Formic Acid Activation in Plants. II. Activation of Formyltetrahydrofolate Synthetase by Magnesium, Potassium, and Other Univalent Cations [1, 2]

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The purification, properties and distribution of the formate activating enzyme, formyltetrahydrofolate synthetase, from plants were described in the preceding paper (5). Studies of the activation of the enzyme by magnesium and univalent cations are described in this paper.

The mechanism of action of magnesium in biological catalysis is reasonably well understood. Most transphosphorylation reactions involving ATP require magnesium ions and it is generally assumed that the reactive species is a magnesium complex of ATP. Knowledge of the mechanism of action of potassium in metabolism, however, is limited. In recent years a number of enzyme catalyzed reactions have been shown to require K^+, NH_4^+ or Rb^+ for activity (4). Evans and coworkers (6, 11) have demonstrated a requirement for K^+, NH_4^+ or Rb^+ by pyruvate kinase and acetic thiokinase from tissues of higher plants. Optimal activity of these enzymes was produced by a K^+ concentration of approximately 0.04 M which is near the normal level of K^+ concentration in plant tissues.

Bertino et al. (1) reported that optimal activity of formyltetrahydrofolate synthetase from erythrocytes required the presence of K^+ or NH_4^+. A detailed study of the K^+ requirement was not made. The purpose of the experiments reported in this paper was to study the activation of the enzyme from plant tissues by potassium ions and to determine the effect of potassium deficiency in plants on the rate of the reaction catalyzed by the enzyme.

Materials and Methods

Preparation of Extracts. Unless otherwise specified the enzyme used in these studies was purified from spinach leaf acetone powders by the procedure reported in the preceding paper (5). The purified enzyme was dialyzed for 6 hours in 0.001 M Tris, pH 7.5, containing 0.01 M mercaptoethanol before use in cation activation studies.

Crude extracts of tobacco leaves were prepared by grinding the leaves with mortar and pestle with an equal weight of 0.05 M Tris buffer, pH 8.0, containing 0.01 M mercaptoethanol. The homogenate was centrifuged at 20,000 × *g* for 15 minutes.

Reagents. ATP and *dl*-tetrahydrofolate were obtained from the Nutritional Biochemicals Corporation. Other chemicals were of reagent grade and were obtained from commercial sources. Tris salts of ATP and *dl*-tetrahydrofolate were prepared by passing solutions of the sodium or potassium salts of these compounds through an exchange column containing Dowex 50 resin (Tris cycle). Tris formate was prepared by neutralizing formic acid to pH 8.0 with Tris.

Assay Procedures. Formyltetrahydrofolate synthetase was assayed as previously described (5). Activity is expressed as mμmoles formyltetrahydrofolate formed per 10 minutes. Tris salts of all compounds were used in order to eliminate K^+ and Na^+ from the assay mixture in experiments in which K^+ was varied.

Tetrahydrofolate was assayed as previously described (5). Protein was determined by the Folin-phenol method of Lowry et al. (10) using bovine albumin as the standard. Potassium was determined with a flame photometer.

Results

Activation by Univalent Cations. The effect of K^+ on the activity of formyltetrahydrofolate synthetase from spinach leaves is shown in figure 1. The optimum concentration of K^+ was 0.2 to 0.3 M; however, 90 % of maximum activity was produced by a K^+ concentration of 0.1 M. The slight activity observed in the absence of added K^+ (fig 1) was probably due to univalent cation contamination of the Tris ATP and Tris tetrahydrofolate, since these compounds were initially in the form of their sodium and potassium salts, respectively. Data from the activation curve were plotted according to the method of Lineweaver and Burk (9) in figure 1 (inset) and the Michaelis activator constant (K_a) for K^+ was determined. The K_a with respect to K^+ was calculated to be 1.3×10^{-2} M.

Other univalent cations also activated formyltetrahydrofolate synthetase. The effect of concentration of chloride salts of other univalent cations on

[1] Received July 31, 1964.

[2] Contribution (Article No. 64-3-50) of the Department of Agronomy, Kentucky Agricultural Experiment Station, Lexington, and published with the approval of the Experiment Station Director.

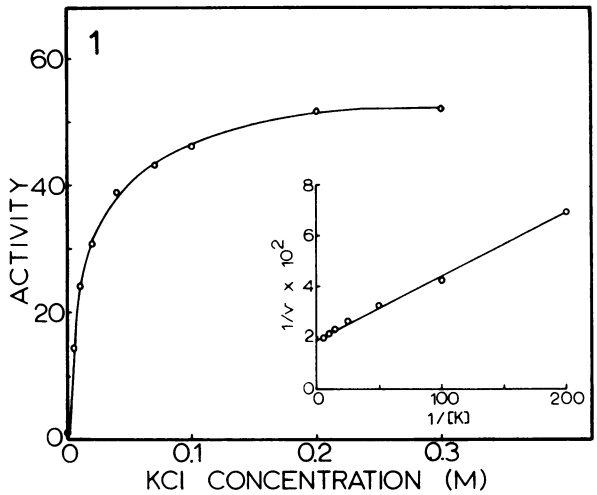

FIG. 1. (Major graph) The influence of K^+ concentration on activity of formyltetrahydrofolate synthetase. The standard assay procedure was used with purified enzyme (28 μg protein) from spinach leaves. Activity is expressed as mμmoles formyltetrahydrofolate formed per 10 minutes. (Inset) Reciprocal plots of reaction velocities versus K^+ concentration indicated in major graph.

activity is shown in table I. Activation by NH_4^+ was almost identical to that of K^+. Rb^+ was more effective than K^+ or NH_4^+ at low concentrations but the maximum activity at optimum concentration was approximately the same for all 3 cations. Na^+ also activated the enzyme but was much less effective than K^+, NH_4^+ or Rb^+.

The K_a and maximum velocity for each of the univalent cations was calculated (table II). The apparent affinity (K_a) of the enzyme for univalent cation

Table I. *Effect of K^+, NH_4^+, Rb^+, and Na^+ on the Activity of Formyltetrahydrofolate Synthetase*

The reaction mixture in a volume of 1 ml contained the following: 100 μmoles triethanolamine buffer, pH 8.0; 150 μmoles Tris formate; 2.5 μmoles $MgCl_2$; 4 μmoles *dl*-tetrahydrofolate; 2 μmoles Tris ATP; purified enzyme extract (28 μg protein) and chloride salts of the indicated univalent cations.

Salt concentration	K^+	NH_4^+	Rb^+	Na^+
	mμmoles formyltetrahydrofolate formed/10 min			
M				
0.00	1	1	1	1
0.01	24	24	28	8
0.02	31	30	37	14
0.04	39	39	45	17
0.07	43	43	48	23
0.10	46	48	50	25
0.20	51	51	52	33
0.30	52	52	53	35

Table II. *K_a and V_{max} Values for Different Univalent Cations in the Activation of Formyltetrahydrofolate Synthetase*

Calculations were made from plots of the data in table I according to the method of Lineweaver and Burk (9).

Cation	V_{max}	K_a
	mμmoles formyltetrahydrofolate formed/10 min	M
K^+	53	1.3×10^{-2}
NH_4^+	53	1.3×10^{-2}
Rb^+	54	8.4×10^{-3}
Na^+	36	3.2×10^{-2}

is highest for Rb^+. The apparent affinity for Na^+ was considerably less than for the other cations.

Effect of Anions. The effect of potassium salts of different anions on enzyme activity is shown in table III. The highest activity was produced in the presence of Cl^-, and NO_3^- markedly decreased activity.

Effect of K^+ on K_m Values for Formate, ATP and Tetrahydrofolate. K_m values for formate, ATP and *dl*-tetrahydrofolate were determined at 2 levels of K^+ concentration in the assay mixture and these values are shown in table IV. The K_m values for formate

Table III. *Effect of Potassium Salts of Different Anions on the Activity of Formyltetrahydrofolate Synthetase*

The reaction mixture in a volume of 1 ml contained the following: 100 μmoles triethanolamine buffer, pH 8.0; 150 μmoles Tris formate; 2.5 μmoles $MgCl_2$; 4 μmoles *dl*-tetrahydrofolate; 2 μmoles Tris ATP; purified enzyme extract (10 μg protein) and potassium salts of the indicated anions.

Salt	Normality of anion	Specific activity
		Units/mg protein
KCl	0.1	1510
	0.2	1960
KBr	0.1	1420
	0.2	1740
KNO_3	0.1	440
	0.2	700
K_2SO_4	0.1	1260
	0.2	1380
K acetate	0.1	1280
	0.2	1240

Table IV. *Effect of K^+ Concentration on K_m Values for Formate, ATP, and dl-Tetrahydrofolate*

The standard assay procedure was used with K^+ varied as indicated.

Substrate	K_m Values in presence of: 0.05 M K^+	0.2 M K^+
	M	M
Formate	3.8×10^{-2}	3.4×10^{-2}
ATP	11.0×10^{-5}	9.0×10^{-5}
dl-Tetrahydrofolate	16.2×10^{-4}	8.9×10^{-4}

and ATP were changed only slightly by changing the K^+ concentration from 0.2 M to 0.05 M. The K_m value for *dl*-tetrahydrofolate, however, was almost doubled by decreasing the K^+ concentration in the assay mixture from 0.2 M to 0.05 M.

Because of the large K^+ effect on the K_m for *dl*-tetrahydrofolate, the effect of *dl*-tetrahydrofolate concentration on the K_a for K^+ was determined. The K_a with respect to K^+ in the presence of 5×10^{-3} and 1×10^{-3} M *dl*-tetrahydrofolate was determined to be 1.2×10^{-2} M and 2.4×10^{-2} M, respectively.

Enzyme Activity in Potassium Deficient Plants. Young tobacco plants were grown for 10 days in nutrient solutions containing Hoagland's solution No. 1 (7) modified to contain 3 different levels of K^+ (table V). Duplicate plants were grown in each treatment. Formyltetrahydrofolate synthetase activity of crude extracts was determined without K^+ added to the assay mixture and in the presence of 0.1 M and 0.2 M K^+. The results are shown in table V.

When K^+ was omitted from the assay mixture, extracts of plants showing moderately severe K^+-deficiency symptoms exhibited much less enzyme activity than did extracts from the plants which exhibited no K^+-deficiency symptoms. When extracts of plants showing K^+-deficiency symptoms were assayed with 0.2 M K^+ in the assay mixture, formyltetrahydrofolate synthetase activity was increased to near the level of activity in normal plants. This recovery of activity when K^+ is added to the assay medium indicates that total formyltetrahydrofolate synthetase was not reduced markedly under these particular conditions of K deficiency. As much as 10-fold stimulation has been produced by adding 0.2 M K^+ to the assay mixture of extracts from selected severely K^+-deficient tobacco leaves.

Activation by Magnesium. The effect of Mg^{++} concentration on the activity of formyltetrahydrofolate synthetase from spinach leaves is shown in figure 2. The concentration of Mg^{++} producing maximum enzyme activity was 10^{-3} to 2×10^{-3} M. Concentrations of Mg^{++} greater than the optimum resulted in inhibition of enzyme activity. Data from the activation curve were plotted according to the method of Lineweaver and Burk (9) in figure 2 (inset) and the Michaelis activator constant (K_a) for Mg^{++} was determined to be 1.1×10^{-4} M.

Table VI. *Effect of Mg^{++}, Mn^{++}, and Ca^{++} on the Activity of Formyltetrahydrofolate Synthetase*

The reaction mixture in a volume of 1 ml contained the following: 100 μmoles triethanolamine buffer, pH 8.0; 150 μmoles Tris formate; 200 μmoles KCl; 4 μmoles *dl*-tetrahydrofolate; 2 μmoles Tris ATP; purified enzyme extract (28 μg protein) and chloride salts of the indicated cations.

Salt concentration	Mg^{++}	Mn^{++}	Ca^{++}
M	mμmoles formyltetrahydrofolate formed/10 min		
0	5.2	4.6	5.3
2.5×10^{-4}	36.5	28.3	14.7
5.0×10^{-4}	40.6	29.0	21.2
1.0×10^{-3}	53.5	27.5	24.0
2.0×10^{-3}	51.5	17.7	26.4
5.0×10^{-3}	49.0	15.4	28.4
7.5×10^{-3}	43.0	14.0	29.0

Mn^{++} and Ca^{++} also activated the enzyme (table VI), but neither cation was as effective as Mg^{++}. Maximum activity was produced by a Mn^{++} concentration of 5×10^{-4} to 10^{-3} M. Higher concentrations of Mn^{++} markedly inhibit the enzyme. Ca^{++} concentrations of at least 7.5×10^{-3} M were required for maximum activity.

Discussion

In common with other enzymes involving ATP, formyltetrahydrofolate synthetase requires Mg^{++} as an activator. In addition K^+ or a related univalent cation is essential for activity. K^+ concentrations of 0.2 to 0.3 M are required for maximum activity. In comparison, other enzyme systems requiring K^+ are usually activated maximally by K^+ concentrations of less than 0.05 M (2, 6, 8, 11, 13, 14). Therefore, the formic acid activating system would probably be one

Table V. *Potassium Concentration and Formyltetrahydrofolate Synthetase Activity of Tobacco Plants Grown in Nutrient Solutions Containing 3 Different Levels of Potassium*

The reaction mixture in a volume of 1 ml contained the following: 100 μmoles triethanolamine buffer, pH 8.0; 150 μmoles Tris formate; 2.5 μmoles $MgCl_2$; 4 μmoles *dl*-tetrahydrofolate; 2 μmoles Tris ATP; 0.2 ml enzyme extract and KCl as indicated.

Treatment	% K in dry matter*	K deficiency symptoms	Specific activity when assayed in presence of:		
			No K^+	0.1 M K^+	0.2 M K^+
meq K/liter			mμmoles formyltetrahydrofolate formed/ 10 min/mg protein		
0	0.7	Moderately severe	9.3	29.4	38.0
1	2.5	None	26.6	36.4	39.2
5	7.5	None	35.0	43.5	43.5

* Average of duplicate samples.

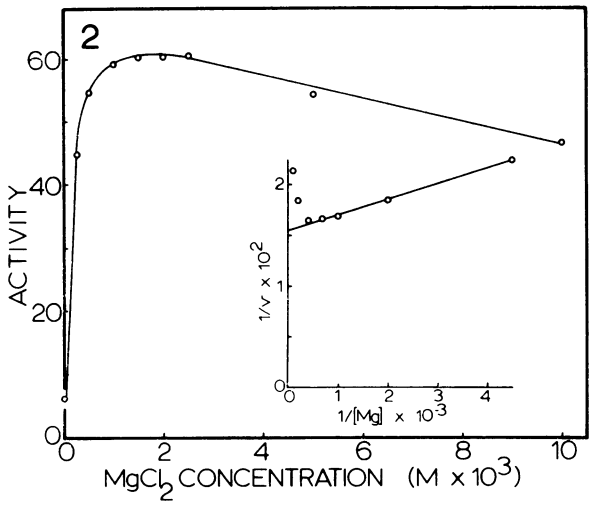

FIG. 2. (Major graph) The influence of Mg^{++} concentration on activity of formyltetrahydrofolate synthetase. The standard assay procedure was used with purified formyltetrahydrofolate synthetase (28 μg protein) from spinach leaves. Activity is expressed as mμmoles formyltetrahydrofolate formed per 10 minutes. (Inset) Reciprocal plots of reaction velocities versus Mg^{++} concentration indicated in major graph.

of the first enzyme systems to be affected under conditions of K^+-deficiency in plants. This is of particular significance because of the important role of formyltetrahydrofolic acid in purine and histidine biosynthesis (3, 12). Thus, the level of K^+ in the tissue might influence the rate of synthesis of such compounds as ATP, GTP, nucleic acids, NAD and NADP.

The concentration of K^+ required for half maximal activity (K_a) is considerably higher for formyltetrahydrofolate synthetase than for other plant enzymes requiring K^+. The K_a with respect to K^+ for formyltetrahydrofolate synthetase is 1.3×10^{-2} M compared to 2.4×10^{-3} M for pyruvate kinase (11) and 9×10^{-3} M for acetic thiokinase (6).

Increasing K^+ concentration of the assay mixture from 0.05 M to 0.2 M results in a marked decrease of the K_m with respect to tetrahydrofolate. Likewise, increasing *dl*-tetrahydrofolate concentration from 10^{-3} M to 5×10^{-3} M results in decreasing the K_a with respect to K^+ by one-half. These data indicate that K^+ increases the apparent affinity of the enzyme for tetrahydrofolate, suggesting that K^+ is required in binding tetrahydrofolate to the enzyme. The possibility that K^+ functions in binding tetrahydrofolate to one of the other substrates cannot be completely eliminated on the basis of these data; however, it would appear that if this were the case, the K_m value for formate or ATP would also be changed by changing the K^+ concentration of the assay mixture. While it has been suggested that K^+ functions in the formation of complexes in biological systems (15), heretofore, no evidence has been presented which implicates K^+ in the binding of a specific compound. Perhaps K^+ functions in a somewhat similar manner in other enzyme systems where K^+ has been shown to be absolutely essential.

Summary

Formyltetrahydrofolate synthetase from plants was shown to require a univalent cation for activity. The univalent cation requirement was satisfied by K^+, NH_4^+, Rb^+, or Na^+ and maximum activity was obtained with 0.2 to 0.3 M concentrations of these cations. Na^+ was least effective in activating the enzyme. Low enzyme reaction rates of extracts from K^+-deficient plants were restored to near normal levels by adding 0.2 M K^+ to the assay mixture.

The influence of several anions on activity was determined. Activity in the presence of NO_3^- was considerably less than activity in the presence of other anions tested.

Increasing the K^+ concentration of the assay medium decreased the K_m with respect to tetrahydrofolate and increasing the concentration of tetrahydrofolate decreased the K_a with respect to K^+. These results suggest that K^+ may function in the binding of tetrahydrofolate to the enzyme. K^+ concentration had little effect on the K_m values with respect to ATP and formate.

Mg^{++} was also required by the enzyme. A Mg^{++} concentration of 1×10^{-3} to 2×10^{-3} M was required for maximum activity. Mn^{++} and Ca^{++} could replace Mg^{++} but were not as effective as Mg^{++} in activating the enzyme.

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Inhibition of Protein Synthesis and of Auxin-Induced Growth by Chloramphenicol [1]

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Introduction

The mode of action of auxin in causing cell enlargement has proven elusive for many years. It is generally accepted that the last phase is a change in the properties of the cell wall which allows it to become extended by osmotic forces with which it was previously in balance. This process usually follows only after a time lag, which may last several minutes or days, depending on the tissue (7, 19, 28). The preceding phases are evidently chemical in nature (see e.g. 28) but evidence about them is limited to the requirement for oxidative energy, and the participation of organic acid metabolism and of one or more sulfhydryl enzymes (see 1, 33). Much of this evidence rests on the effects of inhibitors, which have indicated that a group or chain of chemical events precedes the effects on the cell wall. Since most of the inhibitors act more powerfully on cell enlargement itself than on oxygen consumption, it is probable that the connection between growth and respiration may not be direct. The fact that auxin may in some circumstances stimulate growth without any measurable increase in oxygen consumption points in the same direction (see 33).

In slices of potato and artichoke tubers, the evidence strongly suggests a relationship between cell enlargement and the synthesis of protein (34). Although this has not been borne out in studies on other tissues, at least to the extent that auxin treatment does not always appear to increase the protein synthesis (see 21), it remains possible that there is some connection between growth and protein synthesis. Most of the data, indeed, are consistent with the possibility that auxin promotes directly or indirectly either the synthesis or the turnover of one or more special proteins concerned with cell enlargement. One possible explanation of the differences between the responses of different plant materials is that these proteins may be present in only small amounts in some tissues, but in easily measurable quantities in others.

The present study examines the possibility of a relationship between protein synthesis and cell enlargement, using C^{14}-amino acids and the inhibitor chloramphenicol. In bacteria the action of this antibiotic is specifically exerted on protein synthesis (6), and it has the same action on subcellular particles from plants (10, 11, 18, 27); there is good evidence that it inhibits protein synthesis in plant tissues also (8, 9, 10, 16, 38), although higher concentrations seem to be needed than in particulates or bacteria. Several earlier workers have applied chloramphenicol to excised plant tissues treated with auxin, but either found no inhibition of growth, or else did not ascribe the observed inhibition to the participation of protein synthesis in growth (see 21). Preliminary reports of this work have been presented earlier (20, 21), and Key (13) has recently reported comparable results with inhibitors using 2,4-dichlorophenoxyacetic acid

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[3] Supported in part by a United States Public Health Service Fellowship, No. GF-12,160 to L. D. Noodén from the Division of General Medical Sciences and in part by a grant to Professor K. V. Thimann from the National Science Foundation, No. G21799.