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Synthesis of Methylated Ethanolamine Moieties

Regulation by Choline in Soybean and Carrot

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ABSTRACT

Cultured cell suspensions of both carrot (*Daucus carota* L.) and soybean (*Glycine max*) take up exogenous choline efficiently from their respective growth media. During sustained growth at a concentration near 50 micromolar choline, this compound was taken up at rates which exceeded those at which phosphatidylcholine is synthesized by cells growing in standard (*i.e.* choline-free) media. In 50 micromolar choline, both types of cells metabolized this compound to phosphocholine and phosphatidylcholine, but not to other detected metabolites, and marked accumulations of phosphocholine and choline occurred relative to phosphatidylcholine. Pregrowth in 50 micromolar choline for several doublings decreased the rate at which carrot cells transferred ^3H from L-[^3H_3C] methionine into the network of all methylated derivatives of ethanolamine by some 98%. With soybean cells, a decrease of 77% was observed. In both cell types, transfer of ^3H into *S*-methylmethionine, pectin methyl esters, methylated nucleic acids, and nonpolar lipid continued unabated. Gel-filtered extracts of carrot cells pregrown in 50 micromolar choline had marked decreases in the specific activities of *S*-adenosylmethionine-dependent phosphoethanolamine, phosphomethylethanolamine, and phosphodimethylethanolamine *N*-methyltransferases; extracts of soybean cells had a similar decrease in phosphoethanolamine *N*-methyltransferase. The significance of these findings for regulation of the rate of synthesis of methylated ethanolamine moieties is discussed.

Recent studies have shown that higher plants display a diversity unusual for major biosynthetic pathways in the patterns whereby the methylations involved in formation of PtdCho[2] occur. PtdCho is a dominant phospholipid in higher plants (4, 6, 8, 9). The three methyl groups required to convert an EA moiety to a Cho moiety originate in methionine. In sugarbeet (5) and in *Lemna paucicostata* (3, 11), a committing step involving methylation of P-EA is followed by methylation of P-MEA and P-DMEA. In soybean, the initial methylation also may be that of P-EA, but subsequent methylations utilize only PtdMEA and PtdDMEA as substrates (2, 3). Carrot (2, 3) and, very likely, barley (7) again initially methylate P-EA, but thereafter methylations occur at both the phospho-base and the phosphatidyl base levels. Some contribution from an initial methylation of PtdEA is not ruled out by the evidence available for soybean, carrot, or barley (2, 3).

Clarification of the above patterns sets the stage for investigation of the regulation of the biosynthesis of methylated EA moieties. In a companion paper (13), we present evidence that in *L. paucicostata*, to the extent that Cho is taken up from the medium there is a commensurate decrease in the flow of methyl groups and EA through the AdoMet-dependent methylation of P-EA, resulting in effective down-regulation of the synthesis of methylated EA moieties. In the present paper, we report the results of extension of similar studies to cultured cell suspensions of carrot and soybean. Some of these results have previously been presented in preliminary form (12).

MATERIALS AND METHODS

Plant Materials

Suspension cultures of soybean (*Glycine max* cv Peking) and carrot (*Daucus carota* L. cv Danvers) were grown as described (2). Methods for the production of cells grown in approximate steady states in 50 μM Cho are described under "Results."

Other Methods

Standard procedures for labeling with L-[^3H_3C]methionine, harvesting, and extracting suspension cultures of carrot or soybean, and methods for purification, manipulation, and quantitation of methylated derivatives of EA resulting from such experiments have been described (2). Details of individual experiments are described in the legends of the relevant tables. Chromatographic systems and sources of radioactive compounds were as specified (2). Amounts of radioactivity present in radiolabeled tissue samples as pectin methyl ester were determined from the volatile radioactivity released from the washed methanol-chloroform-insoluble pellet by a brief exposure to 0.1 N KOH at 0°C; in methylated nucleic acids, by the radioactivity solubilized from the deesterified pellet by

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[2] Abbreviations: Cho, choline. The phosphate esters of these compounds are designated by the prefix, P- (*e.g.* P-EA or P-Cho); the corresponding phosphatidyl derivatives, by the prefix, Ptd (*e.g.* PtdEA or PtdCho). AdoMet, *S*-adenosylmethionine; *S*-methylmethionine, *S*-methylmethionine sulfonium salt; EA, ethanolamine; MEA, *N*-methylethanolamine; DMEA, *N,N*-dimethylethanolamine.

treatment with 5% TCA at 90°C for 15 min; and in nonpolar lipid, by the radioactivity running near the solvent front during chromatography with solvent E (10). Tissue samples of *S*-methylmethionine were purified by sequential pH 7 electrophoresis and chromatography with solvent B. ^3H present in samples so purified was rendered volatile by incubation at 100°C at pH 8.3 at exactly the same rate as ^{14}C in samples of authentic [$^{14}CH_3$]*S*-methylmethionine (10).

AdoMet:P-EA-Base *N*-Methyltranferases

These activities were assayed in gel-filtered extracts as described (3), including identification and quantitation of the reaction products by chromatography with solvent C.

RESULTS

Uptake and Metabolism of 50 μM [$^{14}CH_3$]Cho

For the present experiments, it was desired to grow suspension cultures of carrot or soybean to steady states in the presence of a concentration of Cho that did not inhibit growth, yet at which uptake of this compound would at least equal the amount of Cho derivatives endogenously synthesized by cells grown in Cho-free medium. To this end, uptake and metabolism of [$^{14}CH_3$]Cho were studied in preliminary experiments.

Carrot. Suspensions containing 0.006, 0.002, and 0.00067 mL packed cells/mL growth medium were grown for 24 h in the presence of 50 μM [$^{14}CH_3$]Cho. Uptakes of Cho, measured by disappearance of ^{14}C from the media, were, respectively, 66, 30, and 11% of that initially present. The lowest specified concentration of cells is equivalent to approximately 0.2 mg wet weight of cells/mL growth medium. These cells would contain about 1 nmol PtdCho (2). The uptake of 11% of the initial Cho therefore equaled 5.5 nmol/24 h/0.2 mg cells. If sustained at this rate during the 48 h required to double the cell volume, uptake would be 11 nmol, an amount greatly in excess of the rate of endogenous synthesis of PtdCho by cells growing in Cho-free medium. No decrease in the rate of growth was noted at 50 μM Cho. To produce cells grown to an approximate metabolic steady state under these conditions, incubation of the suspension initiated at 0.00067 mL packed cells/mL growth medium was continued for 7 d, during which time concentrated supplements of [$^{14}CH_3$]Cho were added as needed (at approximately 24 h intervals) to prevent the medium Cho from decreasing at any time below 50 μM by more than 18% and, with each addition, to reattain a concentration of 50 μM. At the end of this period the cells were harvested, washed, and extracted. Chromatography of an aliquot of the methanol-water-soluble fraction with solvent B revealed two peaks of ^{14}C only, moving with the mobilities of P-Cho and Cho. These identifications were confirmed by subsequent chromatography of the materials eluted from these areas (solvent C for P-Cho; solvent A for Cho). Chromatography of an aliquot of the chloroform-methanol-soluble fraction with solvent E revealed a single peak of ^{14}C, moving with the mobility of PtdCho. This identification was confirmed by acid hydrolysis of the eluted material and chromatography of the resulting free base with solvent A. The amounts of ^{14}C in P-Cho, PtdCho, and Cho were, respectively, 9.7, 15.2, and 73.7% of the total ^{14}C in the washed tissue sample.

Soybean. Suspensions containing 0.1 and 0.01 mL packed cells/mL growth medium removed from the medium during a 24 h incubation 90 and 15% of an initial concentration of 50 μM [$^{14}CH_3$]Cho. The lighter inoculum is equivalent to 0.56 mg wet weight of cells/mL medium, and these cells would contain 3.9 nmol PtdCho (2). An uptake of 7.5 nmol/24 h/0.56 mg cells sustained over the approximately 72 h required for the soybean cells to double would result in an uptake of 22.5 nmol, again an excess over the rate of endogenous synthesis of PtdCho by these cells. No growth inhibition was noted at 50 μM Cho. Incubation of a cell suspension initiated at 0.01 mL packed cells/mL growth medium was continued for 11 d with periodic refeeding with concentrated [$^{14}CH_3$]Cho to prevent depletion and sustain the concentration near 50 μM, as described above for carrot cells. Analysis of the ^{14}C-containing compounds in the resulting cells (essentially as described above) again revealed only P-Cho, PtdCho, and Cho equal, respectively, to 4.1, 16.4, and 77.8% of the total ^{14}C contents of the washed cells.

Pregrowth of Cells in 50 μM Nonradioactive Cho, and Effect of Such Growth on Metabolism of L-[^3H_3C] Methionine

To determine the effect of pregrowth in 50 μM Cho upon the metabolism of L-[^3H_3C]methionine, suspension cultures of carrot and soybean cells were initiated at 0.00067 and 0.01 mL packed cells/mL in growth media containing 50 μM Cho and were grown for 6.7 and 11 d, respectively. As a guide to the timing and extent of refeeding with Cho needed to sustain the concentration of this compound near 50 μM, parallel cultures were performed under exactly the same conditions with 50 μM [$^{14}CH_3$]Cho substituted for the nonradioactive Cho. Daily additions of Cho or [$^{14}CH_3$]Cho were carried out in the respective cultures, the amounts required being calculated on the basis of measurements of the radioactivity in the media of the ^{14}C-containing cultures. At the end of these growth periods, the cells grown in 50 μM Cho, as well as control cells grown from similar inocula for the same periods, were concentrated, washed, and resuspended in small volumes of their respective growth media, and labeled by incubation with L-[^3H_3C]methionine for 15 min. The results are reported in Tables I (carrot) and II (soybean). In each case there was a striking decrease in the amount of ^3H originating in the methyl group of methionine and entering into the network of all methylated derivatives of EA. In contrast, there was no such decrease in the entry of these methyls into *S*-methylmethionine, pectin methyl ester, methylated nucleic acids, or nonpolar lipid. The implications of these findings are dealt with at greater length in the "Discussion."

Effects of Growth in 50 μM Cho upon the Activities of AdoMet:P-EA-Base *N*-Methyltransferases

The effect of growth of cell suspensions in 50 μM Cho upon the specific activities of the AdoMet-dependent methylations of P-EA, P-MEA, and P-DMEA by cell-free extracts of carrot, and of P-EA by similar extracts of soybean, are reported in

Table I. *Effect of Growth of Carrot Cells in Cho upon Incorporation of Methyl Group Originating in Methionine into Methylated Derivatives of Ethanolamine and into Other Compounds*

Cho-grown cells had been maintained for approximately two doublings in the presence of this compound (see text for details). For labeling, each cell suspension was incubated in its growth medium with L-[^3H_3C]methionine, 6.2 nM, for 15 min, then rapidly harvested, washed, and extracted. Individual methylated compounds were purified and quantitated as described in "Materials and Methods." Total dpm in the washed tissues were 2.02 and 2.67 (each × 10^6) in the order listed. After extraction of nucleic acids, the residual protein pellets contained 8.5 and 11.7% of total tissue radioactivity. This is presumed to have been present chiefly in protein methionine, with a small amount in methylated amino acids (10). The data for the methylated derivatives of EA in the control plants have been reported previously (2) and are repeated here for convenience of comparison.

Compound	Cho Added to Growth Medium	
	None	50 μM
	% total radioactivity in tissue	
P-MEA	2.63	0.047
P-DMEA	1.27	0.015
P-Cho	0.60	0.017
PtdMEA	1.08	0.018
PtdDMEA	2.78	0.037
PtdCho	5.66	0.066
MEA	0.10	0.01
DMEA	0.04	0.003
Cho	0.06	0.015
Total methylated EA	14.22	0.223
S-Methylmethionine	4.1	4.8
Pectin methyl ester	9.9	14.0
Methylated nucleic acid	3.1	4.5
Nonpolar lipid	3.0	3.9

Table II. *Effect of Growth of Soybean Cells in Cho upon Incorporation of Methyl Group Originating in Methionine into Methylated Derivatives of Ethanolamine and into Other Compounds*

Cho-grown cells had been maintained for 2.6 doublings in the presence of this compound. For labeling, each cell suspension was incubated in its growth medium with L-[^3H_3C]methionine, 7.0 nM, for 15 min. Total dpm in the washed tissues were 6.83 and 5.68 (each × 10^6) in the order listed. After extraction of nucleic acids, the residual protein pellets contained 23.0 and 22.9% of total tissue radioactivity. The data for the methylated derivatives of EA in the control plants have been reported previously (2) and are repeated here for convenience of comparison.

Compound	Cho Added to Growth Medium	
	None	50 μM
	% total radioactivity in tissue	
P-MEA	0.80	0.29
P-DMEA	0	0
P-Cho	0	0
PtdMEA	0.53	0.106
PtdDMEA	2.72	0.597
PtdCho	3.45	0.721
MEA	0.01	0.002
DMEA	0.01	0.002
Cho	0.03	0.003
Total methylated EA	7.55	1.72
S-Methylmethionine	2.7	3.6
Pectin methyl ester	26.8	26.5
Methylated nucleic acid	6.3	5.7
Nonpolar lipid	1.9	1.9

Table III. In each case, there was a significant decrease (at least 86%), as compared to the same activity in extracts of cells grown in standard (Cho-free) medium.

DISCUSSION

In the present paper, we have extended studies of the regulatory effects of exogenous Cho upon the synthesis of methylated EA moieties, previously carried out with intact plants of *L. paucicostata*, to cell suspensions of carrot and soybean grown in tissue culture. The results obtained with the latter two systems are similar, and will be discussed together.

The major findings from these studies are those reported in Table I and II. The results in these tables clearly show that pregrowth of either carrot or soybean in 50 μM Cho brings about major down-regulation of the rate at which intact cells transfer methyl groups originating in methionine into the network consisting of all methylated derivatives of EA. For carrot, the decrease in the rate of such transfer (as judged by transfer of ^3H) is some 98%; for soybean, 77%. These decreases in the flow of methyl groups into methylated derivatives of EA are highly specific: radioactive methyl groups originating in methionine continue to enter *S*-methylmethionine, pectin methyl esters, methylated nucleic acids, and nonpolar lipid at undiminished rates.[3] Thus, there is no indication of a generalized failure of methyl transfer reactions due to growth in Cho.

The evidence in Tables I and II is compatible with the Cho-induced blocks in methyl fluxes being localized to the reaction catalyzed by AdoMet:P-EA *N*-methyltransferase. For both carrot and soybean, this is a committing step in biosynthesis of PtdCho. However, to the extent that some of the initial entry of methyl groups into the network of methylated derivatives of EA in these systems may possibly occur also via methylation of PtdEA (2, 3), some regulation may ultimately be found to occur at this putative reaction. The extent of such regulation will be clarified only when the quantitative contributions of the two portals of entry have been more completely evaluated.

Some of the factors which may enable exogenous Cho to be an effective regulator of PtdCho biosynthesis are revealed by observations made during the present work:

[3] As discussed in relation to similar measurements for *Lemna*, the possibility exists that the percentages of total tissue radioactivity in total methylated EA may overestimate the actual residual fluxes into these compounds to the extent that the specific radioactivity of the methyl group of methionine is diluted less, and is consequently higher, in the Cho-grown cells than in the control cells (13). The magnitude of this effect can be judged from the increases in ^3H in *S*-methylmethionine, pectin methyl ester, methylated nucleic acid, and nonpolar lipid in the 50 μM Cho-grown plants as compared to the controls, and is seen to be more marked in the carrot than in the soybean cells (Tables I and II).

Table III. *Effect of Growth in Cho upon S-Adenosylmethionine:Phosphoethanolamine-Base N-*Methyltransferase Activities in Gel-Filtered Extracts from Carrot and Soybean

Cell suspensions were grown either in standard media or for 7 to 8 d (carrot) or 11 d (soybean) with Cho maintained at approximately 50 μM in the media, as described in "Results." Enzyme assays were performed upon gel-filtered extracts as described in "Materials and Methods" with each substrate at the indicated concentration. The results for the cell suspensions grown in standard media have been published (3) and are repeated here for ease of comparison. Note that AdoMet:P-EA *N*-methyltransferase is the only phospho-base *N*-methyltransferase activity which has been demonstrated in extracts of soybean (3).

Tissue	Reaction	Substrate	Enzyme Activity	
			Control cells	50 μM Cho-grown
		μM	*pmole/μg protein/h*	
Carrot	P-EA → P-MEA	225	3.6 (2.6–4.5; 2)[a]	0.34 (0.17–0.50; 2)
	P-MEA → P-DMEA	225	2.5 (2.0–3.1; 2)	0.29 (0.18–0.40; 2)
	P-DMEA → P-Cho	225	3.3 (2.5–4.0; 2)	0.46 (0.28–0.63; 2)
Soybean	P-EA → P-MEA	225	0.083 (0.018–0.18; 4)	0.006 (0.0045–0.008; 2)
	P-EA → P-MEA	675	0.171 (0.042–0.40; 3)	0.015 (0.0128–0.017; 2)

[a] Mean (range; *n*).

(a) Both carrot and soybean cell suspensions take up Cho efficiently from their respective growth media. Thus, at a concentration of 50 μM Cho, an inoculum of 0.00067 mL packed carrot cells removed during 24 h the amount of Cho contained in 0.11 mL, or 164 times the cell volume. Similarly, soybean cells took up 15 times their volume during 24 h. *L. paucicostata* possesses a well characterized active transport system with high structural specificity for Cho. For example, the affinity of the system drops sharply as methyl groups are removed from Cho (*i.e.* affinities are Cho > DMEA > MEA > EA) (1). It may be that similar uptake systems are operative in carrot and soybean.

(b) Experiments with [$^{14}CH_3$]Cho demonstrated that both carrot and soybean cells metabolized this compound to P-Cho and to PtdCho but not to other detected metabolites. As already calculated in "Results," when Cho was maintained near 50 μM in the media, the uptakes of this compound were some 11 and 5 to 6 times the rates of endogenous synthesis of PtdCho by carrot and soybean cells, respectively. Based on the relative amounts of ^{14}C in P-Cho, Cho, and PtdCho in cells labeled virtually to isotopic equilibria during growth in 50 μM [$^{14}CH_3$]Cho (see "Results"), under these conditions the tissue concentrations of P-Cho and Cho were 64 and 485% that of PtdCho in carrot, and 25 and 474% that of PtdCho in soybean. Although the concentrations of these compounds were not measured in cells growing in standard media, all the above findings are consistent with the possibility that in these cells, as in intact *Lemna*, during growth in the absence of exogenous Cho, tissue concentrations of P-Cho and Cho are low relative to PtdCho, and that when excess Cho is taken up from the medium the concentration of PtdCho changes little, the excess accumulating as P-Cho and Cho (13). If this is so, either P-Cho or Cho would be a reasonable candidate to serve as an effector in whatever regulatory changes are brought about by exogenous Cho.

(c) As clearly demonstrated in Table III, during growth in exogenous Cho there was a marked decrease in the specific activity of the AdoMet:P-EA *N*-methyltransferase of both carrot and soybean cells. As discussed above, the findings reported in Tables I and II strongly indicate that down-regulation of the flux through the reaction catalyzed by this enzyme does indeed occur during growth in Cho. The decreases reported in Table III should contribute to such down-regulation. The situation is much the same in the case of *Lemna* (13). Whether other effects which may also contribute in *Lemna*, such as feedback inhibition of AdoMet:P-EA *N*-methyltransferase activity by accumulated P-Cho and further regulatory prevention of build-up of P-EA (13), play roles also in carrot and soybean remains to be investigated.

During their studies of salinized leaves of sugarbeet, Hanson and Rhodes (5) observed that infiltration with a trapping pool of P-Cho decreased the rate of incorporation of ^{14}C originating in [^{14}C]formate (administered during the interval from 60 to 90 min after initial exposure to the P-Cho) into Cho, P-Cho, PtdCho, and betaine, taken together, by as much as 70%. They interpreted these results as "consistent with feedback inhibition by P-Cho on flux through the methylation sequence." Taking these results together with those reported here and in the companion paper (13) implicating regulation of the biosynthesis of methylated EA moieties by exogenous Cho in carrot, soybeans, and *Lemna*, it may not be premature to suggest that such regulation may be widespread throughout higher plants.

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