DISTRIBUTION OF INOSITOL IN SUBCELLULAR FRACTIONS OF YEAST CELLS[1]

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This research was prompted by the fact that inositol has been recognized as an essential yeast nutrilite for many years (Eastcott, 1928), yet it has no established rule in cellular physiology. Some investigators have postulated that the primary function of inositol is that of a building block for some structural component of the cell. This is based upon the observations that inositol is required for yeast growth in much larger amounts (5 to 10 mg per L) than other B vitamins and that it is known to be a component of certain types of lipids.

Analysis of a variety of tissues has shown that inositol is present chiefly as a component of phospholipids (Wittcoff, 1951) and lipo-protein complexes (Folch and Le Baron, 1951). In cereal grains, inositol is found in large amounts of phytin, the calcium, magnesium salt of the hexaphosphate ester. Analysis of yeast cells has shown that most of the inositol is present in a bound form which yields free inositol upon acid hydrolysis (Smith, 1951). Yarbrough and Clark (1957) have shown recently that 80 to 85 per cent of the bound inositol in *Schizosaccharomyces pombe* is lipid in nature. (However, only free inositol can be utilized for growth by inositol dependent yeasts.) (Woolley, 1941*b*).

As part of a study of the role of inositol in the metabolism of yeasts we have determined the distribution of free and combined inositol in various subcellular fractions of yeast cells with the objective of defining more precisely its location within the cell, and its possible role as a structural component. Preliminary observations have also been made upon the nature of the combined forms of inositol in the yeast cell.

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METHODS

Organisms and cultural conditions. The strain of *Saccharomyces carlsbergensis* investigated has an obligate requirement for only biotin, but its growth is stimulated greatly by inositol and slightly by pantothenate. Cells for inositol analysis were grown in a fluid medium containing 1 per cent peptone, 1 per cent yeast extract, and 2 per cent glucose. The strain of *Kloeckera brevis* studied resembles that described by Burkholder *et al.* (1944) since it has an obligate requirement for 6 vitamins, including inositol. Since this organism grows poorly on peptone, yeast extract medium, cells for inositol analysis were grown in a medium of the following composition: glucose, 10 g; L-asparagine, 2 g; $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeCl_3 \cdot 6H_2O$, 0.1 mg; vitamin-free acid-hydrolyzed casein, 1.0 g; niacin, 1 mg; Ca pantothenate, 1 mg; pyridoxine·HCl, 1 mg; thiamin-HCl, 1 mg; biotin, 1 μg; inositol, 10 mg; distilled water, 1 L. The pH was adjusted to 5.0 before autoclaving at 120 C for 10 min.

Both yeasts were grown at 25 C with continuous shaking for 24 to 72 hr, and were washed thoroughly in sterile tap water before analyses.

Inositol assay. The inositol assay method was a modification of that described by Sawyer (1951). The medium described above, but minus the inositol, was made up double strength and autoclaved. Five ml amounts were pipetted into sterile 50-ml Erlenmeyer flasks and varying amounts of sterile inositol solution or samples to be analyzed were added. The volume was adjusted to 10 ml with sterile water, and each flask was inoculated with one drop of a washed suspension of a 24-hr culture of *K. brevis* which had been grown in the complete assay medium. The flasks were incubated for 24 hr at room temperature with continual shaking. The turbidity of the resulting cultures was determined with a Coleman nephelocolorimeter (525 mμ filter), and the amount of inositol present in the samples was

estimated from a curve plotted from standards which were run simultaneously.

Free inositol was determined by assaying extracts obtained with boiling water; total inositol, by assaying hydrolyzates prepared by the method of Woolley (1941*a*); and lipid inositol by assaying similarly prepared hydrolyzates of the washed lipids which were extracted by the method given below.

Lipid extraction. Lipids were extracted by the method of Folch *et al.* (1951) using 20 volumes of 2:1 chloroform-methanol at 4 C. The extracts were freed of nonlipid material as described by these workers. This method of extraction was compared with that of Bloor (1928) and comparable results were obtained with both procedures for total lipid and lipid inositol.

Preparation of subcellular fractions. Preliminary studies on the distribution of inositol in the various fractions employed homogenates which were prepared by shaking 2 g of wet cells, 5 g of pavement marking beads and 5 ml of 1 per cent NaCl in a Mickle disintegrator[3] for 10 min at 4 C. This treatment ruptured approximately 90 per cent of the cells of *S. carlsbergensis* and about 50 per cent of the cells of *K. brevis*.

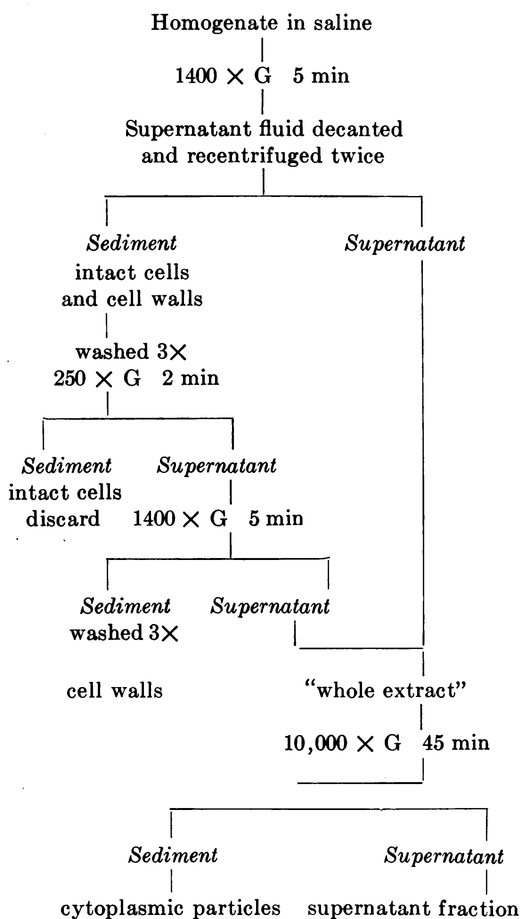

Figure 1. Outine of the preparation of cell fractions.

In experiments where free, total and lipid inositol were determined homogenates were prepared by shaking 20 g of wet cells, 10 ml of 1 per cent NaCl solution, and 60 g of 5 mm glass beads for 1 hr at 4 C, using a shaking machine operating at 300 2-in strokes per minute.

The homogenates were fractionated into cell walls, cytoplasmic particles and supernatant fluid by differential centrifugation as outlined in figure 1. The cell wall fractions obtained in this manner from *S. carlsbergensis* were almost completely free of intact cells, but were slightly contaminated with cytoplasmic granules which remained trapped among the cell walls. Because of the extreme variability in cell size of *K. brevis* a suitable cell wall preparation could not be obtained.

RESULTS AND DISCUSSION

Spectroscopic examination of whole extracts revealed the presence of cytochromes *a*, *b* and *c*, but after sedimentation of the particles only a faint cytochrome *c* band could be detected in the supernatant. The sedimented particles, however, were rich in all three cytochrome components as well as cytochrome oxidase, and suspensions in 1 per cent NaCl took up oxygen with succinate or lactate as substrates. Studies of the enzymatic activity associated with cytoplasmic granules of yeast prepared in a similar fashion have been made by Chantrenne (1943), Slonimski and Ephrussi (1949), Slonimski and Hirsch (1952) and Nossal (1953). These observations indicate that the cytoplasmic granules of yeast are the sites of many oxidative enzymes of the cell and are thus analogous to the mitochondria and microsomes of higher plants and animals.

Table 1 summarizes data on the distribution of total inositol in subcellular fractions of *S. carlsbergensis* and *K. brevis*. The major portion of the inositol of both yeasts is concentrated in the cytoplasmic particles, where it constitutes about

[3] Manufactured by H. Mickle, Hampton, Middlesex, England.

1 per cent of the dry weight of this fraction. It is of interest from the standpoint of comparative biochemistry to recall that comparable granules (mitochondria and microsomes) from animal tissues also possess a high inositol content (Claude, 1946). In contrast to the high concentration of inositol in the particles, the cell walls of *S. carlsbergensis* contain very little, and the small amount found may well be due to contamination with cytoplasmic particles and intact cells.

The total inositol, free inositol and lipid bound inositol of the cytoplasmic particles and the supernatant fraction of *S. carlsbergensis* found in a representative experiment are presented in table 2. All of the inositol in the cytoplasmic particles is bound but only about one-third of this can be extracted with lipid solvents. In the supernatant fluid, approximately 45 per cent of the inositol is free, and of the bound inositol present in this fraction, about 50 per cent is lipid inositol. The difference between our values, 33 per cent of the cellular inositol of *S. carlsbergensis* extractable with lipid solvents, and the higher values, 80 to 85 per cent, reported by Yarbrough and Clark (1957) for *S. pombe*, are probably due to species differences and to culture conditions. We have analyzed cultures of *S. carlsbergensis* grown on a synthetic medium with 0.2 per cent $(NH_4)_2SO_4$ and 0.002 per cent sodium glutamate as the only nitrogen sources and found 100 per cent of the bound inositol of the particles and 78 per cent of the bound inositol of the supernatant fraction extractable with chloroform-methanol.

It is of interest that a large portion of the cellular lipid is also concentrated in the cytoplasmic particles. In our experiments the lipid content of the particle fraction varied from 23 to 35 per cent of the dry weight, which agrees with the values reported by Nyman and Chargaff (1949).

The inositol that could not be extracted with lipid solvents from the cytoplasmic particle of *S. carlsbergensis* could possibly consist of either a phosphate ester such as phytin or a lipid-inositol complex tightly bound to protein such as Folch and Le Baron (1951) have demonstrated in brain tissue. Neither of these complexes would be extracted with lipid solvents but phosphate esters of inositol would be soluble in trichloracetic acid (TCA) (Albaum and Umbreit, 1943). To test this possibility, the cytoplasmic particles of *S. carlsbergensis* were extracted with TCA by the method of Schneider (1945). The extracts were freed of acid by continuous ether extraction for 48 hr and then analyzed for inositol after hydrolysis. Only 13 per cent of the inositol present in the particles was extracted by TCA, and from this it can be concluded that free phosphate esters of inositol do not constitute a major portion of the bound inositol of the cytoplasmic particles. This is in contrast to the results of Smith (1951) but agrees with those of Yarbrough and Clark (1957). It is possible that this fraction is similar to the inositol lipo-protein complex described by Folch and Le Baron (1951).

The demonstration that inositol is concentrated in structural entities within the cell lends support to the hypothesis that inositol is an essential structural component of the cell. The association of much of the inositol of the cytoplasmic granules of yeast with lipids indicates that one function of inositol in the yeast cells is that of a building block for inositol-containing

TABLE 1

The total inositol content of subcellular fractions of Saccharomyces carlsbergensis and Kloeckera brevis

	S. carlsbergensis			*K. brevis*	
	Cell walls	Cytoplasmic particles	Supernatant	Cytoplasmic particles	Supernatant
Fraction, mg dry wt.	144	185	270	240	250
Inositol found, mg	0.12	2.11	0.84	2.2	1.2
Inositol, %	0.08	1.1	0.2	0.9	0.5
Inositol, % of total	4.0	69	27	—	—

TABLE 2

The distribution of lipid and inositol in subcellular fractions of Saccharomyces carlsbergensis

	Whole Extract	Supernatant	Cytoplasmic Particles
Dry wt, mg	796	619	182
Lipid content, mg	91	31	64
Total inositol, mg	4.5	2.0	2.9
Free inositol, mg	0.9	0.9	0
Lipid-bound inositol, mg	1.5	0.47	1.1
Inositol in lipid fraction, % of total	33	24	38

lipids. The role of such lipids in the function of the cytoplasmic granules remains to be determined.

SUMMARY

Analyses of subcellular fractions of *Saccharomyces carlsbergensis* and *Kloeckera brevis* have shown that the 60 to 70 per cent of the cellular inositol is present in the cytoplasmic particles. The cell walls of *S. carlsbergensis* contain little if any inositol. The distribution of lipid between the soluble and particulate components of the cytoplasm was similar to that of inositol.

About 40 per cent of the bound inositol of the cytoplasmic particles can be extracted with cold 2:1 chloroform-methanol or boiling 3:1 ethanol-ether. The nature of the bound inositol which is not extracted by these solvents is not known, but, since very little of it can be extracted with trichloracetic acid, free phosphate esters, such as phytic acid, do not constitute a significant portion of this fraction.

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