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In Vitro Study of the Methylation Pathway of Phosphatidylcholine Synthesis and the Regulation of This Pathway in *Saccharomyces cerevisiae**

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ABSTRACT: A particulate, cell-free preparation has been prepared from *Saccharomyces cerevisiae* capable of incorporating the labeled methyl group of *S*-adenosyl-L-methionine, into phosphatidylcholine, phosphatidyl-*N*-methylethanolamine, phosphatidyl-*N,N*-dimethylethanolamine, and ergosterol. When particles are prepared from yeast grown in the presence of choline, they have a much lower rate of labeling of phosphatidyl-*N*-monomethylethanolamine, phosphatidyl-*N,N*-dimethylethanolamine, and phosphatidylcholine than particles from yeast grown in the absence of choline.

The two preparations are qualitatively similar. They incorporate radioactivity from [*Me*-^{14}C]*S*-adenosyl-L-methionine into phosphatidylcholine at linear rates. Both preparations reach steady-state levels of the methylation intermediates phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N,N*-dimethylethanolamine. The preparation from yeast grown in the absence of choline is more active in the synthesis of phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N,N*-dimethylethanolamine, reaches a higher steady-state level and reaches the steady-state level more rapidly than does the preparation from yeast grown in the presence of choline. The rate of ergosterol labeling is linear and very similar for the two preparations. The two preparations also exhibit the same response to pH and have the same K_m for *S*-adenosyl-L-methionine while the V_{max}'s for the two preparations differ by a factor of 14. The preparations differ in the endogenous concentration of phosphatidylethanolamine, phosphatidyl-*N*-monomethylethanolamine, and phosphatidyl-*N,N*-dimethylethanolamine; however, these differences are not of sufficient magnitude to account for the difference in activity between the preparations. A difference in the amount of enzyme is a likely explanation for the differences observed between the two preparations; this presumably results from the repression of enzyme synthesis in the presence of choline.

The synthesis of phosphatidylcholine (PC)[1] is known to occur *via* a number of metabolic pathways. One pathway, the successive methylation of PE, is the sole mode of PC synthesis demonstrated in bacteria (Kaneshiro and Law, 1964). In mammalian systems both the methylation pathway and the synthesis of PC from CDP–choline and D-α,β-diglyceride have been demonstrated (Bremer *et al.*, 1960; Kennedy and Weiss, 1956). The relative importance of these pathways in PC synthesis has been found to be different in different mammalian organs and between the sexes (Bjornstad and Bremer, 1966). Lombardi *et al.* (1969) observed that the *in vivo* incorporation of [*Me*-^{3}H]AMet was higher in rats fed a choline-deficient diet than in rats fed a choline-supplemented diet.

Saccharomyces cerevisiae is able to synthesize PC by the methylation and CDP–choline pathways (Waechter *et al.*,

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[1] The abbreviations used are: PC, phosphatidylcholine; PDME, phosphatidyl-*N,N*-dimethylethanolamine; PMME, phosphatidyl-*N*-monomethylethanolamine; PE, phosphatidylethanolamine; AMet, *S*-adenosyl-L-methionine; +C, cell-free particulate preparation from cells grown in the presence of choline; −C, cell-free particulate preparation from cells grown in the absence of choline.

1969). Waechter *et al.* (1969) demonstrated that the addition of choline to the growth medium of *S. cerevisiae* greatly reduced the incorporation of [*Me*-^{14}C]methionine into PC by the growing cells. Preliminary *in vitro* studies supported this result. This paper is a more complete report of *in vitro* phospholipid methylation catalyzed by particles prepared from cells grown in the presence or absence of choline in the growth medium.

Materials and Methods

Growth of Yeast. A strain of *S. cerevisiae*, isolated from Fleischmann's Bakers' yeast cake, was used for the study. The yeast were grown overnight on Difco yeast nitrogen base medium supplemented with 4% (w/v) glucose and 48 mM sodium succinate (pH 4.75). [^{32}P]P_i (carrier free, Tracerlab) was added to the growth medium where indicated. The choline concentration was 10 mM when added. The cells were harvested in the exponential phase of growth ($A_{650\ nm}$ 3–5.5) and washed with 0.5% (w/v) KH_2PO_4. The cells were either broken immediately after washing or stored at 4° overnight. The cells were stored overnight in 0.5% KH_2PO_4; 10 mM choline was added to the choline growth cells during storage. The cells which were stored overnight were washed again in 0.5% KH_2PO_4 prior to breakage.

Preparation of a Cell-Free Particulate Fraction from Yeast. Yeast cells were suspended in 0.2 M sucrose–0.05 M potassium phosphate (pH 7.2), approximately 18 ml of solution/g dry weight of cells. The cells were broken in a Ribi cell fractionator (Ivan Sorvall Co.) at 20,000 psi. The suspension of broken cells was centrifuged successively at 500*g* and 1000*g* in order to remove the remaining whole cells. The cell-free supernatant fraction was centrifuged at 90,000*g* for 60 min. The pelleted material was washed once in 0.2 M sucrose–0.05 M potassium phosphate (pH 7.2). The washed pellet was resuspended in the sucrose phosphate solution. Samples were stored at −20°.

Assay for Methylation of Lipid. The enzyme preparation was incubated at 30° in the presence of AMet ([*Me*-^{14}C]AMet, Tracerlab; nonradioactive AMet, Calbiochem.), $MgCl_2$, Tris-Cl, potassium phosphate, and sucrose. The reaction was stopped by the addition, in the cold, of 5.0 ml of 95% ethanol–diethyl ether (3:1, v/v). The mixture was allowed to stand at room temperature for a minimum of 20 min sometimes several hours. Petroleum ether (3 ml, bp 30–80°, Allied Chem.) was added to the mixture followed by 6.0 ml of 1.0 M KCl. The contents of the test tube were mixed after each addition. The extraction mixture was centrifuged; the upper phase was removed and saved. The lower phase was reextracted with 3.0 ml of petroleum ether. The combined upper phases were taken to dryness with a stream of N_2. The lipid was redissolved in 2.0 ml of methanol–toluene (1:1, v/v). Samples of the lipid were taken for phosphorous analysis (Bartlett, 1959) and radioactivity determination by scintillation spectrometry. These figures were used to determine the total nanomoles of label incorporated per micromole of lipid P. The protein content of the +C and −C preparations was determined by a modification of the method of Lowry *et al.* (1951). The relationships between the phospholipid and protein content of the two preparations was found to be 1 μmole of lipid P/4 mg of protein. The distribution of label in the individual lipids was ascertained after thelipid was chromatographed on silicic acid impregnated paper (Whatman SG 81) in chloroform–methanol–concentrated ammonium hydroxide (66:17:3). Autoradiography was used to localize the radioactive areas which were then cut out and counted (Waechter *et al.*, 1969). In all the experiments described in the figures and tables of this paper the exact enzyme assay conditions are stated. All subsequent treatment of the samples, starting with the addition of 95% ethanol–diethyl ether, was as indicated in this section.

Determination of PE, PMME, PDME, and PC in the +C and −C Preparations. The amount of PMME and PDME in the +C preparation is too low for accurate chemical assay; therefore, the determination of these phospholipids requires the use of radioactive isotopes. Yeast were grown on the previously described medium with the addition of carrier-free [^{32}P]P_i, 2.8 mCi in 400 ml of medium. The cell-free preparations were made in the usual manner. For every 1 ml of the particle preparation, 5.0 ml of 95% ethanol–diethyl ether (3:1, v/v) was added and the lipid extraction was performed as described above. In order to determine the amounts of ^{32}P-labeled PE, PMME, PDME, and PC the sample was analyzed directly as the lipid and as the water-soluble deacylation products. Analysis of the deacylation products was necessary to determine the amount of PMME present since radioactive PMME cannot be clearly separated from radioactive PE with the SG 81 chromatography system. The ^{32}P preparations could be assayed in the usual manner since PE does not become labeled with [*Me*-^{14}C]. The lipid was chromatographed on the SG 81 system. A portion of the lipid was deacylated by mild alkaline methanolysis (Lester and Steiner, 1968). The glycerophosphate diesters were separated by anion-exchange column chromatography. The column (0.6 cm inner diameter × 81 cm) was AG 1 × 2, 200–400 mesh resin (Bio-Rad, Laboratories), formate form. The column was equilibrated with 60 ml of ammonium hydroxide (pH 9.0). The sample was applied in a few milliliters and the elution with 0.02 M ammonium formate (pH 9.0) was started. The flow rate was 1.2 ml/min; 4–5-ml samples were collected. The peak elution volumes were: glycerophosphorylcholine, 16 ml; glycerophosphoryl-*N*-methylethanolamine, 75 ml; glycerophosphorylethanolamine, 126 ml; and glycerophosphoryl-*N*,*N*-dimethylethanolamine, 164 ml.

Results

A cell-free preparation from *S. cerevisiae* was found to incorporate the methyl group from [*Me*-^{14}C]AMet into a number of lipids. The labeled lipids were shown by a number of chromatographic techniques to be the phospholipids, PMME, PDME, and PC, and the neutral lipid, ergosterol (Waechter *et al.*, 1969). The assay system consisted of AMet, buffer, $MgCl_2$, sucrose, and the cell-free preparation. It was determined that a washed particulate fraction was active in the above system. Since the assay depends upon endogenous lipids as substrates for methylation, the lipid-free supernatant fraction was not active in this assay system. Addition of the supernatant fraction to the particulate fraction did not enhance the incorporation observed with the particulate fraction alone. Although $MgCl_2$ was not necessary for activity, it was routinely added. The presence of $MgCl_2$ resulted in slight changes in the distribution of radioactivity in lipid. These changes were similar for the +C and −C preparations. The assays of the +C and −C preparations could reflect both the differences in enzymes and substrate.

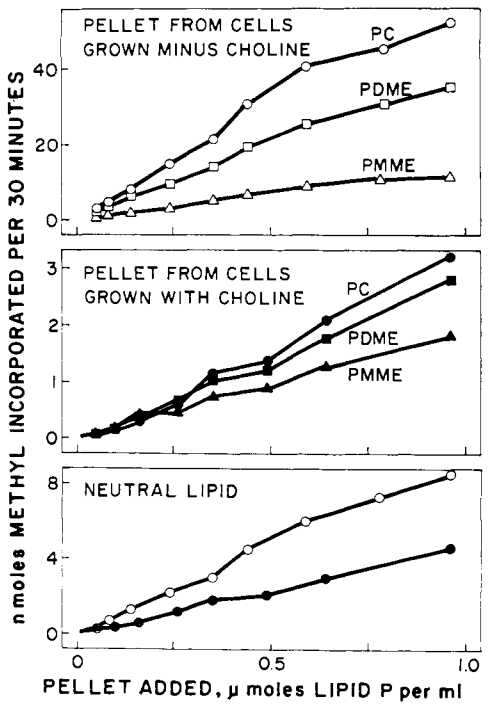

FIGURE 1: The relationship between particle concentration and [*Me*-^{14}C]AMet incorporation into lipid by +C and −C enzyme preparations. Open symbols represent data for the −C preparation and closed symbols represent data for the +C preparation. The assay medium consisted of 2.8 mM $MgCl_2$, 0.3 M Tris-Cl (pH 8.4), 15 mM potassium phosphate (pH 7.2), 60 mM sucrose, 0.45 mM [*Me*-^{14}C]AMet (0.47 μCi), and particles as indicated (1 μmole of lipid P = 4 mg of protein) in 1.0 ml. The reaction was carried out at 30°, for 30 min. Subsequent analysis was carried out as indicated in Methods.

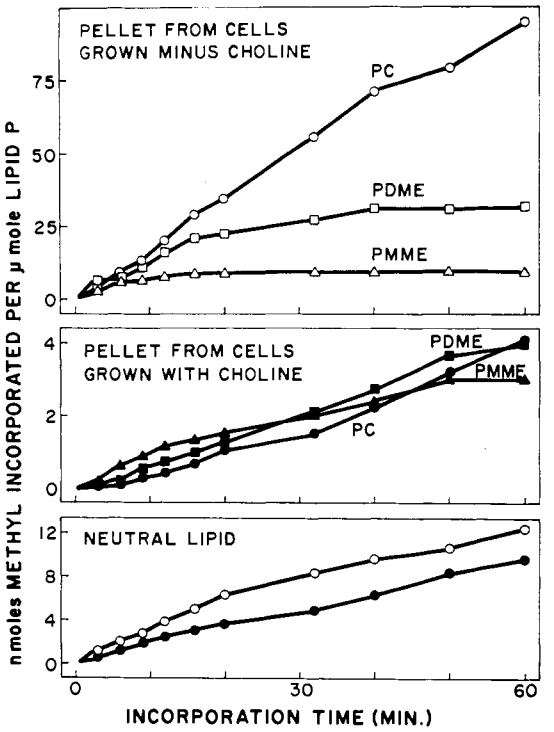

FIGURE 2: The kinetics of incorporation of [*Me*-^{14}C]AMet into lipid by the +C and −C preparations. Open symbols represent data for the −C preparation and closed symbols represent data for the +C preparation. The assay medium consisted of 2.8 mM $MgCl_2$, 0.3 M Tris-Cl (pH 8.4), 15 mM potassium phosphate (pH 7.2), 60 mM sucrose, 0.37 mM [*Me*-^{14}C]AMet (0.35 μCi), and particles (0.25 μmole of lipid P) in 1.0 ml. The reaction was carried out at 30° for the indicated periods of time.

It was observed *in vivo* that the addition of choline to cells which had been grown in the absence or presence of choline had no immediate effect on the methylating ability of the cells. Similarly it was determined *in vitro* that the addition of 20 mM choline in the assay system had no effect upon the methylating ability of the two types of preparations. The absolute rates of methylation observed *in vitro* for the −C and +C preparations, respectively, are quite similar to the rates of incorporation of [*Me*-^{14}C]methionine observed *in vivo* for the cells grown in the absence and presence of choline.

Figure 1 shows the labeling by [*Me*-^{14}C]AMet of various lipids as a function of enzyme concentration. The incorporation of radioactivity into phospholipid is linear for the +C and −C enzyme preparations. The magnitude of the incorporation of radioactivity into total phospholipid and into each phospholipid is very different for the +C and −C preparations. For each phospholipid the −C enzyme preparation has a higher incorporation of methyl groups than does the +C preparation. The synthesis of neutral lipid also increases with enzyme concentration; however, the incorporation of radioactivity is similar for the two enzyme preparations.

Figure 2 shows the incorporation of [*Me*-^{14}C]AMet into lipid as a function of time. The synthesis of PC by both enzyme preparations is linear for the 60 min studied. The labeling of PMME and PDME approach steady-state levels early in the incubation with the −C enzyme preparation. The synthesis of PMME and PDME approach the steady-state levels at the end of the 60-min incubation with the +C preparation. If PE is successively methylated to PC then it would be expected that PMME and PDME would reach steady-state levels while PC would increase linearly in concentration with time. The difference in the rates at which the two enzyme preparations approach the steady-state level reflects the difference in the activity of the two preparations.

The rate of incorporation of radioactivity into phospholipids is much higher for the −C enzyme preparation than for the +C preparation. The incorporation of [*Me*-^{14}C]AMet, after the 60-min incubation, by the −C preparation as compared to the +C preparation gives the following results: PMME 3:1, PDME 8:1, and PC 23:1.

The relative incorporation into PMME and PDME at 60 min reflects the relative steady-state levels rather than the relative rates of synthesis. A comparison of the incorporation of [*Me*-^{14}C]AMet by the two preparations after 3 min would more closely reflect the relative rates of incorporation. The incorporation of label after 3 min by the −C preparation as compared to the +C preparation is PMME 12:1, PDME 38:1.

Figure 3 shows the methylation of lipid as a function of pH. The changes as a function of pH are similar for both enzyme preparations for all the methylated phospholipids. The synthesis of neutral lipid decreases with increasing pH in the pH range studied. The synthesis of PC shows a peak in activity at pH 8.4 for both enzyme preparations. The +C enzyme prep-

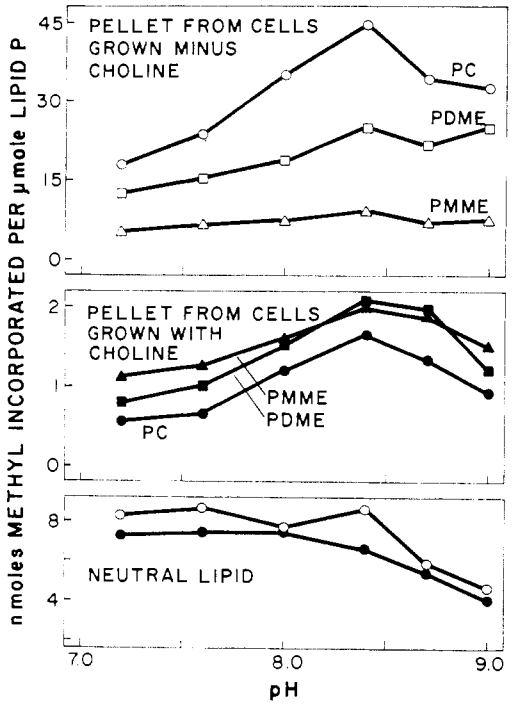

FIGURE 3: The relationship between pH and the incorporation of [*Me*-^{14}C]AMet into lipid by the +C and −C enzyme preparations. Open symbols represent data for the −C preparation and closed symbols represent data for the +C preparation. The assay medium consisted of 2.8 mM $MgCl_2$, 50 mM Tris-Cl (pH as indicated), 2.5 mM potassium phosphate (pH 7.2), 10 mM sucrose, 0.2 mM [*Me*-^{14}C]-AMet (0.35 μC_i), and particles (0.25 μmole of lipid P) in 1.0 ml. The reaction was carried out at 30° for 30 min.

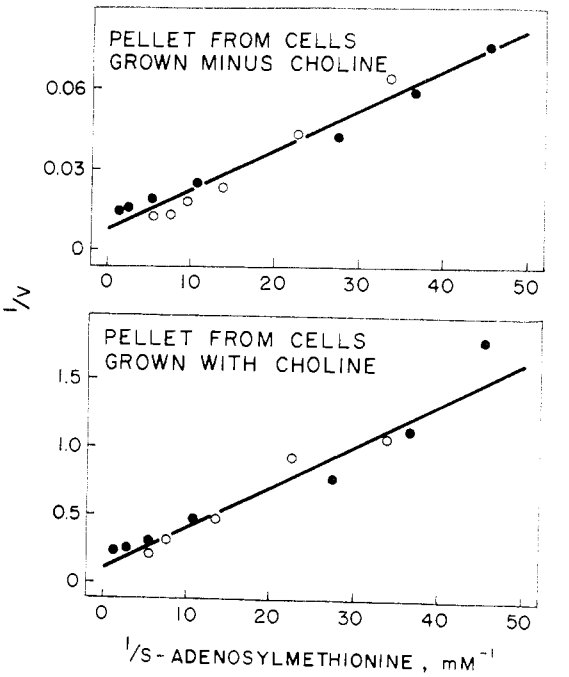

FIGURE 4: Reciprocal plot of the effect of AMet concentration on incorporation of [*Me*-^{14}C]AMet into phospholipid. V = nmoles of *Me*-^{14}C incorporated into phospholipid per μmole of lipid P per 30 min. Closed symbols represent data for an experiment performed with the same particles 1 month after experiment represented by open symbols. The assay medium for the open symbol experiment consisted of 2.9 mM $MgCl_2$, 0.32 mM Tris-Cl (pH 8.4), 2.5 mM potassium phosphate (pH 7.2) 10 mM sucrose, [*Me*-^{14}C]AMet as indicated (3.2 μCi/μmole), and particles (0.25 μmole of lipid P) in 1.0 ml. The assay medium for the closed symbol experiment consisted of 2.8 mM $MgCl_2$, 0.3 M Tris-Cl (pH 8.4), 2.5 mM potassium phosphate, 10 mM sucrose, [*Me*-^{14}C]AMet as indicated (0.88 μC_i/μmole), and particles (0.25 μmole of lipid P) in 1.0 ml. Both assays were performed at 30° for 30 min.

aration also shows a pH optimum at 8.4 in the synthesis of PMME and PDME. This optimum is not observed for PMME and PDME synthesis by the −C enzyme preparation. The difference between the two preparations in respect to PMME and PDME may be related to the different rates at which the two systems reach the steady-state levels of PMME and PDME. Gibson *et al.* (1961) demonstrated that the enzyme(s) of the phospholipid methylation pathway have an alkaline pH optimum. The pH dependence of the phospholipid methylating enzyme(s) shows the same pattern as that found with purified enzyme from *Neurospora crassa* (Scarborough and Nyc, 1967a).

Figure 4 shows the data from two experiments used to calculate the apparent K_m and V_{max} for the two preparations with respect to AMet. The K_m's for the two preparations are 2.7×10^{-4} M for the +C enzyme preparation and 2.3×10^{-4} M for the −C enzyme preparation. The V_{max} for the two preparations are very different. V_{max} for the +C enzyme preparation is 9 nmoles/μmole of lipid P per 30 min, while the V_{max} for the −C enzyme preparation is 133 nmoles/μmole of lipid P per 30 min. The similar K_m values do not support a change in enzyme reactivity as a cause of the difference in activity of the two preparations.

It should be noted (Figure 4), that after storage at −20° for 1 month the enzyme activities were unaffected.

Since the difference in the incorporation of label between the two preparations depends not only upon the difference in enzyme levels but possibly upon the level of endogenous lipid substrates in the assay system, it was necessary to measure the endogenous lipid. Table I indicates the lipid composition of the two preparations. It can be seen that the +C preparation has much less PMME and PDME than the −C preparation. The +C preparation has approximately 60% as much PE as does the −C preparation. If all of the PDME and PC were formed only from PMME and PDME then a large part of the difference in activity of the two preparations could be explained on the basis of the difference in PMME and PDME concentrations. If, however, PE is successively methylated to PC, then the difference in activity cannot simply be a function of the difference in the amount of endogenous lipid.

In order to determine whether or not endogenous PE was successively methylated *in vitro* a pulse-chase experiment was performed. The enzyme preparation was first incubated with a low concentration of high specific activity [*Me*-^{14}C]AMet. The sample was divided into three equal parts. One part was the zero-time sample, *i.e.*, the lipid was isolated at this point. A high concentration of nonradioactive AMet was added to another portion of the sample and the incubation was continued. A control, with the third portion of the sample, was assayed in which no additional AMet was added. Table II shows the results of this experiment for the −C enzyme preparation.

TABLE I: Concentration of Phospholipids and [*Me*-^{14}C]AMe Incorporation into Phospholipid by the −C and +C Enzyme Preparations.[a]

Phospholipid	−C Preparation		+C Preparation	
	nmoles of Phospholipid P per μmole of Total Lipid P[b]	nmoles of [*Me*-^{14}C] Incorporated into Phospholipid per μmole of Total Lipid P	nmoles of Phospholipid P per μmole of Total Lipid P[b]	nmoles of [*Me*-^{14}C] Incorporated into Phospholipid per μmole of Total Lipid P
PE	341		203	
PMME	8.4	11.8	0.3	3.2
PDME	34.7	23.5	3.8	1.1
PC	349	69.2	529	0.6

[a] The concentrations of the phospholipids were determined from the ^{32}P labeling as described in Methods. The assay medium for [*Me*-^{14}C]AMet incorporation into phospholipid consisted of 3.0 mM $MgCl_2$, 0.37 M Tris-Cl (pH 8.4), 7.5 mM potassium phosphate (pH 7.2), 30 mM sucrose, 0.45 mM [*Me*-^{14}C]AMet (4.7 μCi), and particles (0.12 μmole of lipid P) in 1.0 ml. The reaction was carried out at 30° for 2.5 hr. Isolation of the lipid and lipid chromatography were performed as indicated in Methods. [b] These figures refer to the composition of the preparation at the beginning of the assay. The composition of the preparation, at the end of the assay, was determined by lipid chromatography. As stated in the methods [^{32}P]PE and [^{32}P]PMME cannot be clearly separated in the SG 81 lipid chromatography system. No significant changes in the ^{32}P content of the other phospholipids were observed at the end of the assay.

Addition of unlabeled AMet led to a net decrease in the counts per min per μmole of lipid P in PMME and PDME. There was an increase in counts per min per μmole of lipid P in PC which equaled the decrease in PMME plus PDME. It can be seen from the results with neutral lipid that the addition of nonradioactive AMet diluted the remaining radioactive AMet such that no incorporation of radioactivity was observed upon further incubation. The control demonstrates that the preparation retained its methylating ability. If PE could be methylated to PMME and no further, then the counts per min per μmole of lipid P should remain constant upon the addition of nonradioactive AMet. Since there is a decrease in counts per min per μmole of lipid P in PMME, PE must be methylated at least to PDME. By similar reasoning it can be said that PMME can be successively methylated to PC; however, the changes are not large enough to ascertain whether or not PE can be successively methylated to PC. This experiment was not meaningful for the +C enzyme preparation because of the low incorporation under the initial incubation conditions.

The following experiment was performed in order to determine whether or not endogenous PE could be successively methylated *in vitro* by both types of enzyme preparations. Cells were grown, in the presence and absence of choline, with [^{32}P]P_i added to the medium in order to label the phospholipids. The concentrations of PE, PMME, PDME, and PC were determined using the separation methods described in Methods and the ^{32}P label for quantitation. The particulate fractions were assayed with [*Me*-^{14}C]AMet giving rise to the doubly labeled lipids, PMME, PDME and PC. The nmoles of [*Me*-^{14}C] and nmoles of ^{32}P in each of these compounds were measured. The results of these experiments were used to determine whether or not successive methylation of PE had occurred. The ratio of the two radioactive labels (^{32}P from endogenous lipid and ^{14}C from the incorporation of methyl groups) was used to determine the contribution, to the synthesis of PC, of newly synthesized PDME as compared with PDME present at the start of the assay. The results of these experiments are given in Table I.

If all the PC synthesized by the −C preparation were from PMME and PDME then 51.5 nmoles of [*Me*-^{14}C] in PC/μmole of lipid P (2 × 8.4 PMME + 34.7 PDME = 51.5) would be the

TABLE II: Incorporation of Radioactive AMet Followed by Nonradioactive AMet into Lipids by the −C Enzyme Preparation.[a]

Compound	cpm per μmole of Lipid P		
		Addition	
	Zero Time	Nonradioactive AMet[b]	None
All lipids	129,600	128,700	208,740
Neutral lipid	18,290	18,550	42,270
PMME	13,480	5,200	20,480
PDME	50,220	20,950	73,080
PC	47,560	83,980	72,890

[a] The assay medium consisted of 2.9 mM $MgCl_2$, 0.32 M Tris-Cl (pH 8.4), 2.5 mM potassium phosphate (pH 7.2), 10 mM sucrose, 16 μM [*Me*-^{14}C]AMet (0.10 μCi), and particles (0.25 μmole of lipid P) in 0.93 ml. The reaction was carried out at 30° for 10 min. The "zero-time" sample was assayed after this reaction period. [b] Either 2.12 μmoles of AMet in 0.08 ml or 0.08 ml of water was added to the assay medium and the reaction was continued for 20 additional min at 30°.

TABLE III: Calculations to Determine if Successive Methylation of PE Occurs.[a]

	−C Preparation			+C Preparation		
	PMME →	PDME →	PC	PMME →	PDME →	PC
Zero time (nmoles of P/μmole of lipid P)	8.4	34.7		0.3	3.2	
Maximum nmoles of [*Me*-^{14}C] per μmole of lipid P possible after incubation with [^{14}C]AMet if PE is not successively methylated[b]		8.4	51.5		0.3	3.8
Experimental value (nmoles of [*Me*-^{14}C] per μmole of lipid P after incubation with [^{14}C]AMet)		23.5	69.2		3.8	1.1

[a] All assays were performed as described in Table I. [b] Maximum value calculations based on the assumption that all of the PMME is converted into PDME or that all of the PMME and PDME is converted into PC.

maximum PC that could be synthesized. Since there are 69.2 nmoles of [*Me*-^{14}C] in PC/μmole of lipid P then some of the PC must be synthesized by the successive methylation of PE. It should be noted that the total incorporation into PC is less than in previous experiments. This may be related to the fact that the generation time of cells grown on high levels of [^{32}P]P_i is longer than that for cells grown without [^{32}P]P_i. The total incorporation of ^{14}C into PC by the +C preparation is also less than in previous experiments. The incorporation of [*Me*-^{14}C]-AMet into PC is not high enough to determine if there is successive methylation of PE to PC; however, it can be said that there must be successive methylation of PE to PDME since 1.1 nmoles of [*Me*-^{14}C] are incorporated into PDME/μmole of lipid P whereas a maximum of 0.3 nmole of [*Me*-^{14}C] incorporated into PDME/μmole of lipid P is possible if all the PDME is derived from PMME. In a similar experiment with a different, nonradioactive +C preparation, 9.0 nmoles of [*Me*-^{14}C] was incorporated into PC per μmole of lipid P per 2 hr. If this preparation had the same phospholipid composition as the ^{32}P pellet, then successive methylation of PC must have occurred. The amounts of PMME and PDME are too low for accurate chemical assay in the +C preparation so that an experiment cannot be performed with nonradioactive cells. The calculations used to determine whether or not PE is successively methylated are summarized in Table III.

The nmoles of [*Me*-^{14}C] in PDME relative to the nmoles of ^{32}P in PDME is useful in determining how much of the PDME is newly synthesized *vs.* how much is the original endogenous material. If all the PDME is newly synthesized from PE then the nmoles of [*Me*-^{14}C] should equal two times the nmoles of ^{32}P in PDME. It can be seen from Table I that the experimental value for the nmoles of [*Me*-^{14}C] is considerably less than this maximum value. For the following calculations all numbers will be understood to be per μmole of lipid P. If it is assumed that all of the original PMME (8.4 nmoles) is converted into PDME then 8.4 nmoles of [*Me*-^{14}C] involve a single methylation and represent 8.4 nmoles of P converted from PMME into PDME. Since there are 23.5 nmoles of [*Me*-^{14}C] incorporated into PDME, 15.1 nmoles (23.5 − 8.4) of [*Me*-^{14}C] come from PE and involve two methylations. This means that 7.6 nmoles (15.1/2) of P were methylated from PE to PDME. The total number of nmoles of P converted into PDME during the assay is therefore 16.0 nmoles (8.4 + 7.6). The preparation contains 39.1 nmoles of PDME at the end of the assay as measured by the ^{32}P in PDME. Since there are 39.1 nmoles of PDME at the end of the incubation, 23.1 nmoles (39.1–16.0) of PDME represent the endogenous, original PDME. This indicates that two-thirds of the original PDME is still present at the end of the 2.5-hr incubation. If the assumption that all of the original PMME is converted into only PDME is not true, then an even higher fraction of the original PDME would be left at the end of the incubation. This reflects a greater selection, for methylation, of newly synthesized PDME relative to the original PDME. This, in turn, may reflect the same enzyme successively methylating PMME to PC as has been indicated in *N. crassa* (Scarborough and Nyc, 1967a,b). Similar calculations with the data for the experiment with the +C enzyme preparation did not give clear results due to the relatively low synthesis of the methylated phospholipids.

Discussion

The *in vitro* assay of the phospholipid-methylating enzyme (s) supports the *in vivo* findings of the effect of choline in the growth medium on the formation of PC *via* the methylation pathway. The simplest explanation for the observations made is that the +C preparations has reduced levels of the phospholipid-methylating enzyme(s) as compared with the −C preparation. In support of this explanation are the similar pH behavior, the linearity of synthesis with respect to pellet concentration, similar patterns of incorporation of label as a function of length of time of assay and the same K_m for AMet for the two preparations. The close similarity of incorporation of label into neutral lipid under all conditions studied indicates that the concentrations of AMet in the two preparations during the course of the assay are similar. The close similarity of neutral lipid labeling by the two preparations also indicates a similarity between the preparations in metabolism which involves AMet but is not directly related to choline metabolism.

A possible alternative explanation for the different activities of phospholipid methylation by the two preparations is different endogenous lipid substrate concentrations. It has been demonstrated that for both types of preparations PE is successively methylated, at least, to PDME. The difference in

synthetic ability can therefore not be directly related solely to the difference in concentration of PMME. The two enzyme preparations differ by less than a factor of two in the concentration of PE. If the difference in methylating ability is related to substrate concentration then there must be a difference in reactivity of various PE classes. Another alternative explanation, enzyme inhibition, is also not supported by the present observations. The similar response of the two preparations with respect to time, pellet concentration, pH, and in the K_m determination make it seem unlikely that the difference in activity of the two systems is due to inhibition of enzyme activity in the +C preparation.

The activity of this crude preparation from cells grown without choline and assayed with only endogenous lipid substrate compares well with other sources of the methylating enzymes. With a purified enzyme from *N. crassa* and with exogenous substrate Scarborough and Nyc (1967a,b) found a specific activity of 12.1 nmoles of PMME or PDME methylated per mg of protein per 15 min. The yeast system has a specific activity of 16 nmoles of methyl incorporated into PC per mg of protein per 15 min. Other specific activities of purified enzymes with exogenous lipid substrate added are 6.8 nmoles of methyl/mg of protein per 15 min, *Agrobacterium tumefaciens* (Kaneshiro and Law, 1964), and 3.2 nmoles of methyl/mg of protein per 15 min, rat liver (Rehbinder and Greenberg, 1965). This comparison implies that the purified enzyme from yeast would have a high specific activity relative to other purified *N*-methyl transferases and therefore would be a good source of the *N*-methyl transferase(s).

The regulation, by the presence or absence of choline, of the synthesis of PC by the methylation pathway is apparently not limited to *S. cerevisiae*. The effect of choline on the *in vivo* incorporation of [*Me*-^{3}H]AMet in rats appears to be of the same general type of regulation as in the yeast system (Lombardi *et al.*, 1969). A mutant of *N. crassa* which is deficient in its ability to methylate PMME and PDME has been grown in the presence and absence of choline (Crocken and Nyc, 1964). When choline is added to the growth medium the amount of PMME and PDME is decreased relative to the amount found in the cells grown on unsupplemented medium. Since the rates of growth are dependent upon the exact medium composition and the figures reported refer to cells grown for a set period of time, there is a possibility that the differences may be due to comparison at different growth points. It would be of interest to directly compare the synthesis of PC *via* the methylation pathway in *N. crassa* in the presence and absence of choline.

To further characterize the methylation pathway it would be of interest to determine the number of enzymes involved and their intracellular location in yeast. A purified enzyme(s) which is dependent upon exogenous lipid substrate would be of use in the determination of the exact number of enzymes involved in the methylation of PE to PC. If more than one enzyme is involved in the methylation pathway then the effect of choline in the growth medium on each specific enzyme would also be of interest to study. A purified enzyme dependent upon exogenous substrate would also be helpful in clarifying the exact nature of the control mechanism.

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