ROTHAMSTED RESEARCH

Report for 1960

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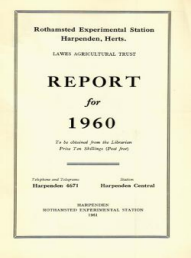

Biochemistry Department

N. W. Pirie

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BIOCHEMISTRY DEPARTMENT

N. W. PIRIE

G. Jenkins resigned and the vacancy was filled by J. W. Sturrock. During the summer Mrs. N. Ayles and Miss S. Herklots joined the staff temporarily to help in cooking leaf protein.

At the invitation of the Jamaican Government, N. W. Pirie visited Jamaica to study the possibilities of making leaf protein there on a large scale; as a result, a local committee was set up and one of our pulpers was sent to Jamaica in October. We hope to send a press soon. N. W. Pirie attended a "Conference on Protein Needs" and the Vth International Congress on Nutrition in Washington.

THE EXTRACTION OF PROTEIN FROM LEAVES

Laboratory scale investigations

As experience increases, our confidence in judging, from the appearance and feel of a leaf, whether protein will be readily extracted from it, also increases. But some batches extract unexpectedly badly, and this cannot always be explained by the usual inhibitory factors, such as acidity or the presence of tannins. Work on the reasons for poor extraction can proceed in two ways: by studying the same species in different physiological states and by comparing species.

Winter wheat was sampled and minced at intervals; the sap was fractionated centrifugally and the ratio of chlorophyll to nitrogen determined on the fibre and sap fractions. In young leaves (less than 170 days from sowing), the ratio was about the same in sap and fibre, which suggests, as did earlier work, that the protein remaining in the fibre is not a special form but remains there for mechanical reasons. Older leaves have relatively more chlorophyll in the fibre, and the chloroplast fraction in the sap is a smaller proportion of the total extracted protein than in young leaves; this suggests that mechanical entrainment of chloroplasts increases as leaves age and that the increased entrainment is associated with diminished extractability of protein. The technique used in the experiment was not adapted to determine which of the changes accompanying maturity was responsible for this extra entrainment. The experiment is being repeated in a more refined manner. No clear pattern emerges from one similar comparison between 10 plant species taken at arbitrary ages. The ratio of chlorophyll in the fibre to chlorophyll in the extract was not correlated with total protein extraction. (Singh.)

Detergents will extract protein that remains associated with the fibre after leaves are minced or extruded through a fine slot. Their action is most apparent on fibre that has already been extracted with alkali because the optimum conditions for extraction can then be arranged without having salts present in high concentration. In

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this way 90–95% of the protein can be extracted even from tobacco leaves up to 15 cm. in width, provided they are still in good condition. (Festenstein.)

Several components of leaves and other tissues are damaged by exposure to air during the first stages of extraction, and several workers have described glove boxes and similar pieces of equipment in which extracts can be made anaerobically. But their use requires very large volumes of inert gas. With slight modification, the arrangement that we use for grinding leaves by forcing them through a narrow slot can be used anaerobically. It gives reproducible results, is quick in action and, because of its small volume, economical with inert gas. (Pirie.)

Gibberellic acid has been used extensively in the treatment of crops, but the reported effects on the dry matter and crude protein yields vary widely. An experiment, using spring vetches (*Vicia sativa* L.), was designed to measure the effect of gibberellic acid on both the dry matter and crude protein yields, and on the extraction of protein from the leaf. A single application (0·08 g. gibberellic acid in 1·0 l. water per 10-foot × 6-foot plot) did not significantly affect yields of dry matter or crude protein from the foliage taken at the first cut, 10 weeks after sowing; there was also no significant difference in the yields of extracted protein at this stage. However, the yields of dry matter and crude protein were both significantly decreased at the second cut, 4 weeks later. Two additional applications of the same amount of gibberellic acid, after the first cut, further decreased these yields at the second cut. The single and triple applications lowered the yields of extracted protein at the second cut enough for the difference in total extracted protein to be significant. Spraying with gibberellic acid before the second cut only, did not affect the dry matter but lowered the yields of both crude protein and extracted protein, though the totalled yields from two cuts were not significantly lowered. It is clear that, with this crop, the yield of protein is not increased by treatment with gibberellic acid. (Byers and Jenkins.)

Large-scale preparations

As in past years, the main work in spring was to produce enough protein for feeding trials. Pig-feeding experiments at the Rowett Research Institute were so successful that comparisons between different leaf species are being undertaken. These results have also interested various laboratories concerned with human nutrition, and 10–20-lb. lots of dry protein have been sent to the Tropical Metabolism Research Unit (Jamaica), Department of Pediatrics (Kampala), Department of Pediatrics of New York University School of Medicine and the Instituto du Nutrición de Centro America y Panama (Guatemala). Preliminary results at the first of these are encouraging.

A new pulper is now in use that is, in principle, the same as the one we have been using for several years, but with various dimensions and details altered to facilitate cleaning and give more economical running. It is satisfactory, and another is being made incorporating still more improvements. (Davys and Pirie.)

The equipment used for large-scale freeze-drying has been radically improved so that we can now freeze 5–8-lb. lots of moist

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protein within a few seconds and get satisfactory drying in 2 days to a soft attractive product. This equipment is being still further improved to meet the increasing demand for protein. The very large surface exposed by this dry material raises oxidation problems, because the leaf lipids are highly unsaturated and give leaf protein a characteristic flavour as they oxidise. Many of the flavours that, by convention, we relish, are also the consequence of oxidation of fats; nevertheless, with a novelty like leaf protein, it seems wise to aim at having as little flavour as possible. Considerable effort has therefore been put into a study of vacuum packing, nitrogen packing, the use of antoxidants and the selection of the best type of container for year-long storage of dry protein. (Morrison and Pirie.)

Lipid content of leaf proteins

A systematic study of the lipid content of bulk preparations has been started. All figures quoted relate to the lipid fraction initially extracted with acid alcohol–ether, and re-extractable in alcohol–ether from the dry state. The figures are much higher than those got by simpler extraction procedures here and elsewhere. The maximum figures for a selection of crops are given in Table 1. The lipid content seemed independent of the age of the crop from which the preparation was made and of the number of times the crop had been cut. From analyses yet made <1% of the total N appears in the lipid fraction.

TABLE 1

Lipid content of leaf protein (% of D.M.)

Crop	Min.	Max.
Maize	25·3	26·8
Mustard	21·9	23·4
Pea vines	26·8	28·3
Red clover	24·2	30·5
Rye	31·9	33·5
Tares	24·0	25·3
Wheat	29·4	31·3

(Byers.)

PLANT ENZYME SYSTEMS

Amines and minor elements in plant metabolism

The inhibition of plant amine oxidase by reagents that react with heavy metals suggests that the enzyme is a metalloprotein. Copper and manganese are present in higher concentration than other metals in preparations purified by the method described last year, which suggests that the enzyme contains one or both of these metals. The copper and manganese compounds in these preparations have now been partially separated by chromatography on diethylaminoethyl-cellulose columns to give a copper fraction (containing 0·08–0·09% Cu and <0·01% Mn) and a manganese fraction (containing 0·10–0·15% Mn and 0·01–0·2% Cu). Most of the amine oxidase activity is in the copper fraction, which also retains the pink colour that we have suggested is a property of the amine oxidase.

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Spectrographic analysis of this fraction (by H. H. Le Riche) showed that, except for zinc, the concentration of which was <0·06%, other trace elements were present at concentrations <0·004%. The results suggest that the enzyme may contain copper but not manganese, and exclude the possibility, suggested last year, that the pink colour comes from a complex of manganic manganese. With hydrazine, the primary reaction product of the pink compound is yellow and absorbs maximally at 420 mμ, which suggests an aromatic carbonyl compound. No evidence was obtained to support the suggestion of other workers that this carbonyl compound is pyridoxal phosphate. Attempts to demonstrate the liberation of pyridoxal phosphate by acid or alkaline treatment of the enzyme preparations failed.

Most of the copper can be removed from the preparations by precipitation with sodium diethyldithiocarbamate. The supernatant solution, from which the excess diethyldithiocarbamate is removed by dialysis, remains pink, but generally shows little amine oxidase activity. Most of the lost activity can be restored by adding Cu^{2+} ions. Conditions have not yet been established under which the enzyme inactivates completely in these experiments. Maximum reactivation is given with concentrations of Cu^{2+} of the order of $10^{-6}M$, though partial reactivation occurs with concentrations $<10^{-7}M$ (0·006 μg Cu^{2+}/ml.). The failure to obtain complete inactivation, therefore, may happen because the reagents used are contaminated with traces of copper.

Preliminary results suggest that the copper-free enzyme is reactivated specifically by Cu^{2+} ions. There is no reactivation with Mn^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+} and Fe^{3+} ions. Copper is apparently present in the preparations in the cupric form; it reacts with 2 : 2′-diquinolyl to form the characteristic lilac cuprous complex only in presence of reducing reagents. Most complexes of cupric copper are blue or green, but the only absorption band shown by the enzyme preparation in the visible part of the spectrum is that from the pink compound. It is therefore suggested that the copper occurs as a complex with the carbonyl compound and that this complex forms the prosthetic group of the enzyme. (Mann.)

The enzymic breakdown of chlorophyll

Considerable progress has been made in the purification of chlorophyllase from sugar-beet leaves. Fractional precipitation of the protein, with acetone, from enzyme-containing extracts gives an active preparation with 50–60% of the original activity. This preparation can be dialysed against dilute neutralised cysteine solution without further loss of activity. Treating the dialysed solution with diethylaminoethyl cellulose removes all colour and some inert protein, with little loss of enzyme activity. The next step is the adsorption of the enzyme on carboxymethyl cellulose and elution with neutral phosphate buffer, which greatly increases the specific activity, but the adsorption is inconsistent and loses much activity. The best preparations have been purified 500-fold, measured on the basis of nitrogen content.

In collaboration with the Botany Department several chemicals, most of which are " growth substances " reported to increase or

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decrease the chlorophyll content of plants, were studied. One of the most interesting is 3-amino-1 : 2 : 4-triazole, which prevents chlorophyll formation in wheat seedlings because chloroplasts do not develop. Chlorophyllase activity, which in normal plants is associated with the chloroplasts, however, is similar in treated and untreated seedlings. The location of the enzyme is being investigated. (Holden.)

Leaf protease

The rapid autolysis of protein in wheat sap, commented on last year, stimulated a more detailed study of the variations in enzyme level with maturity, and a comparison of wheat with other plants. Casein was used as a substrate so as to avoid uncertainties caused by variations in the nature and amount of leaf protein present, and hydrolysis was measured both by increase in non-protein-nitrogen and in free tyrosine. The former was more trustworthy because of the presence of varying amounts of tyrosinase in the extracts. Two experiments, on winter wheat sown in early August and late November, gave similar W-shaped curves when protease activity per mg. of protein N in the sap was plotted as ordinate and age as abscissa; the maximum values were four times the minimum. Of nine species, wheat had the most and maize the least activity. (Singh.)

Leaf mitochondria

Mitochondrial preparations from tobacco leaves retain much of the organisation that they have in the leaf, but they are heavily contaminated with fragments of chloroplasts disrupted during the preparation. Partial separation of chloroplast fragments from mitochondria is achieved by centrifuging the preparations in a " sucrose gradient " made by layering sucrose solutions of decreasing strengths (2–0·3*M*) in the centrifuge tube. Any starch grains and bits of cell wall present quickly sediment to the bottom of the tube, while intact chloroplasts, being less dense, form a compact layer above the most concentrated sucrose solution. Chloroplast fragments and mitochondria occupy two distinct but overlapping zones near the top of the tube.

The centrifugal conditions so far used do not separate the mitochondria and chloroplast fragments completely enough to encourage attempts to prepare chlorophyll-free mitochondria, but the separation is adequate for enzymic activities to be attributed to one type of particle or the other. Thus the distribution of fumarase and cytochrome c oxidase in the centrifuge tubes parallels that of succinic oxidase closely enough to suggest that these activities are associated only with the mitochondria. The glycollic oxidase of the preparations does not sediment at all, and seems, in agreement with last year's results, not to be attached to either of these two types of particle. (Pierpoint.)

Studies on Virus-Infected Leaves

It is often asserted that the first step in a virus infection is the separation of the protein and nucleic acid moieties of the inoculum and that the first step in virus multiplication is the formation of

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nucleic acid which secondarily associates with protein. This is plausible but the evidence is unconvincing.

To study the process of multiplication, leaves infected with tobacco mosaic virus were pulped in the presence of phenol, and phenol was added to pulped leaves at different intervals after pulping. The infectivities of the aqueous part of these extracts, which contains no intact virus particles, were compared; the comparisons were made with leaves pulped at different intervals after inoculation and with systemically infected leaves. Insofar as there were consistent differences, it appeared that extracts from pulp that had lain at room temperature for 10–20 minutes (i.e., conditions in which, according to our earlier work, free virus nucleic acid would be destroyed by leaf ribonuclease) were the more infective. This result conflicts with the idea that any significant amount of the infectivity of an extract is conferred by nucleic acid that was free before the phenol was added.

The infectivity of all these extracts can be increased as much as tenfold by dialysis and, after dialysis, it can be diminished by a dialysate from either infected or uninfected leaves. The nature of this inhibition is still obscure, but it may explain differences between our results and those reported from other laboratories. (Pirie in collaboration with Bawden.)

A possible reason for the difficulties in getting purified infective preparations of some viruses is that the viruses are inactivated by the oxidizing enzymes liberated during the disruption of the leaves. Methods of disrupting the leaves, that preserve many of the intracellular components, prevent some of these reactions, and therefore might stabilise the easily damaged viruses. However, attempts to extract the unstable cucumber mosaic virus from infected tobacco leaves using the sucrose-citrate medium designed to preserve mitochondria and chloroplasts have not given encouraging results. Infective particles of the virus are extracted with it, but much better results are obtained when diethyldithiocarbamate, an inhibitor of some copper containing oxidases, is present. Indeed, in the presence of this substance, edta and citrate, which are essential components of the normal medium, hinder the extraction. The phenomenon is being further studied. (Pierpoint, in collaboration with Harrison.)

Eelworm Hatching Factors

The potato-root eelworm hatching factor

In the 1960 season, 1·34 tons of potato-root diffusate were processed, mostly by the charcoal adsorption method. Attempts to improve the yield at this stage by using ion-exchange resins failed. An average yield of 23% (10 experiments) was obtained, whereas charcoal adsorption gives an average yield of 32%. Improvements in the further purification simplified the process and increased yield by 5–10%.

The hatching factor seems to be a monobasic acid with an equivalent weight of approximately 220. Analytical results are not yet consistent, but they suggest the formula $C_{11}H_{16}O_4$. Solutions of the hatching factor have no optical rotation. Previous workers suggested that a lactone ring is part of the structure of the

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hatching factor, but colour tests diagnostic of αβ- and βγ-unsaturated γ-lactones were negative. Also, the infra-red spectrum (chloroform solution) shows a strong carbonyl absorption band at 1712 cm.$^{-1}$, in keeping with the absorption of a carboxylic acid, but without a maximum corresponding to the carbonyl absorption of an αβ- or βγ-unsaturated lactone.

Beet-root eelworm hatching factors

0·89 tons of root diffusate from rape seedlings grown in soil were processed by the charcoal adsorption procedure. A further 15 gallons of root diffusate from rape seedlings grown without soil was concentrated by evaporation *in vacuo*, or by adsorption on anion-exchange material; this diffusate had about ten times the hatching activity of diffusate from seedlings grown in soil. Attention has been directed solely to the factors present in these root diffusates, which stimulate the hatching of the beet-root eelworm (*Heterodera schachtii*).

Paper chromatograms of partially purified concentrates of rape root diffusates show four to nine regions with hatching activity. The number varies with the solvent system, the concentration at which assayed and the previous treatment of the sample. The results suggest that there are one, or possibly two, substances with high R_F values (0·70–0·90) with strong beet-eelworm hatching activity, which have some similar properties to the potato-root eelworm hatching factor. These compounds are responsible for a greater proportion of the hatching effect with concentrates obtained by direct evaporation, and a lesser proportion with concentrates obtained by adsorption on charcoal. Work has continued on methods of isolation and purification of the more active hatching agents.

Urea, some amino-acids, and sugars stimulate the hatching of the beet eelworm (Wallace, *Rep. Rothamst. exp. Sta.* for 1956, p. 262), but none of these approach the efficacy of the root diffusate at the optimum concentration. Compounds of this type have been reported in root exudates, and the agents with slight hatching activity detected on paper chromatograms may be such compounds. (Clarke.)

Chemical Changes in Self-heated Hay

Samples of good hay (baled at 22% moisture, maximum temperature 40°) and mouldy hay (baled at 46% moisture, maximum temperature 62°) were freeze-dried and analysed for water-soluble solids, glucose, lipids, total and soluble nitrogen. Mouldy hay contained less of all these constituents than good hay; particularly glucose, lipid and soluble nitrogen.

Samples taken at intervals up to 3 weeks after the wet hay was baled, during which time the mould developed, were also analysed to establish when the changes occurred; up to 9 days after baling the samples gave results for water-soluble solids, glucose, lipids, and soluble nitrogen similar to those of good hay, but after 9 days there were appreciable losses, which were associated with a rise in pH from 5·6 to 7·4.

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The increase in acidity, which is characteristic of heating in hay, took place during the first 7 days, when the maximum temperature was reached and the pH fell from 6·5 to 5·5; the iodine values of the total lipids were little changed from 1 to 11 days after baling, indicating that the proportion of unsaturated compounds was not greatly affected during the heating period.

The greatest changes in chemical constituents of this hay occurred well after the temperature had reached its maximum value and had begun to fall, so it can be concluded that the organisms responsible probably differ from those causing the initial fall in pH associated with the rise and maintenance of temperature. (Festenstein, in collaboration with Bunce and Gregory.)

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