Accumulation of Neutral Lipids in *Saccharomyces carlsbergensis* by myo-Inositol Deficiency and Its Mechanism

RECI Procal Regulation of Yeast Acetyl-CoA Carboxylase by Fructose Bisphosphate and Citrate*

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The abnormal accumulation of lipids due to myo-inositol deficiency in *Saccharomyces carlsbergensis*, and the mechanism involved was investigated.

The deficient cells contained much more neutral lipids with a greater ratio of unsaturated fatty acids compared to the supplemented cells, whereas there was no significant change in their phospholipid contents. The biosynthesis of fatty acids and sterols from acetate, and of triacylglycerols and sterol esters from palmitate was markedly augmented in the deficient cells. Acetyl-CoA carboxylase activity of the deficient supernatant was 2- to 5-fold higher than that of the supplemented. However, the activity from both sources was not significantly different after Sephadex G-25 gel filtration of the supernatant, suggesting the presence of low molecular effector(s) in the deficient supernatant. There was a great increase in acid-soluble glycogen, trehalose, and fructose-1,6-P, as well as a drastic decrease in citrate in the deficient cells. Their intracellular levels were calculated so that their effects on acetyl-CoA carboxylase was examined over the range of physiological concentration. Citrate strongly inhibited the enzyme activity of the supernatant, but it had no effect on the preparation after gel filtration. On the other hand, fructose-1,6-P, stimulated the enzyme activity both before and after gel filtration. The acetyl-CoA carboxylase activity in the gel filtrate was measured as a function of citrate concentration at several fixed concentrations of fructose-1,6-P, Citrate counteracted the activation by fructose-1,6-P, in a dose-dependent manner. Citrate lacked the inhibitory effect in the absence of glucose-1,6-P,

It was concluded from these results that neutral lipid accumulation in the deficient cells reflected an increase in the synthesis of fatty acids, at least partly based on an enhancement of acetyl-CoA carboxylase activity, and that the operation of a reciprocal regulation of the enzyme by fructose-1,6-P, and citrate caused a marked elevation of the enzyme activity in the deficient cells with a high fructose-1,6-P, level and a low citrate level.

Although myo-inositol and its phosphatides have been shown to be involved in several important physiological phenomena, myo-inositol is one of the few vitamins whose biochemical functions have not been clearly elucidated. It commonly occurs in nature in its form free, as phosphatides and as phytin. With special conditions it may take the forms of inositol-cyclic phosphate (1), glycercphosphoryl inositol (2), and galactopyranosyl inositol (3). The functions of the phosphatides in excited membranes were comprehensively reviewed by Michell (4).

The role of inositol as a lipotropic factor was first found in 1941 by Gavin and McHenry (5), and a great deal of work has been published since then (6-9). A problem in these studies has been obtaining an inositol-deficient state in animals. Fortunately, we have succeeded in producing an inositol deficiency in rats in a short period of time under a balanced dietary condition. This caused fatty livers (10), and we found that the deposition of triacylglycerols in the livers of the deficient rats resulted from stimulated lipolysis in the adipose tissues (11).

In 1955, Challinor and Rose first observed in microorganism the occurrence of lipid droplets in *Saccharomyces cerevisiae* grown in the absence of inositol (12). Lewin found a similar phenomenon in *Saccharomyces carlsbergensis* (13), and he reported that the deposit of lipids mainly consisted of triacylglycerols (14). Also, Paltauf and Johnston have examined the effects of various carbon sources on the lipid accumulation in inositol-deficient *S. carlsbergensis* (15).

This report deals with the analysis of the accumulated lipids in *S. carlsbergensis* (ATCC 9080), an auxotroph for myo-inositol, grown without inositol, and the mechanism involved in the lipid accumulation. In our studies directed toward clarifying the effect of inositol on lipid metabolism, the usage of a unicellular organism, *S. carlsbergensis* was selected (a) because of the easiness in obtaining an inositol-deficient state, (b) it has simple lipid metabolism, and (c) it is similar to...
animals in the phenomenon of lipid accumulation due to inositol deficiency.

The cultivation of *S. carlsbergensis* in a selected medium, which had been found to produce the most severe biochemical lesions during inositol deprivation (16, 17), resulted in a greater lipid deposition containing sterol esters and acylglycerols than previously reported (14, 15). The accumulation of neutral lipids by inositol deficiency was found to result from an increased synthesis of fatty acids and sterols, with a subsequent accelerated formation of acylglycerols and sterol esters.

The biosynthesis of long chain fatty acids in yeast (18–21) is catalyzed by three enzymatic systems, also present in mammalian cells: acetyl-CoA synthetase, acetyl-CoA carboxylase, and fatty acid synthetase. Although the activity of all these enzymes are known to be varied by nutritional manipulation, acetyl-CoA carboxylase has been the first to be claimed as a rate-limiting enzyme in fatty acid synthesis, and has been most extensively studied in relation to its regulation. Accordingly, our attention has been focused on the change of acetyl-CoA carboxylase activity due to the inositol deficiency.

The enzyme activity in the 100,000 × g supernatant fraction from the deficient cell homogenate has been found to be 2- to 5-fold higher than that from the supplemented. Changes in enzyme activities are caused by alteration in catalytic efficiency by allosteric effectors or by the synthesis or degradation of the enzyme. The results obtained have suggested the existence of low molecular effectors on acetyl-CoA carboxylase in the deficient supernatant fraction rather than an increase in the enzyme protein.

It is well known that in higher animals, carbohydrate metabolism closely interrelates lipid metabolism through changes in the activities of lipogenic enzymes such as acetyl-CoA carboxylase (22–25), fatty acid synthetase (26), glucose-6-P dehydrogenase, 6-P-glucuronate dehydrogenase, pyruvate kinase (27), pyruvate carboxylase (28), citrate-ATP lyase (29, 30), and malate dehydrogenase (27, 30). Furthermore, in the yeast, *S. cerevisiae*, Klein and co-workers (31–33) have studied the regulatory role of glycolytic enzymes on lipid metabolism through the enzyme protein.

The fluctuation in glycolytic and Krebs' cycle intermediates during inositol deficiency was investigated in reference to their possible roles in the regulation of the enzyme. The two intermediates, fructose-1,6-P₂ and citrate found to be markedly increased and decreased, respectively, in the deficient cells, have been shown to exert dual regulatory effects on acetyl-CoA carboxylase over the physiological range of their concentrations. These results will have important implications on the clarification of the mechanisms underlying the neutral lipid accumulation in the inositol-deficient cells.

**Experimental Procedure**

Strain and Growth Condition—Saccharomyces carlsbergensis 4228 (ATCC 30860) was used. The complete growth medium (16) contained per liter: 50 g of glucose, 5 g of potassium citrate, 1 g of citric acid, 5 g of casein hydrolysate, 3.75 g of ammonium sulfate, 0.56 g of KH₂PO₄, 0.4 g of KCl, 130 mg of MgSO₄, 2.5 mg of FeCl₃, 2.5 mg of MnSO₄, 130 mg of CaCl₂·2H₂O, 2.5 mg of calcium pantothenate, 250 μg of pyridoxine, 250 μg of thiamin, 25 μg of biotin, 600 μg of nicotinic acid, and 25 mg of myo-inositol. The pH was adjusted to 5.0 with 5 N HCl. The inoculum for the liquid culture was prepared by static cultivation of the yeast at 30°C for 20 h in the complete medium and 1 ml of suspension of the well-washed cells (absorbance at 620 nm = 1.0) was added to inoculate 200 ml of medium. The cells were collected aerobically in 500-ml Mylar flasks containing 200 ml of medium with and without the addition of inositol at 30°C for the indicated periods. The cells were collected by refrigerated centrifugation and were quickly washed with cold distilled water three times.

Extraction of Lipids and Its Analysis—The total lipids were extracted as follows (34): packed cells were mixed with 0.5 volume of water and 3.75 volumes of a mixture of chloroform/methanol (12:1) and occasionally shaken for 2 h at room temperature. Then, 1.25 volumes of water and chloroform were added and the solution was mixed well. The upper chloroform layer was removed, and the water layer containing the total lipids was filtered through silicone-treated phase-separating paper (Whatman Ipe). This extraction procedure for cell lipids gave the most reproducible result among the several procedures tested. The total lipids were separated into lipid classes by thin layer chromatography on Kiesel Gel H chromatoplate, using petroleum ether/ether/acidic acid (70/30/1) as a developing solvent. Total lipids were measured by the method described by Amenta (35), free fatty acids by Duncombe (36), sterols by Kenny (37), acetylglucosylers by Block and Darret (38), and phospholipids by Harris and Popat (39). The fatty acid composition of each lipid class was analyzed by gas-liquid chromatography as previously described (10).

Analyses Other than Lipids—DNA in the cells was extracted by a modified method of Ogur and Rosen (40), and determined according to the method by Cerioni (41). Protein in enzyme preparations was measured by Lowry's method (42).

**Metabolism of 1-14C Fatty Acids—** The cells of 48-h growth (approximately 400 μg of DNA) were incubated while shaking with 10 μCi of [1-14C] palmitic acid (53.3 mCi/mmol) or [1-14C] oleic acid (50.3 mCi/mmol) in 12 ml of 0.1 M KH₂PO₄ buffer (pH 5.0); the reaction was stopped by chilling. After incubation, the cells were quickly washed 3 times with cold water and the lipids extracted from the cells were hydrolyzed with 2 ml of 33% KOH at 37°C for 1 h. The fraction was extracted with 2 ml of petroleum ether, and after acidification of samples, the fatty acid fraction was similarly extracted. Aliquots of the extracts were transferred to scintillation vials and the radioactivity was counted as described.

**Enzyme Preparation for Acetyl-CoA Carboxylase—** The cells (approximately 5 to 7 g wet weight) harvested at 36- to 48-h cultivation were homogenized with 10 g of glass beads (0.45 to 0.50 mm) at 0°C for 30 s in 10 ml of 0.05 M phosphate buffer (pH 7.2) containing 0.6 mg mannitol and 5 mM disodium ethylenediaminetetraacetate at a Braun homogenizer. The resulting homogenate was consecutively centrifuged at 800 × g for 30 s, and aliquots of the reaction mixture were withdrawn for lipid analysis at the specified time. The lipids extracted from the cells were separated into lipid classes by thin layer chromatography and the radioactivity in the corresponding area was counted as described.

**Standard Assay of Acetyl-CoA Carboxylase—** The enzyme carboxylase was assayed by measuring the recovery of acid-stable radioactivity after incubating extract containing CO₂ from the method of Nakashima and Numa (43). The standard assay medium (total 0.8 ml) contained 50 mM Tris/HCl (pH 7.5), 10 mg of MgCl₂, 2.75 mM glutathione reduced, 3.75 mM ATP, 0.125 mM acetyl-CoA, 0.75 mg/ml of bovine serum albumin, 12.5 mM of NaHCO₃ containing 2 μCi of [1-14C]acetyl-CoA (1.3 to 3 μCi/mmol), and 0.5 ml of Bray's scintillation cocktail (60 g of naphthalene, 4 g of 2,5-diphenylxoxazol (PPO), 0.2 g of 1,4-bis-[2-(5-phenyloxazolyl)] benzene (POPOP), 100 mg of methanol, 20 ml of ethylene glycol in liter dioxane) and the radioactivity was counted. The reaction was linear up to at least 10 mg of protein in 100,000 × g supernatant fraction.

**Extraction of Intermediates—** The cells harvested at the specified time were quickly washed by ice-cold distilled water and 1.5 ml of 10% perchloric acid were immediately added to 1.5 ml of cell suspension in water containing approximately 1.35 mg of DNA cell. The mixture was kept for 30 min at room temperature and centrifuged at 20,000 × g for 10 min. An aliquot of the supernatant fluid was neutralized with a buffered potassium hydroxide mixture (44), the resulting precipitate

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Results

Growth and Lipid Contents in Cells Grown with and without Inositol—As shown in Fig. 1(a), the growth of Saccharomyces carlsbergensis in the absence of inositol was considerably inhibited in comparison to the growth in the presence of inositol. The viability of cells was assessed by methylene blue vital count and a gradual decrease in viability was observed in the inositol-deficient culture with cultivation time. However, within 48 h the dead cell counts seemed to be negligible in the cultures.

The total lipid content during cultivation for 72 h is depicted in Fig. 1(b). The lipid content in the supplemented cells was always unchanged or slightly decreased, while that in the deficient cells progressively increased with cultivation time, resembling the accumulation of acetoin in the deficient culture medium (17).

Composition of Lipid Deposits—The lipid contents of each lipid class extracted from the supplemented and deficient cells of 48-h growth are compared in Table 1. Under our cultivation conditions, the total lipids in the deficient cells were found to be approximately 10 times that in the supplemented cells. The contents of triacylglycerols, diacylglycerols, free fatty acids, sterol esters, and free sterols in the deficient cells were respectively 12-, 3-, 8-, 13-, and g-fold higher than those in the supplemented cells, whereas there was no significant change in their phospholipid content. It should be noted that such a marked increase in the contents of sterol esters and free sterols comparable to the increase in triacylglycerols, has never been observed by the previous workers (14, 15), and furthermore, the deposition of neutral lipids was greater than the values already reported. This may result from the usage of medium found to produce severe metabolic lesions on inositol deprivation (16, 17). The contents of triacylglycerols and sterol esters in the deficient cells gradually increased in a similar manner to the total lipids (Fig. 1(b)) as the cultivation proceeded up to 96 h while that of phospholipids was always within the range of the supplemented cells.

Fatty Acid Profile of Deposited Lipids—The profiles of the fatty acid distribution in each lipid class were compared between the supplemented and deficient cells (Fig. 2). Generally, the deficient cells of 48-h growth contained more unsaturated fatty acids such as palmitoleic and oleic acid, with fewer saturated fatty acids such as palmitic and stearic acid. This tendency was more remarkable in free fatty acid, triacylglycerol, and sterol ester fractions than phospholipid fraction.

Inositol deficiency also increased the ratio of C12 fatty acids to C16 fatty acids, especially in free fatty acid fractions. Although
Paltauf and Johnston (15) observed that the deficient cells contained more fatty acids of medium chain length without any difference in the saturation, our results show that the effect of inositol deficiency on saturation was greater than on the chain length. The unsaturation of fatty acids is known to require reduced pyridine nucleotides and molecular oxygen, therefore the reducing state of a cell is a critical factor. In fact, a morphological mutant of Neurospora crassa with decreased levels of NADPH and NADH has been found to contain only 20% as much of polyunsaturated fatty acids as the wild type in both the phospholipid and neutral lipid fractions (49, 50).

Therefore, pyridine nucleotides were extracted from the supplemented and deficient cells of 48-h growth and the amount was measured enzymatically. The level of pyridine nucleotides was greater in the deficient cells, but the ratio of NADH to NAD+ was not statistically different between the two cells.

**Incorporation of [1-14C]Acetate into Fatty Acid and Sterol Fractions**—The incorporation of [1-14C]acetate into lipids in the two types of cells was examined in order to determine whether the lipid accumulation in the deficient cells reflected an increase in lipogenesis or a decrease in lipid decomposition. For this purpose, the cells separated from the 48-h culture and well-washed, were incubated for 2 h with [1-14C]acetate in phosphate buffer to dissociate the alteration of cells per se, from effects of abnormal metabolites in the culture fluid. The incorporation of [1-14C]acetate into the lipids was linear at least up to 2 h and there was no significant difference in the uptake of [1-14C]acetate between the supplemented and deficient cells. As Table II shows, the incorporation of [1-14C]acetate into fatty acids and sterols was remarkably augmented in the deficient cells compared to the supplemented cells, clearly indicating an increase in fatty acid synthesis and steroidogenesis due to the inositol deficiency. To test the possibility that the abnormal metabolites accumulated in the deficient culture fluid might stimulate incorporation of [1-14C]acetate into the lipids, the washed cells were resuspended in either the supplemented or the deficient culture fluid, and incubated with [1-14C]acetate for 2 h at 30°C. The results obtained in this experiment were similar to those of the experiment with cell suspension in phosphate buffer. Therefore, the deficient cells synthesized more fatty acids and sterols independent of the environmental change.

**Metabolic Fate of [1-14C]Palmitic Acid in Supplemented and Deficient Cells**—Because fatty acid synthesis was enhanced in the deficient cells, the metabolic fate of free fatty acids was investigated by incubating the washed cells of 36-h growth with [1-14C]palmitic acid, a predominant fatty acid in the yeast by measuring the incorporation of the radioactivity into the lipid classes (Table III). Almost all of [1-14C]palmitic acid added to the incubation medium, was taken up into the cells within 2 min. From the initial specific activities of the free fatty acids and radioactivity in the free fatty acid fraction at 15 min (refer to the legend of Table III for the details of calculation), the turnover rates of the free fatty acids for the first 15 min in the supplemented and deficient cells were calculated as 4.97 and 6.91 μmol/mg of DNA, respectively, suggesting that the turnover of fatty acids in the deficient cells was accelerated rather than depressed.

Furthermore, the disappearance rate of fatty acids from the total lipids was calculated from the decrease in the radioactivity in the total lipids and the initial specific activity of the free fatty acids. The radioactivity in the total lipids at 15 min, 5 h, and 12 h of incubation was 1.99 x 10⁶, 1.33 x 10⁶, 1.20 x 10⁶ dpm/mg of DNA in the supplemented cells, and 5.07 x 10⁶, 1.87 x 10⁶, 1.31 x 10⁶ dpm/mg of DNA in the deficient cells. Thus, the disappearance rate of fatty acid in the supplemented cells were 3.77, 4.16, and 4.36 μmol/mg of DNA for 15 min, 5 h, and 12 h, respectively, while those in the deficient cells were 5.35, 8.12, and 8.64 μmol/mg of DNA. These results imply that the delayed catabolism of fatty acids was not primarily involved in the elevation of lipid contents in the deficient cells. The production rates of the phospholipids, triacylglycerols, and sterol esters from fatty acids in the supplemented and deficient cells were estimated as described in the legend of

**Table II**

| Cells                    | Fatty acids | Sterols |
|--------------------------|-------------|---------|
| **Radioactivity incorporated dpm/mg DNA** |              |         |
| Supplemented             | 1.86 x 10⁶  | 2.65 x 10⁶ |
| Deficient                | 7.24 x 10⁴  | 3.22 x 10⁴ |

**Fig. 2.** The fatty acids distribution of the lipids from the inositol-supplemented and -deficient yeast. The lipids extracted from the inositol-supplemented and -deficient yeast of 48-h growth were separated into lipid classes by thin layer chromatography. The fatty acids methylester from each fraction were prepared as described in the text and analyzed by gas-liquid chromatography. Filled columns represent the values for the supplemented cells and open columns those for the deficient cells.
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No significant difference in the production rates of phospholipids was observed between the supplemented and deficient cells whereas the production rates of triacylglycerols and sterol esters at the 15-min incubation were 6 and 3 times greater in the deficient cells than in the supplemented cells. Another experiment with [1-¹⁴C]palmitate in place of [1-¹³C]palmitate produced a similar result. These data indicate that the turnover of fatty acids in the deficient cells was accelerated by fatty acids were deposited primarily as triacylglycerols and sterol esters, consistent with the observations in Table I.

Acetyl-CoA Carboxylase Activity from Supplemented and Deficient Yeast—The results described above strongly indicated that the synthesis of fatty acids was stimulated in the deficient yeast not by an environmental change but by the alteration of a property in the cells. Accordingly, the activity of acetyl-CoA carboxylase which is considered to be a rate-limiting enzyme in fatty acid synthesis in animal (51) was compared in the two cells. The assay of the enzyme activity was performed essentially by the method of Nakanishi and Numa (43), but the addition of citrate into the assay medium and preincubation with it was omitted because unlike the procedure for the animal enzyme it appeared to be unnecessary. The enzyme activity was dependent on ATP, bicarbonate, and acetyl-CoA. The standard assay medium (total volume 0.8 ml) generally contained 2 to 5 mg of protein depending on the enzyme preparations. As anticipated, the acetyl-CoA carboxylase activity in the 10,000 × g supernatant fraction from the deficient cells of 42-h growth was 2- to 5-fold higher than that from the supplemented cells. Since the enzyme activity from the supplemented and deficient cells stayed constant during 37- to 48-h cultivation, the cells harvested during the period were used as enzyme source. Also the dead cell counts were negligible during this period and sufficient lipid deposition was observed in the deficient cells by this time.

In Table IV, the acetyl-CoA carboxylase activity from the two types of cells of 42-h growth are comparatively shown in the various enzyme preparations. As mentioned above, the activities in the 10,000 × g or 100,000 × g supernatant fraction from the deficient cells were approximately 3 times higher than those from the supplemented cells. However, Sephadex G-25 chromatography of the 100,000 × g supernatant fraction decreased the activity from the deficient cells to the level of the activity from the supplemented cells. Overnight dialysis against a phosphate buffer or maintenance of the 100,000 × g supernatant fraction at cold for 24 h produced a similar effect.

Generally, changes in enzyme activities reflect either alterations in catalytic efficiency by allosteric effectors or changes in the amount of the enzymes. These results suggest that the high enzyme activity in the supernatant fraction from the deficient cells was caused by the presence of low molecular effector(s) on acetyl-CoA carboxylase rather than an increase in the enzyme content.

Although the dissociation, or decomposition, or both, of the low molecular effector(s) might be responsible for the observed decrease in the activity of the deficient cells by overnight maintenance at room temperature, the stability of the enzyme might have been reduced by inositol deficiency; for Sullivan and Debusk (52) have reported a reduction in the heat stability of some of the enzymes obtained from N. crassa grown with low inositol concentration.

The heat stability of the enzyme measured by incubating the

Table III

| Lipid Class          | Radioactivity (dpm/mg DNA) | Production rates (nmol/mg DNA) |
|----------------------|----------------------------|-------------------------------|
| Supreme Polysaccharides | 9.48 × 10⁸ (64.5)           | 230                           |
| Free fatty acids     | 1.02 × 10⁹ (51.5)           |                               |
| Triacylglycerols     | 1.68 × 10⁸ (0.837)          | 2.72                          |
| Sterol esters        | 3.32 × 10⁷ (0.166)          | 1.61                          |
| Total lipids         | 1.99 × 10⁸ (100)            |                               |
| Deficient Polysaccharides | 4.73 × 10⁸ (9.28)           | 221                           |
| Free fatty acids     | 4.46 × 10⁸ (89.0)           | 16.9                          |
| Triacylglycerols     | 5.44 × 10⁸ (1.10)           |                               |
| Sterol esters        | 5.03 × 10⁷ (0.099)          | 4.69                          |
| Total lipids         | 5.09 × 10⁸ (100)            |                               |

Table IV

| Enzyme preparation       | Incorporation of [¹⁴C]CO₂ | 10⁻³ × dpm/mg protein |
|--------------------------|--------------------------|----------------------|
| Supreme Polysaccharides  |                          |                      |
| Supreme                  | 2.03 ± 0.034             |                      |
| Deficient                | 6.02 ± 0.311             |                      |
| 100,000 × g supernatant  | 1.78 ± 0.022 (100.0%)    | 5.28 ± 0.201 (100.0%)|
| Sephadex G-25            | 1.73 ± 0.074 (97.2%)     | 1.62 ± 0.020 (30.7%) |
| Dialyzed                 | 0.831 ± 0.022 (46.7%)    | 1.36 ± 0.051 (25.8%) |
| Overnight                | 1.51 ± 0.119 (55.7%)     | 2.10 ± 0.042 (39.8%) |

The data indicate the mean ± S.E. of the triplicates.

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10,000 × g supernatant fraction for 5 to 20 min at 45° was greater with the deficient preparation than with the supplemented. This may result from the protection of acetyl-CoA carboxylase molecule by low molecular effector(s).

Changes in Levels of Metabolites due to Inositol Deficiency—In an attempt to explore the low molecular effectors on acetyl-CoA carboxylase suggested to be in the supernatant fraction from the deficient cell homogenate, the levels of intermediates in carbohydrate metabolism were comparatively examined, because of a known close relation between the activity of lipogenic enzymes and carbohydrate metabolism, and of the occurrence of some altered metabolites in the examined, because of a known close relation between the activity of lipogenic enzymes and carbohydrate metabolism, and of the occurrence of some altered metabolites in the inositol-deficient culture (13, 16, 17, 53–55).

To analyze the intermediates, the fermentation of yeast was immediately stopped by adding 10% perchloric acid (final concentration 5%) into the culture or into the packed cells collected at 36 h after inoculation. Around this time of cultivation, there was a sufficient increase in acetyl-CoA carboxylase activity in the deficient cell preparation. In Table V, the intermediates expressed per mg of DNA from the deficient preparation than with the supplemented cells. Trehalose and fructose-1,6-P₂ in the deficient cells were 7 and 4 times that of the supplemented cells, whereas a drastic decrease in citrate results in the deficient cells compared to the supplemented and deficient cells are compared. Among the intermediates measured, there is a great increase in the content of trehalose and fructose-1,6-P₂ in the deficient cells and fructose-1,6-P₂, whereas a drastic decrease in citrate results in the deficient cells compared to the supplemented and deficient cells. Trehalose and fructose-1,6-P₂ in the deficient cells were 7 and 4 times that of the supplemented cells, respectively, while only one-third of citrate in the supplemented cells was found in the deficient cells. There was also an increase in acid-soluble glycogen and glucan, and a slight but significant increase in P-enolpyruvate and pyruvate in the deficient cells. The cofactors, ATP, ADP, NAD⁺, and NADH, were all significantly higher in the deficient cells than in the supplemented cells. The content of AMP was unchanged. The reduction states (NAD⁺/NADH) in both types of cells were in the same range (1.09 for the supplemented and 1.04 for the deficient).

Measurement of Intracellular Volume in Cells—The extracellular water volume in packed cells was measured by using dextran, and the intracellular volume were obtained by the conventional method as described under “Experimental Procedure.” As shown in Table VI, the whole cell volume expressed relative to DNA of the deficient cells was approximately 40% greater than that of the supplemented, and this increase was exclusively ascribable to an increase in the volume of the cell wall fraction of the deficient cells. No significant change was observed in the volumes of the particulated component and the cytoplasmic fraction of two types of cells. The increase in the volume of cell walls in the deficient cells probably reflects an accumulation of glucan due to inositol deficiency as Ghosh and co-workers (55) have already observed.

Intracellular Concentration of Metabolites—Prior to an investigation of possible regulatory roles of the intermediates on acetyl-CoA carboxylase, the intracellular concentrations of the intermediates were calculated from the data in the first and

### Table V

| Metabolites                  | Cells            |
|------------------------------|------------------|
|                              | Supplemented     | Deficient      |
|                              | µmol/mg DNA      | µmol/mg DNA   |
| Polysaccharides              |                  |
| Glycogen                     |                  |
| Acid-soluble                 | 103 ± 1.0 (4)    | 228 ± 2.2 (4) |
| Alkali-soluble               | 32.4 ± 1.02 (4)  | 39.8 ± 2.50 (4) |
| Glucan                       | 107 ± 1.7 (4)    | 310 ± 1.9 (4) |
| Mannan                       | 87.6 ± 0.79 (4)  | 104 ± 1.5 (4) |
| Trehalose                    | 5.61 ± 0.228 (4) | 38.7 ± 0.34 (4) |
| Glycolytic intermediates     |                  |
| Glucose-1-P                  | Trace            | Trace          |
| Glucose-6-P                  | 0.112 ± 0.0020 (3) | 0.092 ± 0.0005 (3) |
| Fructose-1,6-P₁              | 0.194 ± 0.0052 (6) | 0.539 ± 0.0047 (5) |
| Dihydroxyacetone-P           | 0.037 ± 0.0032 (3) | 0.041 ± 0.0051 (3) |
| Glyceraldehyde-P             | Trace            | Trace          |
| P-enolpyruvate               | 0.044 ± 0.0072 (5) | 0.078 ± 0.0061 (5) |
| Pyruvate                     | 0.085 ± 0.0068 (6) | 0.140 ± 0.0147 (6) |
| Lactate                      | 0.518 ± 0.0310 (4) | 0.683 ± 0.0112 (4) |
| Krebs cycle intermediates    |                  |
| Citrate                      | 3.49 ± 0.085 (6)  | 1.96 ± 0.081 (6) |
| Oxalacetate                  | 0.140 ± 0.0187 (4) | 0.17            |
| Malate                       | 0.419 ± 0.012 (4) | 0.479 ± 0.0330 (6) |
| Adenine & pyridine nucleotides |                |
| ATP                          | 0.122 ± 0.0065 (5) | 0.605 ± 0.0296 (4) |
| ADP                          | 0.275 ± 0.0275 (6) | 0.575 ± 0.0330 (6) |
| AMP                          | 0.962 ± 0.0330 (6) | 1.044 ± 0.0193 (5) |
| NAD⁺                         | 0.082 ± 0.0004 (5) | 0.275 ± 0.0163 (4) |
| NADH                         | 0.075 ± 0.0009 (4) | 0.262 ± 0.0428 (4) |

* Significant (p < 0.01).
* Significant (p < 0.05).
third columns of Tables V and VI, and shown in the second and fourth columns of Table V. It should be noted that there was a big fluctuation between the supplemented and deficient cells in levels of fructose-1,6-P₂ and citrate which have been known to have regulatory functions on a number of enzymes in yeast and mammals.

Effect of Citrate on Acetyl-CoA Carboxylase Activity—In contrast to an augmentation in acetyl-CoA carboxylase activity in the 100,000 × g supernatant fraction from the deficient cells, the level of citrate which is known to activate acetyl-CoA carboxylase from several sources was markedly reduced in the deficient cells. Thus, the effect of citrate on the enzyme preparation from the yeast was examined. The enzyme activity in the 100,000 × g supernatant fraction from the deficient cells, was always 2- to 5-fold higher than that from the supplemented cells depending on the preparations. An addition of 5 to 50 mM citrate into the assay medium strongly inhibited the activity from both sources in a dose-dependent manner as shown in Fig. 3(a), where inhibition is expressed as the percentage of activity relative to that of samples incubated in the absence of citrate. Preincubation with citrate was not needed to cause the inhibitory effect. Furthermore, the magnitude of the inhibition by citrate was much greater with the deficient cells than with the supplemented cells. Fifty per cent inhibition was observed at 25 and 10 mM citrate with the supplemented and deficient enzyme preparations, respectively. Fig. 3(b) represents the effect of citrate on the acetyl-CoA carboxylase activity after Sephadex G-25 chromatography of the 100,000 × g supernatant fractions. By gel filtration, the inhibitory effect of citrate completely diminished up to 20 mM. Citrate neither simulated nor inhibited the acetyl-CoA carboxylase activity in the filtrate, coinciding with the results in brewers’ yeast by Matsushashi and his co-workers (18). A slight inhibition of the activity was observed at 50 mM of citrate. In this experiment, the enzyme activities in the absence of citrate before and after the gel filtration were 5.02 × 10⁻⁴ and 1.60 × 10⁻³ dpm/mg of protein from the deficient versus 1.36 × 10⁻⁴ and 1.22 × 10⁻³ dpm/mg of protein from the supplemented.

Acetyl-CoA carboxylase from rat liver was prepared by overnight dialysis of the 100,000 × g supernatant of the homogenate in 0.25 M sucrose against 0.1 M potassium phosphate (pH 7.0); its responsiveness to citrate was tested in the gel filtration were 5.02 × 10⁻³ and 1.60 × 10⁻³ dpm/mg of protein from the deficient versus 1.36 × 10⁻³ and 1.22 × 10⁻³ dpm/mg of protein from the supplemented.

The responsiveness of the enzyme to stimulation by 2 mM fructose-1,6-P₂ was compared in the various enzyme preparations before and after the gel filtration. The magnitude of the activation by 2 mM fructose-1,6-P₂ was much greater in the supplemented 100,000 × g supernatant fraction (7-fold) than in the deficient (2-fold), and furthermore, the magnitude was

| Table VI |
| --- |
| **Intracellular volume of inositol-supplemented and -deficient yeast** |

The suspension of 36-h growth cells (approximately 2 mg of DNA) was centrifuged at 3,000 rpm for 10 min and the whole cell volume was determined using dextran as described in the text. The cell wall and particulated components were separated by centrifuging the cell homogenate at 800 × g for 10 min and 100,000 × g for 60 min, respectively. The volume of the cytoplasm was calculated by subtraction.

| Intracellular volume (per cent of whole cells) | Supplemented | Deficient |
| --- | --- | --- |
| ml/cells containing 10 mg DNA | | |
| Whole cell | 7.306 (100.0) | 10.272 (100.0) |
| Cell wall | 2.422 (33.2) | 5.178 (50.3) |
| Particulated components | 2.022 (27.7) | 2.994 (20.4) |
| Cytoplasm | 2.861 (39.2) | 3.011 (29.3) |

**Fig. 3.** Acetyl-CoA carboxylase activity as a function of citrate concentration. Acetyl-CoA carboxylase activity in the 100,000 × g supernatant (a) and in the Sephadex G-25 gel filtrate of the 100,000 × g supernatant (b) from the homogenate of 48-h growth cells was measured in the standard assay medium in the presence of various concentrations of citrate. O, activity from the supplemented cells; O, activity from the deficient cells. The enzyme activity from the supplemented and deficient cells in the absence of citrate was 3.92 × 10⁵ dpm/mg of protein and 7.44 × 10⁴ dpm/mg of protein respectively, in (a), and 1.22 × 10⁴ dpm/mg of protein and 1.60 × 10⁴ dpm/mg of protein, respectively, (b). Each point represents the mean of the triplicates.

**Fig. 4.** Acetyl-CoA carboxylase activity as a function of fructose-1,6-P₂ (a) and glucose-6-P (b) concentration. The 100,000 × g supernatant of the 42-h growth cell homogenate was passed through a column of Sephadex G-25, and acetyl-CoA carboxylase activity in the filtrate was measured in a standard assay medium in the presence of various concentrations of fructose-1,6-P₂ or glucose-6-P. O indicates the activity from the supplemented cells and O that from the deficient cells. Each point represents the mean of the triplicates.
unchanged with the supplemented enzyme preparation before and after gel filtration, whereas the degree of the activation was greatly increased with the deficient enzyme preparation after gel filtration to that with the supplemented preparation.

Klein and co-workers have tested various glycolytic intermediates for a stimulatory effect on acetyl-CoA carboxylase (31) and fatty acid synthesis (32, 33) in Saccharomyces cerevisiae, and have observed that fructose-1,6-P₂ has a stimulatory effect on acetyl-CoA carboxylase. But the effect he found was much less than that we found, even above the physiological concentration of fructose-1,6-P₂.

Effects of Glucose-6-P, Glycerol-3-P, Inositol, and Cyclic AMP—Fig. 4(b) represents the acetyl-CoA carboxylase activity as a function of glucose-6-P concentration. Glucose-6-P (0 to 5 mM) slightly stimulated the enzyme activity, and the responsiveness to it was similar in both enzyme preparations. Glucose-6-P is unlikely to be involved in the augmentation of acetyl-CoA carboxylase activity in the deficient yeast. The reasons for this are (a) no change occurred in the levels of glucose-6-P between the supplemented and deficient cells, (b) the concentration required for the activation is much higher than the intracellular concentration, and (c) the degree of activation by glucose-6-P is much less than that by fructose-1,6-P₂, inositol, and cyclic AMP in the presence of 1 mM theophylline failed to affect the acetyl-CoA carboxylase activity from both types of cells. Glycerol-3-P was reported to stimulate acetyl-CoA carboxylase from S. cerevisiae at the low concentration of 2 mM (31, 32).

Cyclic AMP has been shown to inhibit lipogenesis in vivo in rat liver (50), and to inhibit directly acetyl-CoA carboxylase from the same tissue (57, 58), but the effect of cyclic AMP on this enzyme is still controversial.

Counteraction by Citrate of Fructose-1,6-P₂ Activation of Acetyl-CoA Carboxylase—The acetyl-CoA carboxylase activity in the supernatant fraction from the deficient cells was inhibited by citrate to a greater degree (Fig. 3(a)) and stimulated by fructose-1,6-P₂, to a lesser degree than that from the supplemented cells, whereas the enzyme preparation after Sephade G-25 chromatography was not responsive to citrate inhibition (Fig. 3(b)), but was still very sensitive to fructose-1,6-P₂ stimulation (Fig. 4). These results were interpreted to mean that the enzyme in the deficient cells was activated by the presence of some effector(s) one of which may be fructose-1,6-P₂, and that exogenously added citrate reversed the activation by effector(s).

Accordingly, the following experiment was designed to test the hypothesis for a reciprocal regulation of the acetyl-CoA carboxylase by fructose-1,6-P₂ and citrate in vivo. Fig. 5 shows the change of acetyl-CoA carboxylase activity in the gel filtrate fraction from the supplemented cells at variable concentration of fructose-1,6-P₂ and citrate over the physiological range. The supplemented and deficient cells responded to fructose-1,6-P₂ and citrate in an identical manner. In the absence of citrate, an addition of 0.5, 1, and 1.5 mM fructose-1,6-P₂ elevated the activity 1.5-, 4-, and 7-fold, respectively, above the activity without fructose-1,6-P₂. An addition of 5 to 20 mM citrate to this reaction system counteracted the activation by fructose-1,6-P₂ in a dose-dependent manner, and the activation by fructose-1,6-P₂ was completely abolished by a little over 10-fold molar concentration of citrate. The enzyme was nonresponsive to citrate inhibition in the absence of fructose-1,6-P₂. The Hill coefficient roughly estimated from the Hill plot of the inhibition by citrate (abscissa: log[citrate], ordinate: log [(V - V₃)/(V₉₉ - V)]) was 3.3 for both concentrations of 1.5 and 1.0 mM fructose-1,6-P₂. The results from Fig. 5 show an increase in the intracellular levels of fructose-1,6-P₂ from 0.68 to 1.81 mM and a decrease in citrate level from 11.9 to 4.52 mM due to inositol deficiency sufficient to produce a 2- to 5-fold augmentation in the acetyl-CoA carboxylase activity in the deficient supernatant fraction.

These results provide a strong indication that the acetyl-CoA carboxylase from this yeast is controlled by fructose-1,6-P₂ and citrate in a reciprocal manner, that is, a higher fructose-1,6-P₂ level and a lower citrate level cause a marked elevation of the acetyl-CoA carboxylase activity in the deficient cells.

Treatment of 100,000 x g Supernatant Fraction with Aldolase and Citrate Lyase—An attempt has been made to change specifically the level of endogenous fructose-1,6-P₂ or citrate in the 100,000 x g supernatant fractions and to observe the resulting change in the acetyl-CoA carboxylase activity. For this purpose, the supernatant fraction from the deficient cells was incubated for 30 min at 30° with rabbit muscle aldolase (EC 4.1.3.6) which catalyzed the reaction; fructose-1,6-P₂ = dihydroxyacetone-P + glyceraldehyde-3-P, while the supernatant fraction from the supplemented cells was incubated with bacterial citrate lyase (EC 4.1.3.6) which catalyzed decomposition of citrate to oxalacetate and acetate. The changes in the levels of the metabolites and the acetyl-CoA carboxylase activity in the supernatant fraction by these treatments was shown in Table VII.

In the deficient supernatant fraction, the aldolase treatment caused an 81% decrease in fructose-1,6-P₂ with a concomitant 53% decrease in the acetyl-CoA carboxylase activity. On the other hand, the citrate lyase treatment of the supplemented supernatant resulted in a 74% decrease in citrate and a 16% increase in the enzyme activity. In contrast to a marked coordinate change in the enzyme activity with fructose-1,6-P₂ level in the deficient supernatant fraction, the smaller change in the activity corresponding with the change in citrate level in the supplemented supernatant fraction will probably be ascribable to the fact that the enzyme is less sensitive to citrate inhibition at low fructose-1,6-P₂ concentrations. Furthermore, citrate in the supernatant fraction was very unstable during the incubation process so that about 80 to 90% citrate was decomposed by the endogenous enzyme in the control samples. Thus citrate lyase treatment might not have caused an
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Table VII
Changes in levels of fructose-1,6-P$_2$, citrate, and acetyl-CoA carboxylase activity by treatment of supernatant fraction with aldolase and citrate lyase

| Source of 100,000 × g supernatant | Treated with | Fructose-1,6-P$_2$ | Citrate | Acetyl-CoA carboxylase |
|----------------------------------|-------------|-------------------|---------|-----------------------|
|                                  |             | nmol/mg protein   | 10$^{-3}$ × dpm/mg protein |
| Supplemented                     | None        | 5.42 ± 0.178      | 3.54 ± 0.064 | 1.04 ± 0.009 |
|                                  | Citrate lyase| 4.97 ± 0.029      | 0.913 ± 0.0428 | 1.21 ± 0.042 |
| Deficient                        | None        | 32.1 ± 0.27       | 0.550 ± 0.0663 | 5.98 ± 0.140 |
|                                  | Aldolase    | 6.10 ± 0.101      | 0.755 ± 0.0089 | 2.46 ± 0.069 |

Two milliliters of 100,000 × g supernatant fraction from 42-h growth of the supplemented and deficient cells containing approximately 16 mg of protein was incubated at 30° for 30 min with 50 μl of citrate lyase (200 units/25 mg/ml) and 10 μl of aldolase (90 units/10 mg/ml), respectively, in the presence of 2.5 mM MgCl$_2$. The control samples were incubated with equal volume of redistilled water under the identical condition. Upon the completion of incubation, aliquots of the mixture were subjected to the assay of fructose-1,6-P$_2$, citrate after deproteinisation with perchloric acid, and to the assay of acetyl-CoA carboxylase activity by the standard assay method. The data represent the mean ± S.E. for the triplicate.

Effective change in citrate level. Fructose-1,6-P$_2$ was very stable to the incubation process.

The results obtained in this experiment strongly support the assumption that fructose 1,6-P$_2$ and citrate play a critical role in regulation of acetyl-CoA carboxylase.

Discussion

Both the lipid content and composition of yeast are very susceptible to variations in growth conditions such as growth temperature, growth rate, oxygen tension, the nutritional state, etc. (59). A decrease in the growth temperature increases the lipid content in a strain of Candida lipolytica and S. cerevisiae; it also increases the synthesis of lipids containing unsaturated fatty acid residues in almost all types of living organisms. High concentrations of carbon dioxide, sodium chloride, and carbohydrate, and phosphate deficiency in the medium produce cells with a high lipid content, while slower growth rates and lower oxygen tensions affect the degree of saturation and elongation of the fatty acid residues.

A deficiency of the vitamin B complex was observed to produce a marked quantitative and qualitative change in the lipids of yeast auxotrophic for vitamins. Pyridoxine deficiency in Hanseniaspora valbyensis (60) resulted in a decrease of lipid contents affecting the degree of saturation of the fatty acids, while pantothenic acid deficiency decreased the lipid content affecting both chain length and the degree of saturation in H. valbyensis (60) and in S. cerevisiae (61). Biotin deficiency in S. cerevisiae (62) also affected elongation and unsaturation of fatty acids without a change in lipid contents. On the other hand, inositol deficiency in S. cerevisiae (12) and S. carlsbergensis (13), and nicotinic acid deficiency in H. valbyensis increased the lipid content (60). The lipid accumulation due to inositol deficiency occurred independently of any environmental changes, such as pH (10), the presence of abnormal metabolites, the glucose concentration in the medium and growth rate (15). To help evaluate the specificity of changes occurring in inositol-deficient S. carlsbergensis, inositol was added to the culture deprived of inositol at a certain period of incubation and our preliminary data indicated that the addition of inositol alleviated (a) the stimulated synthesis of fatty acids from [1-14C]acetate, (b) the altered profile of lipid classes, and (c) the activation of acetyl-CoA carboxylase.

The cause by which the deficient cells contain more unsaturated fatty acids remains unknown. However, in light of the observation that in the stimulated synthesis of fatty acids by 

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The activation of acetyl-CoA carboxylase.

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observation that in the stimulated synthesis of fatty acids by alleviated (13), and nicotinic acid deficiency in

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saturation and elongation of the fatty acid residues. While pantothenic acid deficiency decreased the lipid content (60). The lipid accumulation due to inositol deficiency occurred independently of any environmental changes, such as pH, the presence of abnormal metabolites, the glucose concentration in the medium and growth rate (15). To help evaluate the specificity of changes occurring in inositol-deficient S. carlsbergensis, inositol was added to the culture deprived of inositol at a certain period of incubation and our preliminary data indicated that the addition of inositol alleviated (a) the stimulated synthesis of fatty acids from [1-14C]acetate, (b) the altered profile of lipid classes, and (c) the activation of acetyl-CoA carboxylase.

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However, except for glycerol 3 P, their effects were tested at concentrations not within the physiological range.

In our experiments, fructose-1,6-P2 activated the acetyl-CoA carboxylase from both cells in a sigmoidal manner and citrate counteracted the fructose-1,6-P2 activation over the range of their physiological concentrations. Citrate alone, however, did not affect the activity. Our preliminary experiments showed that fructose-1,6-P2 would interact with the enzyme protein at three sites and make a conformational change affecting \( K_m \) and \( V_{\text{max}} \) rather than causing a macromolecular change. Fructose-1,6-P2 decreased \( K_m \) and increased \( V_{\text{max}} \) for ATP and acetyl-CoA, while citrate decreased \( V_{\text{max}} \) increased by fructose-1,6-P2. It was demonstrated by Plate and co-workers (26) that the inhibition of fatty acid synthetase by malonyl-CoA could be reversed by fructose-1,6-P2, lowering the \( K_m \) for NADPH which had been raised by malonyl-CoA. Pyruvate kinase is also activated by fructose-1,6-P2, decreasing the \( K_m \) for ATP (68).

In summary, the following conclusions may be drawn for these studies. The accumulation of neutral lipids in the inositol-deficient yeast results, at least partly, from an enhanced acetyl-CoA carboxylase activity caused by low molecular effectors in the cell rather than by an increase in enzyme protein. The greater rate of activation of the enzyme by the higher level of fructose-1,6-P2, and the smaller rate of counteraction by the lower level of citrate, namely a dual control by fructose-1,6-P2 and citrate, result in a marked elevation of acetyl-CoA carboxylase activity in the deficient cells.

We can not assess at this time what causes the fluctuation of these intermediates in the deficient cells, that is, what is the primary effect of the inositol deficiency. To approach this problem, the activities of the related enzymes was compared between the supplemented and deficient cells. Our result suggests a significant increase in the activities of phosphofructokinase and citrate lyase, and a decrease in that of aldolase due to the inositol deficiency. This focus on fructose-1,6-P2 seems to be consistent with the observation of Paltauf and Johnston (15) that when ethanol, pyruvate, or lactate was used for the carbon source, no lipid accumulation occurred in the deficient cells. The high activity of phosphofructokinase in the deficient cells may secondarily result from the lower citrate level. On the other hand, the report by Sullivan and Debush (52) seems to be applicable to the decreased activity of aldolase due to the inositol deficiency. This concept might be plausible if the short life span and accumulation of lipid granules in S. carlsbergensis grown without inositol, are considered as part of the aging mechanism suggested by Hochschuld (79) which involves the leakage of hydrolytic enzymes into the cytoplasm.

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