ROTHAMSTED RESEARCH

Report for 1958

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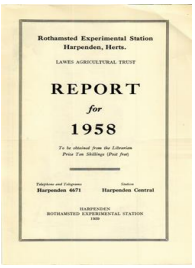

Biochemistry Department

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BIOCHEMISTRY DEPARTMENT

N. W. PIRIE

There have been an unprecedented number of staff changes in the department. M. V. Tracey resigned to become Head of the Commonwealth Scientific and Industrial Research Organization Wheat Research Unit, Sydney, Australia, and the vacancy has been filled by Dr. G. N. Festenstein. D. Fairclough and W. E. Whitman resigned, and these vacancies were filled by R. J. Stephens and Dr. W. A. Vincent. Margaret Holden returned from secondment to the West African Cocoa Research Institute (Tafo, Ghana) and R. H. Kenten has gone to take her place. As a result of a grant of $75,000 from the Rockefeller Foundation, to be spent during five years, two new positions have been created for work on the large-scale production of leaf protein. One has been filled by J. E. Morrison, and the other will be filled in the New Year.

The fourth International Congress of Biochemistry was attended by W. S. Pierpoint and N. W. Pirie; the latter, on the invitation of the Prime Minister, visited Ghana to consider the possibilities of making and using leaf protein there.

Blackening of potatoes after boiling

Samples of Majestic potatoes were taken from the Rothamsted and Woburn six-course rotation and dung NPK experiments during the 1957 harvest. Fertilizer treatments in the six-course rotation experiments consisted of basal PK, NK or NP with and without four levels of N, P or K respectively. In the dung NPK experiments farmyard manure was applied at three levels in all combinations with N, P and K. In both experiments the N, P and K were applied as ammonium sulphate, superphosphate and potassium choride.

Boiling tests were made before and after 5 months' storage. Potatoes from the Woburn DNPK experiment, irrespective of fertilizer treatment, blackened only slightly. In general, those from the other three experiments showed a much more marked blackening. The only clear effect of fertilizer treatment was in the Rothamsted dung NPK experiment, where the potatoes from the plots receiving NPK together with farmyard manure at the highest level blackened little more than those from the Woburn DNPK experiment.

It is now generally believed that the pigment causing the blackening of potatoes after boiling is a complex of ferric iron and dihydroxy phenols. It is suggested that when potatoes are boiled, ferrous iron is liberated and combines with the dihydroxy phenols of the potatoes to give relatively colourless ferrous complexes, which oxidize in air to the intensely coloured ferric complexes. On this hypothesis the intensity of the blackening depends not only on the concentrations of ferrous iron and of dihydroxy phenols but also on those of other compounds present forming relatively colourless iron complexes.

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Citric acid may be of major importance, since its concentration in potato tubers is high compared to that of the dihydroxy phenols.

Observations made here are in agreement with this hypothesis. Blackening was prevented by adding citrate, pyrophosphate or ethylenediamine tetra-acetate, which form relatively colourless complexes with iron, to the water in which the potatoes were boiled, and the colour of potatoes previously blackened by boiling was discharged by treatment with these reagents. Raw potatoes gave no immediate test for ferrous iron with *o*-phenanthroline, but potatoes tested immediately after boiling gave a strongly positive reaction and did not blacken after treatment with the reagent. The formation of ferrous iron on boiling was not confined to the stem end of the potatoes where the characteristic blackening occurs, and also occurred in samples of King Edward potatoes, though these did not blacken after boiling. Blackening of Majestic potatoes was intensified by adding ferrous sulphate to the water in which the potatoes were boiled, while similar treatment of King Edward potatoes caused only a slight yellowing.

Estimations were made of the iron, dihydroxy phenol and citric acid content of the potatoes. In addition, attempts were made to trap and estimate the ferrous iron formed during boiling. While the latter estimation was not put on a satisfactory quantitative basis, the results suggested that much of the total iron was liberated in the ferrous form on boiling. No differences were found in the dihydroxy phenol and iron contents of the Majestic potatoes to account for the observed differences in blackening. In 31 of a total of 33 estimations the stem ends of the potatoes were found to contain more iron than the rose ends. The value (μg. Fe/g. wet wt.) were 4·2–8·7 at the stem end (average 6·3), and 2·6–6·8 at the rose end (average 5·0). The results of the citric acid estimations suggested an inverse relationship between the concentration of citric acid and the intensity of blackening. The concentration of citric acid in the Woburn DNPK potatoes, which blackened only slightly, was generally much greater than that in the potatoes from the other three experiments. As found by other workers, the stem ends of the potatoes, at which blackening typically occurs, always contained much less citric acid than the rose ends. Thus in the Woburn DNPK experiment, the values (mg./g. wet wt.) ranged from 2·8 to 4·7 at the stem end and from 5·8 to 8·3 at the rose end. In the Rothamsted and Woburn six-course rotation experiments the comparable values were 0·8–2·0 (stem) and 3·2–5·1 (rose). In these three experiments no effect of the fertilizer treatments on the citric acid contents was found. In the Rothamsted DNPK experiment the citric acid concentration varied with the fertilizer treatment. The lowest values of 1·1 (stem) and 3·4 (rose) were obtained with the potatoes from the unfertilized plots. The highest values of 3·7 (stem) and 6·5 (rose) were obtained with the potatoes receiving NPK together with the highest level of farmyard manure. These results suggested that the observed differences in the intensity of blackening were due to differences in citric acid content. But in the Rothamsted DNPK experiment many of the other fertilizer treatments caused increases in the citric acid content without decreasing the blackening. This suggested that some other factor was involved, though, at least in part, the

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lack of correlation may have been due to the very variable results of the boiling tests on different samples of the same batch of potatoes. (Mann.)

Plant leaf mitochondria

The medium described last year, containing sucrose, tris buffer, EDTA and citrate, is still the most effective one for the preparation of leaf mitochondria. This year's work has therefore been mainly concerned with its use in the preparation of mitochondria from tobacco.

Citrate, more than any other of the constituents of this medium, has a protective action on the oxidative properties of mitochondria of both tobacco and lupin leaves. The mechanism of this action is not well understood; but the ability of succinate to replace citrate suggests that it is a substrate stabilization of enzymes of the tricarboxylic acid cycle. There is apparently too little citrate in tobacco leaves to exert this protection when the leaves are disrupted. It is generally less than $\frac{1}{5}$ of that added in the extraction medium.

Further evidence has accumulated suggesting that the oxidative reactions of these preparations are components of a tricarboxylic acid cycle similar to that of animal mitochondria. Thus, many of them are inhibited strongly by cyanide, and reversibly by malonate. Also the products of oxidation, identified by paper chromatography, are other substrates of the cycle. Orthophoshate disappears during the oxidation of succinic acid, suggesting that this oxidation at least is connected to the synthesis of " energy-rich " organic phosphates.

The activity of the mitochondria varies with the physiological state of the leaves they are derived from. Thus preparations from the smaller younger leaves of a plant are more active, on a protein N basis, than those of the older leaves. The leaves of 3- and 4-month-old plants which are sending up flower shoots tend to be covered with a sticky exudate, and preparations made from such plants have little activity unless only very small leaves are used. It would be premature, however, to conclude that these activities represent the activities of the mitochondria *in vivo* until more is known about the factors present in disrupted leaves which affect the mitochondria during their isolation. (Pierpoint.)

Chlorophyllase

A study of the enzymic breakdown of chlorophyll in plant tissues has been started. At present remarkably little is known of the processes involved, although chlorophyll disappearance is such a marked feature of ageing in the normal leaf, and of changes associated with virus infections and nutrient deficiencies. Chlorophyllase, the enzyme catalysing the removal of the phytol group from chlorophyll (a comparatively small alteration in the molecule), has been investigated in the leaves of a number of species. Spinach-beet leaves are a particularly good source of the enzyme. So far it has not been possible to make a soluble enzyme preparation, and either acetone powders of fresh leaves or powders (containing chlorophyll) from leaves dried at 40° have been used. It has been confirmed that a high concentration of alcohol, acetone or ether is necessary for enzyme action to take place. Attention has

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been directed to developing a rapid method for measuring enzyme activity, since most of the methods used so far are laborious. A number of surface-active agents had no influence on the rate of enzyme action when tested with either a high or low concentration of acetone present. The enzyme is said to be active in an aqueous system at about 65°, but this has not been satisfactorily confirmed because the substrate was affected at this temperature. The separation by paper chromatography of the breakdown products of chlorophyll is now being studied. (Holden.)

Properties of fragments of tobacco mosaic virus (TMV)

This section of last year's report dealt mainly with the effects of ribonuclease, or extracts whose activity could plausibly be attributed to ribonuclease, on the infectivity of TMV fragments made by treatment with phenol. These experiments have continued, but, in an attempt to get evidence about the widely held view that nucleic acid is solely responsible for the infectivity, most attention has been given to systems in which it would be unreasonable to attribute the inactivation to ribonuclease. The problem has to be posed in this roundabout way because the disinfection of TMV fragments is the most sensitive test for ribonuclease that we know, and the preparations of TMV that we use are not themselves always free from ribonuclease.

Infectivity is destroyed by various oxidizing systems that would not be expected to affect the structure of a nucleic acid built up in the conventional way. Mitochondria made from young tobacco leaves, especially in the presence of ethylenediamine tetra-acetate or some of the Krebs cycle substrates, bring about inactivation. It may be that this is a consequence of oxidizing reactions or of enhancement of ribonuclease activity, but a definite decision on these points has not yet been possible. Neither mechanism offers a plausible explanation of the more rapid loss of infectivity in the complete absence of air or of its loss in the presence of thiamine and thiaminase made from bracken fronds. The latter is a particularly interesting phenomenon, because it may shed light on the function of this curious enzyme; one possibility is that, *in vivo*, it is concerned with the attachment and detachment of groups along the side of a nucleic acid chain.

Studies on the infectivity of TMV fragments have an intrinsic interest, but they may also shed light on the first stages in the normal processes of infection. There is already good evidence that the inoculum is dismantled when it enters a susceptible cell. This has been strengthened by measurements of the distribution of radio-active P in different fractions of the leaf at intervals after inoculation with TMV containing radio-active P. After 3 days more than half of the labelled P appears in low-molecular-weight forms. This is clear evidence for the dismantling of the inoculum, but so far all attempts to get a similar dismantling with a leaf preparation, *in vitro*, have failed. (Pirie in collaboration with Bawden.)

Large-scale production of leaf protein

This work has a two-fold object; to develop techniques for using plants or parts of plants that do not at present contribute to human

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nourishment, and to increase the amount of food that can be got with existing crops from a given area in a given time. We have not yet tackled the problems of selection and husbandry that the first object involves, but, now that the general principles of extraction are becoming stabilized, we have made some progress with the second.

Winter wheat and rye were drilled more closely and fertilized more heavily than in normal farm practice; they are useful crops because they permit work to start at the beginning of May, but the yield of extracted protein (256 and 360 lb./acre respectively) is not as high as that given by spring-sown oats cut in June (471 lb./acre). A second, June, cut from the winter-sown wheat produced half as much protein as the first cut. The dry spring and wet, sunless summer showed that we have still a lot to learn about the management of these heavy, leafy crops. The cereals are convenient, but it is probable that they are far from ideal. Thus, nettles growing more or less wild except for some fertilization, gave 546 lb. of extracted protein per acre, which compares favourably with the sugar-beet figures quoted last year.

In comparing these yield figures with those got by normal methods of agriculture, three points should be borne in mind. With cereals the land is ready for ploughing and re-sowing by the middle of June, the protein that we do not extract is still left in the discarded fibre and available for cattle feeding, and we have only just begun studying the conditions for efficient husbandry.

Only one new crop has been added to our list of protein sources during the year—river weeds. These could be important because 100 miles of river is cleared of weeds each year by just one of the local Drainage Boards, and the product is left to rot in heaps on the bank. We were able to extract 47 per cent of the protein from a rather mature batch.

The standard product was again the 5-lb. block containing 50 per cent water which needed to be deep frozen; but work has continued on other methods of preservation. As extracted, the protein contains a large proportion of lipid, which is readily made rancid by the bacterial flora developed during the extraction process. Solvent extraction, air drying, vacuum and freeze drying, and drying after admixture with various flours and meals have been tried and have been found to be generally successful in preventing this deterioration. It has been necessary to preserve wet samples of protein for dispatch overseas, and where refrigeration was not possible some form of canning was essential. Work has been carried out on direct sterilization by canning, the difficulties found being mainly due to the low rate of heat penetration and the consequent vigorous heat treatment required for a sterile pack. This destroys some of the palatability of the protein, particularly in the outside layers. Work is now being concentrated on the production of a " commercially sterile " pack which can be rendered inert by the bacteriostatic action of salt or antibiotics, on the principle that these agents can easily be removed on opening the packs by washing the insoluble protein with water.

We have not improved on ravioli and cromeskis, described last year, as vehicles for presenting the protein to possible consumers in

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Britain, but several standard Ghanaian dishes have been supplemented with leaf protein and proved acceptable there. More animal feeding experiments have been completed, and some are running at present; they confirm that the protein has a feeding value similar to that of high-grade fish meal. (Byers, Morrison, Pirie, Stephens and Vincent.)

The potato-root eelworm hatching factor

The Nematology Department supplied a record quantity of potato-root diffusate (4¼ tons) during the past season. The bulk of this material has been treated with charcoal under the conditions mentioned in *Rep. Rothamst. exp. Sta. for 1956* (p. 93, Wiltshire), and the active material recovered by elution of the charcoal. The recoveries obtained in the past season have been lower (32 per cent average of 57 experiments), than those obtained by Wiltshire in the 1955 season (39 per cent average of 8 experiments). As noted by Wiltshire, the recovery of activity fluctuates widely, and the causes of this are being investigated. One variable factor which may be involved is the inorganic ion content of the solutions assayed.

Each batch of crude material, obtained by evaporation of the charcoal eluates, has been purified by a three-stage chemical procedure elaborated during the past year. This procedure has given consistent and reproducible results, an average of 85 per cent of the activity in the charcoal eluates being obtained in 9 per cent of the weight. The material obtained is a mixture of organic acids. Chromatographic procedures have been used to resolve this mixture into a number of fractions which are being tested for hatching activity.

The behaviour of the hatching factor on paper chromatograms has been investigated. Six solvent systems have been used, and the R_F values associated with the hatching activity determined in each case. In all the solvent systems used, hatching activity is associated with an elongated fluorescent spot on the chromatogram, but at least some of this fluorescence is due to inactive material. The R_F value of the hatching factor, in one of the solvent systems, phenol–water, is the same as that reported for the hatching factor present in the tomato-root diffusate. The hatching factor is not inactivated by periodate, nor by the digestive juices of *Helix pomatia*, suggesting that a glycosidic moiety is not an essential feature of the active molecule. (Clarke.)

Plant enzyme reactions leading to the formation of heterocyclic compounds

The work on the blackening of potatoes after boiling and on the potato-root eelworm hatching factor has seriously curtailed further study of plant enzyme reactions leading to the formation of heterocyclic compounds, and on the role of plant amine oxidase in the formation of pyrrolidine and piperidine alkaloids. (Clarke and Mann.)

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