Composition of Cells and Cell Walls of Triangular and Ellipsoidal Forms of *Trigonopsis variabilis*

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SUMMARY

Extraction and fractionation of lipids from intact ellipsoidal and triangular forms of *Trigonopsis variabilis* showed both to be rich in lipid, the content in triangular forms being twice that in ellipsoidal forms. Lipid was chiefly present in bound form with which principally choline was associated in triangular cells, whilst in ellipsoidal cells ethanolamine and serine predominated. Very little unsaturated lipid was found in the triangular forms, whereas a threefold greater iodine value was noted with the ellipsoidal forms. Polysaccharides of both cellular forms were extracted and fractionated. The triangular forms were seemingly devoid of glucan or chitin but contained a reducing polysaccharide that formed an unusual copper complex and appeared to be a polymer of glucose and mannose. The ellipsoidal forms contained a small amount of glucan and considerable amounts of glycogen. Cell walls were isolated from both triangular and ellipsoidal forms and found to consist principally of polysaccharide, nearly all of which was alkali-soluble. Walls of triangular forms contained somewhat more lipid than those of ellipsoidal forms, and phospholipid was twice that of ellipsoidal form walls.

INTRODUCTION

In a previous paper (Sentheshanmuganathan & Nickerson, 1962a), the morphological development of *Trigonopsis variabilis* was shown to depend largely on the nutritional environment, especially as pertained to nutrition favouring elaboration of constituents of phospholipids. To obtain further evidence about the involvement of phospholipid synthesis in form development, lipids of whole organisms of both the triangular and ellipsoidal forms of *T. variabilis* have been examined. Striking differences were found between organisms of the two forms as to their contents of free and bound lipids, and the properties of their respective bound lipids. Cell walls were isolated from both cellular forms and the chemical composition of the isolated walls examined with a view to obtaining information permitting interpretation of cellular form in terms of its underlying molecular architecture.

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METHODS

Microbiological methods. The organism used was *Trigonopsis variabilis* (Strain CBS 1040 obtained from the Centraalbureau voor Schimmelcultures, Yeast Division, Delft, Holland). Growth medium and culture conditions used to obtain ellipsoidal or triangular cell forms were exactly as described in the preceding paper (Sentheshanmuganathan & Nickerson, 1962a). Organisms were harvested by centrifugation, washed twice with distilled water and taken for lipid analysis or for isolation of cell walls.

Isolation of cell walls. Freshly harvested, washed organisms were suspended in distilled water, and disrupted by agitation with glass beads in a Waring Blendor (cooled under flowing water) according to the technique of Lamanna & Mallette (1954). About 90 min. agitation was required to obtain complete breakage of organisms. At the end of this period, no whole organisms could be detected microscopically. Cell walls were separated from glass beads and particulate cellular debris by differential centrifugation and repeated washing alternatively with sucrose (8.5 %, w/v) or distilled water (see Falcone & Nickerson, 1956). To avoid contamination in later analyses, washing with phosphate buffer was omitted. After about thirty repetitions of washing and differential centrifugation, a fraction consisting exclusively of cell-wall material was obtained. This was suspended in water and lyophilized. White fluffy preparations were obtained by this procedure, and all the analytical results reported for cell walls are based on such material.

Lipid analysis of whole organisms and cell walls. The use of fresh cell material for lipid analysis is essential; most preliminary treatments cause more or less extensive hydrolytic and oxidative modifications of certain of the lipid fractions, particularly of the phospholipids (Peck, 1947). Therefore, freshly harvested organisms were used for the studies reported here. Unless otherwise mentioned, all extractions were carried out in an atmosphere of nitrogen obtained by bubbling oxygen-free nitrogen through solvents during all operations. Removal of solvents was effected by distillation under reduced pressure; the last traces of solvent were removed by flushing with N_2 gas. Diethyl ether, acetone, chloroform, and ethanol were used as lipid solvents, and were redistilled before use.

Free or readily extractable lipids of whole organisms or of cell walls were extracted by suspending organisms or walls in a mixture of ethanol+ether (1+1, v/v) and allowing to stand at room temperature under an atmosphere of N_2 for 5 days with occasional shaking. Solvent was separated by filtration and the process repeated twice. The extracts were combined, solvent removed, and residues exhaustively extracted with ether for 24 hr. Etheral solutions were dried over anhydrous Na_2SO_4 , and concentrated to small bulk under reduced pressure. A measured sample of concentrate was transferred to a weighed centrifuge tube, and the last traces of ether removed by flowing N_2 . The residue was treated with acetone, and allowed to stand at 2° for 24 hr., whereupon phospholipids precipitated. The insoluble material was packed by centrifugation at 2° for 15 min at 19,600 g. The supernatant fluid was poured off, and adhering acetone drained off as thoroughly as possible. The last traces of solvent were removed in flowing N_2 and the residual material, taken as phospholipid, was weighed.

The residue obtained after the removal of free lipids with ethanol+ether, was

subsequently extracted twice with chloroform for a period of 24 hr. The chloroform extracts were combined to give a second fraction of readily extractable lipids.

Chemically bound and occluded lipids were extracted by subjecting residual material, after washing with aqueous ethanol (25 %, v/v), to a mild digestion with ethanol + ether (1 + 1, v/v) containing HCl (1 %, w/v) at 50° for 5 hr. (Crowder *et al.* 1936; Anderson *et al.* 1937). Cell residues were filtered off and extracted with ether and then with chloroform. All three extracts were combined, concentrated to an aqueous suspension, and extracted with ether for 24 hr. in a liquid-liquid extractor. The ethereal extract was dried over anhydrous Na_2SO_4 , concentrated to small bulk, and made up to a known volume with ether. This fraction is referred to as total bound lipid. Chloroform- and acetone-insoluble fractions were determined on measured samples of total bound lipid, as described above for phospholipids.

Hydrolysis of lipids. To determine nitrogenous constituents of the bound lipids of whole organisms, the material was hydrolysed by refluxing for 3 hr. with 5 ml. 6N-HCl in methanol (Thannhauser, Benotti & Reinstein, 1939). Solvent and HCl were removed at 60° under reduced pressure to obtain a dry residue which was treated with water and again concentrated; this process was repeated twice. Finally, the dry mass was dissolved in a known volume of water and filtered in the cold to give a clear solution.

Selective hydrolysis of bound lipids was performed to distinguish the choline content of lecithin from that of sphingomyelin. Complete hydrolysis liberates choline from both lecithin and sphingomyelin, whilst the selective hydrolysis method of Schmidt, Benotti, Herselman & Thannhauser (1946) liberates choline from lecithin but not from sphingomyelin (Hack, 1947). For details of the method see Schmidt (1957).

Analytical methods. Choline was estimated by converting it to the insoluble enneaiodide with KI_3 , dissolving the precipitate in ethylene dichloride, and determining optical density at 365 m μ (Appleton *et al.* 1953). Whenever an estimation was carried out, two standards were run side by side under the same conditions. Ethanolamine and serine were determined, by the colorimetric method of Axelrod, Reichenthal & Brodie (1953), after conversion into their coloured 2:4-dinitrofluorobenzene derivatives and separation on the basis of their differential solubilities in organic solvents. A mixture containing equal amounts of ethanolamine and serine was used side by side.

Total nitrogen content of bound lipids was determined by the micro-Kjeldahl method of Chibnall, Rees & Williams (1943); NH_3 was distilled in the apparatus of Markham (1942) and trapped in the boric acid reagent of Conway & O'Malley (1942). Total phosphorus was determined by the colorimetric method of Fiske & SubbaRow (1925), described by Umbreit, Burris & Stauffer (1957), after digestion with concentrated H_2SO_4 . Determinations of total reducing sugars of cell walls were carried out on acid hydrolysates by the anthrone method of Chung & Nickerson (1954); hexosamine was estimated by the Blix (1948) modification of the Elson & Morgan method (1933).

Paper chromatography. Amino acids in cell walls were determined by two-dimensional paper chromatography after cell walls had been hydrolysed with 6N-HCl for 24 hr. in a sealed tube at 100° under N_2 . Whatman No. 1 paper was used for chromatograms, which were irrigated for 24 hr. with phenol (containing

8-hydroxyquinoline, 1 mg./2 ml.) + ammonia in one direction. Papers were then dried at 85° for 15 min. to remove phenol and were run in the other direction for 18 hr. with butanol + acetic acid + water (2 + 1 + 2, vol.). Amino acids were detected by spraying with ninhydrin (0.25 %, w/v) in water-saturated butanol and drying papers at 85° for 10 min.

Definition of terms used. Saponification number (SV) is the number of mg. of KOH neutralized by fatty acids, free or combined, in 1 g. lipid when the latter is completely saponified with ethanolic KOH. Iodine number (IV) is the number of g. iodine taken up by 100 g. lipid. Acid number (AV) is the number of mg. of KOH required to neutralize free fatty acids in 1 g. lipid. Ester number (EV) is obtained by difference on subtracting the acid value from the saponification value.

Determination of the above values. The methods described by Baldwin & Bell (1955) were employed. The iodine number of a lipid was determined as follows. To lipid dissolved in chloroform was added 1.0 ml. of pyridine sulphate dibromide reagent; the mixture was shaken thoroughly and allowed to stand at room temperature for 5 min. Residual bromine was determined by titration of iodine liberated from KI with a standard solution of sodium thiosulphate, with starch as indicator. Unsaponifiable material in lipids was estimated as follows. After saponifying a lipid with ethanolic KOH, it was extracted with ether for 5 hr. in a liquid-liquid extractor. The ethereal extracts were dried over anhydrous sodium sulphate, the ether removed, and the residue weighed.

Tests for steroids. Unsaponifiable material was dissolved in chloroform and the following tests performed. Salkowski reaction with conc. H_2SO_4 and Liebermann-Burchard reaction with acetic anhydride and conc. H_2SO_4 (Baldwin & Bell, 1955).

RESULTS

Electron photomicrographs of cell walls of the triangular form of *Trigonopsis variabilis*, prepared as described above, are shown in Pl. 1. The walls appear to be homogeneous (not fibrillar or granular), and essentially free of adhering intracellular material.

Carbohydrate composition

Acid hydrolysis of cell walls resulted in rapid dissolution of the suspension, accompanied by liberation of reducing substances. As hydrolysis proceeded, samples were withdrawn every 30 min. and centrifuged; hexosamine and reducing sugars were estimated in the clear supernatant fluid. It is seen from Fig. 1 that hydrolysis appeared to be complete within 60 min. for both triangular and ellipsoidal wall preparations. When refluxing was continued beyond 90 min., browning of hydrolysates occurred, with a loss of 15 % (w/v) of the estimated reducing carbohydrate. Although the hexosamine content of cell walls of both forms were the same (1.9 %, w/v), total carbohydrate values, as glucose, differed appreciably. With the ellipsoidal form, carbohydrate was 91 % whereas in the triangular walls, it was 81 % (Table 1).

Amino acid composition

Amino acid content of the triangular form walls was almost twice that of the ellipsoidal form walls, as judged by the intensity of ninhydrin spots on paper chromatograms. This agrees with total nitrogen content of the cell walls. As shown in

Table 2, glycine, threonine and alanine were present in major amounts in both forms. Next in order of magnitude were valine, leucine or isoleucine, glutamic and aspartic acids. Lysine was not detected in the hydrolysate of ellipsoidal walls while triangular walls gave only a faint spot. Falcone & Nickerson (1956) obtained evidence for the presence of fifteen amino acids in the cell walls of baker's yeast; similar findings were reported by Eddy (1958); and quantitative analyses of the amino acid composition of proteins in the walls of *Candida albicans* and of baker's yeast were presented by Kessler & Nickerson (1959).

Fig. 1. Release of reducing sugars and hexosamine during acid hydrolysis of cell walls of ellipsoidal and triangular forms of *Trigonopsis variabilis*. 30 mg. cell-wall material hydrolysed with 15.0 ml. 2N-HCl at 100°; samples withdrawn at intervals and centrifuged; total reducing sugar (as glucose) and hexosamine were estimated in the supernatant by methods described in the text. ○····○ = Total carbohydrate (as glucose) in cell walls of ellipsoidal form. ●—● = Hexosamine of ellipsoidal walls. △····△ = Total carbohydrate (as glucose) in cell walls of triangular forms. ▲—▲ = Hexosamine of triangular walls.

Table 1. *Composition of cell walls of the ellipsoidal and triangular forms of Trigonopsis variabilis*

	Cell form	
	Ellipsoidal mg./100 mg. dry wt. organism	Triangular mg./100 mg. dry wt. organism
Moisture	3.0	3.0
Total carbohydrate (as glucose)	91.0	80.6
Hexosamine	1.91	1.87
Nitrogen	1.14	2.34
Phosphorus	0.086	0.153
N:P ratio	13.2	15.3
Free lipids:		
(a) Ether soluble	0.60	1.9
(b) CHCl ₃ soluble	0.12	0.08
Bound lipids:		
(a) Total	4.7	6.7
(b) Acetone insoluble (phospholipids)	0.63	1.4

See text for the methods of isolation of lipids.

Nitrogen and phosphorus

Total nitrogen and phosphorus contents of cell walls of both forms of *Trigonopsis variabilis* are given in Table 1. Not only the nitrogen content, but also the phosphorus of the triangular walls was almost twice that of the ellipsoidal walls. Thus, the N:P ratios of ellipsoidal and triangular walls were 13:2 and 15:3, respectively. In cell walls of baker's yeast, Falcone & Nickerson (1956) reported a phosphorus value of 0.34 %. This higher value may reflect contaminating traces derived from phosphate buffers used in washing of wall preparations. In the present work, this possibility can be excluded since washing with phosphate was avoided. Values of 0.086 % phosphorus (with ellipsoidal walls) and 0.156 % (with triangular walls) were obtained.

Table 2. *Amino acid content of cell walls of ellipsoidal and triangular forms of Trigonopsis variabilis by chromatography*

The chromatogram papers were irrigated for 18 hr. with phenol + water + ammonia soln. (sp.gr. 0.880) (80 + 20 + 1 vol.) in one direction. The papers were dried, solvent removed, and irrigated in the other direction with butanol + acetic acid + water (2 + 1 + 2 vol.). The spots were located by spraying with 0.25% (w/v) ninhydrin in water-saturated butanol. Numbers give relative amounts of each amino acid.

Amino acids	Cell form	
	Ellipsoidal	Triangular
	Relative amount	
Aspartic acid	1	2
Glutamic acid	2	4
Glycine	4	8
Threonine	4	8
Alanine	4	8
Lysine	—	1
Arginine	1	2
Tyrosine	1	2
Valine	2	4
Leucine or isoleucine	2	4

Distribution of phosphorus in cell-wall fractions

To ascertain the distribution of phosphorus in various fractions, cell walls of *Trigonopsis variabilis* were first freed from lipids (free and bound) and then fractionated. To obtain comparable data, the phosphorus content of lipid-free whole organisms was also estimated (Table 3). With lipid-free triangular walls, phosphorus was evenly distributed among the cold perchloric acid (PCA), hot PCA, hot NaOH, and residual fractions. On the other hand, the phosphorus content of ellipsoidal walls was highest in the NaOH-soluble fraction, whilst the other three fractions gave almost similar, lower, values. In fractionation of animal tissues (Schneider, 1945; Schmidt & Thannhauser, 1945), residual material soluble in hot NaOH (2 %, w/v) is usually regarded as phosphoprotein. More recently, Ågren (1958) showed that the 'phosphoprotein fraction' derived from baker's yeast or from *Lactobacillus casei*, indeed, contained phosphorylserine. With lipid-free whole organisms of both triangular and ellipsoidal forms of *T. variabilis*, phosphorus was found principally in the cold PCA extract, and least in the residual fraction. Both forms of whole cells gave almost identical values.

Nucleic acids

Barkulis & Jones (1957) reported the presence of nucleic acid in streptococcal cell walls. These authors mentioned that such nucleic acids were only released on digestion of cell walls with acid, and they did not observe an absorption peak at 260 m μ with aqueous suspensions. On the other hand, Salton & Horne (1951) and Falcone & Nickerson (1956) did not detect nucleic acids in cell walls of *Streptococcus faecalis* and baker's yeast, respectively. Eddy (1958) reported a value of 0.3 % for the nucleic acid content of yeast cell wall but questioned this figure, since his cell-wall preparation was not sufficiently clean, and attributed the finding to the small percentage of unbroken or partially broken cells which remained in the preparation, not to a component of the wall as such. However, isolation of nucleotide components from hydrolysates of clean cell walls of *Mucor rouxii* has recently been reported by Bartnicki-Garcia & Nickerson (1961).

Aqueous suspensions of cell walls, as well as cold and hot perchloric acid extracts of untreated cell walls and of lipid-free cell walls of both forms of *Trigonopsis variabilis* did not show any absorption peak at 260 m μ . Phosphorus in PCA extracts of cell walls of both cell forms (Table 3), therefore, did not derive from nucleic acid.

Table 3. *Distribution of phosphorus in the various fractions of lipid-free whole organisms and cell walls of the two forms of Trigonopsis variabilis*

The materials were suspended in 5 % (w/v) perchloric acid (PCA), kept at 5° for 30 min. and centrifuged. The residue was treated with 5 % (w/v) PCA, boiled for 30 min. at 100° and centrifuged. The sediment was washed twice with water and centrifuged. The residue was finally boiled with 2 % (w/v) NaOH and centrifuged.

Material	Fractionation procedure	Cell form	
		Ellipsoidal	Triangular
		Phosphorus content ($\mu\text{g./100 mg.}$)	
Lipid-free whole organisms	{ None	120.6	133.0
	{ Cold PCA extract	64.0	67.0
	{ Hot PCA extract	20.0	19.4
	{ Hot NaOH extract	25.0	29.0
	{ Residue	13.6	15.8
Lipid-free cell walls	{ None	80.5	136.8
	{ Cold PCA extract	18.1	32.3
	{ Hot PCA extract	13.3	30.5
	{ Hot NaOH extract	28.8	36.9
	{ Residue	15.6	30.0
Cell walls	{ None	86.5	152.9
	{ Cold PCA extract	24.6	38.6
	{ Hot PCA extract	17.6	40.8
	{ Hot NaOH extract	25.2	33.6
	{ Residue	14.5	28.0

Lipids

Total lipids in cell walls of ellipsoidal and triangular forms of *Trigonopsis variabilis* were 5.42 % (w/v) and 8.68 % (w/v), respectively; the major portion of which occurred in a bound or occluded form, which was released only on digestion with

acidic ethanol+ether. The values obtained (Table 1) are comparable to those reported by Kessler & Nickerson (1959) for cell walls of baker's yeast. Intact organisms of *T. variabilis* were found to be unusually rich in lipids, and the contents of ellipsoidal and triangular forms differed appreciably. With the ellipsoidal forms, about 20% of the cellular dry wt. was comprised of lipid, of which nearly all was present in a bound form. With triangular forms, the values were even higher: lipid comprised 43.7% of the dry wt. of which the bulk was chemically bound (Table 4).

Table 4. *Lipid content of whole organisms of ellipsoidal and triangular forms of Trigonopsis variabilis*

Fraction	Cell form	
	Ellipsoidal	Triangular
	Amount (mg./100 mg. dry wt.)	
Free lipids:		
(a) Ether soluble	1.05	3.0
(b) CHCl ₃ soluble	0.20	0.30
(c) Acetone insoluble	Nil	Trace
fraction of (a)		
Bound lipids	18.7	40.4

See text for the method of extraction of the lipids.

Table 5. *Fractionation of bound lipids of whole organisms of both forms of Trigonopsis variabilis*

Bound lipids (g./100 g.)	Cell form	
	Ellipsoidal	Triangular
(a) Acetone soluble	96.8	97.1
(b) Acetone insoluble (phospholipids)	3.2	2.9
(c) CHCl ₃ insoluble	1.56	4.74
Nitrogenous constituents (mg./100 g.)		
(a) Total nitrogen	780.0	560.0
(b) Ethanolamine	70.3	38.6
(c) Choline	37.0	72.0
(d) Serine	66.0	36.0
(e) Choline from lecithin	8.4	29.0
Other properties		
(a) Saponification number	530	315
(b) Acid number	152	86
(c) Ester number (by difference)	378	229
(d) Iodine value	14.0	4.4
(e) Unsaponifiable matter	25 g./100 g.	20.1 g./100 g.
Phosphorus	453	244
	(mg./100 g.)	(mg./100 g.)
N:P ratio	1.72	2.29
Colour test for sterols in unsaponifiable material	+	+++

Properties of bound lipids of whole cells and cell walls

Phospholipids, or the acetone-insoluble fraction of the bound lipids, comprised only a very small fraction of the bound lipid of whole organisms of *Trigonopsis variabilis*; ellipsoidal and triangular forms contained 3.2 and 2.9%, respectively,

whilst the values for ellipsoidal and triangular walls were 13 and 21 %, respectively (Tables 1, 5).

Nitrogen and phosphorus contents of bound lipids of ellipsoidal forms were higher than observed with triangular forms (Table 5), whilst N and P contents of ellipsoidal walls were less than in triangular walls (Table 6). The N:P ratios for bound lipids, not only of whole organisms but also of triangular walls were higher than those observed with ellipsoidal forms. Analysis of nitrogenous constituents of bound lipids extracted from whole organisms revealed that triangular forms contained more choline than did the ellipsoidal forms, whilst ellipsoidal forms possessed more ethanolamine and serine. The use of a selective hydrolysis procedure which, presumably, released choline only from lecithin and not from sphingomyelin, showed that about 40 % of the choline in bound lipid of triangular forms was associated with lecithin, whereas only about 23 % of the choline of ellipsoidal forms was released (Table 5).

Table 6. *Analysis of bound lipids obtained from cell walls of ellipsoidal and triangular forms of Trigonopsis variabilis*

Analysis	Cell form	
	Ellipsoidal	Triangular
	Amount (mg./100 g. bound lipid)	
Nitrogen	120	420
Phosphorus	100	200
N:P ratio	1.2	2.1
Iodine no.	0	0

See text for the analytical methods used.

Other properties of lipids of the two cell forms also differed appreciably (Table 5), the most striking difference being observed in iodine numbers. Most of the fatty acids in triangular form lipids were found to be saturated, whereas those of ellipsoidal forms were unsaturated. In the preceding paper (SentheShanmuganathan & Nickerson, 1962*a*), we reported that the addition of oleic acid or glyceromono-oleate inhibited formation of triangular forms. The results shown in Table 5 are in agreement with the previous observation.

Unsaponifiable material obtained from bound lipids of triangular forms was found to contain almost three times more sterol than found in lipids of ellipsoidal forms (as estimated by the Liebermann & Burchard reaction). Absorption spectra of unsaponifiable material obtained from bound lipids of both forms (Fig. 2) exhibited a marked peak at 242 m μ . Paper chromatography, with propylene glycol + toluene as solvent system, revealed one spot that moved with the solvent front. Ultraviolet (u.v.) absorption spectra of bound lipids are shown in Fig. 3. With both triangular and ellipsoidal forms, a marked peak at 232 m μ is evident, suggesting that a major component of the unsaponifiable fraction is a steroid.

*Polysaccharide composition of ellipsoidal and triangular forms of
Trigonopsis variabilis*

The method of Chung & Nickerson (1954) was used for the fractionation of polysaccharides of ellipsoidal and triangular forms of *T. variabilis* (Table 7). The triangular forms dissolved completely in hot KOH (30 %, w/v), leaving no insoluble material; this finding indicates that these forms were completely devoid of glucan or chitin. With ellipsoidal forms, however, small, but appreciable, amounts of glucan (0.55 $\mu\text{g./mg.}$ cell dry wt.) were found. Moreover, the precipitate obtained with Fehling's solution dissolved completely on the first washing with NaOH (0.1 N), suggesting either that the cells were devoid of mannan, or that the 'copper mannan' precipitate was quite different from that obtained with baker's yeast.

Fig. 2

Fig. 3

Fig. 2. Absorption spectra of the unsaponifiable material obtained from bound lipids of whole cells of *Trigonopsis variabilis* (ellipsoidal and triangular forms). Equal amounts of material were dissolved in CHCl_3 ; absorption spectra measured against the solvent as reference. Note absorption maxima at 242 $\text{m}\mu$. $\times \cdots \times$ = Unsaponifiable material from bound lipids of triangular forms. $\bullet \cdots \bullet$ = Unsaponifiable material from ellipsoidal form lipids.

Fig. 3. Absorption spectra of bound lipids of cell walls of ellipsoidal and triangular forms of *Trigonopsis variabilis*. Bound lipids were dissolved in diethyl ether, and absorption spectra measured in cuvettes fitted with ground glass stoppers. Note absorption maxima at 232 $\text{m}\mu$. $\times \cdots \times$ = Lipids of triangular walls. $\bullet \cdots \bullet$ = Lipids of ellipsoidal walls.

Properties of polysaccharide soluble in KOH. A major portion of the total carbohydrate was found in the KOH fractions from both forms. Polysaccharide soluble in KOH was precipitated with 95 % (v/v) ethanol in water (after removal of lipid by extraction with ether), centrifuged and the supernatant fluid removed. The precipitate thus obtained was washed twice with ethanol, dissolved in water, and reprecipitated with ethanol. This procedure was repeated twice. A sample of the dry residue was dissolved in water and tested with Gram's iodine for glycogen. The polysaccharide obtained from the triangular forms reduced a large amount of iodine, and did not give the colour test for glycogen. On the contrary, polysaccharide from

the ellipsoidal forms did not exhibit reducing properties and gave a typical colour test for glycogen.

Both of these polysaccharides were soluble only with difficulty in hot water; insoluble residues remained even after boiling. Hydrolysis with $N-H_2SO_4$ did not give a clear solution; even after 16 hr. at 100° insoluble residues remained with the polysaccharides from both triangular and ellipsoidal forms. These hydrolysates were neutralized with $Ba(OH)_2$, and the precipitated $BaSO_4$ removed by centrifugation. The supernatant solutions were concentrated under vacuum and spotted on paper (Partridge, 1948). With ellipsoidal forms, glucose was identified, whilst with triangular forms mannose and glucose were found. No quantitative measurements were carried out. The polysaccharides from isolated cell walls of both forms behaved similarly.

Table 7. *Quantitative fractionation and analysis of polysaccharides of cell walls and whole organisms of the two forms of Trigonopsis variabilis*

The fractionations of the organisms were carried out as described by Chung & Nickerson (1954) and the carbohydrate contents estimated by the anthrone method of the same authors.

Fraction	Whole organism		Walls	
	Ellipsoidal	Triangular	Ellipsoidal	Triangular
	Amount (μ g. glucose/mg. dry wt. organism or walls)			
Whole organisms or cell walls	440	405	920	820
Aqueous extract (25° for 30 min.)	0.77	5.4	66	17
Cold TCA* extract	19.30	6.2	23	7
Hot 30% KOH material	321.0	358.0	809	780
Acetic acid extract	0.34	0	—	—
H_2SO_4 digest	0.55	0	—	—
Residue after KOH treatment	—	—	12	10
Alcohol precipitation of KOH soluble material				
Supernatant obtained after precipitation with 95% EtOH	14.0	8.1	—	—
Acid hydrolysate of the ethanol precipitate	274.0	250.0	—	—
Insoluble residue obtained after hydrolysing the alcohol precipitate	60.0	46.2	—	—

* TCA = trichloroacetic acid.

DISCUSSION

Analyses of cellular components of ellipsoidal and triangular cell forms of *Trigonopsis variabilis* emphasize the enrichment of lipids in triangular cells, and the absence of structural constraints imposed by fibrillar wall components of polysaccharide nature. From studies in many laboratories the importance of the cell wall as determinant of cellular form has emerged. This concept is reinforced by the electron-micrograph shown herein (Pl. 1). The triangular or ellipsoidal cellular form results from the mould in which the wall is formed and not, primarily, from the intracellular environment. It must be admitted and emphasized at the outset, that we have been unable to obtain any information that would permit us to hold an opinion about the structural requirements for the triangular form. In the case of the

ellipsoidal form, we have advanced the opinion that a maximally disordered fibrillar structure (conferring a maximal modulus of elasticity) is requisite (Nickerson & Falcone, 1959; Falcone & Nickerson, 1959). Therefore, it is of importance to note that some glucan (fibrillar) component is found in ellipsoidal walls whereas such is not present in triangular walls.

As shown in the preceding paper (Sentheshanmuganathan & Nickerson, 1962*a*), form development in *Trigonopsis variabilis* is subject to nutritional control. Substances such as methionine, that are methyl group donors, favour the development of populations of the triangular form. The role of methyl group donor substances in the biosynthesis of phospholipid moieties is well established, and mediation of the methionine effect on form development through promotion of phospholipid synthesis is strongly suggested by our analytical findings. Total lipid contents of both cellular forms of *T. variabilis* were high and in the triangular forms (43.7 %) was more than twice that in the ellipsoidal forms (20 %). In contrast, the total lipid contents of isolated cell walls (ellipsoidal = 5.42 %, triangular = 8.68 %) were in line with values available for baker's yeast. The proportion of phospholipid relative to total lipid in whole forms and in cell walls of *Trigonopsis* is most striking:

	Whole form	Cell wall
Ellipsoidal form	3.2	11.6
Triangular form	2.9	16.1

Not only are cell walls of the triangular form relatively richer in phospholipid, but the actual amount on a weight basis is twice that of the cell walls of the ellipsoidal form.

Physiological conditions which favour accumulation of lipid in most micro-organisms (e.g. continued incubation after the logarithmic phase of growth in a medium depleted in nitrogen-source, but containing an available carbon-source) are precisely those which lead to development of the triangular form of *Trigonopsis variabilis*. As emphasized in the preceding paper (Sentheshanmuganathan & Nickerson, 1962*a*) triangular populations predominate only after incubation for more than 48 hr. in a medium which permits their development.

Thus, several lines of evidence favour the view that synthesis especially of phospholipids serves as a form-determinant in this yeast. Although such evidence as has been adduced points to the importance of the phospholipid of the cell wall in this connexion, further work is necessary to establish a relationship between the molecular form of such material and cellular shape.

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EXPLANATION OF PLATE

Electron micrograph of cell-wall preparation from triangular form of *Trigonopsis variabilis*. Magnification 21,750.