ROTHAMSTED RESEARCH

Report for 1959

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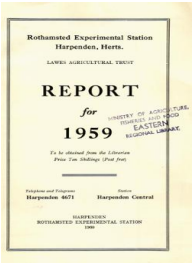

Biochemistry Department

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BIOCHEMISTRY DEPARTMENT

N. W. PIRIE

R. J. Stephens resigned and the vacancy was filled by G. Jenkins; the temporary vacancy during the secondment of R. H. Kenten to the West African Cocoa Research Institute (Tafo, Ghana) was filled by N. Singh; both the appointments made possible by the Rockefeller Foundation grant have now been filled, the second by N. G. Davys. Miss C. Morton and Mrs. W. Duah came for about a month to give us expert help in cooking leaf protein.

At the invitation of the Government of Ghana, Marjorie Byers spent three months in Kumasi studying the extractability of protein from the types of leaf available there; as guest of the Bulgarian Government, N. W. Pirie visited agricultural institutes in Bulgaria.

THE EXTRACTION OF PROTEIN FROM LEAVES

Laboratory scale investigation

The domestic mincer and three-roll ointment mill have been used during the past 20 years to study the extraction of protein from leaves readily available at Rothamsted; this year the study was extended to leaves common in Ghana. The 60 species examined ranged from specially grown crops, through leaves that are a by-product of some other crop (e.g., sweet potato and sugar cane), to wild plants. Eight species yielded high-quality protein and about half the protein of the leaf was extractable; as would be expected, these were mainly legumes with soft leaves. This was a hurried preliminary examination, but it showed that tropical plants have leaves suitable for large-scale extraction of protein and that the texture and appearance of a leaf are fairly reliable guides to its value. (Byers.)

In the hope of getting more reproducible conditions of disintegration, a unit was made in which leaves were forced through a narrow annular slot at pressures up to 10 tons/sq. in. Disintegrating tobacco leaves at different stages of growth in this unit has given some information about the factors that affect the extractability of leaf protein. Slightly alkaline conditions increase the extraction, mainly because of increased breakdown of the chloroplasts so that their fragments separate more fully from the fibre. Protein extraction is not more complete by this method than it is in high-speed grinders of the " Waring Blendor " type, but a larger proportion of what is extracted remains suspended after centrifuging at 1,500 g. It is not yet clear whether the difference arises because one method is breaking up structures originally present in the leaf, or the other causes some protein to coagulate. Much of the protein that remains in the fibre fraction after these processes is liberated by extraction with detergent solutions, and the state of subdivision of the protein in the initial extract is increased by addition of detergents. This may be important in increasing the yield of protein in large-scale extraction. (Festenstein.)

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Extracts made for the large-scale production of leaf protein were also fractionated centrifugally in the laboratory to see whether the losses, when extraction is inadequate, are evenly distributed among all the protein fractions or fall characteristically on one of them. In comparisons between samples of the same crop at different stages of maturity the losses are evenly distributed, but the position is less clear when different species are compared.

For 185 years it has been known that the proteins in leaf extracts precipitate in sequence as the extracts are increasingly heated and that those associated with chlorophyll coagulate first. This is rediscovered from time to time, and it is often suggested that the fact might be applied to make colourless bulk preparations. Several lots of leaf sap from seven different species were examined and, by judicious heating, 30% of the protein extracted from mustard and pea leaves was obtained in a relatively colourless fraction; the process was less satisfactory with other species. (Singh.)

Large-scale preparations

In spring bulk production for a pig-feeding experiment at the Rowett Research Institute took precedence over other work. This was essentially a repetition of last year's experiment at the National Institute for Research in Dairying; both showed that leaf protein is as good as or a little better than fish meal.

Two new crops, *Chenopodium amaranticolor* and turnip tops (*Brassica rapa*), were added to the list of species processed on a large scale. The cereal crops used were confined to wheat, rye and maize. This year the weather was again atypical, with the hot, dry summer a striking contrast to 1958. A good maize crop was grown in these conditions under irrigation, in contrast to the complete failure last year; but the lack of rain affected many of the crops grown for use in July and August. In particular, white clover failed to grow tall enough to be cut mechanically. (Byers and Jenkins.)

A new press was built to our design and works satisfactorily. Leaf pulp is carried on a perforated annulus under a platen with a thick rubber face; the platen is hinged at one side and is lifted and pressed down by cams; during the lifting movement, the platen engages a lever system that moves the annulus round so as to bring a new lot of pulp into position to be pressed. The press is much simpler than the older one, but, like it, is in effect continuous, although pressure is maintained for periods that can be varied from 4 to 15 seconds so as to allow the juice to run away completely.

The unit designed to extract leaf sap in one operation from 100–300-lb. lots of leaves progresses, but we are still not satisfied that we have a satisfactory design to offer to an institute or village wishing to start production in a small way. (Davys and Pirie.)

The techniques of handling leaf extracts to make the standard type of protein remains essentially unchanged, but we have improved the technique of drying the protein so as to get a stable product. Air-dried protein is rather dark and gritty, and freeze-dried protein is not much better when the moist material is frozen slowly in a deep-freeze before being put in the vacuum chamber. When unfrozen protein is used, in lots small enough to freeze within

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a few seconds *in vacuo*, the dried material is pale and soft textured. Four or five pounds of dry protein can be made in a week with our equipment.

Fresh, moist protein was mostly used in cooking, and an extensive repertoire of recipes was devised. Some cooked foods keep well and can be given to visitors to eat at any time, others can be produced from stored pre-mixed ingredients in a few minutes, but some need longer preparation. Tastes vary, but every visitor found at least one of our dishes acceptable. (Morrison and Pirie.)

Studies on Plant Enzyme Systems

Plant-leaf mitochondria

Evidence that mitochondria isolated from tobacco leaves retain much of their structural and chemical organisation came from two sources. First, they catalyse the process of oxidative phosphorylation, which depends on the closely integrated action of a series of enzymes and co-enzymes; secondly, they retain the "soluble" enzyme fumarase, which is released from them by treatments which rupture the external mitochondrial membranes. This chemical evidence for mitochondrial organisation is supported by the appearance of the isolated mitochondria in electron micrographs.

Orthophosphate disappears during the oxidation of many substrates of the tricarboxylic acid cycle. This suggests that there is oxidative phosphorylation, and the suggestion was confirmed when chromatographic and radioactive techniques demonstrated the synthesis of adenosine triphosphate (ATP) from orthophosphate. The phosphorylation reactions are only loosely coupled to the oxidations, and are inhibited by treatments such as "ageing" at 37°, or exposure to 2 : 4-dinitrophenol, which have little effect on the oxygen uptake. The amount of ATP formed depends on the substrate being oxidised. With malate and *iso*citrate about 2 atoms of orthophosphates are esterified per atom of oxygen absorbed, values approximately $\frac{2}{3}$ of the generally accepted theoretical maxima.

The fumarase of tobacco leaves is an –SH dependent enzyme which is competitively inhibited by orthophosphate. Over 90% of the total activity of the leaf extracts is in the washed mitochondrial preparations, but treatments as gentle as repeated freezing and thawing disrupt the mitochondria and release about $\frac{2}{3}$ of the activity in a condition not sedimented by high centrifugal forces (50,000 g. for $2\frac{1}{2}$ hours). Thus, although the link holding most of the fumarase on to the mitochondrial preparation is fairly weak, it successfully withstands the isolation procedure.

How far the cytochrome-linked oxidations of the mitochondrial preparations from tobacco leaves account for the oxygen uptake of intact leaves is a matter for speculation. Recent reports implicated glycollic acid oxidation in the respiration of illuminated leaves, and drew attention to the oxidation of glycollic acid by the tobacco-leaf mitochondrial preparations. This oxidation differs from the oxidation of tricarboxylic acids in a number of respects. It is, for example, not inhibited by cyanide, and it is not coupled to the synthesis of ATP. Moreover, most of this activity in the leaf extracts is not associated with any particles sedimented by high centrifugal

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forces (55,000 g. for 2 hours); less than one-tenth of the total is associated with the mitochondrial preparations. It seems certain that the oxidation is catalysed by glycollic oxidase and not by a cytochrome system. If any portion of the respiration of intact plants is mediated by this enzyme, it will differ from that catalysed by the cytochromes in not being connected to the " high-energy phosphate " synthesising machinery of the mitochondria. (Pierpoint.)

Enzymic degradation of chlorophyll

Chlorophyllase, the enzyme that splits chlorophyll into phytol and chlorophyllide, has hitherto not been separated from cell fragments and so has been studied in suspension rather than in solution. Soluble enzyme preparations were made this year from acetone-powders of the leaves of several species by soaking in sodium citrate solution for 24–48 hours, after the powder was first extracted with ethanol and ether. For unknown reasons, the citrate extract appears to have several times as much enzyme activity as the powder from which it was derived. Sugar-beet leaves have exceptionally high activity and are by far the most useful source of the enzyme. When the activity of a soluble enzyme preparation is being determined, the acetone concentration has to be brought from 60 to 40%, as the higher concentration is strongly inhibitory, whereas it is optimum for insoluble preparations. The enzyme in solution is fairly stable in the cold at pH values between 7 and 9; a variable amount of activity is lost on dialysis. Attempts to concentrate and purify it have so far failed; even precipitation with ammonium sulphate causes a large loss of activity.

The changes in chlorophyllase levels were followed in different parts of pea seedlings grown on moist sand in full light or in the dark. The enzyme level in the cotyledons rose for the first 5 days and then fell. In the seedlings grown in the light, chlorophyllase activity in the shoot increased rapidly. Roots were almost devoid of activity at all stages. When the seedlings were about 2 weeks old over 80% of the total activity was in the leaves, compared with only about 15% of the dry weight. The stems had most of the rest of the activity. In etiolated seedlings the activity in the shoot increased only slightly while they were in the dark, but the level rose sharply when they were transferred to the light.

Attempts to separate the chlorophyllides by paper chromatography using many solvent systems failed. However, several of these systems separated other breakdown products of the chlorophylls, such as phaeophytins and phaeophorbides, satisfactorily. Attempts are being made to separate these compounds on a larger scale on sugar columns. No trace of coloured breakdown products of chlorophylls were detected in extracts of leaves of mature tobacco plants kept in the dark until they were yellow.

It was confirmed that the leaves of barley seedlings have no detectable chlorophyllase activity but that during incubation in aqueous acetone green oxidation products are formed by enzyme action. No enzyme system was found that converts the chlorophylls and their coloured degradation products to colourless compounds. (Holden.)

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Plant amine oxidase—a metalloprotein enzyme

By fractionation on calcium phosphate and aminoethyl cellulose, amine oxidase preparations from pea seedling extracts were purified 1,000-fold. At this stage the enzyme solution is pink, with an absorption band with its maximum at 495 mμ, it is decolorised by dithionite, and the colour is restored by bubbling with oxygen. Carbonyl reagents, such as hydroxylamine, hydrazine, semicarbazide and *iso*nicotinoyl hydrazide, which are known to inhibit the enzyme, also discharge the pink colour. A similar effect of substrate (putrescine), under anaerobic conditions, had previously been reported. These findings suggest that the pink colour is a property of the enzyme.

The presence of copper and manganese in the 300-fold purified preparations was reported in 1954. During the final stages of the purification the copper content increases while the manganese content remains constant or decreases slightly. In highly purified preparations the copper and manganese contents vary from 0·05 to 0·07%. Spectrographic analysis of one preparation (by H. H. Le Riche) confirms that copper and manganese are present in higher concentrations than other heavy metals. The increase in the copper content during purification suggests that the enzyme is a copper protein, as also does its inhibition by agents that chelate copper. Though the manganese content does not increase during purification, it is present finally at the same molar concentration as copper. Manganic manganese forms pink complexes with certain peptides. The light absorption of these manganic peptides closely resembles that of the enzyme preparations, suggesting that the pink colour of the enzyme preparations may be caused by a complex of manganic manganese. Should this prove to be so, the question will arise whether the inhibition of the enzyme by carbonyl reagents, attributed by others to the presence of pyridoxal phosphate in the enzyme, results from the known reactions of these compounds with manganic manganese. (Mann.)

Complexes of manganic manganese with peptides

Manganic manganese is responsible for a pink complex formed when reaction mixtures containing a manganous salt and L-leucylglycine are incubated under alkaline conditions, because it is not formed under anaerobic conditions and manometric experiments show that the intensity of the colour is proportional to the uptake of oxygen. The absorption spectrum of the complex shows a band in the visible region resembling that of manganipyrophosphate but with a maximum at a slightly longer wavelength (495 mμ). The colour is discharged by reducing agents such as dithionite. The complex also oxidises hydrazine with evolution of nitrogen and, as with manganipyrophosphate, this reaction can be used to estimate the manganic manganese of the complex manometrically. Preliminary investigations suggest that some other peptides form complexes with manganic manganese. Positive results were obtained with DL-leucylglycine, glycylglycine and glycyl-DL-alanine and negative results with glycyl-L-leucine. Complexes of manganic manganese probably occur in plants. (Mann.)

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Catalysis of the formation of oxygenated cobalt complexes by peroxidase systems

The oxidation of manganous manganese by hydrogen peroxide is catalysed by peroxidase and certain phenols, and this system also catalyses the formation of reversibly oxygenated complexes of cobalt. When peroxidase is added to reaction mixtures containing a cobaltous salt, histidine, hydrogen peroxide and trace amounts of *p*-cresol, a strong yellow-brown colour forms almost instantaneously. The absorption spectrum of the yellow-brown product shows a band with a maximum at 385 mμ. The colour is partially discharged when the reaction mixture is evacuated in a Thunberg tube, and more completely by acidifying; manometric experiments show that the loss of colour is accompanied by evolution of oxygen. The yellow-brown compound slowly changes to a red irreversibly oxygenated complex. These properties are similar to those previously reported by other workers for the reversibly oxygenated oxy-bis (cobaltodihistidine) complex. (Mann.)

Autolytic enzymes

More nitrogen is always precipitated from a leaf extract by trichloro-acetic acid than by boiling the extract; the difference is sometimes as great as 15%. This has long been known, but the phenomenon remained unexplained. It is now clear that ribonuclease and leaf protease are responsible. Nucleic acid is precipitated by trichloroacetic acid along with protein, but accompanies the protein into the precipitate only when it is intact, and exposure to the ribonuclease in most leaf extracts destroys it. Therefore, when the extract is allowed to age before precipitation there is less intact nucleic acid and, ribonuclease being a relatively thermostable enzyme, nucleic acid is also destroyed during the heating that precedes boiling. This can be demonstrated by determining the nucleic acid in protein precipitates made by both methods after varying amounts of heating and incubation. The protease activity of leaves that do not contain latex is small but not negligible. For example, 40% of the protein in the sap of young wheat becomes unprecipitable by either heat or trichloroacetic acid when the sap is incubated for 2 hours at 37° before precipitation. The effect is less with the sap of the other leaves yet studied, but these two phenomena suffice to account for the observed differences. (Singh.)

Eelworm Hatching Factors

During 1959 only 2·5 tons of potato-root diffusate (supplied by the Nematology Department) were put through the same preliminary stages of purification as last year. By fractionation on silica columns and precipitation from ether : petroleum ether, the melting point of the cream-coloured, microcrystalline, active fraction was raised to 147–149°, but 70–90% of the material is lost in the process, so that the final yield is 50–250 μg. from a litre of diffusate.

The Nematology Department also supplied ⅓ ton of tomato-root diffusate, which, like potato-root diffusate, stimulates eggs of *Heterodera rostochiensis* to hatch. The active material in both diffusates

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seems to be the same, and the purification procedure developed with potato-root diffusate yielded a similar active product from tomato-root diffusate. Furthermore, with six different solvent systems, both products show maximum hatching activity at similar regions of paper chromatograms.

Work was started on the root diffusates from rape, which contains hatching factors for the cabbage-root eelworm (*Heterodera cruciferae*) and the beet-root eelworm (*Heterodera schachtii*).

More bioassays are needed before the results can be assessed properly, but the procedure for isolating the hatching factor from potato-root diffusate gave an end product that stimulated eggs of beet-root eelworm to hatch. The product resembles the potato hatching factor in its solubility properties, fluorescence and chromotographic behaviour, but does not stimulate hatching of eggs of potato-root eelworm. (Clarke.)

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