AN ABSTRACT OF THE THESIS OF

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Title: THE EFFECTS OF UNIVALENT CATIONS ON THE ACTIVITY OF PARTICULATE STARCH SYNTHETASE

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An investigation was conducted to determine the univalent cation requirements of starch synthetase from a variety of plant species of economic importance. The particulate enzyme from sweet corn was shown to have an absolute requirement for potassium, with optimum activation occurring at 0.05 M KCl, and the K_A calculated to be 6 mM. Potassium activation was essentially independent of the anions present although sulfate and iodide cause a maximum inhibition of 15% at concentrations of 0.10 M. Rubidium, cesium and ammonium were 80% as effective as potassium in the activation of this enzyme while sodium and lithium were respectively 21% and 8% as effective as potassium. In the case of the particulate starch synthetase from peas, soybeans, field corn, wheat, bush beans and potatoes, considerable stimulation of enzyme activity was obtained by the addition of potassium to the reaction mixture. In these studies, low enzyme activity was observed in the absence of added potassium, but the content of endogenous

potassium in the starch granules was sufficient to account for the activities observed.

Kinetic analysis of the interactions of potassium and adenosine diphosphate glucose on the apparent affinity of the enzyme for these components was conducted. Potassium, rubidium, cesium and sodium had no effect on the K_m for adenosine diphosphate glucose. Conversely, adenosine diphosphate glucose failed to alter the K_A for potassium. At low levels of potassium and sodium, an additive stimulation of enzyme activity was observed. However, at high levels of sodium, starch synthetase activity was inhibited.

Glutathione failed to activate particulate starch synthetase in the presence or absence of potassium. p-Chloromercuribenzoate inhibited synthetase activity 93%, and this inhibition was reversed 79% by the addition of glutathione. Again, potassium showed no effect on the interaction of p-chloromercuribenzoate and glutathione with the enzyme.

Because of the similarities in starch and glycogen synthesis, experiments were conducted to determine whether potassium is involved in the activity of particulate glycogen synthetase. No positive effect of either potassium or sodium was observed in the investigation with glycogen synthetase isolated from sheep or rat liver.

The Effects of Univalent Cations on the Activity of Particulate Starch Synthetase

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LIST OF ABBREVIATIONS

ADP	Adenosine-5'-diphosphate
ADPG	Adenosine-5'-diphosphate glucose
AMP	Adenosine-5'-monophosphate
ATP	Adenosine-5'-triphosphate
CDPG	Cytidine-5'-diphosphate glucose
DeoxyADPG	Deoxyadenosine-5'-diphosphate glucose
EDTA	Ethylenediaminetetraacetic acid
GDPG	Guanosine-5'-diphosphate glucose
GSH	Glutathione
IDPG	Inosine-5'-diphosphate glucose
K_A	Michaelis constant for activation
K_i	Michaelis constant for inhibition
K_m	Michaelis constant
PCMB	p-Chloromercuribenzoate
TDPG	Thymidine-5'-diphosphate glucose
Tricine	N-tris(hydroxymethyl)methyl glycine
Tris	Tris(hydroxymethyl)aminomethane
UDP	Uridine-5'-diphosphate
UDPG	Uridine-5'-diphosphate glucose
UTP	Uridine-5'-triphosphate
V_{max}	Maximum velocity

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THE EFFECTS OF UNIVALENT CATIONS ON THE ACTIVITY OF PARTICULATE STARCH SYNTHETASE

INTRODUCTION

Potassium is necessary for the normal growth and development of most and perhaps all organisms. The concentration of this cation found in plants is greater than that of any other cation as shown by the extensive study of Beeson (9). The level of potassium in fresh plant tissues ranges from 0.11 N to 0.27 N, while that in deficient tissues is about 0.013 N to 0.17 N (24). Typical plant requirements for potassium are therefore quite large.

Many investigators have attempted to elucidate the role of potassium in plant metabolism. While potassium has been primarily associated with cell membrane permeability and the control of osmotic pressure (76, 103), the main function of this cation appears to be associated with the activation of specific enzymes. In a recent review, Evans and Sorger (24) have compiled the effects of potassium deficiency in vivo and suggest that this cation functions in several different metabolic processes since some 46 different enzymes have been shown to require potassium or other univalent cations. A deficiency of potassium might be expected to affect the overall metabolic activity of the plant by severely reducing the activity of many potassium-requiring enzymes participating in diverse metabolic processes.

Considerable evidence is available which indicates that sodium

is beneficial for plant growth, but generally will not replace potassium entirely (8, 41, 42, 79, 109). Potassium is the best cation activator for most enzymes showing a univalent cation response (24), but sodium can partially substitute for potassium in many of these enzymes. However, sodium fails to activate many potassium-stimulated enzymes and often inhibits enzyme activity. In several halophytic plants and microorganisms, sodium appears to be essential for growth (6, 15, 107).

Rubidium will replace potassium in the growth of several microorganisms and in numerous potassium-activated enzymatic reactions (24, 41, 42, 87). Enzyme activity in the presence of rubidium, however, is not stimulated to the extent that occurs in the presence of potassium. This partial stimulation by rubidium is attributed to the similar physical and chemical properties exhibited by potassium and rubidium cations (41, 42, 87).

Little information is available concerning the interrelation of cesium or lithium in univalent cation activated systems. In most enzyme systems, cesium functions to a lesser degree than either potassium or rubidium, and in some enzyme reactions, this cation either fails to activate or inhibits (24). Lithium appears to be detrimental to the growth of most organisms, and also shows either no activation or inhibition of enzymatic reactions (24).

The effects of potassium deficiency in specific metabolic

pathways have been carefully summarized by Evans and Sorger (24). Metabolic lesions such as the inhibition of protein synthesis (22, 61, 62, 106), decreased photosynthesis (45), reduction of oxidative phosphorylation and photophosphorylation (45, 52, 53), and the reduction of polysaccharide synthesis (16, 17, 58, 81, 96) are all associated with potassium deficiency. A consistent consequence of potassium deficiency in plants is the accumulation of soluble carbohydrates and reducing sugars, and the inhibition of starch synthesis (16, 17, 18, 21, 58, 85). In higher plants, carbohydrate utilization might easily be blocked as a result of the loss of activity of several enzymes known to require potassium or other univalent cations. In the case of starch synthesis, the involvement of potassium at the enzyme level has only recently been suggested.

Since a univalent cation effect in the starch synthesizing systems from different sources has been indicated, it seems that a study of the detailed alkali metal requirements of the starch synthetase enzymes would lead to a better understanding of the factors controlling carbohydrate metabolism. It is, therefore, the objective of this investigation to identify and characterize the metabolic site or sites where potassium deficiency may interfere with the processes associated with starch synthesis from a variety of plant species of economic importance. It is also planned to examine the effects of a series of univalent cations on the activation of starch synthetase, and elucidate

the mode of action of potassium in this system. Since starch and glycogen synthesis procede by similar reactions, the possible involvement of potassium in glycogen synthesis also will be studied.

REVIEW OF LITERATURE

In considering the effect of univalent cations in enzyme activation, it has been proposed that the primary effect involves the interaction of the univalent cation with the apoenzyme or protein molecule (24). While few enzyme systems have been examined for possible explanations of the mechanism involved, the evidence thus far suggests that univalent cations can affect the subunit structure as well as the conformation of the protein involved.

Univalent Cations and Protein Conformation

Kinetic Studies

The kinetics of enzyme reactions have been used as a means of examining the effects of univalent cations on the mechanism of activation. In a detailed analysis of the interactions of potassium, phosphoenolpyruvate, and pyruvic kinase isolated from rabbit muscle, Kachmar and Boyer (47) showed that potassium, ammonium, or rubidium activated pyruvic kinase whereas sodium and lithium counteracted the potassium effects. They also observed that varying the concentration of potassium in the reaction medium affected the V_{max} but not the K_m for substrates. Similar results for other enzyme systems have been obtained by several investigators (23, 38).

From their results, Kachmar and Boyer (47) proposed that phosphoenolpyruvic acid and potassium combined with pyruvic kinase to form an enzyme-activator-substrate complex. The nature of this combination, however, could not be determined.

The difference in the ability of univalent cations to act as activators in this system was suggested to be related to the size of the hydrated ionic radii of these ions (47). The three active ions, potassium, ammonium, and rubidium, have hydrated ionic radii of 5.32 A, 5.37 A, and 5.09 A respectively which are approximately equal. Sodium has a hydrated ionic radius of 7.09 A, and lithium one of 10.03 A. These last two cations function either very poorly or not at all as activators for the pyruvic kinase reaction. The authors (47) felt that the ability of these cations to function as activators or inactivators was dependent on their capacity to combine at a negatively charged site on the enzyme which resulted in the displacement of adjoining structures by some critical amount.

In a similar type of investigation also utilizing pyruvic kinase, Melchoir (64) measured the velocity of the enzyme reaction under various concentrations of ADP, magnesium, and potassium. The kinetics of the reactions studied were analyzed in terms of equilibrium concentrations of the ions in solution. In this study, Melchoir (64) observed that the reaction velocity was directly affected by the concentrations of both potassium and magnesium. Using equilibrium

kinetic analysis, she concluded that both potassium and a magnesium-ADP complex interacted with the enzyme, with potassium activating the enzyme and influencing the binding of the substrate.

Using a tryptophanase from Escherichia coli, Happold and Beechy (38) investigated the effects of univalent cations on the kinetics of this enzyme. This investigation showed that univalent cations affected the organization of the enzyme, and they attributed this effect of the various univalent cations to differences in their hydrated radii.

In studying the univalent cation requirements of acetic thiokinase isolated from spinach leaves, Hiatt (43) showed that potassium or rubidium activated the second step of this two step reaction, but sodium and lithium had no effect. Using a partially purified acetic thiokinase preparation from yeast, Evans, Clark, and Russell (23) obtained similar results. They also found that the V_{max} of the reaction was greatly influenced by the potassium or univalent cation concentration, but no effect was observed on the K_m of the reaction. They concluded that univalent cations must influence either the rate of breakdown of an enzyme-substrate complex or the number of reactive sites on the enzyme. Exposure of additional reactive sites on the enzyme by the univalent cations would be indicative of a change in the conformation of the enzyme protein.

Spectrophotometric Studies

In a series of investigations, Suelter and Melander (101), Kayne and Suelter (48) and Suelter et al. (102) utilized the technique of protein difference spectroscopy to examine the effects of various environmental conditions on the ultraviolet absorption properties of pyruvic kinase. In studying the effects of temperature and univalent and divalent cations on crystalline pyruvic kinase, these authors (48, 101, 102) observed ultraviolet difference spectra which were characteristic for the perturbation of tryptophan residues. The magnitude of the absorption difference at the 295 mμ peak was used as a basis for determining dissociation constants for the various types of cations.

Kayne and Suelter (48) have reported that the difference spectra for pyruvic kinase incubated at high and low temperatures were identical to those spectra obtained when the enzyme was examined in the presence of different concentrations of ionic species. Suelter et al. (102) found that when the difference spectra of pyruvic kinase in a trisCl buffer having univalent or divalent cations was compared to the spectra of the enzyme suspended in trisCl buffer with tetramethylammonium chloride, an ultraviolet difference spectra was obtained which was characteristic for the perturbation of tryptophan residues. If other cations were used in place of tetramethylammonium (e.g. magnesium, manganese, sodium, potassium, cesium, or ammonium),

a shift in the blue region of the spectrum for the enzyme was produced. This shift was attributed to changes in the solvation environment of the protein chromophore. While activator cations of pyruvic kinase were shown to produce these difference spectra, no attempts were made by any of these investigators (48, 101, 102) to relate the changes to the active form of the enzyme.

In a recent paper, Wilson, Evans and Becker (108) investigated the ability of activator cations for pyruvic kinase to perturb tryptophan residues of this enzyme. When pyruvic kinase in 0.1 M KCl was compared to the enzyme in 0.1 M trisCl, absorption differences corresponding to tryptophan perturbation were obtained. If the concentrations of the two cations were increased, the differences in absorbancy between the sample solutions also increased. However, when ultraviolet absorption spectra of the enzyme in the presence of activator cations was compared to that in non-activator cations, no differences in absorption at 295 mμ were evident. The authors concluded that trisCl is responsible for the perturbation of the residues. These authors (108) also reported that if phosphate, arsenate, or sulfate are substituted for chloride as the anion in the comparison of potassium versus tris, inhibition of the perturbation resulted. They concluded that only in the presence of trisCl is perturbation possible and that substitution of these different anions for chloride inhibited the perturbation.

In examining the effects of cation concentrations on the perturbation of tryptophan residues, Wilson, Evans and Becker (108) found that increasing the salt concentration of the different pyruvic kinase samples resulted in a corresponding increase in the absorption differences. At concentrations greater than 0.33 M, activator cations produced greater differences in the spectra than nonactivators. From these results, the authors (108) proposed a model for the conformation of pyruvic kinase under different cation environments. In physiological concentrations of the activator cations, the enzyme was proposed to assume an active conformation while in the presence of nonactivator cations, an inactive conformation was assumed. As the salt concentration is increased interaction of the protein with the nonactivator cations results in perturbation of tryptophan residues, while no such change occurs with activator cations.

Electrophoretic Studies

In a recent report, Sorger, Ford, and Evans (98) examined the effects of univalent cations on the physical organization of pyruvic kinase. Their investigation involved the measuring of changes in immunoelectrophoretic patterns under various environmental conditions. When pyruvic kinase was incubated in an environment of potassium, rubidium, or sidium, and then examined in the electrophoretic gel, the enzyme interacted with antibodies to reproduce a

simple type of pattern. In the presence of tris or lithium, however, a more complicated type of pattern developed. When catalase or sheep serum protein were examined, no differential effects in the immunoelectrophoretic patterns developed.

Sorger, Ford, and Evans (98) interpreted these results to indicate that two major conformational species of pyruvic kinase existed, depending upon the univalent cation present in the environment. In the presence of potassium, rubidium, or sodium, a conformation representative of the active enzyme developed, while in the presence of tris or lithium an inactive enzyme conformation existed. Since no substrate or cations were included in the reaction assay, the differences in the patterns obtained could not be associated with an enzyme-activator-substrate complex as suggested by other authors (48, 101, 102).

More recently, Betts and Evans (10) have examined the effects of univalent cations on the electrophoretic mobility and substrate binding properties of rabbit muscle pyruvic kinase. Their results showed no difference in the mobility of pyruvic kinase when potassium or lithium were present in the electrophoretic medium, indicating a lack of measurable conformational change in the enzyme under these conditions. If magnesium was added to the media, a differential decrease in mobility occurred for the enzyme in the presence of lithium over that for the enzyme in the presence of potassium. This difference was

attributed to a redistribution of the charged groups on the enzyme as a result of the differential binding of magnesium under the experimental conditions.

If the substrate phosphoenolpyruvate was included in the assay medium having magnesium, no effect on the mobility of the enzyme was observed. However, if phosphoenolpyruvate was replaced with ADP, increased mobilities were obtained in the presence of either lithium or potassium. Furthermore, gel filtration studies revealed that two molecules of both substrates are bound to a single enzyme molecule in the presence of either univalent cation. These authors (10) proposed that univalent cation activators initiate only small conformational changes in the enzyme structure either prior to substrate binding or after binding has occurred.

Enzyme Stability

In an investigation on the effects of univalent cations on the interaction of iodoacetate and other thiol inhibitors with acetaldehyde dehydrogenase, Stoppani and Milstein (100) demonstrated that potassium and rubidium, both enzyme activators, protected enzyme activity from these inhibitors. Sodium and lithium, both non-activators and competitive inhibitors of potassium in this system, increased the inactivation exerted by these thiol inhibitors. In a more recent report on the role of univalent cations on acetaldehyde dehydrogenase, Sorger

and Evans (97) showed that increasing concentrations of mercaptoethanol caused a reduction in the concentration of potassium required for maximum enzyme activity. Their investigation also showed that potassium and rubidium protected the enzyme to a greater extent against inactivation at 25° than did sodium or lithium. Furthermore, potassium was shown to restore enzyme activity after prolonged dialysis against tris. These authors (97) interpreted their results as indicating univalent cations are associated with the maintainance of the proper enzyme configuration necessary for activity.

Using pyruvic kinase, Wilson, Evans and Becker (108) examined the stability of this enzyme when incubated in a series of different univalent cation phosphates. In solutions of potassium, rubidium or ammonium, enzyme activity remained relatively high even after 24 hours. In solutions of nonactivator cations, sodium, lithium, and tris, over 50% of the initial activity was lost after 24 hours. Partial restoration of activity after incubation in lithium or tris could be achieved by the addition of potassium to the enzyme solutions. Activator cations appear to stabilize the enzyme whereas nonactivators lead to a nonactive or denatured conformation.

Schneider, Peck and Pawelkiewicz (94) have examined the effects of univalent cations on the subunit structure of glycerol dehydrase from Aerobacter aerogenes. This enzyme is activated by potassium, rubidium or ammonium, but unaffected by sodium. If the

enzyme is passed through a Sephadex G 100 column, equilibrated with sodium, two inactive protein subunits are eluted. If the column is equilibrated with activator univalent cations, glycerol dehydrase is recovered as a single active entity. Furthermore, in the presence of the nonactivator sodium and the substrate glycerol, the enzyme remains associated as a monomeric unit although enzymatically inactive. These authors (94) propose that univalent cation activators are associated with the integrity of the subunit stability and are also associated with other activator functions which, at present, have not been evaluated.

While numerous enzyme systems have been reported to have a univalent cation requirement, only a few of these systems have been examined in detail. Pyruvic kinase appears to be the most thoroughly studied enzyme of those exhibiting a univalent cation requirement, and the mechanism of action of these cations in its activation would appear to be associated with the expression of a particular conformation. In the presence of activator cations, it is postulated from the different studies that the enzyme assumes an active conformation, while in the absence of these cations, an inactive conformation results. The exact mode of this activation, however, has not been established, and may involve several different interactions of enzyme, substrate and activator cation. Wyatt (110) has proposed that these different interactions could serve as metabolic valves in regulating the participation

of different metabolic pathways during the growth, differentiation and development of most organisms.

Starch and Glycogen Synthesis

Initial Developments

For a number of years, the synthesis of starch and glycogen was believed to involve the action of phosphorylase on glucose-1-phosphate. Several inconsistencies associated with this system, however, have created doubt regarding the actual involvement of phosphorylase in polysaccharide biosynthesis. In developing pea seeds, Rowan and Turner (91) reported that the high ratio of inorganic phosphate to glucose-1-phosphate favors the catabolic action of phosphorylase. In the case of glycogen synthesis, Mommaerts et al. (67), Larner and Villar-Palasi (51) and Schmid, Robbins and Traut (93) all showed that agents which increase the phosphorylase concentration in the liver actually cause the breakdown of glycogen.

The concept of the action of phosphorylase in starch and glycogen synthesis has now been replaced with the discovery by Leloir and Cardini (54) of uridine diphosphate glucose:glycogen α-4-glucosyltransferase (glycogen synthetase) in rat liver extracts. The enzyme was shown to catalyze the transfer of the glucose moiety of uridine diphosphate glucose (UDPG) to an $\alpha(1\text{-}4)$ linked glucan acceptor. This

enzyme was found to be tightly bound to the particulate glycogen fraction and appears to be quite specific for UDPG. Partially purified particulate and soluble enzymes have since been isolated from rat liver (49, 56), rabbit muscle (40), pigeon breast muscle (88) and brain tissue (7, 13, 14, 36).

Because of the similarities in structure between starch and glycogen, de Fekete, Leloir and Cardini (19) investigated the possible existence of a similar mechanism for starch synthesis. Utilizing starch granules isolated from beans, these authors were able to demonstrate the presence of a nucleoside diphosphate glucose:α-1, 4-glucan α-4-glucosyltransferase (starch synthetase) which also used UDPG as the glucosyl donor. This particulate enzyme transfers the glucosyl units from UDPG to the non-reducing end of both amylose and amylopectin (57). Recondo and Leloir (86), Murata, Sugiyama and Akazawa (75) and Murata, Minamikawa and Akazawa (72) have demonstrated that adenosine diphosphate glucose (ADPG) was the preferred glucosyl donor, reacting approximately ten times as efficiently as UDPG.

Particulate Starch Synthetase

Since the initial discovery of particulate starch synthetase from beans, this enzyme has been isolated from potatoes (27, 32, 57), sweet corn (30, 57), rice (2, 75), waxy and nonwaxy maize (32, 77),

sweet potato and taro roots (69) and other sources (27, 32, 71). These enzymes are tightly bound to the starch granules and require at least a disaccharide as a primer. Starch granules and oligosaccharides serve as the best glucosyl acceptors, but oligosaccharides of the maltose series (maltose to maltoheptose) also founction competitively as primers indicating that two or more α(1-4) linked glucosyl units will function as acceptor molecules (2, 27, 57). Isomaltose will not function in the reaction, and glucose inhibits this transfer reaction up to 30% (27).

The particulate enzymes show a pH optimum of 7.0 to 7.5 in tris or phosphate buffer, but a pH optimum of 8.0 to 8.5 in glycine buffers (1, 27, 57). With the bean enzyme, a linear relationship was obtained with increasing enzyme concentration at 37°, although maximum activity was obtained at 45° (57). However, in several cases (1, 2, 27, 75), this linear relationship is not well defined and the non-linearity attributed to the particulate nature of the enzyme. The K_m values found for the different enzymes range from 30 mM to 60 mM for UDPG (27, 57) and values from 2 mM to 12 mM for ADPG (1, 32). While ADPG is the preferred substrate, other nucleoside diphosphate sugars (deoxyADPG, CDPG, GDPG, IDPG, TDPG, ADP-maltose, ADP-galactose, UDP-galactose) will function either poorly or not at all (27, 32, 86). The β-anomers of the substrates are ineffective (86). Furthermore, ADPG decreases glucose incorporation from UDPG but

UDPG has no effect on the rate of transfer from ADPG (27).

The addition of EDTA, magnesium, cysteine, GSH, soluble starch, sucrose, glucose-6-phosphate or glucose-1-phosphate had no effect on the activity of starch synthetase from potatoes, sweet corn, sweet potatoes or rice (2, 27, 73). ATP, ADP, AMP and adenosine inhibit UDPG utilization up to 70% but the uridine derivatives have little or no effect on the utilization of ADPG (27). Using starch synthetase from rice, Murata and Akazawa (70) have shown that ADP is a competitive inhibitor of ADPG, with the K_i for ADP calculated to be 10 mM and a K_m for ADP of 3 mM indicating that the apparent affinity for ADP is not larger than that for the substrate (K_m for ADPG = 12 mM). The particulate enzymes are also inhibited by sulfhydryl blocking agents such as iodoacetate or PCMB (2, 27, 68, 73). This type of inhibition can be partially reversed by the addition of adenosine derivatives, with the best protection exhibited by ATP and ADP (73). Less protection is obtained with ADPG (73).

Soluble Starch Synthetase

The starch synthetase enzymes thus far considered have been particulate and several attempts have been made to obtain soluble forms of these enzymes. In an early investigation, Leloir, de Fekete and Cardini (57) attempted to extract the particulate enzyme from the starch granules of beans using different buffers, digitonin or

detergents as well as grinding the granules in the dry state. These treatments all resulted in loss of enzyme activity. In 1964, Frydman and Cardini (28) were successful in obtaining a soluble preparation from sweet corn which transferred glucose from ADPG to phytoglycogen, a soluble polysaccharide related to amylopectin. In a further study (29), these authors were able to partially purify a soluble starch synthetase from tobacco leaves and potato tubers which was capable of transferring glucose from ADPG to an α(1-4) glucan primer.

Since these initial discoveries of the soluble starch synthetase, the enzyme has been isolated from spinach leaf chloroplasts (20, 34, 35), rice and bean leaves (82), potatoes (31), glutinous rice (68, 74), corn (30), peas and beans (33). These soluble enzymes are easily separated from the particulate enzymes and are present in the 100,000 x g supernatant. The enzyme from Chlorella (84) has been purified 25-fold while that from spinach leaf chloroplasts 150-fold (20) by treatments involving ammonium sulfate fractionation, calcium phosphate adsorption and DEAE cellulose chromatography.

The soluble starch synthetases are specific for ADPG or deoxyADPG and other nucleoside diphosphate sugars (UDPG, CDPG, IDPG, TDPG, GDPG, ADP-mannose, ADP-galactose) function either poorly or not at all (20, 31, 33, 34, 35, 84). The β-anomers of ADPG, unreactive in the particulate systems, could act as a glucosyl donor at a lower rate (16%) than ADPG in the soluble enzyme system isolated

from potatoes (31), but had no effect on the incorporation of glucose into starch from ADPG. DeoxyADPG was shown to be a competitive inhibitor of ADPG for the activity of the soluble enzyme from spinach chloroplasts (35) and functioned only 50% as effectively as ADPG in this reaction. This competitive inhibition also was shown for Chlorella starch synthetase (84) and deoxyADPG would function only 13% as effectively as ADPG in this system. The K_m for ADPG is approximately 0.2 mM, while that for deoxyADPG is 0.4 mM (35). Frydman and Cardini (31) observed that different primers can affect the K_m for ADPG for the soluble enzyme from potatoes. In the presence of amylopectin, the K_m was 1.5 mM while in the presence of phytoglycogen, the K_m was calculated to be 0.3 mM.

In an extensive study of the properties of the soluble starch synthetase from spinach chloroplasts, Ghosh and Preiss (35) reported that ADP competitively inhibits this enzyme, and they calculated the K_i to be 0.35 mM. ATP was also inhibitory in this system, but UTP had no effect on enzyme activity. Using the soluble enzyme from potatoes, Frydman and Cardini (31) showed that AMP and ADP inhibit enzyme activity approximately 25% while ATP inhibits only 20%. UDPG has no effect on the chloroplast enzyme characterized by Doi, Doi and Nikumi (20).

The soluble starch synthetases are completely dependent on the presence of a primer or acceptor molecule for activity. The

enzyme from sweet corn (30) utilized only amylopectin and glycogen as primers and amylose, soluble starch and starch granules are ineffective. The soluble enzyme from tobacco leaves (29) could use either amylopectin, glycogen or heated starch granules as acceptor molecules. In their initial report, Frydman and Cardini (29) observed that the soluble starch synthetase from potatoes only functioned with whole starch granules as primer. Amylopectin, glycogen or heated starch granules were inactive in this system. In a later report, Frydman, de Souza and Cardini (33) obtained evidence that the soluble potato enzyme would utilize amylopectin, phytoglycogen or amylose and that starch granules functioned at a much lower capacity than these other polysaccharides. No explanation of this discrepancy is given by these authors.

The soluble enzymes show a pH optimum in the range of 8.5 for glycine buffers. All the enzymes are inhibited by PCMB or iodoacetate, and this inhibition can be reversed by the addition of GSH. However, only in the case of the soluble starch synthetase from spinach chloroplasts (35) has a requirement for sulfhydryl groups for enzyme activity been demonstrated. Glucose, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, phosphoenolpyruvate, 3-phosphoglycerate and pyruvate have no effect on activity of the starch synthetase purified from spinach chloroplasts (20, 35), potatoes (31), and Chlorella (84). Dihydroxyacetone phosphate and

3-phosphoglyceraldehyde inhibit the synthetase from spinach chloroplasts 35% and 97% respectively (35).

Utilizing different plant sources, Frydman and Cardini (32) have been able to solubilize partially the particulate enzyme by use of either urea, α-amylase or mechanical disruption followed by differential centrifugation. Comparison of the activities of the solubilized and bound enzymes showed that the soluble enzyme no longer possessed specificity toward UDPG, but utilized only ADPG as the glucosyl donor. An accompanying increase in the activity and affinity of the enzyme toward ADPG also was observed for the solubilized enzyme. These treatments indicate that modification of the granule structure increases the interaction of the enzyme with the substrate and primers, and that the difference in specificity between the soluble and particulate systems is directly related to the conformation of the enzyme. The authors (32) propose that both the synthetase bound to the granules and the soluble synthetase are different forms of the same enzyme.

An interesting difference between the starch granules present in waxy (glutinous) and those present in nonwaxy varieties of both maize and rice has been established (27, 77, 99). Both varieties contain equal amounts of starch but the waxy varieties have only amylopectin while the nonwaxy varieties contain 20% to 30% amylose in addition to amylopectin. Starch grains prepared from the waxy varieties, however, lack synthetase activity (27, 74, 77).

In a further study of the waxy mutants of maize, Nelson and Tsai (78) and Akatsuka and Nelson (1) demonstrated that nearly all the starch synthetase activity resided in the embryonic tissue and that activity present in the endosperm tissue was due to a separate enzyme system. The two systems differed in their K_m values for ADPG, pH optimum, response to EDTA and inactivation at 60°. These authors indicated that the waxy mutation reduces the endosperm enzyme to a low level but does not effect the activity of the embryo enzyme.

In an initial study on the waxy mutation in rice, Murata, Sugiyama and Akazawa (74) reported that the starch synthetase activity is found almost exclusively in the soluble extract. A further investigation of this system by Murata and Akazawa (3, 68) indicated that the mode of action of the soluble enzyme was identical to that of the particulate enzyme in its primer specificity and its capacity to form glucosidic bonds with these primers. Of greater interest, however, was the fact that the soluble enzyme can be selectively adsorbed onto amylose. The addition of amylose to the soluble preparation resulted in the formation of a precipitate which, after drying and acetone treatment, exhibited considerable starch synthetase activity. The dry enzyme-amylose complex also was stable to heating at 100° for ten minutes and was inhibited by iodoacetamide. The properties of the soluble starch enzyme-amylose complex are similar to those reported for the particulate enzymes from beans (57), maize (1) and nonwaxy

rice (2), and Murata and Akazawa (68) attribute the difference in distribution in the waxy and nonwaxy varieties of rice to the physical nature of the enzymes.

Glycogen Synthetase

Analogous to the starch synthetase systems isolated from plants, glycogen synthetase has been isolated and characterized from several sources (4, 5, 7, 13, 14, 40, 49, 56, 88). In rat liver extracts, the enzyme is strongly adsorbed to the particulate glycogen (55), but soluble forms of the enzyme have been prepared from rabbit muscle (49), rat muscle (56) and sheep brain tissue (7, 36). The synthetase requires the presence of an acceptor polysaccharide and functions by transferring the glucose moiety from UDPG to the acceptor molecule forming an $\alpha(1-4)$ linkage. In most tissues two different forms of glycogen synthetase are present. One form is dependent on the presence of glucose-6-phosphate (D form) for activity while the other (I form) is active in the absence or presence of this cofactor (36, 89, 90, 105). The two forms are interconvertible. ATP, magnesium and a synthetase-I-kinase are required for the conversion of the I form to the D form (25, 26, 36). Conversion of the D form to the I form involves a synthetase-D-phosphatase and a subsequent release of inorganic phosphate (25).

Glycogen synthetase is specific for UDPG and other nucleoside

diphosphate sugars function poorly in the transfer reaction (55). The K_m values for UDPG of the I form and D form of the rat liver enzymes are approximately 1.1 mM and 0.9 mM respectively (44). These values are relative since they are affected by the presence or absence of glucose-6-phosphate in the assay mixture. The K_A for glucose-6-phosphate is 0.6 mM for the D form of the liver enzyme (44). This D enzyme is inhibited 50% by ATP, ADP or AMP while the I form is only inhibited 5% by these compounds (44).

Glycogen synthetases exhibit pH optimums in the range 8.2 to 9.0 in tris buffers and are extremely sensitive to PCMB. Storage stability of the enzyme is two weeks if sulfhydryl reagents are added to the preparations (56). Glycogen appears to be the best primer for the activity of the enzymes but other polysaccharides can function (56). Small polysaccharides of the maltose series are not as effective as starch, amylopectin or amylose (56).

Univalent Cations in Starch and Glycogen Synthesis

While the properties and characteristics of the starch and glycogen synthetase systems have been well established, in only a few studies has the possible involvement of potassium or other univalent cations been considered. In an investigation on the properties of an ADPG-specific glycogen synthetase from Arthrobacter, Greenberg and Preiss (37) showed that GSH, KCl and bovine plasma albumin

consistently stimulated the enzyme activity 15% to 30%. If all three of these components were omitted simultaneously, activity was reduced 60%. Addition of KCl to a final concentration of 25 mM alone resulted in a 24% stimulation in synthetase activity. At the same concentrations, NaCl or NH_4Cl had no effect in this system. In a further study on the ADPG-glycogen synthetase from Escherichia coli, these authors (83) were able to demonstrate that the addition of KCl (25 mM) to the reaction mixture resulted in a 30% stimulation of enzyme activity. The same degree of activation was observed when deoxyADPG was utilized as the glucosyl donor in the E. coli system.

In the characterization of the properties of the soluble starch synthetase from spinach leaf chloroplasts, Ghosh and Preiss (35) demonstrated that glucose transfer from ADPG into starch was stimulated 11% by the addition of KCl to the reaction mixture. If KCl as well as GSH and EDTA were omitted, activity was reduced 40%. In a similar study on the soluble starch synthetase from Chlorella, Preiss and Greenberg (84) obtained a 20% stimulation of enzyme activity by the addition of KCl. Again, if KCl as well as GSH and bovine plasma albumin were omitted, activity was reduced 60%. These effects are similar to those reported for the ADPG-glycogen synthetase from bacteria, although the activation by KCl was not as pronounced.

In 1966, Akatsuka and Nelson (1) demonstrated that potassium

stimulated the activities of both the embryonic and endospermic derived particulate starch synthetase systems from immature maize seeds. In their report, potassium was shown to stimulate both systems when ADPG was the substrate but inhibited at the same level when UDPG was the substrate. EDTA in the presence of potassium, stimulated enzyme activity but was inhibitory when potassium was omitted from the reaction mixture containing the embryonic starch synthetase. Potassium also was shown to have a protective effect against thermal inactivation of the enzyme at 60^{o}. In this report, as well as those previously described, no attempt was made to eliminate potassium from the enzyme components or the reagents. In fact, Akatsuka and Nelson (1) utilized the dipotassium salts of the substrates in their investigation.

Recently, Murata and Akazawa (70) have examined the role of potassium in the starch synthetase from sweet potato roots. In their report, a 7-fold stimulation of the activity of the enzyme was observed by the addition of 0.1 M KCl. From kinetic analysis, the K_A for KCl was determined to be 13 mM, and the enzyme was saturated at 50 mM KCl. No effect of KCl on the K_m for ADPG could be demonstrated by these authors (70). A protective effect of potassium against thermal inactivation of the enzyme also was shown.

The presence of potassium is an important requirement for glycogen synthesis in crude homogenates of pigeon liver and breast

muscle, and rat liver. Several investigators (39, 50, 80, 88, 104) have obtained incorporation of glucose or lactate into glycogen in preparations rich in potassium. Krebs, Dierks and Gascoyne (50), in a study on the synthesis of glycogen from lactate in pigeon liver homogenates, found that the initial rate of lactate incorporation into glycogen was not affected by the presence or absence of sodium or potassium. However, the total glycogen formed in this crude homogenate was greater when potassium was present in the preparation. Nigam and Fridland (80) reported that incorporation of glucose into glycogen by pigeon liver homogenates was effected by the homogenization media utilized. A buffered medium containing sucrose and 0.15 M NaCl was slightly inferior (17%) to a similarly buffered KCl solution. If 0.25 M sucrose was used in place of the univalent cation chlorides, activity was reduced approximately 60%.

Using a crude pigeon breast muscle homogenate, Torres et al. (104) showed that the rate of glycogen labeling from ^{14}C-glucose was markedly stimulated by the addition of potassium to the incubation mixture. Maximum activation of this system was observed at 60 mM KCl, and the system was protected by potassium against heat inactivation at 37°. Isolated glycogen synthetase was stimulated 30% at a concentration of 0.125 M KCl, but this stimulation was not sufficient to account for the increase in glycogen labeling in the crude homogenate.

The involvement of potassium in starch and glycogen synthesis

has thus been suggested. The experimental evidence, however, does not provide an explanation on the essentiality and possible mode of action of potassium or other univalent cations in these systems. It is hoped that the results of this investigation will specify and clarify the manner in which univalent cations function in starch and glycogen biosynthesis.

MATERIALS AND METHODS

Reagents and Glassware

All glassware utilized for the preparation and storage of reagents and for enzyme assays was acid washed with 3 N HCl and thoroughly rinsed with doubly distilled, deionized water. Reagent grade sucrose, EDTA, cysteine, GSH, and glycine were recrystallized before use. Soluble starch and glycogen were washed with ten volumes of deionized water and precipitated by the addition of cold, redistilled ethanol. This procedure was repeated six times and the final precipitates were dried under reduced pressure. All other chemicals also were reagent grade and utilized without further purification. The buffers used throughout this investigation were either tris(hydroxymethyl)aminomethane (tris) or N-tris(hydroxymethyl)-methyl glycine (tricine). The pH of the tris solutions were adjusted with HCl and that of the tricine solutions with tetramethyl-ammonium hydroxide.

The dipotassium and disodium salts of both ADPG and UDPG, and the barium salt of glucose-6-phosphate were purchased from Sigma Chemical Co. (St. Louis, Mo.). The nucleoside diphosphate sugars were converted to their respective tris salts by passing them through a carboxylic cation exchange resin (Amberlite CG-50, 100-

200 mesh) which had been equilibrated with 0.01 M tris buffer at pH 8.0 for ADPG and 8.5 for UDPG. Additional tris buffer was used to elute the substrates. The substrate concentrations in the eluates were determined on the basis of their molar extinction coefficients at the appropriate pH values ($\epsilon_{259\ m\mu} = 15.4 \times 10^3$ for ADPG and $\epsilon_{260\ m\mu} = 1.0 \times 10^4$ for UDPG). Eluates were combined to give the desired final concentration. Tubes having substrate concentrations less than 0.02 M were combined, evaporated to dryness at 0^o under reduced pressure, and reconstituted with water to yield the desired final concentration. The barium salt of glucose-6-phosphate was converted to the tris salt by precipitation of barium with a slight excess of $tris_2SO_4$. These reagents were stored at -15^o until used.

Adenosine diphosphate glucose-^{14}C (glucose-^{14}C U. L.) was purchased from New England Nuclear Corporation (Boston, Mass.). The ethanolic solution of this substrate was evaporated to dryness at 0^o under reduced pressure, and the substrate redissolved in 0.05 M tricine buffer, pH 8.0. Specific radioactivity of this compound was 225 millicuries per mmole.

Enzyme Sources

Sweet corn (Zea mays L. var. Golden Bantam), field corn (Zea mays L. var. Pride 5) and wheat (Triticum aestivum Linn. var. DuChamp) were field grown at the Oregon State University, Department

of Soil's farm near Lebanon, Oregon. Peas (Pisum sativum L. var. Alaska), bush beans (Phaseolus vulgaris L. var. Bountiful) and soybeans (Glycine max L. var. Chippewa 64) were grown under greenhouse conditions in perlite supplemented with a nutrient solution (66). Potato tubers (Solanum tuberosum L.) were purchased from the local market.

Fresh sheep livers were obtained from the Department of Animal Science. One year old white rats were kindly supplied by Dr. Tinsley of the Department of Agricultural Chemistry.

Preparation of Enzymes

Starch granules were prepared from freshly harvested immature seeds and from potato tubers utilizing a modified method of Leloir, de Fekete and Cardini (57). The seeds or tubers were macerated in an Omnimixer for two minutes in four volumes of cold (0 - 4°) deionized water. The homogenate was filtered through four layers of washed cheesecloth to remove excess debris. The milky solution was then centrifuged for five minutes at 1,000 x g in a Sorval refrigerated centrifuge (0-4°). The white pellet was washed four times with cold, deionized water and the final pellet resuspended in four volumes of redistilled acetone at -15°. The starch preparation was collected by filtration on a Buchner funnel at -15°, and was washed four additional times with four volumes of cold acetone. The

final enzyme preparation (starch granules) were dried under reduced pressure at 0° and stored at -20° until utilized. Prior to assaying for starch synthetase activity, the granules (240 mg) were washed at least three times with 6.0 ml portions of 0.1 M tricine buffer, pH 8.0. The final pellet was resuspended in 3.0 ml of 0.05 M tricine buffer, pH 8.0.

Glycogen synthetase was obtained from freshly excised sheep and rat livers by a modified method of Leloir and Goldemberg (55). Sheep were sacrificed and the livers immediately removed and placed on ice. Rats were fed 30% sucrose ad libitum for 12 hours, killed by CO_2 suffocation, the livers immediately removed and placed on ice. The livers were macerated in a Ten Broeck homogenizer with three volumes of cold 0.25 M sucrose containing 0.001 M EDTA. The resulting homogenate was centrifuged at 2,000 x g for ten minutes and the supernatant (crude glycogen synthetase) stored at -15° until assayed. In this form, the enzyme is reported to be stable for at least one month (55).

Further purification of the glycogen synthetase was accomplished by centrifugation of 30 ml of the crude preparation at 25,000 x g for ten minutes at $0 - 4^{\circ}$. The transparent, yellow pellet (particulate glycogen) was suspended in 3.0 ml of 0.05 M tricine buffer, pH 8.5, containing 0.05 M glucose-6-phosphate and 150 mg soluble starch. This suspension was centrifuged at 25,000 x g for ten minutes and the

washing procedure repeated three times. The final pellet was resuspended in 3.0 ml of 0.05 M tricine buffer, pH 8.5, containing 0.05 M glucose-6-phosphate and assayed immediately. A 300-fold purification of glycogen synthetase resulted from the above procedure. Attempts to prepare a stable acetone powder preparation of the enzyme were unsuccessful.

Enzyme Assays

The reaction mixture for the assay of starch synthetase contained the following components in a total volume of 0.2 ml: tricine buffer, pH 8.0, 10 μmoles; tris$_2$ADPG, 1.0 μmole; 4 mg starch granules (20 μg protein); and the appropriate cation concentration. The reaction was initiated by the addition of the substrate, incubated on a shaker for one hour at 37° and terminated by placing the tubes in a boiling water bath for one minute. Neither ADPG nor ADP were hydrolyzed by this boiling treatment.

Glycogen synthetase was assayed using a reaction mixture of the following composition in a total volume of 0.2 ml: 2.0 μmoles glucose-6-phosphate; 1.6 mg glycogen; 10.0 μmoles tricine buffer, pH 8.5; 1.0 μmole UDPG; the appropriate univalent cation concentration; and 0.02 ml of the enzyme preparation containing approximately 1.5 μg of protein. The reaction was initiated by the addition of the substrate, incubated for ten minutes at 37°, and terminated by placing

the reaction tubes in a boiling water bath for one minute.

Nucleoside diphosphates released in the synthetase reactions were determined by means of a modified pyruvic kinase assay (66). Crystalline pyruvic kinase (Calbiochem, Los Angeles, Calif.) having a specific activity of 178 enzyme units per mg was diluted 1:1,000 in 0.05 M potassium phosphate buffer, pH 7.4, containing 0.005 M $MgSO_4$. This solution was prepared fresh for each assay and had a final protein concentration of 2 μg per 0.2 ml. The final reaction mixture of 1.0 ml contained the following final concentrations of constituents: 50 μmoles tris buffer, pH 7.4; 10 μmoles $MgCl_2$; 10 μmoles KCl; 1.5 μmoles phosphoenolpyruvate (cyclohexylammonium salt); 0.2 ml of the pyruvic kinase solution; and the total 0.2 ml synthetase reaction mixtures. The reaction was initiated by the addition of the pyruvic kinase solution and incubated for 20 minutes at 37^{o}. The reaction was stopped by the addition of 1.0 ml of a 0.0125% (w/v) 2, 4-dinitrophenylhydrazine solution followed by 7.0 ml of 0.43 N NaOH. These samples were then centrifuged 15 minutes at 2,000 x g, and the optical density of the supernatant determined at 525 mμ on a Hitachi Perkin-Elmer model 139 spectrophotometer.

Radioisotope Assays

Assay conditions involving the labeled substrate were the same as those previously described for starch synthetase with the exception

that ^{14}C-ADPG was utilized. The reaction was stopped by the addition of 2.0 ml of cold 75% (v/v) methanol. The precipitated starch was washed six times with 2.0 ml aliquots of cold 75% methanol. The final pellet was then hydrolyzed with 2.0 ml of 0.7 N HCl for two hours in a boiling water bath. The final volume after hydrolysis was reconstituted to 2.0 ml with water. A 0.5 ml sample of the hydrolyzate was added to 10 ml of Bray's Solution (12), and the radioactivity was determined with a Packard Model 3375 Tri-Carb Liquid Scintillation Spectrophotometer. The counting efficiency of this system was approximately 65%.

Miscellaneous Assays

Protein was determined by Folin-phenol method (60). The starch granules were boiled for ten minutes in 0.1 ml of 0.1 N KOH prior to protein determination. If this treatment is omitted, measured protein content is reduced by approximately 50%. Protein content per mg of starch granules was approximately 5 μg. In many of the previous reports involving starch synthetase, the specific activities presented were based on starch rather than protein content. This basis fails to take into account the age, size, and enzyme content of the starch granules. Using protein content as a basis for starch synthetase activity eliminates these variations. In the case of glycogen synthetase, the protein was precipitated by 5% trichloroacetic acid prior to

protein determination.

Potassium concentration in the reagents and solutions was determined by flame photometry according to the procedure of Johnson and Ulrich (46). Materials were ashed at 600^{o} in a muffle furnace. The resulting ash was suspended in 10 ml of 1.6 N HNO_3 and filtered through acid washed Whatman #12 filter paper. These samples were then diluted to 100 ml and the percent transmission at 770 mμ was determined using a Beckman DU flame photometer.

All data presented in this investigation is the mean values of triplicate experiments. Lineweaver-Burk type plots were prepared by the method of Least Squares (65).

RESULTS

Starch Synthetase Studies

Validity of the Starch Synthetase Assays

To establish the validity of the starch synthetase assays used in this investigation, experiments were conducted dealing with the effects of incubation time, pH, enzyme and substrate concentrations on the activity of the enzyme. The experimental data presented in Figure 1 represents the incorporation of ^{14}C-glucose into starch as a function of time. The rate of incorporation is linear for 90 minutes. At this time, approximately four percent of the added label had been incorporated into the starch. Assays utilizing the pyruvic kinase method for determining the rate of liberation of ADP from ADPG also yielded comparable results.

The pH optimum for the sweet corn preparation in the presence of excess potassium (0.125 M KCl) was found to be approximately 8.0 (Figure 2). This value is similar to that reported for the activity of the maize enzyme in glycylglycine buffer, but is higher than the optimum pH of 7.5 reported for tris buffer (1). The use of tris buffer in this investigation was avoided when possible because of the reported (11, 108) inhibitory effects of this cation on certain univalent cation activated enzymes.

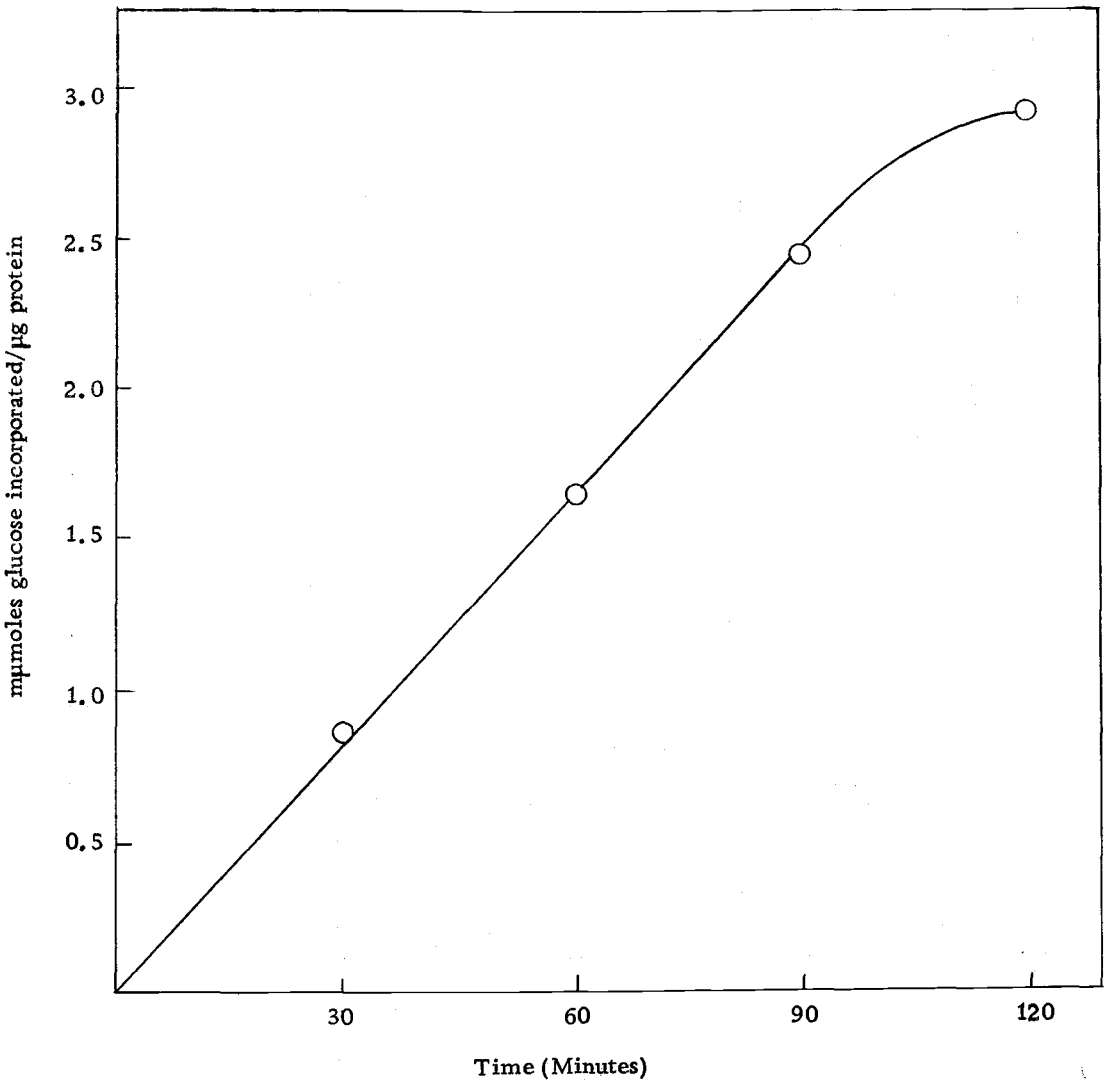

Figure 1. The Incorporation of ^{14}C-Glucose as a Function of Time by Starch Synthetase from Sweet Corn. The reaction mixture contained the following components in a total volume of 0.2 ml: 10 μmoles tricine buffer, pH 8.0; 20 μmoles KCl; 1.0 μmole ADPG (2.6 X 10^5 dpm); and 4 mg starch granules (20 μg protein). Assay procedures are described in the Materials and Methods.

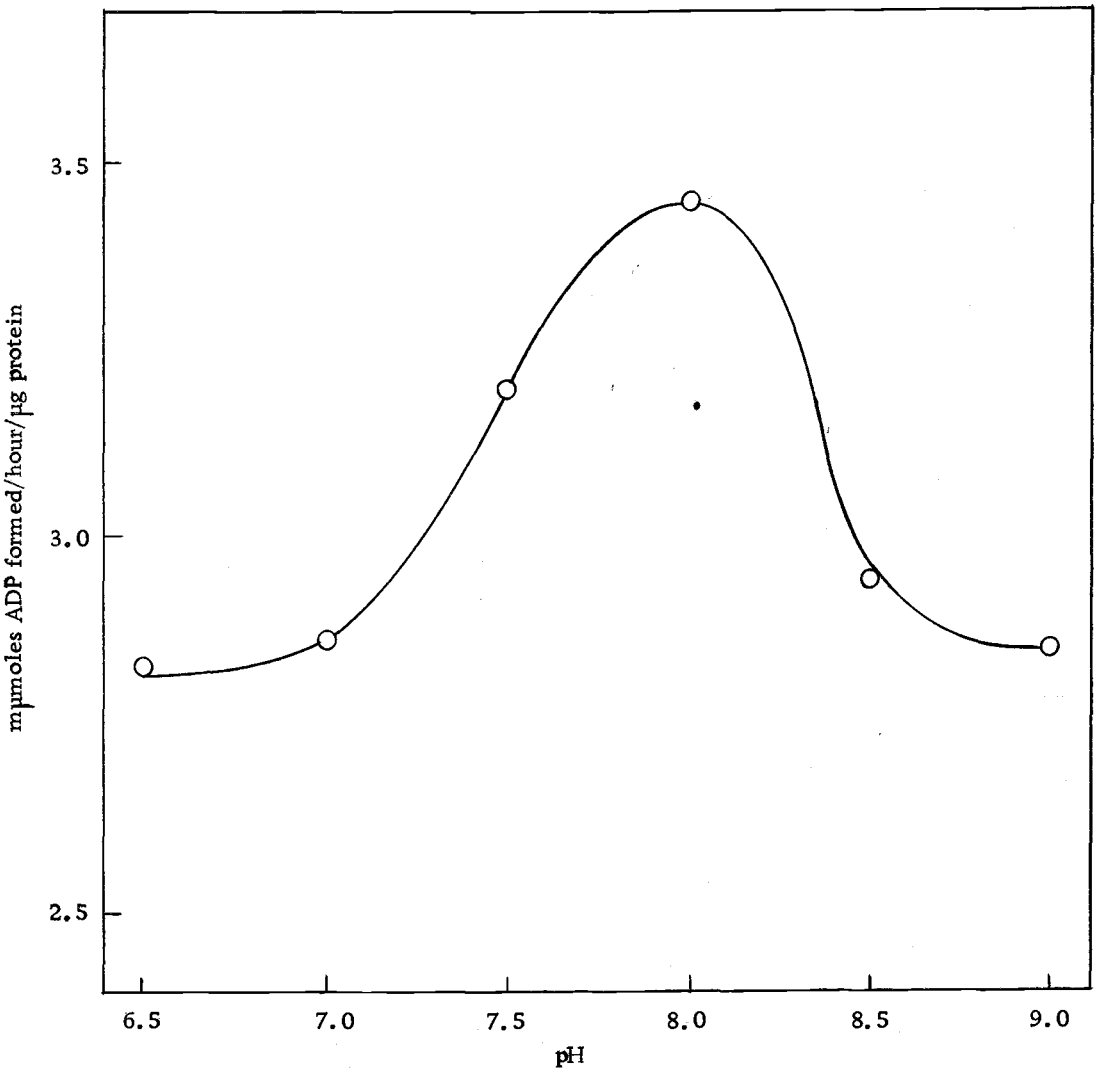

Figure 2. The Effects of pH on the Activity of Particulate Starch Synthetase from Sweet Corn. The pH of 0.05 M tricine was adjusted to the appropriate value with tetramethylammonium hydroxide. Assay procedures are described in the Materials and Methods.

The liberation of ADP in the starch synthetase reaction as a function of enzyme concentration is represented in Figure 3. The slight deviation from linearity may be a result of the particulate nature of the enzyme. Non-linearity also was observed for particulate enzymes from beans (57), rice (2) and potatoes (27). The protein content of the starch granules from sweet corn is somewhat higher than that reported for other enzymes (1, 27, 57) and is probably due to additional protein bound to the granules. The protein content per mg starch, however, was found to be relatively constant throughout the investigation, ranging from three to four μg protein per mg of starch granules.

Figure 4 represents the effects of both $tris_2$ADPG and K_2ADPG on the activity of particulate starch synthetase assayed in the presence of excess potassium (0.125 M KCl). The two curves are quite similar indicating that the tris salt of the substrate had no appreciable inhibitory effects on the activity of the enzyme. The apparent K_m values for the two substrates were determined by kinetic plots (inset of Figure 4), and the values are 1.1 mM for $tris_2$ADPG and 1.0 mM for K_2ADPG. Concentrations of $tris_2$ADPG used in subsequent reaction mixtures were greater than 3.5 mM, and any interference by the tris cation was considered to be of little consequence. It appears that these apparent K_m values are not significantly different under the experimental conditions utilized.

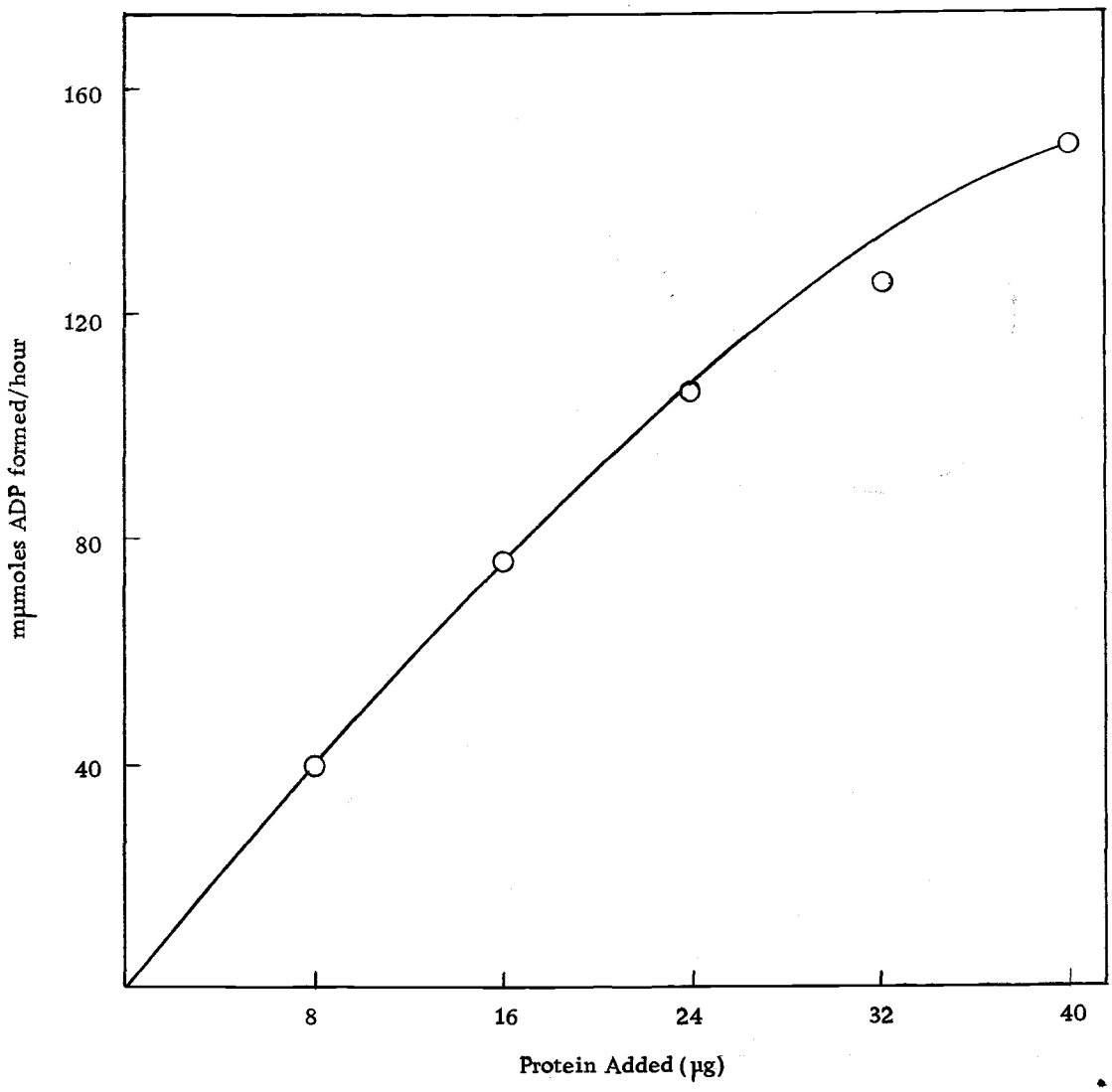

Figure 3. The Effects of Enzyme Concentration on the Activity of Particulate Starch Synthetase from Sweet Corn. Assay conditions are the same as those described in the Materials and Methods except protein concentration was varied as indicated. Reaction mixtures were incubated on a shaker to prevent sedimentation.

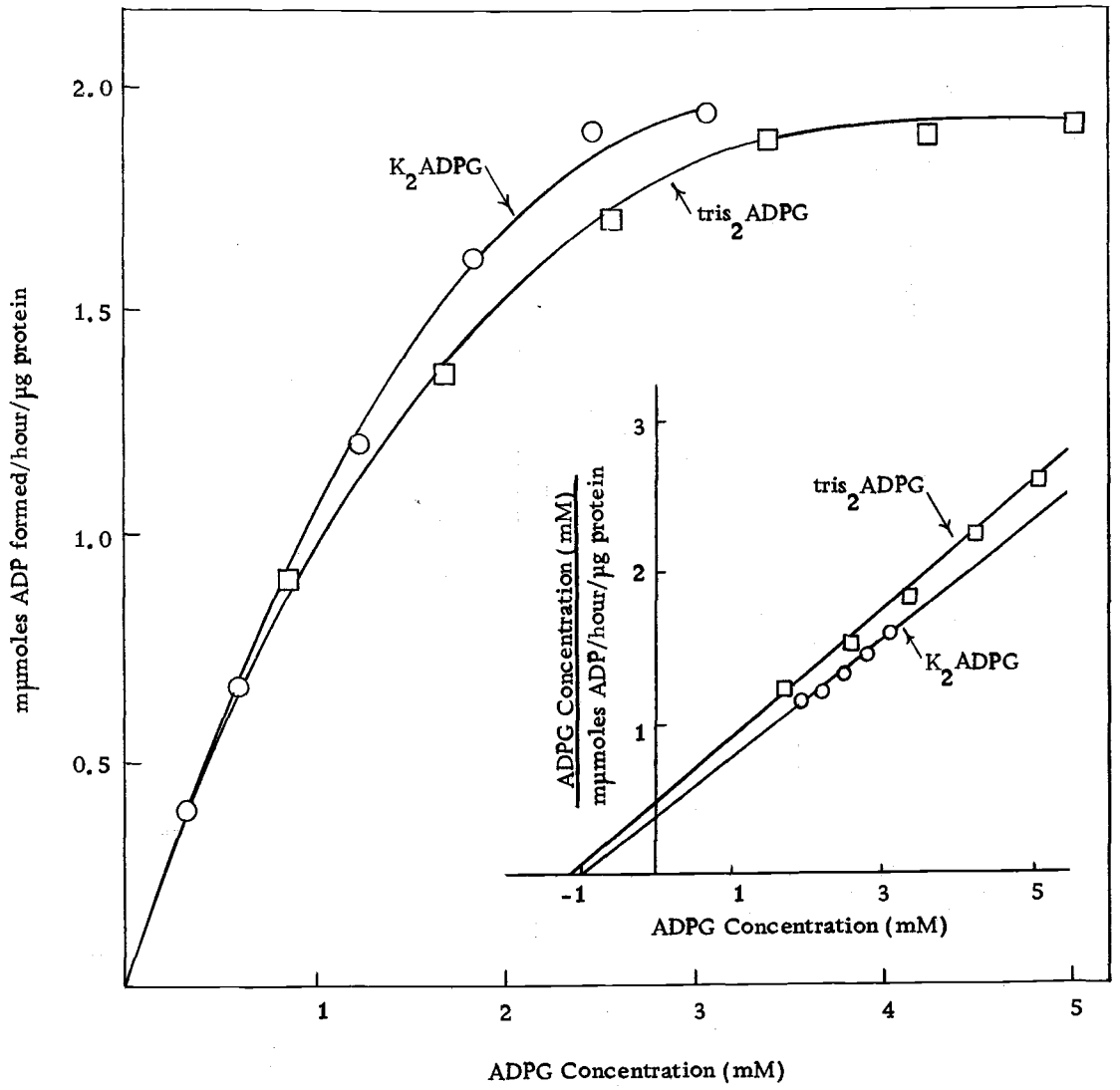

Figure 4. The Effects of the Potassium or Tris Salts of ADPG on the Activity of Starch Synthetase from Sweet Corn. The reaction conditions are the same as those described in the Materials and Methods except substrate concentration was varied. The lines in the modified Lineweaver-Burk plot were located by the method of Least Squares.

Effects of Univalent Cations

The effects of potassium on the incorporation of ^{14}C-glucose from ADPG-^{14}C into starch by the enzyme from sweet corn are presented in Figure 5. An analysis of the kinetics of this reaction (inset of Figure 5) shows an apparent Michaelis constant for activation (K_A) for KCl of 6.1 mM and a V_{max} of 1.67 μmoles glucose incorporated per hour per μg protein. Other experiments involving unlabeled substrate showed potassium saturation occurring at approximately 0.05 M KCl which is a concentration of potassium often found in the cells of plants (24).

The activation of starch synthetase by a series of different univalent cation salts is presented in Figure 6. No activity was observed when univalent cations were omitted from the reaction mixture. The additions of either rubidium, cesium or ammonium cations produced extents of activation similar to that of potassium, but the maximum velocity observed with potassium is greater than that obtained with the other cations (Table I). The addition of sodium or lithium resulted in only slight activation of enzyme activity. The calculated apparent K_A values for these univalent cations are given in Table I. The K_A value for potassium also was determined from experiments involving ADPG-^{14}C and did not differ appreciably from that obtained with the unlabeled substrate. While the ammonium ion

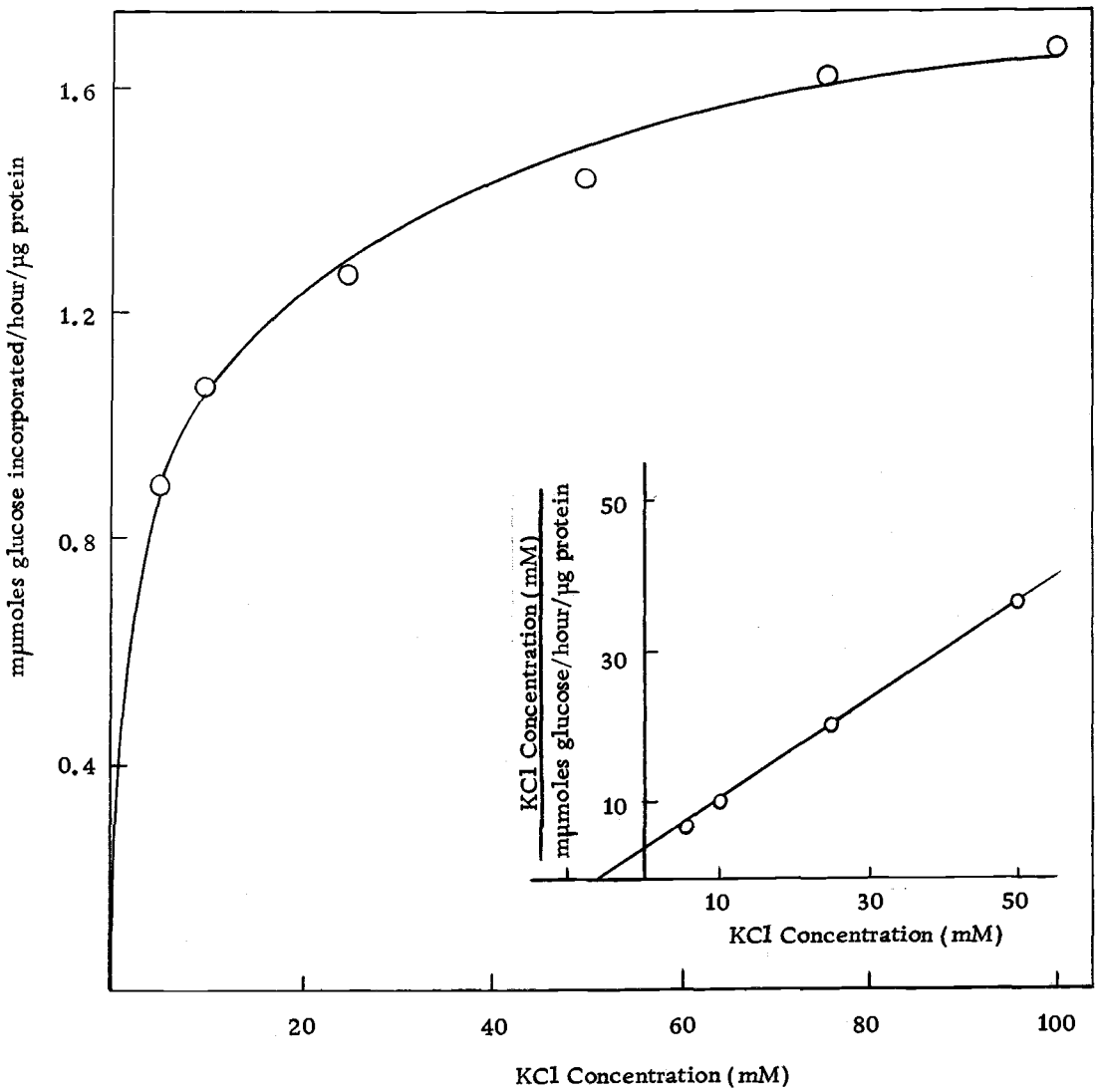

Figure 5. The Effect of Potassium on the Incorporation of ^{14}C-Glucose from ADPG-^{14}C into Starch by Starch Synthetase from Sweet Corn. The reaction mixture as described in the Materials and Methods contained 2.6×10^5 dpm of ADPG-^{14}C and the indicated potassium concentrations. The line in the modified Lineweaver-Burk plot was located by the method of Least Squares.

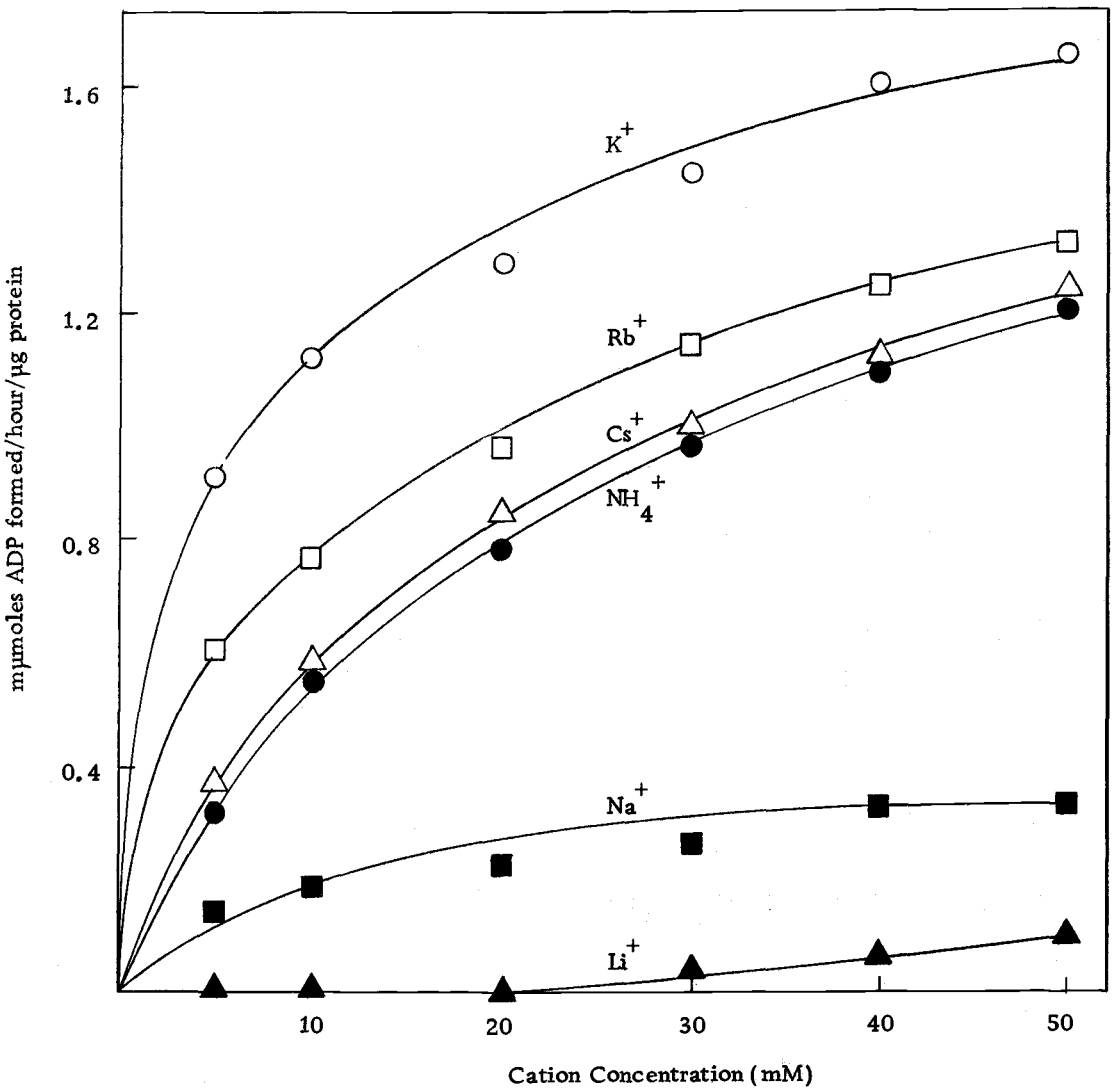

Figure 6. The Effects of Univalent Cation Chlorides on the Activity of Starch Synthetase from Sweet Corn. The basic reaction mixture described in the Materials and Methods was utilized with the exception that the univalent cation concentrations were varied as indicated.

is not a member of the Group I alkali metals, its physical and chemical properties including the radius of the ion, the charge, and its activation of various enzymatic reactions (24) are similar to those of the alkali metal cations and thus it is included in this study.

Table I. The Apparent K_A and V_{max} Values for Different Univalent Cations in the Activation of Starch Synthetase From Sweet Corn.

Cation	K_A	V_{max}
	mM	mμmoles ADP formed/ hour/μg protein
K^+	6	1.7
Rb^+	11	1.5
Cs^+	14	1.4
NH_4^+	19	1.5
Na^+	3	0.4

Note: The values were calculated from the data presented in Figures 5 and 6.

The effects of potassium on the activities of particulate starch synthetase from different sources are presented in Table II. Although the starch granules were washed from three to six times with 0.1 M tricine buffer, pH 8.0, a small degree of activity was observed in most preparations in the absence of added potassium. When potassium was omitted from the reaction mixtures, the starch granules from peas, soybeans and field corn had the lowest residual activities, ranging from 0.6 to 0.9 mμmoles ADP liberated per hour per μg

protein. Analysis of the starch granules from the various sources by flame photometry shows that these preparations contain sufficient endogenous potassium to account for the activities observed in the absence of added KCl (Table III).

Table II. The Effects of KCl on the Activity of Starch Synthetase from Different Plant Sources.

Source of Enzyme	KCl Concentration (mM)						
	0	5	10	25	50	75	100
	mμmoles ADP formed/hour/μg protein						
Sweet corn	0.0	3.8	4.6	5.4	6.5	6.9	7.1
Peas	0.6	2.9	3.5	4.8	5.6	6.8	7.3
Soybeans	0.7	3.6	5.7	9.0	10.0	12.4	13.1
Field corn	0.9	5.5	6.8	7.6	9.0	9.4	9.8
Wheat	2.0	4.5	6.0	6.8	7.2	7.4	7.5
Bush beans	2.1	5.0	7.2	7.4	7.9	8.3	8.3
Potato	2.8	6.1	9.8	12.6	15.2	17.4	17.4

Note: The reaction mixtures contained the following components in a total volume of 0.2 ml: 10 μmoles tricine buffer, pH 8.0; 1.0 μmole ADPG; either 4 mg starch granules (bush bean, potato, wheat, field corn, peas, sweet corn) or 2 mg starch granules (soybeans); and the indicated KCl concentration. Assay procedures are described in the Materials and Methods.

Although an absolute potassium requirement of the starch synthetase was demonstrated only for the enzyme from sweet corn, the activities of the enzymes from the other sources were strikingly stimulated by KCl. The pea, soybean or potato enzymes do not appear to be completely saturated at 0.1 M KCl but all of the other enzymes

were saturated in the range of 0.05 M to 0.075 M KCl. The K_A values for KCl obtained with each of the preparations are presented in Table IV. The uncorrected values are those determined from the data in Table III. The corrected values were calculated by first subtracting the endogenous activities from the other values. These corrected values show a slight change in the K_A values for KCl, and probably represent a more realistic estimate of the K_A of these enzymes. The increased activity of the enzyme from sweet corn (Table III) in comparison to those values previously reported (Figures 5 and 6) is attributed to the use of a new preparation of this enzyme

Table III. Flame Photometric Analysis of the Potassium Content of Starch Granules from the Different Plant Sources.

Source of granules	Potassium content of dry granules (gm/mg of granules)	Potassium concentration in reaction (mM)
Sweet corn	0.1×10^{-7}	0.01
Peas	1.0×10^{-6}	0.50
Field corn	1.0×10^{-6}	0.50
Soybeans	3.8×10^{-6}	1.00
Wheat	5.7×10^{-6}	3.00
Potatoes	7.8×10^{-6}	4.00
Bush beans	5.7×10^{-6}	3.00

Note: Granules were wet ashed at 600° and the material then subjected to flame photometric analysis as described in the Materials and Methods.

Table IV. The Apparent K_A Values for KCl for Starch Synthetase Isolated from Different Plant Sources.

	K_A (mM)	
Plant sources	Uncorrected values	Corrected values
Sweet corn	6	6
Peas	19	33
Soybeans	17	20
Field corn	11	12
Wheat	4	5
Bush beans	7	6
Potatoes	15	18

Note: The values were calculated from the data presented in Table II. The corrected values were determined after subtracting the endogenous values from the initial data.

Since sodium activation of the sweet corn starch synthetase was quite low, an investigation was conducted to determine the possibility of an interaction between sodium and potassium on starch synthetase activity. The effects of four different levels of sodium on activity was determined each at a series of increasing concentrations of potassium and the results are presented in Figure 7. At concentrations of sodium up to 50 mM, enzyme activity was stimulated over the entire range of potassium concentrations. At low levels of potassium, 100 mM sodium resulted in a slight stimulation of activity, but at higher levels of potassium, the effect was inhibitory. The stimulatory effect of sodium at concentrations of 50 mM or less is at least additive and

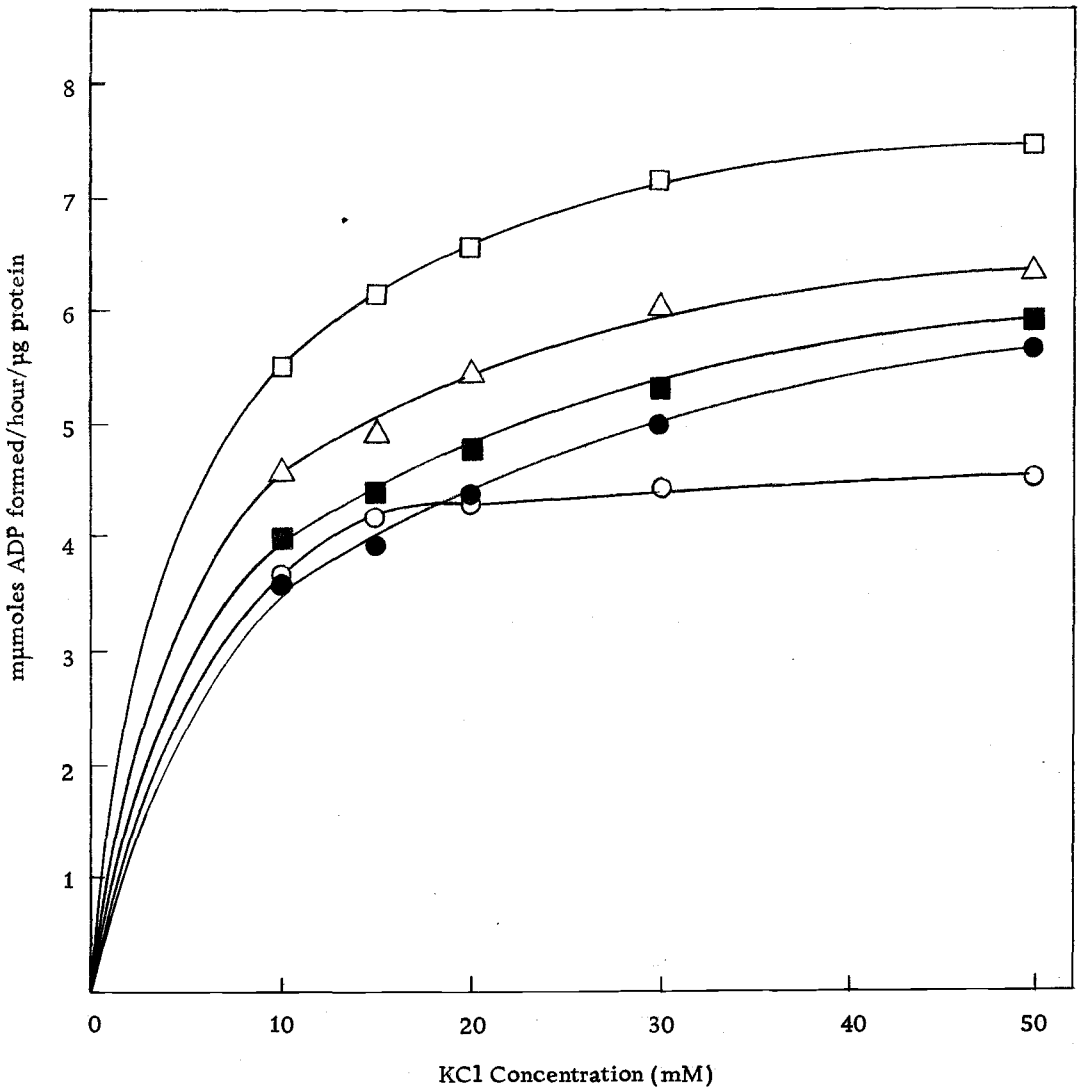

Figure 7. The Interaction of Potassium and Sodium on the Activity of Starch Synthetase from Sweet Corn. The reaction mixture contained the following concentrations of components in a total volume of 0.2 ml: 10 μmoles tricine buffer, pH 8.0; 1.0 μmole ADPG; 4 mg starch granules (20 μg protein); and the appropriate KCl and NaCl concentrations as indicated. No NaCl added: ●——●; 5 mM NaCl: ■——■; 20 mM NaCl: △——△; 50 mM NaCl: □——□; 100 mM NaCl: ○——○.

possibly synergestic since the combined rates of reactions when sodium and potassium were added alone are in some cases less than the rates in the presence of the two cations together. A Lineweaver-Burk plot of the results of this experiment are presented in Figure 8. The interaction of sodium and potassium is not competitive and sodium causes a decrease in the K_A for KCl.

To determine the possible effects of the different univalent cations on the K_m for ADPG, starch synthetase activity was measured as a function of substrate concentration at saturating levels of the different cations. The results of this study are presented in Figure 9 and show that enzyme activation by the univalent cations follows the same type of pattern as observed in the experiments reported in Figure 6. In these experiments (Figure 9), low enzyme activity was observed in the absence of added univalent cations. Analysis of the substrates and the enzyme by flame photometry showed that maximum concentration of potassium present in the reaction mixtures did not exceed 0.01 mM. The reason for the activity in the absence of added univalent cations is unknown.

Analysis of the data presented in Figure 9 by Lineweaver-Burk plots showed that these univalent cations had no appreciable effect on the K_m for ADPG (Figure 10). With the addition of the different univalent cation chloride salts, the K_m values for ADPG fall within the range 1.45 mM to 1.63 mM which are values similar to that obtained

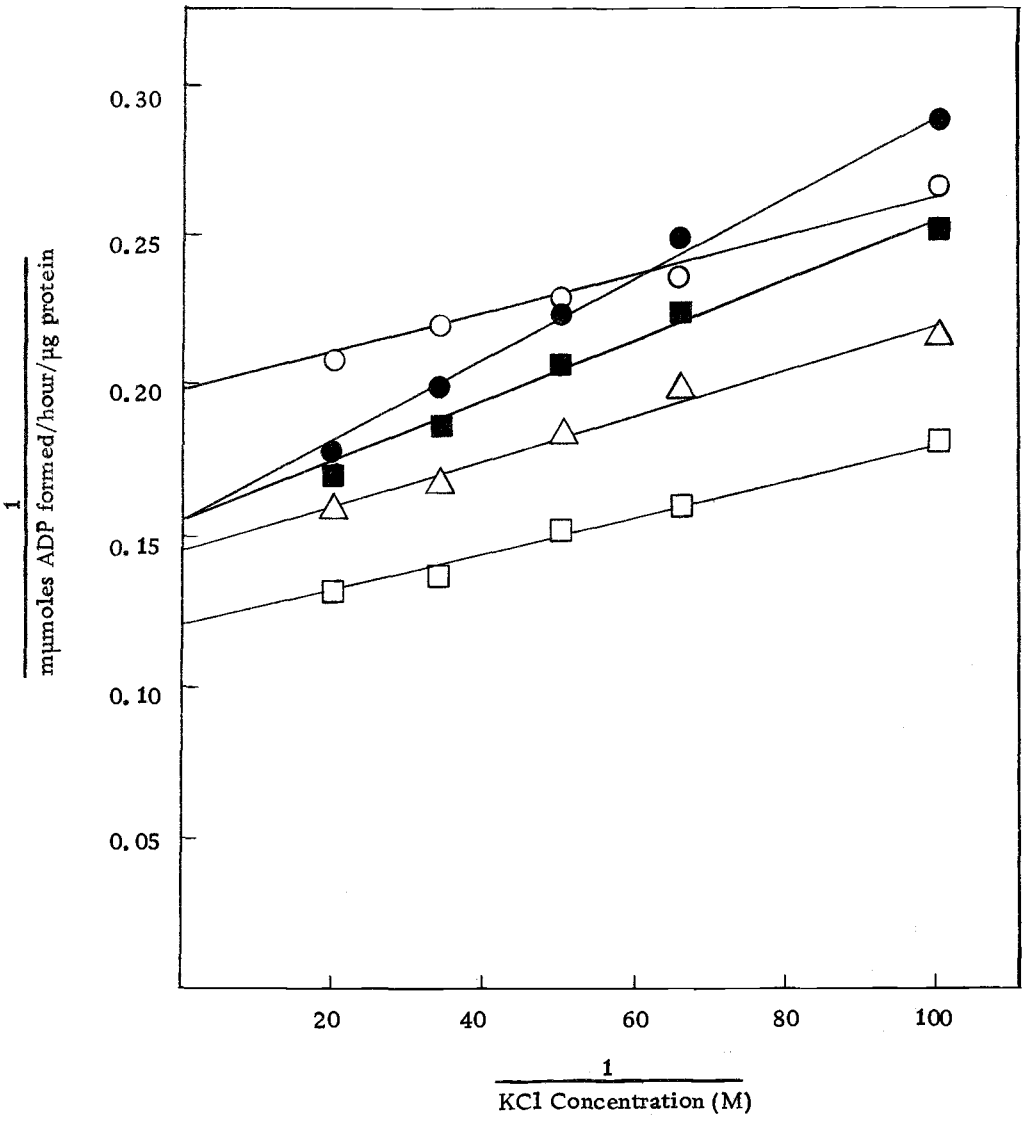

Figure 8. A Lineweaver-Burk Plot of the Interaction of Sodium and Potassium on the Activity of Starch Synthetase from Sweet Corn. The values were obtained from the data in Figure 7, and the lines located by the method of Least Squares. No NaCl: ●——●; 5 mM NaCl: ■——■; 20 mM NaCl: △——△; 50 mM NaCl: □——□; 100 mM NaCl: ○——○.

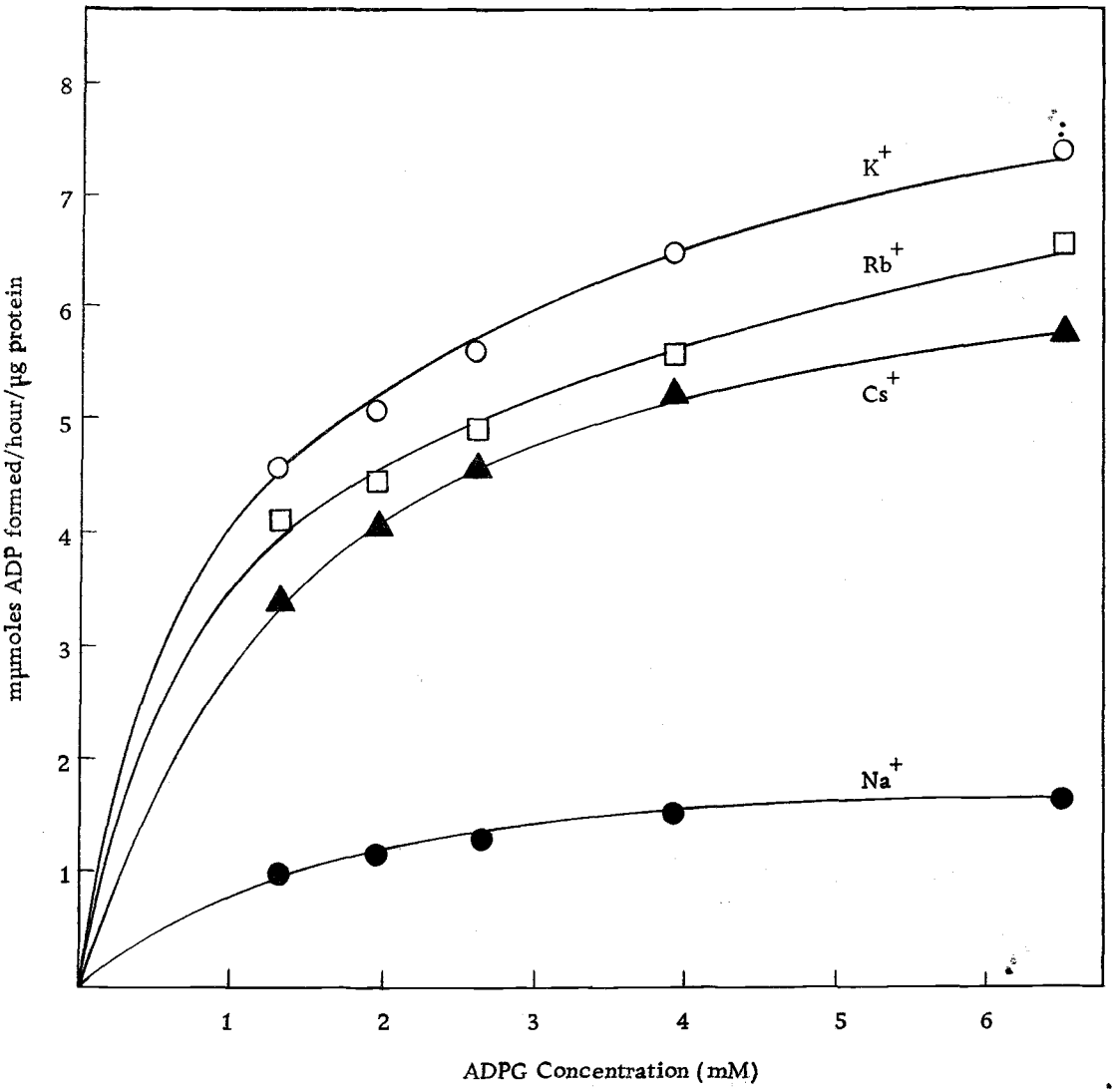

Figure 9. The Effects of Univalent Cations on the K_m for ADPG of Starch Synthetase from Sweet Corn. The reaction mixture contained the following constituents in a total volume of 0.2 ml: 10 μmoles tricine buffer, pH 8.0; 20 μmoles of the indicated univalent cation chlorides; 4 mg starch granules (20 μg protein); and the appropriate ADPG concentration. Assay conditions are those described in the Materials and Methods.

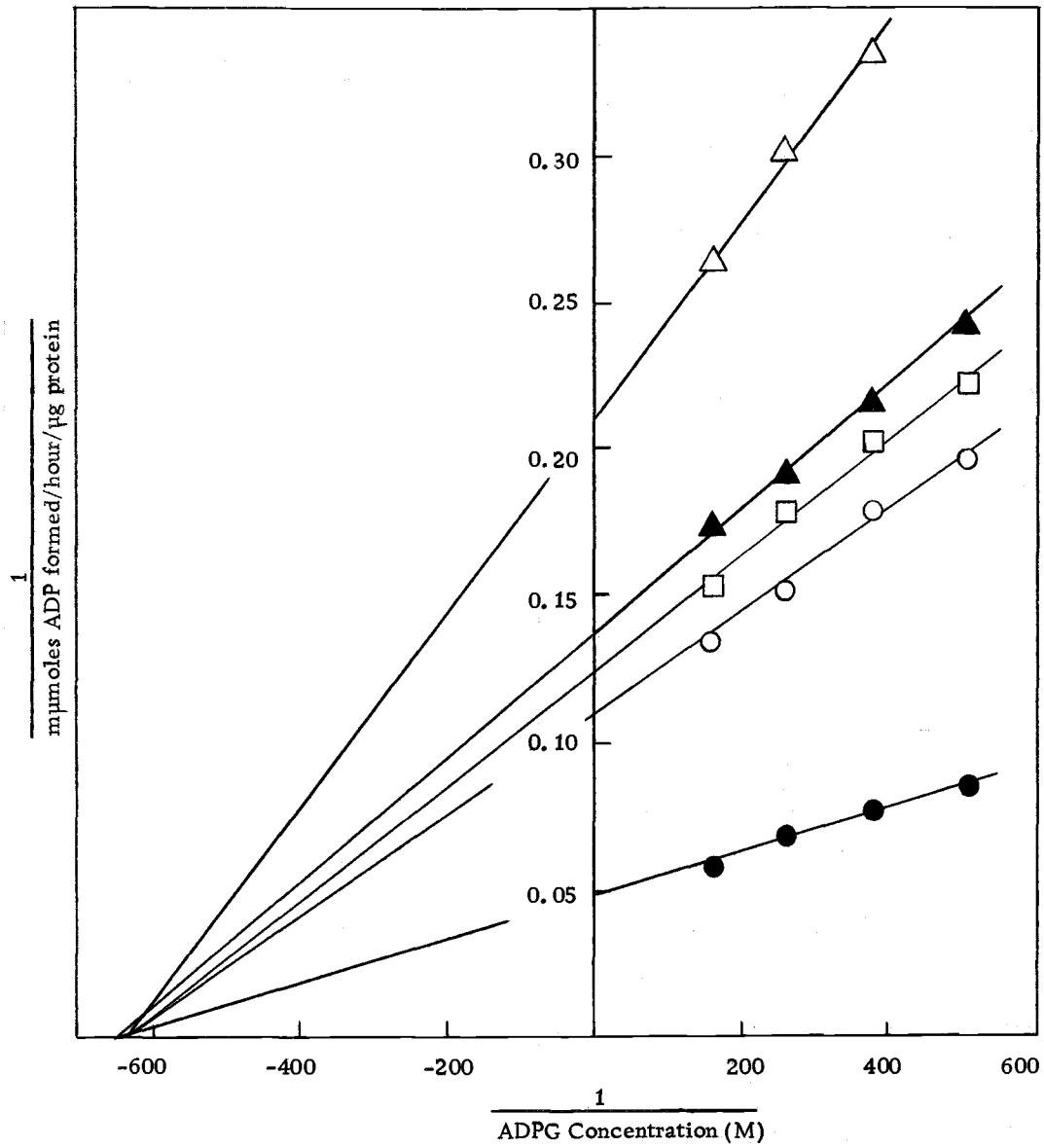

Figure 10. Lineweaver-Burk Plot of the Effects of Univalent Cations on the K_m of Starch Synthetase for ADPG. The values were calculated from the data presented in Figure 9, and the lines located by the method of Least Squares. 1 mM KCl: △—△ ; 100 mM KCl: ○—○ ; 100 mM NaCl: ●—● ; 100 mM RbCl: □—□ ; 100 mM CsCl: ▲—▲ .

in the experiment on the effect of substrate concentration on the activity of starch synthetase from sweet corn (Figure 4). A comparison of the effects of 1.0 mM and 100 mM KCl on the K_m for ADPG (Figure 10) also shows that the value was not altered by the different concentrations of the univalent cation.

Preliminary experiments were conducted to determine the effects of incubation time on enzyme activity at different concentrations of ADPG. The results presented in Figure 11 indicate that a linear relationship exists for at least 20 minutes incubation time for all levels of substrate utilized. At lower substrate concentrations, the rate decreases after this 20 minute period.

Using a 20 minute incubation period, the effects of ADPG on the interaction of potassium and the enzyme were determined, and these results are presented in Figure 12. Stimulation of enzyme activity was observed with increasing substrate and KCl concentrations. Data from this experiment were subjected to kinetic analysis, and the K_A for KCl determined from the slopes of the lines (Figure 13). The K_A values range from 5.8 mM to 6.9 mM and are considered to be sufficiently close to eliminate the possibility that ADPG has an effect on the K_A for KCl.

Since starch synthetase from certain sources will utilize UDPG to a smaller extent than ADPG (Literature Review), an investigation was conducted to determine whether utilization of UDPG was influenced

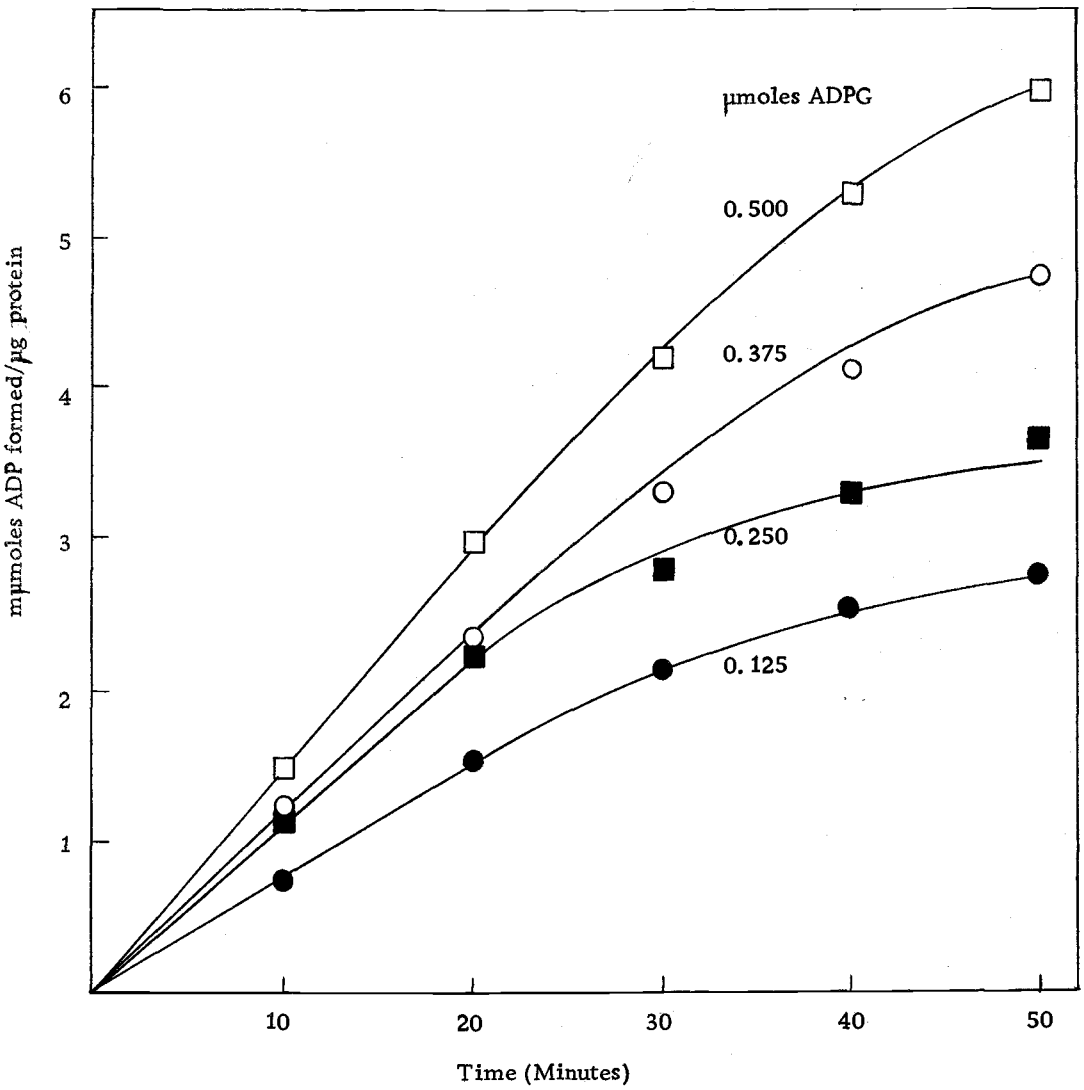

Figure 11. The Effects of Incubation Time and Substrate Concentration on the Activity of Starch Synthetase from Sweet Corn. The reaction mixture of 0.2 ml contained the following components: 10 μmoles tricine buffer, pH 8.0; 20 μmoles KCl; 4 mg starch granules (20 μg protein); and the indicated concentration of ADPG. Assay procedures are described in the Materials and Methods.

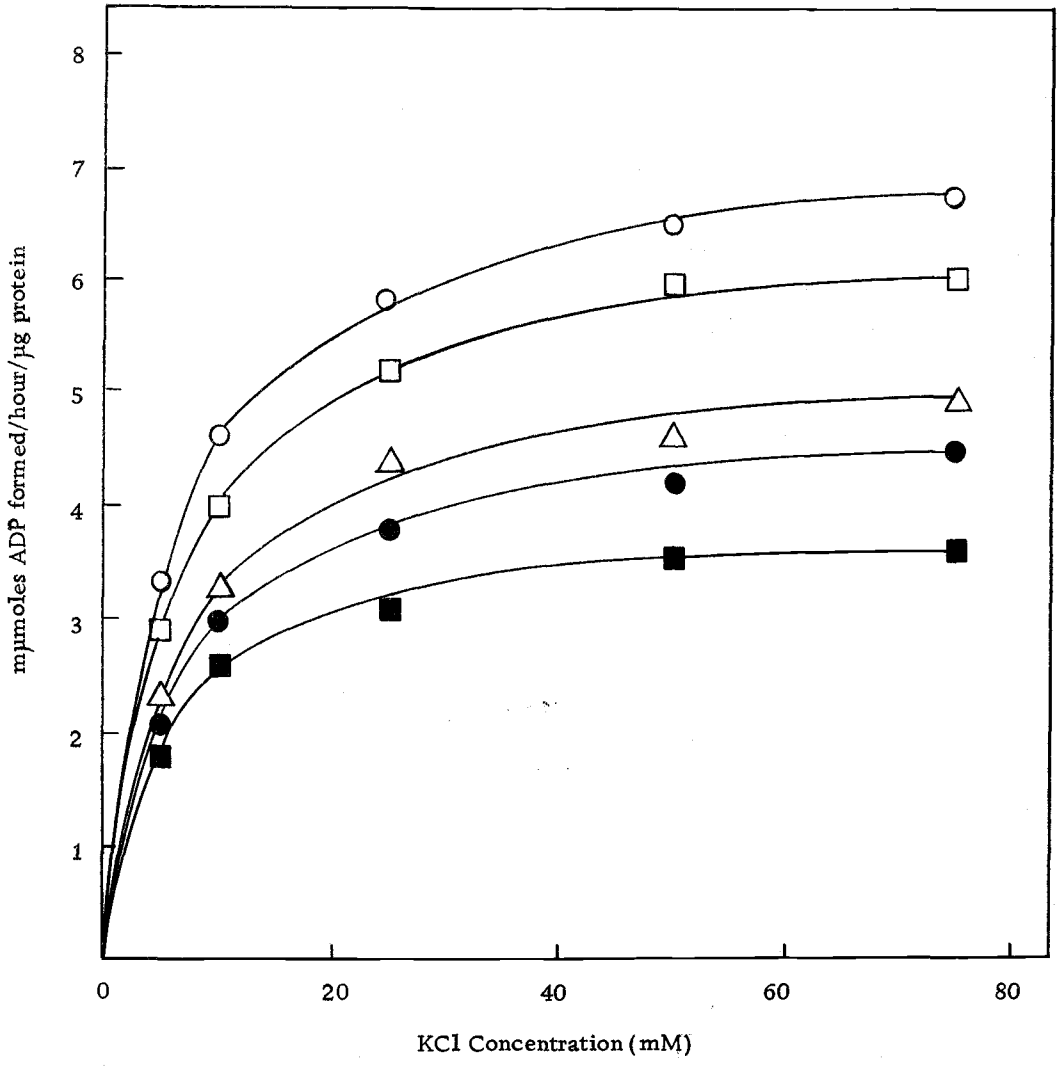

Figure 12. The Effects of ADPG Concentrations on the Potassium Activation of Starch Synthetase. The reaction mixture contained the following components in a total volume of 0.2 ml: 10 μmoles tricine buffer, pH 8.0; 4 mg starch granules (20 μg protein); and the indicated substrate and potassium concentrations. Incubations were for 20 minutes at 37° on a shaker. 1.0 μmoles ADPG O—O; 0.75 μmoles ADPG □—□; 0.50 μmoles ADPG △ △; 0.25 μmoles ADPG ●—●; 0.125 μmoles ADPG ■—■.

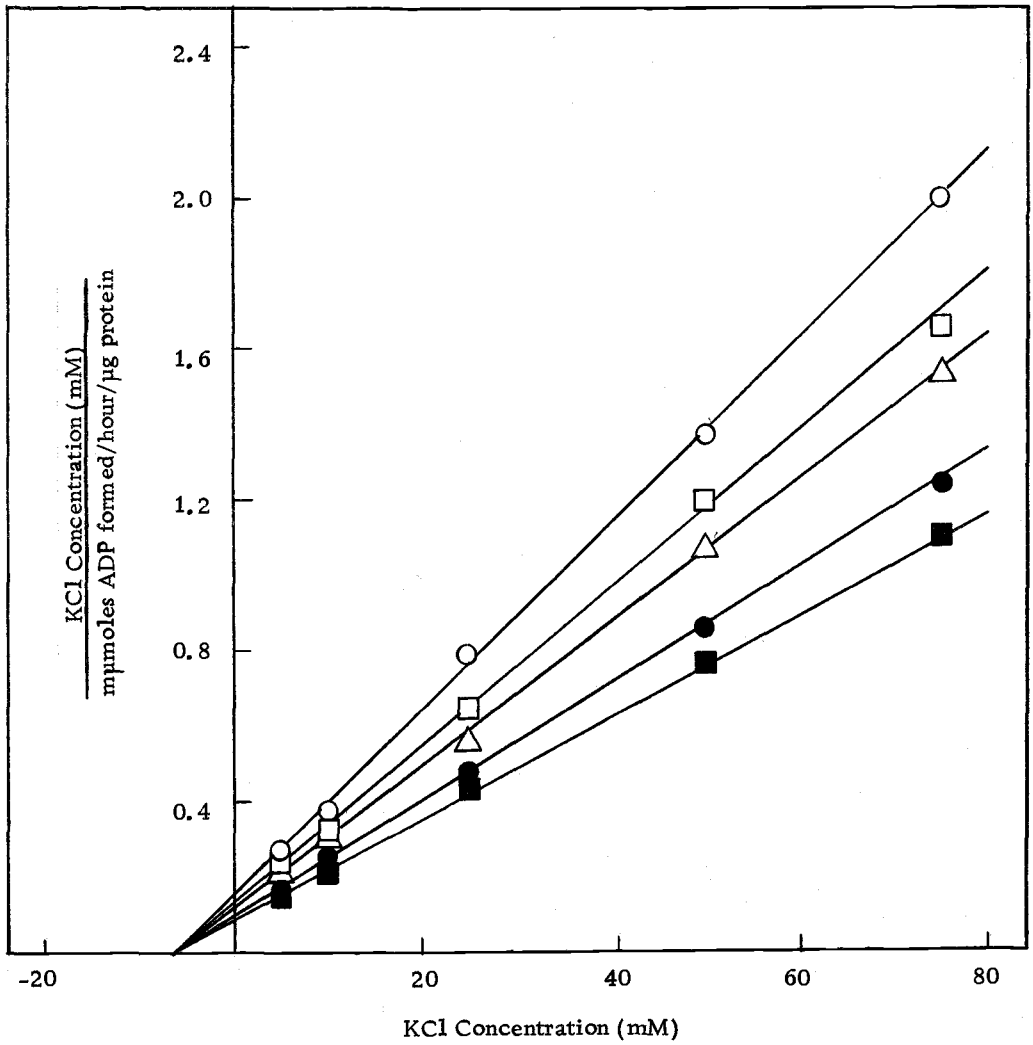

Figure 13. Kinetic Analysis of the Effects of ADPG on the Potassium Activation of Starch Synthetase from Sweet Corn. The values were calculated from the data presented in Figure 12, and the lines located by the method of Least Squares. 0.125 μmole ADPG: ■——■ ; 0.250 μmole ADPG: ●——● ; 0.500 μmole ADPG: △——△ ; 0.750 μmole ADPG: □——□ ; 1.00 μmole ADPG: ○——○ .

by univalent cations. The results (Table V) obtained when ADPG was the substrate show that enzyme activity was stimulated considerably when potassium was present in the reaction mixture, but only slightly when sodium was added. When UDPG was used as the substrate, appreciable enzyme activity was observed in the absence of added cations. When either sodium or potassium was added to this assay mixture, a small stimulation was observed, but this is far less than that observed with ADPG as the substrate.

Table V. The Effects of Univalent Cations on the Utilization of ADPG and UDPG as Substrates for Starch Synthetase from Sweet Corn.

	Enzyme Activity		
Substrate	No salt	NaCl 0.1 M	KCl 0.1 M
	mμmoles ADP or UDP formed/hour/μg protein		
ADPG	0.0	0.8	4.5
UDPG	2.1	2.6	3.7

Note: The reaction mixtures contained the following components in a total volume of 0.2 ml: 10 μmoles tricine buffer, pH 8.0; 1.0 μmole ADPG or UDPG; 4 mg starch granules (20 μg protein); and 20 μmoles KCl or NaCl. Assay procedures are described in the Materials and Methods.

Anion Effects

The results of the effects of different anionic salts of potassium on the activity of starch synthetase from sweet corn are presented in

Table VI. In experiments where chloride, bromide, nitrate, or phosphate salts of potassium were used, no appreciable effect on enzyme activity was observed. When sulfate or iodide salts of potassium were added, a slight inhibition of starch synthetase activity was obtained. This inhibition amounts to only 15% at concentrations of 0.1 M salt.

Table VI. The Effects of Anions on the Activity of Starch Synthetase from Sweet Corn.

	Salt Concentration (mM)						
Salt	0	5	10	25	50	75	100
	mμmoles ADP formed/hour/μg protein						
KCl	0	3.8	4.6	5.4	6.5	6.9	7.1
KBr	0	3.8	4.6	5.5	6.5	7.0	7.4
KI	0	3.6	4.3	5.0	5.6	5.8	5.8
KNO_3	0	3.8	4.5	5.6	6.1	6.4	6.9
K_2SO_4	0	3.3	4.2	5.1	5.3	5.8	5.9
K_2HPO_4 - KH_2PO_4	0	3.8	4.6	5.4	6.1	6.4	6.8

Note: The reaction mixture contained 10 μmoles tricine buffer, pH 8.0; 1.0 μmole ADPG, 4 mg starch granules (20 μg protein), and the indicated salt concentration in a total volume of 0.2 ml. Assay conditions are described in the Materials and Methods.

Since anions have been reported to effect the activity of pyruvic kinase (66), experiments were conducted to ascertain if these anions were affecting the pyruvic kinase portion of the assays. In these studies, the potassium and magnesium concentrations were optimum

for the activity of pyruvic kinase. The results obtained indicated that pyruvic kinase activity was not affected by the presence of any of the added anions (Table VI).

Divalent Cation Effects

Experiments were conducted to determine the effects of divalent cations on the activity of starch synthetase in the presence or absence of added potassium (Table VII). The maximum concentration of divalent cations used in these experiments was 20 mM since higher concentrations, especially for calcium and cobalt, are known to affect the activity of pyruvic kinase (66).

In the presence of potassium, the divalent cations magnesium, manganese, calcium and cobalt all exhibited a slight stimulation of starch synthetase activity over the entire range of concentrations utilized (Table VII). Magnesium exhibited the greatest activation while both nickel and copper inhibited activity. In the absence of potassium, all divalent cation salts except copper resulted in increased enzyme activity. Greatest activation of the enzyme under these conditions was obtained by the addition of manganese. The stimulatory effects of the divalent cations, however, are far less than that obtained from the addition of potassium.

Table VII. The Effects of Divalent Cations on the Starch Synthetase Activity from Sweet Corn in the Presence and Absence of Potassium.

Divalent Cation Salt	KCl 50 mM	Divalent Cation Salt Concentration (mM)				
		0	2.5	5.0	10.0	20.0
		mμmoles ADP formed/hour/μg protein				
$MgCl_2$	+	4.1	4.4	5.2	5.4	6.2
$MnCl_2$	+	4.1	4.2	4.5	4.9	5.8
$CaCl_2$	+	4.0	4.3	4.7	5.0	5.0
$CoCl_2$	+	4.1	4.5	4.9	5.4	5.5
$NiCl_2$	+	4.1	4.2	4.1	3.9	3.6
$CuSO_4$	+	3.9	3.6	3.3	3.1	2.9
$MgCl_2$	-	0.0	0.5	0.5	0.8	0.9
$MnCl_2$	-	0.0	0.9	1.6	2.1	3.4
$CaCl_2$	-	0.0	0.5	1.0	1.2	1.0
$CoCl_2$	-	0.0	0.3	0.5	0.8	0.6
$NiCl_2$	-	0.0	0.2	0.5	0.7	0.6
$CuSO_4$	-	0.0	0.0	0.0	0.0	0.0

Note: The reaction mixtures contained the following in a total volume of 0.2 ml: 10 μmoles tricine buffer, pH 8.0, 1.0 μmole ADPG, 4 mg starch granules (20 μg protein), 20 μmoles KCl (when added), and the indicated divalent cation concentrations. Assays are described in the Materials and Methods.

Effects of Glutathione and p-Chloromercuribenzoate

The addition of sulfhydryl compounds fails to stimulate many of the particulate starch synthetase preparations (Literature Review). However, PCMB or iodoacetate greatly inhibit enzymatic activity (2,

27, 73), and the inhibition may be reversed by the addition of GSH. Murata, et al. (73) showed that ATP, ADP, and ADPG partially protected the enzyme from iodoacetate inhibition. To determine whether a possible interaction between potassium and sulfhydryl compounds occurs with the starch synthetase from sweet corn, experiments were conducted using various concentrations of KCl and GSH. GSH, at concentrations of 1.0 mM and 10.0 mM in the reaction mixture had no effect on enzyme activity in the presence or absence of potassium (Table VIII). The low activities observed in the absence of added potassium is attributed to potassium contamination by the GSH solution. In the presence of either 1.0 mM or 1.0 μM PCMB and 100 mM KCl, starch synthetase activity is inhibited 93% (Table IX). The addition of 10 mM GSH to the enzyme preparation prior to PCMB addition resulted in a marked decrease in the inhibition. The concentration of potassium in the reaction mixture appears to have little or no influence on PCMB inhibition.

Glycogen Synthetase Studies

Validity of Glycogen Synthetase Assays

The appropriate assay conditions for measuring the activity of glycogen synthetase purified from rat liver were determined by a series of experiments. A pH of 8.5 in 0.05 M tricine buffer was

Table VIII. The Effects of Glutathione on the Activity of Starch Synthetase from Sweet Corn in the Presence and Absence of KCl.

GSH Conc.	+KCl	-KCl
	mμmoles ADP/hour/μg protein	
1.0 mM	4.5	0.3
10.0 mM	4.4	0.6
0	4.5	0.0

Note: The reaction mixture contained the following components in a total volume of 0.2 ml: 10 μmoles tricine buffer, pH 8.0; 1.0 μmole ADPG; 4 mg starch granules (20 μg protein); 20 μmoles KCl (when added); and either 0.2 or 2.0 μmoles glutathione. Assay procedures are described in the Materials and Methods.

Table IX. The Effects of p-Chloromercuribenzoate and Glutathione on the Activity of Starch Synthetase in the Presence of High or Low Concentrations of KCl.

Additions	100 mM KCl	1.0 mM KCl
	mμmoles ADP/hour/μg protein	
None	4.2	2.3
PCMB (1.0 mM)	0.3	0.0
PCMB (1.0 μM)	0.3	0.1
PCMB (1.0 mM) + GSH (10 mM)	3.3	1.8

Note: The reaction mixture contained the following components in a total volume of 0.2 ml: 10 μmoles tricine buffer, pH 8.0; 1.0 μmole ADPG; 4 mg starch granules (20 μg protein); either 20 μmoles or 0.2 μmoles KCl; and the indicated concentrations of p-chloromercuribenzoate and glutathione. Assay mixtures were incubated 20 minutes prior to the initiation of the reaction by addition of substrate.

found to be optimum for enzyme activity (Figure 14). This value is similar to the optimum pH for glycine buffer reported by Leloir and Goldemberg (55). The liberation of UDP as a function of enzyme concentration is represented in Figure 15. The non-linearity of the curve can be attributed to the particulate nature of the enzyme (55). In all subsequent experiments, 0.02 ml of the purified enzyme were used which contained approximately 1.5 μg protein. Figure 16 represents the effects of increasing substrate concentration on the activity of rat liver glycogen synthetase. The enzyme is saturated at 7.5 mM UDPG and the K_m for substrate was calculated to be 2.4 mM (inset of Figure 16). This value is somewhat higher than that reported by Leloir and Goldemberg (55), but lies within the range of values reported for other enzyme preparations described in the Literature Review.

Effects of Univalent Cations

The effects of increasing concentrations of potassium or sodium on the activity of rat liver glycogen synthetase in two representative experiments are presented in Table X. No significant difference in activity could be detected at the different levels of potassium or sodium used in these experiments indicating that neither sodium nor potassium has a stimulatory or inhibitory effect on the activity of glycogen synthetase. In other investigations utilizing sheep liver

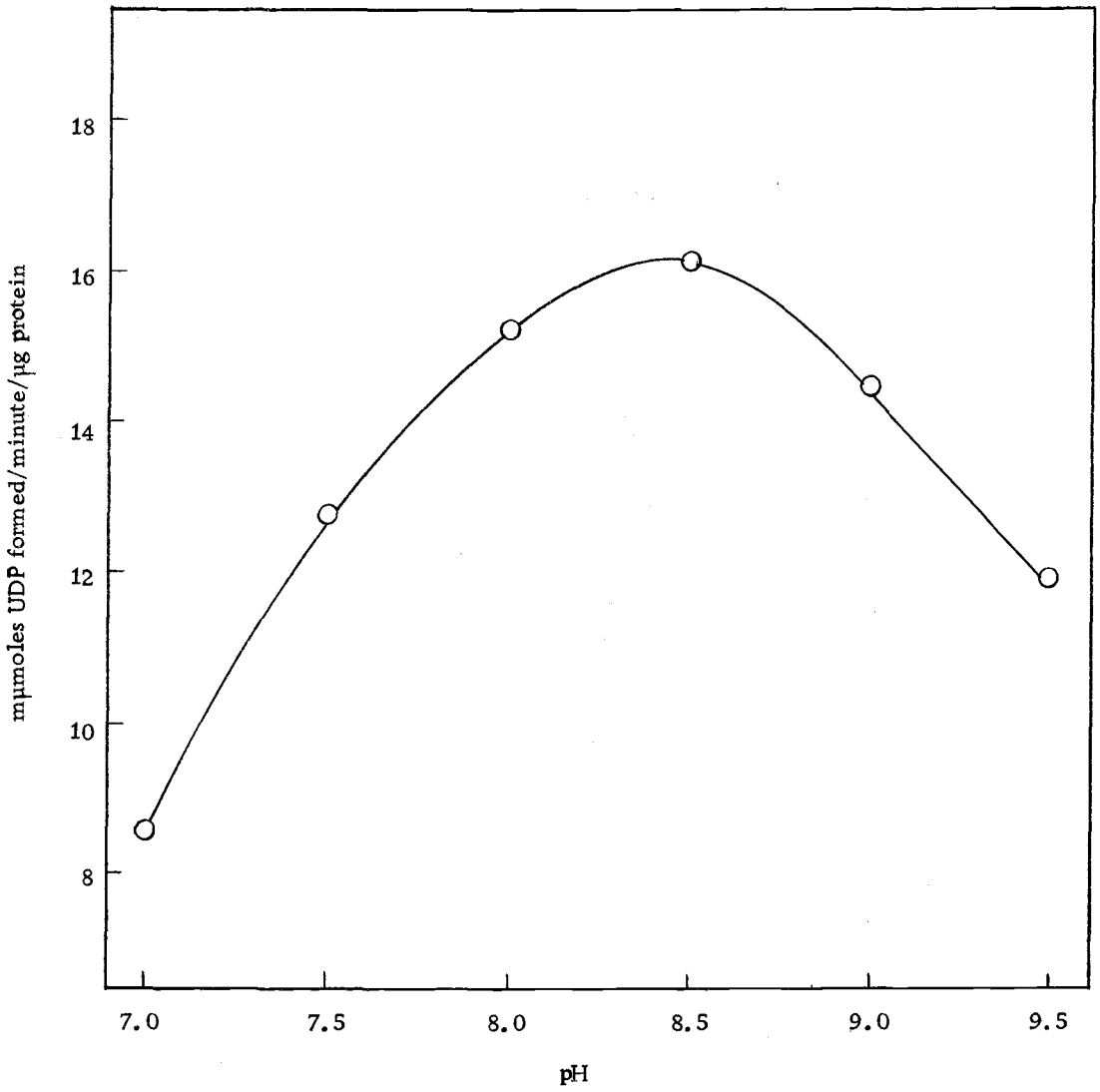

Figure 14. The Effects of pH on the Activity of Glycogen Synthetase from Rat Liver. The pH of 0.05 M tricine was adjusted to the appropriate value with tetramethylammonium hydroxide. Assay procedures are described in the Materials and Methods.

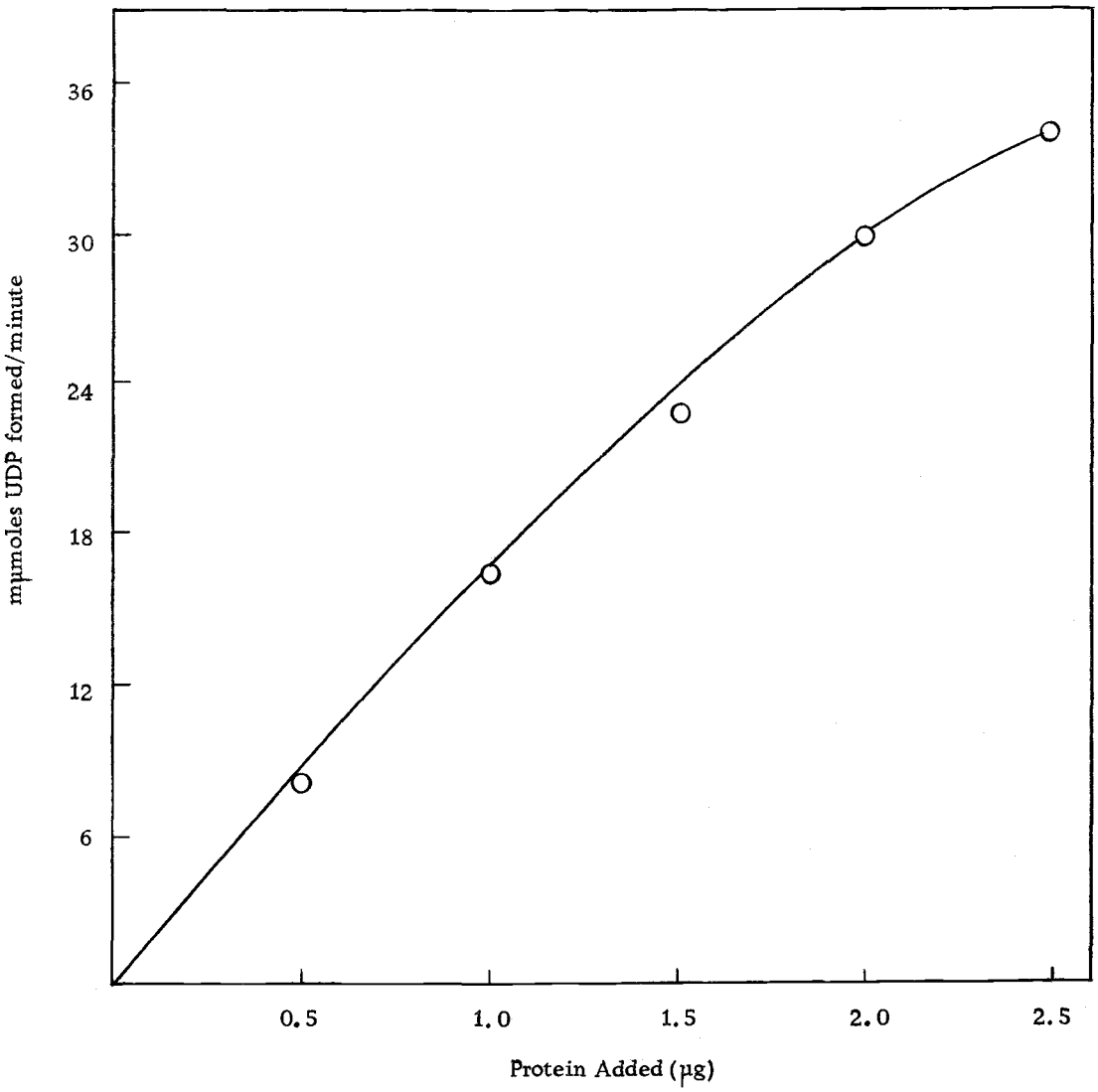

Figure 15. The Effects of Enzyme Concentration on the Activity of Glycogen Synthetase from Rat Liver. Assay procedures are described in the Materials and Methods. Enzyme concentration was varied as indicated.

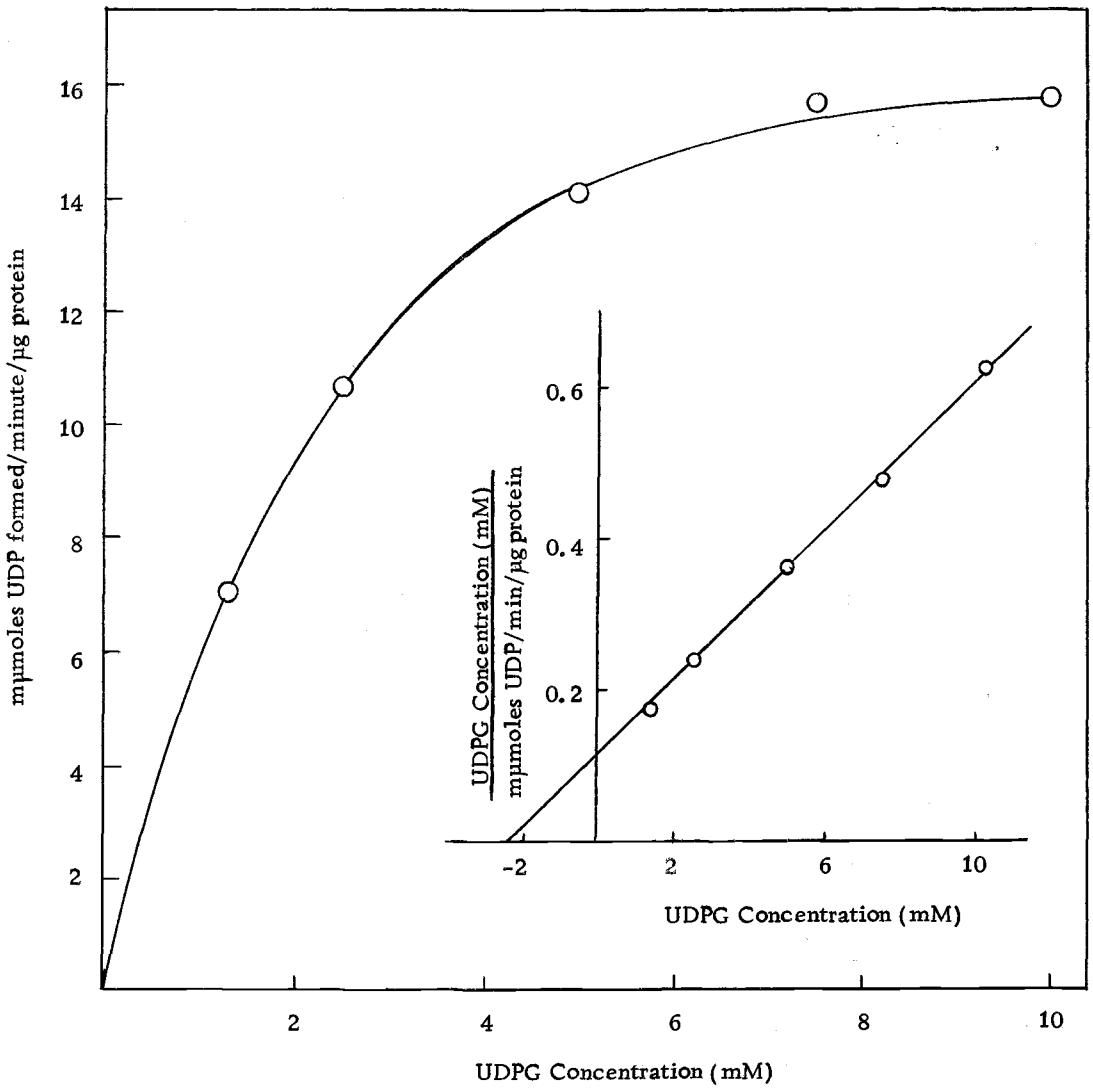

Figure 16. The Effects of UDPG on the Activity of Glycogen Synthetase from Rat Liver. The reaction conditions are those described in the Materials and Methods except that UDPG concentration was varied as indicated. The lines in the modified Lineweaver-Burk plot were located by the method of Least Squares.

glycogen synthetase, the enzyme activity in the presence or absence of potassium was measured and these results are compiled in Table XI. Specific activity was much lower than the rat liver enzyme, but these results also show that potassium fails to stimulate sheep liver glycogen synthetase.

Table X. The Effects of Potassium and Sodium on the Activity of Glycogen Synthetase from Rat Liver.

Salt Addition	Salt Concentration	Glycogen Synthetase Activity	
		Experiment I	Experiment II
	mM	mμmoles UDP/minute/μg protein	
KCl	0.0	8.9	8.0
KCl	12.5	10.0	8.4
KCl	25.0	8.9	8.4
KCl	37.5	9.7	8.2
KCl	50.0	9.7	8.2
NaCl	0.0	8.5	8.0
NaCl	12.5	9.2	8.2
NaCl	25.0	8.5	8.2
NaCl	37.5	8.3	8.8
NaCl	50.0	8.5	8.8

Note: The reaction mixture contained the following components in a total volume of 0.2 ml: 10 μmoles tricine buffer, pH 8.5; 2.0 μmoles glucose-6-phosphate; 1.0 μmole UDPG; 0.6 μmole cysteine; 1.6 mg glycogen; and the indicated univalent cation chloride concentration. Assay procedures are described in the Materials and Methods.

Table XI. The Effects of Potassium on the Activity of Glycogen Synthetase from Sheep Liver.

Experiment	+KCl	-KCl
	mμmoles UDP/minute/μg protein	
I	1.1	1.0
II	1.2	1.0
III	1.1	1.0
IV	1.2	1.2
V	1.1	1.1

Note: The reaction mixture contained the following components in a total volume of 0.2 ml: 10 μmoles tricine buffer, pH 8.5; 2.0 μmoles glucose-6-phosphate; 1.0 μmole UDPG; 0.6 μmole cysteine; 1.6 mg glycogen; 20 μmoles KCl; and 0.02 ml of enzyme preparation (1.5 μg protein). Assay procedures are described in the Materials and Methods.

DISCUSSION

Since potassium deficiency in plants is known to reduce starch accumulation (16, 17, 58, 81, 96), and since potassium markedly affects the activity of isolated particulate starch synthetase, a regulatory role of potassium in the synthesis of this storage carbohydrate is suggested. The results presented in this investigation establish an absolute requirement for potassium in the activity of particulate starch synthetase from sweet corn. This enzyme shows optimum activity in the presence of 0.05 M to 0.10 M KCl and a K_A of 6 mM. Rubidium, cesium or ammonium function approximately 80% as effective as potassium in the activation of starch synthetase while sodium or lithium activated only slightly or not at all. The normal concentrations of univalent cations other than potassium in most plant cells are considered insufficient to be of much importance in the activation of this enzyme. Potassium activation was primarily independent of the anion present although sulfate and iodide anions caused a maximum inhibition of 15% at concentrations of 0.10 M. These results are consistent with the univalent cation activation of many enzymes as summarized by Evans and Sorger (24).

In analyzing the effects of potassium on the activity of starch synthetase from peas, soybeans, field corn, wheat, bush beans and potatoes, a striking stimulatory effect of potassium also was

demonstrated. The enzymes from these sources appear to saturate in the range of 0.05 M to 0.10 M KCl, but their K_A values for potassium are slightly larger than that of the enzyme from sweet corn. Murata and Akazawa (70) have shown that potassium stimulates activity of the particulate starch synthetase from roots of sweet potato, taro, white potato as well as rice grains. Their report as well as the preliminary investigations of others (1, 34, 35, 70, 84) are consistent with the results obtained in this investigation showing the necessity of potassium in the biosynthesis of starch and suggesting a regulatory role of this cation in plants. The optimum concentrations for activation of the starch synthetases from various sources are equivalent to those concentrations of potassium normally found in plant cells (24).

Starch synthetase activity in the presence or absence of potassium was slightly stimulated by the divalent cations magnesium, manganese, calcium and cobalt at concentrations up to 0.02 M (Table VII). Murata and Akazawa (70) have reported that particulate starch synthetase from sweet potato roots showed a maximum stimulation of 50% by calcium and magnesium at concentrations of 5 mM but was inhibited at higher concentrations. In their study, UDPG was utilized as the substrate. It is known that this substrate functions less efficiently than ADPG in the synthetase reactions (72, 75, 86). In reports on the particulate enzymes from potatoes and waxy maize (27) and from beans (57), neither 0.01 M EDTA nor 0.01 M $MgCl_2$

had a stimulatory or inhibitory effect on the incorporation of ^{14}C-glucose from ADPG into starch. Akatsuka and Nelson (1) found that 2 mM EDTA stimulated both the endosperm and embryo starch synthetases from waxy maize in the presence of potassium, but was slightly inhibitory for the embryo synthetase preparation in the absence of potassium. The essentiality of divalent cations for starch synthetase, then, appears to be dependent upon the source of the enzyme and the substrate utilized. The involvement of divalent cations is not absolute, however, and seems only secondary in comparison to the magnitude of stimulation by the univalent cations.

Enzyme activation by univalent cations can occur through a variety of mechanisms, some of which are not well differentiated and often difficult to determine experimentally. While the necessity of potassium in the activity of starch synthetase has been demonstrated in this investigation, the actual mechanism of its activation has yet to be established. Evans and Sorger (24) proposed that univalent cations function by maintaining an active configuration of enzymes and in their absence, an inactive configuration was assumed. Potassium, rubidium and ammonium, having atomic radii of 5.32 A, 5.09 A, and 5.37 A respectively (47) all function as activators whereas sodium and lithium, with atomic radii of 7.9 A and 10.0 A (47) fail to activate. Evans and Sorger (24) indicated that the size of the activating ion played a critical role in the maintainance of the appropriate enzyme

configuration.

In the case of particulate starch synthetase, this theory may also apply. Several other factors, however, must be considered in visualizing this type of control. In addition to enzyme, substrate and univalent cation interactions, starch synthetase is associated with the granules themselves in a manner that has not been defined at present. Also involved in its activity are primer carbohydrates (oligo- and polysaccharides) which serve as glucose acceptors, often more efficiently than the starch granules. Thus, this enzyme system is complex and can involve up to five different components. Several interactions of these components may be involved in the univalent cation activation of the starch synthetase system.

In the analysis of the kinetic studies on the effects of univalent cations on the affinity of the particulate starch synthetase for ADPG (Figure 10), no change in the K_m could be shown for potassium, rubidium, cesium or sodium. At two different concentrations of potassium (1 mM and 100 mM), the K_m for substrate remained constant. Univalent cations appear to be unrelated to the binding of the substrate to the enzyme at the active center. In studying the effects of ADPG on the K_A for potassium (Figure 13), no alteration of the cation affinity was observed at increasing concentrations of substrate. These observations indicate that substrate binding and potassium binding are independent phenomena and no indication of the site or sites of activation is implied.

In considering the role of potassium in the involvement of the proper enzyme configuration, no effect of potassium on the inactivation of the enzyme by PCMB or reactivation by GSH could be demonstrated. Potassium, however, has been shown to protect against heat inactivation of the maize enzymes (1) as well as the particulate enzyme from sweet potatoes (70). Since several authors (94, 97, 100, 108) have implied that univalent cations are involved in stabilizing certain enzymes, the involvement of potassium in protecting particulate starch synthetase against heat inactivation may be interpreted as indicating a possible role of this cation in maintaining a more stable enzyme configuration.

In studying the effect of potassium on the velocity of pyruvic kinase, Melchoir (64) proposed that potassium interacted with ADP and magnesium, and thus influenced the binding of this complex to the enzyme. In the non-enzymatic conversion of ATP and inorganic phosphate to ADP and pyrophosphate, Lowenstein (59) indicated that potassium, in combination with divalent cations, affected the charge-ionic radius of the complex which then altered its activity. Lowenstein (59) felt that this difference in the shape of the potassium-ATP-divalent cation complex influenced the rate of the non-enzymatic reaction. In the case of starch synthetase, the univalent cations failed to alter the affinity of the enzyme for the substrate and the possibility of their interaction with the free substrate seems remote. However, this

does not exclude a possible interaction after the substrate is bound to the enzyme. Potassium could easily alter the position of the bound ADPG at the active site and such a change possibly could bring about an active enzyme conformation. Since UDPG does not function as well as ADPG nor is it stimulated to the extent that ADPG is stimulated, the interaction of potassium with the bound substrate could be ascribed to the purine portion of the ADPG molecule.

Another possible site of involvement of potassium in this enzyme system is the binding of the enzyme to the starch granules. Potassium could function as a weak ionic component necessary for the proper association of the enzyme with the polysaccharides. The binding of iodine (92) and some aliphatic alcohols (95) by amylose is attributed to a helical form of this polysaccharide. Akazawa and Murata (3) have shown that the soluble starch synthetase from rice can be precipitated from solution by the addition of amylose. In this form, the enzyme was stable to acetone treatment and dry heating at 100° for ten minutes. The soluble enzyme UDPG-sucrose transglucosylase was not affected by the addition of amylose indicating that the interaction is specific for starch synthetase. In an attempt to solubilize the particulate enzyme from potatoes, Frydman and Cardini (32) showed that mechanical disruption or treatment with 8 M urea of α-amylase altered the substrate specificity of the enzyme and increased enzyme activity when ADPG was the substrate. These reports lend support to

existence of a polysaccharide-enzyme association and from this, a possible involvement of potassium in these interactions could be suggested.

Further support for the involvement of univalent cations in the association of the enzyme with the polysaccharides is the work of Murata and Akazawa (69) who found that oligosaccharides of the maltose series stimulated sweet potato starch synthetase activity in a manner similar to that observed for potassium. They also showed that this stimulation was distinguishable from the role of oligosaccharides as glucose acceptors. They concluded that the stimulation of potassium alone and that due to the maltooligosaccharides were at least additive, and possibly synergestic. In preliminary experiments by this investigator, studies on the relation of potassium to the particulate nature of the enzyme yielded inconclusive results, and further studies are therefore deemed necessary.

Since the structure and formation of starch and glycogen are similar, experiments were conducted to determine if a potassium requirement existed for the glycogen synthetase system. Previous reports (39, 50, 80, 88, 104) have indicated that potassium affects the incorporation of glucose or lactate into glycogen by crude extracts of pigeon liver and breast muscle. The results obtained in this study of particulate glycogen synthetases from rat or sheep livers showed that neither potassium nor sodium had an effect on the rate of UDP

liberation from UDPG. The reports of Nigam and Fridland (80) and others (39, 50, 88, 104), in which crude homogenates were utilized, cannot be interpreted, therefore, on the basis of a direct univalent cation requirement for glycogen synthetase. The stimulatory effect of potassium on this system as reported may be involved in the conversion of the I form of the enzyme to the D form. This reaction is reported to be catalyzed by a synthetase-I-kinase which requires ATP and magnesium for activity (25, 26, 36). Several kinase reactions with univalent cation requirements already are known (24).

The present experimental results as well as those of other investigators provide no direct answer to the molecular role of potassium in particulate starch synthetase. The fact that it is indispensible for activity for this enzyme and its requirement is comparable to concentrations of potassium present in normal plant tissues are interpreted as indicating a regulatory role in this process. The mechanism of action of potassium, however, appears to be different from that of some other univalent cation-requiring enzymes. It is interesting to note that univalent cations rather than organic molecules serve as activators for starch synthesis whereas in glycogen synthesis, glucose-6-phosphate is an essential cofactor and no cation requirement can be demonstrated.

SUMMARY

An investigation was conducted to determine the univalent cation requirements of particulate starch synthetase from a variety of plant species. The possible mechanism of potassium activation was examined, and the results of these experiments are summarized as follows:

1. The particulate starch synthetase from sweet corn has an absolute requirement for potassium. Optimum activation occurred at 0.05 M KCl and the K_A for potassium was 6 mM.
2. Rubidium, cesium and ammonium functioned approximately 80% as effectively as potassium in the activation of this enzyme while sodium and lithium were respectively 21% and 8% as effective as potassium.
3. The potassium activation of starch synthetase was independent of the anion present although sulfate and iodide caused a maximum inhibition of 15% at concentrations of 0.10 M.
4. The particulate starch synthetase enzymes from peas, soybeans, field corn, wheat, bush beans and potatoes all showed marked stimulation from the addition of potassium. Low enzyme activity was observed for enzymes from these sources in the absence of added potassium, but the endogenous potassium content of the starch granules was

sufficient to account for the activities observed.

5. Potassium, rubidium, cesium or sodium had no effect on the K_m for ADPG and are thus unrelated to the affinity of the enzyme for the substrate.

6. The concentration of ADPG failed to alter the K_A for potassium indicating that substrate and potassium binding are independent phenomena.

7. Enzyme activity was greatly stimulated by potassium when ADPG was the substrate, but only slight activation was observed when UDPG was the substrate.

8. The divalent cations magnesium, manganese, calcium and cobalt produced slight activation of the sweet corn starch synthetase in the presence or absence of potassium, but this stimulation was far less than that observed with potassium.

9. All efforts to show a potassium requirement for particulate glycogen synthetase from rat or sheep liver were negative.

10. The mechanism of potassium activation of starch synthetase appears to be different from that of other univalent cation requiring enzymes.

BIBLIOGRAPHY

1. Akatsuka, Tadami and Oliver E. Nelson. Starch granule-bound adenosine diphosphate glucose-starch glucosyltransferases of maize seeds. Journal of Biological Chemistry 241:2280-2286. 1966.

2. Akazawa, T., T. Minamikawa and T. Murata. Enzymic mechanism of starch synthesis in ripening rice grains. Plant Physiology 39:371-378. 1964.

3. Akazawa, T. and T. Murata. Adsorption of ADPG-starch transglucosylase by amylose. Biochemical and Biophysical Research Communications 19:21-26. 1965.

4. Algranati, Israel E. and Enrico Cabib. The synthesis of glycogen in yeast. Biochimica et Biophysica Acta 43:141-142. 1960.

5. _____ Uridine diphosphate D-glucose-glycogen glucosyltransferase from yeast. Journal of Biological Chemistry 237:1007-1013. 1962.

6. Allen, M. B. and Daniel I. Arnon. Studies on nitrogen-fixing blue-green algae. II. The sodium requirement of Anabaena cylindrica. Physiologia Plantarum 8:653-659. 1955.

7. Basu, D. K. and B. K. Bachhawat. Purification of uridine diphosphoglucose-glycogen transglucosylase from sheep brain. Biochimica et Biophysica Acta 50:123-128. 1961.

8. Bear, Firman E. Sodium symposium. Soil Science 76:1-96. 1953.

9. Beeson, Kenneth C. The mineral composition of crops with particular reference to the soils in which they were grown. Washington, 1941. 164 p. (U. S. Dept. of Agriculture. Miscellaneous Publication 369)

10. Betts, Graham F. and Harold J. Evans. The effect of cations on the electrophoretic mobility and substrate binding properties of pyruvate kinase. Biochimica et Biophysica Acta 167:190-193. 1968.

11. Betts, Graham F. and Harold J. Evans. The inhibition of univalent cation activated enzymes by tris(hydroxymethyl) aminomethane. Biochimica et Biophysica Acta 167:193-196. 1968.

12. Bray, G. A. A simple efficient liquid scintaillator for counting aqueous solutions in a liquid scintillation counter. Analytical Biochemistry 1:279-285. 1960.

13. Breckenridge, Bruce M. and Elizabeth J. Crawford. Glycogen synthesis from uridine diphosphate glucose in brain. Journal of Biological Chemistry 235:3054-3057. 1960.

14. _____ Glycogen synthesis from uridine diphosphoglucose in brain. Federation Proceedings 18:197. 1959.

15. Brownell, P. F. Sodium as an essential micronutrient element for higher plant (Atriplex vesicaria). Plant Physiology 40:460-468. 1965.

16. Buchanan, John M., A. Baird Hastings and Frances B. Nesbett. The effect of the ionic environment on the synthesis of glycogen and total carbohydrate from pyruvate in liver slices. Journal of Biological Chemistry 180:447-455. 1949.

17. _____ The effect of the ionic environment on the synthesis of glycogen from glucose in rat liver slices. Journal of Biological Chemistry 180:435-447. 1949.

18. Cooil, Bruce J. and M. C. Slattery. Effects of potassium deficiency and excess upon certain carbohydrate and nitrogenous constituents in guayule. Plant Physiology 23:425-442. 1948.

19. De Fekete, Maria A. Rongine, Luis F. Leloir and Carlos E. Cardini. Mechanism of starch biosynthesis. Nature 187:918-919. 1960.

20. Doi, Akemi, Kenji Doi and Siro Nikuni. ADP-D-glucose-α-1, 4-glucan α-4-glucosyltransferase in spinach chloroplasts. Partial purification and some properties of the enzyme. Biochimica et Biophysica Acta 113:312-320. 1966.

21. Eaton, S. V. Effects of potassium deficiency on growth and metabolism of sunflower plants. Botanical Gazette 114:165-180. 1952.

22. Ennis, Herbert L. and Martin Lubin. Dissociation of ribonucleic acid and protein synthesis in bacteria deprived of potassium. Biochimica et Biophysica Acta 50:399-402. 1961.

23. Evans, Harold J., R. B. Clark and S. A. Russell. Cation requirements for the acetic thiokinase from yeast. Biochimica et Biophysica Acta 92:582-594. 1964.

24. Evans, Harold J. and George J. Sorger. Role of mineral elements with emphasis on the univalent cations. Annual Review of Plant Physiology 17:47-76. 1966.

25. Friedman, Daniel L. and Joseph Larner. Studies on UDPG-α-glucan transglucosylase. III. Interconversion of two forms of muscle UDPG-α-glucan transglucosylase by a phosphorylation-dephosphorylation reaction sequence. Biochemistry 2:669-675. 1963.

26. _____ Studies on uridine diphosphate glucose: α-1, 4-glucan α-4-glucosyltransferase. VIII. Catalysis of the phosphorylation of muscle phorylase and transferase by separate enzymes. Biochemistry 4:2261-2264. 1965.

27. Frydman, Rosalia B. Starch synthetase of potatoes and waxy maize. Archives of Biochemistry and Biophysics 102:242-248. 1963.

28. Frydman, Rosalia B. and Carlos E. Cardini. Biosynthesis of phytoglycogen from adenosine diphosphate D-glucose in sweet corn. Biochemical and Biophysical Research Communications 14:353-357. 1964.

29. _____ Soluble enzymes related to starch synthesis. Biochemical and Biophysical Research Communications 17:407-411. 1964.

30. _____ Studies on adenosine diphosphate D-glucose: α-1, 4-glucan α-4 glucosyltransferase of sweet-corn endosperm. Biochimica et Biophysica Acta 96:294-303. 1965.

31. _____ Studies on the biosynthesis of starch. I. Isolation and properties of the soluble adenosine diphosphate glucose:starch glucosyltransferase of Solanum tuberosum. Archives of Biochemistry and Biophysics 116:9-18. 1966.

32. Frydman, Rosalia B. and Carlos E. Cardini. Studies on the biosynthesis of starch. II. Some properties of the adenosine diphosphate glucose-starch glucosyltransferase bound to the starch granules. Journal of Biological Chemistry 242:312-317. 1967.

33. Frydman, Rosalia B., Bazilicia C. DeSouza and Carlos E. Cardini. Distribution of adenosine diphosphate D-glucose: α-1, 4-glucan α-4-glucosyltransferase in higher plants. Biochimica et Biophysica Acta 113:620-623. 1966.

34. Ghosh, Hara Prasad and Jack Preiss. The biosynthesis of starch in spinach chloroplasts. Journal of Biological Chemistry 240: 960-962. 1965.

35. _____ Biosynthesis of starch in spinach chloroplasts. Biochemistry 4:1354-1361. 1965.

36. Goldberg, Nelson D. and Ann G. O'Toole. The properties of glycogen synthetase and regulation of glycogen biosynthesis in rat brain. Journal of Biological Chemistry 244:3053-3061. 1969.

37. Greenberg, Elaine and Jack Preiss. Biosynthesis of bacterial glycogen. II. Purification and properties of the adenosine diphosphoglucose:glycogen transglucosylase of Arthrobacter species NRRL B1973. Journal of Biological Chemistry 240: 2341-2348. 1965.

38. Happold, F. C. and R. B. Beechey. Univalent metals and other loose or non-specific activators. In: Metals and enzyme activity, ed. by E. M. Crook. London, Cambridge University, 1958. p. 52-63. (Biochemical Society. Symposium no. 15)

39. Hastings, A. B., J. Ashmore and G. F. Cahill, Jr. Intracellular ionic environment and enzyme activities: carbohydrate metabolism in liver. Archives of Biochemistry and Biophysics 65:78-85. 1956.

40. Hauk, Rosalind and David H. Brown. Preparation and properties of uridinediphosphoglucose-glycogen transferase from rabbit muscle. Biochimica et Biophysica Acta 33:556-559. 1959.

41. Hewitt, E. J. The essential nutrient elements: Requirements and interactions in plants. In: Plant physiology, ed. by F. C. Steward. Vol. 3. New York, Academic, 1963. p. 137-160.

42. Hewitt, E. J. Mineral nutrition of plants in culture media. In: Plant physiology, ed. by F. C. Steward. Vol. 3. New York, Academic, 1963. p. 97-133.

43. Hiatt, A. J. Further studies on the activation of acetic thiokinase by magnesium and univalent cations. Plant Physiology 39:475-479. 1964.

44. Hizukure, Susumn and Joseph Larner. Studies on UDPG: α-1, 4-glucan α-4-glucosyltransferase. VII. Conversion of the enzyme from glucose-6-phosphate-dependent to independent form in liver. Biochemistry 3:1783-1788. 1964.

45. Jackson, William A. and Richard J. Volk. Role of potassium in photosynthesis and respiration. In: The role of potassium in agriculture, ed. by V. J. Kilmer, S. E. Younts and N. C. Brady. Madison, American Society of Agronomy, 1968. p. 109-145.

46. Johnson, Clarence M. and Albert Ulrich. Analytical methods for use in plant analysis. Sacramento, 1959. p. 25-78. (California. Agriculture Experiment Station. Bulletin 766)

47. Kachmar, J. F. and P. D. Boyer. Kinetic analysis of enzyme reactions. II. The potassium activation and calcium inhibition of pyruvic phosphoferase. Journal of Biological Chemistry 200: 669-682. 1953.

48. Kayne, F. J. and C. H. Suelter. Effects of temperature, substrate and activating cations on the conformation of pyruvic kinase in aqueous solution. Journal of the American Chemical Society 87:897-910. 1965.

49. Kornfeld, Rosalind and David H. Brown. Preparation and properties of uridine diphosphate glucose-glycogen transglucosylase. Journal of Biological Chemistry 237:1772-1777. 1962.

50. Krebs, H. A., Christa Dierks and T. Gascoyne. Carbohydrate synthesis from lactate in pigeon-liver homogenate. Biochemical Journal 93:112-121. 1964.

51. Larner, Joseph and Carlos Villar-Palasi. Enzymes in a glycogen storage myopathy. Proceedings of the National Academy of Sciences 45:1234-1235. 1959.

52. Latzko, E. Zur Rolle des K^+ bei der Oxydativen Phosphorylierung. Biochimica et Biophysica Acta 47:350-357. 1961.

53. Latzko, E. and K. L. Mechsner. Bedeutung der Alkaliionen für die Intensität der Lichtphosphorylierung bei Chlorella vulgaris. Naturwissenschaften 45:247-248. 1958.

54. Leloir, L. F. and C. E. Cardini. Biosynthesis of glycogen from uridine diphosphate glucose. Journal of the American Chemical Society 79:6340-6341. 1957.

55. Leloir, L. F. and Sara H. Goldemberg. Synthesis of glycogen from uridine diphosphate glucose in liver. Journal of Biological Chemistry 235:919-923. 1960.

56. Leloir, L. F., J. M. Olavarría, Sara H. Goldemberg and H. Carminatti. Biosynthesis of glycogen from uridine diphosphate glucose. Archives of Biochemistry and Biophysics 81:508-520. 1959.

57. Leloir, L. F., Maria A. Rongine de Fekete and Carlos E. Cardini. Starch and oligosaccharide synthesis from uridine diphosphate glucose. Journal of Biological Chemistry 236:636-641. 1961.

58. Liebhardt, W. C. Effects of potassium on carbohydrate metabolism and translocation. In: The role of potassium in agriculture, ed. by V. J. Kilmer, S. E. Younts and N. C. Brady. Madison, American Society of Agronomy, 1968. p. 147-164.

59. Lowenstein, J. M. The stimulation of transphosphorylation by alkali-metal ions. The Biochemical Journal 75:269-274. 1960.

60. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. Protein measurement with the folin phenol reagent. Journal of Biological Chemistry 193:265-275. 1951.

61. Lubin, Martin. A priming reaction in protein synthesis. Biochimica et Biophysica Acta 72:345-348. 1963.

62. Lubin, Martin and Herbert L. Ennis. On the role of intracellular potassium in protein synthesis. Biochimica et Biophysica Acta 80:614-663. 1964.

63. Luck, David J. L. Glycogen synthesis from uridine diphosphate glucose. The distribution of the enzyme in liver cell fractions. Journal of Biophysical and Biochemical Cytology 10:195-209. 1961.

64. Melchior, J. B. The role of metal ions in the pyruvic kinase reaction. Biochemistry 4:1518-1525. 1965.

65. Mendenhall, William. Introduction to statistics. Belmout, Wadsworth, 1965. 305 p.

66. Miller, Gene and Harold J. Evans. The influence of salts on pyruvic kinase from tissues of higher plants. Plant Physiology 32:346-354. 1957.

67. Mommaerts, W. F. H. M., Barbara Illingworth, Carl M. Pearson, R. J. Guillory and K. Seraydarian. A functional disorder of muscle associated with the absence of phosphorylase. Proceedings of the National Academy of Science 45:791-797. 1959.

68. Murata, Takao and T. Akazawa. Enzymic mechanism of starch synthesis in ripening rice grains. IV. Starch synthesis in glutinous rice grains. Archives of Biochemistry and Biophysics 114:76-87. 1966.

69. _____ Enzymic mechanism of starch synthesis in sweet potato roots. Archives of Biochemistry and Biophysics 130:604-609. 1969.

70. _____ Enzymic mechanism of starch synthesis in sweet potato roots. I. Requirement of potassium ions for starch synthesis. Archives of Biochemistry and Biophysics 126:873-879. 1968.

71. _____ The role of adenosine diphosphate glucose in leaf starch formation. Biochemical and Biophysical Research Communications 16:6-11. 1964.

72. Murata, T., T. Minamikawa and T. Akazawa. Adenosine diphosphate glucose in rice and its role in starch synthesis. Biochemical and Biophysical Research Communications 13:439-443. 1963.

73. Murata, T., N. Nakayama, Y. Tanaka and T. Akazawa. Enzymic mechanism of starch synthesis in ripening rice grains. V. Iodoacetate inhibition of granular starch synthetase activity. Archives of Biochemistry and Biophysics 123:97-103. 1968.

74. Murata, T., T. Sugiyama and T. Akazawa. Enzymic mechanism of starch synthesis in glutinous rice grains. Biochemical and Biophysical Research Communications 18:371-376. 1965.

75. _____ Enzymic mechanism of starch synthesis in ripening rice grains. II. Adenosine diphosphate glucose pathway. Archives of Biochemistry and Biophysics 107:92-101. 1964.

76. Nason, A. and W. D. McElroy. Modes of action of essential mineral elements. In: Plant physiology, ed. by F. C. Steward. Vol. 3. New York, Academic, 1963. p. 449-536.

77. Nelson, Oliver E. and Howard W. Rines. The enzymatic deficiency in the waxy mutant of maize. Biochemical and Biophysical Research Communications 9:297-300. 1962.

78. Nelson, Oliver E. and Chia Yin Tsai. Glucose transfer from adenosone diphosphate-glucose to starch in preparations of waxy seeds. Science 145:1194-1195. 1964.

79. Nicholas, D. J. D. Inorganic nutrient nutrition of microorganisms. In: Plant physiology, ed. by F. C. Steward. Vol. 3. New York, Academic, 1963. p. 363-447.

80. Nigam, V. N. and A. Fridland. Studies on glycogen synthesis in pigeon liver homogenates. Biochemical Journal 105:505-513. 1967.

81. Nightingale, G. T., L. G. Schermerhorn and W. R. Robbins. Some effects of potassium deficiency on histological structure and nitrogenous and carbohydrate constituents of plants. New Brunswick. 1930. 36 p. (New Jersey. Agriculture Experiment Station. Bulletin 499)

82. Nomura, T., N. Nakayama, T. Murata and T. Akazawa. Biosynthesis of starch in chloroplasts. Plant Physiology 42:327-332. 1967.

83. Preiss, Jack and Elaine Greenberg. Biosynthesis of bacterial glycogen. III. The adenosine diphosphate-glucose: α-4 glucosyltransferase of Escherichia coli B. Biochemistry 4:2328-2334. 1965.

84. _____ Biosynthesis of starch in Chlorella Pyrenoidosa. I. Purification and properties of the adenosine diphosphoglucose α-1, 4-glucan, α-4 glucosyl transferase from Chlorella. Archives of Biochemistry and Biophysics 118:702-708. 1967.

85. Pulver, R. and F. Verzár. Connexion between carbohydrate and potassium metabolism in the yeast cell. Nature 145:823-824. 1940.

86. Recondo, Eduardo and Luis F. Leloir. Adenosine diphosphate glucose and starch synthesis. Biochemical and Biophysical Research Communications 6:85-88. 1961.

87. Richards, F. J. Physiological studies in plant nutrition. XI. The effect on growth of rubidium with low potassium supply, and modification of this effect by other nutrients. Annals of Botany, new ser., 8:323-356. 1944.

88. Robbins, Phillips W., Robert R. Traut and Fritz Lipmann. Glycogen synthesis from glucose, glucose-6-phosphate, and uridine diphosphate glucose in muscle preparations. Proceedings of the National Academy of Sciences 45:6-12. 1959.

89. Rosell-Perez, M. and J. Larner. Studies on UDPG-α-glucan transglucosylase. IV. Purification and characterization of two forms from rabbit skeletal muscle. Biochemistry 3:75-81. 1964.

90. Rosell-Perez, M., C. Villar-Palasi and Joseph Larner. Studies on UDPG-glycogen transglucosylase. I. Preparation and differentiation of two activities of UDPG-glycogen transglucosylase from rat skeletal muscle. Biochemistry 1:763-768. 1962.

91. Rowan, K. S. and Donella H. Turner. Physiology of pea fruits. V. Phosphate compounds in the developing seeds. Australian Journal of Biological Sciences 10:414-425. 1957.

92. Rundle, R. E., Joseph F. Foster and R. R. Baldwin. On the nature of the starch-iodine complex. Journal of the American Chemical Society 66:2116-2120. 1944.

93. Schmid, Rudi, Phillips W. Robbins and Robert R. Traut. Glycogen synthesis in muscle lacking phosphorylase. Proceedings of the National Academy of Sciences 45:1236-1240. 1959.

94. Schneider, Z., K. Pech and J. Pawelkiewicz. Enzymic transformation of glycerol to β-hydroxypropionic aldehyde. II. Dissociation of the enzyme into two protein fragments. Bulletin de L'Academie Polonaise des Sciences 14:7-12. 1966.

95. Schoch, Thomas John. The fractionation of starch. Advances in carbohydrate chemistry 1:247-278. 1945.

96. Sideris, C. P. and H. Y. Young. Effects of potassium on chlorophyll, acidity, ascorbic acid, and carbohydrates of Ananas Cosmosus (L.) Merr. Plant Physiology 20:649-670. 1945.

97. Sorger, G. J. and Harold J. Evans. Effect of univalent cations on the properties of yeast NAD^+ acetaldehyde dehydrogenase. Biochimica et Biophysica Acta 118:1-8. 1966.

98. Sorger, G. J., R. E. Ford and Harold J. Evans. Effect of univalent cations on the immunoelectrophoretic behavior of pyruvate kinase. Proceedings of the National Academy of Sciences 54: 1614-1621. 1965.

99. Sprague, G. F., B. Brimhall and R. M. Hixon. Some effects of the waxy gene in corn on the properties of the endosperm starch. Journal of the American Society of Agronomy 35:817-822. 1943.

100. Stopponi, A. O. M. and C. Milstein. Essential role of thiol groups in aldehyde dehydrogenase. Biochemical Journal 67:406-416. 1957.

101. Suelter, C. H. and W. Melander. Use of protein difference spectrophotometry to determine enzyme-cofactor dissociation constants. Journal of Biological Chemistry 238:4108-4109. 1963.

102. Suelter, C. H., Rivers Singleton, Jr., F. J. Kayne, Sandra Arrington, J. Glass and A. S. Mildvan. Studies on the interaction of substrate and monovalent and divalent cations with pyruvate kinase. Biochemistry 5:131-139. 1966.

103. Sutcliffe, J. F. Mineral salts absorption in plants. New York, Pergamon. 1962. 194 p. (International Series of Monographs on Pure and Applied Biology. Division: Plant Physiology. Vol. 1)

104. Torres, Hector N., Lutz Birnbaumer, Maria del Carmen Garcia Fernandez, Elena Bernard and Enrique Belocopitow. Glycogen metabolism in muscle homogenates. I. The effect of potassium ions on glycogen synthesis. Archives of Biochemistry and Biophysics 116:59-68. 1966.

105. Traut, Robert R. and Fritz Lipmann. Activation of glycogen synthetase by glucose 6-phosphate. Journal of Biological Chemistry 238:1213-1221. 1963.

106. Webster, George C. Effects of monovalent cations on the incorporation of amino acids into protein. Biochimica et Biophysica Acta 20:565-566. 1956.

107. Williams, M. Coburn. Effect of sodium and potassium salts on growth and oxalate content of Halogeton. Plant Physiology 35: 500-504. 1960.

108. Wilson, Richard H., Harold J. Evans and R. R. Becker. The effect of univalent cation salts on the stability and on certain physical properties of pyruvic kinase. Journal of Biological Chemistry 242:3825-3832. 1967.

109. Woolley, Joseph T. Sodium and silicon as nutrients for the tomato plant. Plant Physiology 32:317-320. 1957.

110. Wyatt, H. V. Cations, enzymes and control of cell metabolism. Journal of Theoretical Biology 6:441-470. 1964.