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The Chemical Composition of the Egg Shells of the Potato Cyst-Nematode, *Heterodera rostochiensis* Woll.

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1. Eggs of the potato cyst-nematode (*Heterodera rostochiensis* Woll.) were isolated by sieving a suspension of crushed cysts. Eggs were broken open by ultrasonic vibration and the egg shells separated from the released larvae by centrifuging in a potassium tartrate density gradient. About 1mg. of dried egg shells was obtained from 1000 cysts. 2. The major constituent of the egg shells was protein (59%, calculated from nitrogen content). About 80% of the egg shells went into solution on acid hydrolysis. Of the 18 amino acids determined with the Technicon Auto-Analyser, proline was most abundant and, with aspartic acid, glycine and serine, made up about 64% by weight of the total amino acids. The small amounts of aromatic and sulphur-containing amino acids, and the presence of hydroxyproline, indicate a collagen-like protein. 3. The egg shells gave a positive van Wisselingh colour test for chitin, and glucosamine was detected in their acid hydrolysate by chromatography. The glucosamine content of the egg shells, determined by the Elson–Morgan colorimetric method, was 7%, corresponding to about 9% chitin. 4. Dried egg shells contained about 7% of lipid, 6% of carbohydrate and 3% of ash. Polyphenols (3% by weight of the egg shells) were detected in the acid hydrolysates. 5. Neither the collagen nor the chitin showed evidence of crystallinity when examined by X-ray diffraction.

Fairbairn (1957, 1960), Rogers (1962) and Van Gundy (1965) summarized present knowledge of the chemical composition of the egg shells and cuticles of nematodes, based mainly on animal-parasitic species, principally *Ascaris* and *Parascaris*. Because of their smaller size, little is known of the corresponding structures of plant-parasitic nematodes apart from recent work on the genus *Meloidogyne* (Bird, 1958; Bird & Rogers, 1965). The adult female or cyst of the potato cyst-nematode (*Heterodera rostochiensis* Woll.) is roughly spherical, averages 0·4mm. in diameter and contains several hundred eggs themselves 100 μ long and 50 μ wide.

Knowledge of the structure of the egg shells of *Heterodera* spp. may shed light on the mechanism of hatching, diapause, resistance to desiccation and the action of nematicides in this genus. Chitwood (1939) noted that the egg shell of *H. marioni* (= *Meloidogyne* sp.; Chitwood, 1949) gave positive tests for chitin, and contained a vitelline membrane with the properties of a sterol. Doliwa (1956) found that egg shells of *H. rostochiensis* contained protein but not chitin, whereas Tracey (1958) found evidence that chitin was present. We now report on the chemical composition of the egg shells of *H. rostochiensis*.

MATERIALS AND METHODS

Preparation of the egg shells. Batches of 8000 cysts were measured by volume (Jones & Gander, 1962). The cysts were crushed on a channelled aluminium slide (Goodey, 1963) with a glass rod, washed into a glass tube with about 10ml. of water and the suspension stirred mechanically for several minutes to release eggs and larvae from the cysts. Cyst walls were separated by retention on a 61 μ sieve (100 meshes/in.). A 37 μ sieve (400 meshes/in.), placed beneath the first, retained the eggs and larvae, which were then washed into a 10ml. tapered centrifuge tube with water, the excess of which was removed after centrifuging. A suspension of the eggs and larvae in 3ml. of water was transferred to a 10ml. polythene tube and vibrated with an MEL 60w, 20kcyc./sec. ultrasonic disintegrator for 10–15 min., keeping the suspension below 40° meanwhile by cooling in an ice bath. This broke open most of the egg shells and released the larvae. The suspension was next washed thoroughly on a 37 μ sieve where the eggs, egg shells and larvae were retained and the smaller particles of debris passed through. The contents of the sieve were washed into a tube with water and centrifuged, the supernatant liquid was removed and the solids were suspended in 0·5ml. of water.

The egg shells and larvae were separated by centrifuging in a potassium tartrate density gradient, prepared by layering six solutions of decreasing density [1g./ml. (1·5ml.), 0·75g./ml. (1·0ml.), 0·625g./ml. (1·0ml.), 0·5g./ml. (1·0ml.), 0·375g./ml. (1·0ml.) and 0·25g./ml. (1·0ml.)] into a 10ml.

tapered glass centrifuge tube. The 0·5 ml. suspension of egg shells and larvae was pipetted on to the uppermost layer of a freshly prepared gradient and immediately centrifuged at 980*g* for 1 min. in an ENA centrifuge (Chas. Hearson and Co. Ltd.). Most of the egg shells settled as a brown band immediately above the bottom layer (sp.gr. about 1·35). A broad band of free larvae separated in the uppermost layers of the gradient, with another band, a mixture of freed larvae and eggs containing larvae, immediately below it. Soil particles and other heavy debris fell to the bottom of the tube. The layers were removed successively from the tube with a pipette. Microscopic examination of a water suspension of the egg-shell layer showed it was almost entirely composed of the transparent egg shells, together with some tiny fragments of cyst wall and some eggs containing decomposed larvae. This material was washed thoroughly with distilled water on a 37 μ sieve and more of the contaminants eliminated by a further centrifuging in a potassium tartrate gradient. The washed egg shells were finally transferred in suspension, a few millilitres at a time, to an 8 cm. watch glass where, by gentle swirling, the remaining cyst-wall fragments and eggs containing whole or decomposed larvae were brought to the centre of the watch glass. The suspended egg shells, which dispersed throughout the surrounding liquid, were removed with a pipette. Two or three repetitions of this operation removed almost all the contaminants. The resulting suspension was centrifuged and the supernatant liquid removed. The egg shells, dried *in vacuo* over P_2O_5, gave a pale-brown mass with a slightly pearly lustre. The colour of the final product was unaffected by heating the cysts at 100° for 10 min. before releasing the eggs. About 8 mg. of egg shells was obtained from 8000 cysts. The total weight of egg shells analysed was about 100 mg.

Hexosamine. Chitin was detected by the method of Tracey (1955). A modified Elson–Morgan colorimetric method (Allison & Smith, 1965) was used to estimate hexosamine in the hydrolysate obtained by treating the egg shells for 6 hr. with 6 N-HCl in sealed tubes at 100°. Hexosamine was estimated from a standard curve obtained with various amounts of glucosamine treated in the same manner as the egg shells. The method of Gardell (1953) was used to identify the hexosamine present in the hydrolysates. The fractions (1·0 ml.) eluted with 0·3 N-HCl from a column (42 in. × 1 cm.) of Dowex 50W (8% cross-linked; dry mesh 200–400) cation-exchange resin, were evaporated to dryness *in vacuo* and used for hexosamine determinations or ninhydrin degradation (Stoffyn & Jeanloz, 1954) or both.

Carbohydrate. Total reducing and non-reducing saccharides (other than hexosamines) in the egg shells were determined after 2 hr. hydrolysis at 100° with 1·5 N-H_2SO_4 by the method of Dubois, Gilles, Hamilton, Rebers & Smith (1951). The intensity of the colour developed after treatment with phenol–water (4:1, w/w) and conc. H_2SO_4, measured at 490 mμ, was compared with that obtained with a range of concentrations of glucose.

Uronic acid. Uronic acid was measured by the method of Dische (1947).

Polyphenols. Polyphenols in egg-shell hydrolysates (6 N-HCl, 24 hr. at 100° in a sealed tube) were determined by the method of Pro (1952), with a modified Folin–Denis reagent.

Total nitrogen. The total nitrogen in the egg shells was estimated by a micro-Kjeldahl procedure (Mann, 1961).

Paper chromatography. Whatman no. 1 paper was used for the chromatograms, which were developed in two dimensions by downward elution with (*a*) butan-1-ol–acetic acid–water (12:3:5, by vol.) and (*b*) phenol–water (4:1, w/w) containing aq. NH_3 (sp.gr. 0·880) (0·5%, v/v). Ninhydrin degradation products of hexosamine (Stoffyn & Jeanloz, 1954) were applied to Whatman no. 1 paper and developed with (*c*) butan-1-ol–ethanol–water (4:1:1, by vol.).

Amino acids. The amounts of amino acids in hydrolysates of the egg shells were measured with a Technicon Auto-Analyser, a one-column chromatographic system adapted from that of Spackman, Stein & Moore (1958), as described by Hamilton (1963). The separation and determination of the ninhydrin-positive substances in the sample was completed in 20 hr. A known amount of norleucine was added to each sample assayed, to act as an internal standard. The concentrations of the individual amino acids were calculated from the areas of the peaks on the recorder chart (Spackman *et al.* 1958). Corrections were made where necessary in accordance with the variations of the internal standard.

Lipid. The lipid content was estimated from the loss of weight of the dried egg shells after two successive extractions for periods of 24 hr. with chloroform–methanol (1:1, v/v) (10 ml.) at room temperature.

Ash. Ash was determined by heating the egg shells at 500° until constant weight was achieved. The elements present in the residue were detected by spectrographic methods.

X-ray diffraction. Egg-shell material was examined in a single-crystal camera with Cu $K\alpha$-radiation.

EXPERIMENTAL AND RESULTS

The quantitative values given in the following sections are the average of at least two concordant determinations.

Protein and amino acids. The dried egg shells contained 9·5% of nitrogen. Egg shells were heated with 6 N-hydrochloric acid in a sealed tube at 100° for 6, 12 or 24 hr., when the hydrolysates were filtered and dried *in vacuo* over potassium hydroxide. After 6 hr. hydrolysis, 65% by wt. of the egg shells went into solution, and another 15% dissolved during further hydrolysis. The 20% remaining insoluble after hydrolysis for 24 hr. contained about 1·3% of nitrogen.

Column chromatography. The egg-shell hydrolysates were dissolved in 0·1 mM solution of norleucine in 0·1 N-hydrochloric acid before they were added to the column of the Technicon Auto-Analyser, in 1·0 ml. samples containing about 400 μg. of hydrolysate and 13·1 μg. of norleucine. When the elution was completed, the recorded graph was compared with that obtained with a known mixture of amino acids. The two graphs were standardized in terms of the known norleucine content of each mixture. Table 1 lists the amounts of amino acids detected in the 6 hr. and 24 hr. hydrolysates.

The nitrogen content of the 1·0 ml. sample of

Table 1. *Amino acid composition of hydrolysates of the egg shells of* H. rostochiensis

Total amino acids, 60% by weight; total protein [calculated from N (Kjeldahl) × 6·25], 59%.

Amino acid	Amino acid composition (% by wt. of total amino acids) 6hr. hydrolysis	24hr. hydrolysis
Pro	34·2	38·3
Asp	11·4	10·9
Gly	9·8	8·5
Ser	8·3	7·4
Glu	5·2	6·0
Hyp	4·7	5·2
Lys	3·5	2·8
Tyr	3·1	2·1
Ala	2·8	2·3
CyS	2·6	—
Arg	2·2	1·7
Leu	1·9	1·6
Thr	1·9	2·7
Phe	1·8	1·5
His	1·7	2·3
Val	1·5	1·9
NH_3	1·4	0·8
Ile	1·3	1·4
Met	0·6	2·6

hydrolysate calculated from the amounts of the compounds eluted from the column was 33μg., identical with the content of the same hydrolysate determined by the micro-Kjeldahl method, so that all the nitrogen was recovered from the column.

Paper chromatography. Paper chromatography was used to confirm the identity of the amino acids determined quantitatively with the Technicon Auto-Analyser (Table 1), and to confirm the presence of glucosamine. The hydrolysate from about 0·2mg. of egg shells was dissolved in a little water and applied as a single spot to a sheet of Whatman no. 1 paper. The chromatogram was developed in two dimensions by descending flow with the solvent mixtures (*a*) and (*b*). The paper was sprayed with 0·2% ninhydrin in acetone, heated for a few minutes at 105°, and compared with a chromatogram of known amino acids run under identical conditions.

Confirmatory tests were made for several of the amino acids. Developed chromatograms were dipped in 0·2% (w/v) isatin in acetone, and heated at 105° for 3min. Proline was detected as a deep-blue spot and hydroxyproline as a weaker bright-blue spot. Glutamic acid also gave a bluish spot with the same reagent. Glycine was confirmed by a grey-green spot found after treating the chromatogram with 0·2% *o*-phthalaldehyde in acetone, followed by ethanolic potassium hydroxide (Smith, 1958). Ninhydrin–cupric nitrate reagent (Moffat & Lytle, 1959) was used to identify other amino acids.

Hexosamine. Glucosamine was identified in egg-shell hydrolysates by paper chromatography (see above) and with the Technicon Auto-Analyser, and its presence confirmed by column chromatography by the method of Gardell (1953). A single hexosamine band was obtained at the appropriate elution volume for glucosamine. In other tests, the hexosamine-containing fractions from the column were combined and evaporated to dryness, and the hexosamine present converted into the corresponding pentose by oxidative degradation with ninhydrin (Stoffyn & Jeanloz, 1954). After removal of the bulk of the solvents, the reaction products were applied to Whatman no. 1 paper and the chromatogram was developed with solvent system (*c*). Treatment of the paper with silver nitrate–alkali showed that a major product of the ninhydrin degradation had R_F 0·20. Arabinose, and an authentic sample of glucosamine similarly treated with ninhydrin, both gave comparable spots with R_F 0·20. The dried egg shells contained 7% of glucosamine. Hydrolysed larval cuticle and cyst walls of *H. rostochiensis* contained little hexosamine (about 1%).

Chitin. The egg shells but not the larval cuticle or the cyst wall of *H. rostochiensis* reacted positively with the van Wisselingh colour test (Tracey, 1955).

Carbohydrate and uronic acid. The total reducing and non-reducing sugars in hydrolysed egg shells was 5·4% (calculated as glucose). Further hydrolysis of the insoluble material with 6N-sulphuric acid for 6hr. at 100° yielded an additional 0·8% of sugar. Uronic acids were not detected in the acid hydrolysates.

Polyphenols. Dried egg-shell hydrolysates were dissolved in water and the polyphenol content of the solution was determined by comparison with a standard solution of catechol. The egg shells contained 3% of polyphenol (calculated as percentage catechol content).

Lipid. Dried egg shells extracted with chloroform–methanol (1:1, v/v) lost 7% of their weight. Spots of the concentrated extracts on filter paper were stained blue-black by Sudan Black (Gurr, 1958). The colour persisted after washing with aqueous ethanol.

Ash. The dried egg shells gave an ash of 3% of their initial weight. Spectrographic analysis showed the presence of the following elements: calcium, titanium (considerable amounts); aluminium, copper, magnesium, silicon (moderate amounts); iron, manganese (traces). The titanium was probably a contamination from the titanium probe of the ultrasonic disintegrator.

Electron microscopy. In the electron microscope, the teased edges of pieces of egg shell showed a fibrillar structure.

X-ray diffraction. Egg-shell material was examined for evidence of crystallinity in the structure of (*a*) the protein component and (*b*) the chitin component.

(*a*) Attempts to orientate the protein by pressing the egg shells failed; pressed and unpressed specimens gave identical X-ray diffraction patterns. The material gave two very broad haloes, $d = 7$–13 Å and $d = 4$–5 Å, showing that it was largely amorphous. Extraction of the egg shells with chloroform–methanol also left the pattern unchanged.. In similar conditions, fibrous bovine collagen gave the 11·6 Å equatorial reflexion, and the 2·88 Å meridional reflexion characteristic of collagen, together with other weaker reflexions reported for collagen.

(*b*) The small, flocculent, white precipitate, left after boiling egg shells with 5% potassium hydroxide for 24hr. (Rudall, 1955) and repeated washing with cold water, gave a positive van Wisselingh test. Its X-ray diffraction pattern showed two diffuse haloes similar in position to those of the untreated egg shells but narrower and weaker. The material seemed to be largely amorphous, in contrast with chitosan obtained from an authentic sample of chitin, which showed a good ring pattern typical of polycrystalline material.

DISCUSSION

Knowledge of the chemical composition of the egg shells and cuticles of nematodes has been derived principally from *Ascaris* spp. and other species parasitizing animals. The general concept of the nematode egg shell is of a membrane built of layers, an outer layer of chitin, or quinone-tanned protein, or both, and an inner layer of lipid (Fairbairn, 1960; Rogers, 1962; Lee, 1965; Van Gundy, 1965). Kreuzer (1953), for example, found that the egg membrane of *Ascaris* has three layers, the outer one of protein, the middle of chitin and the inner of lipid. Our work shows that egg shells of *H. rostochiensis* also contain the chemical components corresponding to these three layers..

The composition of the *H. rostochiensis* egg shells was: protein (nitrogen content ×6·25), 59%; non-hydrolysed material, 20%; chitin, 9%; lipid, 7%; carbohydrate, 6%; polyphenols, 3%; ash, 3%. The relatively large amount of protein in *H. rostochiensis* egg shells was unexpected, as chitin had been thought to be the principal component (Chitwood, 1951). Of 18 amino acids detected, proline was most plentiful and, with aspartic acid, glycine and serine, made up about 64% by wt. of the total amino acids. A significant amount of hydroxyproline was eluted from the column immediately before aspartic acid. The *trans*-4-hydroxy isomer, which is usual in collagens, is eluted at this stage. Other hydroxyprolines and hydroxylysine were not detected, and there were only small amounts of aromatic and sulphur-containing amino acids.

The amino acid composition suggests the egg shell contains a collagen-like protein, and these proteins have the tough, resilient qualities associated with *H. rostochiensis* egg shells. Collagens have been defined as fibrous proteins with a distinctive amino acid composition and giving a characteristic X-ray diffraction pattern. *H. rostochiensis* egg shells did not give this pattern, although electron micrographs of the shells showed the presence of fibrils. The failure to obtain the diffraction pattern suggests that the sample has a less organized structure than is usual with collagens. Other invertebrate collagens investigated, including *Ascaris* cuticle (Fauré-Fremiet & Garrault, 1944; Picken, Pryor & Swann, 1947; Watson & Silvester, 1959), had fibrillar structure and gave wide-angle X-ray diffraction patterns comparable with those obtained with vertebrate collagen. The composition of *Ascaris* egg shells has not been determined, but paper chromatography of hydrolysates showed the presence of all the amino acids obtained from *H. rostochiensis* egg shells, including hydroxyproline (Kreuzer, 1953; Jaskoski, 1962). Quantitative amino acid analyses on the hydrolysates of *Ascaris* cuticle (Bird, 1957; Watson & Silvester, 1959) showed proline to be the most abundant, with glycine, arginine and lysine next in amount; together they accounted for about 62% of the amino acid nitrogen, and there was also 1·58% of hydroxyproline. Collagen-like proteins occur in the cuticle of other nematode species, e.g. *Toxocara mystex*, *Strongylus equinus* (Bird, 1956) and *Aspicularis tetraptera* (Anya, 1966), and the identification of a collagen as the major constituent of *H. rostochiensis* egg shells extends the range of structures and nematode species in which this protein is known to be a structural material.

Some protein in nematode cuticle and egg shell is probably tanned. The dried egg shells of *H. rostochiensis* were light-brown, suggesting tanning, and the colour was unaffected by heating the cysts before isolating the egg shells. The egg-shell hydrolysates contained only very small amounts of the aromatic and, more particularly, the hydroxy aromatic amino acids, and the shells contained polyphenols customarily associated with tanning processes. The considerable amount of humin, i.e. material insoluble after acid hydrolysis (about 20% of the dry weight of the egg shells), probably includes material derived from the tanned protein. The egg shells were not appreciably changed by boiling in water for 24hr., which also suggests that the collagen fibres are firmly bound.

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REFERENCES

Allison, D. J. & Smith, Q. T. (1965). *Analyt. Biochem.* **13**, 510.
Anya, A. O. (1966). *Parasitology*, **56**, 179.
Bird, A. F. (1956). *Exp. Parasit.* **5**, 350.
Bird, A. F. (1957). *Exp. Parasit.* **6**, 383.
Bird, A. F. (1958). *Nematologica*, **3**, 205.
Bird, A. F. & Rogers, G. E. (1965). *Nematologica*, **11**, 224.
Chitwood, B. G. (1939). *Proc. helm. Soc. Wash.* **5**, 68.
Chitwood, B. G. (1949). *Proc. helm. Soc. Wash.* **16**, 90.
Chitwood, B. G. (1951). *Circ. U.S. Dep. Agric.* no. 875, p. 17.
Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
Doliwa, U. (1956). *Wiss. Z. Univ. Rostock*, **5**, 133.
Dubois, M., Gilles, K., Hamilton, J. K., Rebers, P. A. & Smith, F. (1951). *Nature, Lond.*, **168**, 167.
Fairbairn, D. (1957). *Exp. Parasit.* **6**, 491.
Fairbairn, D. (1960). In *Nematology*, p. 267. Ed. by Sasser, J. N. & Jenkins, W. R. Chapel Hill: University of North Carolina Press.
Fauré-Fremiet, E. & Garrault, H. (1944). *Bull. biol.* **78**, 206.
Gardell, S. (1953). *Acta chem. scand.* **7**, 207.
Goodey, J. B. (1963). *Tech. Bull. Minist. Agric. Fish. Fd. no. 2: Laboratory Methods for Work with Plant and Soil Nematodes*, 4th ed., p. 16. London: Her Majesty's Stationery Office.
Gurr, E. (1958). *Methods of Analytical Histology and Histochemistry*, p. 122. London: Leonard Hill Ltd.
Hamilton, P. B. (1963). *Analyt. Chem.* **35**, 2055.
Jaskoski, B. J. (1962). *Exp. Parasit.* **12**, 19.
Jones, F. G. W. & Gander, M. C. (1962). *Nematologica*, **8**, 39.
Kreuzer, L. (1953). *Z. vergl. Physiol.* **35**, 13.
Lee, D. L. (1965). *The Physiology of Nematodes*, p. 81. Edinburgh: Oliver and Boyd.
Mann, P. J. G. (1961). *Biochem. J.* **79**, 623.
Moffat, E. D. & Lytle, R. I. (1959). *Analyt. Chem.* **31**, 926.
Picken, L. E. R., Pryor, M. G. M. & Swann, M. M. (1947). *Nature, Lond.*, **159**, 434.
Pro, M. J. (1952). *J. Ass. off. agric. Chem. Wash.*, **35**, 255.
Rogers, W. P. (1962). In *Theoretical and Experimental Biology vol. 2: The Nature of Parasitism.* Ed. by Danielli, J. F. New York: Academic Press Inc.
Rudall, K. M. (1955). *Symp. Soc. exp. Biol.* **9**, 49.
Smith, I. (1958). In *Chromatographic Techniques*, p. 59. Ed. by Smith, I. London: W. Heinemann (Medical Books) Ltd.
Spackman, D. H., Stein, W. H. & Moore, S. (1958). *Analyt. Chem.* **30**, 1190.
Stoffyn, P. J. & Jeanloz, R. W. (1954). *Arch. Biochem. Biophys.* **52**, 373.
Tracey, M. V. (1955). In *Modern Methods of Plant Analysis*, vol. 2, p. 264. Ed. by Paech, K. & Tracey, M. V. Berlin: Springer-Verlag.
Tracey, M. V. (1958). *Nematologica*, **3**, 179.
Van Gundy, S. D. (1965). *Annu. Rev. Phytopath.* **3**, 43.
Watson, M. R. & Silvester, N. R. (1959). *Biochem. J.* **71**, 578.