Synthesis of Methylated Ethanolamine Moieties

Regulation by Choline in *Lemna*

S. Harvey Mudd*† and Anne H. Datko

Laboratory of General and Comparative Biochemistry, National Institute of Mental Health, Bethesda, Maryland 20892

**ABSTRACT**

The results of experiments in which intact plants of *Lemna paucicostata* were labeled with either L-[4H3]methionine, L-[^13C5]methionine, or [1,2-14C]ethanolamine support the conclusion that growth in concentrations of choline of 3.0 micromolar or above brings about marked decreases in the rate of biosynthesis of methylated forms of ethanolamine (normally present chiefly as phosophatidylycholine, with lesser amounts of choline and phosphocholine). The in vivo locus of the block is at the committing step in the biosynthetic sequence at which phosphoethanolamine is methylated by S-adenosylmethionine to form phosphomethylethanolamine. The block is highly specific: flow of methyl groups originating in methionine continues into S-adenosylmethionine, S-methylmethionine, the methyl moieties of pectin methyl ester, and other methylated metabolites. When choline uptake is less than the total that would be synthesized by control plants, phosphoethanolamine methylation is down-regulated to balance the uptake; total plant content of choline and its derivatives remains essentially constant. At maximum down-regulation, phosphoethanolamine methylation continues at 5 to 10% of normal. A specific decrease in the total available activity of AdoMet: phosphoethanolamine N-methyltransferase, as well as feedback inhibition of this enzyme by phosphocholine, and prevention of accumulation of phosphoethanolamine by down-regulation of ethanolamine synthesis may each contribute to effective control of phosphoethanolamine methylation. This down-regulation may necessitate major changes in S-adenosylmethionine metabolism. Such changes are discussed.

In *Lemna paucicostata* growing rapidly in dim light in a medium containing inorganic salts and sucrose (the standard conditions used in our laboratory [6]), some 55 to 60% of the total methionine methyl groups used for methyl transfer reactions are devoted to the biosynthesis of methylated forms of EA^2^ (14). These accumulate chiefly in the form of PtdCho (some 89% of the methyl groups in question) with 7% present as free Cho, 4% as P-Chol (9), and very minor amounts as P-MEA and P-DMEA (15). We have recently presented evidence, derived from both in vivo labeling (15) and enzyme (5) studies, that these N-methylations occur almost exclusively with P-EA-bases as substrates, converting P-EA successively to P-MEA, P-DMEA, and P-Chol. The latter is converted to CDP-Chol, and finally to PtdCho. *L. paucicostata* possesses an active transport system with high structural specificity for Cho. Uptake is sufficiently active so that at external Cho concentrations of 0.65 μM, or higher, uptake of this compound would equal, or surpass, the total amount endogenously synthesized by plants growing in Cho-free medium (3). Together, these findings suggested to us that *Lemna*, if provided with an external source of Cho, might down-regulate methyl group utilization for biosynthesis of Cho and its derivatives. (In this paper the term ‘Cho synthesis’ will be used in the broad sense to signify the synthesis of any Cho moiety, whether or not this moiety is derivatized at the time it is synthesized. As already mentioned, in *L. paucicostata* virtually all synthesis of Cho moieties occurs by successive methylations of P-EA, so that the Cho moiety is actually formed initially as P-Chol [5, 15]). Here, we present in vivo evidence that indeed such down-regulation does occur, that its locus is at the committing step in which P-EA is N-methylated to P-MEA, and that the activity of the N-methyltransferase catalyzing this reaction is decreased by growth of *Lemna* in the presence of exogenous Cho. Some of these results have been communicated previously in brief form (16).

**MATERIALS AND METHODS**

**Plant Materials**

*Lemna paucicostata* Hegelm. 6746 was grown under standard conditions in medium 4 containing 20 μM SO^4^2− and Mes

3 In all higher plants investigated to date, available evidence is compatible with N-methylation of P-EA being a committing step on the pathway to PtdCho (5). However, the substrates for subsequent methylations differ: in sugarbeet (11), as in *Lemna* (5, 15), P-EA-bases are used; in soybean, subsequent methylations take place almost exclusively with PtdMEA and PtdDMEA as substrates (4, 5); in carrot and barley, both of these pathways may be utilized (4, 5, 12). For some plants, a supplemental role for a pathway commencing with the N-methylation of PtdEA has not been excluded (4).

---

1 Reprint requests should be addressed to the authors at Building 36, Room 3DO6, National Institute of Mental Health, Bethesda, MD 20892.

2 Abbreviations: EA, ethanolamine; MEA, N-methyl ethanolamine; DMEA, N,N-dimethyl ethanolamine; Cho, choline. The phosphate esters of these compounds are designated by the prefix, P- (e.g. P-EA or P-Chol); the corresponding phosphatidyl derivatives, by the prefix, Ptd (e.g. PtdEa or PtdChol). AdoMet, S-adenosylmethionine sulfonium salt; S-methylmethionine, S-methyl methionine sulfonium salt.
buffer (6). To produce altered steady states with respect to
Cho uptake, plants were grown for at least 4.7 doublings in
standard medium with Cho added at various concentrations
(specified in the individual experiments). The volume of
medium and the total frond day of growth in that medium
were adjusted so that in no case did the Cho concentration
of the medium decrease during the course of the experiment
more than 21% from its initial concentration.

Isotopic Labeling of Intact Plants and Measurement of
Labeled Compounds

For labeling to isotopic equilibrium, *Lemma* plants were
grown for at least 4.7 doublings in the presence of the specified
radioiodelabeled compound. When $^{32}$PO$_4^{3-}$ or $^{35}$SO$_4^{2-}$ was used,
the chemical concentration of the ion in question was the
same as that in the standard medium. [1,2-$^{14}$C]EA or [1$^4$CH$_3$]
Cho were added to the standard medium at concentrations
specified in the individual experiments. As described above,
during all such experiments growth conditions were adjusted
so that during the entire labeling period the concentration of
the radiolabeled compound in the medium changed less than
21%.

Plants growing under steady state conditions were labeled
continuously for specified relatively brief periods with either
L-$^{14}$CH$_3$]methionine or L-$^{1}$H$_2$C]methionine as described (14,
15).

Labeled plants were processed to yield a washed methanol-
chloroform-insoluble pellet and components soluble in either
methanol-water or chloroform-methanol. EA derivatives labeled
with either $^{14}$CH$_3$]Cho, L-$^{14}$CH$_3$]methionine, L-$^{1}$H$_2$C
methionine, or $^{32}$PO$_4$ were purified, identified, and quanti-
tated essentially as described (4). Minor variations in these
standard procedures are specified for each experiment.

For plants labeled with [1,2-$^{14}$C]EA, the procedures
described above were modified so that EA, P-EA, and PdEA
could be included in the analyses: EA and P-EA were isolated
from a chromatogram of an aliquot of the methanol-water-
soluble fraction with solvent B. With this solvent, cleanly
separated peaks (in order of increased mobility) were obtained
of P-EA, P-Cho (together with any P-MEA or P-DMEA), EA,
and Cho (together with any MEA or DMEA). The methylated
phospho-bases could then be separated by our standard pro-
cedures (e.g. chromatography with solvent C); the methylated
free bases, by chromatography with solvent A. PdEA was
isolated from a chromatogram of an aliquot of the chloro-
form-methanol-soluble fraction. With this solvent, PdEA cleanly
separated from PdCho. PdEA could then be sub-
jected to acid hydrolysis to form EA, the radio-purity of which
was demonstrated by chromatography with solvent A.

$^{35}$S-Labeled AdoMet, S-methylmethionine, or methionine
were measured as described (1).

Other Methods

Chromatographic systems and solvents previously design-
nated as A, B, C, and E were used as described (4). Methods
for protein determination, paper electrophoresis, mild alka-
line deacylation, acid hydrolysis of phosphatidyl derivatives
or glycerylphospho-bases to the free bases, utilization of au-
thentic internal radioactive standards as markers, location of
radioactive compounds on chromatograms and electropho-
etrograms, elution of such materials, and calculation of the
amounts of radioactivity in particular compounds which had
been subjected to several sequential purification steps have
been described (4, 5, 14, 15).

Chemicals

Sources or methods for preparation of most compounds
have been specified (4, 5, 15). [1,2-$^{14}$C]EA was purchased
from ICN Radiochemicals.

Enzyme Assays

AdoMet:PA-base N-methyltransferase activities were gen-
erally assayed in centrifuged, gel-filtered crude extracts using
the standard conditions and procedures previously described
(5). In one series of experiments (noted in the text) a total
crude brei was used. The concentration of the phospho-base
substrate is specified for each experiment.

CTP:Cho cytidyltransferase activities were assayed in
crude breis as described (5).

Cho kinase was assayed in an extract prepared by homog-
enization of one part wet weight of plants in two parts of
a medium containing Tris-HCl (pH 7.4), 0.01 M sucrose, 0.32
M; and MgCl$_2$, 0.3 mM (19). After centrifugation, the super-
natant fluid was gel-filtered through a small column of Sep-
hadex G-25 equilibrated with the extracting fluid (diluted with
approximately 0.5 volume of water to allow for the dilution
brought about by tissue water). The reaction mixture (a modi-
fication of that used by Tanaka et al. [18]) contained (in
$\mu$mol): Tris-HCl (pH 8.5), 20; MgCl$_2$, 1.6; ATP, 1.6; [1$^4$CH$_3$]
Cho, 124 nmol, $5 \times 10^3$ dpm; and 0.09 mL enzyme (or equiva-
lent diluted extracting fluid) in a final volume of 0.20
mL. After 20 min at 30$^\circ$C, 1.0 mL ice-cold water was added,
and an aliquot of 1.0 mL was quickly removed and applied
to a column of Dowex 50-NH$_4^+$ (0.8 $\times$ 3.0 cm) and was
washed through with water to a final volume of 5 mL.
Separate experiments demonstrated that during this pro-
cedure, [1$^4$CH$_3$]Cho was retained on the column, whereas
[1$^4$CH$_3$]P-Cho came through in the water wash. Radioactivity
was measured in an aliquot of the latter, and the remainder
was used for chromatographic identification of the product.

EA kinase was assayed in extracts homogenized, centri-
fuged, and gel-filtered in the same manner as those used for
assay of Cho kinase. The reaction mixture (similar to that
used by Macher and Mudd [13], but with KCl added as by
Wharfe and Harwood [19], and without carrier EA) contained
(in $\mu$mol): Tris-HCl (pH 8.5), 20; MgCl$_2$, 0.36; ATP, 0.18;
KCl, 0.04; [1,2-$^{14}$C]EA, 6.3 nmol, $10^7$ dpm; and 0.11
mL enzyme (or equivalent diluted extracting fluid) in a final
volume of 0.20 mL. After 30 min at 30$^\circ$C, 1.0 mL ice-cold
water was added, and an aliquot of 1.0 mL was quickly
removed and applied to a column of Dowex 50-H$^+$ (0.8 $\times$ 3.0
cm). The flow-through was collected together with an eluate
of 0.1 n HCl to a total volume of 10 mL. As shown previously
(5), this procedure eluted [1$^4$C]P-EA, while leaving [1$^4$C]EA

Downloaded from on August 18, 2017 - Published by www.plantphysiol.org
Copyright © 1989 American Society of Plant Biologists. All rights reserved.
RESULTS
Down-Regulation of the Biosynthesis of Methylated EA Moieties during Growth in the Presence of External Cho

In early experiments we studied the effects of pregrowth in 25 μM Cho upon the rate of transfer of the methyl group originating in methionine into the network consisting of all methylated derivatives of EA. This concentration of Cho was chosen initially because: (a) it does not affect the growth rate of L. paucicostata (3); and (b), at this concentration, as shown previously (3), and confirmed during the present work, the uptake of Cho is approximately 5 nmol/frond-doubling, or 6 to 7 times the total content of Cho and its derivatives in plants grown in the absence of added Cho (3). Thus, more than enough preformed Cho should be available to the plants under these conditions for realization of any potential for down-regulation of the biosynthesis of this compound.

The results of one such experiment are summarized in Table I. As expected (14, 15), in control plants after the short labeling period used in this experiment, most of the radioactivity in the network of methylated derivatives of EA was found in the methylated phospho-bases. Prior growth in Cho decreased the entry of methionine methyls into the network of methylated derivatives of EA to 1 part in 14.9, or 7% of the rate observed with control plants.

The possibility was considered that the decrease in radioactivity in methylated derivatives of EA in the tissues of plants grown in Cho resulted from preferential excretion of such compounds into the medium. The media from the incubations with L-[^3H]methionine (those from both the control and the Cho-grown plants) were examined by a combination of column and paper chromatographic methods. No [^3H]HPCho was detected. The total ^3H-containing material which was not separated from Cho amounted in neither case to as much as 3% of the ^3H content of the tissue P-Cho (data not presented). It was concluded that excretion of methylated derivatives of EA could not account for the difference observed between the control plants and those grown in Cho.

The specificity of the decrease in methylation of EA derivatives in plants grown in Cho was supported by two observations: first, the unlikely possibility that the decrease in methylation of EA derivatives resulted from a failure to form AdoMet was eliminated by the demonstration that in control plants 12% of total tissue radioactivity was found in AdoMet, whereas in Cho-grown plants this value was 19%. Second, evidence against a generalized failure of transmethylation reactions was provided by the finding that in control plants S'-methylenethionine contained 7.2% of total tissue radioactivity, whereas in Cho-grown plants this compound contained 12%.

To extend these results, a similar experiment was performed in which incubations with methyl-labeled methionine were carried out for several periods, up to 4 h, and in which a number of additional methyl-containing metabolites of methionine were analyzed (Table II). It has previously been demonstrated that as labeling time with methionine increases, the portion of methyls accumulating in PtdCho rises (15). At all three incubation times there were marked decreases in the Cho-grown plants in the percent of tissue radioactivity found in the network of methylated derivatives of EA. Residual rates of methyl entry varied from 9.5 to 7.7%. In contrast, the portion of radioactivity found in each of the other measured methylated metabolites of methionine was invariably increased.

Table I. Effect of Growth in Cho upon Incorporation of Methyl Group Originating in Methionine into Methylated Derivatives of Ethanolamine

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cho Added to Growth Medium</th>
<th>% total radioactivity in tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>25 μM</td>
<td></td>
</tr>
<tr>
<td>P-MEA</td>
<td>3.2</td>
<td>0.25</td>
</tr>
<tr>
<td>P-DMEA</td>
<td>3.3</td>
<td>0.27</td>
</tr>
<tr>
<td>P-Chol</td>
<td>7.2</td>
<td>0.44</td>
</tr>
<tr>
<td>PtdCho</td>
<td>0.37</td>
<td>0.011</td>
</tr>
<tr>
<td>Cho, MEA, DMEA</td>
<td>0.67</td>
<td>0.03</td>
</tr>
<tr>
<td>PtdMEA</td>
<td>0.032</td>
<td>ND^a</td>
</tr>
<tr>
<td>PtdDMEA</td>
<td>0.16</td>
<td>ND^a</td>
</tr>
<tr>
<td>Total methylated</td>
<td>14.9</td>
<td>1.0</td>
</tr>
<tr>
<td>EA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a Not detected.

Cho Regulation and Tissue Concentrations of Cho Derivatives as Functions of Cho Uptake

To further extend the above results, it was desired to determine the effects of growth in lower concentrations of Cho, so that the uptake of this compound would be less in relation to endogenous Cho synthesis. Plants were grown to isotopic equilibria in steady states brought about by the addition of [^14CH]Cho to the growth media at concentrations ranging from 0.01 to 25 μM. The experimental design was based upon several considerations: Cho moieties in L. paucicostata are synthesized almost exclusively in the form of P-Chol, which is the precursor of PtdCho (5, 15). The biosynthesis origin of free Cho is less certain. It probably arises largely from PtdCho (or possibly, and to a lesser extent) from P-Chol (15). Nevertheless, L. paucicostata possesses an active Cho kinase (as documented later in this paper), and experiments
with \([\text{L-}^{3}\text{CH}_3\text{Cho}]\) have shown that upon being taken up from medium this compound is rapidly converted to \([\text{L-}^{3}\text{CH}_3\text{P-Cho}]\) (2), and that, after a suitable period of chase, the \(^{3}\text{C}\) becomes distributed between \text{P-Cho}, \text{PtdCho}, and free Cho itself in proportions which subsequently change little with time (15). Thus, it seems reasonable to assume that in plants grown for at least several doublings with a constant external source of radiolabeled Cho, the available Cho moieties, whether taken up from the medium, or formed biosynthetically as P-Cho, will come to be distributed between P-Cho, PtdCho, and Cho in such a manner that each of these compounds will arrive at a constant, and equal, specific radioactivity. The specific activity in question will be a function of the specific activity of the radiolabeled Cho in the medium, the uptake of this compound, and the rate of synthesis of Cho in the plants, as specified in Eq. (1) (10):

\[
T = \frac{MU}{U+S'}
\]

where \(T\) = specific activity of a tissue Cho-containing compound \((\text{dpm/nmol})\), \(M\) = specific activity of Cho in the medium \((\text{dpm/nmol})\), \(U\) = uptake of Cho from the medium \((\text{nmol/frond-doubling})\), \(S\) = endogenous synthesis of Cho \((\text{nmol/frond-doubling})\). Eq. 1 may be rearranged to solve for \(S\):

\[
S = \frac{U(M - T)}{T}
\]

The results of these experiments are summarized in Table III. Values required for solution of Eq. 2 for each plant sample were arrived at as follows: uptakes of Cho were calculated either according to the removal of \([\text{L-}^{3}\text{CH}_3\text{Cho}]\) from the medium (row a), or the total amount of \(^{3}\text{C}\) recovered in the tissues (row b). In almost all instances, values calculated according to these alternative methods agreed satisfactorily. Values for the specific activity of \([\text{L-}^{3}\text{CH}_3\text{Cho}]\) in the medium (row c) were calculated from the amounts of labeled and unlabeled Cho added. Of the various tissue Cho-containing compounds, in practice it was simplest to determine the specific radioactivity of PtdCho. This was so because experiments with \(^{32}\text{P}\)-labeled plants (row e) demonstrated that, within experimental error (see row f), the tissue concentration of PtdCho was the same in plants grown in media containing anywhere from 0.01 to 25 \(\mu\text{M}\) Cho. Therefore, it could be assumed that plants grown at intermediate concentrations of Cho contained the same amount of PtdCho. A constant mean PtdCho content of 0.669 nmol/frond was then used to calculate the specific activity of each tissue sample of PtdCho (row d). Using the values for \(U\), \(M\), and \(T\) displayed in Table III, rates of synthesis of Cho were then calculated from Eq. 2. Again, alternative calculations were performed, using uptake values based either on removal of \([\text{L-}^{3}\text{CH}_3\text{Cho}]\) from the medium (row g), or, alternatively, upon \(^{3}\text{C}\) recovered in the tissues (row h).

The above analysis explains any change in the specific radioactivity of Cho taken up from the medium to a lower specific radioactivity in a Cho-moiety-containing compound in tissue as due to dilution by plant synthesis of nonradilabeled Cho moieties. The less the decrease in specific radioactivity, the less the rate of plant synthesis of Cho moieties. The results in Table III show that, as Cho was raised in the medium from 0.01 to 0.65 \(\mu\text{M}\), there was a concomitant diminution in the decrease in the specific radioactivity from that of medium Cho to that of tissue PtdCho (compare rows c and d for each sample). This diminution is reflected by the lower calculated rates of Cho synthesis (rows g or h). At medium Cho concentrations of 1.4 \(\mu\text{M}\), and above, the two specific radioactivities in question became the same within experimental error, and plant Cho synthesis became undetectable by the present

---

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

Plants were pregrown as explained in the legend to Table I, then labeled in similar media by incubation with \(\text{L-}^{3}\text{CH}_3\text{methionine}, 4.0\) nm, for the times indicated. After incubation, total dpm in the washed plants (in the order listed) were 5.71, 5.37, 14.1, 13.4, 23.3, and 22.7 (each \(\times 10^3\)). Methylated derivatives of P-EA, PtdEA, and EA were purified as described in the legend to Table I, except that chromatography with solvent C was used only to separate phosphobases where indicated, and that, for the phosphatidyl compounds, steps after the mild alkaline decylation were omitted. Radioactivity in AdoMet or S-methymethionine was quantitated after pH 7.0 electrophoresis of an aliquot of the methanol-water-soluble fraction (14). Radioactivity in pectin methyl ester was measured by the methanol-soluble, volatile \(^{14}\text{C}\) liberated from the washed methanol-chloroform-insoluble pellet by a brief treatment with 0.1 n KOH at 0°C (14); that in nonpolar lipid, by the \(^{14}\text{C}\) traveling near the solvent front (approximately 30 cm) after chromatography of an aliquot of the chloroform-methanol-insoluble fraction with solvent E (14); that in the de-esterified pellet by the methanol-insoluble \(^{14}\text{C}\) after the de-esterification procedure specified above (14). The values for methylated EA derivatives in the control plants shown in this table have been published previously (15), and are included here for ease of comparison.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time of Incubation with L-[\text{3}CH_3\text{methionine]}</th>
<th>% total radioactivity in tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0 h</td>
<td>2.5 h</td>
</tr>
<tr>
<td>P-MEA</td>
<td>2.4</td>
<td>1.4</td>
</tr>
<tr>
<td>P-DMEA</td>
<td>1.5</td>
<td>*</td>
</tr>
<tr>
<td>P-Chol</td>
<td>17.6</td>
<td>2.4</td>
</tr>
<tr>
<td>PtdCho</td>
<td>7.9</td>
<td>0.4</td>
</tr>
<tr>
<td>Cho, MEA, DMEA</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>PtdME</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PtdDMAE</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Total methylated EA</td>
<td>29.5</td>
<td>2.8</td>
</tr>
<tr>
<td>AdoMet</td>
<td>3.0</td>
<td>9.9</td>
</tr>
<tr>
<td>S-Methionine</td>
<td>8.2</td>
<td>12.7</td>
</tr>
<tr>
<td>Pectin methyl ester</td>
<td>9.4</td>
<td>11.0</td>
</tr>
<tr>
<td>Nonpolar lipid</td>
<td>9.4</td>
<td>12.9</td>
</tr>
<tr>
<td>De-esterified pellet</td>
<td>31.3</td>
<td>33.1</td>
</tr>
</tbody>
</table>

* Not separated from P-Chol; would be included in value for that compound. * Not detected.
method. (The calculated value of synthesis for plants grown at 25 μM Cho is regarded as the fortuitous mathematical result of multiplication of a large uptake \( U \) by a small difference in specific radioactivities \( [M - T] \), and therefore is considered as not reliably different from zero.)

Rows i and j in Table III list the tissue contents of P-Cho and of free Cho, calculated according to the assumption that, as discussed above, these compounds had the same specific radioactivity as did PtdCho. The tissue total for all Cho derivatives (row k) was then arrived at by summing P-Cho, free Cho, and PtdCho. The implications of these findings, as well as those arising from the rates of Cho synthesis reported in Table III, are considered in detail in the “Discussion.”

**Labeling to Isotopic Equilibria with \([1,2,14C]EA\): Effects of Cho or Methionine**

The results reported above suggest that exogenous Cho brings about a down-regulation of the rate of transfer of methionine methyls into the network of methylated derivatives of EA. If this is so, a concomitant decrease in the rate of entry of P-EA into this same network would be expected. To test this prediction, experiments were carried out in which plants were grown to isotopic equilibrium in the presence of a low external concentration of \([1,2,14C]EA\). The effect on the resultant labeling pattern brought about by addition of 3 μM Cho to the growth medium was examined. The results (Table IV) confirm the prediction: plants growing in Cho converted only 9.7% of the \( 14C \) entering the tissues in the form of \([1,2,14C]EA\) into the network of methylated derivatives of EA, as compared to 65.4% in the control plants. This amounts to a decrease to a residual rate of 14.8% of that observed in the control plants. This decrease was balanced by an increase in the flow of \( 14C \) into unmethylated derivatives of EA itself (78.7% in the Cho-grown plants, compared to 30.5% in the controls). The largest part of this increase was accounted for by PtdEA.

In these studies, we wished to measure the flux of radioactivity originating in EA into only the ethanolamine moiety of methylated EA derivatives. It was possible that some portion of the administered \([1,2,14C]EA\) might be metabolized to a ‘one-carbon’ fragment which, in turn might label the methyl group of methionine, and so eventually label methylated EA derivatives in their methyl, rather than in their EA, moieties. To evaluate this possibility, an experiment was carried out with 2 μM methionine in the growth medium. Work from this laboratory has previously shown that, in *Lemma*, growth in this concentration of methionine brings about a decrease in the *de novo* synthesis of methionine. Further, under these
REGULATION OF SYNTHESIS OF METHYLATED ETHANOLAMINE MOIETIES

Table IV. Labeling Patterns with [1,2-14C]EA: Effect of Cho or Methionine

<table>
<thead>
<tr>
<th>Compound</th>
<th>Addition to Growth Medium</th>
<th>% of total radioactivity in tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>Cho</td>
</tr>
<tr>
<td>EA</td>
<td>6.3</td>
<td>15.5</td>
</tr>
<tr>
<td>P-EA</td>
<td>1.6</td>
<td>5.3</td>
</tr>
<tr>
<td>PtdEA</td>
<td>22.6</td>
<td>57.9</td>
</tr>
<tr>
<td>Total unmethylated EA derivatives</td>
<td>30.5</td>
<td>78.7</td>
</tr>
<tr>
<td>P-MEA</td>
<td>^a^</td>
<td>^a^</td>
</tr>
<tr>
<td>P-DMEA</td>
<td>0.2</td>
<td>^a^</td>
</tr>
<tr>
<td>P-Ch</td>
<td>2.5</td>
<td>3.0</td>
</tr>
<tr>
<td>PtdCho</td>
<td>57.1</td>
<td>4.6</td>
</tr>
<tr>
<td>Cho</td>
<td>5.6</td>
<td>2.1</td>
</tr>
<tr>
<td>PtdDMEA</td>
<td>ND^b^</td>
<td>ND</td>
</tr>
<tr>
<td>MEA</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DMEA</td>
<td>ND</td>
<td>c ND</td>
</tr>
<tr>
<td>Total methylated EA derivatives</td>
<td>65.4</td>
<td>9.7</td>
</tr>
</tbody>
</table>

^a^ Not separated from P-Ch; would be included in value for that compound.  
^b^ Not detected.  
^c^ Not separated from Cho; would be included in value for that compound.

conditions the pool of soluble methionine expands greatly, and acts as a trap for any radioactivity which does enter the methyl group of methionine (8). Together, these effects should minimize any potential flow of 14C originating in EA into the methyl moieties of methylated EA derivatives. The results of this experiment (last column of Table IV) demonstrate that growth in methionine essentially did not alter the labeling pattern obtained with [1,2-14C]EA from that in control plants. It was concluded that the patterns reported in Table IV are due virtually entirely to incorporation of the EA moiety, rather than to incorporation of methionine methyl groups containing radioactivity originating in EA.

Effects of Growth in Cho upon Various Enzyme Activities

AdoMet:P-EA-base N-methyltransferase Activities

In an initial experiment, plants were pregrown to steady state in 25 µM Cho, and AdoMet:P-EA N-methyltransferase activity was measured (using 100 µM P-EA in the reaction mix). The brei from the Cho-grown plants had only 21% the activity of a brei from control plants (expressed on a frond-equivalent basis). Centrifugation of the breis, followed by gel-filtration of the supernatant fluids, led to 81 and 80% recovery of the activity in the initial brei for the control and Cho-grown plants, respectively. Mixture of a brei from control plants with one from Cho-grown plants resulted in an activity which was 84% of that predicted on the basis of simple additivity of activities. Thus, there is no evidence that the observed difference in activities was due to either inhibitory or stimulatory factors in the extracts.

To determine the range of external Cho concentrations over which the decrease in P-EA N-methyltransferase activity occurs, plants were pregrown to steady states at several Cho concentrations (Table V). Activity was significantly decreased when Cho was 0.3 µM in the growth medium, and, at a concentration of 1.4 µM had decreased essentially as much as would be the case even at higher concentrations.

Activities of all three AdoMet:P-EA-base N-methyltransferases were then assayed in gel-filtered extracts from plants which had been pregrown in 3 µM Cho, and compared to those from control plants. The results (Table VI) suggest that the decrease produced by growth in Cho is relatively specific for the methylation of P-EA. Whereas the rate of this methylation reaction was decreased some 80%, the rates of methylation of P-MEA and P-DMEA were decreased by only 30 and 40%, respectively.

Cho Kinase

Preliminary experiments with gel-filtered extracts from control plants demonstrated that under the standard conditions used to assay this enzyme, activity was linear with enzyme and time of incubation (up to 60 min). Product formation was completely dependent upon addition of ATP to the reaction mix. The product of the reaction was identified by its comigration with authentic [3H]P-Cho during chromatography with solvent B.

Cho kinase activities were then compared in extracts from control plants and plants pregrown in 3 µM Cho (Table VI). No significant difference was observed.

EA Kinase

Again, preliminary experiments demonstrated that under the standard conditions used, this activity was almost linear with enzyme and time (up to 60 min). Product formation was completely dependent upon the addition of ATP and MgCl2 to the reaction mix, but was not affected by omission of KCl. The reaction product was identified by its comigration with authentic P-EA during chromatography with solvent B.

The results summarized in Table VI show that EA kinase

Table V. Effects of Growth in Various Concentrations of Cho upon AdoMet:P-EA N-Methyltransferase Activity

<table>
<thead>
<tr>
<th>Cho in medium (µM)</th>
<th>P-EA N-methyltransferase Activity (µmol/60 min/µg protein)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.04</td>
<td>100</td>
</tr>
<tr>
<td>0.3</td>
<td>0.63</td>
<td>61</td>
</tr>
<tr>
<td>0.65</td>
<td>0.46</td>
<td>44</td>
</tr>
<tr>
<td>1.4</td>
<td>0.31 (0.24–0.37)*</td>
<td>30 (23–36)*</td>
</tr>
<tr>
<td>3.0</td>
<td>0.28</td>
<td>27</td>
</tr>
</tbody>
</table>

* Range with two independent plant preparations.
activity was not decreased in extracts from plants which had been pregrown in 3 \mu M Cho.

**CTP-P-Cho Cytidylyltransferase**

This activity was assayed in brei only (5). In one experiment, activity in a brei from plants pregrown in 25 \mu M Cho was 88% of that in a brei from control plants. The reaction rate in the standard assay is judged by the rate of conversion of \[^{14}\text{C}]\text{P-Cho}, \text{present at a relatively low concentration, to} \[^{14}\text{C}]\text{CDP-Cho}. \text{This conversion rate of} \[^{14}\text{C}]\text{ Cho will be decreased if sufficiently large amounts of carrier P-Cho are added to the reaction mixture (5). Plants pregrown in 25 \mu M Cho are known to contain greatly increased amounts of P-Cho (Table II). Correction of the rate observed with the Cho-grown plants in accord with the effect estimated to result from the amount of P-Cho added with the brei from these plants restored the calculated rate of cytidylyltransferase activity to the control value.}

**Effects of Growth in Cho upon the Tissue Concentrations of AdoMet and S-Methylmethionine**

Plants were labeled to isotopic equilibria with \(^{35}\text{SO}_{4}^{2-}\) of known specific activity by growth in either standard or 25 \mu M Cho-containing media. The tissue concentrations of AdoMet and S-methylmethionine were then measured from the \(^{35}\text{S}\) contents of these compounds. AdoMet in control plants was 3.2 to 5.0 pmol/frond (range in two independent plant samples); in Cho-grown plants, 5.0 pmol/frond. S-Methylmethionine in control plants was 4.9 to 7.4 pmol/frond; in Cho-grown plants, 4.8 pmol/frond.

**In Vitro Effects of Various Compounds upon AdoMet:P-EA N-Methyltransferase Activity**

A few studies were conducted of possible inhibitory effects on the activity of AdoMet:P-EA N-methyltransferase. P-EA was 100 \mu M. Using a brei as enzyme, the following inhibitions were observed: P-Cho, 70 \mu M, 17%; P-Cho, 175 \mu M, 27%; Cho, 400 \mu M, 0%; EA, 175 \mu M, 0%; using a gel-filtered supernatant fluid, P-Cho, 1 mm, 73%; Cho, 10 mm, 26%; EA, 2 mm, 23%.

**DISCUSSION**

Lines of evidence reported in this paper, and derived from labeling intact *Lemna* plants with L-[\(^{3}\text{H}\)]methionine (Table I), L-[\(^{14}\text{CH}_{3}\)]methionine (Table II), or [1,2-\(^{14}\text{C}\)]EA (Table IV), each support the conclusion that growth in Cho brings about a marked decrease in the rate of biosynthesis of methylated EA moieties by these plants. The in vivo loci of the block may be placed with confidence at the committing step in the biosynthetic sequence at which P-EA is methylated by AdoMet to form P-MEA. Thus, in Cho-grown plants, radioactive methyl groups from methionine rapidly enter AdoMet but do not progress normally to enter P-MEA or any of its subsequent methylated derivatives up to, and including, PtdCho. Likewise, radioactive EA moieties enter P-EA and PtdEA, but not P-MEA or subsequent methylated derivatives.

This block in the methylation of P-EA is highly specific (Table II): radioactive methyl groups derived from methionine continue to enter AdoMet, S-methylmethionine and the methyl moieties of pectin methyl ester. Radioactivity from methionine continues also to label nonpolar lipid. In control plants, somewhat more than half of this radioactivity has been shown to be in the methyl moiety of Cho methyl ester; the remainder, in a chemical form which is as yet uncertain (14). Finally, the major flux of radioactivity into compounds located in the de-esterified pellet also continues. In control plants some 25% of such radioactivity has been shown to be present in methylated derivatives of nucleic acids and basic protein amino acids, with the remainder in protein methionine itself (14). In all instances, the portion of total tissue radioactivity found in these compounds was higher in choline.

---

**Table VI. Effect of Growth in 3 \mu M Cho upon Various Enzyme Activities**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cho Added to Growth Medium</th>
<th>Relative Rate in Cho Plants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>3 \mu M</td>
</tr>
<tr>
<td>P-EA methylpherase(^a)</td>
<td>1.4 (1.2–1.7; 3)(^b)</td>
<td>0.27 (0.25–0.28; 2)</td>
</tr>
<tr>
<td>P-MEA methylpherase</td>
<td>3.4 (3.3–3.5; 3)(^b)</td>
<td>2.4 (2.4–2.5; 2)</td>
</tr>
<tr>
<td>P-DMEA methylpherase</td>
<td>3.3 (3.0–3.6; 3)(^b)</td>
<td>2.0 (1.8–2.2; 2)</td>
</tr>
<tr>
<td>Cho kinase</td>
<td>65 (64–65; 2)</td>
<td>69 (67–71; 2)</td>
</tr>
<tr>
<td>EA kinase</td>
<td>14 (11–15; 3)</td>
<td>18 (17–18; 2)</td>
</tr>
</tbody>
</table>

\(^a\) Methylpherase has been used as an abbreviation for N-methyltransferase. \(^b\) Values taken from Table II of our previous publication (5).
grown plants than in controls. This observation is likely to be explained, not by a higher rate of synthesis of each of these compounds in the choline-grown plants, but rather by a higher specific radioactivity of the pool of soluble methionine which is a precursor of each. Work from this laboratory has shown that, in \textit{Lemna}, the specific radioactivity of the methyl group of administered methionine is diminished in the plant tissue due to dilution by newly formed nonradioactive methyl moieties which are incorporated into methionine \cite{7,9}. The extent of this dilution is determined by the demand of methyl groups required for transmethylation reactions. In the Cho-grown plants, in which the utilization of methionine methyls for methylation of P-EA is diminished, such dilution will be less, and the specific radioactivity of the methyl group of methionine will remain higher.

The results of the experiments reported in Tables I, II, and IV agree also in indicating that the block at the conversion of P-EA to P-MEA, although marked, is not complete. Radioactivity derived from methionine methyls continued to flow through this step at some 7 to 10\% of the control rate. Indeed, according to the considerations outlined in the paragraph above, this rate of flow of radioactivity probably somewhat overestimates the true rate of residual methylation of P-EA, because the specific radioactivity of methionine methyl is higher in the Cho-grown than in the control plants.

Insight into the extent of down-regulation of Cho biosynthesis as a function of the amount of Cho taken up from the medium is provided by the isotope dilution experiments reported in Table III. We note that the rates of Cho synthesis calculated in these experiments are subject to considerable uncertainty. For example, if the alternative estimates of Cho uptake differ, the estimates of synthesis will vary accordingly. (A case in point is provided by the values for plants grown at 0.01 $\mu$M Cho. In such cases, we think the value based upon tissue $^{14}$C content \cite{Table III, row h} is probably more reliable, since the calculation of removal of $^{14}$C from the medium was often based on decreases of only 10\%–15\%.) Furthermore, as already mentioned in "Results," in cases in which the specific activity of the medium Cho is close to (or even below) that of tissue PtdCho (as was true for all plant samples grown at 1.4 $\mu$M Cho, or above), the calculated rates of synthesis will also be less certain. Bearing in mind these limitations, we think, nevertheless, that the results in Table III support several important conclusions. Plants grown at a concentration of 0.3 $\mu$M Cho took up some 0.39 nmol/frond-doubling of this compound, and decreased the rate of its synthesis by 0.730 – 0.414 = 0.32 nmol/frond-doubling (as compared to plants provided with only a trivial amount of Cho from the medium, \textit{i.e.} those grown at 0.01 $\mu$M Cho). (The somewhat more reliable values from rows b and h \cite{Table III} have been used to carry out these and the following calculations.) Plants grown at 0.65 $\mu$M Cho took up 0.715 nmol and decreased their rate of synthesis by 0.730 – 0.156 = 0.57 nmol. In both instances, the increment in uptake has been almost balanced by a down-regulation of synthesis. The same conclusion is supported by the observation that the total amount of tissue Cho derivatives \cite{Table III, row k} increased relatively little up to 0.65 $\mu$M Cho in the medium. Again, it appears that uptake has been compensated by almost commensurate down-regulation of synthesis. At higher concentrations of medium Cho, rates of uptake exceeded the rate of synthesis in unsupplemented plants, and endogenous synthesis became undetectable by the technique used in these experiments. Total tissue Cho then virtually tracked Cho uptake. (This would be the case if the plants do not metabolically degrade Cho moieties. Previous results from our laboratory have demonstrated that this is so for plants grown without exogenous Cho \cite{9}. The absence of any detected $^{14}$C-containing products formed from [$^{14}$CH$_3$] Cho [other than those containing the Cho moiety, itself! \cite{see legend to Table III} support the likelihood that this is so even for plants grown in, and accumulating, relatively high concentrations of P-Cho and Cho \cite{Table III, rows i and j}.)

The data in Table III provide, also, some insight into which compound (or compounds) might be effectors in bringing about the down-regulation of P-EA methylation attendant upon growth in Cho. Over the critical range from 0.01 to 1.4 $\mu$M external Cho, in which this down-regulation took place, the tissue concentration of P-Cho increased markedly (about 10-fold), but thereafter increased relatively little (about 1.7-fold). Certainly, this observation is consistent with P-Cho being an effector. The concentration of free Cho also increased (about 5-fold) as medium Cho was increased to 1.4 $\mu$M. Although free Cho continued to increase (another 15-fold) as medium Cho was raised over the range in which further down-regulation of P-EA methylation was largely absent, the data do not preclude free Cho (or perhaps some tissue pool containing only a portion of this compound) from playing a role in the regulatory phenomenon under discussion.

One mechanism which almost certainly contributes to the down-regulation of P-EA methylation brought about by growth in Cho is a decrease in the total available activity of AdoMet:P-EA N-methyltransferase itself. This decrease occurs over the appropriate range of external Cho concentrations \cite{Table V}, is in reasonable quantitative agreement with the physiological extent of the down-regulation, and is at least partially specific for this enzyme activity \cite{Table VI}. A decision as to whether this decrease is due to a down-regulation of the number of active enzyme molecules, or to the production of an enzyme with diminished specific activity, must await the results of further experimentation, as must an understanding of the relationship of this decrease to the less marked effects on AdoMet:P-MEA and P-DMEA N-methyltransferase activities \cite{Table VI}.

A second mechanism which may also contribute to reduction of P-EA methylation in plants grown in Cho is feedback inhibition of AdoMet:P-EA N-methyltransferase activity by P-Cho. After growth in 1.4 $\mu$M Cho, P-Cho rose to a concentration of 0.296 nmol/frond \cite{Table III}. Since the average volume of a frond is close to 500 nL \cite{2}, this is equivalent to 590 $\mu$M (assuming P-Cho is uniformly distributed in plant tissue). Based on the results of the \textit{in vitro} studies of inhibition, this concentration would produce a major inhibitory effect on P-EA N-methyltransferase activity.

An objection may be raised to the conclusion that a decrease in the activity of AdoMet:P-EA N-methyltransferase (whether due to a decrease in the amount of active enzyme, and/or to feedback inhibition) will result in a decrease in the rate of P-EA methylation. This objection follows from the observation...
that this methyltransferase, at least under the in vitro conditions studied, has a high $K_m$ for P-EA ($>675$ μM) (5). If the enzyme normally functions below saturation with P-EA, and a decrease in its activity led to accumulation of this substrate, the net result might be restoration of flux back almost to its original value, negating effective regulation at this step.

To evaluate the weight of this objection, several steps were taken. First, the concentration of P-EA in control plants was calculated using the percent values in the first column of Table IV and the reasonable assumption that all EA derivatives in this column have the same specific radioactivity. Based upon a concentration of 0.69 nmol/frond for PtdCho (see Table III), the resulting calculated concentration for P-EA was 0.019 nmol/frond,$^4$ or 38 μM (assuming uniform tissue distribution), well below the apparent $K_m$ of the N-methyltransferase for this substrate. The concentration of P-EA in plants grown in choline cannot be specified with the same certainty. However, we have observed (SH Mudd, AH Datko, unpublished data) that Lemna paucicostata plants grown in our standard medium with 300 μM EA added, and taking up EA at a rate of some 3 nmol/frond·doubling (and increasing their concentration of P-EA 30-fold, or more, do not significantly change their content of PtdEA. Therefore, it seems reasonable to assume that the plants grown in 3 μM Cho (Table IV) likewise will contain an unchanged concentration of PtdEA. Based on this assumption (and the data in Table IV), one can calculate concentrations for the plants grown in 3 μM Cho of 0.025 nmol/frond for P-EA (and 0.072 nmol/frond for EA), concentrations which are essentially unchanged from those in the control plants. Thus, it appears likely that during growth in Cho, EA synthesis is down-regulated sufficiently to compensate for the decreased utilization of P-EA, and that the latter compound does not accumulate enough to negate the regulatory effect of the decreased activity of AdoMet:P-EA N-methyltransferase.

Regulatory control of the biosynthesis of methylated EA moieties at the step involving P-EA methylation, reported here for L. paucicostata, appears not to be restricted to that plant. Feedback inhibition and down-regulation of AdoMet:P-EA N-methyltransferase activity have each been implicated in studies of other plant systems. Hanson and Rhodes (11) infiltrated salinized sugarbeet leaves with a trap of P-Chol. Sixty min later the leaves were infiltrated with [14C]formate, and incubated for an additional 30 min. The result was a decrease of as much as 70% in the accumulation of 14C.

$^4$ Similar calculations for other values in Table IV gave the following results (in nmol/frond): EA: 0.076; PtdCho, 0.273; total unmethylated EA derivatives, 0.369; total methylated EA derivatives, 0.790. The validity of the values calculated by this means is supported by their consistency with similar values arrived at in different ways. Thus, the calculated value for PtdCho, 0.27 nmol/frond, agrees well with 0.26 nmol/frond, the value for PtdCho measured on the basis of an experiment in which control plants were labeled to isotopic equilibrium with $^{32}P$O$_4^-$ (15). Further, the calculated total for methylated EA derivatives, 0.79 nmol/frond agrees well with the value in Table III, 0.75 nmol/frond, for total tissue Cho derivatives (which constitute by far the major portion of the total methylated EA derivatives).

in Cho, P-Chol, PtdCho, and betaine, taken together. These results, as well as those of computer modeling of the data, were interpreted as consistent with feedback inhibition by P-Chol on flux through the methylation sequence (11). We have carried out with intact tissue-cultured cells of both soybean and carrot in vivo labeling studies with L-[3H]methylmethionine similar to those reported here for Lemna. The results, reported in a companion paper (17), indicate that pregrowth of these preparations in Cho brings about effective down-regulation of the flux of methyls originating in methionine into the network of methylated derivatives of P-EA. This down-regulation is accompanied by decreases in the activity of AdoMet:P-EA N-methyltransferase, much as occurs with Lemna.

Finally, it is noteworthy that the down-regulation of the biosynthesis of methylated EA moieties documented in this paper as resulting from growth in Cho may require the plant to make major adjustments in its AdoMet metabolism. Cho biosynthesis in L. paucicostata growing under our standard conditions accounts for 55% of the entire quantity of methionine methyl groups utilized for transmethylation (14). During growth in Cho, which down-regulates P-EA methylation by 90 to 95%, the overall demand for transmethylation is then alleviated to the extent of 55% × 0.90 (or 0.95) = 50% (or 52%). This amounts to a decrease in AdoMet consumption of some 2.2 nmol/frond.$^3$ Relative to other methionine derivatives, this is a very large amount. For example, total protein methionine is about 1.05 nmol/frond (14), and soluble methionine 0.007 nmol/frond (1). Yet, as shown here, during growth in Cho the tissue concentration of AdoMet remained virtually the same as that in control plants, 0.005 nmol/frond. Clearly, the plant possesses a mechanism (or mechanisms) to avoid accumulation of this compound in the face of major variations in the rate at which it is being utilized. Not only does this avoid possible perturbations in the rates of other transmethylation reactions, but it also avoids tying up an undue amount of methionine in the form of AdoMet. Maintenance of the pool of soluble methionine (which, as emphasized above, is very small relative to its turnover) is certainly necessary to permit ongoing protein synthesis. To explain the avoidance of AdoMet accumulation, it would be worthwhile to search for down-regulation of the enzyme responsible for synthesis of this compound, ATP:methionine adenosyltransferase, during growth in Cho. We suggest, also, that the working of what might be called the ‘S'-methylmethionine cycle' may provide an additional mechanism for fine-tuning the concentration of AdoMet. In this cycle, AdoMet and

$^3$ The same value for decreased AdoMet consumption is arrived at by consideration of the data on P-EA utilization. Thus, from the values for total un-methylated and total methylated EA derivatives in footnote 4, it can be calculated that total EA synthesis in control plants is 0.369 + 0.790 = 1.16 nmol/frond. Of this, 0.790 + 1.16 × 100% = 68% is normally methylated (in the form of P-EA). During growth in Cho, such methylation is decreased by 90% (to 95%), reducing the utilization of P-EA by 0.79 × 0.90 (to 0.95) = 0.71 (to 0.75) nmol/frond. Since conversion of each P-EA to P-Chol requires three methyl groups, the resulting decrease in AdoMet utilization will be 3 × 0.71 (to 0.75) = 2.1 (to 2.3) nmol/frond.
methionine are utilized to form $S$-methylmethionine and $S$-adenosylhomocysteine. The latter is cleaved to homocysteine, which then reacts with $S$-methylmethionine to form two molecules of methionine (14; SH Mudd, AH Datko, unpublished observations). As a result, AdoMet is converted back to methionine without net consumption of methyl groups. The possible physiological advantage of such a cycle under conditions in which the plant may tend to have more AdoMet, and less methionine, than is desirable is obvious.

**LITERATURE CITED**