The Cyst Wall of *Colpoda steinii*

A SUBSTANCE RICH IN GLUTAMIC ACID RESIDUES

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1. The cyst wall of *Colpoda steinii* has been isolated and its chemical nature examined. It had a nitrogen content $13.9 \pm 0.2\%$ (S.D.) and an ash $8.6 \pm 1.6\%$ (S.D.). After lipid and hot-acid extraction there was a variable residual phosphorus of 0.19–0.64%. The protein nature, indicated by infrared and ultraviolet absorption, was confirmed when 100 μ g. of hydrolysed wall gave a ninhydrin colour equivalent to that given by 0.88–1.01 μ moles of glycine. Hexosamine, hexose, pentose, lipid and dipicolinic acid were absent. 2. Paper chromatography of hydrolysates, besides showing the presence of the usual protein amino acids and three unidentified ninhydrin-reacting spots, indicated the presence of large amounts of glutamic acid. Estimated by chromatography, the amount present was 52.9 ± 0.6 (S.D.) g./100g. of ash-free wall; manometric estimation of L-glutamic acid with L-glutamate 1-carboxy-lyase gave 46.5 ± 0.9 (S.D.) g./100g. 3. Free carboxyl groups were estimated by titration as 0.159 ± 0.011 (S.D.) mole/100g. and those present as amide as 0.154 ± 0.004 (S.D.) mole/100g., and the total was compared with the dicarboxylic acid content 0.360 ± 0.010 (S.D.) mole/100g. 4. After treatment with 98% formic acid 25–30% of the wall material could be extracted by 0.05 M-sodium carbonate solution (extract 1); after treatment of the residue with performic acid a further 62–63% based on the original weight could be extracted by 0.05 M-sodium carbonate (extract 2). 5. The average values found for the glutamic acid contents were 21.7g./100g. for extract 1 and 58.0g./100g. for extract 2. The cysteine acid content of whole oxidized wall was about 5.8g./100g. and of extract 2 also about 5.8g./100g. The glutamic acid and cysteine acid contents of the final residue were also investigated. 6. The significance of these extraction experiments in relation to the wall structure is discussed.

The various species of the free-living protozoan *Colpoda* may be stimulated by unfavourable changes in environment to produce resistant encysted forms. The most important of these changes is the shortage of food (Taylor & Strickland, 1938) and cells will become rounded, absorb their surface structures and begin the secretion of a thick protective wall within a few minutes of washing away the culture fluid. Many other Protozoa show a similar behaviour and the dramatic change in morphology and metabolism accompanying the encystment process may form a useful model in a study of cellular differentiation (Trager, 1963).

Older work on the nature of the cyst walls of free-living Protozoa (Stein, 1854; Fabré, 1885; Goodey, 1913) stressed the possible presence of carbohydrate materials, as did the observations of Sebestyén (1935) on *Diplopsalis acuta*. More recently cellulose has been identified as a component of the wall of *Acanthamoeba* sp. (Tomlinson & Jones, 1962) and

chitin has been claimed to be present in the cyst membranes of *Pelomyxa illinoisensis* (Sachs, 1956).

This paper describes the growing in substantial quantities of the organism *Colpoda steinii* and the isolation and chemical investigation of wall preparations from the encysted form of this organism. Short accounts of some aspects of the work have already been published (Tibbs, 1964, 1965).

METHODS

Isolation of Colpoda steinii. *C. steinii* was isolated from dry pond grass which had been exposed to air as the result of drought. This grass was immersed in water in a Petri dish and left for 1–2 hr., when excystment of *Colpoda* occurred, the larger forms being easily distinguishable under the dissecting microscope from the smaller *C. steinii* and *C. aspera* (Burt, 1940). One of the smaller forms was picked out with a Pasteur pipette and washed by transferring it through several drops of water in cavities on microscope slides. The organism was then placed in a drop of *Cloaca*

cloacae suspension and kept in a humid atmosphere at room temperature. After a period of rapid multiplication encystment took place on the third to fourth days. The cysts adhered to the glass surface and the supernatant was removed and replaced by a drop of hay infusion. Within less than 2 hr. the drop was rich in motile forms of *Colpoda*.

The organisms in this drop were used for species identification and for further subculturing and all the work described in this paper has been carried out on descendants of one cell isolated as described. A detailed morphological analysis has not been carried out but the organism averaged about 30μ in length as indicated by Burt (1940) for *C. steinii*, and possessed the 'beard' of pseudomembranelles together with the long caudal cilia which are characteristic and unique for this species.

Culture of *Colpoda steinii*. The organism was grown in quantity by using *Cloaca cloacae* as food in the following manner. Ten litres of a solution of 0.2% Difco Bacto-Tryptone and 0.2% Difco yeast extract were distributed amongst a number of litre conical flasks so that each flask contained approximately 300 ml. of medium. After stoppering and autoclaving the flasks were inoculated with *Cloaca cloacae* (Challoner P11, N.C.T.C. 9238) and incubated for 24 hr. at 37°. The bacteria were then harvested by using a Sharples continuous centrifuge and washed by following the medium through the centrifuge with 2 l. of water. The concentrated suspension was filtered through coarse sintered glass to remove cotton-wool fibres etc. and made up to 2 l. with water. Suspensions so obtained contained 1.2–1.8 mg. dry wt. of bacteria/ml. and this concentration was optimum for subsequent growth of *C. steinii*. If the bacteria were suspended in a larger volume of water the subsequent yield of *Colpoda* was no greater and handling difficulties were increased. If the volume of the final suspension was made considerably less than this, growth was not rapid and final yields were reduced.

The suspension was now inoculated with a washed motile preparation of *C. steinii* obtained by decanting the supernatant fluid from an encysted culture and treating this with hay infusion. With cultures aged about 2 weeks excystment occurred in 1½–2 hr. at 25°. With older cultures or cultures which had been allowed to dry out excystment took longer.

The suspension was now spread on flat-bottomed Pyrex dishes; the depth of liquid in each dish was kept to a minimum (about 1 mm.), for growth in deeper layers was slow, encystment uncertain and yields were poor. The dishes were made level and kept at 25°. *C. steinii* multiplied rapidly and on the third to fourth days after inoculation most of the cells encysted fairly synchronously within an hour or so. The trays were kept a further 7 days to allow the encystment process to reach completion. The growth temperature was not critical.

Preparation of cyst wall. The contents of the dishes were now harvested, and centrifuged at about 1000g for 5 min. to spin down the cysts. These cysts were transferred to two 20 ml. tubes of a Mickle high-speed shaker and shaken with an equal volume of grade 4 Ballotini beads (approx. 1.0 mm. diam.) in a Mickle shaker for 15 min. The contents were then examined under phase contrast to make sure that cell rupture was complete. Occasionally the organic matter in the tubes was more concentrated and more viscous than usual and a longer period of shaking was necessary. Excessive shaking was inadvisable because powdered glass was produced and the wall itself became fragmented. The

size of bead was important and other sizes from 0.1 to 2.0 mm. diam. were less effective.

The homogenate was filtered through grade 2 sintered glass (which retained the beads but allowed the wall material to pass through) and the filtrate spun at 500g for 5 min. The supernatant was discarded and the residue, consisting mainly of wall material, was washed several times by centrifugation until the supernatants were clear. The residue, which consisted of wall material to which a certain amount of particulate matter adhered, was suspended in a small volume of water and dispersed by a 10 sec. treatment with the probe of a 13 kcy. ultrasonic oscillator (Ultrasonics Ltd., Otley, Yorks.). This short treatment dislodged much of the small particulate matter but did not further rupture the walls. The walls were again spun down at 500g for 5 min. and washed several times with water. The final suspension (Plate 1b) was freeze-dried for storage. Yields were 30–40 mg.

Maintenance of species. It was found that if sucrose solution (final concn. 0.25–0.75M) was added to a cyst suspension in water the suspension could be frozen at –10° and the cells remained viable for many months. In the absence of sucrose, after a few days at –10° only a small proportion of cells was still viable. Repeated thawing and freezing of cultures stored with sucrose caused no obvious reduction in viability, which was tested by thawing, removing the supernatant by centrifugation and spreading the cysts with hay infusion in a Petri dish. With a preparation of viable cysts the dish, after an hour or two, would be teeming with motile organisms.

Analyses. Some ash contents and elementary analyses were carried out on the material dried for 2 hr. at 105° by Weiler and Strauss, Oxford. For analyses in the laboratory preliminary drying for 2 hr. at 105° was also employed. The ash content was taken as the residue obtained after heating at 550° for 2 hr. Total nitrogen was estimated by the micro-Kjeldahl method and phosphorus by the Allen (1940) procedure.

For the detection of hexosamine in the cyst wall an acid digest (16 hr. at 105° in 6N-HCl) was dried *in vacuo* and an amount equivalent to 1 mg. applied as a spot to a strip of Whatman no. 1 filter paper. The chromatogram was developed in propan-2-ol-water (4:1, v/v), dried, and sprayed with the hexosamine (Elson-Morgan) reagent (Smith, 1960, p. 252). An amount of glucosamine equal to 2% by wt. of the cyst wall was added to a further sample of wall which was hydrolysed and chromatographed as just described. There was no difficulty in detecting glucosamine on this control chromatogram.

With glucose as standard, hexose was determined by the anthrone reaction (Trevelyan & Harrison, 1952). A 10 min. heating period with the reagent was used (Yemm & Willis, 1954) and during this the cyst wall was dissolved. With xylose as standard the reaction with orcinol (Albaum & Umbreit, 1947) was used to detect pentose. Lipid was estimated by measuring the loss in weight on extracting wall (10–20 mg.) at room temperature once with ethanol, twice with ethanol-chloroform (3:1, v/v), once with ethanol-diethyl ether (3:1, v/v) and once with diethyl ether.

The amount of material dissolving in two 10 min. extractions at room temperature with 0.5N-perchloric acid was also determined. These extracts were made up to a known volume and the ultraviolet-absorption curve was plotted. Wall preparations were also tested for the spore compound

Plate 1. (a) Photomicrograph of partially ruptured *Colpoda steinii* cysts as seen in phase contrast. Cells were mounted in culture fluid and broken by applying pressure to the coverslip. On the left-hand side are three cells, one of which is highly swollen, in which the inner wall has been fractured but the cytoplasm has been retained by an external membrane. On the right-hand side are cells in which both the inner wall and external membrane have been fractured and from which the cytoplasm has been partially expelled. ($\times 800$) (b) Photomicrograph of *C. steinii* wall preparation in water as seen in phase contrast. The thick inner layer of the wall complex is the major component. ($\times 800$).

dipicolinic acid by measuring the absorption of a hot aqueous extract (Powell, 1953). The ultraviolet absorption of the cyst wall itself at high and low pH values was recorded as follows. Wall (10 mg.) was extracted twice with 0.5N-perchloric acid at 70° for 20 min. to remove nucleotide and nucleic acid absorbing material and then treated with 5 ml. of N-NaOH for 20 hr. at 37°. Undissolved material was removed by centrifugation at 15000g for 10 min. and discarded. This was dark-coloured and gritty, had a negligible nitrogen content and was assumed to consist of contaminating inorganic matter. Portions of the supernatant were taken for nitrogen estimation and for dilution with water or acid to give solutions, the pH values and ultraviolet-absorption curves of which were measured.

Paper chromatography was used to detect amino acids. Cyst walls and cyst-wall fractions were hydrolysed by boiling with 6N-HCl for 24 hr. Humin was removed and after evaporating to dryness under partial vacuum hydrolysates were dissolved in water for chromatography. For two-dimensional work an amount of hydrolysate corresponding to 100–150 µg. of wall was used. Chromatography at 25° was by the ascending method and with a frame of the type described by Datta, Dent & Harris (1950). Solvents employed were butan-1-ol-acetic acid and phenol-NH₃. They were used in that order and made up as described by Smith (1960, p. 83). After drying chromatograms were sprayed with ninhydrin as described by Moffat & Lytle (1959). Desalting before chromatography was not necessary. Unidentified ninhydrin-reacting spots were further investigated by the pyridoxal transamination method of Kalyankar & Snell (1957).

Several amino acids (cysteic acid, aspartic acid, glutamic acid and glycine) were estimated by running one-dimensional descending chromatograms in phenol-NH₃. Loading of the chromatograms was carried out so that the acids to be estimated were present in amounts of 3–10 µg. and standards were run on the same sheet. After 24 hr. development at 25° phenol was removed and amino acids were estimated according to Kay, Harris & Entenmann (1956).

To determine the optical configuration of amino acids present in the cyst-wall hydrolysate the paper-chromatography method described by Boulanger & Osteux (1963) was used. The D-amino acid oxidase (EC 1.4.3.3) was a hog-kidney preparation from Sigma Chemical Co. and the catalase (EC 1.11.1.6) was from British Drug Houses Ltd. The same procedure was also used with an L-amino acid oxidase preparation (EC 1.4.3.2) (from snake venom, supplied by Sigma Chemical Co.). L-Glutamic acid was determined manometrically in hydrolysates with glutamate decarboxylase (EC 4.1.1.15) (Gale, 1963). The enzyme preparation was a crude acetone-dried *Escherichia coli* powder obtained from Sigma Chemical Co.

Ninhydrin reactions in solution were performed by the Moore & Stein (1948) procedure. Amide nitrogen was estimated by heating 5 mg. samples with 2N-HCl in sealed tubes at 100° for 1–6 hr. The tubes were opened and HCl was removed *in vacuo* over P₂O₅ and NaOH. The NH₃ in each tube was estimated by the micro-diffusion technique (Conway, 1957) and the values were extrapolated to zero time to find the true amide nitrogen.

Each point on the titration curve was obtained as follows. A known amount of wall (approx. 10 mg.) was dispersed in 2 ml. of M-KCl and a known volume of 0.05N-HCl or 0.05N-KOH (both in M-KCl) added. Nitrogen was used to

exclude CO₂ when the final pH was greater than 5. The tube containing the wall was sealed and incubated for 2 days at 30° and, after an accurate estimate of the volume of the liquid phase had been obtained by weighing, was centrifuged. A portion (1 ml.) of the supernatant was taken for titration of the free acid if the final pH was less than 4; between pH 4 and 10 the free acid was calculated from the pH of the supernatant. Reversibility was verified by adding that volume of titrant calculated to bring the system back to the original pH and 2 days at 30° was allowed for this equilibrium to be established.

Oxidation and extraction of wall preparations. Oxidation with performic acid was carried out as described by Thompson & O'Donnell (1959) for 16 hr. at 0°. The freeze-dried material was extracted for 48 hr. at 37° with 0.05M-Na₂CO₃ (1 ml. to every 2 mg. of material). A drop of toluene was included in the suspension to reduce any bacterial contamination. The extract was removed by centrifuging at 50000g for 30 min. and the residue washed with water. Salt was removed from the combined extract and washings by dialysis and the extract and insoluble residue were freeze-dried. From two such experiments the diffusate was freeze-dried, refluxed with 6N-HCl for 24 hr. and, after removal of the acid *in vacuo*, made up to a known volume and the reaction with ninhydrin investigated (Moore & Stein, 1948). Individual amino acids present in the hydrolysed diffusate were detected, after desalting in an electrolytic desalter, by the chromatographic procedure described above.

RESULTS

Disruption of cysts. The cyst wall of *Colpoda* consists of more than one layer. In intact cysts, but especially in partially disrupted suspensions, it is possible to see a smooth rigid wall within a thin membrane. The inner wall is fractured more easily than the outer membrane and a half-way stage in cyst rupture can frequently be seen, where the inner wall has been broken and allows cytoplasm to pass through, but the external membrane is still intact and retains the cytoplasm. When cysts are crushed under a coverslip both these layers may be broken and although the cytoplasm is released into the external medium it is not always immediately dispersed but often remains coherent with a smooth external surface. Presumably yet another membrane is involved. All these points may be seen in Plate 1(a). Plate 1(b) shows that the smooth rigid and comparatively thick component forms a major part of the final preparation, whereas the origin of some thinner and less rigid material that is also present is less certain.

The final preparation contained a small amount of particulate matter which was not removed by further washing with water, buffers or salt solutions.

General nature of cyst-wall material. The percentages of ash, carbon and hydrogen as determined on five different preparations by Weiler and Strauss were 8.6 ± 1.6 (S.D.), 42.1 ± 1.1 (S.D.) and 6.3 ± 0.2

(s.d.) respectively. The nitrogen content determined in this Laboratory on six batches of material was 13.9 ± 0.2 (s.d.). Hexosamine, if present, was less than 2% and determinations of hexose on four batches of wall gave values ranging from 1.8 to 3.0%. Similarly the pentose contents of four batches were between 0.9 and 1.5%. These small figures have doubtful significance and therefore hexosamine, hexose and pentose are essentially absent from the walls of *Colpoda* cysts. Lipid also appeared to be absent. Up to 6% loss in weight occurred during extraction of four batches of wall with cold acid. This extract absorbed in the ultraviolet with a maximum at $255m\mu$ and presumably was nucleotide. Estimated as adenylic acid and by using a molar extinction coefficient 15.0×10^3 (Bock, Ling, Morell & Lipton, 1956), the amount varied in four different batches but was always less than 0.7%. Hot-water extracts of wall (5mg.) showed some ultraviolet absorption with maxima again at $255m\mu$ with no suggestion of a peak at about $270m\mu$; this excludes the presence of the bacterial spore compound dipicolinic acid (Powell, 1953).

Eleven batches of material contained 1.0–1.5% of phosphorus. Although much of this was extracted by treatment with lipid solvents and hot 0.5N-perchloric acid some was left behind. The amount remaining was variable and, based on the original weight of unextracted wall, was 0.2–0.6%.

Hydrolysates of three batches of cyst wall (100 μ g. quantities, corrected for ash) gave colours with ninhydrin equivalent to 0.96, 0.88 and 1.01 μ moles of glycine. The ultraviolet-absorption curves measured at pH 0.6 and 12.7 on a batch of wall freed from nucleotide or nucleic acid absorbing material showed the profile of protein containing both tyrosine and tryptophan. The method of Beaven & Holiday (1952) could not be used to calculate with any confidence the amounts of these two amino acids, for when the two curves were corrected as suggested by these authors to take into account any non-specific absorption they crossed at $288m\mu$ and not between 277 and $281m\mu$ as would be expected for a normal protein. This, together with the observation that the curve in alkali showed what appeared to be an exceptionally high absorption at the longer wavelengths around $320m\mu$, suggests the presence of some other absorbing material and makes a calculation of the tyrosine and tryptophan content valueless. The infrared-absorption curves of two wall preparations made up as Nujol mulls were recorded with a Unicam SP.200 spectrophotometer and showed strong absorption at about 3300, 1660 and 1550cm^{-1} . These absorptions are typical of a protein (Fraser, 1953).

Dr K. M. Rudall of the Astbury Department of

Biophysics in the University of Leeds examined the X-ray powder diagram of a wall preparation and found that because of the presence of strong diffractions arising from inorganic material it was not possible to define with certainty any characteristic diffractions from the organic material. A wall preparation with a low ash content of 6.4% (not sulphated) was examined qualitatively by the Department of Spectrochemistry, Macaulay Institute for Soil Research, Aberdeen, and 25 elements were detected in the ash. Silicon and magnesium were possibly the main constituents but copper, lead, tin and zinc were also present in relatively large amounts. Such elements as calcium, potassium and sodium made only a minor contribution.

Ninhydrin-reacting substances present in hydrolysates. Chromatograms of cyst-wall hydrolysates showed considerable quantities of glutamic acid. Also present were aspartic acid, glycine, serine, threonine, alanine, tyrosine, lysine, arginine, histidine, valine, phenylalanine, leucine and proline. Three other unidentified ninhydrin-reacting spots were detected and the pyridoxal method of Kalyankar & Snell (1957) indicated that these spots were not due to α -amino acids.

The glutamic acid content of five preparations was 52.9 ± 0.6 (s.d.) g. from 100g. of ash-free wall. This value is high, for with the solvent system used (phenol-ammonia) glutamic acid would not have separated from one of the unknown ninhydrin-reacting spots. This spot was not strong and the error introduced is not serious.

A D-amino acid oxidase preparation when sprayed on to a chromatogram had no effect on a D-glutamic acid control spot, as judged by the lack of fluorescence on spraying with *o*-phenylenediamine in trichloroacetic acid but attacked D-alanine without difficulty. The enzyme had no detectable effect on a chromatogram of a cyst-wall hydrolysate. The L-amino acid oxidase preparation attacked both L-alanine and L-glutamic acid and after treating a chromatogram with this enzyme all major amino acid spots fluoresced when sprayed as before. It was concluded that much of the glutamic acid was in the L-configuration and that most likely all the other amino acids were completely in the L-form. Manometric estimation of the L-glutamic acid gave, for three batches of wall, 45.2, 46.6 and 47.7 g. from 100g. of ash-free wall. The corresponding figures for glutamic acid, obtained by chromatography, were 51.8, 53.5, and 52.8. The higher values may result from the contamination of the glutamic acid spot already mentioned and a small degree of racemization taking place during hydrolysis.

There was no difference in the apparent amide content when the period of heating in 2N-hydrochloric acid varied from 1 to 6hr. Five determina-

Fig. 1. Titration curve of cyst wall carried out in M -KCl. The zero on the bound proton scale has arbitrarily been set to pH 6. See the text for experimental details.

tions gave 2.16 ± 0.05 (s.d.) as the percentage of amide nitrogen in the ash-free wall. This value is equivalent to 0.154 ± 0.004 mole of amide/100 g. of material.

The titration curve is shown in Fig. 1; the substantial scatter of the points is due in part to the small quantities of material and volumes used. With the zero set at pH 6 the protons bound at pH 2 and below under conditions of high ionic strength are equivalent to the free carboxyl groups (Cannan, 1942). Thirteen points lying at or below pH 2 gave as their mean value 0.159 ± 0.011 (s.d.) mole of bound proton/100 g. of ash-free wall.

Solubilization and fractionation of wall. Phase contrast was used to examine the effect on the wall of a variety of reagents. At room temperature N -hydrochloric acid, N -sodium hydroxide, $8M$ -urea, $0.1M$ -EDTA, pH 7.9, followed by $8M$ -urea, cupriethylene diamine (Coleman & Howitt, 1947), lithium isothiocyanate (50%, w/v) (Signer & Strässle, 1947), m -cresol and dichloroacetic acid, were without apparent action even after several days. Formic acid (98%) had some effect and the outlines of the walls became less well defined. At a concentration 1 mg./ml. in $0.05M$ -phosphate, pH 7.4, the enzyme from *Streptomyces griseus* (Pronase)

obtained from Sigma Chemical Co. as their type V purified grade, and pancreatin supplied by the British Drug Houses Ltd., were without obvious effect after 1 week at 37° .

After oxidation with peracetic acid (Alexander & Earland, 1950) or performic acid (Thompson & O'Donnell, 1959) the wall was no longer inert; even water dissolved a considerable amount of material and after 48 hr. only thin membranes remained. A higher pH seemed to be even more effective in dissolving the oxidized wall. Conditions suitable for the complete oxidation of cystine in another insoluble protein, wool keratin (Thompson & O'Donnell, 1959), were chosen for the routine oxidation of wall and extraction was carried out at 37° by using $0.05M$ -sodium carbonate. Five extractions of different batches gave, on an ash-free basis, $23 \pm 3\%$ (s.d.) as the proportion undissolved.

During these experiments the weight of extract recovered plus the weight of residue was about 10% less than the original weight of material taken. Some loss was due to handling but some material might also have escaped through the bag during dialysis of the extract. From two fractionation experiments the diffusates after concentration and hydrolysis contained ninhydrin-reacting material giving a colour equivalent to 3.4 and 2.5% by wt. of the original wall extracted. After removal of salt these hydrolysates were chromatographed and contained at least 13 of the common amino acids.

Since 98% formic acid appeared to dissolve some wall material and since the performic acid used for oxidation contained excess of formic acid, in making much of the wall soluble performic acid was acting in two ways. A two-stage extraction was carried out in which wall was first treated with 98% formic acid for 16 hr. at 0° and, after freeze-drying, was extracted with $0.05M$ -sodium carbonate: 30, 25 and 27% of material was extracted on an ash-free basis from three preparations (extract 1). When the residues from the batches which had released 25 and 27% into solution were oxidized and extracted a further 62 and 63% respectively, based on the original weight of material, was dissolved (extract 2). In these experiments the amount extracted was taken as the difference between the amounts of ash-free starting material and ash-free residue.

Composition of wall fractions. Qualitative paper chromatography showed that all spots were present in all fractions. Extract 1 contained much less glutamic acid than did the intact wall. Cysteic acid and large quantities of glutamic acid but considerably less of the three basic amino acids were present in extract 2. The final residue contained large amounts of glutamic acid but less cysteic acid than extract 2 and was rich in the three basic amino acids.

Some of these differences are shown in the

Table 1. *Amount of some amino acids in preparations and fractions of the wall of Colpoda steinii cysts*

For methods of estimation and wall fractionation see the text. Several results under any one heading refer to analyses of different batches of material. Each result is the average of duplicate estimations carried out on a hydrolysate of known concentration and individual estimations did not differ from the mean by more than 5%. Results indicated by A are expressed as g. of acid from 100g. of ash-free material and those indicated by B are expressed as ninhydrin colours as a percentage of the total hydrolysate ninhydrin colour. Values under B were calculated from knowledge of the amino acid content (from chromatography) of a particular hydrolysate, the total ninhydrin colour in solution given by the same hydrolysate and the ninhydrin colour given under the same conditions by a known amount of the amino acid. For any particular acid where figures appear directly above and below each other corresponding to sub-headings A and B these figures refer to analyses on the same hydrolysate.

		Whole wall	Whole oxidized wall	Extract (1)	Extract (2)	Final residue
Glutamic acid	A	52.9 ± 0.6 (5)	51.7, 55.5, 50.5	25.3, 16.9, 23.0	57.1, 58.8	—
	B	—	37.0, 36.7	21.7, 19.1	49.9	42.9
Cysteic acid	A	—	6.4, 4.9, 6.1	—	6.1, 5.5	—
	B	—	4.4, 3.4	—	4.6	1.9
Aspartic acid	A	4.9, 6.6	5.7, 6.4, 5.5, 6.6	9.7, 9.6	3.8, 5.5	—
	B	—	4.1, 5.0	11.0	3.3	—
Glycine	A	8.9, 8.0, 8.7	—	—	—	—
	B	—	—	—	—	—

quantitative results summarized in Table 1. Two ways, explained in the legend, were chosen to express the results. The advantages of describing an amino acid content by the ninhydrin colour as a percentage of the total hydrolysate ninhydrin colour are that in addition to giving a better idea of the molar proportion of the acid it makes it possible to discuss the content of the final residue. This contained so much inorganic matter (two such fractions had non-sulphated ash values of 25 and 38%) that correction for this was almost impossible and expressing the amino acid content on a weight basis was meaningless.

The most important feature of these results is that a glutamic acid-rich fraction is not extractable as the result of treatment with formic acid but is extractable after oxidation.

DISCUSSION

The wall of the intact cyst appears to be made of several layers and although the final preparation may have contained more than one type of membrane the thick rigid layer was (by weight) the principal component.

The ash contained many elements; some, e.g. lead and tin, present in fair amounts are not likely to have had a biological origin and almost certainly arose from contamination. There is no evidence for an important degree of wall mineralization. Much of the inorganic content of the isolated material may be located in the small amounts of particulate matter which are always present. Some of these

particles are probably glass fragments for small particles of glass are released into the suspension from the glass beads and container during the disruption process.

Other protozoan cyst walls which have been examined are those of an *Acanthamoeba* (Tomlinson & Jones, 1962) and *Pelomyxa illinoisensis* (Sachs, 1956), where cellulose and chitin respectively seem to be major components. The fact that *Colpoda* cyst walls are almost pure protein and very different from these other two types stresses the diversity that exists amongst Protozoa. In composition *Colpoda* cyst walls are more like bacterial spore walls, which also contain large amounts of protein (Strange & Dark, 1956; Warth, Ohye & Murrell, 1963). Small amounts of residual phosphorus were found in the preparations described here and the spore walls of some bacteria also contain residual phosphorus (Strange & Dark, 1956; Warth *et al.* 1963).

The glutamic acid content of the wall estimated by manometry was 46.5 ± 0.7 g. or 0.316 ± 0.005 mole from 100g. of ash-free wall, and the aspartic acid content obtained by chromatography was 0.046 ± 0.005 mole/100g. This gives 0.360 ± 0.010 mole/100g. as the total dicarboxylic acid content, which may be compared with 0.313 ± 0.015 mole/100g., the total number of carboxyl groups present either as amide or in the free acid form. These two figures do not quite agree but the difference may not be significant and it is concluded that the bulk of the carboxylic acid side chains have been accounted for.

As judged by observation under the microscope

wall preparations appeared to resist the action of Pronase and pancreatin. This resistance may be due to the highly acidic nature of the protein, for work in this Laboratory (J. Tibbs & P. M. Whyte, unpublished work) has shown that soluble fractions of the wall and poly- α -L-glutamate are all relatively inert towards these enzymes. Resistance to proteolytic enzymes would also be expected if a substantial proportion of the glutamyl residues were peptide-bonded through their γ -carboxyl groups.

An average value, from Table 1, for the cysteic acid content of oxidized wall is 5.8 g. from 100 g., and this corresponds to a cystine content of 4.1 g./100 g. The insoluble nature of the wall appears to be largely due to the presence of disulphide bonds, for a major proportion (just over 60%) is made soluble only when these bonds are broken. That much of the glutamic acid exists as free poly- α -L-glutamate is unlikely for this substance is soluble in 0.05M-sodium carbonate. The 60% of the wall which is made soluble only when the disulphide bonds are ruptured contains about 58.0 g. of glutamic acid/100 g. (the average of the two values shown in Table 1) and contains about 67% of the total glutamic acid of the untreated wall preparation. This glutamic acid must be involved in direct or indirect linkage to cystine residues. It is not known whether merely glutamic acid-rich protein(s) is being dealt with or whether much of the glutamic acid exists as polyglutamate linked to a polypeptide chain of mixed amino acids through cystine residues.

When cells excyst the thick inner wall disappears and the trophic form reappears within, and finally bursts its way through, a thin, expandable, boundary membrane. If cells which are excysting are studied under phase contrast in the form of both live and crushed preparations the conclusion seems inescapable that the membrane which imprisons the trophic form at a late stage of excystment is derived from and is possibly identical with the external wall layer of the fully encysted cell. The thick inner wall appears to be digested away, and in view of the chemical nature of the preparations described in this paper, which are largely composed of this thick inner wall, this digestion should result in a supply of amino acids and in particular glutamic acid. These acids could be used for the synthesis of necessary protein and the large amount of glutamic acid could be readily deaminated to provide a source of energy.

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