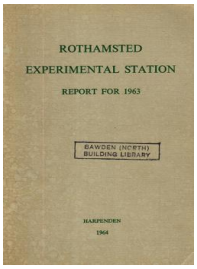
ROTHAMSTED RESEARCH

Rothamsted Report for 1963

Full Table of Content



Biochemistry Department

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BIOCHEMISTRY DEPARTMENT

N. W. PIRIE

Pamela Bell and S. F. H. Shah left the department and M. F. Bacon joined it. Hilary Searle was on the staff for six months. Mr. J. J. Bohannan worked on large-scale protein extraction for two weeks. W. S. Pierpoint accepted an invitation to spend most of the year at the University of California (Los Angeles). N. W. Pirie attended a conference in the U.S.A. on "The origin of Prebiological Systems" and a meeting in Edinburgh of the Planning Committee of the International Biological Programme.

Studies on Plant Proteins

Plant diamine oxidase. We are now satisfied that there is copper in the prosthetic group of diamine oxidase, and attention has shifted to the identity of the carbonyl compound that apparently also forms a part of the prosthetic group. Other workers have suggested that this is pyridoxal phosphate. Attempts to liberate pyridoxal phosphate from the enzyme by methods used with known pyridoxal phosphate-containing enzymes have so far failed, which suggests that either it is bound very firmly to the enzyme protein or that the carbonyl compound is not pyridoxal phosphate.

The claim that the prosthetic group contains pyridoxal phosphate was based on the observation that, when plant saps containing the diamine oxidase were dialysed, with the object of removing the prosthetic group, their rates of oxygen uptake and ammonia formation in the presence of added amines were increased by adding pyridoxal. Clarke & Mann (*Rothamsted Report* for 1956, p. 90) reported that the increased oxygen uptakes were caused, at least in part, by oxidation of the pyridoxal rather than by activation of the apoenzyme. This oxidation is brought about by the hydrogen peroxide produced during the oxidation of the amines and is catalysed by the peroxidase present in the plant saps. Diamine oxidase is inactivated during the reactions it catalyses; this reaction inactivation is attributed to the hydrogen peroxide formed during the oxidation of the amines, and is partially prevented by catalase. Experiments with purified enzyme preparations show that reaction inactivation of the diamine oxidase can be produced by adding peroxidase at concentrations of the same order as in the plant saps; this inactivation is not prevented by catalase. Peroxidase substrates, including pyridoxal, protect the diamine oxidase from this inactivation in so far as they are preferentially oxidised. These observations suggest that the increase in diamine oxidase activity produced by adding pyridoxal to systems of dialysed plant saps is not caused by reactivation of the apoenzyme but by preventing the inactivation of the holoenzyme.

Pyridoxal increases the rate pea-seedling extracts take up oxygen without added amines; when Mn^{2+} ions are also added there is a rapid and large uptake of oxygen. Carbon dioxide and ammonia are among the reaction

https://doi.org/10.23637/ERADOC-1-56

products. Preliminary investigations suggest that the reaction may be an example of the known peroxidase-catalysed oxidative decarboxylation of amino acids by pyridoxal and Mn^{2+} ions. Therefore, there is no evidence that the carbonyl compound in the diamine oxidase is pyridoxal, but the possibility has not been excluded. (Hill and Mann)

Manganese complexes in pea-seedling extracts. During the preparation of diamine oxidase, from pea-seedling extracts, partially purified preparations were found to contain more manganese than copper. During the final stages of purification, by column chromatography, the manganese complexes separate from the enzyme and appear in two main fractions, which were partially purified to give preparations with manganese contents of 0·14% and 0·08% respectively, based on the protein contents. No enzymic activity has so far been found in these fractions. (Hill and Mann)

The distribution of amine oxidases in higher plants. Manometric and colorimetric techniques detected only diamine oxidase in the plants yet tested, and there was no evidence for a monoamine oxidase or a specific histaminase, spermine oxidase or benzylamine oxidase. Diamine oxidase occurs in several plants, especially in the *Leguminosae* and *Labiatae*, but of several mature plants examined it was only detected manometrically in *Lavandula spicata* and pea root nodules. It occurs in week-old seedlings of several legumes, e.g., *Pisum sativum*, *Vicia faba*, *Phaseolus mungo*, *Phaseolus aureus* and *Trifolium pratense*. Enzyme activity was not detected manometrically in the extract of entire seedlings of field bean (*Vicia faba* var. Spring Tick), but when the seedlings were divided into root, cotyledons, epicotyl and shoot tip it was readily detected in the shoot-tip extract. Diamine oxidase was detected in the other extracts only by a more sensitive colorimetric method, depending on the formation of the yellow dihydroquinazolinium compound when *o*-amino benzaldehyde reacted with the Δ^1-pyrroline produced by the oxidative deamination of putrescine.

The results with *Vicia faba* suggest that diamine oxidase might be detected in mature plants provided these were subdivided into separate parts. In some of the early experiments by other workers who reported diamine oxidase, the enzyme was detected by the disappearance of a known amount of histamine added to the plant extract; the disappearance of histamine was followed pharmacologically. Histamine may have been destroyed by reactions other than oxidative deamination. Monoamine oxidase has been reported in several higher plants, including *Papaver rhoeas* and *Chelidonium majus*. No evidence was obtained manometrically for the presence of monoamine oxidase in mature specimens of either of these two plants. (Hill)

Tobacco-leaf polyphenoloxidase. Cucumber mosaic virus (CMV) is stable in extracts of infected tobacco leaves in which polyphenoloxidase is inhibited by diethyldithiocarbamate (DIECA) and from which polyphenols have been dialysed away. When chlorogenic acid is added, and the oxidase reactivated with copper sulphate, the virus is inactivated. But virus in many preparations is inactivated by chlorogenic acid alone, suggesting that

https://doi.org/10.23637/ERADOC-1-56

the oxidase recovers spontaneously from inhibition by DIECA without requiring added copper. This spontaneous recovery of the polyphenol-oxidase of some extracts is not caused by accidental contamination with copper; it has prompted a manometric study of the enzyme in tobacco leaf extracts.

Extracts of CMV-infected leaves, prepared in buffered DIECA and then dialysed, when incubated with copper sulphate and chlorogenic acid, absorbed almost 2 atoms of oxygen per molecule of chlorogenic acid present. The rate of oxidation usually depends on added copper and is diminished to a tenth at copper concentrations below 3·6 μM. However, extracts made from the leaves of plants that had been infected with CMV for many weeks are active without added copper sulphate. Thus, an extract made from leaves infected for 8 weeks rapidly oxidised chlorogenic acid, and the rate was increased only 10% by 0·36 mM copper sulphate.

Extracts of leaves infected for long periods contain three or four times as much copper as young leaves, and twice as much iron. This seemed a possible cause of the spontaneous reactivation of the polyphenoloxidase, but increasing the copper and iron content of younger leaves, either by infiltrating metal salts or adding them after the leaves were disrupted, did not reactivate the oxidase. Moreover, mixing extracts containing inhibited enzyme with extracts containing reactivated enzyme at different stages in their preparation did not reactivate the inhibited enzyme.

As the reactivation in leaves infected for long periods is not explained by their larger copper content, it seemed more likely to be due to differences in properties of the enzyme protein in leaves of different ages. This was supported by the values found for the Michaelis constant (K_m) of the enzyme in different extracts. In all extracts made from leaves of plants infected for long periods the K_m was approximately $2{\cdot}7 \times 10^{-3}$ M, but in some of those made from recently infected plants, and which took up little oxygen unless copper was added, the K_m was smaller. Extracts made from recently infected plants were also far less stable on storing at 2° or −5° C than were those from plants infected for long periods.

DIECA may inhibit polyphenoloxidase in two ways: by physically removing copper from the enzyme, or by forming a complex with the copper still attached to the enzyme. If it forms a complex the inhibition should be reversed by such treatments as exhaustive dialysis, incubation for short periods at 30° C at neutral or acid pH, or dialysis against salts of other heavy metals. All these methods were tried, but none reversed the DIECA inhibition of tobacco-leaf polyphenoloxidase. Thus it appears that inhibition involves actual removal of the copper, which makes the spontaneous reactivation of polyphenoloxidase in DIECA extracts of some leaves difficult to explain. (Bell and Pierpoint)

Cucumber mosaic virus. Cucumber mosaic virus (CMV) has been separated from most of the protein in extracts of infected tobacco leaves by filtering through columns of granulated agar gel. A gel of 3% agar is forced twice through 50 mesh gauze and the granules suspended in the 0·01*M* tris buffer (pH 7) used to prepare the column. Infective extracts are made in buffered sodium diethyldithiocarbamate (DIECA) and washed

https://doi.org/10.23637/ERADOC-1-56

through the column with the same buffer. Infectivity is mainly associated with a small peak of material which absorbs light at 258 mμ and is eluted immediately after the void volume; however, there is evidence that not all this material is virus. Most of the plant protein is eluted in a second peak which has little or no infectivity. The enzyme polyphenoloxidase, which causes inactivation of CMV in extracts not containing DIECA, is eluted after the virus peak, but a little before the bulk of the protein. The behaviour of CMV on agar gel is that expected from a spherical particle at least 30 mμ in diameter. (Bell, in collaboration with Harrison, Plant Pathology Department)

Large-scale production of leaf protein. The arrangements for running the three protein extraction units that have been sent to other institutions (in Lincolnshire, Lucknow and Jamaica) are still not altogether satisfactory, but it is reasonable to expect that in time proper arrangements will be made. A pulper has been sent to the Department of Agriculture at Makerere College so that they can start to study the prospects for using leaf protein in Uganda.

The principle underlying the extraction process has not been altered, but there have been many improvements in detail. The crop can now be fed into the pulper automatically from a large tank full of water; this helps to remove dirt and flints, ensures a more even feed and an optimal amount of water in the pulp. The smaller pulper, referred to in the Report for 1962, works well and, with a smaller expenditure of power, has as great an output as the larger pulpers. The belt press, referred to in the Report for 1961, was completely rebuilt so that it can be integrated with the pulper. This makes the unit more compact and easier to clean. The outstanding remaining problem is to make the arrangement for getting the crop from the top of the elevator into the pulper less cumbersome; some progress has been made on this during the year. (Davys and Pirie)

Two probably independent factors control the suitability of a cultural technique, and crop, for leaf-protein extraction: the amount of protein that accumulates in the leaf, and the readiness with which it can be extracted. Last year's results on barley were confirmed this year; "Nitro-Chalk" at 20 cwt/acre gave a leaf containing 3·55% N (in the dry matter) compared to 2·8% when "Nitro-Chalk" was used at 10 cwt/acre, but the yield of dry matter was smaller, as was the percentage extraction, so that the use of the extra fertiliser was slightly detrimental.

Maize is a useful crop, because its date of harvest allows continuity of production throughout the summer and autumn. Extraction from it is never so large as from wheat or barley (30–40% instead of 50–60%), but the percentage extraction does not diminish as the leaf matures, so that full use can be made of the late growth. Protein was extracted from the following new species this year: *Agrostemma githago milas* (corncockle), *Althaea rosea*, *Amaranthus caudatus*, *Euphorbia lathyrus*, *Lavatera trimestris*, *Nicandra physaloides* and *Perilla folii atropurpurea*, but only the corncockle gave a reasonable yield. This species will repay further study, as it gives the largest recorded yield of dry matter over a short period. (Byers and Sturrock)

Feeding experiments in other laboratories have shown that what is

https://doi.org/10.23637/ERADOC-1-56

loosely called cytoplasmic protein has better nutritive value than chloroplast protein in spite of apparently trivial differences in amino-acid composition. In an attempt to interpret the better nutritive value of protein from mature rather than young leaves, which was commented on in the Report for 1962, predominantly chloroplast protein fractions have been made by heating sap to 53° C and separating the curd; the cytoplasmic protein is then coagulated at 80° C. For this purpose a heat exchanger was made with a scraped surface maintained at 60° C. Preparations were made from 10 different species; the ratio in which protein appeared in the two fractions differed greatly—from 44% in the cytoplasmic fraction with lupins to 4% with maize. Some of these fractions are now being tested for their nutritive value at the National Institute for Research in Dairying. (Byers and Davys)

There is little digestion of leaf protein *in vitro* by papain at 37° C under conditions that completely hydrolyse casein. By increasing the amount of papain, while keeping the other factors constant, digestion was increased, but raising the temperature had a much greater effect on both the initial rate of reaction and the final amount of hydrolysis. 70° C is the optimum temperature. KCN was the most effective of the various substances used to activate the enzyme. Maximum papainic digestion of freeze-dried maize (*Zea mays*) protein occurred at pH 6·6 using the KCN-activated enzyme; similar results were obtained using vetch (*Vicia sativa*) protein. Work is continuing on other proteins and with the enzymes of the mammalian digestive tract because the slow hydrolysis *in vitro* seems to be in conflict with the satisfactory digestion found *in vivo*. (Byers)

The Tropical Metabolism Research Unit in Jamaica asked for labelled protein with a larger proportion of ^{15}N than that made last year. Wheat was therefore grown in sand culture with a mixture of calcium and potassium nitrates containing 30% of the N as ^{15}N. Because of the cost of these labelled salts, they were applied at the smallest concentration compatible with growth, and the effluent was assiduously recycled. Not unexpectedly, growth was poor, and half the N remained in the fibre after extraction. But the N content of the fibre was surprisingly great; 4·35% on the first harvest instead of the 1·5–2·0% usual with wheat. There is no obvious explanation of this, and more experiments are being made on a small scale. The total nitrogen recovered in all the fractions from three harvests exceeded the amount of nitrogen added by nearly 4 g, and the percentage of ^{15}N was smaller in successive harvests. Clearly, therefore, there was some source of assimilable nitrogen (amounting to about 10 lb/acre) other than the nitrates added. (Byers and Sturrock)

During the last few years we have noticed that many batches of freeze-dried protein absorbed so much of the air in the sealed plastic pouches in which the protein is usually stored that the pouch shrank on to its contents and became rigid. It was difficult to account for this merely by the absorption of the 21% of O_2 in air, and it was reasonable to conclude that N_2 was being absorbed too. This absorption was confirmed by measurements of the fall in pressure within sealed cans of protein kept at 100° C and there was an increase in the nitrogen content, determined by Kjeldahl, of the lipids extracted from the protein after this treatment. Experiments done in

https://doi.org/10.23637/ERADOC-1-56 pp 6

this way, however, could give misleading results if protein breakdown gave rise to nitrogen compounds soluble in lipid solvents. The wheat fibre, containing some protein and lipid, which was a by-product of the preparation of protein labelled with ^{15}N, was used to get unequivocal evidence of N_2 absorption. The percentage of ^{15}N in the lipids extracted from fibre that had been kept for 5 weeks in air at 100° C was 17·9, and it was 18·3 when the material was stored in commercial N_2, whereas the original percentage was 19·8.

Commercial antioxidants have hitherto failed to increase the shelf-life of dried-leaf protein. Dried and powdered amla fruit (*Emblica officinalis*) is used in Pakistan as an antoxidant. The O_2 uptake of wheat or maize protein mixed with 2·5% of amla powder was only half that found normally. Amla fruit is also a rich source of vitamin C. (Shah)

Chlorophyll

Enzymic bleaching of chlorophyll. The seeds of several legumes have strong bleaching activity; one of them, Calvary clover (*Medicago echinus*), has greater bleaching and lipoxidase activity than soya bean. Bleaching in the presence of linoleic acid is inhibited by commercial antioxidants (Santoquin, Progallin P and nor-dihydroguaiaretic acid). Bleaching takes place not only with unsaturated acids such as linoleic or linolenic, which are substrates for lipoxidase, but also with acids that are not substrates for this enzyme. A number of acids of different types were tested on legume seeds and extracts of soya flour. With unsaturated acids such as maleic and cinnamic and with saturated acids such as glycollic, succinic, malic, citric and propionic bleaching was negligible. But with long-chain saturated fatty acids there was much bleaching, and with oleic acid it was of the same order as with linoleic acid.

With extracts from shoots of barley and pea seedlings it was confirmed that bleaching of chlorophyll accompanies enzymic oxidation of glycollate. This process is much slower than with cotyledons in the presence of unsaturated fatty acids. (Holden and Bacon)

Separation and purification of chlorophylls. Polyethylene powder has recently been used for preparing pure chlorophyll *a*. Crude extracts of plant pigments can be chromatographed on it without preliminary fractionation, and it is thus obviously preferable to any absorbent used hitherto. With a sample of this material, from the Dow Chemical Co., larger quantities of pure chlorophylls *a* and *b* were made more quickly than before. An aqueous acetone extract (70%) of bean leaves was chromatographed on a column of the powder. The xanthophylls were washed through, the chlorophylls were eluted with 85% acetone and the carotene was left on the column. The chlorophylls were transferred from the acetone eluate to petroleum ether and then chromatographed on a column of Whatman crystalline cellulose. Chlorophylls *a* and *b* were well separated on this adsorbent and crystallisable preparation obtained. (Holden)

Separation of chlorophylls *a* and *b* and associated pigments by thin-layer chromatography on cellulose. Catalytic activity, leading to degradation of

https://doi.org/10.23637/ERADOC-1-56 pp 7

xanthophylls, for example, has been reported with some of the inorganic adsorbents commonly used in thin-layer chromatography. The use of cellulose for separating chlorophylls and chlorophyll derivatives was therefore preferred. Development on this adsorbent with petroleum spirit (b.p. 60–80° C)–acetone–*n*-propanol (90 : 10 : 0·45 by vol.) resulted in separation of pheophytins *a* and *b*, chlorophylls *a* and *b*, pheophorbide *a* and chlorophyllide *a* from each other and from chlorophyllide *b* and pheophorbide *b*. Most of the spots, however, were long and comet-shaped, and this resulted in limited "tailing" of chlorophyll *b* into pheophorbide *a*, and of chlorophyllide *a* into chlorophyllide *b* and pheophorbide *b*. Some of the carotenoids present in plant extracts were also separated from the chlorophylls. The resolution on layers applied to microscope slides was almost as good as with plates of normal size, the apparatus required is extremely simple, and the development time is only 80 seconds. (Bacon)

Chemical Changes in Self-heated Hay

Mouldy hay and farmer's lung disease. Analysis of results of 1961 field experiments with hay in Great Knott I showed that the development of the actinomycete antigen important in farmer's lung disease (F.L.H. antigen) was positively correlated with increases in pH, and soluble and volatile nitrogen in the hay. In contrast, development of fungal antigen was correlated with a decrease in soluble nitrogen.

The thermophilic actinomycetes *Thermopolyspora polyspora* and *Micromonospora vulgaris*, which produce F.L.H. antigen (see Report of the Soil Microbiology Department, p. 71), grow better when the pH is greater than 7; *Micromonospora vulgaris* does not grow on hay initially at pH 6, but *T. polyspora* does, making it possible to assess the biochemical changes produced in hay by *T. polyspora*, the richer source of F.L.H. antigen.

Hay sterilised with propylene oxide was inoculated with a suspension of *T. polyspora* in enough sterile water to raise the water content of the hay to 40%, and then incubated at 40° C for 2 weeks; the pH increased from 6·0 to 7·4, and soluble and volatile nitrogen increased greatly, confirming the indications for F.L.H. antigen found in the field experiments. The effects of sterilisation with propylene oxide and incubation of sterile hay with water alone were studied in separate experiments. Sterilised hay incubated at 40° C showed few changes, but in presence of 40% water soluble and volatile nitrogen increased and non-reducing sugar (sucrose and fructosan) was converted to reducing sugar (glucose and fructose); the total soluble sugar (reducing + non-reducing sugar) was unchanged. Incubation with *T. polyspora* gave larger increases in soluble and volatile nitrogen than incubation with water alone; much reducing sugar, particularly glucose, was lost, but there was little extra change in non-reducing sugar. Thus glucose is used preferentially to fructose, and fructosan is little used by *T. polyspora*.

The changes in the sterile hay were apparently caused by plant enzymes acting in presence of the water added. Incubation of the hay with sucrose solution at 37° C gave substantial hydrolysis of the sucrose, confirming invertase activity in the hay even after two year's storage. Thus water

https://doi.org/10.23637/ERADOC-1-56

added to stored hay not only makes plant constituents available to micro-organisms but to residual plant enzymes as well.

Laboratory experiments on self-heating. The experiments of Miehe in 1906, on the heating of hay in wide-mouthed vacuum flasks were extended to examine further the effects of water and aeration on the self-heating process. A maximum temperature of 49° C was reached when 250 g of the chopped hay used in experiments on F.L.H. antigen (see Report of the Soil Microbiology Department, p. 71) was moistened to 40% water content and allowed to self-heat in a 4 litre thermos flask. The temperature reached 58° C with 500 g hay in the flask and also with 15 kg hay placed in a barrel lagged with glass-fibre sheet. The final products were all found by Dr. J. Pepys and Mr. P. A. Jenkins to be rich in F.L.H. antigen, considerably more so than any hay obtained in the field. With only 25% water the maximum temperature was 30° C.

The effect of aeration on heating was studied with a sparger tube passing down to the bottom of the thermos flask. Aeration of 350 g hay at 25 ml/min had no effect on the progress of self-heating until after the maximum temperature was reached; then the temperature declined faster than in the non-aerated flask. Aeration of 500 g hay at 25 ml/min had no effect on the temperature pattern at all when the flask was closed with a layer of cottonwool at the top, the usual procedure; when closed with a lid, the maximum was decreased by 10° C, indicating the importance of inward diffusion of air.

Barley grain moistened to 35% water content self-heated to a maximum temperature of 59° C and after 55 days contained F.L.H. antigen. (Festenstein in collaboration with Gregory and Lacey, Plant Pathology Department, and Skinner, Soil Microbiology Department)

The Potato-root Eelworm Hatching Factor

In 1963 4½ tons of potato-root diffusate were processed, and the crude material accumulated is now in the course of purification. The yield of hatching factor, of the degree of purity reached so far, is 12 μg/litre.

Investigations into the structure of the hatching factor have continued, but progress is limited by the small amount of material obtained, its instability and its strongly hygroscopic properties, which make quantitative analytical determinations difficult on the free hatching factor. The factor can be obtained in the form of an amorphous barium compound, albeit in small yield (about one-third of the weight of hatching factor used). This compound can more readily be transferred and weighed, and is being used for various analyses. A single carbon analysis, however, consumes some 20% of the year's supply of the hatching factor.

The ultra-violet, infra-red and nuclear magnetic resonance spectra of the factor were recorded and are being interpreted. The molecular weight was determined by allowing a solution of a known compound to equilibrate by isothermic distillation with a solution of the factor. This gave a value of 272, but was accompanied by some decomposition during the determination.

https://doi.org/10.23637/ERADOC-1-56

On titration with alkali the hatching factor gives a simple titration curve (pK = 4·17). After treatment of the factor with excess alkali a neutral and an acidic fraction can be recovered. The unpurified acidic fraction has an apparent equivalent weight of 161.

Oxidation of the factor with alkaline permanganate gave a mixture of at least three acids in small yields. Preliminary chromatographic studies showed that these acids are not members of the simple mono- and di-carboxylic acid series. One of the unknown acids behaved similarly to, but not identically with, aconitic acid on paper chromatography.

Work on synthetic analogues of hatching factors continued, and is discussed in the report of the Nematology Department (Clarke and Shepherd). Several compounds required for this work were synthesised by established procedures. The screening of compounds for activity in stimulating hatch of eggs of *Heterodera schactii* was largely completed. (Clarke)

https://doi.org/10.23637/ERADOC-1-56 *pp 10*