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Rothamsted Experimental Station Report for 1965

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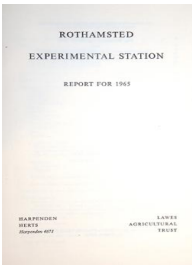

Biochemistry

N. W. Pirie

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BIOCHEMISTRY DEPARTMENT

N. W. PIRIE

After seven years of secondment in West Africa, R. H. Kenten has been appointed to one of the posts created for overseas research and is now a member of the Plant Pathology Department. Mr. D. W. P. Murty, of the Department of Agriculture, Stock and Fisheries in Papua and New Guinea, and Dr. O. L. Oke, of the Department of Chemistry at the University of Ife in Nigeria, spent a few weeks with us learning the use of our machinery for the large-scale extraction of leaf protein. Mr. J. I. Ahmad has come as a voluntary worker to work on problems connected with leaf protein. At the invitation of the Australian Academy of Science, N. W. Pirie visited various research centres in Australia and New Guinea; at the request of the Royal Society he attended a N.A.S.A. conference on International Participation in Biomedical Experiments in Space held in Houston (U.S.A.); as a member of the COSPAR committee on "Life Science and Space Research" he attended the annual symposium in Mar del Plata (Argentina); and, as a member of the international committee for section U.M. of the International Biological Program (I.B.P.), he attended the section meeting in Paris. Margaret Holden and M. F. Bacon attended a NATO Advanced Study Institute on the "Biochemistry of Chloroplasts" in Aberystwyth.

Work on the selection of crops that would be suitable for the large-scale production of leaf protein, and the improvement of machinery for this purpose has more immediate application to agriculture than the rest of our work; it has now been accepted both nationally and internationally as a suitable project for the I.B.P. and another extraction unit has been sent abroad—to Bubia in New Guinea. Spontaneous combustion of damp hay is a real but rare phenomenon; we are beginning to understand the conditions of insulation and rate of access of air that are needed before it can happen. The breakdown of chlorophyll is an index of senility, of infection in the living plant, and of poor processing conditions in stored or preserved vegetables. The changes are complex, but it is now possible to distinguish the separate roles of fission of the chlorophyll : protein complex, the phytol : chlorophyllide compound, and the conjugated double-bond system in the free chlorophyll. Although the losses caused by virus infection are well known, little is known about the mechanism by which viruses injure or kill the host. Increased oxidation is often an early sign of infection, and some progress has been made towards identifying the particular enzyme processes involved in this initial stimulation. It is reasonable to assume that the deleterious effects of many trace-element deficiencies depend on the failure of enzyme processes in which these elements are taking part. Our studies on diamine oxidase, which contains copper, and on the oxidations brought about by peroxidase in the presence of manganese, are part of an attempt to unravel the normal function of these enzyme reactions. Progress in determining the structure of nematode hatching factor has been

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recorded in earlier Reports, and attention is now directed towards the material with which the hatching factor may be reacting.

Production of Leaf Protein as a Human Food

The cold summer frustrated our attempt to get a production rate of 1000 kg of extracted protein per hectare; with three cuts of wheat and two sowings of mustard the yield was 967 kg. On a more fertile site a single late cut of wheat gave more than 900 kg. With a suitable site and cropping sequence, and favourable weather, we now think 1,500 kg/ha attainable. The obvious advantages of a legume when the primary object is protein production caused us to re-examine white clover. Once established, it yields 700 kg/ha, but the yield was much smaller during the first year. Lupins yielded >300 kg/ha, in 3 months; unlike most legumes, the protein extracts readily from the leaves, even when the plant is mature and stalky.

Ten new species were tested: *Sorghum vulgare*, *Vaccaria pyramidata* Med. (soapwort), *Silybum marianum*, *Salvia coccinea*, *Carthamus tinctorius*, *Rudbeckia bicolor superba*, *Kochia scoparia trichophylla*, *Leonurus sibericus*, *Scabiosa atropurpurea* and *Ricinus communis*. The first two are the most promising. The others either extracted poorly, were inconveniently mucilaginous (*S. atropurpurea*) or gave a black and unattractive product (*S. coccinea*). Because of success with a sample of quinoa (*Chenopodium quinoa*) last year, enough was grown this year for a large-scale run. In yield and growing time it resembles maize, sorghum and mustard and could be a useful crop in some environments, for it grows luxuriously at high altitudes. The new variety of barley, Maris Badger, gave about twice as much extracted protein as Proctor at each level of fertiliser nitrogen, largely because it produced more foliage; average extraction rate was also greater (63 compared to 56%).

Improvements to machinery. The loading tank described last year works well, and an extension to the elevator allows it to feed the pulper directly without an intermediate chopper-blower. The new pulper, the seventh in the series of pulpers involving radical changes from their predecessors, runs evenly and is easy to clean. Its throughput is less than the older pulpers, and it takes as much power; probably for this reason the percentage of protein it extracts is greater—it is simply doing a more complete job of pulping. A nylon and P.V.C. belt with polyurethane splice was fitted to the press and ran throughout the season under about twice the tension that damaged its predecessor. A still stronger belt can be made, so the usefulness of this type of press seems certain. As a tentative step towards estimating the cost of making leaf protein, the pulper power consumption was measured with a wide range of crops. It ranged from 0·64 kwh to extract 1 kg of protein from the soft-textured quinoa, to 5·5 with the much harsher maize. About half as much protein comes out on the second extraction as on the first, and only about half as much power per kg is needed to get this.

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Comparisons between the Large Unit and Village Unit. The "village unit" (*Rothamsted Report* for 1961, p. 98) is convenient for making protein in 5–10 kg lots that would be suitable for tests with small animals, or preliminary trials with people, but it does not extract such a large proportion of the protein of the leaf as the large pulper. The difference varies with the crop; thus, from rape, even when mature, the "village unit" extracts 73% of the protein got with the large unit, but with white clover only 31%. Laboratory scale experiments show that this difference does not arise from the delay in the "village unit" between the first damage to the leaf and the final extraction. We think therefore that, in its present form, the "village unit", though useful, does not disintegrate the leaf sufficiently. This is reflected in its smaller power consumption per ton of crop processed, and we are trying to increase the amount of effective work done in it.

Labelling with ^{15}N. As a result of the work on ^{15}N-labelled leaf protein described in earlier Reports, the Medical Research Council paid for a further supply of ^{15}N to make labelled wheat grain from which gluten is to be made for work on celiac disease. The isotope, in the form of KNO_3 and $Ca(NO_3)_2$ with 31% enrichment with ^{15}N, was given from 9 June, when flower initials were just forming, to wheat growing on sand. No fresh solution was added after 13 July, but the effluent was recycled until harvest on 23 August. 58% of the N was recovered in the crop; 42% of this was in the grain, 42% in the straw and 16% in the chaff and roots. There were interesting differences in labelling, presumably showing the extent to which structures had laid down relatively immobile protein before the ^{15}N was given; thus, the chaff had 6·7% enrichment, the roots and straw 9% and individual grains ranged from 9 to 17%. Unfortunately, the position of the heavily and lightly labelled grains in the ear was not recorded in this first experiment. (Byers, Davys, Pirie and Sturrock)

Self-heating of Hay

Conditions in a stack, where self-heating hay is surrounded by hot hay that heats and moistens incoming air, were simulated on a small scale by experiments in aerated Dewar flasks. Hay moistened to different water contents and aerated by diffusion through a cotton-wool plug and also hay in a flask with a lid, aerated through a tube to the bottom, did not self-heat beyond 70° C, the maximum temperature reached by thermophilic micro-organisms. Temperatures of 100° C and over have been reached recently in experiments elsewhere.

Moistened hay in a 4-litre Dewar flask in an incubator was aerated at 35 cc/min with humidified air; it self-heated to 68° C when the temperature of the incubator was adjusted manually to just below that of the Dewar flask, but reached 100° C when the temperature of the incubator was adjusted automatically to 0·5° C below that in the flask in apparatus built with Currie (Physics Department). Temperature, O_2 consumption and CO_2 production were measured automatically.

Hay of 45% water content (96–98% relative humidity) was generally

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used, but hay of 63% water content (99% relative humidity) in one experiment also reached 100° C. The air was humidified by passing through one bottle of H_2SO_4 and two of NaCl at the appropriate relative humidity in the air incubator. Experiments with humidified air passed into the bottom of the flask or allowed to diffuse downwards from the top gave similar results. The temperature reached 100° C in 3–4 days and remained there for a day or two; subsequent aeration with dry air caused the temperature to fall, but the fall could be arrested at any point by stopping the airflow. Aeration with dry air from the outset gave self-heating to only 68° C.

Hay examined at the end of the experiments was dark brown, and at pH 4·5–5·6 there was no visible sign of mould growth. It thus resembled the hay of self-heated stacks. The dry matter losses were 4–7%. Assuming that the dry matter lost is carbohydrate, the energy required to heat the mass to 90° C, in experiments in which there is no gain or loss of water, would be two-thirds of that provided by oxidation of that amount of dry matter. If the assumption is correct, this calculation illustrates how small the available surplus of energy is and why self-heating to inflammation can happen only when the loss of heat in escaping warm air, and by evaporation of water, is narrowly controlled. (Festenstein in collaboration with Currie, Physics Department)

Chlorophyll Breakdown

The development of a rapid thin-layer chromatographic technique for separating chloroplast pigments (*Rothamsted Report* for 1964, p. 118) has made it possible to study quantitatively the changes in leaves treated in various ways. Some of these changes are enzymic, others not; but both are likely to be important in the processing of green vegetables, where the preservation of a fresh colour is essential.

When leaves are heated in water, several changes take place in the chlorophylls, depending on the temperature and the duration of heating. At temperatures between about 50° C and 80° C chlorophyllides form in the leaves of most species because of chlorophyllase action, whereas at 100° C the enzyme is inactivated and there is no conversion to chlorophyllide. Chlorophylls *a* and *b* are partly converted into the isomers *a′* and *b′* at temperatures above 70° C. The chemical nature of this change is not yet fully understood. Loss of magnesium to give pheophytin also occurs to a small extent, the amount formed increasing with the temperature and duration of heating. The amount of chlorophyll extractable by petroleum ether, which is less than 5% in unheated leaves, is increased to about 40% by pre-treatment at 70° C and to over 70% at 100° C. In addition to these changes, up to 20% of the total green pigment is also lost.

When leaves that have been heated to temperatures between 60° C and 100° C are kept in an aqueous medium at a pH above 8, in the dark with air present, chlorophylls *a* and *b* are each gradually converted into two additional pigments. These have colours and visible absorption spectra similar to those of the original chlorophylls, but can be separated from them by chromatography. Their R_f values and lack of extractibility into 0·02*N*-KOH from ether solution imply that the phytyl group has not been re-

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moved and that they have no free carboxylic acid groups. They do not give colour changes when methanolic potash is added; the cyclopentanone ring (or its substituents) has therefore probably been altered, perhaps by oxidation. The magnesium-free derivatives of the four changed chlorophylls differ from each other and from pheophytins *a* and *b*. The chlorophylls in unheated leaves kept in an alkaline solution do not form coloured breakdown products, but disappear slowly to give unidentified colourless compounds. Thus, as a prelude to this reaction, apparently the leaves must first be damaged in some way. Damage by freezing or freeze-drying, however, does not lead to the subsequent formation of changed chlorophylls.

Changed chlorophylls also form when unboiled leaves are kept in aqueous organic solvents in the dark. The pigments seem to be identical with those formed in boiled leaves under alkaline conditions. In 50% acetone solution *Heracleum* leaves give much chlorophyllide and little of the changed chlorophylls, whereas barley seedlings give no chlorophyllide, and much of the green pigment still present is in the form of the changed chlorophylls. Some of the chlorophyll is converted into colourless compounds; the amount of bleaching depends on the concentration of acetone and is greatest at about 45%. Mature *Heracleum* leaves lose only about one-third of their chlorophyll in 24 hours, but barley seedlings lose more than half in 3 hours. The bleaching seems to be enzymic, as it is prevented by boiling. Freezing before incubation in acetone does not greatly inhibit the bleaching. This is in contrast to the effect on the chlorophyll-breakdown system in detached leaves left on water, where pre-freezing prevents the subsequent formation of colourless compounds.

In senescing leaves the disappearance of chlorophyll parallels protein breakdown and, probably, fat. Chloroplast preparations were incubated in various conditions with trypsin and lipase to see whether the usual very slow breakdown of chlorophyll could be speeded. Except for a decrease in the absorption at 660 mμ, because of pheophytin formation, there was little evidence for increased chlorophyll destruction.

We have so far been unable to confirm reports that in late autumn the pigments in deciduous leaves that are indistinguishable from chlorophylls *a* and *b* on chromatograms have altered absorption spectra.

Protochlorophyll is readily bleached by the legume seed–fatty acid system described in earlier Reports. Mesoporphyrin IX and protoporphyrin IX dimethylesters are only slightly bleached, perhaps because of a difference in solubility rather than in structure. Bleaching of pure chlorophylls *a* and *b* differs in some respects from the bleaching observed with crude chlorophyll preparations. With purified pigments, oleic acid is very much less effective than linoleic acid. In the absence of enzyme, pure chlorophyll *a* is converted into pheophytin *a* by linoleic and oleic acids, whereas pure chlorophyll *b* and crude, mixed chlorophylls are not affected. (Bacon and Holden)

Layer Chromatography

Chlorophyllides *a* and *b* and pheophorbides *a* and *b* are satisfactorily separated from each other and from chlorophylls and pheophytins by thin-layer chromatography on cellulose with petroleum spirit (b.p. 60–80° C)—

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acetone (80 : 20 v/v) as developing solvent. A narrow tank, designed for developing layer chromatograms, requires less than 10 ml of solvent, minimises loss of volatile solvents and can be used for continuous development. The sample applicator described previously was modified for use in preparative layer chromatography. A sample larger than before can now be applied faster and at various rates. (Bacon)

Mechanisms of Oxidation

Polyphenoloxidase of tobacco leaves. There is now good evidence that the first product of the oxidation of chlorogenic acid by tobacco-leaf polyphenoloxidase is a quinone. As this quinone may be responsible for the inactivation of cucumber mosaic virus, some of its reactions have been studied.

Like simpler quinones, it is readily substituted by compounds containing $-NH_2$, $>NH$, $>CH_2$ and $-SH$ groups. Some $-SH$ and $>CH_2$ compounds produce stable derivatives of chlorogenic acid by reducing the carbonyl groups of the quinone to phenolic groups and introducing substituents linked by $-S-$ or $-CH_2-$ groups into the aromatic ring. Amino acids form analogous compounds with $=N-$ linkages, but these are further oxidised to substituted quinones that may, in some conditions, catalyse the oxidation of excess amino acid. Sulphur-containing amino acids react with the quinone mainly through their S atoms, although judging by the range of trace products revealed by chromatography, they also probably react to some extent through their amino groups.

Except when the quinone reacts with some amines and indoles, only one substitution into the aromatic ring occurs. By analogy with simpler quinones this is likely to be at the 6′ position. Evidence that this is so in the sulphone formed from the quinone and benzene sulphinic acid has been derived from an examination (by N. F. Janes, Insecticides Department) of the Nuclear Magnetic Resonance spectrum of this compound. An alternative suggestion, that the quinone reacts with proteins and amines through the 5′ position, has recently been made but seems less likely.

Potassium ethyl xanthate, an inhibitor of polyphenoloxidase, was shown last year to react with the quinone produced by this enzyme from chlorogenic acid. So do other inhibitors of polyphenoloxidase such as diethyldithiocarbamate and ascorbate. In appropriate conditions the enzyme, partially inhibited by these compounds, is reactivated, as the quinone formed reacts with, and so removes, the inhibitors. This reversal of inhibition ought to be borne in mind when these inhibitors are used to implicate copper-containing enzymes, such as polyphenoloxidase, in the metabolic processes of plant tissues. (Pierpoint)

Leaf mitochondria. The increased respiration that follows inoculating leaves of *Nicotiana glutinosa* with tobacco mosaic virus (Owen, P. C., *Ann. appl. Biol.* (1958) **46**, 198) was confirmed. Assuming that most of the respiration of uninfected leaves is mediated by mitochondria, there are three likely explanations: (1) the mitochondrial enzymes may function faster; (2) they may increase in amount; (3) a non-mitochondrial respiration may

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develop based, for example, on polyphenoloxidase, which makes little or no contribution to the respiration of uninfected leaves. An examination of the oxidative systems in extracts of infected and uninfected leaves may decide between these alternatives.

Extracts of infected leaves contain the same amount of protein, but slightly less chlorophyll and glycollic oxidase, than do extracts from the same dry weight of healthy leaves; this probably reflects cytoplasmic damage that develops during infection. They also contain slightly less cytochrome oxidase than do extracts of healthy leaves, but, in contrast, two to three times as much polyphenoloxidase. Although these preliminary results do not indicate whether the first or third explanation is correct, they argue against an increase in mitochondrial material following infection. (Pierpoint)

The oxidation of Schiff's bases of pyridoxal and related compounds by Mn^{2+}ions, peroxidase and light. The oxidation of mixtures of pyridoxal or pyridoxal phosphate and amino acids by Mn^{2+}ions, which is activated by peroxidase and by light, depends on the formation of the Schiff's bases between the pyridoxal or pyridoxal phosphate and the amino acids. The oxidation of these bases is mediated by a manganese oxidation–reduction cycle; there is a lag period because of the time needed for manganic manganese to reach a threshold concentration. The activating effects of peroxidase and of light are attributed to catalysis of manganese oxidation by these agents. The work links up with that of Kenten and Mann (*Rothamsted Reports* for 1949 and 1954, pp. 65 and 75 respectively) on the oxidation of manganese by peroxidase systems and by illuminated chloroplasts.

Other workers have reported the oxidative decarboxylation of methionine and tryptophan by the system with the formation of the corresponding amides, and attribute catalytic functions to the pyridoxal and pyridoxal phosphate in the reactions. Our results show that both decarboxylation and deamination of the amino acids may occur. The amounts of carbon dioxide and ammonia produced depend on the amino acid reacting. Phenylalanine yields benzaldehyde, which was identified by isolation as the 2,4-dinitrophenylhydrazone; this suggests that phenylpyruvic acid may be an intermediate, because it is oxidatively decarboxylated to benzaldehyde by systems of Mn^{2+}ions and peroxidase. Spectrophotometry and paper chromatography show that the pyridoxal and pyridoxal phosphate are also changed during the reactions; they may be oxidised, but the products have not yet been identified.

The Schiff's bases formed between pyridoxamine and α-oxo acids are also oxidised by systems of Mn^{2+}ions and peroxidase. Most experiments were made with pyruvic acid, but preliminary results suggest that α-oxobutyric, α-oxoglutaric and glyoxylic acids are also active in the system. Mn^{2+}ions cause little or no oxygen uptake in neutral solutions of pyridoxamine or of pyruvate separately, but when added to mixtures of pyridoxamine and pyruvate they cause oxygen to be taken up rapidly after lag periods which depend on the concentrations of the reactants and range from a few minutes to several hours. This suggests that it is the Schiff's base formed between the pyridoxamine and the pyruvate that is oxidised.

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Peroxidase activates the reactions by shortening the lag periods. The concentration of pyruvate, determined enzymically with the system of lactic dehydrogenase and NADH or spectrophotometrically after conversion to the 2,4-dinitrophenylhydrazone and isolation by paper chromatography, does not change much during the reaction. Pyruvate therefore acts catalytically in the system. The total oxygen uptakes in the reactions are proportional to the pyridoxamine added. The absorption spectrum of the initial reaction mixtures shows the band at 327 mμ characteristic of pyridoxamine; in the incubated reaction mixtures the peak shifts to 318 mμ, where pyridoxal absorbs maximally. The disappearance of pyridoxamine and formation of pyridoxal was confirmed by paper chromatography. Spectrophotometric estimations of pyridoxamine with picrylsulphonic acid, and of pyridoxal as the phenylhydrazone and as the Schiff's base with ethanolamine, suggest that all the pyridoxamine disappears and about half of it is accounted for as pyridoxal. The amount of ammonia finally present is about two-thirds of that formed by the oxidative deamination of all the pyridoxamine to pyridoxal; the remaining third is liberated by treating the reaction mixtures with strong alkali, suggesting the possibility that an amide may be a reaction product. The results suggest that the primary reaction catalysed is the oxidative deamination of pyridoxamine to pyridoxal and that peroxidase, Mn^{2+}ions and pyruvate together constitute a pyridoxamine oxidase system. (Hill and Mann)

The carboxyl component of the prosthetic group of pea-seedling diamine oxidase. Several amine oxidases of animal and bacterial origin contain pyridoxal phosphate as a component of their prosthetic groups. It has been claimed that pyridoxal phosphate occurs in pea-seedling diamine oxidase, but much of this evidence, based on activation of diamine oxidase in crude extracts, has been shown to be incorrect (*Rothamsted Report* for 1964, p. 120). A direct search was therefore made for pyridoxal or related compounds in purified pea-seedling diamine oxidase. The supernatant fluid, after treating the enzyme with either trichloroacetic acid or perchloric acid, did not show the pyridoxal phosphate absorption spectrum. Attempts to remove the carbonyl component from the pea-seedling diamine oxidase, by treatment with hydrazine followed by precipitation of the protein by heat, did not reveal the presence of pyridoxal compounds. Prolonged dialysis of the diamine oxidase for up to 28 days against distilled water resulted in only slight loss of enzymic activity, and this loss was not restored by adding FMN, FAD, pyridoxal or pyridoxal phosphate. Enzyme activity lost by dialysis against hydroxylamine solution was not restored by adding pyridoxal phosphate or pyridoxal phosphate plus cupric ions. Digestion of the pea-seedling diamine oxidase with chymotrypsin and trypsin similarly showed no pyridoxal. All this suggests that, if the prosthetic group of pea-seedling diamine oxidase does contain pyridoxal or pyridoxal phosphate, this must be very firmly bound.

Digestion of the pea-seedling diamine oxidase with Pronase, a protease from *Streptomyces griseus*, gives a product showing a shoulder in its absorption spectrum at 320–330 mμ. The carbinolamine form of pyridoxal phosphate (i.e., $\geqslant$CCH(OH) NHR) absorbs in this region and work

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is in progress, using Pronase and hydrochloric acid hydrolysates of the diamine oxidase, to isolate the compound and to determine whether this band is caused by a pyridoxal compound. (Hill)

The Potato-root Eelworm

Hatching factor. The modifications in the methods used for the initial treatment of potato-root diffusate are described in the Report by the Nematology Department. These have enabled us to process 20 tons of diffusate—three times as much as in 1964. Small-scale trials show that 80% of the hatching activity can be recovered when the diffusate is concentrated by evaporation *in vacuo*, whereas the older procedure of charcoal adsorption recovers only about 28%. As a result, a large-scale evaporator is being installed.

In the later phases of purification, silica proves to be a satisfactory supporting material for thin-layer chromatography with benzene–chloroform as the mobile phase. After development, the chromatoplates were sprayed with sulphuric acid and heated at 110° C. The purified hatching factor was revealed as a single brown spot, which fluoresced in ultraviolet light.

The values previously reported for the molecular weight of hatching factor, measured on the Mass Spectrometer, now seem to be doubtful because of the non-volatility of the sample or its strong adsorption on the surface of the target. (Clarke)

Chemical composition of the egg-shells of the potato cyst-nematode, *Heterodera rostochiensis*. We do not yet understand the action of hatching agents on the eggs of cyst-nematodes, but they could either affect the metabolism of the quiescent larva directly, or indirectly through their action on the eggshell or larval cuticle. The eggshell of potato cyst-nematode (*H. rostochiensis*) was therefore studied as a first step in elucidating the mechanism. Eggs were isolated by sieving a suspension of the crushed cysts. The eggshells were broken open by ultrasonic vibration and separated from the released larvae by centrifuging in a potassium tartrate density gradient. Eggs containing whole or decomposed larvae were removed by hand. About 8 mg of dried eggshells were obtained from 8,000 cysts. They were light brown, with a pearly lustre.

The eggshells contained about 9% of nitrogen. On acid hydrolysis, 60–75% of the material went into solution. The constituent amino acids of the hydrolysed protein were determined, first qualitatively by two-dimensional paper chromatography, and secondly, quantitatively by column chromatography using the Technicon Auto-Analyser. Eighteen amino acids were detected and account for 90% of the nitrogen. Proline was dominant, with glycine, aspartic acid and serine next in importance; these four amino acids made up about 64% by weight of the total amino acids. Aromatic and sulphur-containing amino acids were present in small amounts. This, together with the presence of hydroxyproline, suggested a collagen-like protein in the eggshell. The eggshells of the cyst-nematodes

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have been described as "chitinoid". The eggshells of the potato cyst-nematode gave a positive van Wisselingh colour test for chitin, and glucosamine was detected by paper chromatography after acid hydrolysis. The glucosamine content of the eggshells, determined by the Elson–Morgan colorimetric method, was 8–10%. Total carbohydrate (other than glucosamine) in the eggshells was 6%. Extraction of the eggshells with chloroform–methanol (1 : 1 v/v) showed that they contained 30–50% of lipids. (Clarke in collaboration with Cox and Shepherd, Nematology Department)

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