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Rothamsted Report for 1966

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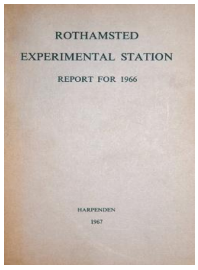

Biochemistry Department

N. W. Pirie

N. W. Pirie (1967) *Biochemistry Department ;* Rothamsted Report For 1966, pp 98 - 109 **- DOI: https://doi.org/10.23637/ERADOC-1-14**

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J. W. Sturrock resigned and D. B. Arkcoll and R. Olsson were appointed. R. A. Buchanan, from the C.S.I.R.O. Division of Dairy Research (Australia) has come as a temporary worker and G. Street has come on a grant from the International Biological Program (I.B.P.). R. G. Carlsson, from the Institute of Plant Physiology, Lund, and O. M. Anderson, from the Swedish Seed Association, Svalöv, spent a few weeks learning our methods of handling leaf protein because work on it is in the Swedish programme for the I.B.P. N. W. Pirie organised an I.B.P. Working Group meeting on "Novel Protein Sources" during the 2nd International Congress of Food Technology in Warsaw and attended the I.B.P. General Assembly in Paris; he also attended the 9th COSPAR meeting in Vienna and the 17th International Astronautical Congress in Madrid.

Enzyme actions

Enzyme systems in virus-infected leaves

Mitochondrial enzymes. Mitochondria of leaf cells contain most of the tricarboxylic acid-cycle enzymes and are likely to be responsible for most of the respiration of the leaves. A possible explanation of the increased respiration of virus-infected leaves is that infection leads to an increase in the mitochondrial material of leaves. Our previous attempts to confirm published evidence supporting this explanation failed. These have now been repeated using more refined methods. Discs were used, cut from *Nicotiana glutinosa* leaves, immediately after inoculation with tobacco mosaic virus, and floated on nutrient solution containing antibiotics to check bacterial contamination. However, no increase was observed in the mitochondrial protein or in such mitochondrial enzymes as cytochrome oxidase. In contrast, an increase in the polyphenoloxidase of this tissue has been repeatedly confirmed.

Another possible explanation of the increased respiration is that virus infection affects the control mechanisms of leaf mitochondria so that they oxidise substrate faster. This possibility is difficult to test, as most of the factors thought to control the specific activity of mitochondrial enzymes inside the cell are either removed or altered during extraction. A recent suggestion that the thiamine pyrophosphate (TPP) content of mitochondria limits their oxidative activities also seemed open to the objection that the TPP content of mitochondria could be altered during their isolation. In particular, it seemed possible that the serum albumin and cysteine, commonly used in the isolation medium, could remove mitochondrial TPP either by absorbing it or reacting with it.

We find no evidence that serum albumin absorbs TPP either from solution or from mitochondrial preparations. However, cysteine reacts with TPP in alkaline conditions where TPP exists as a sulphydryl compound

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and might be expected to form a mixed disulphide. Quantitative study of the reaction shows that it proceeds slowly and only to a small extent. Moreover, cysteine does not inactivate such TPP-requiring enzymes as carboxylase. There is therefore no reason to consider that the use of an extraction medium containing serum albumin and cysteine greatly alters the TPP content of isolated mitochondria, and this medium can be used to test the possibility that virus infection stimulates respiration by increasing TPP. (Pierpoint)

Photosynthetic enzymes. Changes in enzyme systems, other than respiratory ones, were studied in discs of *Nicotiana glutinosa* leaves after infection with tobacco mosaic virus. Photosynthetic reactions of isolated chloroplasts were chosen first, because we have experience in assaying these reactions in other plants, and also because it should be possible to correlate their activity with known changes in the photosynthetic capacity of infected leaves. However, it has proved unexpectedly difficult to assay photosynthetic processes such as the light-induced reduction of nicotinamide adenine dinucleotide phosphate in chloroplast preparations from discs of *N. glutinosa*. The reason is possibly the ease with which photosynthetic enzymes are eluted from these chloroplasts during their isolation; attempts to prevent this are being made. (Olsson)

Studies on uninfected leaves and leaf enzymes

Carbonyl component of pea-seedling diamine oxidase. The carbonyl component of pea-seedling diamine oxidase has not yet been isolated, but evidence is accumulating that, contrary to the claim of other workers, it is unlikely to be either pyridoxal or pyridoxal phosphate. Purified pea-seedling diamine oxidase was hydrolysed both with acid (constant boiling HCl or HCl–formic acid 50/50 w/w) at 110° C and with pronase (a protease from *Streptomyces griseus*) at 37° C). Both the native enzyme and its hydrolysates were examined for pyridoxal and pyridoxal phosphate using: (1) absorption spectrophotometry at different pH values in the region from 300 to 600 nm; (2) treatment with phenylhydrazine; (3) thin-layer, paper and column chromatography; (4) the apo-enzyme of tyrosine decarboxylase in the presence of ATP. The last method is sensitive and specific for pyridoxal and pyridoxal phosphate.

Neither pyridoxal nor pyridoxal phosphate were detected in the diamine oxidase or its hydrolysates. Pyridoxal phosphate was easily detected in the hydrolysates when added to diamine oxidase at the concentration expected in the free enzyme (1 mole pyridoxal phosphate per mole enzyme, assuming a molecular weight of about 80,000). In control experiments with tyrosine decarboxylase (an enzyme that contains pyridoxal phosphate) and casein (a protein not containing pyridoxal phosphate), pyridoxal was easily detected in hydrolysates of the former, but was not found in the latter unless added to the protein before hydrolysis.

Distribution of metalloproteins in plants. In an attempt to interpret the observed signs of trace-element deficiency, work was started on the distribution of metalloproteins in various plant parts; particular attention is being paid to enzymes containing copper, zinc or manganese. (Hill)

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The oxidation of Schiff bases of pyridoxamine with α-oxo acids by Mn^{2+} ions and peroxidase. Systems containing Mn^{2+} ions and peroxidase catalyse the oxidation of Schiff bases of pyridoxal or pyridoxal phosphate with amino acids, and of pyridoxamine with α-oxo acids. Such Schiff bases are intermediates in the reactions catalysed by pyridoxal phosphate-containing enzymes, and evidence is accumulating that some enzymes may contain both metal ions and pyridoxal phosphate. In the oxidation of mixtures of pyridoxamine and pyruvate by Mn^{2+} ions and peroxidase the main reaction is the oxidative deamination of pyridoxamine to pyridoxal; pyruvate acts catalytically. At pH 7 the reaction is catalysed by Mn^{2+} ions alone after lag periods that are prolonged by diluting the initial concentrations of the reactants. Peroxidase shortens the lag period, and its effect is greatest with dilute reactants. At pH 5 the reaction is not catalysed by Mn^{2+} ions alone, or only after very long lag periods, even when the initial concentrations of the reactants are large. When peroxidase is also present the reaction is as fast at pH 5 as at pH 7.

Because pyridoxal is a reaction product, the rate and extent of oxygen uptake is increased by adding amino acids. The increase is caused by the oxidation, by Mn^{2+} ions and peroxidase, of the Schiff bases of the pyridoxal with the amino acids, and results in the oxidative deamination and decarboxylation of the amino acids.

Mn^{2+} ions and peroxidase also catalyse the oxidation of Schiff bases of pyridoxamine with α-oxo acids other than pyruvic acid. With α-oxobutyrate and glyoxylate, results were as with pyruvate except that the reactions were slower and the yields of pyridoxal were smaller. With α-oxoglutarate and phenylpyruvate the reactions were more complex because peroxidase and Mn^{2+} ions catalyse the oxidative decarboxylation of these acids. Adding pyridoxamine increased the rate and extent of oxygen uptake; pyridoxal and ammonia were reaction products.

Light can replace peroxidase in initiating the oxidation of Schiff bases of pyridoxal and amino acids, but not those of pyridoxamine and α-oxo acids. We suggested that the oxidations are mediated by a manganese oxidation–reduction cycle and attributed the initiation of the reaction by peroxidase to its catalysis of manganese oxidation; the lag period would then be the time necessary for manganic manganese to reach a threshold concentration. The observation that peroxidase can be replaced by traces of colloidal manganese dioxide (10^{-5}–10^{-6} M) supports this suggestion. Once started in this way, the reactions proceed to completion without adding more manganese dioxide. These amounts of manganese dioxide cause little or no oxygen uptake in reaction mixtures free from Mn^{2+} ions. It is suggested that manganic manganese is the initiator of the reactions, oxidising the Schiff bases to products (possibly free radicals) that react directly with molecular oxygen yielding compounds able to oxidise manganous manganese without the intervention of peroxidase. (Hill and Mann)

The oxidation of amines by extracts of barley seedlings. Putrescine slightly increased the rate of oxygen uptake by extracts of 8–12-day-old barley seedlings. The increase was accompanied by formation of Δ′-pyrroline (estimated with *o*-aminobenzaldehyde), which is presumably

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formed by oxidation of the putrescine. The active factor in the extracts was concentrated by precipitation with ammonium sulphate followed by dialysis. These preparations oxidised the diamines putrescine and cadaverine more readily than phenylalkylamines, such as β-phenylethylamine. In substrate specificity they thus resemble the diamine oxidase of pea seedlings. Further work is necessary to find out whether the active factor is an amine oxidase or the oxygen uptake depends on more complex reactions, possibly involving transamination followed by oxidation of a reaction product. Monocotyledonous plants, including barley seedlings, have been reported not to contain diamine oxidase, whereas amine transaminases are widely distributed in plants. (Hill and Mann)

Chlorophyll bleaching. Chlorophyll is bleached in suspensions of barley seedling leaves kept in aqueous acetone (*Rothamsted Report* for 1965, p. 109). In wheat and rye seedlings, but not in oat, it is bleached as much as in barley. Lipoxidase activity has been reported in germinating cereal seedlings, and it seemed possible that chlorophyll bleaching in aqueous acetone might be associated with a lipoxidase–lipoperoxidase system, as it is in legume-seed extracts.

The leaves of wheat, barley and rye seedlings all have lipoxidase activity, but oat seedlings have very little. A survey was therefore made of chlorophyll bleaching and lipoxidase activity in the leaves of about 50 different species from a wide range of families, and on 35 members of the Compositae.

A few species (e.g. *Endymion non-scriptus*, *Lamium album*) showed no loss of chlorophyll. At least 25% of the chlorophyll of most other species was bleached, and more than this was bleached in many (e.g. *Centaurea montana*, *Digitalis purpurea*), though with some of these it occurred only in leaf discs and not in ground tissue (e.g. *Urtica dioica*, *Alliaria petiolata*). Lipoxidase was detectable in all the leaves tested, but in some the activity was extremely small. Extracts from leaves with an active chlorophyll-bleaching system were all rich in lipoxidase. In the Compositae the lipoxidase activity agreed closely with the conventional classification into tribes. The activity was weak in members of the Anthemideae (e.g. *Artemisia abrotanum*), but strong in the Cynareae (e.g. *Cirsium vulgare*).

Lipoxidase was rapidly inactivated in crude extracts of many species, even when substances such as cysteine, DIECA and Polyclar AT were added, but it was stable in cereal-seedling extracts. Etiolated seedlings had much more activity than green. Lipoxidase is associated with a fraction that sediments at 3,000 g, and much of it can be extracted with 0·5% Triton-X 100. The enzyme is fairly stable on freezing, dialysis and precipitation with ammonium sulphate. It acts optimally near pH 6, which is more acid than the optimum for lipoxidase from legume seeds.

Loss of chlorophyll from senescing leaves. In the search for a species that has a particularly active chlorophyll-degrading enzyme system the rate was measured at which chlorophyll is lost from leaf discs kept moist and in the dark. The extracts were also examined by thin-layer chromatography on cellulose for the possible presence of coloured breakdown products of chlorophyll.

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The rate at which chlorophyll disappeared differed greatly. *Paeonia* and *Syringa* leaves, picked at the end of May, lost none in 10 days and *Chamaenerion angustifolium* only 5%. Leaves of cereals, including oat, lost their chlorophyll comparatively quickly, but the most rapid loss was from purple sprouting broccoli leaves, in which only 13% of the chlorophyll remained after 5 days. Coloured degradation products did not accumulate in any species. The only coloured porphyrins detected were occasional traces of pheophytin, pheophorbide and chlorophyllide. There seems little or no relation between the amount of chlorophyll bleaching in aqueous acetone and the rate of chlorophyll loss in senescing leaves, suggesting that different enzymes are involved in the two systems. (Holden)

Chlorophyllase. As a prelude to a study of the substrate specificity of chlorophyllase, further attempts were made to purify the enzyme. A preparation of sugar-beet chlorophyllase, partially purified by acetone precipitation and by chromatography on DE 50 cellulose, was separated into two main protein fractions by gel-filtration through Sephadex G-100. The faster-running material, of larger molecular weight, had only slight chlorophyllase activity. The position of maximum chlorophyllase activity, estimated by a rapid chromatographic procedure, coincided with the slower material. Comparison with the gel-filtration behaviour of ovalbumin and chymotrypsinogen suggests that the molecular weight of this chlorophyllase is about 37,000. (Bacon and Holden)

Chlorophyll

"Changed" chlorophylls. Altered chlorophylls are produced by alkaline treatment of heated leaves or by the action of aqueous acetone or methanol on fresh leaves (*Rothamsted Report* for 1965, p. 108). "Changed" chlorophylls *a*-1, *a*-2, *b*-1 and *b*-2 are spectroscopically similar to chlorophylls *a* and *b* and contain phytol; they are probably oxidation products. "Changed" chlorophylls *a*-1 and *a*-2 can be produced in the brown alga *Fucus serratus*, which contains no chlorophyll *b*; they are therefore derived from chlorophyll *a*. Neither is identical with pyrochlorophyll *a*.

Pigments similar to, and probably identical with, the "changed" chlorophylls are also produced when fresh leaves are left in alkaline brine, when leaf extracts containing petroleum spirit are washed with sodium chloride solution and left moist or when purified chlorophylls *a* and *b* are kept in 50% aqueous acetone. Altered chlorophylls are therefore produced under various conditions, and the composition of leaf extracts assumed to contain chlorophylls *a* and *b* only should be checked chromatographically.

Other "changed" chlorophylls are also produced under the above conditions. Two of these, termed "changed" chlorophylls *a*-3 and *b*-3, absorb maximally at 417 and 653 nm, and 443 and 631 nm, respectively. Faster-running compounds with similar spectra are formed when purified chlorophylls *a* and *b* are kept in methanol containing only traces of water.

"Changed" chlorophylls *a*-1 and *b*-1 were converted into phytol-free compounds by a crude chlorophyllase preparation, but *a*-2 and *b*-2 seemed not to be affected. (Bacon and Holden)

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Artefacts from chromatography. The presence of unusual chlorophyll-like pigments in ripening green peppers has been reported recently. It seemed that these might be relevant to the problem of chlorophyll degradation in ageing leaves, but chlorophylls *a* and *b* were found to be converted readily on some chromatographic adsorbents into "changed" chlorophylls *a*-1, *a*-2, *a*-3, *b*-1, *b*-2 and *b*-3. The unusual pepper pigments have properties very similar to those of the "changed" chlorophylls, and they are probably artefacts of chromatography, rather than products of the breakdown of chlorophyll in the plant.

Conversion into "changed" chlorophylls is fastest on thin layers of silica gel, but also occurs readily on Celite 545 and kieselguhr G. The reaction is inhibited by an atmosphere of nitrogen. The "changed" chlorophylls are also formed on Whatman No. 1 paper, but negligible amounts are produced by thin-layer chromatography on cellulose under rapid conditions of working. This method is therefore suitable for checking the identity of pigments separated on other adsorbents. The conclusions reached in the preceding section were come to in the light of this knowledge of the risk of artefact formation. (Bacon)

Extraction of leaf protein

Agronomic experiments. Because of the interest in leaf-protein production now being shown in many countries, the effects of different forms of husbandry on the yield of extractable protein were examined in more detail than hitherto. Except in experiments on small plots, conventional farm crops were used, although these may not ultimately be chosen in the temperate zone, and will probably not be chosen in the tropics. Experimentation here is intended to uncover the general nature of the factors that govern extraction so that advice can be given to those working in regions where experimentation is more difficult.

Most crops gave larger yields than in the past, partly because of improved extraction resulting from double pulping. More than 1,000 kg/ha of protein was extracted from a sequence of winter wheat followed by each of three fodder crops, and also from red clover on its own.

Red clover grew well on a fertile plot and avoided the establishment year needed with white clover, although 3 months were needed for the first 200 kg/ha of protein. This comparatively wasteful period can probably be diminished by sowing the crop along with a cereal. An attempt to do this with vetch in spring wheat proved worthwhile, giving more than 600 kg/ha of extracted protein in 3½ months. Problems arise in keeping a pure stand free from weeds and in harvesting a short and slippery (clovers) or entwined (vetch) crop. Undersowing helps keep down weeds in the clovers and provides a frame to hold up the vetch.

Protein yields from wheat seem to follow dry-matter production and reach a sharp maximum when floral development starts to dominate vegetative growth as the ears emerge. This pattern is shown by several other crops, although maize and sorghum produce vegetative growth until floral development is almost complete.

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Regrowth patterns are less obvious, and conflicting results were obtained within individual crops. Red clover gives better results with regrowths from older, and wheat from younger plants. During the last two years barley regrew rather better than wheat, in contradiction to previous conclusions. The barley varietal trial showed little difference between the yields of Maris Badger and Proctor, thus further emphasising the enormous effect of season and plot fertility shown by the 1964 and 1965 results, and those of wheat in 1965 and 1966.

Quicker growth in the early maize variety "Inra 200" enabled it without top dressing to yield as much protein as "Caldera 402" that had been top-dressed with 100 units of nitrogen. Removing the cobs at the milky stage showed that the foliage was still worth processing and gave yields of about 300 kg/ha. More protein would be obtained for consumption by harvesting both cobs and leaves in this way, and the combination would probably have a greater biological value because the grain protein is rich in methionine. (Arkcoll)

Large-scale extraction. Few changes were made in the equipment. Devices to agitate the crop on its way into the pulper were fitted to prevent the "bridging" that sometimes caused trouble. In commercial production the machine would probably be fed by a chopper-blower; this change is of interest under experimental conditions only. The press was fitted with a new belt strong enough to withstand the stresses put on it.

Significant economies in the power needed to extract a kg of protein can be made by selecting the optimal pulper speed and arrangement of beaters inside the pulper. In most of the experiments designed to find these optima the residue was repulped with added water. Sometimes the second extract yielded more protein than the first; it usually yielded one-third as much. This is several times as much extra protein as is extracted by the simpler arrangement used hitherto, but the extraction consumes a proportionately greater amount of power. Maize is an exception to this generalisation, for with it simple extraction is adequate. The economic advisability of making a second extract depends on the cost of power and the use that is to be made of the ultimate leaf residue. (Arkcoll, Byers and Davys)

Medium-scale extraction. As mentioned last year, the "village unit" extracts less of the protein from the leaf than the large unit, but it is more economical of power. Various sizes and arrangements of ribs on the roller were tried, but these brought about no general improvement; we gained the impression that there may be a different optimal arrangement for each texture of crop, but the differences are small. By hanging weights on the rotating frame of the unit performance was improved and juice ran out much earlier when two disc coulters were fitted on an adjustable mounting behind the roller. We noticed some years ago that the unit was relatively inefficient at bringing material with long stalks, e.g. maize and kale, to a working consistency. The discs avoid the need for preliminary chopping.

Another "village unit" was made and sent to the University of Ife in Ibadan. (Ahmad, Davys and Pirie)

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Laboratory-scale extraction

Effects of pretreatment on protein extraction. The bulk extraction of protein is favoured by alkaline conditions, and many pretreatments are known that promote the extraction of particular proteins, e.g. ribosomes or enzymes. The amount of protein extracted was slightly increased by infiltrating clover with 0·1–0·2 N, NH_3, KCN or polyvinylpyrrolidone; it was decreased by infiltration with some metal chelating agents and antioxidants. So far no pretreatment has been found that could be used on a large scale to increase the extractability of protein from species, such as some of the clovers, that often extract poorly. (Ahmad)

Systematisation of extraction technique. Institutes in many parts of the world are publishing statements about the percentage of protein that can be extracted from the leaves of different plant species. We have often commented on the need to supplement these statements with more agronomic information; more consistent methods of extraction are also needed. An IBP grant was given to develop an extraction technique that would give repeatable results when used by different people, and that would be comparable in principle with large-scale extraction.

A press was made in which 800–900 g of pulp in a cloth is pressed between two vertical grooved platens by a bell-crank lever giving a 40:1 increase in pressure. This permits a ton to be quickly applied over a platen area 23 cm × 23 cm in a unit that could easily be taken into the field. With this arrangement the volumes of juice expressed from successive lots of the same pulp differ by $<5\%$. Because the extent to which fibre gets packed on the cloth varies, making a filter surface of varying porosity, the protein content of successive fractions of juice, and of juices made with different loads on the lever, also varies somewhat. Work is in progress to diminish this variation. (Davys, Pirie and Street)

Extraction from new species and digestibility studies. Hemlock (*Conium maculatum*), the species from which Rouelle made the first sample of leaf protein in 1773, was added to our list along with Chinese cabbage (*Brassica chinensis*), endive (*Chicorium endive*), groundnut (*Arachis hypogaea*), soyabean (*Glycine max*) and Kudzu vine (*Pueraria lobata*). Of these only groundnut and soya seem promising.

Laboratory-prepared barley protein, precipitated at 80° and washed in 0·25*N*-HCl, was less well digested than either water-washed material or the corresponding large-scale preparation (acid-washed at pH 4·0). The digestibility of the water-washed protein is increased by removing part of the lipid by extraction with 3:1 alcohol:ether, but similar treatment of freeze-dried large-scale preparations does not improve digestibility to the same extent. We have other reasons for thinking that the resistance of leaf proteins towards proteolysis depends in part on the formation of lipid complexes.

The amino-acid composition of lupin "cytoplasmic" protein, prepared by controlled heating of the leaf extract, resembles that of a marrow "cytoplasmic" fraction, prepared by the same method, and analysed by Stahmann (*J. Agric. Fd Chem.* (1965), **13**, 139). In spite of the increased

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nutritive value of protein made from older plants, increasing age did not affect the amino-acid composition. (Byers)

Is N_2 absorbed during storage? We suggested (*Rothamsted Report* for 1963, p. 96) that the lipids in leaf-protein preparations absorbed atmospheric nitrogen, but further work indicates the various lines of evidence for this suggestion are inconclusive.

Sealed plastic pouches containing freeze-dried leaf protein often shrink on to the contents and become rigid, but the protein is so porous, and so occupies such a large volume, that the shrinkage can be accounted for by absorption of oxygen alone. There may be a fall in pressure within sealed cans of leaf protein stored at 100° C, but this can be accounted for by tiny leaks in the cans which allow gas to escape during the prolonged heating but not to return during the brief period before the pressure is measured. Such leaks were demonstrated in cans containing leaf protein or leaf fibre, by the diminution in weight of the unopened can after a few days' heating. Leaks were also demonstrated in 450-ml cans containing 3 ml of water which after being at 100° C for 4 days developed vacuums ranging from 1 to 27·5 in. of mercury.

The "lipid" nitrogen content of freeze-dried leaf protein stored at 100° C for long periods increases, but this is not accompanied by comparable increases in total N. When wheat fibre labelled with ^{15}N is stored at 100° C a small diminution in the percentage of ^{15}N in the "lipid" nitrogen can be accounted for by a redistribution of nitrogen compounds soluble in lipid solvents after storage, because the plant tissues may not be uniformly labelled with ^{15}N.

Although there is still no positive evidence for N_2 absorption by leaf-protein preparations, some suggestions of its occurrence remain, and absorption by leaf lipids and other vegetable oils is being investigated.

Manometric observations on various freeze-dried leaf proteins suggest that oxidation, as measured by oxygen absorption, is fastest in highly coloured samples, which contain much lipid, and/or more than 5% moisture. (Buchanan)

Silage production from fibre residues. Several small batches of vacuum silage were made and found acceptable by cattle in the field. Clovers proved the best crops and gave a fine and easily compressible fibre, which kept for several months with very little obvious microbial spoilage. Samples were successfully made with added molasses, the protein-free juice or water, and with the fibre alone.

Maize fibre was drier than clover, and the lack of soluble fermentable carbohydrates resulted in about 10–20% spoilage when ensiled without the protein-free juice. Some fibre was successfully dried in the sun. (Arkcoll)

Self-heating of hay

The conditions were studied in which hay self-heats beyond 70° C, the maximum temperature reached by thermophilic micro-organisms, and can lead eventually to self-ignition. 125 g of hay, moistened to 45% water content, was kept in a 1-litre Dewar flask in an automatically controlled

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incubator maintained at a temperature just below that attained by the hay. When aerated with humidified air at 35 cc/min, it self-heated to 100° C in 3–4 days, and hay aerated with dry air at 10 cc/min self-heated to 68° C in 3 days, but did not exceed 70° C, even after 18 days. However, hay aerated with dry air at 10 cc/min until 70° C was reached, and then aerated more slowly, self-heated to 89° C in 8 days; on curtailing the air-flow further, the temperature rose to 100° C in a further 8 days. In another experiment, once 70° C was reached, the air-flow was decreased and the temperature control was set so as to respond only to rises in temperature. In this system the temperature rose to 84° C in 18 days, with water being continuously lost; normal control was resumed, and water continued to be lost for another 18 days, when the temperature rose at an increasing rate from 90° to 168° C during 3 days.

When air was circulated in a closed system with continuous removal of water and CO_2 more than 10 litres of air were needed for the temperature to reach 68° C, and a further 5 litres provided air enough for the temperature to reach 168° C. By judicious adjustment of the air flow the temperature rose from 62° to 100° C in 22 days and then rapidly to 192° C. When the experiment was stopped the temperature was rising at 10°/h.

The heated samples were analysed for total N, soluble carbohydrate, pentosan and cellulose. Samples that had heated to 68° C showed losses of soluble carbohydrate and cellulose, but little loss of pentosan, even after 18 days at 68° C. Samples that had heated to 100° C in 3–4 days showed losses of pentosan; after longer periods above 70° C this became the main constituent affected. More than half the pentosan disappeared.

Fructosan constituted more than half of the soluble carbohydrate initially. After self-heating at 68° C fructosan was converted to fructose, but 20% fructosan still remained after 18 days at 68° C. Hay that had self-heated to 100° C in 3–4 days, however, contained no fructosan. Besides increases in fructose, self-heated hays gave extracts containing pentose (not present in extracts from the unheated hay) there was no increase in glucose. Hydrolysates of the water extracts with N-H_2SO_4 gave increased reducing sugar. This was fructose where fructosan was present, but pentose also occurred, and increased appreciably in the samples that had self-heated over 70° C and showed losses of pentosan. Glucose did not increase in the hydrolysed extracts. The loss of pentosan above 70° C and the increase in combined pentose products found in extracts of these samples suggest the importance of the oxidation of pentose-containing compounds in the self-heating process above 70° C. (Festenstein with Currie, Physics Department)

The potato cyst-nematode

Hatching factor. The modifications introduced in the initial treatment of potato-root diffusate (*Rothamsted Report* for 1965, p. 149) caused difficulties in subsequent bioassays of the crude material because it contained substances that inhibit hatch. These inhibitors persisted until the final stages of purification. Difficulties arise in attempts to fractionate the factor by electrophoresis because traces of the buffering agents affect hatching.

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The hatching factor did not migrate in buffer at pH 1·96, but did in buffers at both pH 4·8 and 6·1. This behaviour is in keeping with that of a moderately strong organic acid (*Rothamsted Report* for 1963 gave 4·17 as the pK_a).

Attempts were made to make derivatives suitable for analysis, including mass spectrometry, using reagents likely to react with the known functional groups (–OH and –COOH) of the factor. None of the reactions tried (methylation, acetylation and trimethylsilylation) yielded crystalline material, but infra-red and nuclear magnetic resonance spectra suggest that derivatives were formed. These reactions are being further investigated. The factor decomposed when treated with thionyl chloride or acetic anhydride as preliminary steps in the preparation of other derivatives. Hatching activity could not be regenerated from the reaction products.

The factor was insufficiently volatile to give a significant peak on gas–liquid chromatography (SE 30 column, 60–240° C), and decomposed at the higher temperatures. However, treatment of purified hatching factor with boron trifluoride–methanol gave a product that showed one major peak on G.L.C. together with two minor peaks.

The N.M.R. spectrum of various samples of the purified hatching factor (in deuterochloroform solution) showed three major bands at 7·35, 7·82 and 8·75 that arise from C–H protons, with relative intensities of about 1:2:3. Diacetone alcohol ($(CH_3)_2C(OH)CH_2COCH_3$) gave bands at 7·37, 7·82 and 8·74, with relative intensities of 1:2:3. Diacetone alcohol is a condensation product of acetone which is extensively used as a solvent in various stages of the purification procedure. A typical sample of purified hatching factor would contain about 10% diacetone alcohol as a contaminant if this were solely responsible for the three N.M.R. bands. (Clarke)

The composition of the eggshells and cyst walls of the potato-cyst nematode. Electron micrographs (P. Dart, Soil Microbiology Department) of the eggshells of *Heterodera rostochiensis* showed two main layers. The outer thicker layer, which may correspond to the protein component of the eggshells, is formed of fibrils; these did not show the axial periodicity characteristic of most collagens. The second main layer may be chitin. Chromatography of eggshell hydrolysates on ion-exchange columns gave nearly quantitative recoveries of the hexosamines, and confirmed that glucosamine was the principal hexosamine present. There was also a small amount of another amino sugar, probably galactosamine, which amounted to about 8% of the total hexosamine present, and about 0·5% by weight of the eggshells. Uronic acids were not detected, but there is about 3% of polyphenol (calculated as percentage catechol content).

Kaul (*Nematologia* (1962), **8**, 288–292) suggested that hatching from cysts of *H. rostochiensis* was subject to seasonal self-regulation through changes in the content of an inhibitory catechin-like substance present in the cyst wall. However, Shepherd and Cox found that the number of larvae that emerged from diapausing *H. rostochiensis* eggs almost doubled in the presence of pieces of newly tanned cyst wall. Soluble compounds were therefore extracted from the cyst wall. Aqueous alcohol extracts gave a

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dark brown gum, sparingly soluble in water. A solution of this gum in water, or in potato-root-diffusate, gave hatches appreciably less than those obtained with the corresponding control solution. Preliminary examination by paper chromatography showed that the gum contained several blue fluorescent polyphenols. (Clarke with Shepherd, Nematology Department)

Infection with the fission products of tobacco mosaic virus

To get evidence about the early phases of virus infection, and about the mechanism by which viruses spread within the infected plant, attempts have been made here and elsewhere to arrest the processes at different stages and then to examine the components of the infected leaf. When phenol is used as the arresting agent, and infectivity measurements are used as an index of the amount of nucleic acid derived from virus, various other components of the inoculum greatly affect the number of lesions produced on test plants. At physiological concentrations a polysaccharide and Ca^{2+} ions are partly responsible (*Rothamsted Report* for 1960, p. 112; for 1964, p. 123).

Some of the agents are inhibitors of infectivity, that is to say, infectivity is diminished by their presence but is restored when they are removed, but others seem to be inactivators of the nucleic acid. We do not know the composition of the substance(s) responsible, but concentrates active at 50 mg/l have been made by fractionation on ion-exchange resins and on Sephadex. Activity is not destroyed by heating in neutral solution, but is by heating in dilute acid. Inactivation is not immediate, but increases with the duration of contact between nucleic acid preparations and the partially purified agent.

The assumption underlying this work is that changes in the concentration or distribution of some of these agents may be responsible for the great variations in the susceptibility of leaves to infection according to their age, position and physiological state, but this aspect of the problem cannot be tackled until more is known about the number of agents involved. (Pirie, with Bawden)

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