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ROTHAMSTED RESEARCH

Report for 1962

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Biochemistry

Rothamsted Research

Rothamsted Research (1963) *Biochemistry ;* Report For 1962, pp 98 - 106 **- DOI: https://doi.org/10.23637/ERADOC-1-95**

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N. Singh and J. E. Morrison left the department and Pamela Bell joined it. Mr. W. H. Boshoff (Makerere College, Uganda), Dr. Stefania Manys (Institute of Bast Fibres, Poznan) and Dr. H. Graham (Council of Scientific Research, Jamaica), spent two to three weeks with us learning the methods used in making leaf protein in bulk. Margaret Holden attended the "Symposium on Metabolism of Chlorophyll Pigments" at St. Trond (Belgium). N. W. Pirie was a member of the international mission advising on the future of the Imperial College of Tropical Agriculture (Trinidad); he also attended a symposium on food quality in Bari (Italy) and the first meeting of the working parties for the International Biological Year held in Rome.

Studies on Plant Proteins

Enzymic bleaching of chlorophyll. An enzyme that catalyses the conversion of chlorophyll into colourless compounds is most active in the cotyledons of soya beans and germinating peas. The enzyme also occurs in the shoots and roots of pea and wheat seedlings and in the seeds of many legumes; it has so far not been detected in mature tissues.

When cotyledons are ground for 3 minutes with a solution containing chlorophyll, acetate buffer pH 6 and 20% acetone, the absorption at 660 mμ diminishes by as much as 90%. The little pigment remaining is chlorophyll *b*, all the chlorophyll *a* having been destroyed. At pH 8·5 bleaching occurs only when an unsaturated fatty acid such as linoleic acid is added. At both pH values the bleaching is associated with a large increase in the absorption of ultraviolet radiation, probably because of peroxidation of unsaturated fatty acids. Chlorophyll-bleaching activity of tissues is correlated with lipoxidase activity, which is responsible for the formation of fatty acid peroxides. The bleaching probably happens only during lipoxidase action and is not caused by the products of the reaction. The colourless compounds formed from chlorophyll have not been identified; the loss of colour implies that the porphin ring system has been broken, or the sequence of double bonds interrupted, probably in more than one place.

Purification and properties of chlorophyllase. The method for preparing partly purified chlorophyllase was simplified and the yield improved, but the purity was not enhanced.

Chlorophyllase in chlorophyll biosynthesis. The final stages in the biosynthesis of chlorophyll in etiolated seedlings, when transferred to the light, are the reduction of protochlorophyllide to chlorophyllide *a* and the attachment of the phytyl side chain to form chlorophyll *a*. The last step is

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assumed to be catalysed by chlorophyllase, but Willstatter and Stoll's experiments of 50 years ago, in which chlorophyll was obtained by incubating chlorophyllide with phytol and the chlorophyllase-containing meal from dried leaves, have never been confirmed. Crude and purified preparations of sugar-beet leaf chlorophyllase were therefore tested for their ability to phytylate chlorophyllide. Using various ratios of phytol to chlorophyllide at pH values between 5 and 9, and with acetone concentrations from 20 to 60%, formation of chlorophyll was not observed. Protochlorophyllide holochrome from etiolated bean seedlings is now being prepared to investigate the possibility that the pigment can be phytylated when it is attached to protein.

Using the differential solubility of the phytylated and unphytylated pigments in acetone and petroleum ether as a criterion, about 90% of the protochlorophyll was not phytylated in etiolated wheat seedlings 10 days old. The chlorophyllase activity of dark-grown seedlings was small, but phytylation was rapid on exposure to light. After only a few seconds some chlorophyllide had been formed, but an appreciable amount of chlorophyll was already present; a trace of protochlorophyllide was still detectable, but this had usually disappeared after 1 minute in the light. After 5 minutes in the light about two-thirds of the total chlorophyll was phytylated, and after 1 hour all the pigment absorbing at 660 mμ was extracted by petroleum ether from the acetone solution, i.e., was chlorophyll, and chlorophyllide was no longer present.

The statement that protochlorophyll from cucurbit seeds is not a substrate for chlorophyllase was confirmed. In old, but still viable marrow seeds, the protochlorophyll is completely converted into protopheophytin, which also is not a substrate for the enzyme. (Holden)

Plant amine oxidase. Further investigations on highly purified preparations of the amine oxidase of pea seedlings by manometry, spectrophotometry and analytical centrifugation support the suggestion that their pink colour comes from the prosthetic group of the enzyme and that this is a copper complex of a carbonyl compound. Ultracentrifugal analysis, by H. L. Nixon, of the purest preparation so far obtained suggests that it is centrifugally homogenous. It therefore appears that, although the preparation catalyses the oxidative deamination of aliphatic mono- and diamines, phenyl-alkylamines, histamine, spermidine and agmatine, and of the dibasic amino acids ornithine and lysine, one enzyme of wide substrate specificity is responsible. The enzyme is a metalloprotein containing 0·08–0·09% of firmly bound copper. When the copper is split off by incubating the preparations with sodium diethyldithiocarbamate the catalytic activity towards all the substrates is lost but is restored by adding Cu^{2+} ions.

The only absorption band in the visible part of the spectrum has its maximum at about 500 mμ; this produces the pink colour. Carbonyl reagents such as hydrazine, hydroxylamine and isonicotinyl hydrazide inhibit the enzyme and also convert the pink compound to yellow products. Thus, with hydrazine, the primary product absorbs most at 420 and 350 mμ, suggesting that the pink compound contains a carbonyl group. The

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pink compound also reacts with the substrates of the enzyme to give yellow products absorbing maximally at 465, 435 and 350 mμ. These reactions are so rapid that it has not yet been possible to determine their rates precisely. Preliminary results suggest that the rates vary with the different substrates according to the affinities of the substrate for the enzyme. It is suggested that these yellow products are the enzyme-substrate compounds and may be copper complexes of Schiff bases. They are stable only under anaerobic conditions and rapidly oxidise in the air to give the reaction products and free enzyme. The inactive copper-free protein obtained by incubating the preparations with sodium diethyldithiocarbamate remains pink but absorbs maximally at about 490 mμ. It still reacts with hydrazine but does not form the yellow enzyme-substrate compounds until it is reactivated by Cu^{2+} ions; the maximum absorption then reverts to 500 mμ. These results suggest that the prosthetic group of the enzyme is a copper complex of a carbonyl compound.

The purified enzyme is reasonably stable in the absence of substrate, but is rapidly inactivated during the reactions it catalyses. This reaction inactivation is, in part at least, caused by hydrogen peroxide, which is a reaction product. Catalase partially prevents this type of inactivation. The enzyme is not inactivated by small concentrations of hydrogen peroxide unless a substrate is also present. Thus, hydrogen peroxide or putrescine at mM concentrations cause little or no inactivation when incubated separately with the enzyme for 30 minutes in nitrogen, but together they produce almost complete inactivation. The experiments on reactivation of the copper-free preparations by Cu^{2+} ions are complicated by the fact that Cu^{2+} ions also cause a rapid reaction-inactivation. The suggestion was made last year that Cu^{2+} ions catalyse the inactivation of the enzyme by hydrogen peroxide, but the yellow enzyme-substrate compounds, which are normally stable under anaerobic conditions, are very unstable in presence of Cu^{2+} ions. The rapid disappearance, in presence of Cu^{2+} ions, of the bands at 465, 435 and 350 mμ, which are characteristic of the enzyme substrate compounds, is not accompanied by the reappearance of the enzyme band at 500 mμ, and is correlated with inactivation of the enzyme. These experiments were done anaerobically, so the inactivation produced by Cu^{2+} ions was independent of hydrogen peroxide. The results suggest that Cu^{2+} ions and hydrogen peroxide act independently, not on the enzyme or substrate, but on the enzyme-substrate compound, which is a labile intermediate in the enzyme-catalysed reaction. (Hill and Mann)

Leaf mitochondria. Work on the estimation and intracellular location of respiratory enzymes of healthy plant leaves was ended with a study of succinate dehydrogenase, malate dehydrogenase and aconitate hydratase. The experience gained in the last few years will be used to follow the changes that occur during the ageing of leaves and during virus infection. These changes may throw some light on the respiratory systems and the organisational states of leaf cells during these processes.

In tobacco leaves succinate dehydrogenase is confined to mitochondria, and it is not separated from the succinate oxidising system by either

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differential or sucrose gradient centrifugation. Nor is it easily washed out of these particles. In this respect it differs from malate dehydrogenase and aconitate hydratase, which although associated to some extent with mitochondria, are extensively washed off during disruption of the leaves in aqueous solutions. Some malate dehydrogenase is also located in the chloroplasts, where its function is so far unknown; this, too, is largely eluted during leaf disruption.

Succinate dehydrogenase is a difficult enzyme to estimate, as it is activated and inactivated by many different agents. Preliminary measurements suggest that it could be the limiting factor in the mitochondrial oxidation of succinate. This, together with its firm attachment to the mitochondria, make it a useful indicator of mitochondrial integrity and function.

As a preliminary to studying these enzymes in ageing and diseased plants, the respiration was measured of leaf discs from healthy tobacco plants and from plants infected with tobacco severe etch virus. The measurements were mainly concerned with standardising techniques, but they showed that disks from young leaves respired about four to six times as much as disks from older leaves. They also tentatively confirm the 40–60% increase in respiration when lesions become visible in virus-infected leaves. (Pierpoint)

Cucumber mosaic virus. Cucumber mosaic virus, extracted in the presence of diethyldithiocarbamate (dieca) and then dialysed, is inactivated when incubated with copper and chlorogenic acid. The character of this inactivation, such as the need for oxygen, sensitivity to dieca and the effectiveness of other polyphenols, clearly indicates that the inactivation is brought about by the polyphenoloxidase present in the extracts. Some evidence suggests that short-lived intermediates rather than the final products of the oxidation bring about the inactivation, and they may act on the nucleic acid of the virus rather than its protein coat; both these suggestions, however, need confirmation. Either ferrous or ferric iron, but not some other metals, will replace copper in the inactivating system. As iron does not restore the activity of polyphenoloxidase once it has been inactivated by dieca, and as it forms a complex with chlorogenic acid, the mechanism of this inactivation must be quite different from the one involving copper.

The inactivation of this virus by polyphenoloxidase is probably responsible for the loss of infectivity when infected leaves are disrupted in buffer or water. Highly infective extracts have so far been obtained only by grinding in the presence of dieca or potassium ethyl xanthate. Some other metal chelating agents that do not inhibit the enzyme do not prevent virus inactivation. A polyphenol substrate of the oxidase was extracted from leaves disrupted either in a nitrogen atmosphere or in acid. Although it has not been characterised properly, it is likely to be chlorogenic acid or one of its isomers. Estimations showed that there is enough of these compounds in tobacco leaves to account approximately for the virus inactivation.

Few other plant viruses seem susceptible to this inactivating system.

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Adding dieca does not, for instance, affect the infectivity of extracts containing lucerne mosaic, arabis mosaic, tomato black ring or tobacco mosaic virus. Nor are these viruses affected when leaf extracts are incubated *in vitro* with chlorogenic acid and copper. The special properties of cucumber mosaic virus that make it susceptible may emerge from a knowledge of the mechanism of this inactivation. (Pierpoint, in collaboration with Harrison, Plant Pathology Department)

Large-scale production of leaf protein. The dry spring and cold summer seriously interfered with work on the extraction unit that is on loan to British Crop Driers (Navenby, Lincs), staffing changes interfered with the unit in Jamaica, and shipping delays caused a very late start with the unit in Lucknow. There is good reason to think that each of these outside centres of work on leaf protein will be more productive next year.

The redesigned "village unit" for extracting juice from 300–500-lb lots of leaf in one operation worked well and were another made, it would be essentially a copy of the present one.

The new self-loader eliminates much of the labour of feeding the large pulper and gives a more even feed; the insertion of a chopper-blower between the feeder and pulper allows the pulper to be fed through an opening with only a quarter the area needed hitherto. These two improvements stimulated the design of a smaller and cheaper pulper, now being built, which should have the same performance as the existing ones but take less power. (Davys and Pirie)

There are two reasons for harvesting leafy crops at different stages of maturity: to find the rhythm of growth, regrowth and replacement by another crop that leads to maximum yields, and to examine the differences in quality and extractability of protein from leaves of different ages. It is now well established that the extractability of leaf protein usually diminishes with increasing maturity of the leaf; nutritional studies at the National Institute for Research in Dairying and the Rowett Research Institute suggest that there are significant differences in the biological values of protein from the same species when taken at different stages of growth and that, with wheat at least, the older leaves give the better protein. There is therefore an optimum time of harvest when both extractability and biological value are satisfactory. This year's work on wheat showed that the yield of extractable protein fell from 389 kg/ha to 247 between the middle of May and the middle of June, and regrowth after the early cut gave a further 192 kg/ha.

Similar studies were made with maize (*Zea mays*) in an experiment that, presumably because of differences in weather, gave results significantly different from those last year. Three of the four varieties tested this year gave a steady increase in the yield of extractable protein during the growing season. Amino-acid analyses (done in collaboration with Prof. M. A. Stahmann) on last year's samples showed striking differences in the ratio of methionine to methionine sulphoxide, although the total was reasonably constant. This year some of the extracts were subdivided, and

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the separation and purification procedures were varied as extensively as was compatible with getting a reasonably good final product. These batches of protein are being analysed to see whether the differences in Me/MeO are controlled by the physiology of the leaf, the process of isolation or by variations in analytical technique. Last year protein fractions were made by heating extracts of marrow (*Cucurbita ovifera*) leaves first to 60° and then to 80°. These have now been analysed and differences found between their amino-acid compositions. Extracts from rape (*Brassica napus*) and lupin (*Lupinus albus*) were therefore subjected to stepwise coagulation this year, and the protein is being analysed.

Several more species were grown for experimental small-scale extraction of protein; of these the most successful were white lupin (*Lupinus albus*), woad (*Isatis tinctoria*), berseem or Egyptian clover (*Trifolium alexandrinum*), *Amaranthus melancholicus ruber* and duckweed (*Lemna minor*). *Musa balbisiana*, related to the common banana, *M. sapientum*, was a poor source of protein.

The demand for protein labelled uniformly with ^{15}N increased and is expected to increase still more; it is used in human feeding trials in Jamaica and as a means of getting ^{15}N-labelled eggs. A frame was therefore built in which crops can be grown in sand culture and partly protected from rain; the effluent is recirculated to economise ^{15}N. Work started late in the season, but 120 g of protein, containing 10–11·4% N was made from wheat and mustard. With the facilities here it is easier to make N-labelled protein in this way than by growing micro-organisms or algae. (Byers and Sturrock)

Experience shows that the protein in leaf extracts coagulates spontaneously. There is a risk, therefore, that the "village unit" may be less suitable than the large unit for some leaves, because of the delay in separating juice from fibre and the precipitation of protein in an unextractable form on the fibre. The protein contents of successive samples of juice from runs on marrow, maize, nasturtium (*Tropaeolum majus*), rape and kale (*Brassica oleracea*) were therefore determined; the juice running out during the later stages of extraction sometimes contained more protein than the initial samples, but usually contained 10–15% less. With these species, and our ambient temperatures, the risk of loss seems small, but it may be greater in hotter climates. (Byers, Davys and Pirie)

No new technique for presenting leaf protein on the table was developed, but several methods for preserving protein without drying or heating were tested. The simplest was the most successful. Moist (40% DM) protein from a filter stocking or filter press was crumbled and mixed with 90 g of sodium chloride for each kg of protein; this produces a concentration of 15% in the water when diffusion is complete. The mixture rammed tightly into a jar and covered with wax to exclude air seems to keep indefinitely. Provided other salted foods are not being eaten, the amount of salt present is not excessive; 10 g (dry weight) of protein, which is a reasonable amount to take in a day, would be accompanied by 2·25 g of salt; only a quarter of the amount usually eaten. (Morrison and Pirie)

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Drying or storage of leaf protein at moderately high temperature greatly decreases its nutritive value and digestibility. As unsaturated lipids present in the protein are very susceptible to oxidation, the changes taking place in the lipids and in their association with the protein were studied after storage under different conditions. Oven-dried (100°) clover, kale and wheat protein was extracted with chloroform–methanol (2 : 1) mixture, and the residue was soaked in a range of protein-softening reagents before re-extraction with chloroform–methanol. Soaking the residue in 90% phenol, glacial acetic acid, 5% perchloric acid or acidified chloroform–methanol released some of the bound lipid; formic acid (90%) and 10% TCA extracted more of this lipid, whereas 8M urea and formamide extracted less.

The quantity of lipid soluble in chloroform–methanol decreased from 22 to 11% when freeze-dried leaf protein was stored at 100° in Kilner jars for 52 days. This was accompanied by a large decrease in the iodine number, phosphorus and chlorophyll content of the lipid. The change in extractability of the lipid, and in its iodine number, was small when the protein was kept at 100° in nitrogen.

There was a strong smell of sulphur compounds in samples kept at 100° either in air or in nitrogen. There was also an increase in the water-soluble and lipid nitrogen in both the samples, but the increase was greater with protein kept in nitrogen; this suggests that products of lipid oxidation combine with protein breakdown products and become insoluble. Freeze-dried protein kept at 55° and 37° deteriorated less and samples stored at 4° kept well.

Estimation of water-soluble phosphorus before and after storage of protein gave the same results, indicating that no phosphorus had been split off from the protein. The lipid phosphorus that became unextractable with chloroform–methanol mixture was extracted with chloroform–methanol–12*N*–HCl (100 : 50 : 1). The atomic ratio of N/P in the extract was 1·3. The phosphatides may form phosphatidopeptides with the proteins present or salts with the minerals present in the protein.

There was much less than the usual amount of chlorophyll in lipid extracts made from protein that had been kept at 100°. (Shah)

Chemical Changes in Self-heated Hay

The biochemical changes that take place in hay that has been baled or stacked when it is too wet are being studied because such hay may be a cause of human disease; that aspect is described in the Soil Microbiology Department report. The spontaneous combustion of hay raises many points of biochemical interest. Thermophiles offer a ready explanation of a rise in temperature to 65–70°, but no plausible mechanism has been proposed for raising the temperature several hundred degrees further to the combustion point. Conditions that will lead to spontaneous combustion have not yet been defined, but some trials were made, and they will be continued.

The stack of wet hay built in June 1961, which reached a maximum temperature of 65°, was opened after 185 days and the different layers

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analysed; the inner core of brown hay was surrounded by regions of mouldy hay; the brown hay was mainly at pH 4·8 and was mould-free, but there were a few small intensely mouldy white areas with pH 7·4–7·8 and very little soluble sugar. The unheated green hay on the outside of the stack, with a moisture content of 27%, also had a high pH and very little soluble sugar; it contained very many bacteria that grew at 25°, but very few that grew at 40° and 60°. The percentage of ash in the dry matter of most of the layers was only 10% more than in the hay initially, but it was 45% more in the small regions of white mould in the brown hay, representing 30% loss of dry matter. This suggests active respiratory loss of carbon in these regions.

The small loss of dry matter in the core of brown hay, which had heated considerably, may be explained by conversion of carbohydrate to acid, rather than oxidation to volatile CO_2. The sequential samples from bales and stacks obtained from Great Field in 1961 were analysed for total acidity, and large values were correlated with low pH; samples from the centre of the stack were more acid than samples from the bales that also contained 30% moisture initially.

Non-reducing sugars decreased sharply during wilting, probably from enzyme activity, and continued to fall after baling and stacking. The reducing sugar increased during wilting, accompanying the decrease in non-reducing sugar, but fell after baling and stacking, associated with the general rise in micro-organisms, especially fungi. (Festenstein in collaboration with Gregory & Maureen E. Lacey, Plant Pathology Department, and Skinner, Soil Microbiology Department)

The Potato-root Eelworm Hatching Factor

About the same quantity (700 gal) of potato-root diffusate as last year was collected and processed. Charcoal deactivated with stearic acid has been used as an adsorbent for the hatching factor. Small-scale experiments gave good recoveries, but gave only a 15% yield when applied on a large scale. The conditions necessary for purifying larger batches of crude material eluted from the charcoal were established, and this has greatly shortened the time spent at this stage. The active solid material (m.p. 147–149°), described in earlier reports, is not homogenous; it is largely a complex of high-melting-point material, and the hatching factor in approximately equal proportions. Chromatographic procedures were developed by which the high-melting material is removed and the hatching factor obtained as a colourless resin in a final yield of 10–30 μg per litre of root diffusate. The resin can be converted into a solvated low-melting form which readily reverts to the resinous state. The results of a preliminary X-ray examination suggest that the solid is micro-crystalline.

Work is in progress to repeat and extend the earlier chemical evidence obtained with less pure material.

The ultra-violet absorption spectrum of the purified material has no maximum, but shows weak shoulders at 252 and 300 mμ. This absorption might be from a conjugated aromatic ring or a highly hindered αβ-unsaturated carbonyl compound. Some caution is needed in drawing

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inferences from the spectrum, as the absorption is of low intensity, and it might arise from trace amounts of strongly absorbing material rather than from weakly absorbing groups in a pure substance.

Hartwell, Dahlstrom and Neal (*Phytopathology* (1960), **50**, 612) state that the potato-root eelworm hatching factor obtained from tomato-root diffusate does not contain a free carboxyl group. There is, however, strong evidence for the existence of a carboxyl group (or a potential carboxyl group) in the hatching factor from potato-root diffusate. Thus, it can be extracted with organic solvents from acid but not alkaline solutions, and it can be converted into active salts. The infra-red spectrum of the hatching factor (as a film) shows, in addition to a carbonyl band at 1712 cm^{-1}, bands at 2650 and 3500 cm^{-1} associated with carboxyl–OH stretching vibrations. In contrast, when the hatching factor is converted to the barium salt the spectrum shows a diminished carbonyl band, and strong new bands at 1550 and 1408 cm^{-1}. Changes of this sort are typically associated with salt formation by carboxylic acids.

The bioassay still remains the only method of detecting the hatching substance. The introduction and modification of the purification procedures mentioned above have involved the testing of over 2,000 samples by the Nematology Department. Work on analogues of the hatching factor are described in the report of that department. (Clarke)

https://doi.org/10.23637/ERADOC-1-95