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ROTHAMSTED RESEARCH

Report for 1964

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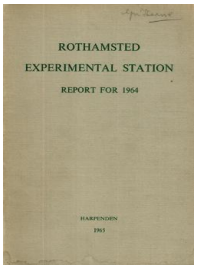

Biochemistry Department

N. W. Pirie

N. W. Pirie (1965) *Biochemistry Department ;* Report For 1964, pp 115 - 125 **- DOI: https://doi.org/10.23637/ERADOC-1-57**

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BIOCHEMISTRY DEPARTMENT N. W. PIRIE

Mrs. S. H. Green joined the staff for the summer and autumn. Dr. D. M. Samuel spent four months here as a Colombo Plan Fellow learning to use the large-scale equipment for extracting leaf protein that is being sent to his Institute in India. Professor C. R. Stocking, Botany Department of the University of California (Davis), was also with us for four months studying the extraction of leaf mitochondria in water-free media. N. W. Pirie attended the First General Meeting of the International Biological Program, in Paris, and the Sixth International Biochemical Congress, in New York.

Much of the work of the department is done to increase general understanding of biochemical processes in plants, both healthy and diseased, but some is on problems of immediate importance to agriculture. Although the work on extracting leaf protein may not be applied in the near future in Britain, it is an essential part of any serious attempt to alleviate protein deficiency in the diet eaten in many wet tropical regions, and it is our national policy to work on problems that could benefit less-favoured countries. The practical application of studies on farmer's lung disease are obvious, and the kind of conditions that make hay hazardous can now be stated with fair precision. Cyst-forming eelworms might be eradicated if all the cysts could be made to hatch in soil free from any susceptible plants. Substances that stimulate hatching, and cheap enough to be applied to large areas of land, may be found by testing chemicals more or less at random, but knowledge of the structure of the natural hatching factors is likely to be a useful guide in the search. Work on the components of plants that affect their susceptibility to infection by viruses also has a practical bearing on the spread of infection both within a plant and between plants.

Metabolic Processes in Plants

Chloroplasts and mitochondria. Chloroplasts can be isolated (Stocking, *Pl. Physiol., Lancaster* (1959), **34**, 56) from freeze-dried leaves in a non-aqueous environment. Because they have not been leached of their water-soluble components, they are useful in studying chloroplast composition and the location of the primary products of photosynthesis. This method of preparation was examined to see to what extent the chloroplasts are contaminated with other cell components, and whether it could be adapted to isolate mitochondria.

Tobacco leaves were freeze-dried and disrupted in a mixture of hexane and carbon tetrachloride of appropriate composition. The extracts were filtered and centrifuged on "density gradients" made by layering hexane–carbon tetrachloride mixtures of decreasing densities in centrifuge tubes. The green material was usually resolved into three fractions: a large band of density about 1·34, a smaller one of density about 1·29 and a variable

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amount of heavy material at the bottom of the tube. The lightest fraction consists of "intact" chloroplasts with little microscopically visible contamination. It contained only 8–20% of the total chlorophyll of the extracts. This small yield of purified chloroplasts was not increased by many modifications to the procedure, so it is difficult to compare these preparations with those made elsewhere. This fraction of purified chloroplasts always contained cytochrome oxidase. As this enzyme is thought to be exclusive to the mitochondria, it is likely that 2–10% of the mitochondria accompany these chloroplast preparations.

Most of the cytochrome oxidase of the preparations sediments to the bottom of the centrifuge tubes. Although it has not yet been separated from the bulk of the glycollic oxidase and pyruvic kinase of the preparation, these preliminary results are encouraging for it should be possible to devise density gradients in which the particles carrying these three types of enzyme can be separated by centrifuging. (Pierpoint)

Polyphenoloxidase of tobacco leaves. The intermediates formed when polyphenols are oxidatively polymerised inactivate or bind some of the more labile enzymes and viruses. In particular, cucumber mosaic virus is inactivated in extracts of infected tobacco leaves during the oxidation of chlorogenic acid, and getting infected extracts requires extraction methods that prevent this oxidation. It is unknown which of the products of the oxidation is the inactivator or which parts of the virus are sensitive. The first step in answering these questions is a knowledge of the mechanism by which chlorogenic acid is oxidised.

If the oxidation follows a similar course to the oxidation of catechol by mushroom phenolase the initial step would be the formation of a quinone with the uptake of one molecule of oxygen per molecule of quinone formed. A series of non-enzymic oxidation–reductions could then convert half of the quinone to a hydroxyquinone, and regenerate chlorogenic acid from the rest: polymerisation of the hydroxyquinone would produce the brown end products. There is evidence compatible with this model. Thus the formation of hydroxyquinones during the enzymic oxidation of chlorogenic acid is indicated by the effects of amines. Deeply coloured pigments are formed and the amount of oxygen absorbed depends on whether the amine is primary, secondary or tertiary. The formation of a quinone with the absorption of one molecule of oxygen is indicated by the effect of quinone-trapping reagents on the reaction. One such reagent, benzene sulphinic acid, gives a complex with the quinone that is stable and easily handled. It has been extracted from the reaction mixture and is being characterised.

With this knowledge of the way in which chlorogenic acid is oxidised, the action of some inhibitors of the process were studied. Attention was given to substances that protect the infectivity of cucumber mosaic virus in leaf extracts, principally diethyldithiocarbamate and potassium ethyl xanthate. The carbamate powerfully inhibits polyphenoloxidase, so that oxygen is not taken up and intermediates are not formed. The xanthate also inhibits the enzymes, but in a less complete and more complex manner; small amounts produce proportionally more inhibition than larger ones,

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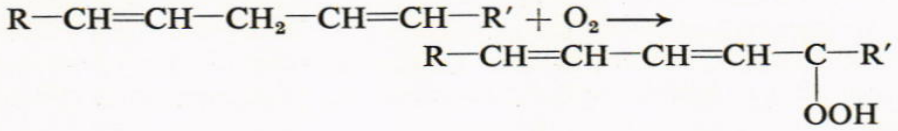
although very small amounts of xanthate apparently stimulate the enzyme slightly. Apart from these effects on the enzyme, xanthate powerfully reacts with one of the first products of chlorogenic acid oxidation. The compound formed was extracted and some of its properties noted. These two distinct effects of xanthate explain the previously puzzling observation that some concentrations of xanthate stabilise cucumber mosaic virus in leaf extracts without completely inhibiting polyphenoloxidase. (Pierpoint)

Enzymic breakdown of chlorophyll. The chlorophyll-bleaching activity of legume seed extracts was correlated with the formation of fatty acid hydroperoxide



catalysed by lipoxidase (*Rothamsted Report* for 1962, p. 98). Further work, however, suggested that other factors were also involved, and it seems that chlorophyll is bleached during breakdown of peroxides rather than during their formation. Although bleaching and lipoxidase activity were related, the two did not parallel each other exactly, and the optimum pH for bleaching was near 6, whereas for linoleate hydroperoxide formation it was near 8. In addition, various long-chain fatty acids that are not substrates for lipoxidase also bleached chlorophyll when added to ground seeds or seed extracts. Blain and Styles (*Nature, Lond.* (1959), **184**, 1141) found a lipoperoxidase in soya-flour extracts that catalysed the decomposition of linoleate hydroperoxides and concurrently bleached carotene. This enzyme seems also to be one of the components of the chlorophyll-bleaching system.

With small volumes of seed extracts, linoleate hydroperoxide was formed at a rate proportional to the volume of extract. But to bleach chlorophyll it was necessary to add a large volume of extract; the amount of hydroperoxide in the reaction mixture decreased after having reached a maximum soon after the start of incubation. Under the same conditions oleic acid formed much less hydroperoxide, and this was quickly broken down. Incubating a seed extract with fatty acid before adding chlorophyll diminished the bleaching because the bleaching factor was inactivated.

Commercial preparations of purified lipoxidase catalysed chlorophyll bleaching only very slightly by themselves, but when added to seed extracts plus linoleic acid, the bleaching was increased as much as threefold. The likely explanation for this is that, because the amount of hydroperoxide formed is increased, more can react before the lipoperoxidase is inactivated.

Material with chlorophyll-bleaching activity is precipitated from soya-flour and pea-seed extracts by ammonium sulphate, but all other treatments tried lead to the loss of some or all of the activity.

A search for the presence in yellowing leaves of a chlorophyll-bleaching system similar to that in legume seeds has failed. (Holden)

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Thin-layer chromatography of chlorophylls *a* and *b* and related compounds. Mixtures of chlorophylls *a* and *b* and pheophytins *a* and *b* can be determined quantitatively by thin-layer chromatography on cellulose. The separated pigments are eluted with acetone and estimated spectrophotometrically. Rapid work in the minimum of light gives recoveries $> 90\%$. The pigments were separated more sharply on the thin-layer cellulose than on paper. Water must be removed from sample solutions before they are applied, otherwise some of the chlorophyll remains on the baseline and might be mistaken for chlorophyllide. The method is probably also suitable for estimating pheophorbides and chlorophyllides.

Qualitative results can be obtained rapidly, particularly when using layers applied to microscope slides. This sensitive technique is proving useful in studies on the products of enzymic breakdown of chlorophyll. Various pigments move on the cellulose layers in the order (slowest first): chlorophyllide *b*, chlorophyllide *a*, pheophorbide *b*, pheophorbide *a*, chlorophyll *b*, protochlorophyll, chlorophyll *a*, pheophytin *b* and pheophytin *a*.

Acetone extracts of leaves turn brown when acid, because the chlorophylls are converted to pheophytins. Phenol–acetic acid–water extracts are green, and it seemed that pheophytins might not be formed in this system. Pheophytin absorption spectra can, however, change considerably with pH, and a combination of spectrophotometry and thin-layer chromatography showed that pheophytins are produced in phenol–acetic acid–water. In this solvent a solution of pheophytin *a* is blue, pheophytin *b* is yellow and a mixture of the two pigments is green. The spectrum of pheophytin *a* in aqueous acetone is the same at pH 1·5 as at neutrality, but differs considerably from its spectrum in phenol–acetic acid–water.

An apparatus was designed to apply the sample quantitatively and uniformly as a streak to the baseline of thin-layer or paper chromatograms. It is suitable for both preparative and quantitative chromatography. (Bacon)

Oxidative deamination and decarboxylation of amino acids by plant extracts. Pyridoxal phosphate slightly accelerates the uptake of oxygen by pea-seedling extracts (*Pisum sativum*). When Mn^{2+} ions are also added, oxygen is taken up rapidly; no other metal ions tested were active. The total increase in oxygen consumed is proportional to the pyridoxal phosphate added and exceeds 2 moles of O_2/mole of pyridoxal phosphate; rather more than 1 mole each of carbon dioxide and ammonia/mole of pyridoxal phosphate were formed as reaction products. The reactions are oxidative deaminations and decarboxylations of the amino acids in the extracts, catalysed by peroxidase in presence of pyridoxal phosphate and Mn^{2+} ions. Such peroxidase systems catalyse the oxidative decarboxylation of amino acids. Similar results were obtained with pyridoxal but not with pyridoxine, pyridoxamine or pyridoxic acid, suggesting that the reactions involve the aldehyde group of pyridoxal and pyridoxal phosphate; other aldehydes tested were inactive. The pyridoxal and pyridoxal phosphate disappear during the reaction. The uptake of oxygen is probably partly explained by the oxidation of these compounds, but the products have not been identified.

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The pea-seedling extracts contain two factors essential for the reaction, in addition to Mn^{2+} ions. These factors are separable by dialysis. The non-diffusible material contains a thermolabile factor, replaceable in the reaction by peroxidase. The diffusate contains a thermostable factor replaceable by amino acids. Preparations of the thermostable factor were also made from ethanolic extracts of pea seedlings. The plant extract can be replaced in the reaction by a mixture of peroxidase, Mn^{2+} ions and amino acids. When pyridoxal phosphate is added to such mixtures oxygen is rapidly taken up, pyridoxal phosphate disappears and carbon dioxide and ammonia are formed, as in the experiments with plant extracts. Evidence was also obtained that the reactions are activated by light.

After adding pyridoxal phosphate to the mixture of plant extract and Mn^{2+} ions, there is a lag period before oxygen starts to be taken up rapidly. The lag period ranges from a few minutes with pea-seedling extracts to an hour or more with bean-seedling extracts (*Phaseolus vulgaris* and *multiflorus*). In identical experiments at different times with the system of peroxidase and thermostable factor, the lag period varied between 5 and 90 minutes. This variation was related to variation in light intensity. The lag period was shortened by light, by traces of hydrogen peroxide or by treatment of the thermostable factor preparation with charcoal; it was lengthened by excluding light, or by adding catalase or traces of catechol. The lag is probably partly caused by the presence of inhibitors, probably phenolic compounds, that are peroxidase substrates and are adsorbed by charcoal. Light causes the formation of a peroxide, which is used by peroxidase to oxidise the inhibitors. Even after the inhibitors are removed as completely as possible by repeated charcoal treatment, activation by light is still demonstrable. Activation by light is also observed with systems of purified peroxidase, amino acids, pyridoxal phosphate and Mn^{2+} ions. This suggests that in addition to its indirect effect in removing inhibitors light also directly activates the reaction.

The compounds oxidised in the reactions may be the Schiff's bases formed between the pyridoxal phosphate and the amino acids. The reactions proceed readily in very dilute solutions of pyridoxal phosphate, but this substance is consumed, so these reactions can have little significance in the metabolism of amino acids, unless the modification of pyridoxal phosphate is reversible *in vivo* so as to enable this compound to act catalytically in the system. However, as Schiff's bases are intermediates in the action of all the pyridoxal phosphate-containing enzymes involved in amino-acid metabolism, the fact that they are attacked by systems of peroxidase and Mn^{2+} ions is significant. (Hill and Mann)

Distribution of amine oxidases. Measurements of oxygen uptake and ammonia production show that diamine oxidase occurs in the shoot tips, cotyledons and root nodules of many legumes; it has now been found in *Glycine max*, *Phaseolus vulgaris* and *Phaseolus multiflorus*, but not in the non-legumes *Datura stramonium*, *Momordica balsamina*, Hemp (*Cannabis sativa*) and Hazel (*Corylus avellana*). Oxygen uptakes were increased with extracts of Cuckoo Pint (*Arum maculatum*), Honeysuckle (*Lonicera periclymenum*) and Red Deadnettle (*Lamium purpureum*) when putrescine

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was used as the substrate, but ammonia was not detected by the techniques employed.

The colorimetric test for diamine oxidase based on the formation of the yellow quinazolinium complex with *o*-amino benzaldehyde when putrescine is used as the substrate proved unreliable. Without the complementary evidence of oxygen uptake or ammonia production this method will not distinguish between diamine oxidase, diamine dehydrogenases or diamine transaminases. (Hill)

Activation of diamine oxidase. When cadaverine is used as the substrate, adding pyridoxal phosphate to dialysed clover-seedling extracts increases the oxygen uptake. It is also known, both by measurement of the oxygen uptake and by the ammonia produced, that other substances apparently unrelated to pyridoxal phosphate, e.g., catechol, ascorbate, cysteine and L-ketoglutarate, seem to increase diamine oxidase activity towards cadaverine. In spite of this, the apparent activation by pyridoxal of diamine oxidase in dialysed clover extracts is still quoted as evidence for the prosthetic group of this enzyme containing a pyridoxal component. Mann and Clarke (*Rothamsted Report* for 1956, p. 90) showed that peroxidase catalysed the oxidation of pyridoxal, a reaction that could account for the increased oxygen uptake but not for the increase in ammonia when pyridoxal was present.

Diamine oxidase is inhibited by peroxidase (Hill and Mann, *Rothamsted Report* for 1963, p. 92), and this inhibition can be prevented by peroxidase substrates. This inhibition was found to be specific for cadaverine, little or no inhibition occurring when other amines, e.g., putrescine and histamine, are used as diamine oxidase substrates. The peroxidase-catalysed inhibition of diamine oxidase, when cadaverine is used as the substrate, is prevented by most of those compounds; they apparently activate diamine oxidase in crude plant extracts, and have little effect on oxidation of putrescine and other diamines. These results confirm last year's observations that the effects of adding catechol, etc., to plant extracts are not caused by any activation of diamine oxidase but by preventing the peroxidase-catalysed formation of an inhibitor, when cadaverine is the substrate. (Hill)

Production of Leaf Protein as a Human Food

Effects of age and fertilisation on extraction. When extra fertiliser nitrogen was applied in early spring to wheat and barley plants harvested at different ages the yield of protein from young plants was proportional to the amount of fertiliser N up to the most applied, but as the plants aged the response apparently became equal from all treatments.

Maize, harvested at approximately weekly intervals from the stage of ear emergence, gave less extractable protein as the plants aged. The results, together with previous ones, suggest that yield of extracted protein increases to a maximum when the dry matter reaches about 15% and then diminishes as the dry matter of the leaf increases and the plants mature.

Yields from other crops were similar to those in previous years, except

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rape, which yielded exceptionally well, probably because it was sown early. Alsike clover, reintroduced this year, suffered from drought, and the protein extracted poorly from the small yield of foliage.

Nettles gave a very small yield of protein compared to other plants and other years. However, this weed was growing without fertiliser and in a shaded woodland. (Sturrock)

Improvements in machinery. The process of leaf-protein extraction is now sufficiently well understood and accepted for us to concentrate on refinements that will ultimately determine the circumstances in which production is economic. To save labour, and to remove dust and stones from the crop, an elevator collects the crop from the surface of a tank of water. Paddle wheels, and an arrangement of dams, keep the water circulating so that the crop is always pressing against the bottom of the elevator; this gives an unprecedentedly uniform feed.

The reserve pulper was sent to the Agricultural Research Institute at Coimbatore, and a new one was made to our design. In it the beaters are evenly spaced, because stroboscopic study of an older model, in which the use of a standard hub necessitated an irregular spacing, showed zones in which mounds of pulp built up and then collapsed. This improvement gives more even running; the pulper also costs less.

As a result of these improvements, tough crops can be pulped twice as fast as in 1955–57, for the same power consumption. With soft, easily pulped crops the capacity of the press limits the rate of working. Part of this improvement can probably be attributed to the pre-chopping that the crop gets when it comes off the automatic elevator. Arrangements for feeding the pulper are being further improved. The belt press described in previous reports works well, but the belt is unlikely to be strong enough to stand up to commercial use. Other belting materials and methods of manufacture are being investigated.

Storage. Storage raises no novel problems, and on the scale on which leaf protein is likely to be produced during the next decade there is no reason for storing it rather than using it as it is produced. However, freeze-dried protein is made so that it can be sent overseas for experimentation. Satisfactory laminated pouches have been found that protect it from air. In the normal method of preparation the protein is heated to 80° C and cools slowly from that temperature. Several batches of protein were heated at 100° C for 0·5 hour during the preparation to see whether more complete enzyme inactivation would give products that absorb oxygen more slowly. Unexpectedly, absorption was significantly more rapid. (Davys and Pirie)

The fractionation of proteins by controlled heating. An attempt to study the ratios of fractions from extracts of maize leaves of different ages was inconclusive, as there was $< 5\%$ "cytoplasmic" protein at all stages of growth. This year three species known to contain more "cytoplasmic" protein, rape, lupin and marrow, were examined regularly over several weeks, and the percentage distribution of the "chloroplastic" and "cytoplasmic" fractions was independent of leaf age. Wheat, examined to see

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whether the result with maize was typical of cereals, yielded $< 5\%$ "cytoplasmic" protein at all ages over a 12-week test period.

Freeze-dried "cytoplasmic" fractions from rape, lupin and marrow are much more easily digested by KCN-activated papain than the corresponding "chloroplastic" preparations and are usually hydrolysed more than the unfractionated protein from the same extract. However, when the two fractions are recombined in the correct proportions to simulate whole protein, the mixture is less digestible than the original. There was little difference in either the rate of digestion or the final amount of hydrolysis of variously precipitated freeze-dried tare (*Vicia sativa*) proteins by KCN-activated papain. The digestibility of freeze-dried maize protein was not affected by 7 days dry heating (60° C) or by 3 days at 100° C, but 6 days at 100° C decreased the percentage of soluble N and the proportion of it that was TCA-soluble in a 48-hour digest.

Protein was extracted, on a laboratory scale, from the following new species this year: *Asperula azurea*, *Anchusa capensis*, *Polygonum baldschuanicum*, none of which gave good yields, and *Chenopodium quinoa* and *C. nuttalliae*. These last two species were raised at The John Innes Institute from seed harvested in Bolivia (two varieties from different altitudes) and Mexico respectively, where they are common grain plants. Between 40 and 50% of the nitrogen in the leaf was extracted as protein N from these plants, and it is proposed to grow them in the field next year. (Byers)

Presentation on the table. People who wish to disparage leaf protein often say that it will cost too much to make and be inedible when made. Improvements in the efficiency of production are noted in the section on machinery; progress has also been made in presentation. After preliminary trials to find suitable ratios for the different components, several dishes were cooked from weighed and analysed materials and part of the final product was dried, ground and analysed. Satisfactory agreement between the expected nitrogen content and that determined make it probable that the proportion of the total nitrogen in the dish that is contributed by leaf protein can be calculated from the weights and compositions of the ingredients. Ten palatable, or even appealing, dishes containing $> 20\%$ of protein, half of it leaf protein, were made. (Byers, Green and Pirie)

Proteins labelled with ^{15}N. Last year the poor extraction of protein from wheat leaves grown on sand with minimal nitrogen and enhanced ^{15}N was commented on. This protein was wanted for nutritional studies in Jamaica. Because more labelled protein might be called for, the reasons for the poor recovery of a very expensive fertiliser were sought. In another experiment, in a glasshouse in winter, protein was extracted normally both with meagre fertilisation and with enhanced ^{15}N. The earlier result has not therefore been confirmed, but the wheat was then grown in the open in summer so that it was physiologically more mature at harvest.

At the request of a team interested in celiac disease, wheat labelled with ^{15}N is now being grown to prepare gluten. (Pirie and Sturrock)

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Infection with the Fission Products of Tobacco Mosaic Virus

The infectivity of the extract made from infected tobacco leaves with saturated phenol depends greatly on the technique used in making the extract and for removing the phenol, so that the inoculum can be put on test plants (*Rothamsted Report* for 1960, p. 112). Many components of these extracts seem to be implicated; the dominant pair are Ca^{2+} ions and an indiffusible polysaccharide. When present in physiological concentrations in the inoculum, either of these can diminish the number of lesions produced by purified tobacco mosaic virus nucleic acid to a fifth, and at greater concentrations they make the inoculum non-infective. Other so-far unidentified components of sap inhibit the process of infection in a similar way. It is not known whether this is a consequence of the co-action of such known components as chlorogenic acid, hexenal and nicotine, all of which are inhibitors when used at concentrations greater than those in sap, or whether there are further naturally occurring inhibitors active at physiological concentrations. (Pirie and Bawden)

Chemical Changes in Self-heated Hay

For the self-heating of hay in Dewar flasks, aeration by diffusion through a cotton-wool plug at the top of the flask was adequate with 500 g hay moistened to 40% water content in a 4-litre flask, but additional aeration at 50 ml/min was required for maximum self-heating of the lower half of a mass of 1,250 g hay in a 10-litre flask.

The effect of water content was studied in 4-litre flasks with 500 g hay. Good-quality hay usually showed one broad temperature maximum, but secondary temperature peaks developed on storage. The times taken to reach maximum temperatures differed greatly, depending on the quality of the hay. Water content determined the maximum temperatures reached by different hays, but did not alter the heating pattern of any particular hay.

The final products were usually visibly mouldy throughout the mass with pH values near 7, thus resembling hay from mouldy bales rather than brown hay from self-heated stacks, which is usually mould-free and acid. Samples from the flasks were extracted with acetone and examined by Dr. J. Pepys and Dr. P. A. Jenkins, of the Institute of Diseases of the Chest, for antigen associated with farmer's lung disease (F.L.H. antigen); other samples were analysed by Lacey (Plant Pathology Department) for their microflora. The critical water content for producing F.L.H. antigen is 29–34%. In this range hay self-heated to 33–55° C and differed considerably in antigen content. Hay with a multi-peaked heating pattern produced F.L.H. antigen more readily than hay with only one peak. Hay containing less than 29% water self-heated little and produced little antigen, so that hay containing less than 25% water seems unlikely to develop antigen at all. Although hay containing 40% water invariably produced F.L.H. antigen, hay containing 47–68% water, which self-heated to 61–65° C, contained less antigen the wetter the hay, particularly in the lower parts of the flasks.

Sequential events during self-heating were studied by filling several

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flasks with 500 g hay moistened to 40% water content and emptying them at intervals. F.L.H. antigen first appeared faintly on the third day, and strongly thereafter. Reducing sugar decreased rapidly, 80% was lost by the third day. Pentosan was affected on the third day and cellulose on the fifth day, with final losses on the ninth day of 16% and 14% respectively. The pH values rose from 6·2 to a maximum of 8·7 on the fifth day and then declined to near 8.

Experiments with moistened barley grain were continued and extended to oats. F.L.H. antigen was usually in the middle of the mass in the Dewar flask, but not in the drier, upper regions or below, where water accumulated. Thus, as with hay, F.L.H. antigen does not occur when the grain is either too dry or too wet. Excessive water would exclude air, and this probably adversely affects F.L.H. antigen production. (Festenstein in collaboration with Lacey, Plant Pathology Department, and Skinner, Soil Microbiology Department)

The Potato-root Eelworm Hatching Factor

A record quantity of potato-root diffusate, 7 tons, was treated in 1964 and yielded 30 mg of purified material and twice as much crude. The purity of the end-product was tested by paper chromatography using the six solvent systems that were used for the chromatography of partially purified material. The R_F of purified material often differed from that of less pure material. The conclusion that the purified material is now largely homogeneous is supported by its improved chromatographic characteristics, i.e., it travels as a compact well-defined spot rather than as the diffuse elongated one previously obtained.

5-Methoxytryptamine has been recommended for the characterisation of organic acids; the salts obtained are usually crystalline and non-hygroscopic. However, the product obtained with this compound and the hatching factor remains solid only in the solvated state. The ready change of state from a stable solvated solid to a gum is typical of the hatching factor itself, as well as of several of its salts.

The size of the hatching-factor molecule is at present uncertain. Indirect evidence (from the nuclear magnetic resonance spectrum) suggests that the molecule is larger than had been thought, with a molecular weight of perhaps 600–700. A value of 272 was reported earlier (*Rothamsted Report* for 1963, p. 99), but the determination was accompanied by some decomposition. Arrangements have been made for a sample to be examined in a mass spectrometer. The spectrum obtained should give a molecular weight, in addition to other information.

Some tentative ideas about the structure of the hatching factor can now be formed. The molecule seems to contain a non-aromatic alicyclic ring system, to which is attached a long alkyl side chain (at least 18 carbon atoms). It contains methyl groups, perhaps 9 if the larger molecular weight is correct. The dominant feature of the molecule is the presence of carbonyl groups. One of these forms part of an acidic group, presumably a carboxyl group (*Rothamsted Report* for 1963, p. 100), although there was no indication of a carboxyl group in the N.M.R. spectrum. This carbonyl group has

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another carbonyl group in the β-position. Two carbonyl groups occur in the form of enolic groups, but these do not give any pronounced colour with ferric chloride. Another carbonyl group probably occurs as an acetyl group. There seems to be no methoxyl or other ether groups.

Collaborative work on synthetic hatching factors is described in the report of the Nematology Department. (Clarke)

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