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Regulation of Phosphatidylcholine Biosynthesis in *Saccharomyces cerevisiae*

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Evidence is presented which indicates that the biosynthesis of phosphatidylcholine by the methylation pathway in growing cultures of *Saccharomyces cerevisiae* is repressed by the presence of choline in the growth medium. This result, obtained previously for glucose-grown cells, was also observed for lactate-grown cells, of which half of the phosphatidylcholine is mitochondrial. A respiration-deficient mutant of the parent wild-type strain has been studied, and its inability to form functional mitochondria cannot be due to an impaired methylation pathway, as it has been shown to incorporate ^{14}C-CH_3-methionine into all of the methylated glycerophosphatides. The incorporation rate is depressed by the inclusion of 1 mM choline in the growth medium, suggesting a regulatory effect similar to that demonstrated for the wild-type strain. The effects of choline on the glycerophospholipid composition of lactate and glucose-grown cells is presented. The repressive effects of the two related bases, mono- and dimethylethanolamine, were examined, and reduced levels of ^{14}C-CH_3-methionine incorporation were found for cells grown in the presence of these bases. The effect of choline on the methylation rates is reversible and glucose-grown cells regain the nonrepressed level of methylation activity in 60 to 80 min after removal of choline from the growth medium.

One pathway for the biosynthesis of phosphatidylcholine (PC) involves the stepwise addition of three methyl groups to phosphatidylethanolamine (PE) with *S*-adenosylmethionine (*S*-AdMe) as the methyl donor.

PE $\xrightarrow{S\text{-AdMe}}$ phosphatidylmonomethylethanolamine (PMME) $\xrightarrow{S\text{-AdMe}}$ phosphatidyldimethylethanolamine (PDME) $\xrightarrow{S\text{-AdMe}}$ PC

Exogenous choline can also serve as a precursor for PC. As shown by Kennedy and Weiss (19), this major pathway involves conversion of choline to cytidine diphosphate (CDP)-choline which reacts with diglyceride to form PC. There is evidence for the presence of the methylation pathway in animals (4, 7, 11) and in microorganisms (12, 17, 25). A brief report from this laboratory (27) showed that, during growth on a defined medium with glucose as the carbon source, baker's yeast cells readily incorporated label from ^{14}C-CH_3-methionine into all three methylated glycerophospholipids. Cells grown in the presence of choline had a much lower incorporation rate than cells grown in the absence of choline. Measurements of the rate of phospholipid methylation were also carried out with a cell-free extract by using ^{14}C-H_3-*S*-adenosylmethionine as the methyl donor. These in vitro studies (26, 27) showed much lower methylation rates for cell extracts prepared from cells grown in the presence of choline. Thus, it was concluded that the synthesis of the enzyme(s) catalyzing the methyl transfers was repressed when choline was in the growth medium, and PC synthesis proceeded mainly by the CDP-choline pathway.

In this paper we report further in vivo studies concerning the regulation of the methylation pathway. Since the mitochondria of the yeast cell contain a substantial fraction of the cellular PC, we thought it was of interest to examine whether the overall pattern of regulation is affected when comparing cells in which the quantity of mitochondria is altered by the nature of the carbon source and in which the quality of the mitochondria is altered by mutation. The effect of choline in the growth medium on the total phospholipid composition has been examined for respiring cells and those which are repressed with respect to mitochondrial biosynthesis. In addition, we stud-

[1] This work represents part of a doctoral dissertation to be submitted by C.J.W.

ied the efficacy of monomethylethanolamine (MME) and dimethylethanolamine (DME) in repressing the synthesis of the enzyme(s) involved in phospholipid methylation. A study of the kinetics of derepression is also presented.

MATERIALS AND METHODS

Organism. The yeast studied in these experiments was isolated from Fleischmann bakers' yeast cake. The respiration-deficient mutant was a spontaneous mutant of the same strain which showed a growth requirement for a fermentable carbon source and formed characteristic "petite" colonies when grown on agar plates.

Growth of yeast. In all experiments, the initial cultures were grown aerobically in shake flasks at 30 C on Yeast Nitrogen Base for Carbon Assimilation (Difco) which contained 20 mg of DL-methionine per liter. The carbon source was 4% glucose unless otherwise stated. MME, DME, or choline was added to a final concentration of 1 mM where indicated. The cell density was estimated by measuring the optical density at 650 nm (OD_{650}).

Assay for methyl incorporation into lipid. Cultures were grown as described above to an OD_{650} of 1.0 to 2.0. Cells were harvested by centrifugation at room temperature and washed twice with fresh media not containing the supplemental carbon source. Cell suspensions were then prepared with fresh media containing the appropriate carbon source to a final OD_{650} of between 1.0 to 2.0. After shaking for 10 min at 30 C, 5 μCi of ^{14}C-CH_3-methionine (11 mCi per mmole; New England Nuclear Corp., Boston, Mass.) was added to each 10-ml suspension. Isotope incorporation was stopped by the addition of trichloroacetic acid to a final concentration of 5%. After the lipids were extracted (20), portions of the extract were taken to measure the amount of lipid phosphorus by an adaptation of the Bartlett procedure (6). A portion was also used to determine the amount of radioactivity incorporated into lipid. These samples were counted in vials with 10 ml of a 0.4% 2,5-bis 2-(5-*t*-butylbenzoxazolyl) thiophene solution in toluene-ethanol-water (50:50:4, v/v) in a Packard Tri-Carb scintillation spectrometer. The labeled lipids were resolved by chromatography on Whatman SG-81 paper by using chloroform-methanol-concentrated ammonium hydroxide (66:17:3) as the developing solvent. The chromatographic pattern of the methylated lipids is depicted in Fig. 1. The procedure for verifying the identity of the labeled lipids and determining the percentage of radioactivity in each zone has been previously outlined (27). These percentages multiplied by the total amount of isotope in each lipid extract gave the amount of label in each methylated lipid. The methylation rates of neutral lipids, assayed as one spot, provided a control for effects on the specific activity of the methyl pool. Calculations of all rates were based on the specific activity of exogenous methionine.

RESULTS

Alterations of glycerophosphatide composition by presence of choline in the growth medium. Since the presence of choline in the growth medium affected the rate of phospholipid methylation, it was of interest to determine whether there were any related changes in the net amount of the various cellular phospholipids. The glycerophosphatides were measured in cells from populations growing exponentially with either glucose or lactate as carbon source, plus and minus choline (Table 1). With both carbon sources, choline in the growth medium results in drastically lowered levels of the two methylated precursors of PC. For the glucose-grown cells, this finding is consistent with the already reported (27) repressive effect of choline on the methylation pathway and suggests that a similar regulatory effect is also present in lactate-grown cells. The presence of choline in the growth medium also leads to a con-

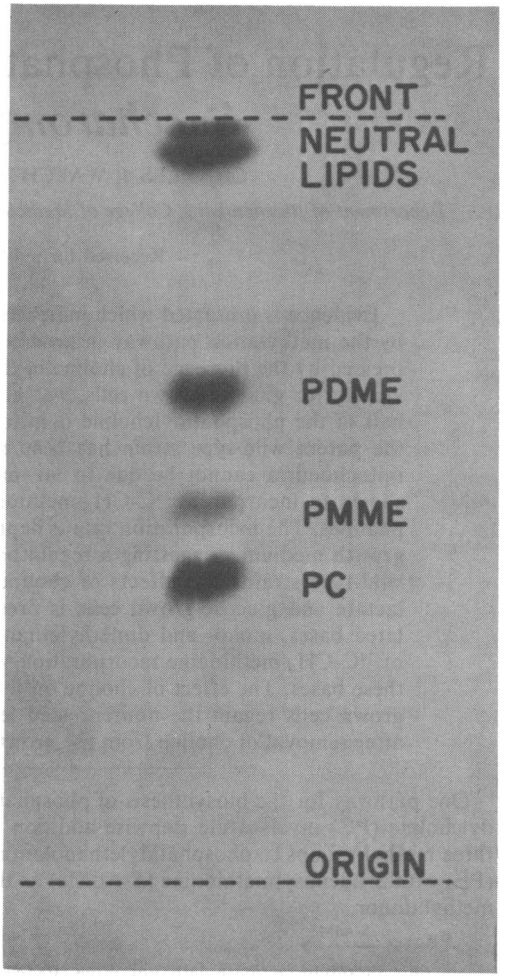

FIG. 1. *Autoradiogram of Whatman SG-81 paper chromatogram of lipids labeled with ^{14}C-CH_3-methionine. The developing solvent was chloroform-methanol-concentrated ammonium hydroxide (66:17:3). The autoradiogram was prepared by exposing the paper chromatogram to Kodak No-Screen X-ray film.*

TABLE 1. *Phospholipid composition of S. cerevisiae grown in the presence and absence of choline*[a]

Addition to basal medium	Carbon source	Optical density at harvest	Phospholipid content (μmoles of phosphorus per gram, dry wt)											
			Total	GP-choline	GP-DME	GP-MME	GPE	GP-serine	GPI	GPIP	GP	GPGP	GPG	$(GP)_2G$
None	Glucose	0.79	19.7	7.10	.662	.177	5.87	0.670	4.19	.020	.049	.028	.022	0.544
None	Glucose	2.95	20.9	8.27	.531	.226	6.21	0.612	3.95	.017	.063	.019	.019	0.665
None	Glucose	5.2	22.1	7.89	.774	.201	7.16	0.619	4.17	.015	.093	.018	.018	0.953
Choline	Glucose	0.98	26.7	14.0	.051	.02	5.74	1.00	4.59	.011	.069	.029	.021	0.865
Choline	Glucose	3.87	26.3	13.4	.045	.02	5.25	1.49	5.04	.021	.134	.039	.021	0.721
Choline	Glucose	7.3	25.1	13.4	.058	.01	6.66	0.906	3.00	.015	.080	.020	.013	0.818
None	Lactate	1.96	39.9	18.2	.140	<.012	12.38	0.71	4.41		.044		.104	3.57
Choline	Lactate	1.88	46.4	25.8	.014	<.014	9.47	1.29	5.64		.060		.413	3.44

[a] Cultures were grown on yeast nitrogen base medium with 4% glucose or 1% lactate as carbon source; each was supplemented with 1 mCi ^{32}P-inorganic orthophosphate (carrier free, Tracer Lab, Waltham, Mass.) per liter and with 1 mM choline as indicated. The cells were inoculated at an optical density of less than 0.001 and harvested at the absorbancies listed. The samples were washed twice with cold distilled water, and the lipid was extracted (20). The total lipid phosphorus was estimated chemically (6). Two-dimensional thin-layer chromatography of the water-soluble phospholipid deacylation products was performed (26). After autoradiography, the spots were cut out, and the ^{32}P was measured; the fraction of the total counts in each spot multiplied by the total phospholipid phosphorus, estimated chemically, gave the above results.

The column designations, in order, refer to glycerophosphorylcholine, glycerophosphoryldimethylethylethanolamine, glycerophosphorylmonomethylethanolamine, glycerophosphorylethanolamine, glycerophosphorylserine, glycerophosphorylinositol, glycerophosphorylinositolphosphate, glycerophosphate, glycerophosphorylglycerophosphate, glycerophosphorylglycerol, and diglycerophosphorylglycerol.

siderable increase in the PC level, indicating that when PC is formed predominantly by the CDP-choline pathway (19), higher amounts of PC accumulate than when synthesis occurs solely by the methylation of PE.

The data also suggest regulation of the PE level in glucose-grown cells. The level of PE is not affected by the presence of choline, although all of the PC is formed from PE in the absence of choline, whereas very little is converted in the presence of choline. It can also be seen that the non-nitrogenous phosphatides are unaffected by the presence of choline in the growth medium. The phosphatide composition for glucose-grown cells was found to be essentially constant throughout the portion of the exponential growth phase which was sampled.

Effect of choline on the level of lipid methylation activity in cells grown on 1% lactate. Yeast cells grown on a nonfermentable carbon source such as lactate are derepressed with respect to mitochondrial biosynthesis, and, as anticipated, the cardiolipin level and the total phospholipid level is higher in lactate-grown cells (Table 1). Assuming that the cardiolipin is ca. 15 to 20% of the mitochondrial phospholipid P (22), the increase of ca. 3 μmoles of cardiolipin P per g (dry weight) should be associated with a total change of 15 to 20 μmoles of phospholipid P per g (dry weight) owing to increased mitochondria. This is roughly the increase observed comparing lactate and glucose-grown cells (Table 1), suggesting that most of the total phospholipid difference is due to increased mitochondrial levels and that about half of the PC of lactate-grown cells is mitochondrial. The rate of phospholipid methylation has been measured in cells grown by using 1% lactate as carbon source in the presence and absence of 1 mM choline to determine whether the additional PC formed is subject to the same regulatory control as in the glucose-grown cells. There is a much lower incorporation of label into phospholipids of choline-grown cells, and the magnitude of the effect offers no support to the idea that the pathway for synthesis of PC associated with mitochondria is uniquely regulated (Fig. 2).

Effect of choline on the methylation of lipids in a respiration-deficient mutant. A cytoplasmic mutation ("petite") of very high frequency can yield a respiratory-deficient cell with aberrant mitochondria lacking cristae (19). It was felt worthwhile, therefore, to study the possibility that the altered morphology reflected a general derangement in phospholipid biosynthesis. In addition, the presence of "petites" (approximately 5%) in our wild-type strain could conceivably offer an explanation for the low, non-repressible level of methylation activity found for choline-grown cells with 4% glucose as the carbon source. The incorporation studies shown in Fig. 3 show that the methylation pathway is not impaired and that the level of these enzyme(s) is regulated by the presence of choline in the growth medium. These results with the mutant are quantitatively similar to those reported for the parent wild-type strain grown on glucose (27).

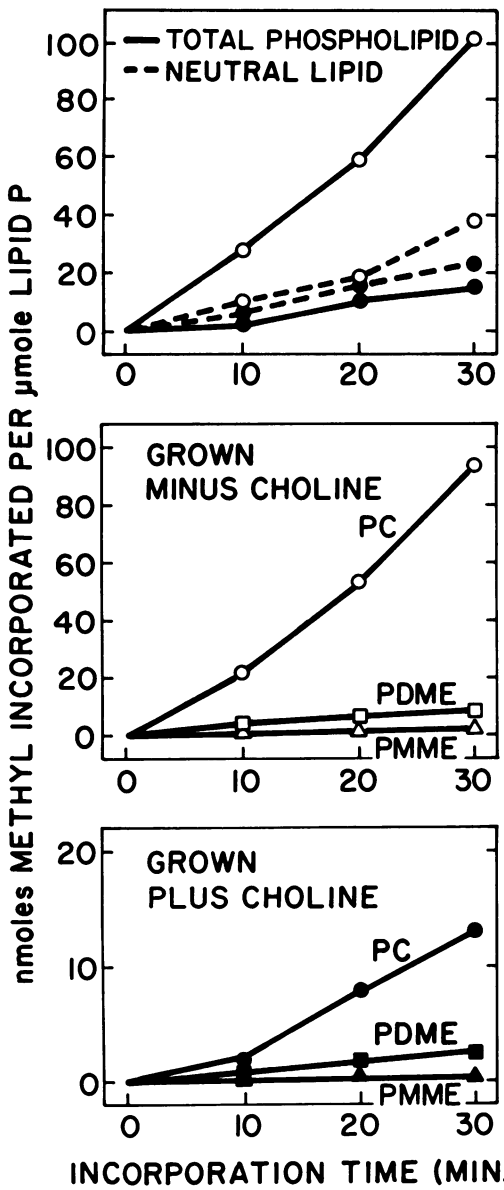

FIG. 2. *Kinetics of ^{14}C-CH_3-methionine incorporation into lipids of cells grown plus and minus 1 mM choline, utilizing 1% lactate as the carbon source. The experimental procedure described in the text was followed. Symbols: ○, △, □, data for cells grown without choline; ●, ▲, ■, data for cells grown in the presence of 1 mM choline.*

Kinetics of derepression. If the lower methylation activity of choline-grown cells is due to a repression of synthesis of the *N*-methyl transferase(s), the cells should be able to regain the normal level, when incubated under growth conditions, after the removal of choline. The recovery of methylation activity has been followed,

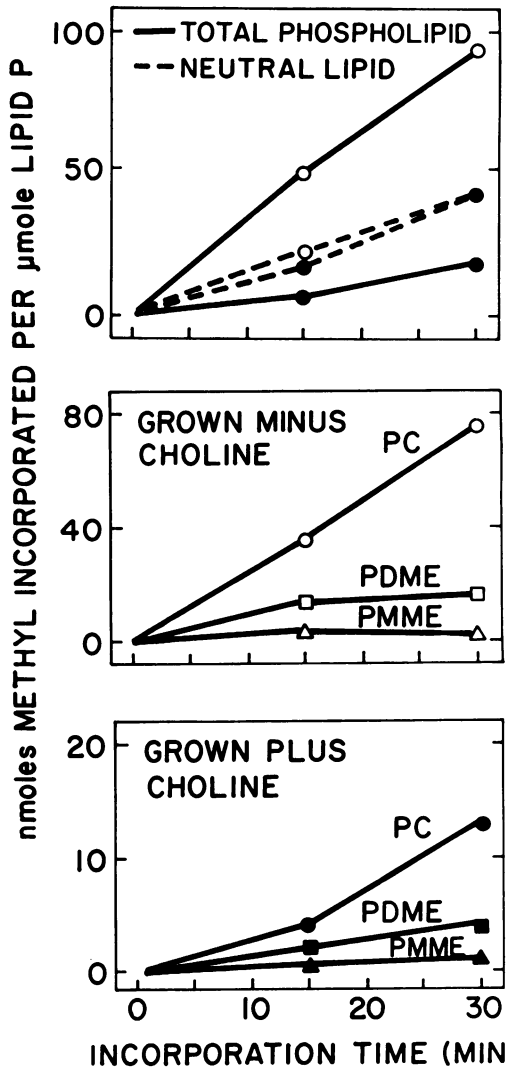

FIG. 3. *Kinetics of ^{14}C-CH_3-methionine incorporation into lipids of a respiration-deficient mutant grown with and without 1 mM choline, utilizing 4% glucose as the carbon source. Symbols: ○, △, □, data for cells grown without choline; ●, ▲, ■, data for cells grown in the presence of 1 mM choline.*

and the results are given in Fig. 4. The data indicate that cells grown in choline-containing medium, harvested, washed, and resuspended in fresh media lacking choline, regain the derepressed level of *N*-methyl transferase activity in slightly less than one generation period, 60 to 80 min. The sterol methylation rates as shown in Fig. 4 remained relatively constant throughout the growth phase studied.

Effect of DME and MME in the growth medium on methylation activity. The mechanism of the repressive action of choline remains to be ex-

plored. The identification of the effector molecule, i.e., choline or some derivative such as PC, and the precise step(s) in the formation of active *N*-methyl transferase(s) being affected are two prominent questions. It is worthwhile to note that lowered methylating activity results from growth in the presence of DME and MME (Table 2). This indicates that at least exogenous choline per se is not required for the regulatory effect. Also, the quantitative effect of these bases, as compared with choline, on the labeling of the three methylated phospholipids raises the possibility that the steps of methyl transfer may be individually regulated. The methylation of neutral lipids is independent of the presence of these natural aminoalcohols in the growth medium (Table 2).

DISCUSSION

All of the evidence suggests very strongly that the inclusion of the free-base choline in the growth medium results in a repression of synthesis of the specific phospholipid *N*-methyl transferase(s) in *S. cerevisiae* (26, 27).

Since the incorporation studies might be assaying only a small and possibly misleading percentage of the actual cellular activity, a calculation was made to evaluate how much PC a given mass of cells could synthesize if they methylated at a rate equal to the in vivo rates obtained. A mass of cells containing 1 μmole of phospholipid have 330 nmoles of PC (Table 1). From the incorporation rate for PC in Table 2, for cells grown in the absence of any added bases, cells containing 1 μmole of lipid phosphorus can make 1.4 nmoles of PC per min, or 140 nmoles in a generation period of 100 min. Thus, it appears that the in vivo rates obtained represent a substantial amount of the actual cellular activity, and that the assumptions that the specific activity of intracellular ^{14}C-H_3-*S*-adenosylmethionine is the same as exogenous methionine and that turnover is not excessive are good approximations.

TABLE 2. *Effect of monomethylethanolamine and dimethylethanolamine in the growth medium on the methylation of phospholipid*[a]

Base added to growth medium	Incorporation of ^{14}C-CH_3-methionine into lipid products (nmoles per min per μmole of lipid phosphorus)				
	PMME	PDME	PC	Total phospholipid	Neutral lipid
No base added ...	.238	1.27	4.13	5.64	2.22
MME	.399	1.65	1.60	3.65	2.11
DME	.105	.070	.940	1.12	2.37
Choline	.094	.219	.251	.564	2.60

[a] These cultures were grown and assayed by the procedures described in the text. The bases were added as denoted at a concentration of 1 mM in all cases. The isotope incorporations were carried out for 20 min in the absence of any methylated aminoalcohols. Abbreviations: PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyldimethylethanolamine; PC, phosphatidylcholine; MME, monomethylethanolamine; DME, dimethylethanolamine.

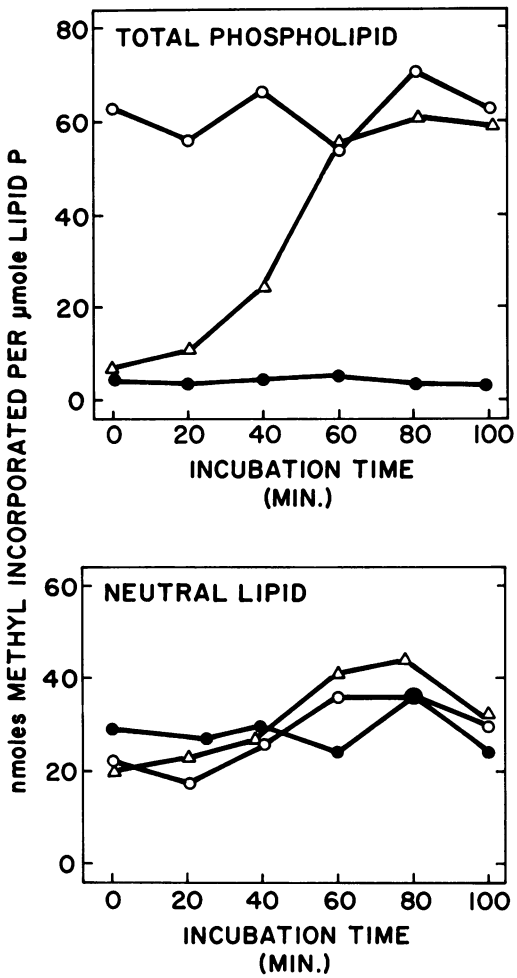

FIG. 4. *Derepression kinetics of the N-methyl transferase(s) involved in phosphatidylcholine biosynthesis. Cells were grown as described in the text, plus and minus choline. They were harvested, washed, and transferred to fresh medium. Beginning at zero time, 10-ml samples were assayed for ^{14}C-CH_3-methionine incorporation at 20-min intervals. All isotope incorporations were carried out for 10 min in the absence of choline. Symbols:* ○, *cells grown and incubated in the absence of choline;* ●, *cells grown and incubated in the presence of 1 mM choline;* △, *cells grown in the presence of 1 mM choline and incubated in the absence of choline. All three cultures grew at the same rate throughout the 100-min incubation period.*

The labeling of neutral lipids, which are predominantly ergosterol and its methylated precursors (1, 2, 10, 22), has provided a means for detecting any substantial changes in the specific activity of the intracellular methyl pool resulting from the presence of choline in the growth medium. The compositional data emphasize the repression effect by revealing that the lower incorporation of label from ^{14}C-CH_3-methionine into PC occurs even though the cell accumulates nearly twice as much PC as cells grown in the absence of choline. This result suggests that there is no unique level of PC required for normal cell growth. As pointed out earlier (27), the pathway for the incorporation of exogenous choline into PC is apparently uninfluenced by the exogenous supply of choline. This suggests that the CDP-choline pathway still fulfills an important function despite de novo synthesis of PC via methylation. In recent years there have been several investigations which reported that in rat liver the molecular species of PC formed by the cytidine dinucleotide pathway differ in fatty acid composition from those formed via the methylation pathway (5, 18, 24). It would be interesting to compare the fatty acid composition of PC formed by the two pathways in yeast. The data have also confirmed an earlier observation that respiring cells, which have a higher content of cytochromes, contain a higher level of cardiolipin (R. L. Lester, Fed. Proc. **22**:415, 1963).

A study of the derepression kinetics demonstrated that the effect of choline is reversible and that, when choline is removed and the cells are incubated under growth conditions, the nonrepressed level of methylation activity is rapidly regained.

The methylation pathway of lactate-grown cells in which half of the PC is mitochondrial and a respiration-deficient mutant appears to be subject to the same regulatory effect of choline in the growth medium (Fig. 2 and 3).

The incorporation of phosphate esters of MME and DME via their CDP intermediates into phospholipids of rat liver and brain dispersions has been demonstrated by Ansell and Chojnacki (3). It was also observed many years ago that some choline-requiring mutants of *N. crassa* could grow with exogenous supplements of MME or DME, or both (13, 14, 16), indicating that these aminoalcohols are incorporated into phospholipid, by cytidine dinucleotide intermediates analogous to CDP-choline or perhaps by an exchange reaction with PE (8, 15). It was deemed important to study the effects of MME and DME in the growth medium on the methylation rates because of their intrinsic interest as precursors of the choline moiety of PC. The labeling pattern of the individual methylated phospholipids of cells grown in the presence of 1 mM DME is compatible with the conclusion that there is a much lower level of the enzyme(s) catalyzing the first two methyl transfers. It is also possible that DME is repressing all three enzymatic steps by virtue of its molecular relationship to choline, and that the incorporation of label into PC that does occur is a result of a higher level of endogenous PDME formed by a cytidine dinucleotide pathway. In fact, we found (*unpublished data*) that labeled DME is incorporated into PDME and subsequently methylated to form PC. The high level of incorporation of label into PDME for MME-grown cells may be due to the higher level of PMME formed by the incorporation of MME into phospholipid by a cytidine dinucleotide pathway. It also seems that the presence of 1 mM MME in the growth medium results in a slight decrease in the labeling of PC. Unfortunately, the data obtained to date do not allow an unequivocal interpretation. One prominent problem is in deciding whether an increased level of one of the precursors is due to an increased level of endogenous phospholipid substrate or to a reduced level of the enzyme catalyzing the ensuing methyl transfer. To obtain a better understanding of the precise regulatory effects of MME, DME, and choline, it will be necessary to conduct extensive in vitro studies with specific phospholipid substrates. Hopefully, these studies will help to answer the question of how many methyl transferases are involved in the methylation pathway and whether they are separately regulated.

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