INOSITOL-REQUIRING MUTANTS OF *SACCHAROMYCES CEREVISIAE*

MICHAEL R. CULBERTSON AND SUSAN A. HENRY

Department of Genetics, Albert Einstein College of Medicine, Bronx, New York 10461

Manuscript received November 18, 1974

ABSTRACT

Fifty-two inositol-requiring mutants of *Saccharomyces cerevisiae* were isolated following mutagenesis with ethyl methanesulfonate. Complementation and tetrad analysis revealed ten major complementation classes, representing ten independently segregating loci (designated *ino1* through *ino10*) which recombined freely with their respective centromeres. Members of any given complementation class segregated as alleles of a single locus. Thirteen complementation subclasses were identified among thirty-six mutants which behaved as alleles of the *ino1 locus*. The complementation map for these mutants was circular.——Dramatic cell viability losses indicative of unbalanced growth were observed in liquid cultures of representative mutants under conditions of inositol starvation. Investigation of the timing, kinetics, and extent of cell death revealed that losses in cell viability in the range of 2–4 log orders could be prevented by the addition of inositol to the medium or by disruption of protein synthesis with cycloheximide. Mutants defective in nine of the ten loci identified in this study displayed these unusual characteristics. The results suggest an important physiological role for inositol that may be related to its cellular localization and function in membrane phospholipids. The possibility is discussed that inositol deficiency initiates the process of unbalanced growth leading to cell death through the loss of normal assembly, function, or integrity of biomembranes.——Part of this work has been reported in preliminary form (CULBERTSON and HENRY 1974).

AUXOTROPHIC fatty acid mutants of *Saccharomyces cerevisiae* have provided valuable information on the central roles fatty acids play as structural and functional components in cellular membranes (WISNIESKI, KEITH and RESNICK 1970; SCHWEIZER and BOLLING 1970; HENRY and KEITH 1971, HENRY and FOGEL 1971; PROUDLOCK, HASLAM and LINNANE 1971; HENRY 1973; CULBERTSON and HENRY 1973). Since fatty acid auxotrophs are pleiotrophically defective for all membrane-associated phospholipids, direct experimental control over membrane composition can be achieved by supplementing the mutants with various molar ratios of fatty acids having different chain lengths and degrees of saturation. The effects of altered membrane composition can then be studied in relation to other aspects of cell physiology. However, it has not been possible using these mutants to study the roles of individual classes of lipids independently of each other. The isolation and characterization of mutants auxotrophic for inositol, a major carbohydrate moiety present in a limited number of membrane phospholipids of yeast, may provide an approach to this problem. Prudent exploitation of inositol-requiring mutants may be expected to illuminate more

definitively the relationships between structure and function of specific phospholipids and the elements which regulate their activities in biomembranes.

Seven inositol-containing lipids have been detected chromatographically in cell extracts of *S. cerevisiae*, including three membrane-associated phospholipids (phosphatidylinositol, diphosphoinositide, and triphosphoinositide) and four sphingolipids which appear to be cell wall-associated (LESTER and STEINER 1968; STEINER *et al.* 1969; STEINER and LESTER 1972a). The major phospholipid, phosphatidylinositol, is synthesized from CDP-diglyceride and free myo-inositol precursors. The product of this reaction serves in subsequent steps as the precursor for the polyphosphoinositides and the sphingolipids (ANGUS and LESTER 1972; STEINER and LESTER 1972b).

Several lines of evidence point to an important role for inositol in membranes. For example, high turnover rates of phosphomonoesters in polyphosphoinositides of yeast (STEINER and LESTER 1972c) and various animal tissues (DAWSON 1966) suggest a role for these compounds in membrane transport phenomena. In addition, an inositol auxotroph of *S. cerevisiae* which, due to a second mutation, is able to grow in the presence of the inositol analog myo-inosose-2, displays altered membrane properties observed by nitroxide electron-spin labelling under conditions where the membranes contain a high proportion of myo-inosose-2 (WATKINS *et al.* unpublished observations).

It has also been observed that inositol auxotrophs of Neurospora (LESTER and GROSS 1959) and inositol-requiring species of yeast (RIDGWAY and DOUGLAS 1958) die logarithmically when starved for inositol. Saturated fatty acid auxotrophs behave similarly during fatty acid starvation (HENRY 1973) as do biotin- or pantothenate-requiring organisms when starved for their requirements (PONTECORVO *et al.* 1953; KURAISHI *et al.* 1971; SHIMIDA, KURAISHI and AIDA 1972). Since biotin and pantothenate are required for fatty acid synthesis LYNEN, 1967, VAGELOS *et al.* 1966) and fatty acids are required for phosphatidylinositol synthesis, "unbalanced growth" in all these examples may result from defective synthesis of membranes lacking essential lipids.

The present work concerns a genetic analysis of fifty-two ethyl methanesulfonate-induced mutants of *S. cerevisiae* that require myo-inositol for growth. The number and distribution of genetic loci are determined by complementation and tetrad analysis. Evidence is presented that starvation of the mutants for inositol results in unbalanced growth.

MATERIALS AND METHODS

Strains: Parental strains in mutagenesis were heterothallic, adenine-requiring (*ade5*), haploid strains of *Saccharomyces cerevisiae* derived in both mating types from crosses involving strains originally obtained from H. O. HALVORSON. The following strains were obtained from the Berkeley collection: P49 (*α lys2*); S856C (**a**, *ade1*, *lys2*); X2928-4C (*α*, *ade1*, *gal1*, *trp1*, *ura3*, *his2*, *leu1*, *met14*); 4335-7-3A (**a**, *lys1-1*, *ade2-1*, *trp1-1*, *thr1 CUP1*, *suc1*); 4335-7-3B (same as 4335-7-3A but opposite mating type).

Growth Conditions: Unless otherwise indicated, all growth trials were at 30°.

Media: Mutants were maintained on YEPD (2% glucose, 2% bactopeptone, 2% agar, 1% Yeast extract). Complete synthetic medium contained the following components: 2% glucose, Difco vitamin-free yeast nitrogen base (6.7 gm/L), biotin (2 μg/L), calcium pantothenate

(400 μg/L), folic acid (2 μg/L), niacin (400μg/L), p-aminobenzoic acid (200 μg/L), pyridoxine hydrochloride (400 μg/L), myo-inositol (2000 μg/L), lysine (20 mg/L), arginine (10 mg/L), methionine (10 mg/L), threonine (60 mg/L), tryptophan (10 mg/L), leucine (10 mg/L), histidine (10 mg/L), adenine (10 mg/L), uracil (10 mg/L). Inositol-less medium was identical with complete except for the omission of inositol. Auxotrophic requirements of various strains for amino acids, purines, or pyrimidines were tested by omitting the appropriate component from complete medium. Glucose-free medium was prepared by adding the various salts, trace elements, and nitrogen source individually. This was necessary, since Difco vitamin-free YNB contains a small amount of glucose.

Genetic methods: Haploid strains were crossed by mixing cells of opposite mating type on YEPD. Following overnight incubation the mixture was replica plated to media appropriate for the selection of diploids. Prior to sporulation cells were transferred back to YEPD, incubated overnight, and replica plated to sporulation medium containing 2% potassium acetate (FOGEL and HURST 1967). Mature asci were dissected by micromanipulation, following a ten-minute treatment with a 1:10 dilute solution of glusulase. Ascospore clones were tested for their requirements by replica plating to appropriate defined media. Unambiguous identification of genotype was provided where required by allele-specific complementation tests.

Induction and recovery of inositol-requiring mutants: Mutants were induced by treating cultures of parental strains with EMS (ethyl methanesulfonate), according to the procedure of LINDEGREN *et al.* (1965). Ninety percent of the cells were killed following seventy minutes' exposure to the mutagen. Cells were plated at an appropriate dilution on YEPD and incubated for several days. Colonies were replica plated to synthetic media with and without inositol. Colonies able to grow only on the supplemented medium were retrieved for analysis. Approximately 50,000 colonies were screened.

Preliminary analysis: Primary mutants derived from the **a** mating type parent were crossed through two successive generations with strain P49. Those derived from the α parent were similarly crossed with S856C. The twofold purpose of these crosses was to confirm the mendelian segregation of the inositol requirement for each mutant and to remove, as much as possible, the mutagenized background. All experiments were performed subsequent to this initial screening procedure.

Complementation: The test for complementation was performed by streaking samples of the mutants in parallel lines on YEPD. Following overnight incubation these plates were cross-stamped by replica plating onto fresh YEPD plates such that strains of opposite mating type intersected at right angles with each other. These plates were incubated overnight and replica plated to inositol-less medium. The complementation response was measured by observing the amount of growth at mutant × mutant intersections where diploids had formed.

The petite phenotype of some primary mutants and the possibility of poor mating efficiency were considered potential problems which might interfere with complementation and impose limitations on the reliability of the results. The petite phenotype was eliminated where necessary by the recovery from tetrads of respiratory-sufficient mutants following outcrossing to standard, respiratory-sufficient stocks. In addition, a qualitative assay for mating efficiency was designed and implemented as a control. Strains derived from each primary mutant were constructed in both mating types such that all **a**-derivatives required adenine (*ade1 or ade5*) and all α-derivatives required lysine (*lys2*). Selection based on these markers provides an assay for diploid growth independent of the inositol requirement. Since vigorous diploid growth occurred at all mutant × mutant intersections on this control medium, the amount of diploid growth due to complementation of the inositol genes could be standardized against this control. Using this procedure, the results obtained are free of ambiguities due to mating inefficiency.

Cross-feeding: An assay for cross-feeding reactions utilized the same procedure as that for the complementation test except that mutants of the same mating type were employed. In addition, wild-type strains were tested by a similar procedure for their ability to cross-feed inositol-requiring mutants.

Centromere linkage: Linkage of inositol genes with any centromere was estimated by including known centromere-linked markers in crosses. Second division segregation frequencies for inositol genes were calculated from the relationship:

$$T = X + Y - 3/2XY$$

where T = observed frequency of tetratype asci, X = second division segregation frequency of the inositol gene, and Y = known second division segregation frequency for a centromere-linked gene (PERKINS 1949). Second division segregation frequencies for centromere-linked markers used in this study are *ade1*, 10.1; *leu1*, 3.9 (HAWTHORNE and MORTIMER 1960). Centromere linkage of *ade1* and *leu1* was verified in those crosses where both markers were segregating simulataneously. The observed frequency of tetratype asci for these markers (12.7) was not statistically different from the predicted value (13.1), which was calculated from the known second division segregation frequencies.

Suppressibility: The suppressibility of mutations conferring the inositol requirement was determined by crossing the mutants with nonsense suppressors selected in strains 4335-7-3A and 3B, which carry known ochre (*lys1-1*, *ade2-1*) and amber (*trp1-1*) nonsense mutations (HAWTHORNE 1969). Presumptive suppression in these crosses was indicated by 4+:0— and 3+:1— segregations for the inositol requirement in tetrads. The segregation of the suppressors in mutant × suppressor crosses could be unambiguously identified only in those asci which were parental ditype with respect to a suppressor and a known suppressible mutation. In crosses presumed by these criteria to involve suppressible *ino* mutations, these parental ditype asci were carefully inspected to ensure that no suppressor-carrying spore clones were simultaneously inositol-requiring.

For each presumptive suppressible mutant, spore clones of the following genotype were isolated: *ino*$^-$, *lys1-1*, *ade2-1*, *trp1-1*, *sup*. Lawns of these strains were replica plated to media lacking individually the four components: inositol, lysine, adenine, and tryptophan. Co-reversion of the inositol marker with the known ochre or amber mutations was taken as further evidence for suppressibility.

Inositol starvation: Representative mutants were tested for growth in liquid synthetic medium inoculated from overnight YEPD precultures. Growth was examined in the following media: complete, inositol-less, lysine-less, inositol-less plus 3% tergitol NP40 (Sigma), and inositol-less plus 100 μg/ml cycloheximide (Sigma). It was found that cycloheximide at this concentration prevents growth of the mutants but does not result in loss of viability in complete medium.

Preculture cells were washed twice in minimal medium and inoculated at a density of approximately 10^6 cells/ml. For the cycloheximide experiments, cells were washed, inoculated in complete medium, and allowed to grow until there was a perceptible increase in optical density. Cycloheximide was added and the cells were harvested one-half hour later, washed twice in minimal medium plus cycloheximide, and resuspended in cycloheximide-containing inositol-less medium at 10^6 cells/ml.

Cell growth was followed by four methods: optical density, plating for viable cells, estimation of petite frequency, and estimation of percentage dead cells by staining with methylene blue. Petite frequencies were determined by replica plating colonies to medium containing glycerol as sole carbon source. To calculate the percentage of dead cells, a solution of methylene blue (0.6%) was mixed in a 1:5 ratio with samples of the liquid yeast cultures. After five minutes in the stain, the cells were examined under the microscope and the percentage of dead cells was determined (LINDEGREN 1949). When precise numbers were required, the cells were counted in a hemocytometer.

RESULTS

Fifty-two inositol-requiring mutants were recovered from mutagenesis with ethyl methanesulfonate. The mutants are designated throughout this report by laboratory stock numbers MC-1 through MC-52. One mutant (MC-13) was isolated and provided to us for genetic analysis by T. WATKINS and A. KEITH.

With the exception of one (MC-16), to be described in a later section, the inositol requirement of each mutant segregated in tetrads derived from heterozygous diploids (*ino⁻/INO*) according to the normal mendelian 2+:2− ratio expected of nuclear genes. All mutants were recessive to wild-type in diploid hybrids. Hybrids involving the various mutants are referred to by laboratory stock numbers MRC-1 through MRC-147.

Petite phenotype: In addition to requiring inositol for growth, several primary mutants required a fermentable carbon source such as glucose. These mutants failed to grow when glycerol was provided as sole carbon source. In all such mutants the petite phenotype either failed to segregate, as expected for cytoplasmic petites, or it segregated independently of the inositol requirement, as expected for unlinked, nuclear petites. However, evidence will be presented that under conditions of starvation for inositol, a relationship does exist between the primary lesion (inability to synthesize inositol) and the secondary accumulation of petites.

Complementation: Complementation responses were tabulated for all 2704 pairwise combinations of the mutants according to the procedures outlined in MATERIALS AND METHODS. The complementation data summarized in Figure 1 provide an estimate for the number of genetic loci represented. Ten major complementation classes were identified. The distribution of mutants among these classes was highly asymmetric in the sense that seven of the ten classes were represented only once (III, V, VI, VII, VIII, IX, X), while the largest class (I) was represented by thirty-six of the fifty-two mutants. This large class could be subdivided into thirteen subclasses on the basis of numerous complementation responses occurring within it. Among class I mutants it was not possible to draw a complementation map without invoking circular overlaps.

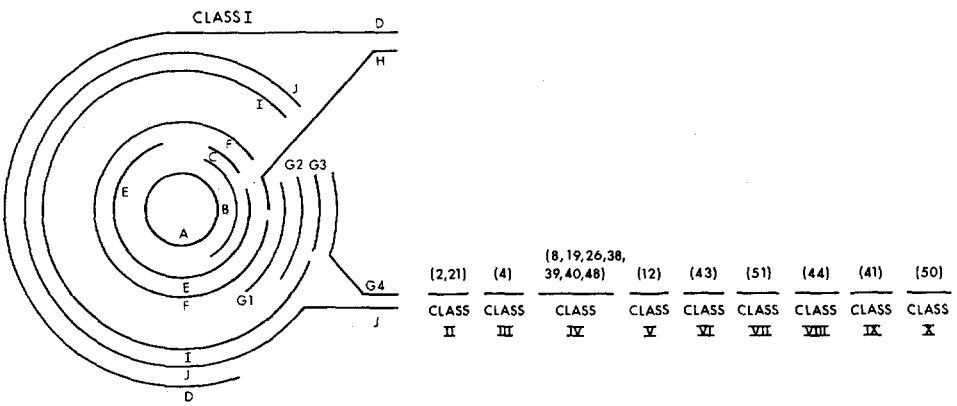

FIGURE 1.—Complementation among 52 inositol-requiring mutants. Complementing mutants are represented on non-overlapping lines. Roman numerals refer to major complementation classes. Arabic numerals refer to laboratory stock numbers for mutants representing complementation classes II through X (stock numbers for the 36 mutants of class I are omitted from the figure). Capital letters in class I refer to the various subclasses which were identified.

Three qualitatively distinct types of complementation responses were observed: vigorous complementation, weak complementation, and complementation failure. Vigorous responses, equivalent in intensity to control responses (*lys* × *ade*), were observed among pairs of mutants representing different major complementation classes and could be easily scored one day after replica plating. As shown in Table 1, vigorous responses were also observed among pairs of mutants representing several subclasses of class I (ID × IG1, ID × IC, ID × IB, IG1 × IC, IC × IE).

The response was considered weak when perceptible growth was observed at diploid intersections three to four days following replica plating. Weak responses were only observed among certain pairs of mutants in class I (Table 1). This type of response was never equivalent in intensity to control responses, but was noticeably different from cases of complementation failure.

When no visible growth took place at diploid intersections after four days' incubation following replica plating, complementation failure was scored. Mutants of the same complementation class, such as MC-2 and MC-21 (class II), failed to complement each other, although they vigorously complemented mutants of all other complementation classes. In addition, mutants representing subclass IA of class I, while failing to complement all mutants in class I, vigorously complemented mutants representing other major complementation classes.

As shown in Table 1, all three types of responses were observed among mutants of class I. Figure 2 is presented as an aid in visualizing the complexities of this

TABLE 1

Subclasses of complementation class I

Subclass	Allelic representatives	Complementing alleles within class I	Response intensity*
IA	1,5,9,13,15,17,18,24,29,30,33,36,42	NONE	—
IB	6,20,27	14	+
IC	23	3,7,10,11,14,16,28,32,34,35,37, 45,46,49,52	+
ID	14	6,7,11,20,23,27,28,32,34,35,37, 45,46,49,52	+
IE	3,10	23	+
IF	22,47	16	±
IG1	28,37,46	14,23	+
IG2	32,49,52	14,23,31	±
IG3	11,34,35	14,23,25,31	±
IG4	7,45	14,23,25	±
IH	16	22,23,25,31,47	±
II	25	7,11,16,34,35,45	±
IJ	31	11,16,32,34,35,49,52	±

* A positive response (+) was scored when vigorous growth was observed at mutant × mutant intersections 24 hours following replica plating. A negative response (—) was scored when no growth was observed 96 hours following replica plating. An intermediate response (±) was scored when perceptible growth was observed 72 hours hours following replica plating.

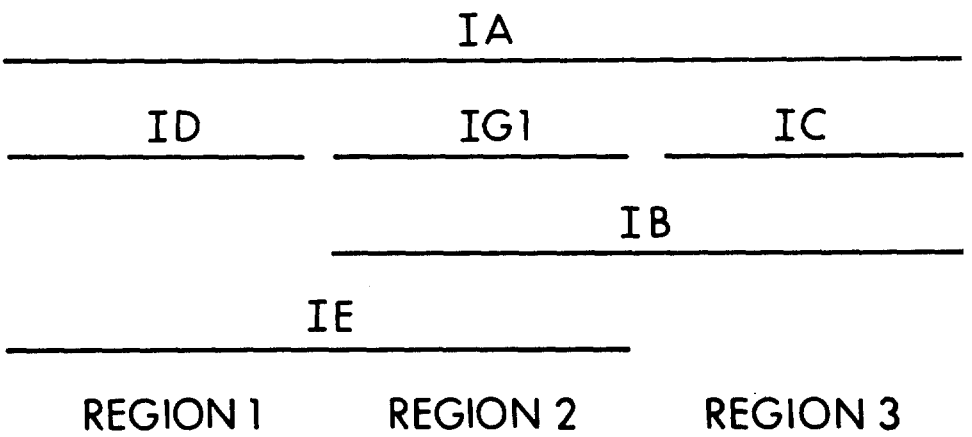

FIGURE 2.—Partial complementation map for class I mutants. As an aid to visualizing the complexities of this class of mutants, the figure includes only those subclasses listed in Table 1 which are defined by mutants giving vigorous complementation responses (+) and mutants failing to complement all representatives of class I (—).

class. This simplified complementation map includes only those subclasses represented by non-complementing and vigorously complementing mutants. When viewed in this manner, three distinct regions are apparent, defined by the subclasses ID, IG1, and IC. The possible significance of these regions will be considered in the DISCUSSION.

Cross-feeding: The results of cross-feeding analysis were negative at all mutant × mutant intersections. In addition, when wild-type strains were tested for their ability to feed inositol-requiring mutants, no visible growth of the mutants was observed.

Centromere linkage: The combined data for crosses of inositol-requiring mutants to strains carrying centromere markers indicate that among mutations chosen as representatives of the ten major complementation classes, none are centromere-linked. In each case the PD:NPD:T ratio approximates the 1:1:4 ratio expected of genes which are at least 34 centimorgans from any centromere.

Linkage relations between complementation classes: Recombinational analysis of relevant pairwise mutant combinations indicates that each major complementation class presented in Figure 1 represents an independently segregating gene (Table 2). Accordingly, the ten genes identified in this study shall be designated by genetic nomenclature which is consistent with the complementation map presented in Figure 1 (i.e., MC-1 in class I shall be called *ino1-1*, MC-2 in class II shall be called *ino2-2*, etc.). Among the 44 pairwise combinations of mutants which were necessary for a complete linkage study, five crosses were unsuccessful due to extremely poor spore survival.

Linkage relations within complementation class I: Because of the numerous examples of complementation between alleles of *ino1* (class I), linkage relations within this group were studied. The results of ascus dissection and tetrad analysis of diploids heterozygous for mutations representing the various subclasses of

TABLE 2

Linkage relations between complementation classes

Hybrid no. MRC*	Parental strains	Complementation classes represented	Number of asci† PD 0+:4−	NPD 2+:2−	T 1+:3−	Other 2+:2− segregations
90	MC13 × MC2	I × II	25	24	93	*ade1,lys2*
18,91	MC13 × MC4	I × III	10	13	74	*ade1,lys2*
51,90,91	MC13 × MC8	I × IV	31	44	165	*ade1,lys2*
57,91	MC13 × MC12	I × V	24	33	96	*ade1,lys2*
89	MC2 × MC4	II × III	17	12	66	*ade1,lys2*
90,95	MC2 × MC8	II × IV	45	49	167	*ade1,lys2*
64,89	MC2 × MC12	II × V	15	15	72	*ade1,lys2*
91	MC4 × MC8	III × IV	6	17	59	*ade1,lys2*
65,89,91	MC4 × MC12	III × V	40	27	143	*ade1,lys2*
91	MC8 × MC12	IV × V	10	10	61	*ade1,lys2*
103	MC43 × MC13	VI × I	2	2	12	*ade1,lys2*
104	MC43 × MC2	VI × II	1	3	12	*ade1,lys2*
105‡	MC43 × MC4	VI× III	—	—	—	—
106	MC43 × MC8	VI × IV	0	1	14	*ade1,lys2*
107	MC43 × MC12	VI × V	3	6	9	*ade1,lys2*
108,114	MC43 × MC51	VI × VII	5	8	24	*ade1,lys2*
132	MC43 × MC44	VI × VIII	1	1	6	*ade1,lys2*
130	MC43 × MC41	VI × IX	3	0	4	*ade1,lys2*
125	MC43 × MC50	VI × X	0	1	7	*ade1,lys2*
109	MC51 × MC13	VII × I	1	3	10	*ade1,lys2*
110	MC51 × MC2	VII × II	0	7	8	*ade1,lys2*
111‡	MC51 × MC4	VII × III	—	—	—	—
112	MC51 × MC8	VII × IV	0	4	9	*ade5,lys2*
113	MC51 × MC12	VII × V	2	2	8	*ade1,lys2*
133	MC51 × MC44	VII × VIII	1	1	6	*ade1,lys2*
131	MC51 × MC41	VII × IX	2	1	5	*ade1,lys2*
126	MC51 × MC50	VII × X	2	0	4	*ade1,lys2*
136	MC44 × MC13	VIII × I	0	2	5	*ade1,lys2*
137	MC44 × MC2	VIII × II	3	3	4	*ade1,lys2*
138‡	MC44 × MC4	VIII × III	—	—	—	—
100	MC44 × MC8	VIII × IV	0	0	7	*ade1,lys2*
139	MC44 × MC12	VIII × V	0	2	3	*ade1,lys2*
127	MC44 × MC41	VIII × IX	2	2	3	*ade1,ade5,lys2*
123	MC44 × MC50	VIII × X	1	1	6	*ade1,lys2*
140	MC41 × MC13	IX × I	3	2	5	*ade1,lys2*
141	MC41 × MC2	IX × II	2	3	6	*ade1,lys2*
142‡	MC41 × MC4	IX × III	—	—	—	—
143	MC41 × MC12	IX × V	1	0	10	*ade1,lys2*
99	MC41 × MC8	IX × IV	1	1	6	*ade1,lys2*
144	MC50 × MC13	X × I	1	1	7	*ade1,lys2*
145	MC50 × MC2	X × II	2	1	5	*ade1,lys2*
146‡	MC50 × MC4	X × III	—	—	—	—
101	MC50 × MC8	X × IV	0	2	5	*ade1,lys2*
147	MC50 × MC12	X × V	1	3	4	*ade1,lys2*

* Diploid MRC 89 was constructed by crossing MC2 with a recombinant double mutant (MC4,MC12), which was derived from MRC 65. MRC 90 was similarly constructed, using MC2 and a double mutant (MC8,MC13) derived from MRC 51. MRC 91 was heterozygous at four sites (MC4,MC8,MC12,MC13). All other MRC strains were constructed by regular mutant × mutant crosses.

TABLE 3

Linkage relations within complementation class I

Hybrid no.	Parental strains	Class I subgroups	Complementation response	Number of asci*,† PD 0+:4−	NPD 2+:2−	T 1+:3−	Other 2+:2− segregations
MRC 10	MC1 × MC6	IA × IB	—	10	0	0	*ade5,lys2*
MRC 34	MC5 × MC6	IA × IB	—	12	0	0	*ade1,ade5,lys2*
MRC 43	MC13 × MC6	IA × IB	—	20	0	0	*ade5,lys2*
MRC 44	MC9 × MC6	IA × IB	—	2	0	0	*ade1,ade5,lys2*
MRC 15	MC1 × MC7	IA × IG4	—	12	0	0	*ade5,lys2*
MRC 33	MC5 × MC7	IA × IG4	—	15	0	0	*ade5,lys2*
MRC 50	MC13 × MC7	IA × IG4	—	8	0	0	*ade5,lys2*
MRC 66	MC13 × MC14	IA × ID	—	10	0	0	*ade1,ade5,lys2*
MRC 58	MC6 × MC14	IB × ID	+	18	0	0	*ade1,ade5,lys2*
MRC 78	MC20 × MC14	IB × ID	+	10	0	0	*ade1,lys2*
MRC 80	MC27 × MC14	IB × ID	+	10	0	0	*ade1,lys2*
MRC 40	MC6 × MC7	IB × IG4	—	14	0	0	*ade5,lys2*
MRC 73	MC23 × MC14	IC × ID	+	75	0	1‡	*ade5,lys2*
MRC 87	MC23 × MC10	IC × IE	+	11	0	0	*ade5,lys2*
MRC 29	MC6 × MC3	IB × IE	—	16	0	0	*ade1,ade5,lys2*
MRC 26	MC7 × MC3	IG4 × IE	—	13	0	0	*ade5,lys2*
MRC 74	MC23 × MC32	IC × IG2	±	11	0	0	*ade5,lys2*
MRC 75	MC23 × MC34	IC × IG3	±	11	0	0	*ade5,lys2*
MRC 76	MC23 × MC35	IC × IG3	±	10	0	0	*ade5,lys2*
MRC 77	MC23 × MC37	IC × IG1	+	11	0	0	*ade5,lys2*
MRC 54	MC14 × MC11	ID × IG3	±	17	0	0	*ade1,ade5,lys2*
MRC 59	MC14 × MC7	ID × IG4	±	16	0	0	*ade1,ade5,lys2*
MRC 83	MC14 × MC32	ID × IG2	±	11	0	0	*ade1,lys2*
MRC 84	MC14 × MC34	ID × IG3	±	10	0	0	*ade1,lys2*
MRC 85	MC14 × MC35	ID × IG3	±	11	0	0	*ade1,lys2*
MRC 86	MC14 × MC37	ID × IG1	+	11	0	0	*ade1,lys2*
MRC 81	MC14 × MC28	ID × IG1	+	11	0	0	*ade1,lys2*

* Allele identification was performed as described in MATERIALS AND METHODS. Spore genotypes were unambiguously identified in crosses involving complementing alleles. PD asci were identified on the basis of phenotype in crosses involving noncomplementing alleles and allele tests resulted in negative diploid growth at all intersections.

† PD=parental ditype. NPD=nonparental ditype. T=tetratype. 4th spore was deduced where required.

‡ Both alleles segregated 2+:2−.

class I are presented in Table 3. In all crosses analyzed the alleles of *ino1* were tightly linked, as indicated by PD:NPD:T ratios of 1:0:0. Only one tetratype ascus was detected among the tetrads from crosses listed in Table 3 (MC-23 × MC-14). Allele tests confirmed that the single prototrophic spore clone of this ascus arose through a reciprocal recombination event. In general, non-reciprocal recombination in crosses involving class I mutants was rare. The preliminary data suggest that gene conversion rates for most of the mutants are on the order of 1% or less.

† 4th spore deduced where required. PD=parental ditype. NPD=nonparental ditype. T=tetratype.

‡ Insufficient spore survival (less than 10%).

Suppressibility: All mutants except MC-16, which will be discussed below, were characterized for suppressibility by both amber and ochre suppressors. Tetrad analysis of diploids heterozygous for an amber suppressor did not reveal any evidence for amber-suppressible inositol auxotrophy. In each cross the inositol requirement segregated normally (2+:2−), although the known amber-suppressible mutation *trp1-1* (HAWTHORNE 1969), segregating in the presence of the amber suppressor, gave the expected 4+:0−, 3+:1−, and 2+:2− ascal classes. Among tetrads from diploids heterozygous for an ochre suppressor, the following inositol-requiring mutants met the criteria for ochre suppressibility: MC-8, MC-9, MC-15, MC-17, MC-21, MC-23, MC-26, MC-41, MC-43, MC-50. However, only one mutant, MC-21 (*ino2-21*), consistently co-reverted with the known ochre-suppressible mutations. Further studies will be required to confirm the suppressibility of other presumptive ochre inositol mutants.

Two-gene segregation in a primary mutant: One mutant (MC-16), for which the primary isolate was clearly auxotrophic, behaved differently from all other mutants in diagnostic tests. Dissection and tetrad analysis of heterozygous diploids constructed by a cross between the primary mutant isolate and a wild-type strain (S856C) revealed aberrant segregation of the inositol requirement. Although some asci from the diploid gave clear 2+:2− segregations, many others gave 3+:1− and 4+:0− segregations. The three prototrophs of 3+:1− asci were not phenotypically identical. Generally, two of the three grew vigorously and resembled growth on complete medium, while the remaining prototroph grew very poorly but was clearly distinguishable from the mutant spore clone. Among 4+:0− asci, two of the spore clones grew poorly.

The possibility of a suppressor introduced through the S856C parent was considered a likely explanation for the aberrancy in tetrads. However, numerous independent crosses with the mutant, including a backcross to the parental strain used in mutagenesis as well as crosses to several other standard laboratory strains, provided evidence contrary to this explanation. The same segregational pattern was observed regardless of the strain to which the mutant was crossed, suggesting that some property of the mutant strain itself was responsible for the aberrant segregations.

The combined data from these crosses indicated that 2+:2−, 4+:0−, and 3+:1− segregations occurred at a frequency approximating the 1:1:4 ratio expected for a two-gene segregation. The following working hypothesis was proposed as an explanation of these preliminary results. The primary mutant, MC-16, according to this explanation, is a double mutant containing an allele of *ino1* which confers a leaky inositol requirement and an independently segregating gene which acts to abolish the leaky phenotype to give a strongly mutant phenotype. If the leaky inositol requirement is designated as *ino1-16* and the modifying gene as *r*, then non-growing mutants would have the genotype *ino1-16 r*, leaky mutants would have the genotype *ino1-16 R*, and prototrophs would either be wild-type for both genes (*INO R*) or mutant for the modifying gene (*INO r*). The observed pattern of segregation in tetrads is explained as outlined in Table 4A.

TABLE 4

Evidence for an independent gene (r) *causing modified expression of a leaky inositol auxotroph* (ino1-16)

A. Expected result from the cross *ino1-16 r* × *INO R*

Ascal type	Meiotic products A	B	C	D	
Parental	*ino1-16 r*	*ino1-16 r*	*INO R*	*INO R*	genotype
ditype	—	—	+	+	phenotype
Nonparental	*ino1-16 R*	*ino1-16 R*	*INO r*	*INO r*	genotype
ditype	±	±	+	+	phenotype
Tetratype	*ino1-16 r*	*ino1-16 R*	*INO r*	*INO R*	genotype
	—	±	+	+	phenotype

B. Observed results demonstrating a 2-gene segregation

Cross*	Number of tetrads: Ascal phenotypes 2+:2−	2+:2±	2+:1±:1−	2±:2−	0+:4−	0+:4±	4+:0−	1±:3−
ino1-16 r × *INO R*	3	3	9	0	0	0	0	0
ino1-16 r × *ino1-16 r*	0	0	0	0	8	0	0	0
ino1-16 r × *ino1-16 R*	0	0	0	8	0	0	0	0
ino1-16 r × *INO r*	8	0	0	0	0	0	0	0
ino1-16 R × *INO R*	0	9	0	0	0	0	0	0
ino1-16 R × *ino1-16 R*	0	0	0	0	0	8	0	0
ino1-16 R × *INO r*	2	2	6	0	0	0	0	0
INO R × *INO R*	0	0	0	0	0	0	10	0
INO R × *INO r*	0	0	0	0	0	0	8	0
ino1-16 r × *ino1-13 R*	0	0	0	2	3	0	0	3
ino1-16 r × *ino1-25 R*	0	0	0	1	1	0	0	6
ino1-16 R × *ino1-25 R*	0	0	0	8	0	0	0	0

* Strains of genotypes *ino1-16 R* and *INO r* were obtained from NPD asci as explained in the text. *INO R* was obtained from a PD ascus. The original isolate (MC-16) was *ino1-16 r*.

The results of crosses designed to test this hypothesis are given in Table 4B. On the basis of phenotype, strains were constructed of the following presumptive genotypes: *ino1-16 r* (phenotype = non-growing mutant), *ino1-16 R* (phenotype = leaky mutant), *INO r* (phenotype = wild type), and *INO R* (phenotype = wild type). The two strains which were phenotypically wild-type were distinguished on the basis of the types of tetrads from which they were derived.

These derived strains were crossed with each other in various combinations. The salient features of the results of these crosses (Table 4B) are as follows: (1) The cross *ino1-16 r* × *ino1-16 R* gives a diploid which is homozygous for *ino1-16* and heterozygous for *r*. Detection of eight 2±:2− tetrads demonstrates mendelian segregation for *r*. (2) The cross, *ino1-16 r* × *INO r*, gives a diploid which is heterozygous for *ino1-16* and homozygous for *r*. Detection of eight 2+:2− tetrads demonstrates mendelian segregation for *ino1-16*. (3) The cross *ino1-16 r* × *INO R* gives a diploid which is heterozygous for both genes. Detection of three 2+:2−, three 2+:2±, and nine 2+:1±:1− tetrads demonstrates the independent segregation of the two genes. The cross *ino1-16 R* × *INO r* gives the same result, as expected.

An attempt was made to determine which of the two genes detected in the original MC-16 isolate is linked with mutants representing complementation class I (see Table 1). In particular, the last cross listed in Table 4B (*ino1-16 R* × *ino1-25 R*) is constructed such that linkage pertains to the auxotrophic requirements conferred by *ino1-16* and *ino1-25* and not to the modifying gene *r*, since the latter is present in homozygous wild-type condition (*R*/*R*). Detection of eight parental ditype asci (2+:2−) from this cross demonstrates that *ino1-16* and *ino1-25* are linked. Accordingly, the independently segregating gene *r* is not linked with class I mutants.

Inositol starvation: Cell growth and viability were examined under several defined sets of conditions for mutants representing the ten major complementation classes. The following mutants, which require inositol and lysine (*lys2*), were tested: *ino1-13* (class I), *ino2-2* (class II), *ino3-4* (class III), *ino4-8* (class IV), *ino5-12* (class V), *ino6-43* (class VI), *ino7-51* (class VII), *ino8-44* (class VIII), *ino9-41* (class IX), *ino10-50* (class X).

Precipitous loss of cell viability was observed under conditions of inositol starvation in cultures of nine of the ten mutants tested. The exceptional mutant, *ino5-12*, is discussed below. The results given in Figure 3 for strain MC-2 (*ino2-2*, *lys2*) are typical of the results obtained for other strains. Differences in growth kinetics between strains could in general be attributed to differences in doubling time or rate of reversion to wild type. Figure 3 depicts three parameters: viable cells, culture optical density, and petite frequency in the cultures.

Starvation of strain MC-2 for its lysine requirement in the presence of inositol results neither in cell death nor in cell growth. Under these conditions the strain is remarkably stable over a twenty-four-hour period. However, under conditions

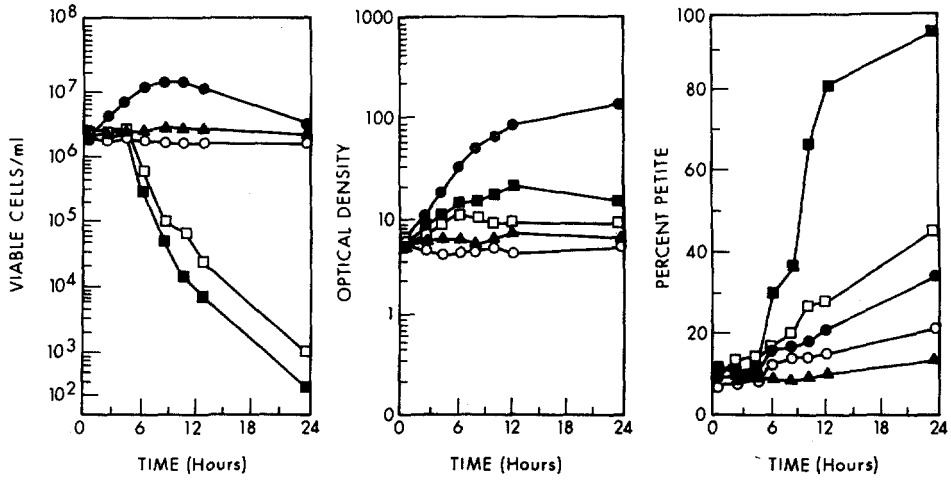

FIGURE 3.—Effect of inositol starvation. The data presented are for strain MC-2 (*a*, *lys2*, *ino2-2*). Complete synthetic medium (●), −inositol +lysine (■), −inositol +lysine + 100μg/ml cycloheximide (○), −inositol +lysine + 3% tergitol NP-40 (□), +inositol −lysine (▲).

of starvation for inositol in the presence of lysine, the culture becomes unstable. During the first several hours of starvation the culture increases in optical density while the viable cell number remains constant. At approximately four hours, inositol-starved cells begin to lose viability at an increasingly precipitous rate. Cell death reaches its maximum rate at approximately eight hours and by twenty-four hours the cells have undergone four log orders of death from a starting density of 10^6 viable cells/ml to a final density of 200 viable cells/ml. As shown in the figure, this dramatic loss in cell viability can be prevented by the addition of inositol or by the interruption of protein synthesis with cycloheximide. Tergitol NP40, which was shown to accelerate the rate of death in starved, saturated fatty acid-requiring strains (HENRY 1973), has no significant effect on the rate of death of starved inositol-requiring mutants.

Optical density profiles, as shown in Figure 3, do not correlate with the loss of cell viability under conditions of inositol starvation. However, estimates of the percentage of dead cells determined during the course of starvation by the methylene blue staining procedure outlined in MATERIALS AND METHODS closely correlate with estimates of viability based on the ability of cells to form colonies. Inspection of starved cells under the microscope revealed that the majority of unstained and presumably viable cells were slightly enlarged and unbudded or they were present as complete doublets. Stained cells, which are presumably inviable, were present at variable stages of development with respect to the budding process. Otherwise the cells appeared morphologically normal and there was little evidence of lysis during the most rapid period of cell death.

The majority of surviving cells in inositol-starved cultures were found to be respiratory-deficient (petite). During the course of inositol starvation, as shown in Figure 3, the frequency of petites increases with time from an initial low level of 5–10% to a final frequency approaching 100% of the surviving cells after twenty-four hours of starvation.

Severe difficulties were encountered with one mutant, MC-12 (*ino5-12*), the sole representative of complementation class V. When tested on solid medium, this mutant was clearly auxotrophic for inositol, although growth on inositol-supplemented medium was poor. Growth tests on media containing inositol and various alternative carbon sources indicated that non-fermentable carbon sources were better able to support growth than glucose. In tetrads these strain characteristics consistently segregated with the inositol requirement. For these reasons the mutant was tested in liquid media in the presence and absence of inositol with either glucose or glycerol as sole carbon source. In general, the results of these experiments support the conclusions that the strain requires inositol and that glucose exerts some inhibitory effect on growth. However, starvation of MC-12 for inositol does not result in precipitous cell death. Further experiments will be required to unravel the complexities of growth displayed by this mutant.

DISCUSSION

Complementation and linkage analysis of fifty-two inositol-requiring mutants of *Saccharomyces cerevisiae* enabled the identification of ten independently

segregating loci which recombined freely with their respective centromeres. This provides a minimal estimate for the number of genes which may be involved in inositol biosynthesis. The inositol-requiring mutants reported in this study are presumably defective in the soluble enzyme system which was shown to catalyze the stereo-specific formation of myo-inositol from glucose-6-phospate in the yeast *Candida utilis* (CHARALOMPOUS and CHEN 1966), as follows:

$$\text{glucose-6-phosphate} \xrightarrow{\text{synthetase}} \text{inositol-1-phosphate}$$

$$\text{inositol-1-phosphate} \xrightarrow{\text{phosphatase}} \text{inositol} + P_i$$

No biochemical evidence is yet available to indicate whether the synthetase represents the action of a single enzyme or whether *in vivo* the phosphatase is strictly specific for inositol-1-phosphate. It is entirely possible that cyclization of the hemi-acetal of glucose-6-phosphate to the fully reduced, six-membered carbon ring of inositol requires participation of a greater number of enzymes than is implied in the above scheme. This becomes evident on considering the complexity of the synthetase reaction, which must involve at least two steps (ring opening and ring closure) and which requires, in addition, the participation of NAD without net oxido-reduction. The identification in this study of ten loci among a series of inositol-requiring mutants lends further support to these ideas and suggests that at least ten polypeptides are involved in the enzymatic synthesis of inositol. Further investigation will concentrate on elucidating the roles of these ten loci in the inositol pathway.

The ino1 *locus*: Thirty-six mutants were shown to contain defects at the *ino1* locus. The complementation relationships between these mutants proved to be complex. The circular complementation map for *ino1* alleles (Figure 1) consisted of thirteen subclasses (Table 1), and could be simplified by excluding weak complementation responses to a linear map consisting of three definitive regions (Figure 2). These results can be interpreted according to two alternative hypotheses. The simplest view assumes that *ino1* alleles represent defects in a single gene which codes for the synthesis of an enzyme having identical, repeating subunits. Through the formation of a hybrid enzyme in heteroallelic diploids, compensating interactions might nullify the effects of mutations causing alterations at different sites along the polypeptide, resulting in an enzyme whose conformation is compatible with partial activity. According to this hypothesis, complementation responses among pairs of *ino1* alleles would be intracistronic (FINCHAM and DAY 1963).

The evidence from complementation analysis is also compatible with the assumption that *ino1* alleles represent defects in a series of adjacent genes having related functions. Weak and vigorous complementation responses could be explained by assuming that the former represent intracistronic while the latter represent intercistronic complementation. Previous studies on other systems lend credence to this interpretation (GILES 1963; FINK 1966).

Distinguishing between these possibilities will require a combination of genetic and biochemical evidence derived from the further characterization of nonsense mutations and the assignment of specific enzyme defects in the mutants.

Cross-feeding: Cross-feeding reactions depend upon the accumulation of chemical intermediates prior to the site of mutational blocks and the diffusion of such intermediates across cell membranes. The nature of intermediates in the inositol biosynthetic pathway provides a plausible explanation for the absence of cross-feeding between inositol-requiring mutants. Except for the end product of the reaction sequence, the intermediates are phosphorylated and would not be expected to cross membrane barriers (CHARALOMPOUS and CHEN 1966). The inability of wild-type strains to cross-feed the mutants can be explained by the small pool size for free inositol in wild-type cells (ANGUS and LESTER 1972) and the large amount of exogenous inositol required to support growth of the mutants (WATKINS *et al.* unpublished observations).

Isolation of a potential regulatory mutant: Evidence has been presented for the existence of an independently segregating gene (*r*) which modifies the expression of a leaky inositol auxotroph (*ino1-16*) (Table 4). While the recessive gene *r* has no detectable effect on growth in strains which are not auxotrophic for inositol, it results in the complete failure of growth when combined with an allele of the *ino1* locus which, by itself, results in leaky growth. We offer the hypothesis that the product of gene *r* may influence either the rate of synthesis or the level of activity of an enzyme controlled by the *ino1* locus. Enzymological studies concerned with the effect of this potential regulatory mutation on enzyme activity are in progress.

Inositol-less death: Evidence has been presented that inositol-requiring yeast cells die precipitously when starved for inositol (Figure 3). The important features of this phenomenon are: (a) disruption of protein synthesis with cycloheximide prevents cell death in inositol-less medium; (b) starvation for an amino acid requirement in the same strain under conditions where inositol is provided in the medium does not result in cell death; and (c) the efficiency of survival among respiratory-deficient petites is much greater than among respiratory-competent cells.

Calculations based on the actual numbers of viable petites at various points in time in inositol-starved cultures suggest that, irrespective of the increase in petite frequency relative to total viable cells, inositol starvation does not appear to induce the formation of new petites, but rather it provides strong pressure for selective enrichment of preexisting petites and petites derived from them through the process of mitotic budding.

These properties of inositol-requiring mutants may provide a means of selective enrichment for other types of auxotrophs. Since disruption of protein synthesis prevents cell death, co-starvation for inositol and any given amino acid should result in the enrichment of amino acid auxotrophs among surviving cells. The high efficiency of enrichment already demonstrated in mutant selection based on inositol-less death in Neurospora (LESTER and GROSS 1959) and fatty acid-less death in yeast (HENRY and HOROWITZ 1975) indicates that it will be

possible to obtain high yields of auxotrophic and other catagories of mutants in yeast. Development of methods for mutant enrichment are currently in progress.

Similar results demonstrating death of yeast cells have been obtained in studies of saturated fatty acid-requiring mutants (HENRY 1973). Since both inositol and fatty acids are known to be directly involved in the lipid portion of membranes, the combined results from these two systems raise fundamental questions regarding the integration of lipid synthesis with macromolecular synthesis in a program of balanced growth. A strong indication that protein and lipid synthesis are not tightly coupled is provided by the results of two converse situations: (a) inhibition of protein synthesis results in a shift toward a stable maintenance state with preservation of cell integrity and viability and (b) inhibition of lipid synthesis by starvation for an essential lipid requirement results in unbalanced growth and cell death. In the latter case the cells are apparently unable to enter a stable resting state, which may have implications for mechanisms of cell cycle control.

It is tempting to speculate that in the case of inositol starvation of an inositol auxotroph or fatty acid starvation of a fatty acid auxotroph, membrane synthesis ceases in the absence of required phospholipid precursors without any relay system which communicates the defect to the internal cell machinery. According to this tentative hypothesis, on-going protein synthesis in a cell which is incapable of synthesizing new membrane would eventually be lethal.

We wish to express our appreciation to T. WATKINS and A. KEITH, who isolated the first inositol requirer and who first elicited our interest in this problem.

This study was supported by grants from the N.I.H., GM 19629 and GRSG 5 SOI RRO5397. MICHAEL R. CULBERTSON is a trainee in genetics supported by an N.I.H. Training Grant in Genetics, GM000110. This work is from a dissertation to be submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in the Sue Golding Graduate Division of the Albert Einstein College of Medicine.

LITERATURE CITED

ANGUS, W. W. and R. L. LESTER, 1972 Turnover of inositol and phosphorus containing lipids in *Saccharomyces cerevisiae*; extracellular accumulation of glycerophosphorylinositol derived from phosphatidylinositol. Arch. Biochem. Biophys. **151**: 483–495.

CHARALOMPOUS, F. C. and I. CHEN, 1966 Inositol-1-phosphate synthetase and inositol-1-phosphate phosphatase from yeast. pp. 698–704. In: *Methods in Enzymology*, Vol. IX. Edited by S. P. COLOWICK and N. O. KAPLAN. Academic Press, New York.

CULBERTSON, M. R. and S. A. HENRY, 1973 Genetic analysis of hybrid strains trisomic for the chromosome containing a fatty acid synthetase gene complex (*fas1*) in yeast. Genetics **75**: 441–458. ——, 1974 Inositol mutants in yeast. Genetics **77**: s15–16.

DAWSON, R. M. C., 1966 The metabolism and function of polyphosphoinositides in the nervous system. pp. 57–67. In: *Cyclotols and Phosphoinositides*. Edited by H. KINDL. Pergamon Press, Oxford.

FINCHAM, J. R. S. and P. R. DAY, 1963 *Fungal Genetics*. Edited by W. O. JAMES. F. A. DAVIS Company, Philadelphia.

FINK, G. R., 1966 A cluster of genes controlling histidine biosynthesis in *Saccharomyces cerevisiae*. Genetics **53**: 445–459.

FOGEL, S. and D. D. HURST, 1967 Meiotic gene conversion in yeast tetrads and the theory of recombination. Genetics **57**: 455–481.

GILES, N. H., 1963 Genetic fine structure in relation to function in *Neurospora*. Proc. 11*th* Intern. Congr. Genetics **2**: 17–30.

HAWTHORNE, D. C., 1969 Identification of nonsense codons in yeast. J. Molec. Biol. **43**: 17–75.

HAWTHORNE, D. C. and R. K. MORTIMER, 1960 Chromosome mapping in Saccharomyces centromere-linked genes. Genetics **45**: 1085–1110.

HENRY, S. A., 1973 Death resulting from fatty acid starvation in yeast. J. Bacteriol. **116**: 1293–1303.

HENRY, S. A. and B. HOROWITZ, 1975 A new method for mutant selection in *Saccharomyces cerevisiae*. Genetics (In press.)

HENRY, S. A. and S. FOGEL, 1971 Saturated fatty acid mutants in yeast. Molec. Gen. Genet. **113**: 1–19.

HENRY, S. A. and A. D. KEITH, 1971 Membrane properties of saturated fatty acid mutants of yeast revealed by spin labels. Chem. Phys. Lipids **7**: 245–265.

KURAISHI, H., Y. TAKAMURA, T. MIZUNAGA and T. UEMURA, 1971 Factors influencing death of biotin deficient yeast cells. J. Gen. Appl. Microbiol. **17**: 29–42.

LESTER, H. E. and S. R. GROSS, 1959 Efficient method for selection of auxotrophs in *Neurospora*. Science **139**: 572.

LESTER, R. L. and M. R. STEINER, 1968 The occurrence of diphosphoinositide and triphosphoinositide in *Saccharomyces cerevisiae*. J. Biol. Chem. **243**: 4889–4893.

LINDEGREN, C. C., 1949 *The Yeast Cell: Its Genetics and Cytology*. Educational Publishers, Inc., St. Louis.

LINDEGREN, G., Y. L. HWANG, Y. OSHIMA and C. C. LINDEGREN, 1965 Genetical mutants induced by ethyl methanesulfonate in *Saccharomyces*. Can. J. Genet. Cytol. **7**: 491–499.

LYNEN, F., 1967 The role of biotin dependent carboxylations in biosynthetic reactions. Biochem. J. **102**: 381–400.

PERKINS, D. D., 1949 Biochemical mutants in the smut fungus *Ustilago maydis*. Genetics **34**: 607–626.

PONTECORVO, G., J. A. ROPER, L. M. HEMMONS, K. D. MACDONALD and A. W. J. BUFTON, 1953 The genetics of *Aspergillus nidulans*. Advan. Genet. **5**: 141–238.

PROUDLOCK, J. W., J. M. HASLAM and A. W. LINNANE, 1971 Biogenesis of mitochondria. The effects of unsaturated fatty acid depletion on lipid composition and energy metabolism of a fatty acid desaturase mutant of *Saccharomyces cerevisiae*. Bioenergetics **2**: 327–349.

RIDGWAY, C. J. and H. C. DOUGLAS, 1958 Unbalanced growth of yeast due to inositol deficiency. J. Bacteriol. **76**: 163–166.

SCHWEIZER, E. and H. BOLLING, 1970 A *Saccharomyces cerevisiae* mutant defective in saturated fatty acid biosynthesis. Proc. Natl. Acad. Sci. U.S. **67**: 660–666.

SHIMIDA, S., H. KURAISHI and K. AIDA, 1972 Unbalanced growth and death of yeast due to pantothenate deficiency. J. Gen. Appl. Microbiol. **18**: 383–397.

STEINER, S., S. SMITH, C. J. WAECHTER and R. L. LESTER, 1969 Isolation and partial characterization of a major inositol-containing lipid in baker's yeast, mannosyl-diinositol, diphosphoryl-ceremide. Proc. Natl. Acad. Sci. U.S. **64**: 1042–1048.

STEINER, S. and R. L. LESTER, 1972a Studies on the diversity of inositol-containing yeast phospholipids: incorporation of 2-deoxyglucose into lipid. J. Bacteriol. **109**: 81–88. ——, 1972b *In vitro* studies as phospholipid biosynthesis in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **260**: 222–243. ——, 1972c Metabolism of diphosphoinositide and triphosphoinositide in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **260**: 82–87.

VAGELOS, P. R., P. W. MAJERUS, A. W. ALBERTS, A. R. LARABEE and B. P. AIHAUD, 1966 Structure and function of acyl carrier protein, Fed. Proc **25:** 1485–1495.

WATKINS, T., M. A. WILLIAMS, A. D. KEITH, M. R. CULBERTSON and S. A. HENRY, unpublished observations.

WISNIESKI, B. J., A. D. KEITH and M. R. RESNICK, 1970 Double bond requirement in a fatty acid desaturase mutant of *Saccharomyces cerevisiae*. J. Bacteriol. **101:** 160–165.

Corresponding editor: F. SHERMAN