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^{14}C Tracer Evidence for Synthesis of Choline and Betaine via Phosphoryl Base Intermediates in Salinized Sugarbeet Leaves[1]

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ABSTRACT

Like other chenopods, sugarbeets (*Beta vulgaris* L. cv Great Western D-2) accumulate glycine betaine when salinized; this may be an adaptive response to stress. The pathway of betaine synthesis in leaves of salinized (150–200 millimolar NaCl) sugarbeet plants was investigated by supplying [^{14}C]formate, phosphoryl[^{14}C]monomethylethanolamine ([^{14}C]Ⓟ MME) or phosphoryl[^{14}C]choline ([^{14}C]Ⓟ choline) to leaf discs and following ^{14}C incorporation into prospective intermediates. The ^{14}C kinetic data were used to develop a computer model of the betaine pathway.

When [^{14}C]formate was fed, Ⓟ MME, phosphoryldimethylethanolamine (Ⓟ DME) and Ⓟ choline were the most prominent methylated products at short labeling times, after which ^{14}C appeared in free choline and in betaine. Phosphatidylcholine labeled more slowly than Ⓟ choline, choline, and betaine, and behaved as a minor end product. Very little ^{14}C entered the free methylethanolamines. When [^{14}C]Ⓟ MME was supplied, a small amount was hydrolyzed to the free base but the major fate was conversion to Ⓟ DME, Ⓟ choline, free choline, and betaine; label also accumulated slowly in phosphatidylcholine. Label from supplied [^{14}C]Ⓟ choline entered choline and betaine rapidly, while phosphatidylcholine labeled only slowly and to a small extent.

These results are consistent with the pathway Ⓟ MME → Ⓟ DME → Ⓟ choline → choline → → betaine, with a minor side branch leading from Ⓟ choline into phosphatidylcholine. This contrasts markedly (a) with the pathway of stress-induced choline and betaine synthesis in barley, in which phosphatidylcholine apparently acts as an intermediate (Hitz, Rhodes, Hanson 1981, Plant Physiol 68: 814–822); (b) with choline biogenesis in mammalian liver and microorganisms. Computer modeling of the experimental data pointed strongly to regulation at the Ⓟ choline → choline step, and also indicated that the rate of Ⓟ choline synthesis is subject to feedback inhibition by Ⓟ choline.

The levels of betaine[3] in shoots of chenopods increase in response to salinization or water deficit. This stress response is found in the crop species sugarbeet (11) and spinach (2, 17) as well as in wild halophytic Chenopodiaceae (11, 20). Betaine accumulation is also a metabolic response to water or salt stress among certain wild and cultivated Gramineae, including barley (8, 12, 25), *Spartina* (19), and the tropical pasture species green panic and buffel grass (5). It has been suggested that stress-induced betaine accumulation is adaptive, with betaine acting as a nontoxic cytoplasmic osmoticum and/or as a protectant against enzyme inactivation (16, 24).

In neither beets (11) nor barley (14) is betaine readily catabolized by any organ, and its accumulation in stressed plants results from enhanced *de novo* synthesis via choline, principally or solely in the leaves (2, 10, 11). Whereas betaine is apparently a secondary product accumulated only in some taxa, its precursor choline is universally present in plants in the form of PC, if not also as free choline and Ⓟ choline. Although it is clear that biosynthesis of choline and betaine in plants entails methylation of ethanolamine (2, 3, 10), little is known of the intermediates or enzymes involved in the methylations. In animals and microorganisms, the methylations are believed to occur predominantly at the level of phospholipid-bound bases (reviews, 6 and 7) so that turnover of phospholipid head groups is an integral part of *de novo* choline synthesis. Although there is evidence that spinach leaves and castor bean endosperm contain enzymes capable of methylating PE and its mono- and dimethyl derivatives, the quantitative significance of this pathway as a source of choline *in vivo* is unclear (review, 15; see also 2 and 7). Radiotracer studies of water-stressed barley leaves have implicated an active alternate methylation pathway via phosphoryl bases, although the phospholipid PC appeared to be involved because ^{14}C from Ⓟ choline passed via PC before entering a free choline pool destined for oxidation to betaine (13). Similar ^{14}C tracer experiments on choline and betaine synthesis in leaves of spinach (2), and sugarbeet (11) have recently indicated that these chenopods may differ from barley in that there is no participation of phospholipids at all; however, in neither of these studies were the intermediates in the soluble methylation pathway identified. In the present experiments, we therefore sought to find the earliest methylation products of choline and betaine biosynthesis in salinized sugarbeet, by using [^{14}C]formate as a source of [^{14}C]methyl groups. We then synthesized the prospective intermediates [^{14}C]Ⓟ MME and [^{14}C]Ⓟ choline, and followed their metabolism by salinized leaf tissue. Results were interpreted using a computer simulation model, which enabled estimation of metabolite fluxes and pool sizes.

MATERIALS AND METHODS

Plants and Growing Conditions. Plants of the commercial sugarbeet (*Beta vulgaris* L. cv Great Western D-2) were grown in 7-cm diameter plastic pots of vermiculite (3 plants/pot) in growth chambers as described previously (11). Salinization with 25 mM NaCl added to the nutrient solution was started 3 weeks after

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[3] Abbreviations: betaine, glycine betaine (*N,N,N*-trimethylglycine); MME, monomethylethanolamine; DME, dimethylethanolamine; Ⓟ ethanolamine, phosphorylethanolamine; Ⓟ MME, phosphorylmonomethylethanolamine; Ⓟ DME, phosphoryldimethylethanolamine; Ⓟ choline, phosphorylcholine; PE, phosphatidylethanolamine; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyldimethylethanolamine; PC, phosphatidylcholine; CDP, cytidine diphosphate; QAC, quaternary ammonium compound(s); ψ_s, solute potential.

planting, and the NaCl concentration was then raised in 25 mM steps every 3 d (11). Unless otherwise stated, leaves were harvested for labeling experiments when the NaCl concentration was 150–200 mM, after 3 d at the concentration specified. Leaf solute potential (ψ_s) was estimated on frozen/thawed leaf discs (11).

Radiochemicals and Labeling Experiments. [^{14}C]Formate (55 or 59 μCi μmol^{-1}) and [2-^{14}C]Ⓟ ethanolamine (33 μCi μmol^{-1}) were from Amersham Corp.; [*methyl*-^{14}C]CDP-choline (53 μCi μmol^{-1}) was from New England Nuclear. [^{14}C]Ⓟ MME and [^{14}C]Ⓟ choline (9.5 μCi μmol^{-1}) were synthesized by methylating [^{14}C]Ⓟ ethanolamine (5 μmol, 47 μCi) with 30 μmol of methyl iodide under conditions given in Reference 13.

Leaf discs (11 mm diameter) for labeling were punched from leaves close to full expansion, infiltrated with 1-μl droplets containing ^{14}C-compounds, and incubated in darkness (11). In some experiments, an unlabeled trapping pool of Ⓟ choline (Ca^{2+} salt, aqueous solution, pH 7–8) was infiltrated 1 h before infiltration with [^{14}C]formate. In all experiments, samples comprised a batch of 3 discs, each from a different leaf. Incubations were stopped by freezing discs in liquid N_2.

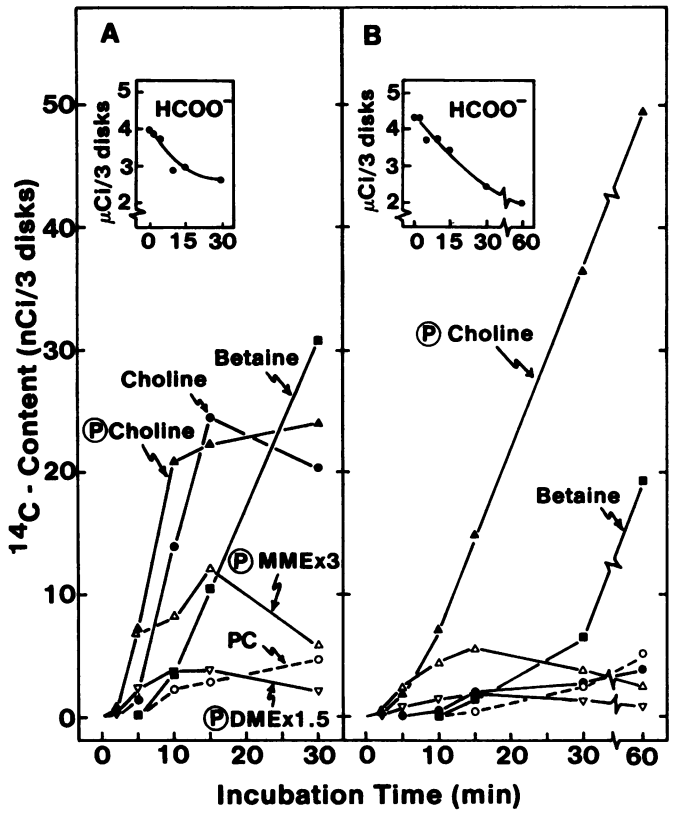

FIG. 1. Representative time courses for labeling of QAC and related metabolites during incubation in darkenss of salinized sugarbeet leaf discs infiltrated with [^{14}C]formate. Zero time was taken as the midpoint of the infiltration process, which took 1 to 2 min. Data points are for single batches of three discs. A, Plants salinized to 150 mM NaCl, leaf $\psi_s = -15$ bars. [^{14}C]Formate dose = 24 nmol, 1.3 μCi/disc. B, Plants salinized to 200 mM NaCl, leaf $\psi_s = -18$ bars. [^{14}C]Formate dose = 26 nmol, 1.4 μCi/disc. Symbols in A and B are the same. Insets are maximum estimates of [^{14}C]formate remaining in the discs (equal to aqueous phase ^{14}C not accounted for by metabolites). Throughout both experiments, the TLC zones corresponding to free MME, DME, and their phospholipid derivatives contained very little ^{14}C (≤0.5–1 nCi/3 discs). For purposes of comparison, ^{14}C contents of Ⓟ MME were multiplied by 3, those of Ⓟ DME by 1.5, based on the assumption that these compounds contained respectively one and two [^{14}C]methyl groups, and that the QAC contained three.

Extraction, separation, identification, and determination of labeled and unlabeled compounds were carried out using the procedures given in References 11 and 13, with these modifications: (a) carrier Ⓟ choline (1 μmol/sample) was routinely added before extraction, to improve [^{14}C]Ⓟ choline recovery. (b) For experiments in which [^{14}C]Ⓟ choline and [^{14}C]Ⓟ MME were fed, labeled metabolites in the anion fraction eluted from the AG 1 resin were separated by TLC on Silica Gel G developed with methanol:acetone:concentrated HCl (90:10:4, v/v) (TLC system 1). (c) For experiments in which [^{14}C]CDP-choline was sought, samples received 2 μmol of unlabeled carrier CDP-choline and were extracted with the methanol/chloroform/water procedure given in Reference 9. The aqueous phase was freeze-dried, redissolved in water, and applied to a 1-ml column of AG 1 (OH^-) resin. This column was eluted with 4 ml 1 N HCl; the eluate was freeze-dried, taken up in 0.2 ml of water, and separated using TLC system 1.

Because the separation methods used for labeled metabolites involved several sequential microscale steps, ^{14}C-metabolites present in very small chemical amounts were not recovered quantitatively from extracts. The recoveries of ^{14}C-labeled Ⓟ MME, Ⓟ DME, and Ⓟ choline were checked by spiking unlabeled leaf disc samples with 2 to 7 nmol of these compounds before extraction; 1 μmol unlabeled carrier Ⓟ choline was also always added. Average recoveries were: [^{14}C]Ⓟ MME, 68% (three trials); [^{14}C]Ⓟ DME, 52 % (two trials); [^{14}C]Ⓟ choline, 62% (seven trials; range, 53–72%). The recovery values for [^{14}C]betaine (99%) and [^{14}C]choline (48%) have been reported elsewhere (11). Corrections for incomplete recovery were not applied to the data of Figures 1, 3, and 4, except in the case of amounts of fed [^{14}C]Ⓟ MME (Fig. 3, inset) or fed [^{14}C]Ⓟ choline (Fig. 4, inset) remaining unmetabolized.

Computer Modeling of ^{14}C-Labeling Kinetics. The kinetics of ^{14}C metabolism were simulated iteratively by a computer program analogous to that used by Hitz *et al.* (13) which derives from the mathematical treatment of isotopic labeling in steady state metabolic pathways given by Folkes and Sims (4). The principles are as follows. The rate of isotope accumulation in a specific intermediate of a biosynthetic pathway is determined by the pool size of the intermediate, its rate of synthesis, its rate of utilization, and the specific activity of its precursor. Distortions of labeling kinetics can arise when two or more pools of an intermediate exist within the tissue. Computer models were constructed such that pool sizes, rates of synthesis, rates of utilization, precursor-product relationships, compartmentation of pools, and transport (equilibrium) rates between pools could be parametized. Within the computer program, during short time intervals (0.001 h of the incubation period), material of known specific activity was drawn into, mixed with, and then withdrawn from pools of estimated initial size at estimated rates. During each time interval, new values of pool sizes and specific activities were computed, and utilized to perform the next repetitive influx/efflux operations throughout a programmed reaction sequence. This iterative process generated simulated pool sizes and specific activities for each component of the pathway throughout the entire time course of the required incubation period. Values of pool sizes, synthesis, utilization, uptake and transport rates were adjusted in the model until simulated labeling kinetics closely matched the observed data. Computer models were executed on a HP 3354 computer (Hewlett-Packard, Palo Alto, CA).

RESULTS AND DISCUSSION

[^{14}C]Formate-Labeling Experiments. Figure 1 shows two representative short time courses of labeling of methylated compounds. They share the following features. (a) At 2 to 5 min, Ⓟ MME, Ⓟ DME, and Ⓟ choline were major labeled products, but

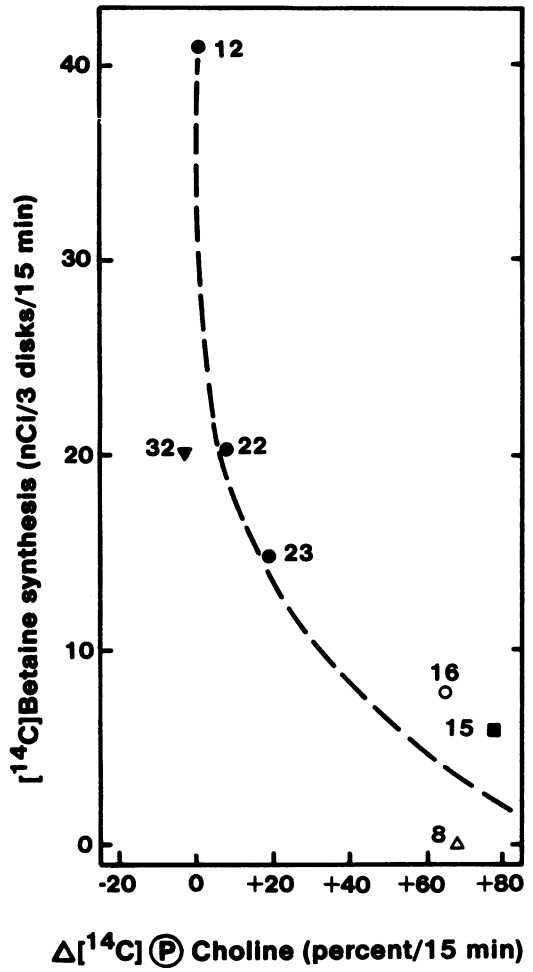

FIG. 2. Scatter plot relating the rates of [^{14}C]betaine synthesis (y) and change in the pool size of [^{14}C]Ⓟ choline (x) between 15 and 30 or 60 min during incubation of sugarbeet leaf discs with [^{14}C]formate. Data points are from Figure 1 and from five similar experiments carried out over a 2-year period. Numbers next to points give the [^{14}C]Ⓟ choline pool size (nCi/3 discs) at 15 min. Plants received the following NaCl treatments: (●) salinized to 150 mM; (△) salinized to 150 mM and held at this level for a total of 6 d; (○) salinized to 150 mM, then rewatered for 3 d; (▲) salinized to 175 mM; (■) salinized to 200 mM. The broken trend line was hand-drawn. The coefficient of linear correlation (r) between y and x was −0.82, significant at the 5% level.

the corresponding free bases and phosphatidyl bases were not appreciably labeled (Fig. 1). (b) The ^{14}C content of Ⓟ MME and Ⓟ DME increased until 10 to 15 min and declined thereafter. This decline paralleled depletion of the [^{14}C]formate pulse, of which at least one-third had been metabolized by 30 min (insets, Fig. 1). (c) Free choline began to label only after Ⓟ choline was already labeled, and accumulated little or no further label after 15 min. (d) A lag period of 5 to 10 min preceded appearance of significant ^{14}C in betaine, after which the rate of [^{14}C]betaine synthesis quickly became linear. (e) PC acquired very little ^{14}C until about 10 min, and then began to accumulate ^{14}C slowly and continuously.

There are notable differences between the experiments of Figure 1, A and B, in the labeling patterns of Ⓟ choline and betaine. In A, the level of [^{14}C]Ⓟ choline changed little after 10 min whereas in B it continued to increase steadily throughout the experiment and [^{14}C]Ⓟ choline was the most heavily labeled product at 60 min. The rate of [^{14}C]betaine synthesis was more than 3-fold higher in A, and [^{14}C]betaine had become the major ^{14}C-QAC by

Table I. *Levels of [^{14}C]CDP-Choline in Salinized Sugarbeet Leaf Discs Exposed to [^{14}C]Formate for 15 Minutes*

In all experiments, carrier CDP-choline (2 μmol) was added before extraction to duplicate batches of three leaf discs. In a check experiment, 0.8 nmol (44 nCi) of authentic [^{14}C]CDP-choline was added to batches of unlabeled discs before adding carrier CDP-choline; 14 nCi of [^{14}C]CDP-choline (32%) was recovered.

Experiment	CDP-Choline		[^{14}C] Ⓟ Choline
	Carrier recovery[a]	^{14}C content[b]	
	%	*nCi/3 discs*	*nCi/3 discs*
1	33	≤0.8	32
2	30	≤0.4	10
3	20	≤0.1	15

[a] Carrier recovery was determined spectrophotometrically by $A_{280\ nm}$ in Experiment 1. In Experiments 2 and 3, carrier recovery was estimated from the size and intensity of UV-absorbing bands on TLC relative to a range of CDP-choline standards.

[b] No discrete ^{14}C band corresponding to CDP-choline was detectable by autoradiography; most of the ^{14}C recovered in the CDP-choline zone represented slight tailing of the heavily labeled Ⓟcholine band which ran ahead of CDP-choline in TLC system 1.

30 min. Figure 2 demonstrates that these differences conform to a statistically significant trend in the results of various [^{14}C]formate experiments. High [^{14}C]betaine synthesis rates occurred only in cases where the level of [^{14}C]Ⓟ choline remained fairly constant after 15 min, whereas low [^{14}C]betaine synthesis rates were associated with a continuous increase in the amount of [^{14}C]Ⓟ choline. Although some of the experiment-to-experiment variation described in Figure 2 is apparently random, it is of interest that neither rewatered plants nor plants kept for a prolonged period at 150 mM NaCl showed high rates of [^{14}C]betaine synthesis.

If it is assumed that the main pathway of choline and betaine synthesis in sugarbeet involves the free bases (MME → DME → choline → → betaine), as proposed by Coughlan and Wyn Jones for spinach (2), then the following predictions can be made about labeling kinetics. (a) Because the free bases MME and DME acquire little ^{14}C (Fig. 1), the pool sizes of these intermediates must be extremely small and any substantial flux through these pools would lead to rapid isotope incorporation into choline with virtually no lag. (b) Label should not enter Ⓟ choline before appearing in free choline. Neither of these predictions is satisfied by the results of Figure 1. Rather, the results of Figure 1 suggest operation of a methylation pathway at the level of phosphoryl bases (Ⓟ MME → Ⓟ DME → Ⓟ choline) with hydrolysis of Ⓟ choline to choline followed by oxidation of choline to betaine. The results also indicate that, in beet leaf discs, Ⓟ choline can behave not only as an intermediate, but also—when betaine synthesis rates are low—as an end product. Although the kinetic data conform with the view that Ⓟ choline is the precursor of PC, they do not implicate PC as an intermediate between Ⓟ choline and free choline as in barley (10, 13), because PC only began to acquire label after choline became appreciably labeled.

The extraction and fractionation procedures used left open the possibility that the [^{14}C]Ⓟ choline and other phosphoryl bases were, in whole or in part, hydrolysis products of more complex derivatives such as nucleotide bases (7). To check this point for [^{14}C]Ⓟ choline, mild extraction and fractionation procedures were applied to [^{14}C]formate-fed samples containing carrier CDP-choline (Table I). Little ^{14}C was recovered as CDP-choline (no more than 3–13% of that in Ⓟ choline at 15 min), which argues against heavy ^{14}C traffic through CDP-choline (and other CDP-bases) relative to that through Ⓟcholine.

In all experiments with [^{14}C]formate, the $CHCl_3$ fraction contained an unknown ^{14}C-metabolite that ran close to PC; this

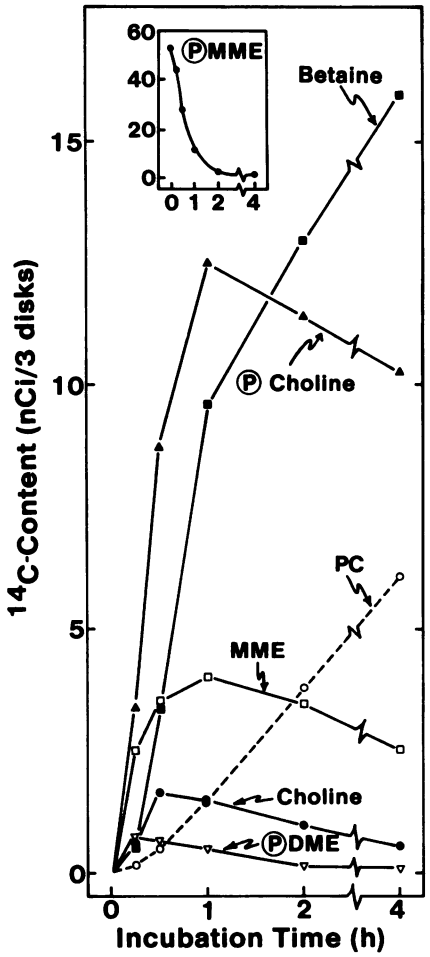

FIG. 3. Time courses for labeling of metabolites of [^{14}C]Ⓟ MME in salinized sugarbeet leaf discs. Plants were salinized to 150 mM NaCl (leaf ψ_s = −18 bars). The [^{14}C]Ⓟ MME dose was 53 nCi (5.6 nmol)/3 discs. Data points are for single batches of three discs. The values for free [^{14}C] MME were corrected for 0.8% hydrolysis of [^{14}C]Ⓟ MME during processing. The TLC zones corresponding to PMME and PDME contained only very small amounts of ^{14}C (≤0.1 nCi/3 discs) at all sampling times. No labeling of free DME was detected by autoradiography (detection limit about 0.2 nCi/3 discs). Inset shows [^{14}C]Ⓟ MME remaining, corrected for incomplete recovery (68%).

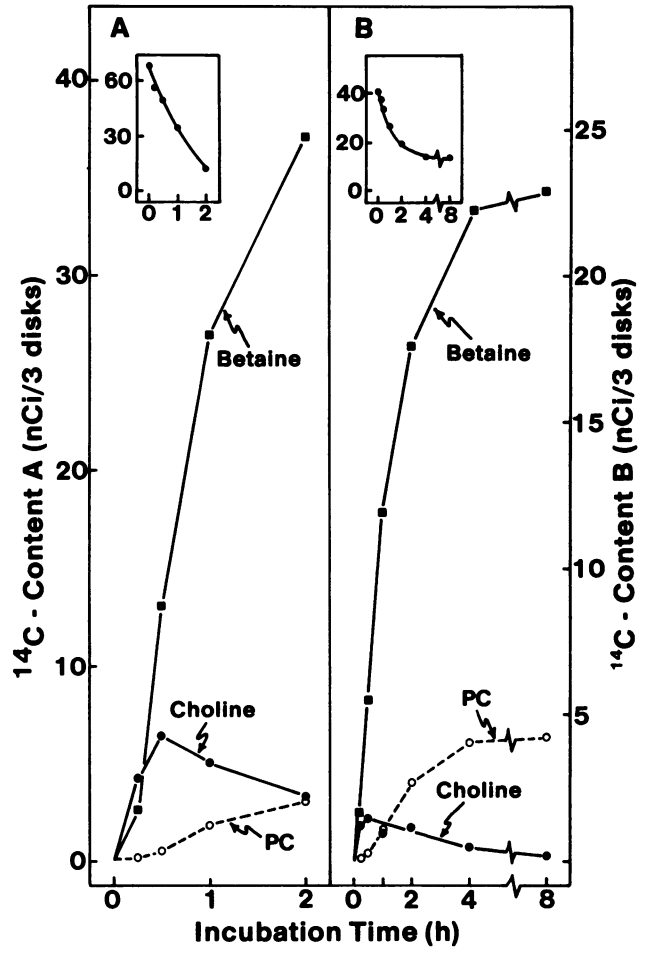

FIG. 4. Time courses for labeling of free choline, betaine, and PC from [^{14}C]Ⓟ choline in salinized sugarbeet leaf discs. Plants were salinized to 150 mM NaCl (leaf ψ_s = −15 bars). [^{14}C]Ⓟ choline doses per three discs were: A, 68 nCi (7.2 nmol); B, 41 nCi (4.3 nmol). Data points are for single batches of three discs. The values for free [^{14}C]choline were corrected for hydrolysis of [^{14}C]Ⓟ choline that occurred during processing; hydrolysis was 2% in A, 4.5% in B. No label was detected by autoradiography in PDME, PMME, Ⓟ DME, Ⓟ MME, DME, or MME; detection limits (nCi/3 discs) were 0.05 to 0.1 for phospholipids and 0.3 to 0.5 for phosphoryl bases and free bases. Insets show [^{14}C]Ⓟ choline remaining, corrected for incomplete recovery (71% in A, 55% in B).

metabolite was most prominent at 5 to 15 min, when it contained up to 1 to 2 nCi/3 discs. Acid hydrolysis (4 N HCl, 20 h, 100°C) converted all the ^{14}C to water-soluble form, which migrated as a band close to the solvent front in TLC system 1. The occurrence of this metabolite may have led to overestimation of label in PC, particularly at earlier time points, so that the data for PC in Figure 1 can be viewed as maximum values. This argues further against a role for PC as an intermediate in choline and betaine synthesis.

[^{14}C]Ⓟ MME Metabolism. To test the conclusions from [^{14}C] formate-labeling experiments, the prospective intermediates [^{14}C] Ⓟ MME and [^{14}C]Ⓟ choline were synthesized and supplied to leaf discs. The discs readily absorbed and metabolized a small dose (1.9 nmol/disc) of [^{14}C]Ⓟ MME; by 2 h, 95% of the ^{14}C had been converted to other compounds (Fig. 3). The labeling patterns of Ⓟ DME, Ⓟ choline, and free choline were consistent with roles for these compounds as intermediates in betaine synthesis: all were labeled at least as heavily as betaine at the earliest time point (15 min); their ^{14}C contents peaked at 30 to 60 min, and then fell as the supplied [^{14}C]Ⓟ MME was used up. In contrast, the phospholipids PMME and PDME acquired very little ^{14}C, and PC accumulated ^{14}C steadily after an initial lag. Free MME contained almost as much label as Ⓟ choline at 15 min, and remained quite prominent throughout the experiment. Free DME, however, was not significantly labeled at any time. This indicates that the MME produced by Ⓟ MME hydrolysis is unlikely to be a substrate for methylation so that its production represents a metabolic side branch not related to Ⓟ MME conversion to QAC. This side branch is probably reversible, because plant tissues can incorporate exogenous MME and DME into the corresponding phospholipids (13, 23), presumably via phosphoryl base intermediates. Rephosphorylation could explain the decline in [^{14}C]MME after 1 h in Figure 3. The rate and extent of [^{14}C]Ⓟ MME conversion to QAC show that the supplied [^{14}C]Ⓟ MME had a very good access to an internal, metabolically active Ⓟ MME pool, and that this pool was small relative to the traffic through it; the absence of a significant storage pool of Ⓟ MME is also implied.

[^{14}C]Ⓟ Choline Metabolism. Small doses of [^{14}C]Ⓟ choline (2.4 or 1.4 nmol/disc) were metabolized to free choline, betaine, and PC (Fig. 4). As in the experiment with [^{14}C]Ⓟ MME, the labeling pattern of choline indicated that it was an intermediate between Ⓟ choline and betaine, whereas the labeling pattern of PC was

that of an end product, with no net loss of ^{14}C from PC occurring even when [^{14}C]Ⓟ choline utilization had ceased (Fig. 4B, 4–8 h). Two further points arise from the two experiments of Figure 4. Firstly, no labeled demethylation products—free DME, MME, and their derivatives—were found in either experiment, indicating that the methylation reaction sequence is irreversible, as in barley (13). Secondly, in neither experiment was the [^{14}C]Ⓟ choline depleted as rapidly as was a [^{14}C]Ⓟ MME dose of similar size (Fig. 3). Also, comparing experiments A and B of Figure 4, the maximum rates of [^{14}C]Ⓟ choline depletion and [^{14}C]betaine synthesis in B were about one-half those in A, and in B less of the supplied label was accessible for metabolism. These observations might be explained either by slow and incomplete absorption of [^{14}C]Ⓟ choline from free space, or by co-existence of, and flux between, metabolically active and storage pools of Ⓟ choline.

Trapping Experiments with Ⓟ Choline. If Ⓟ choline is an intermediate in betaine synthesis, a trapping pool of Ⓟ choline should reduce the incorporation of ^{14}C from [^{14}C]formate into betaine. Table II summarizes results with traps of 0.5 and 0.1 μmol/disc, infiltrated 1 h before [^{14}C]formate. Three effects were evident. (a) The 0.5- and 0.1-μmol traps reduced total ^{14}C-QAC synthesis by about 70 and 50%, respectively. (b) Of the ^{14}C-QAC that were synthesized, the percentage in [^{14}C]betaine was much lower in the presence of traps. (c) Conversely, the percentages of ^{14}C-QAC in the forms of [^{14}C]Ⓟ choline and [^{14}C]choline increased in the presence of traps. In the case of the 0.1-μmol trap, the increase in [^{14}C]choline was absolute (25 *versus* 15 nCi) as well as relative; this absolute increase was statistically significant ($P = 0.01$). Effects b and c are consistent with Ⓟ choline as an intermediate in betaine synthesis, provided that the traps were absorbed and suffered hydrolysis during the incubation. In experiments where a 0.5- or 0.1-μmol dose of ^{14}C-labeled Ⓟ choline was infiltrated, both doses were taken up satisfactorily and both were converted to choline and betaine, very extensively so in the case of the smaller dose (Table II). Effect a was unexpected. It was not due to depression of ^{14}C-QAC synthesis by the trap infiltration pretreatment *per se*, because the control discs were infiltrated with water. One possible interpretation is that Ⓟ choline has a feedback effect on its own synthesis.

Computer Modeling of Betaine Synthesis via Phosphoryl Base Intermediates. Our model was based on this hypothetical biosynthetic scheme:

Ⓟ ethanolamine → Ⓟ MME → Ⓟ DME → Ⓟ choline →
↓
CDP-choline
↓
PC
choline → betaine aldehyde → betaine

Only the proposed methylation sequence leading to Ⓟ choline is novel; for all reactions after Ⓟ choline, there is good biochemical and/or radiotracer evidence in plants (7, 15).

Estimates of rates of betaine synthesis and degradation used in initial model building were drawn from our published data on sugarbeet, as shown in Table III. The rates of betaine synthesis (and of all other metabolite fluxes through pathways) were assumed to remain constant after cutting discs and throughout the incubation period with ^{14}C-substrates. Values for levels of choline, Ⓟ choline, and PC in sugar beet leaves were measured; literature values for other plants were used to assign upper bounds to two noncritical parameters (Table III).

The primary aim of the modeling work was to detect inconsistencies in data from diverse ^{14}C kinetic experiments that would require rejection of the biosynthetic scheme above. A secondary aim was to detect potential regulatory mechanisms in choline and betaine synthesis. There is circumstantial evidence for precise regulation of the rate of betaine synthesis in salinized beet: betaine level is a linear function of ψ_s in leaves of salinized plants, and since such plants cannot degrade betaine, this control over betaine level must be via modulation of the rate of betaine synthesis relative to growth (11). In our ^{14}C kinetic experiments, a decrease in the amount or activity of a regulatory enzyme (or slowed transport of substrate across a compartment boundary) should provoke an accumulation of ^{14}C in at least the first intermediate upstream of the regulatory step, and a drop in ^{14}C flux downstream from this step. Also, an expanded or expanding pool size of a metabolite that exerts feedback control over the rate of its own synthesis should be associated with a decreased ^{14}C flux through steps upstream of the metabolite.

The model of Figure 5 was constructed by starting with the [^{14}C]Ⓟ choline data of Figure 4, and then proceeding to the [^{14}C]Ⓟ MME results of Figure 3; the rate of depletion of label from Ⓟ MME and the amount of label recovered in Ⓟ DME suggested that the endogenous pools of Ⓟ MME and Ⓟ DME were very small. The model was then applied to whole leaf [^{14}C]ethanol-

Table II. *Effects of Trapping Pools of Ⓟ Choline on [^{14}C]Formate Incorporation into QAC by Salinized Sugarbeet Leaf Discs*

Plants were salinized to 150 mM NaCl (leaf $\Psi s = -15$ or -17 bars). Triplicate batches of three discs were infiltrated with 1 μl of H_2O (controls) or 1 μl of Ⓟcholine solution per disc, followed 1 h later by [^{14}C]formate (0.4 μCi, 7 nmol/disc). Incubation with [^{14}C]formate was for 30 min. In concurrent 90-min experiments, the uptake and metabolism of 0.5 or 0.1 μmol of [^{14}C] Ⓟcholine/disc (13 nCi/μmol) were checked using duplicate batches of three discs, which were washed in 10 ml of 1 mM Ⓟcholine before extraction. The ^{14}C activity washed out within 5 min was taken as label not absorbed from free space.

Treatment	Total ^{14}C-QAC[a]	^{14}C Distribution among QAC			
		Ⓟcholine	Choline	Betaine	PC
	nCi/3 discs	*% total ^{14}C-QAC*			
Control	49	57	6	29	7
+0.05 μmol Ⓟcholine[b]	15***	75*	13**	6**	5*
Control	79	18	18	53	12
+0.1 μmol Ⓟcholine[c]	40**	24	62***	8***	6**

[a] ^{14}C contents of Ⓟ choline + choline + betaine + PC; differences between control and trap treatments significant at the 5, 1, or 0.1% levels are marked *, **, or ***, respectively.

[b] In the companion experiment with 0.5 μmol [^{14}C]Ⓟcholine, 35% was not absorbed; of the ^{14}C recovered in the washed discs, 83% was in Ⓟcholine, 4% in choline, and 13% in betaine.

[c] In the companion experiment with 0.1 μmol [^{14}C]Ⓟcholine, 21% was not absorbed; of the ^{14}C recovered in the washed discs, 22% was in Ⓟcholine, 32% in choline, and 44% in betaine.

Table III. *Experimentally Determined Values for Parameters Used for Initial Model Construction*

When necessary, values were converted to units of nmol/3 discs by taking batches of three discs to have fresh and dry weights of about 90 and 9 mg, respectively. All values for sugarbeet refer to cv Great Western D-2.

Parameter	Species and Tissue	Treatment	Value	Reference
			nmol/3 discs	
Choline content[a]	Sugarbeet leaves	NaCl, 150 mM	100	
Ⓟcholine content[a]	Sugarbeet shoots	NaCl, 150 mM	<10	
	Sugarbeet leaves	NaCl, 150 mM	<10	
PC content[b]	Sugarbeet leaf discs	NaCl, 150 mM	63	
Betaine content	Sugarbeet shoots	NaCl, 150 mM	2100	11
Betaine synthesis rate	Sugarbeet leaves	NaCl, 150 mM	8 h^{-1}	11
Betaine degradation rate	Sugarbeet leaves	NaCl, 150 mM	0 h^{-1}	11
Betaine aldehyde content	Spinach leaves	NaCl, 0 or 300 mM	<10	2
	Barley leaves	Turgid or wilted	<10	10
CDP-choline content	Spinach leaves	NaCl, 0 or 300 mM	<10	2

[a] Determined on ion exchange fractions from extracts of freeze-dried leaf or whole shoot material by isotope dilution analysis, using the Dragendorff spray reaction on thin-layer electrophoresis or TLE plates to estimate the chemical amount of choline or Ⓟcholine.

[b] Determined from the phosphate content of the PC zone on TLC plates (13).

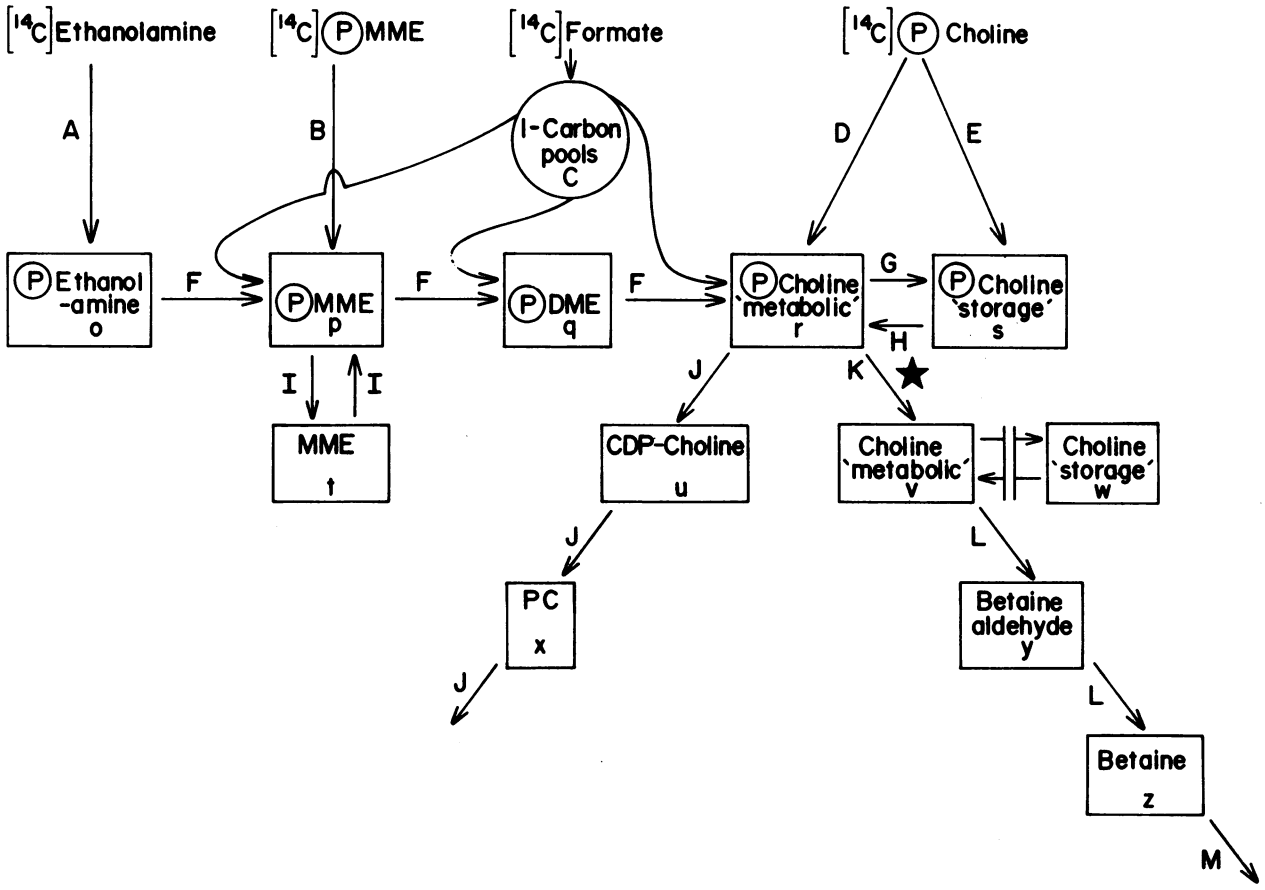

FIG. 5. A model to account for the kinetics of incorporation of ^{14}C from various ^{14}C-precursors into QAC and related metabolites in salinized sugarbeet leaf tissue. The star adjacent to the Ⓟ choline → choline flux designates a prospective control point. Parameters A, B, and D through N represent flux rates (nmol/h·3 discs) in various experiments. Parameter C is a specific radioactivity value. Parameters n through z are pool sizes (nmol/3 discs). The values for these parameters specified in Table IV are those which provided the closest agreement between computer-simulated and observed labeling kinetics of each component in the reaction scheme for each independent experiment. The known quantities and specific activities of the supplied ^{14}C-precursors and their kinetics of utilization were used first to generate estimates of uptake rates (A, B, D, E) and the flux (F) to Ⓟ choline. The pool sizes of the various intermediates of the pathway and their rates of synthesis and utilization were then progressively modulated within the computer program (using the experimental values of Table III as initial estimates, and respecting upper bounds specified therein) until a close match between observed and simulated time courses of ^{14}C incorporation was accomplished for all measured components of the system.

amine-labeling data (Fig. 3B of Ref. 11), and lastly to studies with [^{14}C]formate (Fig. 1). For this last application, the flux of [^{14}C] formate carbon into the pools of 1-carbon intermediates supplying methyl groups to the betaine pathway was taken to be small in comparison to fluxes from endogenous, unlabeled sources of 1-carbon fragments, so that the specific activity of the 1-carbon

Table IV. *Theoretical Values for Parameters Derived or Assumed during Computer Modeling*

Parameter	^{14}C-Precursor and Experimental Data Source					
	Ethanolamine (Ref. 11, Fig. 3B)	Ⓟ MME[a] (Fig. 3)	Formate[b]		Ⓟ Choline	
			Fig. 1A	Fig. 1B	Fig. 4A	Fig. 4B
Flux rates (nmol/3 discs·h)						
A	>20					
B		20				
C[c]			8.8	5.0		
D					10.0	2.0
E					0.6	1.1
F	9.3	7.0	10.0	7.0	10.0	10.0
G	<0.1	3.3	0.1	3.0	0.1	0.1
H	<0.1	0.3	0.1	0.1	0.1	0.1
I		1.5				
J	1.0	1.0	1.5	1.5	1.5	1.5
K	8.3	3.0	8.5	2.6	8.5	8.5
L	8.3	2.7	8.5	2.6	7.5	8.2
M	0	0	0	0	0	0
Initial pools (nmol/3 discs)						
o	1.7					
p	0.2	0.2	1.3	0.1		
q	0.2	0.2	0.2	0.1		
r	0.5	0.9	0.9	0.6	1.5	1.5
s	2.7	7.0	3.0	4.5	3.0	3.0
t		2.0				
u	1.0	1.0	0.3	1.0	1.0	1.0
v[d]	0.5	0.2	1.4	0.3	0.7	0.5
w	100	100	100	100	100	100
x	63	63	63	63	63	63
y	0.01	0.2	0.2	0.4	0.2	0.2
z	2100	2100	2100	2100	2100	2100

[a] In this experiment only, the simulated [^{14}C]Ⓟcholine values were reduced by a factor of 0.67 to accommodate a 67% recovery of Ⓟcholine.

[b] Because an unidentified [^{14}C]formate metabolite (see text) ran close to PC in TLC, experimental values for [^{14}C]PC at $t < 15$ min were subject to overestimation and so were not used in modeling.

[c] Parameter C is not itself a flux rate, but the specific radioactivity (dimensions, nCi/nmol) of the 1-carbon pools supplying methyl groups; the flux rate from these pools into each methylation step equals F.

[d] The model could not distinguish between ^{14}C accumulation in choline due to slight expansion of the metabolic pool *versus* slow entry of [^{14}C]choline into the large storage pool. For simplicity, the former situation was assumed, so that v, not w, was allowed to increase.

pools was far lower than that of the supplied [^{14}C]formate (1). The specific activity of the 1-carbon pools was assumed to reach a constant value after a 1 to 2 min lag. All three methylation reactions were taken to draw on the same pool of methyl donor. It was further assumed that the 'metabolic' pools of Ⓟ MME and Ⓟ DME were so small in relation to traffic through them that their specific radioactivities rapidly approached the specific radioactivity of the methyl donor pools x1, and x2, respectively. Theoretical parameters required to accommodate the isotopic labeling of each component of the reaction scheme for individual experiments are summarized in Table IV; these values were derived by progressively adjusting flux rates and pool sizes until a close approximation between observed and simulated labeling kinetics was achieved. Note that we use the term 'storage' pool not to imply a storage function but to distinguish this pool from another (metabolic) pool of the same substance through which ^{14}C flux is far greater.

Within the limits of the pool sizes and rates specified in Table IV, the model of Figure 5 gave adequate simulations of all experimental data. The observed rate of betaine synthesis could be accounted for without invoking additional methylation pathways operating on phospholipid-bound bases or free bases and it was unnecessary to consider PC as a significant source of free choline for betaine synthesis. Although a significant phospholipid methylation pathway to which supplied bases and phosphoryl bases (ethanolamine, Ⓟ MME, Ⓟ choline) had poor access could have been overlooked in experiments with such precursors, it would almost certainly have been revealed by the [^{14}C]formate experiments so that the model developed for the other ^{14}C-precursors would have been inadequate to account for the [^{14}C]formate data. These findings are in line with our earlier suggestion (11) that betaine synthesis in sugarbeet does not involve phospholipids, a view reached also for spinach by Coughlan and Wyn Jones (2). Although the latter authors mooted a methylation sequence at the level of free bases, the evidence supporting this was indirect, being based on trapping experiments with free MME and DME, and the phosphoryl bases were not analyzed in detail. It seems in any case certain that the chenopods sugarbeet and spinach have a soluble pathway of choline and betaine synthesis quite unlike the phospholipid path believed to operate in mammals and microorganisms. (It is interesting to note that radiotracer results implicated a soluble choline synthesis path in rat liver [18], although the

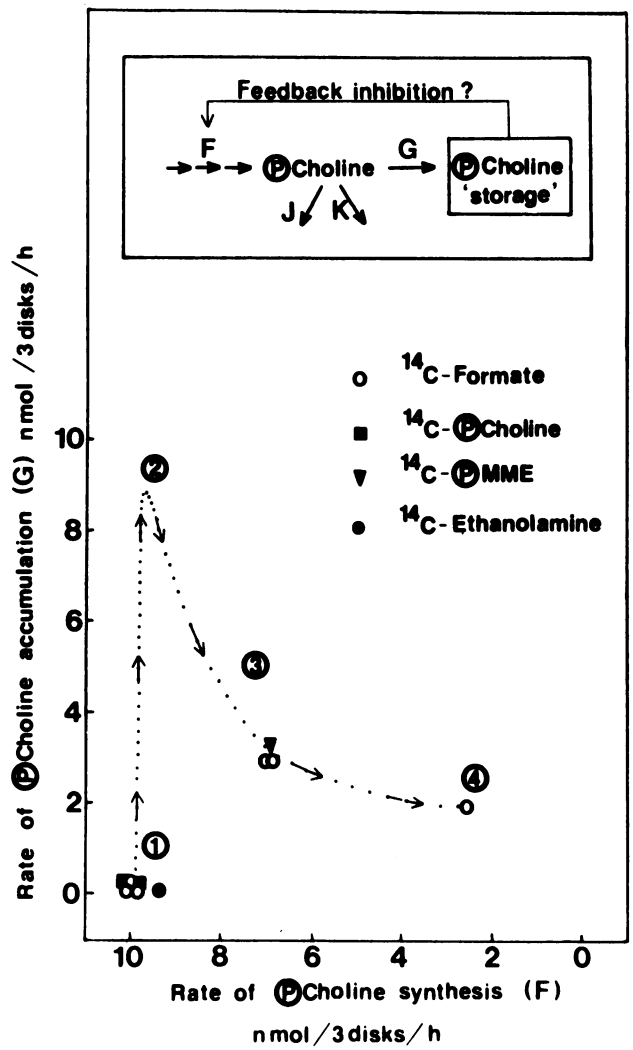

FIG. 6. A qualitative interpretation of model-generated data in terms of regulation of the rate of Ⓟ choline synthesis (F) by the size of the storage pool of Ⓟ choline, as shown in the simplified diagram in the inset. Note that the model of Figure 5 can give a reliable estimate of the rate of increase in the size of the Ⓟ choline pool (=G), but not of the actual size of this pool. Thus, our interpretation entails inferences about a pool size drawn from estimates of its first derivative with respect to time. Data points are for parameters F and G taken from Table IV, and from four additional [^{14}C]formate experiments (not shown). The arrowed line and numbers one through four trace the possible impact of a reduction in the rate of the Ⓟ choline → choline step (K) on the rates of Ⓟ choline synthesis (F) and accumulation (G). One–two, Leaf tissue is initially synthesizing choline and betaine at a high rate and the Ⓟ choline pool size is constant. The rate of Ⓟ choline utilization (K) falls: Ⓟ choline then begins to accumulate rapidly; the maximum rate that G could reach equals F–J. Two–three, If Ⓟ choline inhibits its own synthesis, then Ⓟ choline accumulation begins to depress F. Three–four, As the Ⓟ choline pool expands further, F and G both tend to zero.

evidence was challenged later [21].) It seems also probable that the chenopods have methylation of phosphoryl bases in common with barley, but differ in that they produce free choline for betaine synthesis simply by hydrolyzing Ⓟ choline rather than after first incorporating Ⓟ choline into PC.

Two features of the model implicate the Ⓟ choline → choline step as a control point, although they shed no light on the nature of the control. First, the model requires that there be both a metabolic pool and a storage pool of Ⓟ choline, and that *carbon flux into the storage pool be highly variable from experiment to experiment.* When carbon flux into the storage pool of Ⓟ choline is elevated, there is a substantial reduction in the rate of betaine synthesis, but not the rate of PC synthesis (Table IV). As discussed above, such behavior would be predicted were the Ⓟ choline → choline step a control point in betaine synthesis. The second model feature consistent with regulation at this step is the very small size of the metabolic pool of free choline, which is less than 1% of the total choline of the tissue. Apparently, in beet leaves, only choline recently released from Ⓟ choline is available for oxidation to betaine. From theory, the Ⓟ choline → choline step seems a likely control point since this hydrolysis can be viewed as the committed step in betaine synthesis; in sugarbeet leaves, free choline appears to have no intrinsic importance and metabolic requirements for choline can be met via Ⓟ choline. Note also that were Ⓟ choline able to control its own synthesis –a possibility raised by the data of Table II—then overall control of the betaine pathway could be achieved by modulation of the Ⓟ choline → choline reaction. As explained in Figure 6, certain of the modeling results are consistent with feedback inhibition by Ⓟ choline on flux through the methylation sequence.

One prediction from our model is that betaine-synthesizing tissues in chenopods have a specific Ⓟ choline phosphatase, perhaps spatially related to a metabolic pool of choline. The existence of such an enzyme is quite consistent with the resistance of Ⓟ choline to hydrolysis by the ubiquitous, nonspecific acid phosphatases of plants (22). A second prediction is that at least the flux-generating enzyme step in Ⓟ choline synthesis is subject to feedback inhibition by Ⓟ choline.

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