ROTHAMSTED RESEARCH

Report for 1961

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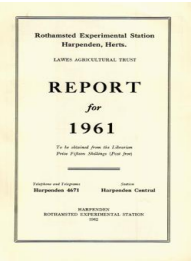

Biochemistry Department

N. W. Pirie

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BIOCHEMISTRY DEPARTMENT

N. W. PIRIE

Mr. F. H. Shah (Pakistan Council of Scientific and Industrial Research) is working in the department during the tenure of a Colombo Plan Fellowship, and Dr. F-T Khouw was here for some months with a fellowship from the Food and Agriculture Organisation. Drs. P. M. Sankaran (Ministry of Defence, India) and Mumtaz Hussain (Ministry of Defence, Pakistan) visited briefly to examine the process of extracting leaf protein on a large scale.

Six members of the department attended the 5th International Congress of Biochemistry in Moscow. N. W. Pirie spent six weeks in India, at the request of the Indian Government and the Colombo Plan, reporting on the prospects of making leaf protein there. He also visited the U.S.A. to take part in a symposium on " Nutrition for an expanding world population ", organised by the Society for Economic Botany, and the " Woodring Conference on the Origins of Life ", organised by the U.S. Geological Survey.

STUDIES ON PLANT PROTEINS

The breakdown of chlorophyll

About 50 kg. sugar-beet leaves were processed to prepare chlorophyllase. Little further progress was made in purifying the enzyme, but preparations with high specific activity can now be made quickly and simply in reasonable yield. Cellulose phosphate is a much more efficient adsorbent than carboxymethyl-cellulose, and therefore gives a higher yield, but it is less specific, so gives preparations with lower specific activity. Electrophoresis shows the presence of three components in a 200-fold purified preparation.

Up to 40% of the chlorophyll of some leaves has been stated to be extracted by non-polar solvents, but preliminary investigation of its extractability by polar and non-polar solvents failed to confirm this. With most of the species tested less than 5%, and with none more than 10%, of the total chlorophyll was soluble in petroleum ether. Only young leaves have so far been examined, and solubility may change on ageing.

Chlorophylls *a* and *b*, pheophytins *a* and *b*, chlorophyllides *a* and *b* and pheophorbides *a* and *b* were all separated from each other by suitable variations of the proportions of petroleum ether, benzene and acetone in the running solvent and in the atmosphere of the tank in which the paper chromatograms are run.

Acetone extracts from French bean leaves give a red crystalline precipitate on standing in the cold. A tentative identification of this as carotene was confirmed by Dr. V. Booth of the Dunn Nutritional Laboratory. Carotene precipitation also occurred in acetone extracts from the leaves of several other species, but in amounts small compared with those from bean leaves grown in a glasshouse with extra illumination, from which as much as 0·1% of the dry

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matter of the leaf will precipitate out of extracts containing about 80% acetone. (Holden.)

Plant amine oxidase, a copper-protein enzyme

A study of the inhibition of plant amine oxidase by chelating agents confirmed the suggestion, made last year, that the enzyme is a copper protein. The chelating agents used were 1 : 10-phenanthroline, 2 : 2-dipyridyl, 8-hydroxyquinoline, sodium diethyldithiocarbamate and potassium ethyl xanthate. At concentrations of 30–300 μM all these reagents completely inhibited the enzyme. The inhibitions could be prevented by adding metal ions to the reagents, to form the respective metal chelates, before the enzyme was added. Several metal ions, including Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} ions, partially or completely prevented the inhibition with all the reagents except potassium ethyl xanthate. Cu^{2+} ions generally appeared less effective than Co^{2+} or Ni^{2+} ions, because the enzyme was rapidly inactivated by Cu^{2+} ions during the course of the reactions it catalyses. When this inactivation by Cu^{2+} ions was prevented, by adding pyrophosphate to the reaction medium, Cu^{2+} ions completely prevented the inhibition by all the chelating agents and were the only metal ions that prevented the inhibition by potassium ethyl xanthate.

Except with sodium diethyldithiocarbamate, the same metal ions which prevented the inhibitions also reversed the inhibitions produced by preincubating the enzyme with the chelating agents. The inhibition of the enzyme by sodium diethyldithiocarbamate was partially or completely prevented by Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} ions, but was reversed only by Cu^{2+} ions.

Estimations of the copper content of the enzyme preparations, after dialysis against the chelating agents, showed that copper was split off from the preparations by sodium diethyldithiocarbamate but not by the other chelating agents. Dialysis against water, to remove the chelating agents, reversed the inhibitions except that produced by sodium diethyldithiocarbamate, which was reversed only by Cu^{2+} ions.

The primary reaction producing the inhibition is probably the combination of the chelating agent with enzyme-bound copper to form an enzyme–inhibitor complex. These complexes are dissociable and generally break down, on dialysis or on adding metal ions with which the inhibitor forms a complex, to liberate the copper-protein enzyme. The enzyme–sodium diethyldithiocarbamate complex dissociates into copper-free protein and copper diethyldithiocarbamate complex; only Cu^{2+} ions can then reverse the inhibition.

The copper is firmly bound to the enzyme protein, and the amine oxidase may therefore be classified as a metalloenzyme. The enzyme is inactivated during the reactions it catalyses by hydrogen peroxide, which is one of the reaction products. Preliminary results suggest that Cu^{2+} ions catalyse this reaction–inactivation. Other copper-containing oxidases are subject to rapid reaction–inactivation and with one of them—ascorbic acid oxidase—the reaction–inactivation is also catalysed by Cu^{2+} ions. (Hill and Mann.)

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Leaf protease

Protease activity per g. protein N in young leaves of spring wheat sown in a glasshouse in December or winter wheat sown in January increased rapidly when the shoot apex changed from the vegetative into the reproductive state; after this the activity soon fell to about half of the maximum. Activation and inhibition studies show that leaf protease belongs to the SH-group of enzymes. The starting activity of leaf extracts is so low that they are unpromising sources from which to purify protein-splitting enzymes, and the protein could not be purified more than five-fold. (Singh.)

Leaf mitochondria

Mitochondria are completely separated from chloroplasts by centrifuging leaf extracts in a " sucrose gradient "; separation from fragments of broken chloroplasts is less complete. This year a slight modification of the procedure increased the separations a little, still not enough to produce chlorophyll-free mitochondria, but enough to show whether particular enzymic activities are associated with mitochondria or chloroplasts. There is increasing interest in the enzymic capabilities both of purified chloroplasts and of mitochondria, so it seemed worth while to find what factors limited the usefulness of this method of separation.

Exposure to the high osmotic pressures of the sucrose solutions that form the gradient does not appreciably affect the activity of glycollic oxidase or the mitochondrial enzymes fumarase and cytochrome oxidase. Nor does it appear to affect the attachment or non-attachment of these enzymes to cellular particles. This is not so with malic dehydrogenase, another enzyme of the tricarboxylic acid cycle. The small amounts of activity present in crude mitochondrial or chloroplast preparations appear to be eluted during sedimentation in concentrated sucrose solutions. Such an elution of very loosely bound components is obviously a major limitation to the usefulness of the technique in its present form.

The cytochrome oxidase activity of tobacco-leaf extracts increases 2-4-fold after standing on ice for 24 hours. The effect of certain surface-active agents on this activation suggests that it may be caused by the slow disorganisation of the lipid-containing mitochondrial membranes, so allowing freer approach of the enzyme to its substrate, reduced cytochrome c. This increase resembles the activation of chlorophyllase that occurs when it is extracted from acetone powders of leaves, and also, but perhaps less closely, the activation of polyphenolase of broad-bean leaf extracts. These effects emphasise a difficulty in estimating enzymes in crude leaf extracts, especially those enzymes, like chlorophyllase and cytochrome oxidase, that are surrounded by, or are built into, membranes of cellular structures. (Pierpoint.)

VIRUSES

Highly infective preparations of cucumber mosaic virus were prepared from tobacco leaves disrupted in the presence of the copper chelating compound sodium diethyldithiocarbamate, but not otherwise. To study the copper-dependent inactivation of the virus,

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attempts were made to construct model inactivating systems *in vitro*. One, produced simply by adding copper and chlorogenic acid to the dialysed leaf extract, probably depends on the polyphenol-oxidase present in the extract. It was extremely effective against virus preparations made in the spring and, as it is likely to operate during the disruption of infected leaves, most attention was given to it. However, progress was hindered, as it was much less active against virus preparations made later in the year. This difference may reflect some uncontrolled part of our technique or some seasonal difference in the virus preparations. (Pierpoint, in collaboration with Harrison, Plant Pathology Department.)

Large-scale Production of Leaf Protein

Progress is being made in establishing leaf-protein production at places other than Rothamsted. A pulper and press sent to Jamaica are being used under the guidance of a committee set up by the Scientific Research Council. A pulper and press are on loan to British Crop Driers (Navenby, Lincs.); the unit is not yet fully installed, but some runs were made in late autumn, and full production should start next spring. The " village unit " mentioned in the 1959 Report was extensively redesigned and now works satisfactorily on 100–200-lb. batches of leaf. Juice is extracted in one operation, and the process takes about an hour and requires 1–2 h.p. The prototype, made for belt drive, will be sent to the National Botanic Gardens (Lucknow), where work on the extraction of leaf protein is about to start. A somewhat simplified machine now being made will be adapted for drive either by a belt or a built-in electric motor.

Our presses, working on the principle of intermittent application of pressure by a platen to a mass of pulp that moves forward as the platen rises, are still satisfactory, but there are defects in intermittent action. We have therefore made a continuous press in which pulp is fed on to the inner surface of a belt that carries it continuously between the belt and a perforated drum. Performance exceeds expectation, and we are now modifying the pulper so that the two machines can be properly integrated. (Davys and Pirie.)

Three new crops, Alsike clover (*Trifolium hybridum*), strawberry clover (*Trifolium fragiferum*) and rape (*Brassica napus*), were grown here for large-scale extraction, and tomato (*Solanum lycopersicum*), comfrey (*Symphytum peregrinum*) and *Tagetes minuta* were got from outside sources. None seems to be preferable to the crops with which we normally work. On the laboratory scale, many species were extracted for the first time during 1960 and 1961: *Melilotus alba, Lupinus luteus, Medicago lupulina, Tropaeolum lobbianum, Atriplex hortensis rubra, Stellaria media, Petroselinum crispum, Lotus corniculatus, Anthyllis vulneraria, Trifolium dubium, Alliaria officinalis, Glechoma hederacea, Anthriscus sylvestris, Heracleum spondylium, Galium aparine, Mercurialis perennis, Tanacetum vulgare, Carduus arvensis, Ranunculus repens, Lamium album, Pennisetum glaucum.* Only the first seven gave very good results, but all were better than the following, which are not recommended: *Onobrychis sativa, Glycine max, Aegopodium podagraria, Arctium lappa, Ranunculus*

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ficaria, Chamaenerion angustifolium, Tussilago farfara, Galeopsis tetrahit, Fagopyrum esculentum, Cichorium intybus, Poterium sanguisorba, Buphthalmum sp., Centauraea sp., Lactuca sativa, Oenothera lamarkiana, Calendula officinalis, Chrysanthemum coronarium, Hedysarum coronarium, Campanula rapunculus, Arum maculatum and Salvinia auriculata.

Four varieties of maize (*Zea mays*) were harvested at three dates; striking differences appeared in the yield of extractable protein per acre and the apparent optimal date for harvesting. They need not be discussed now, because the experiment will be repeated next year. One result worth noting, however, is that Caldera 331 photosynthesised at the rate of 40 tons of dry matter per acre per year during the periods 27 July to 16 August. The amino-acid compositions of the twelve batches of protein are being determined by Prof. M. A. Stahmann (Wisconsin), because some earlier results suggested that these may differ with variety and age as well as species; any such differences might explain some disagreement between feeding experiments done on different batches of protein made at different times from the same species.

At the request of the Medical Research Council Tropical Metabolism Unit, Jamaica, some protein labelled with ^{15}N was prepared. ^{15}N, as $K^{15}NO_3$, was fed in nutrient solution to a mildew-resistant variety of wheat, grown in sand in the greenhouse, and cut twice, 9 weeks after sowing and 4 weeks later. First growth yielded 59 g. containing 10% N with 0·7% atom excess of ^{15}N: the excess on the product from the second growth was smaller. This protein was freeze-dried and is now being used in human metabolism experiments. (Byers and Sturrock.)

Overheating leaf protein during drying diminishes its feeding value for animals; experience during the last 10 years suggests that storage also affects it and that the lipids became less readily extracted after a time. Quantitative tests showed that heating affects the lipids; thus, from a batch of clover protein 24·7% of lipid was extracted by a mixture of chloroform and methanol from fresh or freeze-dried material but only 20·7% after drying in an oven at 100°. The effect may be physical, because lipid can be extracted fully from oven-dried protein softened with phenol before extraction. (Shah.)

We prepared less protein than last year because an adequate stock to meet such feeding trials as were foreseen was built up early in the year. The freeze-drier was further improved; its capacity is now 5 kg. of ice per cycle, and the duration of a cycle is shorter. This allows a substantial proportion of the protein from each run to be stored in such a way that it can be reconstituted into a paste very similar to the normal fresh product. Freeze-drying also allows protein to be sent economically to many parts of the world, but it is not envisaged as part of non-experimental production.

Dried protein keeps well with little change in flavour when tightly packed in a sealed container to diminish access of air, but for prolonged storage it is packed in N_2. Various methods of preservation by fermentation in the presence of oatmeal, diastase, malt extract and cabbage (as in sauerkraut) were tried. They have not, so far, been wholly successful, but technique is improving; there is room for

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differences of opinion on the merits of the traditional products of fermentation so that criteria of success are uncertain. (Morrison and Pirie.)

The performance of the " village unit " was studied at various stages of its evolution to decide the best weight for the roller and distribution of the bars on it. Many runs were made with the final version, in which the rate of liberation of juice and consumption of power were measured. Protein made in the unit resembles that made by the larger apparatus. (Singh.)

The Potato-root Eelworm Hatching Factor

700 gallons of potato-root diffusate were processed in 1961. Diethylaminoethyl-cellulose failed to give satisfactory recoveries of the hatching factor from potato-root diffusate, so the complex behaviour of the hatching factor during absorption on and desorption from charcoal is being studied to improve the yields.

The purified hatching factor is more difficult to handle in the solid form than was first expected, because it is unstable and hygroscopic. The pale-green copper salt and a cream-coloured calcium salt are both highly active and suitable for analysis. The hatching factor shows no evidence of free carbonyl or hydroxyl groups. Attempts to obtain crystalline derivatives (esters and amides) of the acidic group of the hatching factor failed. The hatching factor gives a positive iodoform reaction and a negative Zimmerman test. Both reactions are given by the CH_3CO-group, but groups readily oxidisable to a CH_3CO-group also give a positive iodoform reaction.

Drastic methods of attack, such as alkali fusion, zinc dust distillation, hydrogen iodide–red phosphorus reduction, etc., proved uninformative, because the products were resinous and not amenable to purification. The products from acid hydrolysis of the hatching factor, however, can be separated into a steam-volatile portion, an ether-extractable acidic fraction, an ether-extractable neutral fraction and a water-soluble residue. Each of these fractions gives a mixture of products when treated with 2 : 4-dinitrophenylhydrazine; five compounds and traces of two others were found in the steam volatile fraction. Progress was also made in separating and purifying the 2 : 4-dinitrophenylhydrazones from the other fractions. The recrystallised derivatives are being characterised by various methods, including X-ray powder photographs (by G. Brown, Pedology Department), a particularly valuable method with the small amounts of material available (0·5–6 mg.).

Experiments with purified material showed that inactivation at room temperature is very rapid (within minutes) at pH $\geqslant$ 11, and more slow at a lower pH. The inactivation is complete and irreversible. The alkali-treated hatching factor has an increased acid function, which previous workers attributed to the opening of a lactone ring. An alternative possibility now being examined is that inactivation and the increased acid function arise from the action of alkali on a readily hydrolysable ester group. Acid hydrolysis after an initial alkaline treatment of the hatching factor appears to give fragments similar to those given by direct acid hydrolysis.

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Beet-root eelworm hatching factors

Rape seedlings grown in nutrient solution are one of the most convenient sources of root diffusate with high hatching activity, but hatching curves with such diffusates are abnormal, possibly because they contain inhibitors, and the efficacy of subsequent treatments cannot readily be followed. The diffusate from rape seedlings grown in soil is less active, but gives a normal hatching curve and so was used as starting material to develop isolation procedures. Concentrating the hatching factor in the diffusate by direct evaporation *in vacuo* is more effective in producing active concentrates than is absorption on charcoal. (Clarke.)

Chemical Changes in Self-heated Hay

Studies on the chemical changes in baled wet hay from Great Field, begun in 1960, were extended to include frequent analyses of hay, both baled and stacked at 30% (" wet ") and 15% (" dry ") moisture content. The wet stack was initially about 12 feet high on a 15-foot-diameter base, and the other about 8 feet high. The maximum temperatures reached were 55° and 27° for the wet and dry bales respectively and 65° and 23° for the wet and dry stacks respectively. The stacks were sampled through pipes penetrating into the interior.

Samples taken at intervals up to 90 days were freeze-dried, stored at —20° and analysed for ash, glucose, total, soluble and steam-volatile nitrogen (distilled at pH 9·5, chiefly ammonia); pH was determined on suspensions of undried samples from the same batches, which were also used for bacterial studies (Soil Microbiology Department) and fungal studies (Plant Pathology Department).

Mould developed unevenly in the wet bales, and the analyses showed corresponding variations: the pH values varied from 5·5 to 6·8, but the glucose content dropped sharply 10 days after baling and remained low. Soluble nitrogen was little in some samples, but more than in the mouldier samples of 1960. Steam-volatile nitrogen increased 5 days after baling and remained high.

The pH of samples from the middle of the stack of wet hay fell from the original 6·2 to 5·3 in 3 days, and the values remained between 4·5 and 5·0 after 18 days; the soluble nitrogen rose sharply after 2 days and remained high, and steam-volatile nitrogen likewise increased. Glucose values were lower than those of dry hay, but higher than those of wet bales. The changes resemble those occurring in silage.

Respiration continued during the 5 days of wilting before baling or stacking and led to loss of dry matter, which was measured by the increase in ash content. After this there was only a slight increase in ash content, although the high temperature maintained shows that metabolism continues, but it does not lead to the formation of a volatile product such as CO_2.

During the 90-day period the changes in the dry bales and stack were small.

Hay from Great Knott 1 baled at 35% moisture content (max. temp. 60°) rapidly developed mould, and its pH was 7·9 ten days

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after baling. The samples varied more than those from Great Field (there were different grasses present), and though the analyses gave roughly parallel results, steam-volatile nitrogen increased greatly in only a few samples. (Festenstein in collaboration with Gregory, Plant Pathology Department, and Skinner, Soil Microbiology Department.)

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