable in a digestion mixture. (Buchanan and Byers)

Amino acid composition of leaf proteins. It is established that the composition of bulk preparations of proteins extracted from leaves of different species is similar, and varies little with leaf age, so routine analyses on unfractionated leaf protein seem unnecessary. However, preparations made from *Amaranthus gangeticus*, *Momordica charantia* and *Vigna sinensis* were analysed because there was no information about protein extracted from tropical leaves: they had compositions similar to those of all other unfractionated proteins.

Analyses on some 'cytoplasmic' fractions of lupin made by heat fractionation were reported in 1966. This year fractions were made from lupin and barley leaves by precipitating with acid from the supernatant fluids after centrifuging extracts at different speeds. The lupin fraction sedimenting at 800 × g, which contains almost all the chloroplasts, contained less methionine, histidine, proline and lysine, and more phenylalanine and leucine than the unfractionated protein. The protein that remains insoluble after digestion with papain contains less aspartic and glutamic acid, proline and lysine, and more leucine, iso-leucine and phenylalanine than the original protein. (Byers)

Physical structure of the protein. Leaf protein is more slowly digested *in vitro* than most other food proteins. It seems improbable that this is an intrinsic property of the protein because it is a mixture of many different proteins that probably all have different digestion rates. It is more probable that the protein is shielded from enzyme attack in some way. Electronmicrography shows that very few intact chloroplasts remain in heat-coagulated protein, so the protein is not completely enveloped. Attempts to increase the rate of *in vitro* digestion by removing part of the carbo-

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hydrate present by digestion with fungal amyloglucosidases failed. (von Hofsten)

By-products and co-operation with other institutes. Not unexpectedly, the liquor that runs away from heat-coagulated protein varies in composition according to the species, age and fertiliser treatment of the crop. Dry matter ranged from 12 to 41 g/l, nitrogen from 0·25 to 1·2 and total carbohydrate from 2·5 to 22. Average values for the composition of the dry matter are 3·1 % N and 40 % carbohydrate. With the grasses, fructosans and other reserve polysaccharides accounted for about half the carbohydrate; the proportion from wheat and some other crops was smaller. When heated for a few days at 100° C the fructosan in hay breaks down to fructose; the fructosan in leaf extracts could be broken down in a similar way. Nevertheless, the carbohydrate in leaf extracts is of questionable nutritional value to single-stomached animals and much of the N has little nutritional value. We therefore disagree with the policy, now advocated in some quarters as an improvement on our method, of drying the whole extract rather than separating the protein. Ten to twenty times as much water would have to be removed by evaporation, the product would have a strong flavour, it would be much more subject to 'Maillard reactions', and would be nutritionally impaired. It is preferable to use the liquor as a substrate for the growth of micro-organisms. During the past year we have gone to the other extreme and have systematically analysed the fluid that is discarded when the protein is resuspended and washed. Washing, in our opinion, is inadequate unless this fluid contains less than 3 g dry matter per litre; a press-cake containing 70 % water would then be contaminated with only 1 % of soluble material. (Festenstein)

The extracted fibres from all those species used for bulk protein production were dried in a current of warm air and sent to the School of Agriculture at Newcastle where they will be used in an experiment to determine their nutritive value for sheep, both alone and when supplemented with urea. Although the fibre contains, on average, only 1·8 % N, its nutritional value is likely to be greater than that of a 'super-hay' with that N content because the crop providing the fibre would have been younger and less lignified.

Many gallons of liquor from lucerne were sent to the Department of Pathology at Cambridge for use in research on the control of cholesterol metabolism. About 120 kg of protein was sent to twelve different institutes, the largest consignment to the University of Rhode Island for use in a feeding experiment comparing various novel proteins. The last of the large-scale pulpers, made during the period when design was fluid, was sent to the Rowett Research Institute.

Studies on pigments

Relationship between xanthophylls and growth substances. The naturally-occurring growth substance abscisic acid (dormin, abscissin II) has a similar chemical structure to xanthophylls, violaxanthin and neoxanthin, which contain epoxide groups. Taylor and Smith (*Nature, Lond.* (1967),

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215, 1513) reported that violaxanthin, after irradiation with a tungsten lamp, produced a substance that inhibited the germination of cress seeds. We did some preliminary experiments to see whether leaf xanthophylls can be converted into substances that inhibit plant growth.

Xanthophylls were prepared from sugar beet, wheat and bean leaves. Chlorophylls were removed from acetone extracts either by saponifying with methanolic potash or by chromatography on a polyethylene column. In addition to lutein, violaxanthin and neoxanthin, several other pigments that seem not to be artifacts occurred in smaller amounts. The major xanthophylls were separated from each other by chromatographing on a column of cellulose powder using petroleum ether/acetone (85:15) as developing solvent. Lutein fractions from several columns were pooled; on standing at 2° C an orange precipitate of pure lutein separated. Neoxanthin was almost free from other pigments, but the violaxanthin fraction contained a minor xanthophyll and it was usually partly converted to luteoxanthin, though sometimes almost completely to aureoxanthin. Antioxidants could not be used to prevent this because they might interfere with subsequent assays for growth substances.

Measured amounts of pigment were put on discs of filter paper. After the solvent had dried, half the papers were irradiated by a 60-watt lamp at a distance of 15 cm. All the papers were moistened with distilled water and cress seeds placed on each. After 48 hours the lengths of the radicles were measured and the amount of inhibition estimated by comparison with growth of controls on papers without pigments. Even before being irradiated, both violaxanthin (two epoxy groups) and neoxanthin (one epoxy group) seemed to inhibit seedling growth to about the same extent. Irradiation destroyed the pigments, as shown by a progressive change in the absorption spectra as the irradiation was continued, and the amount of inhibition of radicle growth was usually increased. Lutein, which does not have an epoxy group, did not inhibit growth of cress seedlings before it was irradiated, but when exposed to tungsten light the colour disappeared and growth was inhibited. (Holden and Humphries)

Changed chlorophylls. Chlorophyll derivatives with colours similar to the parent compounds are formed in leaves and leaf extracts *in vitro* under various conditions. At least some of these derivatives, termed 'changed' chlorophylls, seem to be produced by oxidation of Ring V of the chlorophylls. The properties and conditions of formation of such pigments were studied further to compare with altered chlorophylls observed in processed vegetables.

'Changed' chlorophylls *a*-1, *a*-2, *a*-3, *b*-1, *b*-2 and *b*-3, produced from chlorophylls *a* and *b* by conversion on silica-gel G, were purified by thin layer chromatography on cellulose and characterised by measuring the rates at which fractions with different absorption spectra 'run'. Alterations to the chlorophylls when left 12 days in the dark at 20° C in 80% acetone, 0·01M in sodium bicarbonate, were also examined. About 50% of chlorophyll *a* was converted to 'changed' chlorophyll *a*-3. With pheophytin *a*, about 30% of Mg-free *a*-3 was formed, together with 10% of a compound with absorption maxima in diethyl ether at 666 and 400 nm, and 5% of a

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pigment or mixture of pigments showing maxima at 686, 662 and 404 nm. The *b* pigments were altered faster than the *a* pigments. Ten per cent of chlorophyll *b* was converted to a compound absorbing maximally at 649 and 443 nm (678 and 431 nm with magnesium removed). In addition, much pigment spectroscopically and chromatographically identical with 'changed' chlorophyll *b*-3 was formed, together with 10% of a compound with the same spectrum but a smaller R_F value. The spectra of the two pigments after removing magnesium showed maxima at about 665 (a broad peak), 426 and 414 nm, and suggested more than one component in each. When chlorophyll *b* was left for three days only, there was 40% conversion to a compound that co-chromatographed with *b*-3 but absorbed slightly differently (absorption maxima at 635 and 445 nm; at 655 and 426 nm with magnesium removed). With pheophytin *b*, the two products formed absorbed maximally at 653 and 427 nm and at 655 and 427 nm, and had R_F values slightly smaller than pheophytin *b* and chlorophyll *a*, respectively.

These results indicate that the range of altered chlorophylls is even wider than observed previously. Precise characterisation of 'changed' chlorophyll *b*-3 and its magnesium-free derivative is difficult because of the chromatographic and spectral similarity of some of the pigments described above. (Bacon)

Preliminary results suggest that chromatography of plant extracts on polyethylene powder can lead to altered chlorophylls or chlorophyllides forming rapidly when the water-acetone developing solvent contains 60% or less of acetone. There is probably little alteration with 70% or more of acetone, unless chromatography is prolonged or the eluted pigments are stored without removing water. A similar trend was observed with purified chlorophylls left in aqueous acetone, where conversion to altered chlorophylls was slower in 80% of acetone than in 50%. (Bacon and Holden)

Staff and visiting workers

R. A. Buchanan returned to Australia and J. I. Ahmad to India after gaining Ph.D degrees of London University for studies on leaf protein. Dr. B. von Hofsten (from the Institute of Biochemistry at the University of Uppsala), the secretary of the Swedish International Biological Program, spent three months with us. Mr. M. A. Fafunso came here for two months at the expense of the U.K., I.B.P. to learn how to use the laboratory-scale pulper and press that were being sent jointly to the Departments of Chemistry at the University of Ife and Biochemistry at the University of Ibadan. We had shorter visits from Dr. Rajammal P. Devadas, the Principal of the Home Science College at Coimbatore, Mr. M. F. Maguire, of the Agricultural Institute near Dublin, and Mr. R. J. Bobic who hopes later to work on leaf protein in India.

N. W. Pirie attended I.B.P. symposia on 'The biological basis of productivity' in Bulgaria and on 'The evaluation of novel protein products' in Sweden. J. M. Hill was awarded the M.Phil. degree of London University.

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