Plant Physiol. (1966) *41*, 313–318

Effect of Cycocel Derivatives and Gibberellin on Choline Kinase and Choline Metabolism[1]

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Received June 21, 1965.

Summary. Cycocel stimulated the activity of partial purified choline kinase from spinach or squash leaves, but it inhibited the activity of yeast choline kinase. The activity of different Cycocel analogs on plant growth corresponded to their stimulatory effect on the isolated choline kinase. Cycocel had no effect upon the activity of a plant phosphatase which hydrolyzed phosphorylcholine nor upon adenosine triphosphatase from wheat roots or leaves.

Gibberellin A_3 inhibited choline kinase activity and reversed the stimulatory effect of Cycocel on the kinase.

Total choline kinase activity per squash plant was not greatly increased by Cycocel treatment. However, on the basis of fresh weight, total kinase activity was increased by Cycocel treatment. Gibberellin A_3 partially reversed these increases. Treatment with Cycocel plus indoleacetic acid resulted in a large increase in choline kinase activity.

The same distribution of tracer among phosphorylcholine, choline and betaine was observed when either phosphorylcholine-C^{14} or choline-C^{14} was fed to barley or wheat roots. Cycocel stimulated the incorporation of choline-C^{14} into the insoluble fraction and into lipids. Cycocel inhibited phosphorylcholine uptake by roots.

Thus Cycocel stimulated choline kinase activity and the utilization of choline-C^{14}. The effect of Cycocel upon kinase activity in vivo and in vitro was reversed by gibberellin A_3.

The growth of plants with short internodes is the primary alteration after treatment with 2-chloroethyltrimethylammonium chloride[3] or Cycocel (2, 8). This effect (2, 9) is completely reversed by gibberellin A_3. Since Cycocel is an analog of choline, we have investigated the effect of Cycocel on choline and phosphorylcholine metabolism in plants.

Materials and Methods

Materials. Choline-$C^{14}H_3$ chloride (2 mc/mmole) and choline-1,2-C^{14} (1.3 mc/mmole) were purchased from NiChem Incorporated. The barium salt of phosphorylcholine-1,2-C^{14}, 0.4 mc/mmole, was purchased from Nuclear Research Chemicals, and the sodium salt was prepared by treatment with IRC 50 (Na^+). Each compound chromatographed as a single component in a 2-dimensional solvent system of water-saturated phenol followed by butanol-propionic acid-water (1). The preparation of Cycocel and other related derivatives has been described (8, 10).

Choline Kinase Preparation and Assay. Units of activity and choline kinase preparation were the same as described in the accompanying paper (7). For in vitro studies in this report, the plant choline kinase preparations was the fraction obtained by precipitation between 28 to 37 g of $(NH_4)_2SO_4$ per 100 ml of spinach leaf sap and then dialysis against a dilute $MgCl_2$ solution (7). In the assay 0.01 ml Cycocel (to give a designated final concentration) was added to 0.4 ml of enzyme, and then 0.05 ml of a stock solution of substrate and factors was added. This stock solution contained 0.5 ml of choline-1,2-C^{14} (1.5×10^{-2} M), 0.4 ml of 0.12 M ATP (pH 9), 0.3 ml of 0.16 M $MgCl_2$ and 1.5 ml of 0.2 M glycine buffer at pH 9.6. The reaction mixture was incubated for 1 hour at 30° and the experiments terminated by heating for 3 minutes at 100°. Control experiments were run with boiled enzymes.

The yeast choline kinase preparation and spectrophotometric assay were also described in the previous paper (7).

Spinach leaves were purchased in local grocery stores. Table Queen squash plants were grown in a greenhouse in flats of soil supplemented with Hoagland nutrient. The squash plants were treated

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[1] This investigation was supported by Public Health Service Research Grant AM 05059. It has been approved for publication as journal article No. 3672 of the Michigan Agricultural Experiment Station.

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[3] Abbreviations: 2-Chloroethyltrimethylammonium chloride: Cycocel which was originally abbreviated CCC (7).

at the first leaf stage of growth with substances as designated in the results and then harvested at the sixth leaf stage. Except for the cotyledons the whole plant about 12 mm above the soil level was used for enzyme assay.

Choline-C^{14} and Phosphorylcholine-C^{14} Metabolism Experiments. Thatcher wheat or Traill barley seedling were used for choline-C^{14} metabolism studies. Wheat seeds were surface sterilized with 2 % NaOCl for 30 seconds, rinsed 3 times with sterile distilled water, and germinated on filter papers in a sterile petri dish. These minimal conditions for obtaining sterile seedlings were developed by germination on sterile agar plates and examination for growth of microorganisms.

After 4 days for germination, 2 seedlings were aseptically transferred in an innoculation box to a black plastic rack from which the roots extended into 300 ml of sterile 0.2 strength Hoagland nutrient solution. The seedlings were grown inside a large glass desiccator and the nutrient was aerated with sterile compressed air. Exhaust air was passed through 2 N KOH in a Vigeraux column in order to absorb any $C^{14}O_2$. Unless rigorously sterile conditions were maintained both choline-C^{14} and phosphorylcholine-C^{14} in the nutrient culture were converted in a few hours to $C^{14}O_2$. When this occurred the experiments were abandoned.

After transplanting the seedlings into the desiccator jars, they were placed in a plant growth chamber at about 21° day temperature, 13° night temperature and 1500 ft-c of light. Rapid aeration kept the temperature inside the desiccators at less than 6° above the chamber temperature. After 3 days enough sterile 0.1 M Cycocel was injected into the nutrient solution to produce a final concentration of 10^{-3}M. Twenty-four hours later solutions containing the labeled substrates were also injected into the culture. The choline-C^{14} solutions were prepared in 50 % (v/v) ethanol, and before use the ethanol was removed by evaporation and the choline-C^{14} redissolved in cool sterile water.

The roots of the wheat seedlings remained in the choline-C^{14} solutions for 1 to 24 hours. When the plants were removed, the roots were washed off with water and the plants divided into roots and epicotyl. Each plant tissue was extracted with boiling 95 % ethanol and then 80 % (v/v) ethanol, and both solutions were combined and referred to as the alcohol soluble material. The tissue was further extracted with boiling water. The residual tissue was dried and combusted to $C^{14}O_2$ (12) which was collected in an alkali trap. All fractions were counted by liquid scintillation and corrected for counting efficiency.

The distribution of C^{14} among the soluble products was determined by 2-dimensional paper chromatography using water-saturated phenol first and then butanol-propionic acid-water (1). To prepare chromatograms it was necessary to reduce the extract to near dryness and redissolve the components with a small amount of 80 % ethanol. This procedure probably resulted in the loss of some of the soluble lipoidal material.

Results

Stimulatory Effect of Cycocel on Plant Choline Kinase Activity. With dialyzed ammonium sulfate preparations of the choline kinase from spinach leaves, the activity of the enzyme was doubled by optimal amounts of Cycocel (fig 1). In these experimental conditions (see Methods) there was 1.5 × 10^{-3} M choline, 1 × 10^{-2} M ATP and 1 × 10^{-2} M $MgCl_2$. Optimal stimulation of kinase activity occurred with 1 × 10^{-2} M Cycocel. This stimulation of the kinase activity was abolished when the Cycocel was removed by dialysis, and the enzyme activity returned to its original level. Similar results were obtained when the kinase was prepared from either wheat, squash or spinach leaves.

The nature of the Cycocel stimulation of the kinase activity is unknown. As discussed in the previous paper, the kinase activity in the plant is not great and the choline-C^{14} isotope assay does not lend itself to development of good kinetic analyses. In exploratory experiments, maximum Cycocel stimulation of activity occurred when there was approximately a 5-fold excess of Cycocel concentration over the substrate choline concentration.

A yeast choline kinase preparation was obtained with at least 100-fold higher specific activity on a

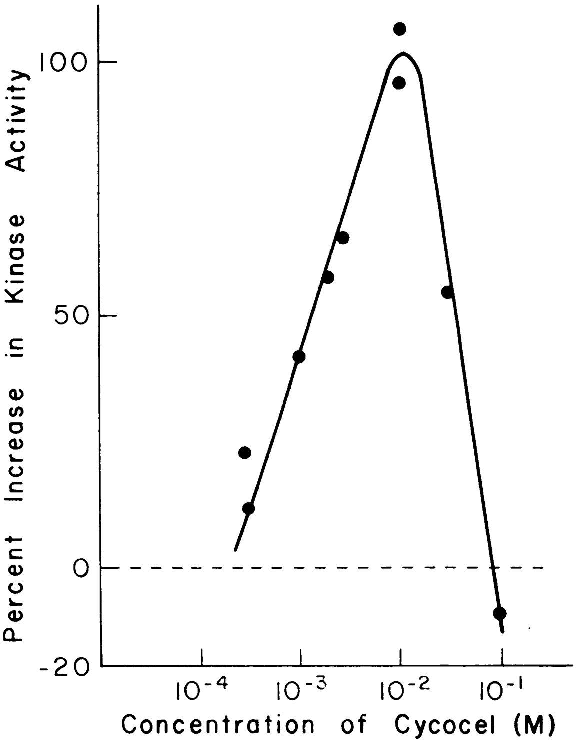


FIG. 1. Stimulation by Cycocel of partially purified choline kinase from spinach leaves.

protein basis than could be obtained for the plant kinase experiments. The yeast kinase preparation also contained little adenosine triphosphatase activity, while the plant kinase was mixed with much adenosine triphosphatase. The activity of the yeast choline kinase was not stimulated by addition of Cycocel, rather, the activity was partially inhibited by Cycocel. Similar results were obtained when the assay was run by either the choline-C^{14} or the spectrophotometric procedure (table I). Thus, the kinase from leaves was stimulated by Cycocel, and the kinase from yeast was partially inhibited by Cycocel.

Adenosine triphosphatase activity from both wheat roots and epicotyl was partially purified. No inhibition or stimulation was observed over a range of 10^{-5} to 10^{-2} M Cycocel at pH 6 or 8.4 in the presence of calcium ions.

Effect of Other Cycocel Derivatives on Choline Kinase Activity. This laboratory has tested approximately 200 derivatives of Cycocel for biological activity as measured by growth of plants with shorter internodal distance (8, 10). The effect of 7 of the most active Cycocel derivatives were measured on spinach choline kinase activity in vitro and all were found to have a stimulatory effect on the enzyme activity (table II). Three analogs which were not active in vivo as growth retardants were also tested, and these derivatives did not stimulate the kinase activity in vitro. The inactivity of these derivatives as growth substances has been attributed to the removal of 1 methyl group, so that these compounds were not trimethylammonium salts, although the side chain had the proper structural configuration for activity. Thus, the in vivo biological activity or inactivity of Cycocel analogs corresponds to the observed in vitro stimulation of choline kinase activity.

Table I. *Effect of Cycocel on Yeast Choline Kinase*

The protein concentration of the enzyme preparations ranged between 0.04 to 0.06 mg/ml.

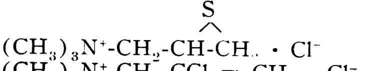
	C^{14} Chromatographic assay		Spectrophotometric assay	
Addition	Phosphorylcholine formed*	Relative activity	△ OD at 340 mμ**	Relative activity
	%			
None	15.9	100	0.32	100
10^{-2} M	13.3	67***	0.19	59

* As percent of total choline-C^{14} added.

** OD change in 10 minutes and corrected for a control without substrate.

*** Values ranging between 50 and 84 were obtained.

Table II. *Effect of Cycocel Derivatives on Choline Kinase Activity*

Cycocel derivatives	Relative activity on plant growth*	Relative in vitro kinase activity**
None	...	100
$(CH_3)_3N^+\text{-}CH_2\text{-}CH_2Cl \cdot Cl^-$	+4	228
$(CH_3)_3N^+\text{-}CH_2\text{-}CH_2Br \cdot Br^-$	+4	185
$(CH_3)_2N\text{-}CH_2\text{-}CH_2Br \cdot HBr^-$	None	89
$(CH_3)_3N^+\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}Cl \cdot Cl^-$	+2	160
$(CH_3)_3N^+\text{-}CH_2\text{-}CH{=}CH_2 \cdot Cl^-$	+4	141
$(CH_3)_2N\text{-}CH_2\text{-}CH{=}CH_2 \cdot HCl$	None	76
$(CH_3)_3N^+\text{-}CH_2\text{-}CH_2\text{-}CH_3 \cdot Br^-$	+3	205
$(CH_3)_2N\text{-}CH_2\text{-}CH_2\text{-}CH_3 \cdot HBr$	None	110
 $(CH_3)_3N^+\text{-}CH_2\text{-}\overset{S}{\widehat{CH\text{-}CH_2}} \cdot Cl^-$	+3	178
$(CH_3)_3N^+\text{-}CH_2\text{-}CCl = CH_2 \cdot Cl^-$	+3	124

* Summary of data from references 8 and 10.

** Enzyme activity in the standard assay (7) without addition of Cycocel was equated to 100.

Reversal by Gibberellin in Cycocel Stimulation of Choline Kinase Activity. Because gibberellin A_3 reverses the long-term growth effect induced by Cycocel, the effect of gibberellin A_3 by itself was tested in vitro on choline kinase activity as well as the effect of gibberellin A_3 on the Cycocel stimulation of the kinase activity (table III). Gibberellin A_3 inhibited the isolated plant choline kinase activity. Gibberellin A_3 (4×10^{-4} M) reversed nearly all of the stimulatory effect from 10^{-2} M Cycocel, and in the presence of 10^{-2} M gibberellin A_3 and 10^{-2} M Cycocel a normal rate of kinase activity was measured. Thus gibberellin A_3 inhibited the enzyme activity, Cycocel stimulated the activity, and when combined there was no effect. This in vitro phenomenon is yet another example of the mutually antagonistic effect between gibberellin and Cycocel which has been observed for the in vivo growth and development of plants (2, 9, 10).

Effect of Cycocel and Gibberellin A_3 on the Amount of Kinase in Squash Plants. In this study the amount of the choline kinase enzyme was determined in Table Queen squash plants which were grown with Cycocel, gibberellin A_3, IAA and combinations of Cycocel with gibberellin A_3 or IAA. The internode length of the plants which were treated with 2×10^{-3} M Cycocel was reduced to about 10 % of normal height. Leaf development was not altered by the treatments, and after the sixth leaf stage, the entire plant tops were harvested and from each group of 8 plants a dialyzed ammonium sulfate precipitate of the kinase was prepared as described for spinach leaves.

Table III. *Effect of Cycocel and Gibberellin A_3 on Activity of Choline Kinase*

Additions	Relative activity
None	100
1×10^{-2} M Cycocel	162
4×10^{-4} M Gibberellin A_3	74
1×10^{-2} M Gibberellin A_3	66
1×10^{-2} M Cycocel + 4×10^{-4} M gibberellin A_3	114
1×10^{-2} M Cycocel + 1×10^{-2} M gibberellin A_3	103

From the 2×10^{-3} M Cycocel treatment alone, the fresh weight and mg of the protein in the crude enzyme fraction, which contained the kinase, was reduced about half in comparison with the untreated plants (table IV). On the other hand the specific activity of the kinase from the Cycocel-treated plants was nearly doubled that found in the control plants. Thus the total units of kinase activity in the Cycocel-treated plants was nearly the same as in the control plants. The reduction from Cycocel treatment in weight and in the protein content of the enzyme preparation had occurred without loss of total kinase activity on a per plant basis. Until the enzyme is more purified, it can not be determined whether these changes involve a change in specific activity of the enzyme. As shown in table IV, the kinase activity from the Cycocel-treated plants could also be stimulated in vitro by addition by Cycocel to the same extent as the kinase from untreated plants.

In another series of experiments, squash seedlings were initially treated with 1×10^{-3} M Cycocel and then a week later with gibberellin A_3 or IAA (table V). In this experiment with half as much Cycocel as used in the previous test, growth retardation was less. The total units of kinase per each plant treated with Cycocel had increased. The units of kinase activity per 100 g of fresh tissue increased even more due to a decrease in weight of the Cycocel-treated plants. This increase in units of kinase activity did not occur with gibberellin A_3 treatment alone. Plants treated with both Cycocel and gibberellin A_3 contained less choline kinase activity than Cycocel-treated plants. However the kinase level had not dropped to that in untreated plants, probably because the gibberellin treatment occurred a week after the Cycocel treatment.

Table IV. *Formation of Choline Kinase in Squash Plants Treated With Cycocel*

	Control	Cycocel treated*
Weight of 8 plants	64.5 g	35.5 g
Total protein in enzyme fraction	200 mg	107 mg
Specific activity as units/mg protein	0.013	0.025
Total units	2.8	2.7
Increase in activity upon adding 3×10^{-3} M Cycocel to enzyme fraction	65 %	54 %

* Treatment consisted of 1 liter of 2×10^{-3} M Cycocel per flat of 8 plants.

In vivo treatment with IAA caused reduced growth of the plants. The units of kinase per plant did not change significantly, but the units of activity per g fresh weight of the plant increased. A combined treatment of Cycocel plus IAA significantly stimulate the amount of kinase on the basis of activity per plant and activity per fresh weight. This stimulation from the combined treatment suggests that further in vivo growth experiments with combinations of these 2 substances should be made. A synergistic effect of Cycocel with IAA on the growth of parthenocarpic tomato fruit has already been observed (13).

Metabolism of Choline-1,2-C^{14}. Cycocel stimulated greatly the amount of choline-1,2-C^{14} incorporation into residual material of wheat and barley roots. Results shown in table VI are for plants which had been treated with Cycocel 24 hours prior to adding choline-C^{14}. When the Cycocel and choline-C^{14} were added simultaneously (data not shown) a similar trend in results were obtained, but the difference between the controls and treated plants was less. Cycocel treatment of wheat generally inhibited choline-C^{14} uptake, but this latter effect may not be of physiological significance. The nature of the residual material containing the C^{14} has not been determined, but it was not extractable by hot 95 % alcohol, 80 % alcohol or boiling water. Similar results were also obtained with choline-$C^{14}H_3$, but the results are not detailed, because as much as 25 % of the C^{14} in the choline-$C^{14}H_3$ were unknown radiation decomposition products. Since C^{14} from choline-1,2-C^{14} rapidly appeared in the residual fraction, the choline molecules were probably incorporated intact.

The products in the alcohol soluble fraction from choline-1,2-C^{14} metabolism by barley roots consisted of choline, phosphorylcholine, betaine and lipids (table VII). Cycocel-treated plants contained little

Table V. *Choline Kinase Activity in 8 Squash Plants after in Vivo Treatment With Growth Substances*

In each plot 8 plants were treated after development of the first true leaf. Cycocel treatment consisted of 1 soil drench with 1 liter of 1×10^{-3} M solution. Gibberellin A_3 (2.6×10^{-4} M) and IAA (5.7×10^{-4} M) were sprayed on the leaves at the beginning of the second week and again 4 days later.

Treatment		Fr wt	Kinase activity	
First Week	Second week	g	Units/plants	Units/100 g
None	None	75	2.6	3.5
Cycocel	None	60	3.6*	6.0*
None	Gibberellin A_3	69	2.3	3.3
Cycocel	Gibberellin A_3	61	3.1	5.1*
None	IAA	53	3.2	6.1*
Cycocel	IAA	54	4.2*	7.8*

* Significant in comparison with controls.

Table VI. *Choline-1,2-C^{14} Incorporation Into Insoluble Fraction of Roots*

Plants	Time after adding choline	Control plants Total	Control plants In residue		Cycocel treated plants* Total	Cycocel treated plants* In residue	
		cpm	cpm	%	cpm	cpm	%
Wheat	1 hr	8000	710	8.9	3900	2990	50.9
	3 hr	32,900	3490	10.6	12,700	6060	47.7
Barley	1 hr	12,100	1340	11.1	12,700	7280	57.3
	3 hr	44,100	5560	12.6	62,900	34,340	54.6

* Plants in nutrient culture were treated with 10^{-3} M Cycocel 24 hours prior to addition of choline-1,2-C^{14}.

Table VII. *Choline-1,2-C^{14} Metabolism In Barley Roots*

Values are percent distribution of C^{14} among the constituents of an 80 % alcohol extract of barley roots.

Treatment	Choline	Phosphorylcholine	Betaine	Lipids
	%	%	%	%
Choline-1,2-C^{14}	16	30	47	5
Choline-1,2-C^{14} + 10^{-3} M Cycocel	1	30	52	15
Phosphorylcholine-1,2-C^{14}	16	30	45	5

of the choline-C^{14} substrate; there was a significant increase in C^{14} incorporation into the lipids. Thus Cycocel seemed to stimulate choline incorporation into lipids and the insoluable residue.

Metabolism of Phosphorylcholine-C^{14}. The initial rate of uptake of phosphorylcholine-$C^{14}H_3$ or phosphorylcholine-1,2-C^{14} by wheat seedling was very slow; in the order of 0.1 the rate of choline-C^{14} uptake. This result was unexpected since tracer P^{32} labeled phosphorylcholine had been readily absorbed by roots (5). Perhaps only the phosphate part of the molecules is rapidly exchanged.

The slow rate of uptake of phosphorylcholine by wheat seedlings was further severely inhibited by 10^{-3} M Cycocel in the nutrient culture. The reason for this inhibition is not known. Thus metabolic data on phosphorylcholine-C^{14} metabolism in the presence of Cycocel was not obtained. In untreated plants identical distribution of C^{14} among the metabolic products was found after feeding phosphorylcholine-1,2-C^{14} as after giving choline-1,2-C^{14} (table VII).

Discussion

Cycocel stimulated the specific activity of choline kinase in vitro, and the stimulation was reversed by low concentrations of gibberellin A_3. The kinase was inhibited by gibberellin A_3. This is the only isolated enzyme system so far reported to be effected by both Cycocel and gibberellin. However, Cycocel also stimulated the polynucleotide phosphorylase activity of wheat roots (3). In vivo Cycocel plus IAA resulted in the formation of an increased amount of choline kinase. Cycocel alone in vivo resulted in increased choline kinase activity on a fresh weight basis, although the amount of enzyme per plant did not show a large increase. Consistent with the enzymatic studies, Cycocel markedly increased the in vivo incorporation of choline-C^{14} into lipids and insoluble constituents of the plant. This might be accounted for by increased activity of the kinase.

A difference in choline kinase from spinach and yeast was indicated by Cycocel which stimulated the plant enzyme and inhibited the yeast enzyme. This difference might be due in part to the higher specific activity of the yeast kinase. However the most purified spinach kinase which we prepared (7) was also stimulated by Cycocel, although it was not used for most of the Cycocel studies reported in this manuscript.

Cycocel and gibberellin treatments result in opposite growth responses. The growth alteration from a large amount of Cycocel can be reversed by a small amount of gibberellin (2, 10). The Cycocel remains in the plant unchanged (unpublished data). A logical explanation for these effects has been documented by data which indicate that gibberellin synthesis is inhibited by Cycocel (4, 6). The research in this manuscript was done before the inhibition of gibberellin synthesis by Cycocel was known. The question now arises as to whether the Cycocel stimulation of choline kinase and polynucleotide phosphorylase reflects a primary or indirect mechanism of action of Cycocel. Stimulation of lipid synthesis would be consistent with growth of sturdier and more resistant plants after Cycocel treatment. In the presence of excess Cycocel the normal regulation of cellular synthesis by gibberellin might be prevented since the hormone would be absent. Then an additional direct effect from excess Cycocel on certain enzymes might intensify the growth retardation effect of Cycocel.

The inhibition by Cycocel of choline-C^{14} and particularly of phosphorylcholine-C^{14} uptake by wheat and barley roots is an effect of Cycocel which has not been further investigated. Phosphorylcholine has been implicated as an organic form of phosphorus storage and transport (5,11). The absorption of the compound from nutrient culture may not be a normal physiological process. However the nearly complete inhibition of phosphorylcholine uptake by roots treated with Cycocel indicate that the absorption was an active process and that Cycocel may effect other in vivo sites which function in choline metabolism.

Very rapid labeling of phosphorylcholine with P^{32} orthophosphate was a unique physiological property of this compound (5,11). In the present study with C^{14} labeled substrates, the same distribution of tracer among phosphorylcholine and betaine was found when either phosphorylcholine-C^{14} or choline-C^{14} was fed. It appeared that during or after absorption, a rapid equilibration occurred between choline and its phosphorylated form. These results are consistent with the hypothesis that phosphorylcholine may participate in phosphate transport in the plant.

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