HEXOKINASE AND SUCROSE SYNTHASE ACTIVITY DURING KERNEL DEVELOPMENT IN TRITICALE, WHEAT AND RYE

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TABLE OF CONTENTS

	PAGE
LIST OF TABLES	v
LIST OF FIGURES	vi
ABBREVIATIONS	ix
ABSTRACT	xi
INTRODUCTION	1
LITERATURE REVIEW	2
Starch Formation in Cereals	2
Maize	3
Wheat	9
Other cereals	14
Hexokinase	16
Sucrose synthase	18
MATERIALS AND METHODS	20
Chemicals	20
Growth and Collection of Plant Materials	20
Preparation of Crude Extract	21
Hexokinase Assay	22
Sucrose Synthase Assay	23
Alkaline Phosphatase Assay	24
Inorganic Phosphate Assay	24
Protein Determination	25
Glucose and Sucrose Determination	26
RESULTS AND DISCUSSION	27
Optimization of Procedures	27
The hexokinase assay	27
Distribution and stability of enzyme activity	50
Hexokinase	50
Sucrose synthase	56

TABLE OF CONTENTS - Continued

	PAGE
Dry Weight and Fresh Weight	59
Soluble Protein	72
Sucrose Synthase and Sucrose	79
Hexokinase and Glucose	90
Substrate Specificity of Crude Extract Hexokinase	92
GENERAL DISCUSSION	95
LIST OF REFERENCES	98

LIST OF TABLES

TABLE		PAGE
1	Effect of washing ^{14}C-glucose with 0.17M NH_4OH	34
2	Effect of various chemicals on alkaline phosphatase activity from crude extract	43
3	Inhibition of phosphatase from undialyzed crude extract by inorganic phosphate using o-carboxyphenyl phosphate as substrate	44
4	Effect of inorganic phosphate on hexokinase activity from undialyzed crude extract	45
5	Hexokinase activity of crude extract after various treatments	49
6	Distribution and stability of hexokinase activity in various crude extract fractions	55
7	Rate of ^{14}C-glucose and ^{14}C-fructose utilization by 30 day post-anthesis crude extract hexokinase	93

LIST OF FIGURES

FIGURE		PAGE
1	Hypothetical scheme of sucrose-starch conversion during maize endosperm development	7
2	Hypothetical scheme of starch synthesis in wheat	11
3	Formation of glucose-6-phosphate with time catalyzed by yeast hexokinase	29
4	Formation of glucose-6-phosphate with time catalyzed by hexokinase from undialyzed crude extract	31
5	Reaction velocity as a function of the volume of untreated crude extract hexokinase	33
6	Relationship between rate of o-carboxyphenyl phosphate hydrolysis and volume of undialyzed crude extract from kernels of 18 day post-anthesis Manitou wheat	37
7	Time course of the production of inorganic phosphate from glucose-6-P in the presence of crude extract from 18 day Manitou wheat kernels	39
8	Rate of P_i production from glucose-6-P in the presence of varying quantities of crude extract from 18 day post-anthesis Manitou wheat kernels	41
9	Effect of dialysis on the rate of glucose-6-P formation as a function of crude extract volume	48
10	Rate of glucose-6-P and fructose-6-P formation with crude extract volume using the most active dialyzed crude extract	52
11	Effect of varying substrate concentration on rate of product formation catalyzed by crude extract hexokinase	54

LIST OF FIGURES - Continued

FIGURE		PAGE
12	Rate of sucrose conversion to UDP glucose with crude extract volume	58
13	Dry weight accumulation with kernel development for Manitou wheat	61
14	Dry weight accumulation with kernel development for 6A190 triticale	61
15	Dry weight accumulation with kernel development for 6A250 triticale	63
16	Dry weight accumulation with kernel development for Stewart 63 wheat	63
17	Dry weight accumulation with kernel development for Prolific rye	65
18	Fresh weight change with kernel development for Manitou wheat	67
19	Fresh weight change with kernel development for 6A190 triticale	67
20	Fresh weight change with kernel development for 6A250 triticale	69
21	Fresh weight change with kernel development for Stewart 63 wheat	69
22	Fresh weight change with kernel development for Prolific rye	71
23	Changes in soluble protein with kernel development for Manitou wheat	74
24	Changes in soluble protein with kernel development for 6A190 triticale	74
25	Changes in soluble protein with kernel development for 6A250 triticale	76

LIST OF FIGURES - Continued

FIGURE		PAGE
26	Changes in soluble protein with kernel development for Stewart 63 wheat	76
27	Changes in soluble protein with kernel development for Prolific rye	78
28A	Changes in hexokinase and sucrose synthase activity with kernel development for Manitou wheat	81
28B	Changes in glucose and sucrose levels with kernel development for Manitou wheat	81
29A	Changes in hexokinase and sucrose synthase activity with kernel development for 6A190 triticale	83
29B	Changes in glucose and sucrose levels with kernel development for 6A190 triticale	83
30A	Changes in hexokinase and sucrose synthase activity with kernel development for 6A250 triticale	85
30B	Changes in glucose and sucrose levels with kernel development for 6A250 triticale	85
31A	Changes in hexokinase and sucrose synthase activity with kernel development for Stewart 63 wheat	87
31B	Changes in glucose and sucrose levels with kernel development for Stewart 63 wheat	87
32A	Changes in hexokinase and sucrose synthase activity with kernel development for Prolific rye	89
32B	Changes in glucose and sucrose levels with kernel development for Prolific rye	89

ABBREVIATIONS

ADP	adenosine-5'-diphosphate
ADP glucose pyrophosphorylase	ATP:α-D-glucose 1-phosphate adenyltransferase
ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
CDP	cytidine-5'-diphosphate
o-CPP	o-carboxyphenyl phosphate
DPM	disintegrations per minute
DTT	dithiothreitol
EDTA	ethylenediamine tetra-acetic acid
F-6-P	fructose-6-phosphate
G-1-P	glucose-1-phosphate
G-6-P	glucose-6-phosphate
GDP	guanosine-5'-diphosphate
IBB	isobutanol:benzene
NAD	nicotinamide-adenine dinucleotide
NEN	New England Nuclear
phosphorylase	α-1,4-glucose:orthophosphate glucosyl transferase
P_i	inorganic phosphate
PVP	polyvinylpolypyrrolidone
starch synthase	ADP glucose:α-1,4-glucose α-4-glucosyl transferase

ABBREVIATIONS - Continued

TCA	trichloroacetic acid
TDP	thymidine-5'-diphosphate
UDP	uridine-5'-diphosphate
UTP	uridine-5'-triphosphate

ABSTRACT

Saari, Leonard Lyle. M.Sc., The University of Manitoba, February, 1977. Hexokinase and Sucrose Synthase Activity During Kernel Development in Triticale, Wheat and Rye. Major Professor: Dr. Robert D. Hill.

Developing triticale, wheat and rye grains were studied from 6 to 42 days post-anthesis with respect to hexokinase and sucrose synthase activity. The two enzymes were compared with levels of sucrose, glucose and protein as well as changes in fresh and dry weight. The objective was to investigate the relationship of these parameters to the shrivelling problem associated with mature grains of certain triticale lines.

Crude extracts of the seed samples were prepared by grinding and centrifugation. Dialysis of the homogenate was necessary to remove an inhibitor of hexokinase which was probably anionic and of low molecular weight. Phosphatase activity, which was prevalent in the crude extract, was inhibited by the addition of 65 mM P_i to the assay media.

Sucrose synthase correlated well with dry weight gain in all of the cereals tested suggesting a role for this enzyme in dry matter accumulation. Atypical sucrose and sucrose synthase levels occurred during kernel development in 6A190, a shrivelled triticale. Dry weight accumulation during the same period remained unaltered; thus, it appears doubtful that the sucrose and sucrose synthase aberrations are directly responsible for kernel shrivelling in 6A190.

For all of the cereals tested, hexokinase was present throughout seed formation, but increased substantially with the decline of sucrose

synthase and cessation of dry matter accumulation. Hexokinase from 30 day post-anthesis samples utilized glucose more rapidly than fructose.

INTRODUCTION

The combination of the genomes of rye (*Secale*) and wheat (*Triticum*) resulted in the formation of triticale. This new crop species offered the potential high quality of wheat along with the hardiness of rye.

Agronomic problems have hindered the further commercial development of triticale. Abnormal kernel development resulting in shrivelled seeds at maturity has been one of the most persistent difficulties.

The resultant endosperm collapse in some triticale lines at maturity has been associated with decreased starch accumulation (Hill *et al.*, 1974). This could result from either a poor supply of nutrients to the developing kernel or an inefficient utilization of the nutrients. Assuming that translocation to the developing endosperm is not limiting, then some aspect of starch metabolism is likely aberrant in triticale. This line of investigation has already shown that during development and at maturity, highly shrivelled triticale lines possessed greater levels of α-amylase, an enzyme which hydrolyzes starch, than non-shrivelled lines (Hill *et al.*, 1974). The shrivelling may be due, in part, to precocious germination.

Another possibility is that the biosynthesis of starch is abnormal in shrivelled triticale. To investigate this hypothesis, sucrose synthase and hexokinase were compared with carbohydrate content and dry weight accumulation as kernel development progressed.

LITERATURE REVIEW

Starch Formation in Cereals

The ability of plants to produce starch from UDP glucose was demonstrated by De Fekete *et al.* (1960) and Leloir *et al.* (1961). ADP glucose was found to donate glucose to starch at a faster rate than UDP glucose (Recondo and Leloir, 1961). These discoveries and subsequent findings led to the general adoption of ADP glucose-α-1,4-glucan 4-α-glucosyl transferase (starch synthase) as the primary catalyst in the synthesis of α-1,4 glucosidic linkages in higher plants (Preiss *et al.*, 1973; Turner and Turner, 1975).

Another enzyme, starch phosphorylase, has the ability to transfer glucosyl units from glucose-1-phosphate to the glucan. Its presence has been shown in many plant tissues producing starch, but the role of this enzyme in starch metabolism is unclear. However, glucose-1-phosphate has been shown to effectively donate glucosyl units to a proteic acceptor in the absence of primer from studies with non-sedimentable potato tuber fractions (Tandecarz *et al.*, 1975). The intermediate glucoprotein or the resultant α-1,4-glucan can then serve as primer for the transglucosylating enzymes.

The biosynthetic mechanism of starch production has been studied in wheat, rice, maize, and barley. Further research has been conducted on wheat to see whether the biosynthetic system, photosynthetic capacity, or translocation is limiting starch accumulation. These investigations will be discussed in the following sections.

Maize

De Fekete and Cardini (1964) demonstrated the presence of all the enzymes in maize endosperm catalyzing the following reactions:

sucrose $\rightleftharpoons$ ADP glucose (or UDP glucose) $\rightarrow$ starch

sucrose $\rightleftharpoons$ UDP glucose $\rightleftharpoons$ glucose-1-P $\rightleftharpoons$ ADP glucose $\rightarrow$ starch

This proposal for starch biosynthesis in maize led to investigations to see whether sucrose itself was transported directly to the site of synthesis.

Shannon (1968) reported that before or during sucrose movement from the terminal phloem elements in the pedicel and pericarp tissues of corn, sucrose was hydrolyzed to glucose and fructose. Upon arrival at the endosperm, the monosaccharides were quickly converted to sucrose which seemingly was the storage form of carbohydrate prior to starch synthesis. Subsequent investigation supported these findings (Shannon, 1972). Radioautographs were made of serial sections cut from maize kernels after 1 to 6 hours exposure to $^{14}CO_2$. The results showed that translocated sugars entered the endosperm as glucose and fructose while 30 percent of the dry weight in the pedicel was sugar, mostly in the form of sucrose. Shannon hypothesized that after sucrose hydrolysis in the placento-chalazal tissues, the monosaccharides moved through the free space of the cell walls to various parts of the endosperm. The cited evidence was that the monosaccharides moved at approximately the rate of diffusion from the base of the endosperm to the crown without being metabolized. Another investigation supported this hypothesis (Shannon and Dougherty, 1972). Invertases of the placento-

chalazal and pedicel tissues were more active than pericarp invertase in maize kernels 12 to 40 days post-anthesis. The presence of sucrose synthase was not found thereby discounting its role in the sucrose breakdown.

Proposed mechanisms for starch synthesis in maize have been aided by the use of the shrunken-2 (sh_2) and the brittle-2 (bt_2) mutants of maize. Mature sh_2 seeds weighed only 75 percent of normal, and the starch content was only 25 to 30 percent of normal endosperms (Tsai and Nelson, 1966). The reduced starch content resulted in collapsed endosperms. Activities of ADP glucose pyrophosphyorylase were found to be 12 and 17 percent of normal in sh_2 and bt_2, respectively (Dickinson and Preiss, 1969). These results implicated ADP glucose pyrophosphorylase in normal starch synthesis.

The deficient ADP glucose pyrophosphorylase in sh_2 mutant maize provided an explanation for the prolonged hexokinase synthesis with endosperm development in the sh_2 (Cox and Dickinson, 1973b). Lower activities of ADP glucose pyrophosphorylase may have resulted in increased sucrose levels from the accumulation of more hexose phosphates. Castor bean hexokinase levels increased greatly by the application of externally applied sucrose, glucose, and fructose (Marré *et al.*, 1965) although the mechanism was unknown. In the same way, prolonged hexokinase synthesis may be induced by greater sugar concentrations.

A more intensive investigation of enzyme activities in maize endosperm was carried out by Tsai *et al.* (1970). At 12 to 14 days post-anthesis, prior to the most rapid starch synthesis, activity was

detected for sucrose-UDP glucosyl transferase, soluble ADP glucose-starch glucosyl transferase, ADP-glucose pyrophosphorylase, and phosphorylases II and III. Normal starch production was shown to be dependent on these enzymes (Dickinson and Preiss, 1970). The enzymes studied by Tsai *et al.* (1970) were not detectable from 10 to 12 days after pollination, a period of rapid starch synthesis. Since nucleoside diphosphate glucose-starch glucosyl transferase and UDP-glucose pyrophosphorylase were observed before and during this interval, it was suggested that early starch synthesis in maize occurred via these two enzymes. Phosphorylase was also present at an early stage in seed development (8 days post-anthesis) and may have contributed to early starch production.

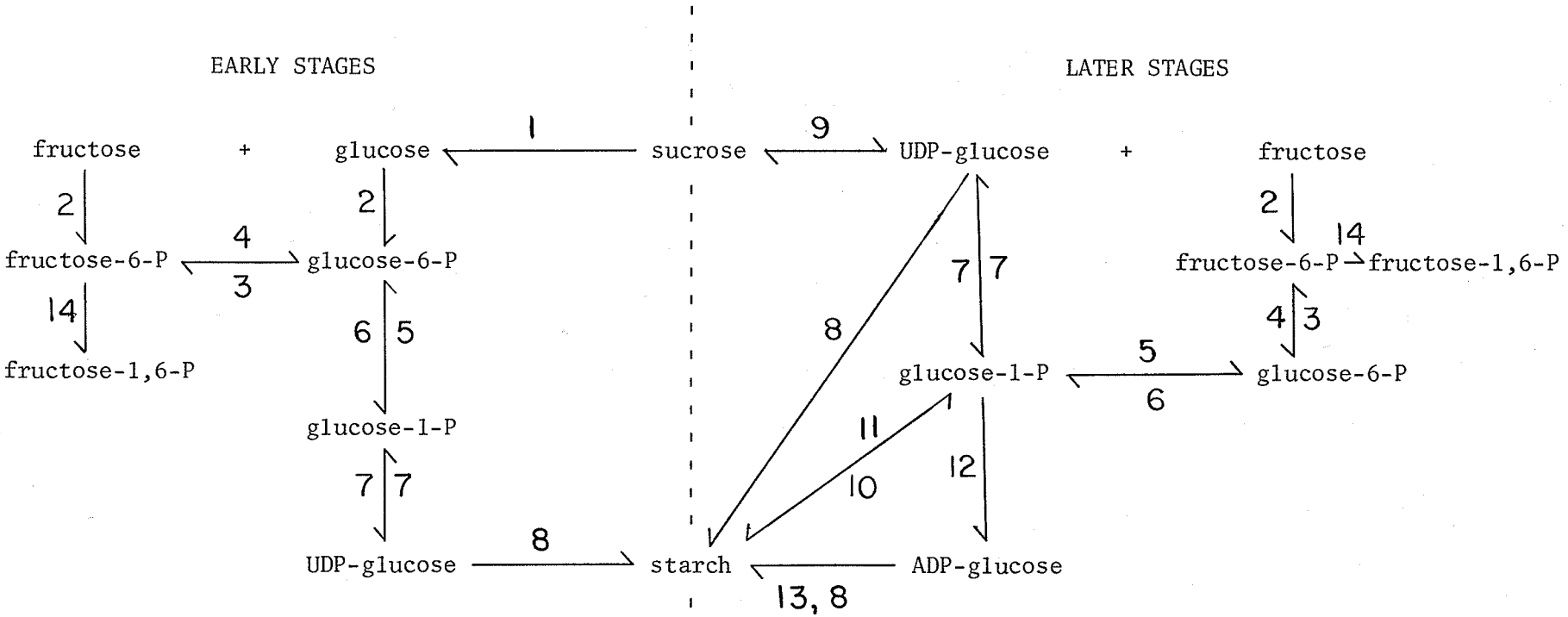
The presence of many other enzymes in developing maize was shown as well by Tsai *et al.* (1970). Activities for nucleoside diphosphokinase, phosphoglucomutase, glucose phosphate isomerase, hexokinase, and invertase were demonstrated. The role of these enzymes in starch synthesis can be seen in a mechanism proposed by the same workers (Fig. 1).

The proposal was made (Tsai *et al.*, 1970) that sucrose-UDP glucosyl transferase seemed to be a key enzyme in starch synthesis since it increased dramatically at 12 days post-anthesis coinciding with the beginning of rapid starch synthesis and a decrease in invertase activity. Also, the function of sucrose synthase was suggested to be the cleavage of sucrose rather than the reverse reaction. Supporting the idea was the observation that at the physiological pH found in maize, potato sucrose synthase favored UDP glucose formation

FIGURE 1

Hypothetical scheme of sucrose-starch conversion during maize endosperm development.

Reference number used in figure	Enzyme name
1	Invertase
2	Hexokinase
3	Glucose phosphate isomerase I
4	Glucose phosphate isomerase II
5	Phosphoglucomutase, soluble
6	Phosphoglucomutase, starch-bound
7	UDP glucose pyrophosphorylase
8	Starch granule-bound nucleoside diphosphate glucose-starch glucosyl transferase
9	Sucrose-UDP glucosyl transferase
10	Phosphorylase I
11	Phosphorylases II and III
12	ADP glucose pyrophosphorylase
13	Soluble ADP glucose-starch glucosyl transferase
14	Phosphofructokinase


EARLY STAGES
LATER STAGES
fructose
+
glucose
1
sucrose
9
UDP-glucose
+
fructose
2
2
2
14
fructose-6-P
4
3
glucose-6-P
fructose-6-P
fructose-1,6-P
14
6
5
4
3
fructose-1,6-P
8
7
7
glucose-1-P
5
6
glucose-6-P
glucose-1-P
11
10
12
7
7
UDP-glucose
8
starch
ADP-glucose
13, 8

(Pressey, 1969). At later stages of kernel development, Tsai *et al.* (1970) doubted that sucrose-UDP glucosyl transferase directly synthesized ADP glucose since the affinity of this enzyme for UDP was much greater than for ADP. Also, uridine nucleotides inhibited the usage of ADP (De Fekete and Cardini, 1964).

An opposing view was held by Ozbun *et al.* (1973). Their results cited sufficient activity of ADP glucose pyrophosphorylase and starch synthase in maize kernels from 8 to 28 days post-anthesis to account for starch synthesis at all stages. Previously, Ozbun *et al.* (1971) had shown the ability of an ADP glucose-α glucan-4-α glucosyl transferase to synthesize polyglucan in the absence of primer. UDP glucose pyrophosphorylase and phosphorylase were also found to be relatively high (Ozbun *et al.*, 1973). The authors felt that UDP glucose pyrophosphorylase was involved in cell wall synthesis. Phosphorylase contributions to starch synthesis in maize at early stages of development were discounted due to the low activities of ADP glucose pyrophosphorylase, starch synthase and UDP glucose pyrophosphorylase in sh_4 endosperms during early periods of synthesis. Previously, only phosphorylase activity had been shown to be low (30 percent of normal) in sh_4 endosperms relative to normal dent corn (Tsai and Nelson, 1969a), and the argument that this enzyme was responsible for starch initiation seemed valid (Tsai and Nelson, 1969b). However, the previously cited findings of Ozbun *et al.* (1973) implicated ADP glucose pyrophosphorylase and starch synthase in initiating starch synthesis and diminished the idea of early starch accumulation via phosphorylase.

An attempt to resolve the conflicting views of Tsai *et al.* (1970) and Ozbun *et al.* (1973) with respect to early starch synthesis in maize was made by Shannon (1974). Developing maize kernels were found to be composed of cells with varying physiological ages. Assuming that smaller starch granules were indicative of younger cells, an *in vivo* study of ^{14}C incorporation into starch granules of differing sizes was made to see whether a difference in the rate of starch synthesis existed. Smaller granules were shown to contain less ^{14}C per granule or per granule surface area. This result suggested to Shannon that early starch synthesis was via less efficient enzymes such as the ones suggested by Tsai *et al.* (1970) and later synthesis was via the ADP glucose pathway.

Wheat

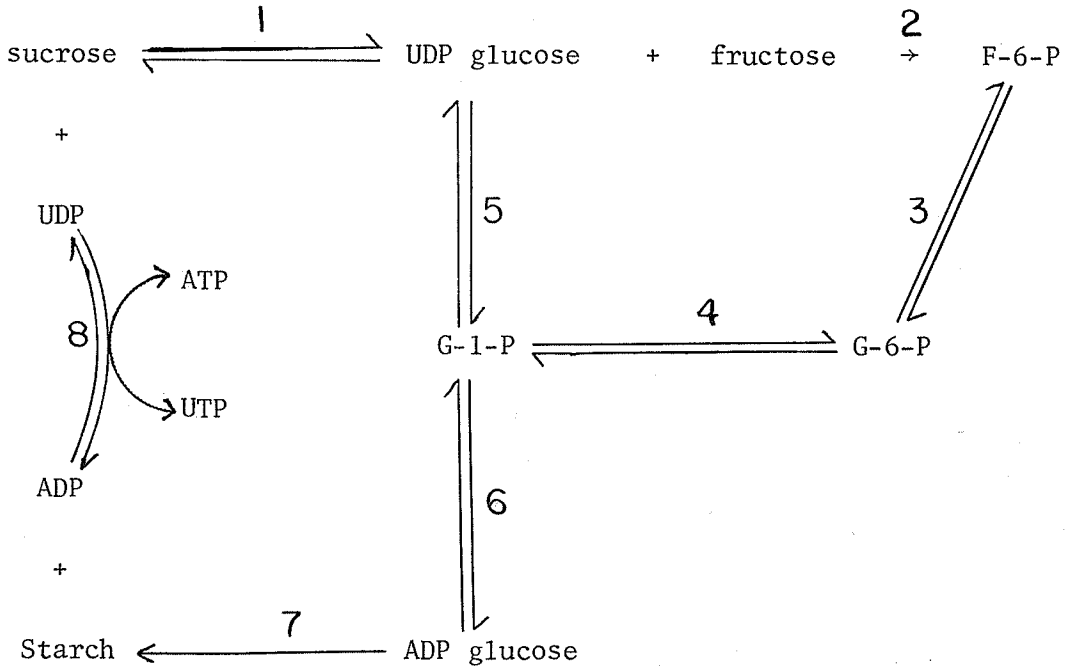
The enzymatic basis of starch synthesis in wheat has not been so intensively researched as in maize. Rather, the main effort in wheat has been to investigate whether photosynthetic capacity, translocation, or the starch biosynthetic potential is limiting starch production in the grain.

Turner (1969), however, has proposed an enzymatic scheme for starch synthesis in wheat (Fig. 2). The mechanism was very similar to the one proposed for maize (Tsai *et al.*, 1970). In Turner's study, both UDP glucose pyrophosphorylase and ADP glucose pyrophosphorylase were found to increase with increasing starch synthesis. From these results and the known nucleotide specificities of starch synthase (Recondo and Leloir, 1961) and sucrose synthase (De Fekete and Cardini, 1964), ADP

FIGURE 2

Hypothetical scheme of starch synthesis in wheat.

Reference number used in figure	Enzyme name
1	Sucrose synthase
2	Hexokinase
3	Glucose phosphate isomerase
4	Phosphoglucomutase
5	UDP glucose pyrophosphorylase
6	ADP glucose pyrophosphorylase
7	Starch synthase
8	Nucleoside diphosphokinase


sucrose
1
UDP glucose
+
fructose
2
F-6-P
+
UDP
5
3
8
ATP
4
G-1-P
G-6-P
UTP
ADP
6
+
Starch
7
ADP glucose

glucose was suggested to be the main substrate for starch synthesis in wheat (Turner, 1969).

One of the earlier experiments investigating the types of limitation to starch production was performed by Jenner (1968). Varying the light intensity from darkness to 1500 foot candles had no effect on the rate of starch synthesis in detached ears of wheat if enough exogenous sucrose was supplied. This suggested that the rate of synthesis was determined by the ear itself.

In subsequent trials, sucrose concentration in the endosperm was highly correlated with the rate of starch synthesis (Jenner, 1970). Sucrose concentration in the endosperm was suggested to regulate the rate of starch synthesis. A linear relationship existed between external sucrose levels and the sucrose concentration in the floral organs and rachis. However, in the endosperm, the maximum sucrose level was much lower than the concentration in the rachis implying that sucrose transport through the rachilla and vascular bundles to the pericarp was limiting, thus impeding starch accumulation.

Defoliation experiments and culturing detached ears of wheat on sucrose further amplified the implications of Jenner (1970). Photosynthetic capacity did not seem to be limiting from defoliation experiments (Jenner and Rathjen, 1972a). Further, the amount of sucrose in the grain changed relatively little with defoliation although this was somewhat dependent on the length of the period between treatment and observation (Jenner and Rathjen, 1972b). By supplying sucrose to ears of wheat, sucrose accumulated to concentrations higher than normal in all parts of the ear except the grain. The sucrose levels found in the grain were similar to those found in normal

plants. Jenner and Rathjen (1972b) concluded that photosynthesis provided adequate assimilate and that the limits to sucrose levels in the grain were due to the final stages of sugar transport. Translocation studies in wheat (Wardlaw and Moncur, 1976) supported this hypothesis. These workers demonstrated the ability of wheat to increase the movement of C^{14}-photosynthate to the head when the demand for assimilate in the ear increased.

Since sucrose availability to the wheat grain seemed to ultimately dictate the rate of starch synthesis, the mechanism by which this sugar transport occurred was studied. Initial studies using radioactive sugar solutions to culture wheat grains suggested that extracellular hydrolysis of sucrose was not necessary for absorption into wheat grain and intracellular hydrolysis of sucrose before incorporation into starch was doubtful (Jenner, 1974). This contrasted with sucrose transport in corn (Shannon, 1972). Conflicting results were obtained when the flag leaf from a wheat plant was exposed to $^{14}CO_2$ and the distribution of the label in the kernel observed (Sakri and Shannon, 1975). A lower proportion of label in sucrose appeared in the pericarp and endosperm relative to glucose and fructose, which was interpreted as an indication that sucrose hydrolysis was necessary for movement from the phloem to the endosperm. Other results obtained by Sakri and Shannon (1975) did not support a simple sucrose inversion prior to endosperm absorption. Injection of ^{14}C-fructosyl-sucrose into the peduncle resulted in very little randomization of the label between the glucose and fructose of sucrose extracted from the pericarp and endosperm when compared to extracted sucrose from the rachis.

The assumption was that glucose and ^{14}C-fructose were at equilibrium and the isomerizing enzymes would transfer more of the label to the glucose in the endosperm before sucrose resynthesis. Sakri and Shannon suggested that if hydrolysis was necessary for transport, then fructose and glucose were not available for isomerization. This would occur by diffusion into the endosperm via the apoplast so contact with the cytoplasmic isomerizing enzymes (Kriedman and Beevers, 1967) would be avoided. Jenner and Rathjen (1975) lately proposed that in the maturing grain, the fall in starch accumulation was caused by a decrease in the synthetic capacity of the endosperm and not by reduced assimilate supply to the grain. They found that dissected wheat grains allowed to take up sucrose under non-limiting substrate conditions decreased in the rate of starch accumulation after a given period of development.

Other Cereals

Interest in starch metabolism has been prevalent in rice as well as wheat and maize. An early investigation of the fate of UDP glucose-C^{14} when incubated with starch granules from rice synthesizing amylose and amylopectin led to a proposed mechanism for starch production (Akazawa *et al.*, 1964). This scheme employed UDP glucose as the donor for the growing starch molecule. Murata *et al.* (1964) showed a lower level of UDP than of ADP in developing rice grain and higher UDP glucose levels in the grain than ADP glucose levels. As in corn (De Fekete and Cardini, 1964), a specific inhibition of sucrose-ADP glucose glucosyl transferase by UDP was demonstrated (Murata *et al.*, 1966). Perez *et al.* (1975) reported on the levels of reducing and non-reducing sugars,

starch, soluble protein and several enzymes metabolizing sucrose, glucose-1-P, and glucose nucleotides in developing rice grain. From these results, the authors proposed a scheme for starch synthesis in rice very similar to the one forwarded by Turner (1969) in wheat.

Other work in starch metabolism has been conducted on barley. Only UDP glucose-linked starch synthesis was observed in barley amyloplasts up to 12 days post-anthesis. Before 12 days, ADP glucose was ineffective but became the main glucosyl donor after this time (Baxter and Duffus, 1971). Unprimed phosphorylase activity was demonstrated in endosperm extracts from barley between 2 and 10 days post-anthesis (Baxter and Duffus, 1973a). The initiation of α-1,2 glucans as primers for starch synthase was suggested as the role of phosphorylase in young (less than 10 days) barley endosperm.

Many enzymes associated with starch production were surveyed in developing barley endosperm (Baxter and Duffus, 1973b). Sucrose-UDP glucosyl transferase was found to be relatively high in young endosperm suggesting that incoming sucrose was mainly converted to UDP glucose. In maize, this enzyme was undetectable until 12 days after flowering (Tsai *et al.*, 1970). Starch synthesis in barley was proposed to be initiated by phosphorylase activity and UDP glucose starch synthetase. From 10 to 15 days after flowering, the presence of UDP glucose pyrophosphorylase, ADP glucose pyrophosphorylase, and nucleoside diphosphokinase suggested that starch production proceeded via the UDP glucose → G-1-P → ADP glucose → starch route (Baxter and Duffus, 1973b).

Hexokinase

The principal route for carbohydrate degradation in living organisms is glycolysis. The incorporation of hexoses into this metabolism scheme occurs via a nonequilibrium reaction catalyzed by hexokinase (E.C.2.7.1.1). In mammalian systems, hexose phosphorylation is inhibited by glucose-6-phosphate but partially relieved by orthophosphate (Newsholme *et al.*, 1968). Metabolic control of phosphofructokinase regulates the glucose-6-phosphate levels in these tissues which in turn mediate hexokinase activity (Turner and Turner, 1975). Yeast (Colowick, 1973) and mammalian (Purich and Fromm, 1971) hexokinase have been studied extensively. Yeast hexokinase is not very senstitive to glucose-6-phosphate inhibition and therefore, it is unlikely that this metabolite regulates hexokinase in this organism (Turner and Turner, 1975). Several metabolites including citrate, malate, and 3-phosphoglycerate activate yeast hexokinase (Kosow and Rose, 1970).

A wide variety of plant species and tissues have exhibited hexokinase activity (Cox and Dickinson, 1971). Hexokinase in plants has been briefly mentioned in a recent review (Turner and Turner, 1975). Wheat germ hexokinase has been purified (Meunier *et al.*, 1971) and studied (Meunier, 1975), but for the most part, kinetic data on higher plant hexokinases is limited. Cox and Dickinson (1973a), however, reported K_m values for glucose of 0.1 - 0.2 mM for endosperm hexokinase and 3.4 mM for the scutellum form.

More attention has been given to hexokinase activities in developing cereal grains. In barley, the enzyme was detectable 7 days after anthesis in both the soluble portion and the amyloplast fraction (Baxter

and Duffus, 1973b). Both forms increased in activity until 21 days post-anthesis and levelled off. No samples were checked after 26 days. Amyloplast activity was approximately one half of the soluble portion.

Hexokinase extracted from developing rice grains increased after flowering to a maximum at 11-12 days post-anthesis and then declined (Perez *et al.*, 1975).

Tsai *et al.* (1970) observed numerous enzymes in developing maize endosperm including hexokinase. The hexokinase activity increased from 8 days to a maximum at 22 days and then declined. Similarly, Cox and Dickinson (1971) found an increase in hexokinase activity in maize endosperm up to 27 days after pollination before a decrease ensued. Finally, the activity remained constant at approximately 35 percent of the peak activity up to 50 days post-anthesis. Germination studies up to 9 days after planting were also conducted. The results suggested that the hexokinase of germinating maize was a residual enzyme from developing endosperm since the activity level at one day after planting was about one half of the final hexokinase activity at 50 days post-anthesis. This activity continued to decrease up to 9 days after planting.

Later, these same authors surveyed hexokinase activity in the developing endosperms of shrunken-2 (sh_2) mutants of maize (Cox and Dickinson, 1973b). This mutant has approximately 75 percent of the kernel weight of dent and about 25-30 percent of the starch content. Up to 18 days post-anthesis, both the dent and sh_2 maize increased in hexokinase activity at the same rate. Dent corn reached a maximum in activity at 22 days and declined thereafter. Hexokinase activity in the sh_2 mutant increased to 30 days and then dropped.

Sucrose Synthase

Sucrose synthase (UDP-glucose:D-fructose 2-glucosyl transferase E.C.2.4.1.13) was discovered in 1953 (Cardini and Leloir, 1953). Since then, the enzyme has been demonstrated in a number of plants (Pressey, 1969; Avigad, 1964; Grimes *et al.*, 1970; Tsai *et al.*, 1970; Baxter and Duffus, 1973b; Perez *et al.*, 1975).

Most of the plant tissues showing sucrose synthase activity were non-photosynthetic suggesting that this reversible enzyme was involved in the degradation of translocated sucrose from photosynthetic organs rather than in the synthesis of sucrose *per se* (Delmer, 1972). Indeed, much evidence now supports this idea. For instance, at physiological pH, potato sucrose synthase more readily cleaves sucrose (Pressey, 1969).

UDP seems to be the preferred nucleotide for sucrose synthase activity. In sweet corn, this enzyme has a lower affinity for ADP than UDP (De Fekete and Cardini, 1964). The same authors demonstrated that sucrose cleavage with ADP is inhibited by UDP. In the direction of sucrose synthesis, Grimes *et al.* (1970) have shown that although sucrose synthase from *Phaseolus aureus* could utilize ADP glucose, TDP glucose, CDP glucose, or GDP glucose as substrate, UDP glucose was preferred and effectively inhibited sucrose synthesis via the other nucleoside diphosphate glucoses. Also, sucrose-UDP glucosyl transferase in developing rice grain had a much higher activity than sucrose-ADP glucosyl transferase (Perez *et al.*, 1975). Murata *et al.* (1966) showed that UDP inhibited sucrose-ADP glucosyl transferase and that UDP levels were consistently lower and UDP glucose levels higher than the respective ADP and ADP glucose levels in developing rice grain.

Changes in activity associated with maturation have been studied in rice (Perez *et al.*, 1975), barley (Baxter and Duffus, 1973b), maize Tsai *et al.*, 1970), and potato (Pressey, 1969). Patterns of sucrose-UDP glucosyl transferase activity were similar for the three cereals mentioned when assayed in the direction of sucrose cleavage. Soluble endosperm extracts from barley exhibited sucrose synthase activity at 5-7 days and increased to a high at 25 days. Thereafter, activity declined. Maize endosperm enzyme gained activity up to 22 days and decreased slightly to the 28 day stage. Activity in rice grain extract peaked at 11-12 days post-anthesis and fell quickly to 16 percent of the maximum activity at 21 days.

MATERIALS AND METHODS

Chemicals

The chemicals used were of reagent grade and were dissolved in glass distilled water. The chemicals purchased from Sigma Chemical Company were tris (hydroxymethyl) aminomethane (tris buffer), glucose-6-P, ATP, o-carboxyphenyl phosphate, dithiothreitol, NAD, UDP, bovine serum albumin, UDP glucose dehydrogenase E.C.1.1.1.22 (type III from bovine liver), hexokinase E.C.2.7.1.1 (type C-300 and type C-130 from yeast), and alkaline phosphatase E.C.3.1.3.1 (type V from chicken intestinal mucosa). Glucose, sucrose, Na_2HPO_4, NaH_2PO_4, EDTA, $MgCl_2$, PVP, NH_4OH, TCA and HCl were purchased from Fisher Scientific Company. The Dowex 2X-8 anion exchanger, 50-100 mesh, and the AG I1 A8 Biorad ion retardation resin were obtained from BIO-RAD Laboratories. Glucostat reagent was bought from Worthington Biochemical Corporation. Uniformally labelled D-(^{14}C)-glucose, 200 mCi/mmole and D-(^{14}C)-fructose, 200 mCi/mmole were purchased from New England Nuclear and Amershan/Searle Corporation. Nitex monofilament screen cloth was obtained from B. and S. H. Thompson Company, Ltd.

Growth and Collection of Plant Materials

Wheat (*Triticum aestivum* L., cv. Manitou and *Triticum durum* L., cv. Stewart 63), rye (*Secale cereale* L., cv. Prolific, and triticale (*X Triticosecale* Wittmack, lines 6A190 and 6A250) were field grown in the summer of 1975. Beginning at 6 days after anthesis, samples were harvested every 4 days up to 42 days post-anthesis. The heads

were kept in ice while kernels from the central portion of the head were removed. The sample of kernels was then frozen in liquid nitrogen and stored at -20°C until prepared for assay.

Portions of the frozen samples were freeze-dried. The seeds were counted and weighed after freeze drying to determine the dry weight. These freeze-dried samples were used for the glucose and sucrose determinations.

Preparation of Crude Extract

Duplicate samples of twenty frozen seeds were taken for each varietal date. For most of the preliminary tests on the assay, 18 day post-anthesis Manitou was used. Fresh weight was determined at this point in the procedure. The twenty seeds were ground to a slurry with 5.0 ml of 50 mM phosphate buffer, pH 7.2, which contained 0.1 mM EDTA and 2 mM DTT. All procedures were carried out at 0 to 4°C. PVP (0.75 g) was added at this point for some experiments. The homogenate was squeezed through Nitex #86 nylon monofilament screen cloth and then centrifuged at 10,000 g for 10 minutes. The pellet formed was resuspended in the same phosphate buffer as used for the extraction when assaying the pellet activity.

Early experiments employed the supernatant directly as the main source of hexokinase activity. Later, 1.0 ml of the supernatant was dialyzed with stirring against 250 ml of the phosphate buffer containing EDTA and DTT for 4 hours. The buffer media was then changed and the crude extract was subsequently dialyzed overnight. Finally, the dialysate was washed from the dialysis tubing with cold buffer and made to 4.0 ml. This dialyzed crude extract was used for hexokinase,

sucrose synthase, and protein determinations.

Hexokinase Assay

A modification of the radioassay of Gots and Bessman (1973) was used to determine hexokinase activity in the crude extract.

The final reaction mixture consisted of phosphate buffer, pH 7.6, 65 mM; $MgCl_2$, 11.5 mM; ATP, 5.38 mM; and ^{14}C-glucose, 1.6 mM and 0.8 mM. The specific activity of the radioactive glucose was varied from 0.4 to 1.0 µCi/µmole in different experiments. 100 µl of dialyzed crude extract was incubated in this reaction mixture at 25°C for 15 minutes in a final volume of 0.625 ml. The reaction was terminated by the addition of 4 ml of 1M glucose in 0.17M NH_4OH. Zero time controls were run in which crude extract was added and the glucose/NH_4OH solution immediately added. Occasionally, controls which contained no ATP were run. These showed no reaction. Each crude extract from a 20 seed sample had two reaction tubes along with a control tube. After termination of the reaction, 0.25 g of untreated dry Dowex 2X8 - 100 anion exchange resin, 50-100 mesh, was added to each test tube. In a 5 minute period, the mixture was vigorously agitated 3 times on a Vortex mixer. The supernatant was aspirated and discarded. The resin was washed 4 times with 4 ml of 0.17M NH_4OH. After the final wash, 1.0 ml of 1.0N HCl was added and the resin agitated on the Vortex mixer every two minutes for eight minutes. A 0.20 ml aliquot of the acid eluant was counted in 10 ml of a commercial scintillator such as Aquasol (NEN). The channels ratio method (Wang and Willis, 1965) was used to determine quenching. Hexokinase activity in the crude extract was determined by

the following formula:

$$\frac{\text{micromoles formed}}{\text{minute}} = \frac{(\text{dpm sample} - \text{dpm control}) \times \text{dilution factor}}{\text{specific activity of substrate} \times \text{assay time}}$$

where dpm is disintegrations per minute, the dilution factor is 5 (0.20 ml of acid eluant counted out of a total volume of 1.0 ml) and the specific activity expressed as dpm/micromole of substrate glucose. The amount of enzyme that catalyzed the conversion of one micromole glucose per minute was defined as an enzyme unit.

The same procedure was used when ^{14}C-fructose was substrate.

Sucrose Synthase Assay

Assay II for sucrose synthase outlined by Avigad (1964) was used with modification. One solution (solution A) was made up containing 50 mM tris buffer, 0.1 mM EDTA, 1.0 mM $MgCl_2$ and 40 mM sucrose. The pH was adjusted to 8.4. Another solution (solution B) was prepared fresh daily consisting of 2 mM UDP and 1.4 mM NAD. For each run, 0.6 ml solution A was mixed with 0.2 ml solution B. Immediately before assay, an excess (over 100 units) of UDP glucose dehydrogenase was added. The activity of the UDP glucose dehydrogenase was periodically checked by adding an excess of UDP glucose. The addition of 25-100 µl of crude extract initiated the reaction. Control assays incubated without sucrose or UDP or with heated crude extract gave no reaction.

The NAD reduction at 340 nm was observed using the Cary Model 15 recording spectrophotometer.

Enzyme activity of the crude extract was calculated using the following formula:

$$\frac{\text{micromoles UDP glucose formed}}{\text{minute}} = \frac{\Delta A_{340}/\text{minute} \times \text{reaction volume}}{6.22 \times 2}$$

where the molar extinction coefficient of NADH was employed in the denominator. An enzyme unit was defined as that amount of enzyme which catalyzed the conversion of one micromole of sucrose per minute to UDP glucose.

Alkaline Phosphatase Assay

The procedure outlined in the Worthington Enzyme Manual (1972) which used o-carboxyphenyl phosphate as substrate was used to assay phosphatase from crude extract. For inorganic phosphate inhibition studies of alkaline phosphatase, phosphate buffer at various concentrations, pH 7.5, was used instead of tris buffer. Activity was calculated with the following formula:

$$\frac{\text{nanomoles o-CPP hydrolyzed}}{\text{per minute}} = \frac{\Delta A_{300}/\text{minute} \times \text{reaction volume} \times 10^6}{3500}$$

where 3500 is the molar absorbancy index of salicylic acid at 300 nm.

Inorganic Phosphate Assay

The basic outline of the assay employed was taken from Mozersky *et al.* (1966). A 1.1 ml volume of a mixture of 0.19M tris, pH 7.5, 59 mM $MgCl_2$ and 2.9 mM glucose was added to 0.5 ml of 68 mM glucose-6-phosphate. The $MgCl_2$, glucose and tris were used to simulate conditions

in the hexokinase assay. The reaction was initiated with 0.2 ml crude extract which was incubated at 25°C for 20 minutes. Termination of the reaction was accomplished by adding 1.0 ml of 0.9M TCA. The test tubes were placed in ice. Subsequent steps were carried out at 0 - 4°C. Five microliters of fresh 30% H_2O_2 were added to each tube to oxidize the dithiothreitol present. The samples were then centrifuged at 5000 g for 10 minutes.

A 2.0 ml volume of 1.5 percent ammonium molybdate in 0.5N H_2SO_4 was added to 2.0 ml of the supernatant left after centrifugation. After mixing, 4 ml of a water saturated mixture of 1:1 isobutanol-benzene were added. The two phases were mixed on a Vortex mixer for 30 seconds and then allowed to stand on ice for 10 to 15 minutes. The upper layer was removed with a Pasteur pipette and brought to room temperature before taking an absorbance reading at 370 nm. A standard curve was prepared using Na_2HPO_4 at pH 7.5.

Protein Determination

Duplicate samples of 0.5 ml crude extract were mixed with 0.5 ml of 10 percent TCA and allowed to stand in ice for 30 minutes. A 3000 g x 5 minute centrifugation followed. The supernatant was decanted and the centrifuge tubes inverted on filter paper to remove the TCA. The precipitate was then dissolved in 0.5 ml of 0.75N NaOH which was subsequently diluted to 4.0 ml total. One ml aliquots were taken and assayed by the method of Lowry *et al.* (1951). Bovine serum albumin was used as the protein standard.

Glucose and Sucrose Determination

Lyophilized seed samples were ground in a Wiley mill and four 0.1 g subsamples were taken and weighed for the glucose determination. The samples were boiled in 5 ml of 75 percent ethanol for 10 minutes and then centrifuged. The supernatant was poured off and the extraction was repeated. The ethanol was removed from the combined supernatant fractions by evaporation under N_2 gas. The residue was taken up in 5 ml H_2O and subsequently used for assay.

Duplicate samples of this preparation and glucose standards were assayed for glucose using the Worthington Glucostat Reagent Set (Worthington Biochemical Corporation).

Sucrose was determined by incubating samples with invertase and then assaying for glucose using the glucostat reagent set. By calculating the differences in glucose levels before and after invertase treatment, the sucrose content was determined.

One mg of invertase was dissolved in 1 ml of 0.1M acetate buffer at pH 4.6. A 0.5 ml sample from the ethanol extractions was incubated overnight with 0.5 ml of invertase in acetate buffer. After incubation, the pH was adjusted to pH 7 with 0.1N KOH. This preparation was then assayed for glucose using the Worthington Glucostat Reagent Set. The glucose value obtained before invertase incubation was then subtracted from the glucose value obtained after invertase treatment. Actual sucrose levels were obtained from interpolation of glucose levels observed after incubation of sucrose standards with invertase.

RESULTS AND DISCUSSION

Optimization of Procedures

The Hexokinase Assay

The procedure developed by Gots and Bessman (1973) was employed to assay hexokinase from crude extract. The radioassay displayed linear product formation with time for both yeast hexokinase (Fig. 3) and undialyzed crude extract hexokinase (Fig. 4). The foremost problem was that of achieving linearity between reaction velocity and increasing crude extract. A typical relationship between the two parameters using untreated crude extract is shown in Figure 5. The following discussion deals with the treatments attempted to achieve a linear relationship between the rate of glucose-6-phosphate formation and the quantity of crude extract analyzed.

One of the first items investigated was the radioassay itself. In the method, four portions of 4 ml 0.17M NH_4OH were used to wash unreacted ^{14}C-glucose from the Dowex resin. The washings removed over 99 percent of the applied radioactivity (Table 1). Addition of 4 ml of 1M glucose in 0.17M NH_4OH effectively stopped the hexokinase reaction, and no detectable differences in activity were observed for zero-time controls containing either no crude extract or varying amounts of crude extract. Thus, the washing system and zero-time controls were useable without modification.

The response observed in glucose-6-phosphate synthesis to increasing crude extract levels suggested that either an inhibitor might be present in the crude extract or that breakdown of glucose-6-phosphate was

FIGURE 3

Formation of glucose-6-phosphate with time catalyzed by yeast hexokinase.

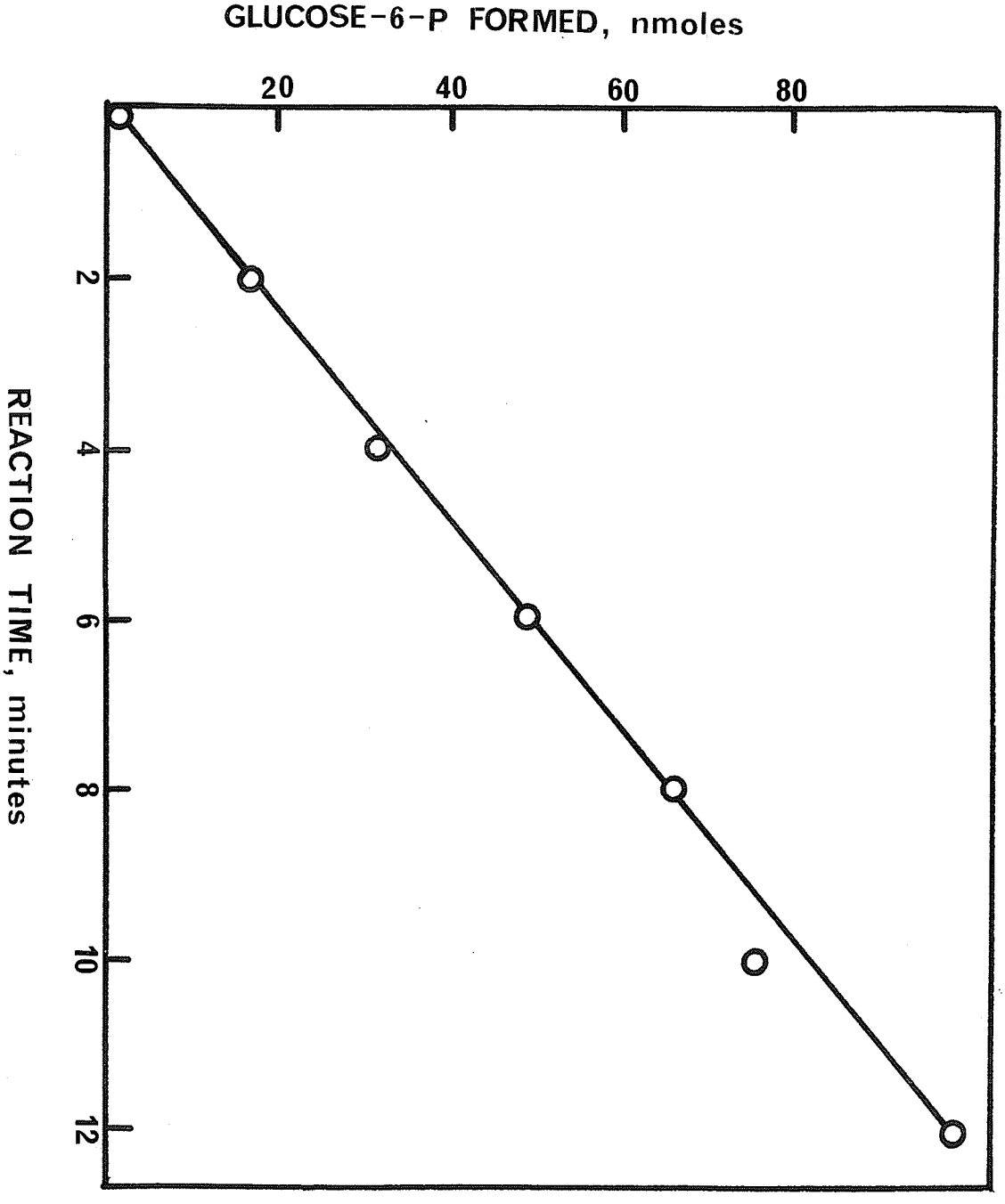

GLUCOSE-6-P FORMED, nmoles
20
40
60
80
REACTION TIME, minutes
2
4
6
8
10
12

FIGURE 4

Formation of glucose-6-phosphate with time catalyzed by hexokinase from undialyzed crude extract.

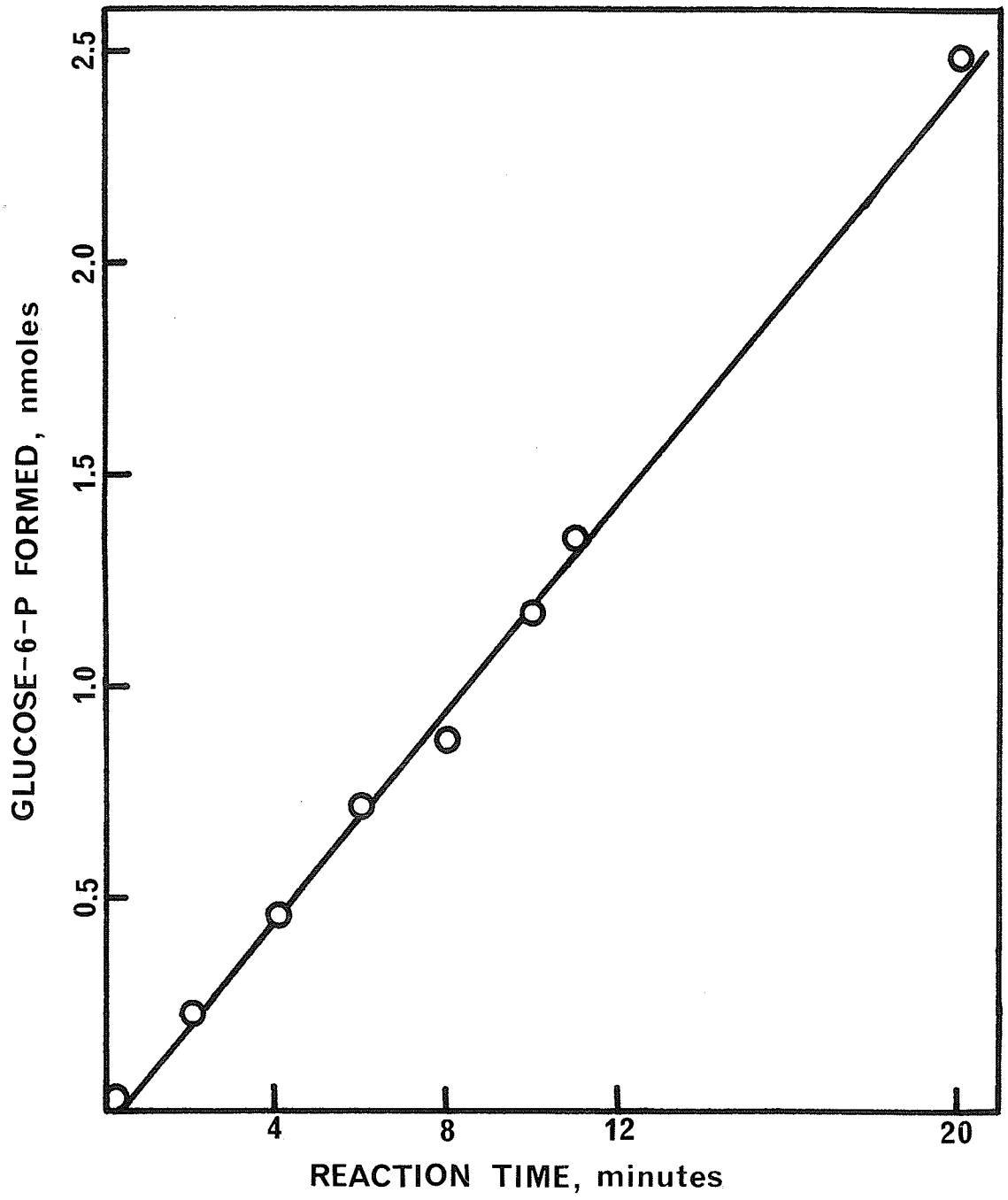

GLUCOSE-6-P FORMED, nmoles
0.5
1.0
1.5
2.0
2.5
4
8
12
20
REACTION TIME, minutes

FIGURE 5

Reaction velocity as a function of the volume of untreated crude extract hexokinase. Total reaction volume for each trial was 0.725 ml.

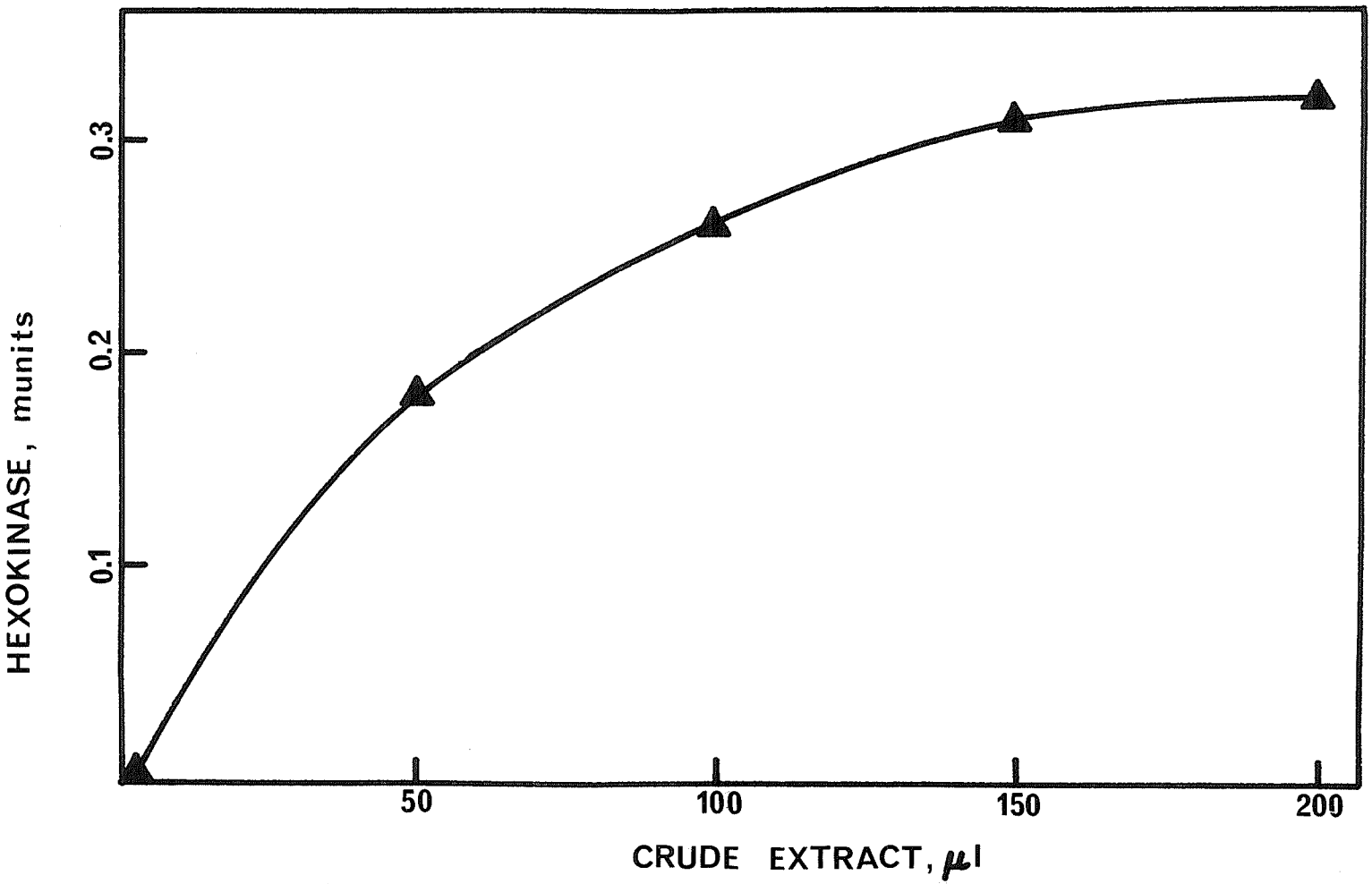

HEXOKINASE, munits
0.1
0.2
0.3
50
100
150
200
CRUDE EXTRACT, µl

TABLE 1. Effect of washing ^{14}C-glucose with 0.17M NH_4OH.

After a 15 minute incubation, the reaction catalyzed by hexokinase was terminated with 4.0 ml of 1.0M glucose in 0.17M NH_4OH. A series of 4 washes of 4 ml of 0.17M NH_4OH followed.

Total DPMs applied = 909,000

Fraction	Activity in effluent	
	DPMs	% of total activity applied
4.0 ml glucose in NH_4OH	754,000	84
1st wash of NH_4OH	129,000	14
2nd wash	12,600	1
3rd wash	3,360	< 1
4th wash	2,840	< 1
total	902,000	99

occurring from other enzymes in the extract.

Possibly, glucose-6-P breakdown was occurring because of a phosphatase. Cox and Dickinson (1973a) had already noted interference in hexokinase assays by phosphatase when using preparations from maize crude extract. Using the o-carboxyphenyl phosphate assay for alkaline phosphatase (Worthington Enzyme Manual, 1972) a linear relationship was established for crystalline enzyme from chicken intestine and for crude extract (undialyzed) from 18 day post-anthesis Manitou wheat (Fig. 6).

Using o-carboxyphenyl phosphate as substrate, a rate of 24 nmoles/minute/seed was observed in the absence of any exogenous inorganic phosphate. Using the usual extraction procedure for hexokinase with phosphate buffer, the reaction rate was 6 nmoles o-carboxyphenyl phosphate/minute/seed. Corresponding hexokinase activities were approximately 0.3 nmoles glucose-6-phosphate formed/minute/seed. Although the phosphatase and hexokinase activities were not directly comparable because of the different substrates employed, the results did indicate a substantial amount of phosphatase activity. Of interest, was the observation that the inorganic phosphate from the extraction buffer inhibited the phosphatase activity by 75 percent.

The assay of Mozersky *et al.* (1966) for inorganic phosphate was used to check phosphatase activity with glucose-6-phosphate as substrate and to confirm the results from the o-carboxyphenyl phosphate assay. The amount of inorganic phosphate liberated with time (Fig. 7) using crude extract and the reaction velocity versus crude extract volume (Fig. 8) were both shown to be linear. About 10 nmoles glucose-6-phosphate/minute/seed were hydrolyzed by endogenous phosphatase in the

FIGURE 6

Relationship between rate of o-carboxyphenyl phosphate hydrolysis and volume of undialyzed crude extract from kernels of 18 day post-anthesis Manitou wheat.

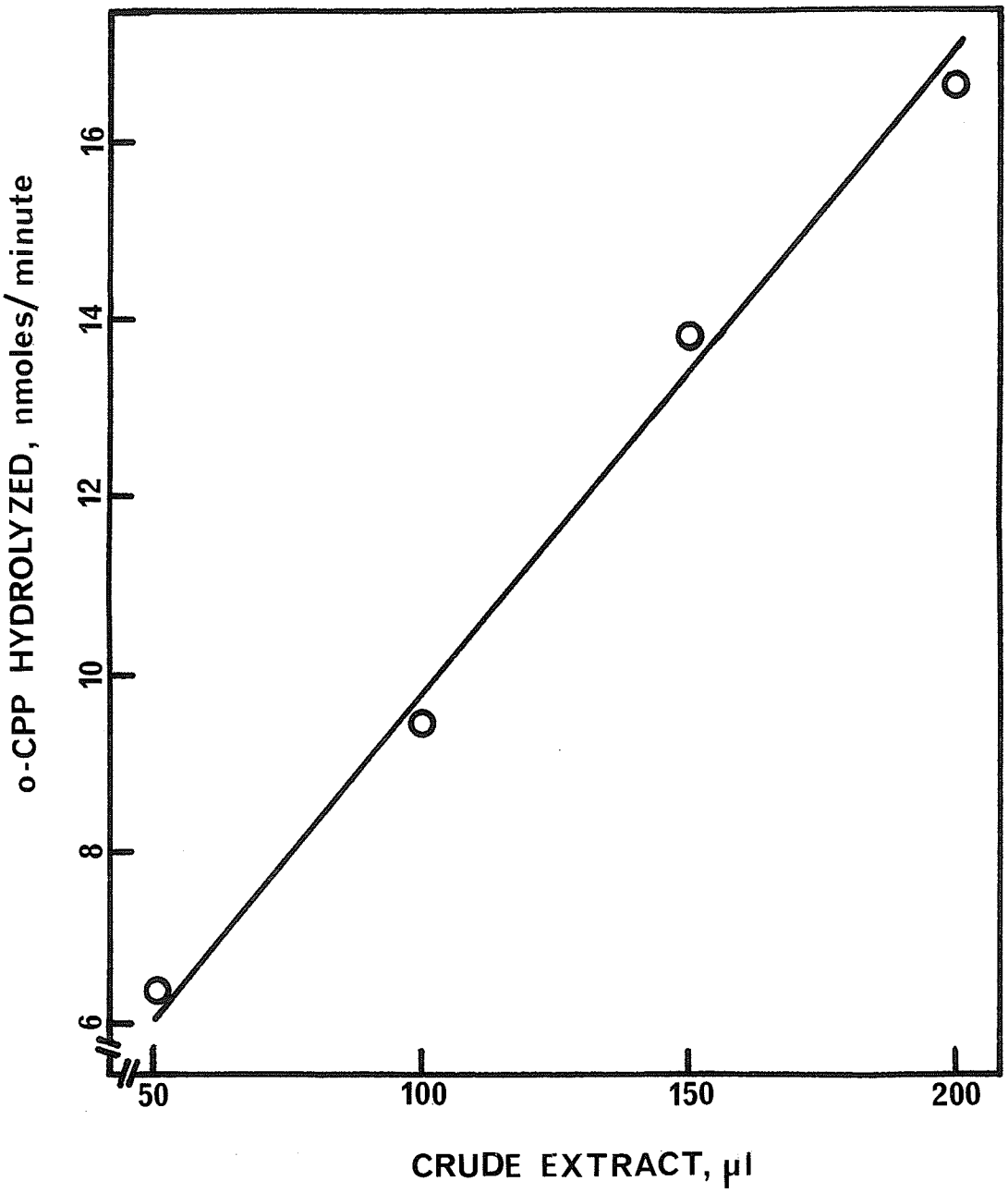

o-CPP HYDROLYZED, nmoles/minute
6
8
10
12
14
16
50
100
150
200
CRUDE EXTRACT, µl

FIGURE 7

Time course of the production of inorganic phosphate from glucose-6-P in the presence of crude extract from 18 day Manitou wheat kernels.

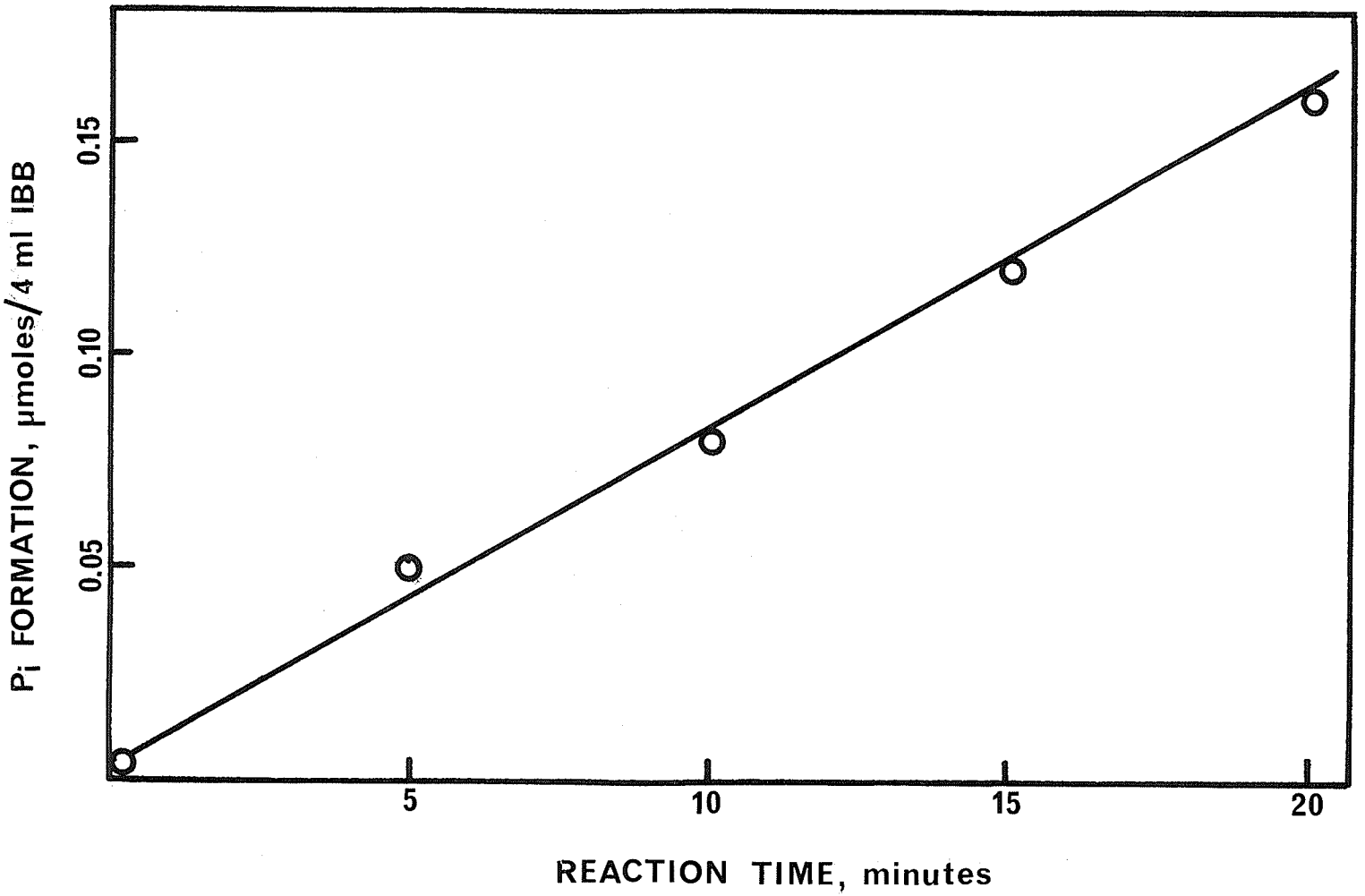
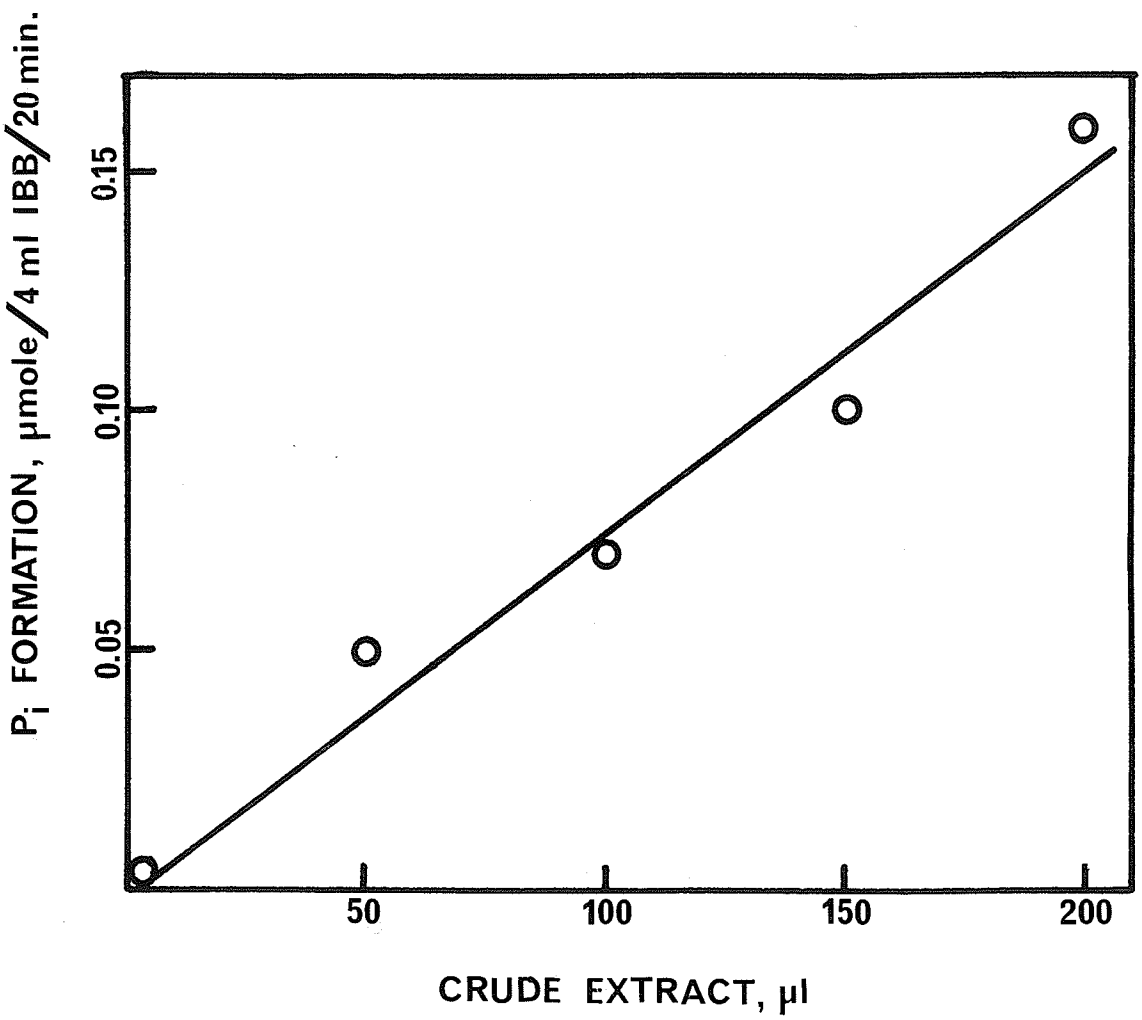

P$_i$ FORMATION, µmoles/4 ml IBB
0.05
0.10
0.15
5
10
15
20
REACTION TIME, minutes

FIGURE 8

Rate of P_i production from glucose-6-P in the presence of varying quantities of crude extract from 18 day post-anthesis Manitou wheat kernels.


P_i FORMATION, µmole/4 ml IBB/20 min.
0.05
0.10
0.15
50
100
150
200
CRUDE EXTRACT, µl

absence of added inorganic phosphate using this assay compared to 24 nmoles/minute/seed from the o-carboxyphenyl phosphate assay. Since both assays had indicated considerable phosphatase activity in comparison to glucose-6-phosphate formation via hexokinase, a search for an inhibitor of phosphatase commenced.

The alkaline phosphatase of chicken intestine has been reported to be a zinc metallo-enzyme (Worthington Enzyme Manual, 1972). When pre-incubated with crystalline chicken intestine phosphatase, 5 mM EDTA was found to inhibit activity by over 90 percent. The activity was restored by the addition of $ZnSO_4$. However, the addition of EDTA showed no effect in altering the rate of o-carboxyphenyl phosphate hydrolysis when crude extract was used. The effect of various compounds on phosphatase activity from crude extract is shown in Table 2. EDTA (2.3 mM) did not inhibit the phosphatase whereas pyrophosphate (2.1 mM) inhibited the hydrolysis of o-carboxyphenyl phosphate by crude extract by 42 percent. Inorganic phosphate (2.6 mM) and copper sulfate (36 mM) were the most effective inhibitors. Both compounds effectively inhibited phosphatase activity by over 80 percent. In these experiments, tris buffer was used instead of phosphate buffer so the only inorganic phosphate present initially was endogenous. Phosphate was chosen as an inhibitor because of its effective inhibition at low concentration and because it was already a component of the extraction media. Table 3 demonstrates that greater than 90 percent inhibition of phosphatase was obtained with concentrations of 12 mM phosphate. No phosphatase activity was observed at 67 mM phosphate. Table 4 illustrates that hexokinase activity was affected by varying the concentration of inorganic phosphate. For convenience, a concentration of 65 mM was chosen since it probably inhibited

TABLE 2. The effect of various chemicals on alkaline phosphatase activity from crude extract.

Inhibitor	Final concentration (mM)	Inhibition of alkaline phosphatase (%)
EDTA	2.3	0
Pyrophosphate	2.1	42
Phosphate	2.6	80
$CuSO_4$	36	83

TABLE 3. Inhibition of phosphatase from undialyzed crude extract by inorganic phosphate using o-carboxyphenyl phosphate as substrate.

Final P_i concentration (mM)	Phosphatase activity (nmol/min)	Inhibition (%)
0*	17.2	-
12	1.03	94
24	0.5	97
46	0.2	99
67	0	100

*Tris buffer used.

TABLE 4. Effect of inorganic phosphate on hexokinase activity from undialyzed crude extract.

Final P_i concentration (mM)	Hexokinase activity	
	munits	Increase (%)
0*	0.45	-
50	0.62	38
99	0.54	20
124	0.51	13

*Tris buffer used.

phosphatase completely and was in the range of concentrations of inorganic phosphate which elevated hexokinase activity. However, when the relationship between velocity and enzyme concentration was observed using 65 mM phosphate in the reaction mixture, a hyperbolic curve was again obtained. Because it was found that inclusion of phosphate inhibited phosphatase activity in the crude extracts, 65 mM P_i was used in all hexokinase assays in spite of its inability to correct the observed non-linear relationship.

In order to remove possible low molecular weight inhibitors, the crude extract was dialyzed. Using the dialyzed extract, the plot of glucose-6-phosphate formed versus volume of crude extract was linear suggesting that dialysis did remove a small molecular weight inhibitor(s) (Fig. 9).

Further treatments of the crude extract (Table 5) showed this molecule to be heat stable and probably anionic. Crude extract treated with either an anion or combined anion-cation exchanger partially removed component(s) that were inhibitory. Addition of either extract to dialyzed extract yielded 80 percent of the activity expected assuming total removal of the inhibitor from the dialyzed extract (Table 5, treatments 5 and 6). The addition of undialyzed boiled extract to dialyzed extract yielded only 40 percent of the expected value. These results illustrate the partial removal of inhibitor by ion exchange whereas boiling the extract failed to alter the inhibition.

To separate the crude extract hexokinase and inhibitor, gel filtration was used. Two peaks of absorption at 280 nm were seen as the elution proceeded. The first peak displayed hexokinase activity, and the second peak contained the inhibitor. A 5 drop addition from the

FIGURE 9

Effect of dialysis on the rate of glucose-6-P formation as a function of crude extract volume. Total reaction volume for each trial was 0.725 ml.

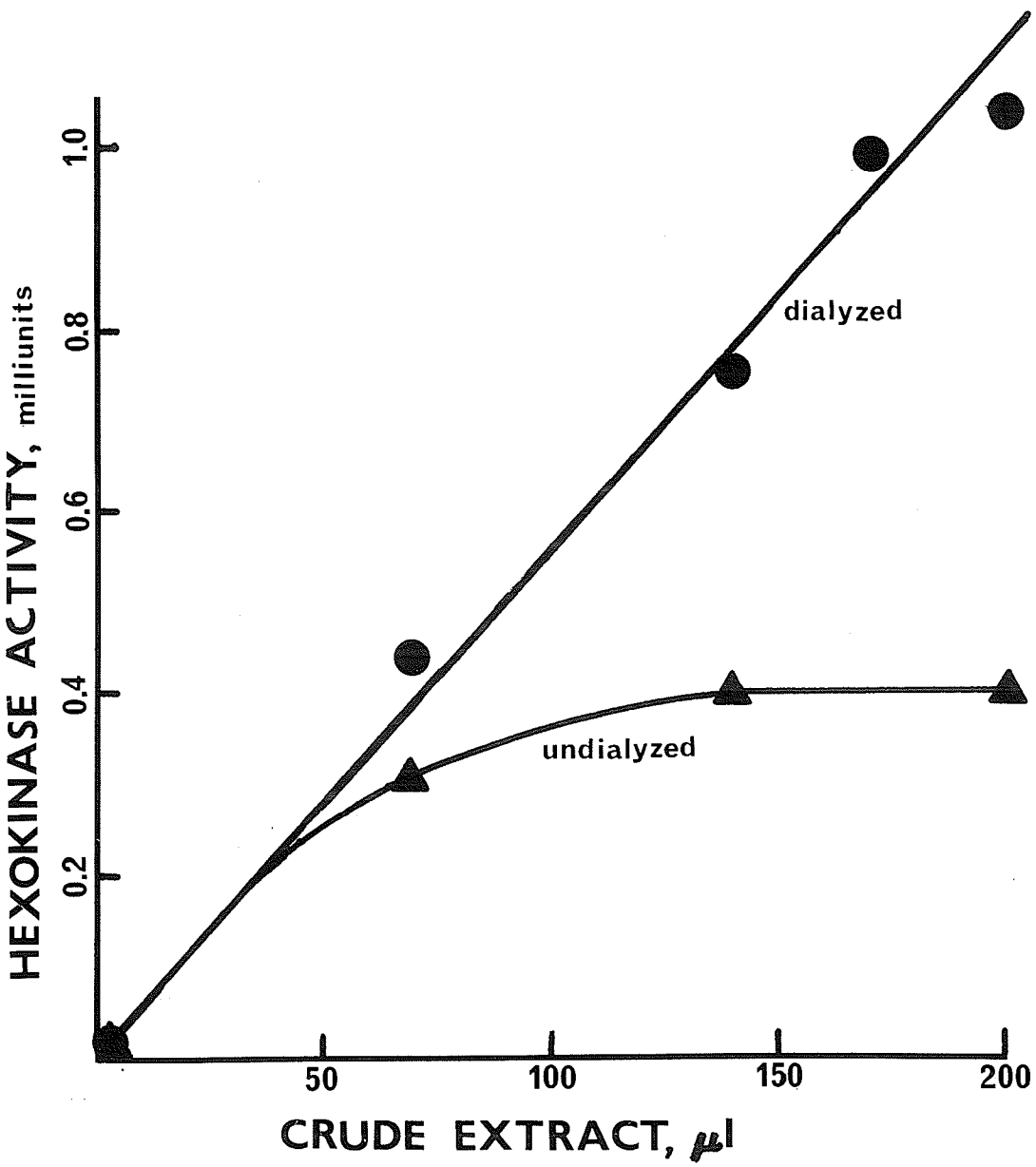

HEXOKINASE ACTIVITY, milliunits
0.2
0.4
0.6
0.8
1.0
dialyzed
undialyzed
50
100
150
200
CRUDE EXTRACT, µl

TABLE 5. Hexokinase activity of crude extract after the following treatments.

Condition	Observed relative activity	Expected relative activity
1. Dialyzed crude extract	1.0	-
2. Undialyzed crude extract after passing through AGIIA8 (cation-anion exhchanger)	0.44	-
3. Undialyzed crude extract after passing through Dowex 2X-8 (anion exhanger)	0.39	-
4. Boiled undialyzed crude extract	0.0	-
5. Equal volumes #1 and 2	0.57	$\frac{1.0 + 0.44}{2} = 0.72$
6. Equal volumes #1 and 3	0.56	0.70
7. Equal volumes #1 and 4	0.20	0.50

second peak sample to 1.0 ml of the first peak eluant showed an inhibition of 20 percent. A wavelength scan of the trailing peak was identical to ones observed for adenine nucleotides.

ADP has been shown to be inhibitory in mammalian systems (Colowick, 1973). Glucose-6-phosphate is also a strong inhibitor of mammalian hexokinase, and the inhibition is relieved by P_i (Turner and Turner, 1975) as is the case with crude extract hexokinase (Table 4). Either of these molecules would be anionic above pH 7 and could function as the inhibitor. The main objective was to alleviate hexokinase inhibition, and this was accomplished by dialysis.

With the inhibitor removed by dialysis, the most active preparation of hexokinase, 6A250 42 days post-anthesis, exhibited linear reaction velocity with crude extract volume for both ^{14}C-glucose and ^{14}C-fructose as substrate up to 200 µl of crude extract volume (Fig. 10). Varying the substrate glucose on reaction velocity gave an apparent K_m of 1.6×10^{-4}M (Fig. 11). This value is of the same magnitude as other commonly reported values which range from 1 to 4×10^{-4}M for hexokinase from mammalian and yeast sources (Gots and Bessman, 1973).

These results along with the effect of inhibitor removal by dialysis permitted the further investigation of hexokinase activities with respect to kernel development.

Distribution and Stability of Enzyme Activity

Hexokinase. Table 6 illustrates the distribution of hexokinase from crude extract between the supernatant and pellet. The resuspended pellet contained 17 percent of the total activity (dithiothreitol dialyzed

FIGURE 10

Rate of glucose-6-P and fructose-6-P formation with crude extract volume using the most active dialyzed crude extract (6A250 42 day post-anthesis). Both ^{14}C-glucose and ^{14}C-fructose were employed as substrates. Total reaction volume for each trial was 0.725 ml.

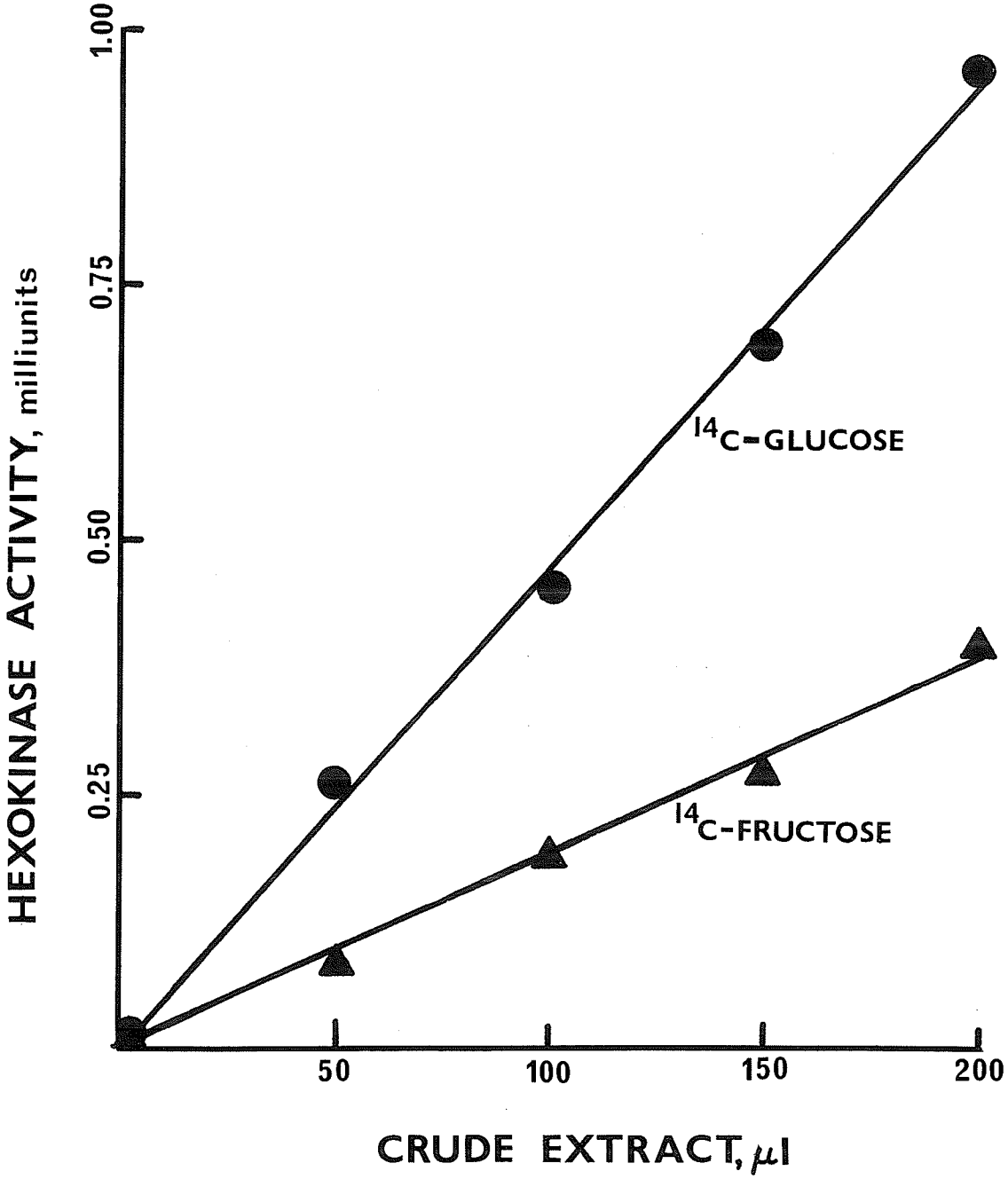

HEXOKINASE ACTIVITY, milliunits
1.00
0.75
0.50
0.25
^{14}C-GLUCOSE
^{14}C-FRUCTOSE
50
100
150
200
CRUDE EXTRACT, µl

FIGURE 11

Effect of varying substrate concentration on rate of product formation catalyzed by crude extract hexokinase. For each substrate concentration, the reaction was initiated by the addition of 100 µl of crude extract from 42 day post-anthesis Stewart 63.

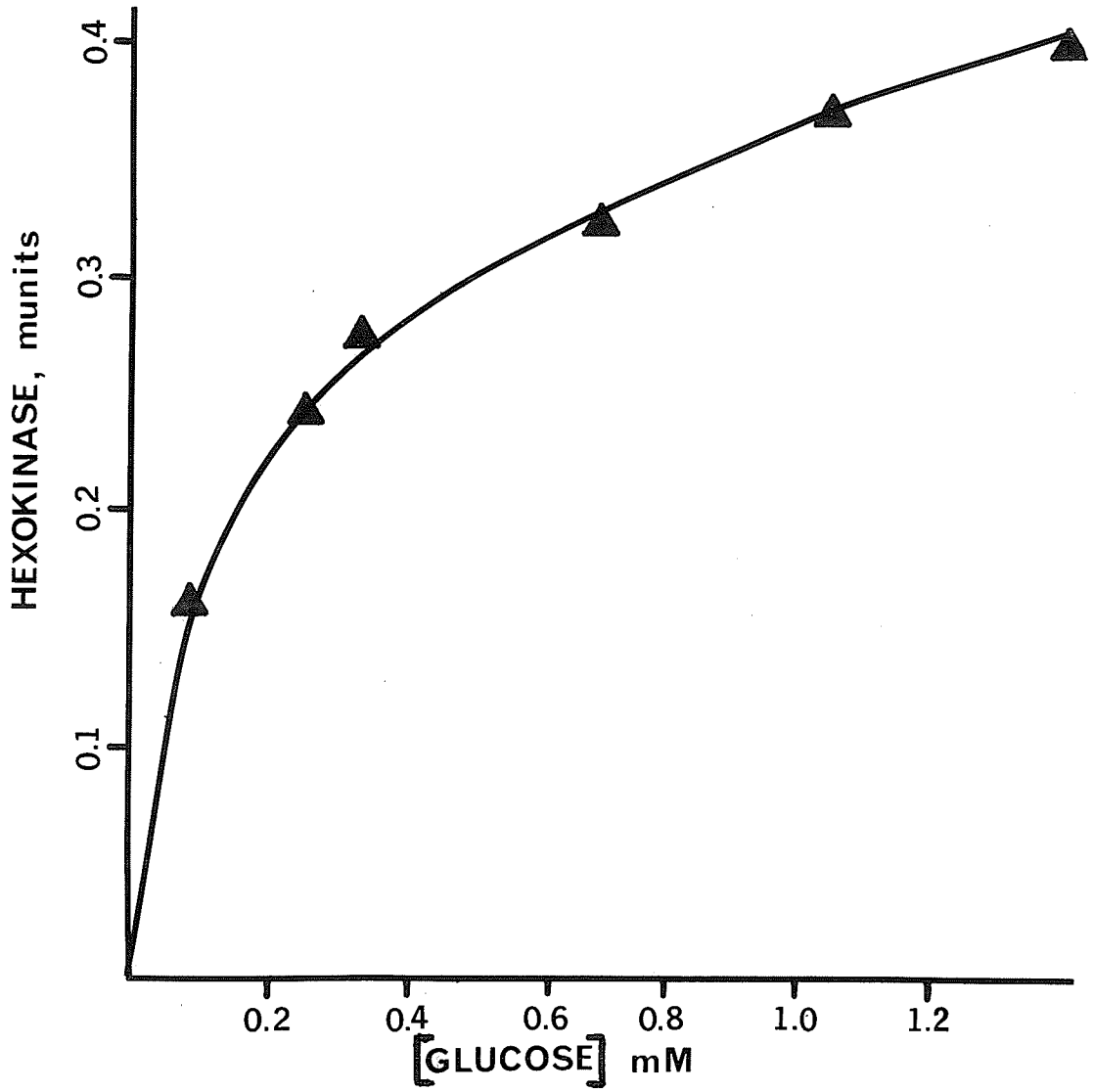

HEXOKINASE, munits
0.1
0.2
0.3
0.4
0.2
0.4
0.6
0.8
1.0
1.2
[GLUCOSE] mM

TABLE 6. Distribution and stability of hexokinase activity in various crude extract fractions.

Fraction	Activity (munits/seed)	Relative activity
Supernatant (dialyzed in presence of 5 mM DDT)	1.60	1.0
Supernatant (dialyzed without DTT in dialysis media)	1.13	0.71
Supernatant (dialyzed with DTT but assayed 48 hours later)	1.51	0.94
Resuspended 10,000 g pellet	0.34	0.21

supernatant activity plus pellet activity). This may affect the interpretation of the present results since maize endosperm was found to increase somewhat in soluble hexokinase with kernel development. The soluble extract, however, was considered to be indicative of endosperm activity (Cox and Dickinson, 1973b).

The absence of dithiothreitol in the dialysis media caused loss of hexokinase activity from crude extract by 42 percent (Table 6). Presumably, the dithiothreitol prevented the oxidation of the sulfhydryl groups of hexokinase thereby lessening enzyme inactivation.

Table 6 also gives an indication of crude extract hexokinase stability. After 48 hours storage at 0-4^{o}C, the crude extract lost only 6 percent hexokinase activity.

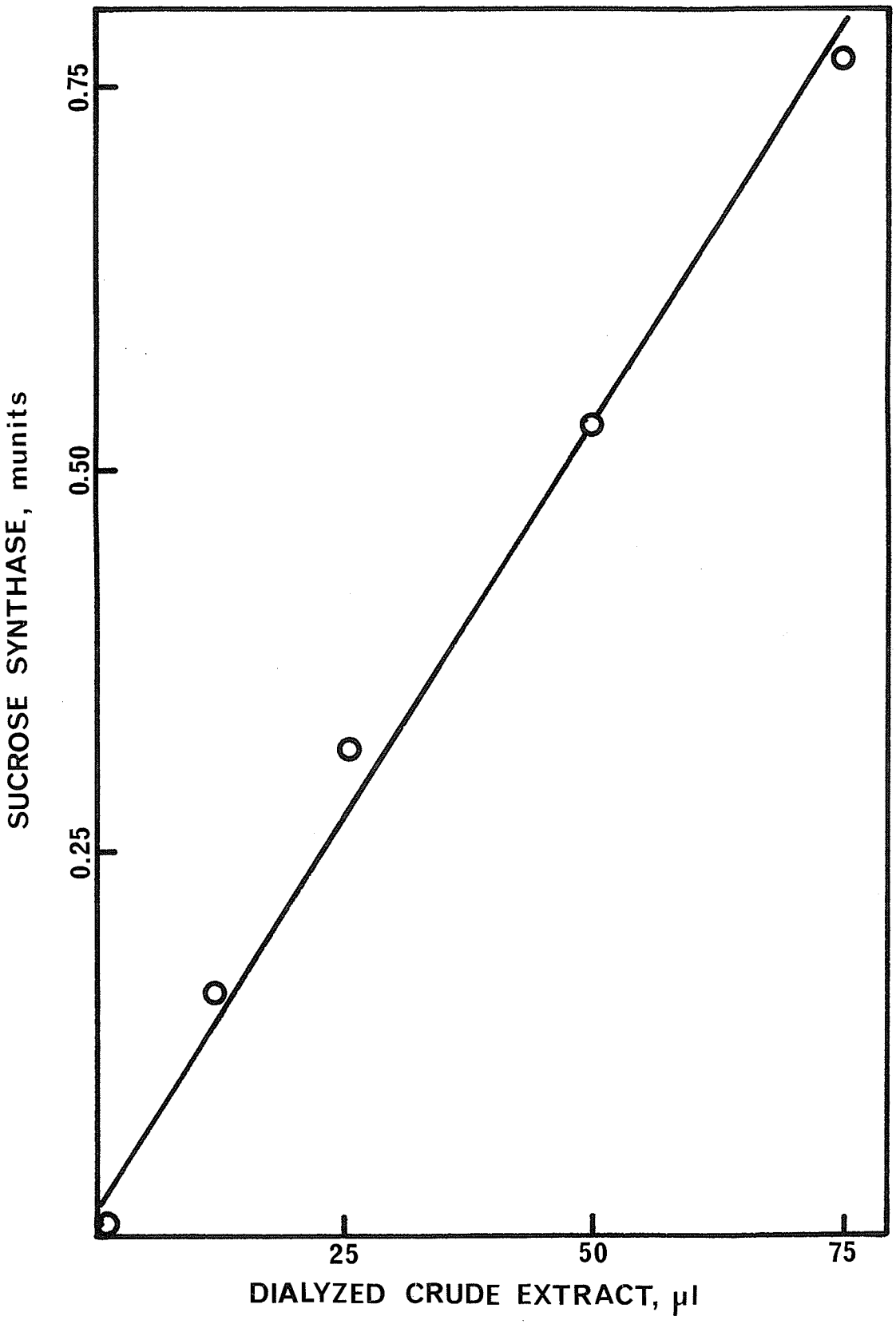
Sucrose synthase. There was a linear relationship between sucrose synthase reaction velocity and increasing crude extract volume after dialysis (Fig. 12).

Sucrose synthase was extremely stable with time after dialysis. The dialyzed crude extract when left at room temperature for 18 hours retained full activity. However, dithiothreitol was required in the dialysis media to retain maximal activity. Experiments where crude extract was dialyzed in the absence and presence of 5 mM dithiothreitol showed 42 percent inactivation in the absence of dithiothreitol. Varying the concentration of dithiothreitol from 1 to 5 mM in the dialysis media had no effect on sucrose synthase activity.

Assaying the resuspended 10,000 g pellet was not possible using this assay because the addition of the turbid suspension to the small volumes in the spectrophotometric cells resulted in non-quantitative results.

FIGURE 12

Rate of sucrose conversion to UDP glucose with extract volume. The source of sucrose synthase was dialyzed crude extract from 18 day post-anthesis Manitou.


SUCROSE SYNTHASE, munits
0.25
0.50
0.75
25
50
75
DIALYZED CRUDE EXTRACT, µl

Overall, both hexokinase and sucrose synthase from crude extract were found to be quite stable especially when dialyzed in the presence of dithiothreitol.

Dry Weight and Fresh Weight

The dry weight of all the cereals investigated increased linearly from 6 days to approximately 30 days post-anthesis (Figs. 13-17). The dry weight increase for Manitou halted at 26 days while 6A190 and Prolific rye ceased dry matter accumulation at 33 and 34 days, respectively. Stewart 63 and 6A250 no longer increased in dry weight after 30 days post-anthesis. Although all dry matter accumulation ceased around 30 days, the difference between 6A190 and Manitou seems large enough to suggest that starch deposition ends earlier in Manitou than in the shrivelled triticale, 6A190. The duration of starch synthesis then may not be the limiting factor for normal kernel development in 6A190.

A slight loss in dry weight occurred in all of the cereals after the maximum dry weight had been attained. Possibly, an export of substrates of starch synthesis occurred after the synthetic processes had ceased functioning.

The same cereals all displayed an initially fast increase in fresh weight up to 14 days post-anthesis, but patterns varied considerably thereafter (Figs. 18-22). For example, Manitou and 6A250 began dessicating at 26 days and 30 days post-anthesis, respectively. Prolific rye and 6A190 both started losing moisture by 22 days after flowering. In 6A250 and Manitou, the decline in fresh weight coincided with the cessation of dry weight accumulation. The other cereals did not display

FIGURE 13

Dry weight accumulation with kernel development for Manitou wheat.

FIGURE 14

Dry weight accumulation with kernel development for 6A190 triticale.

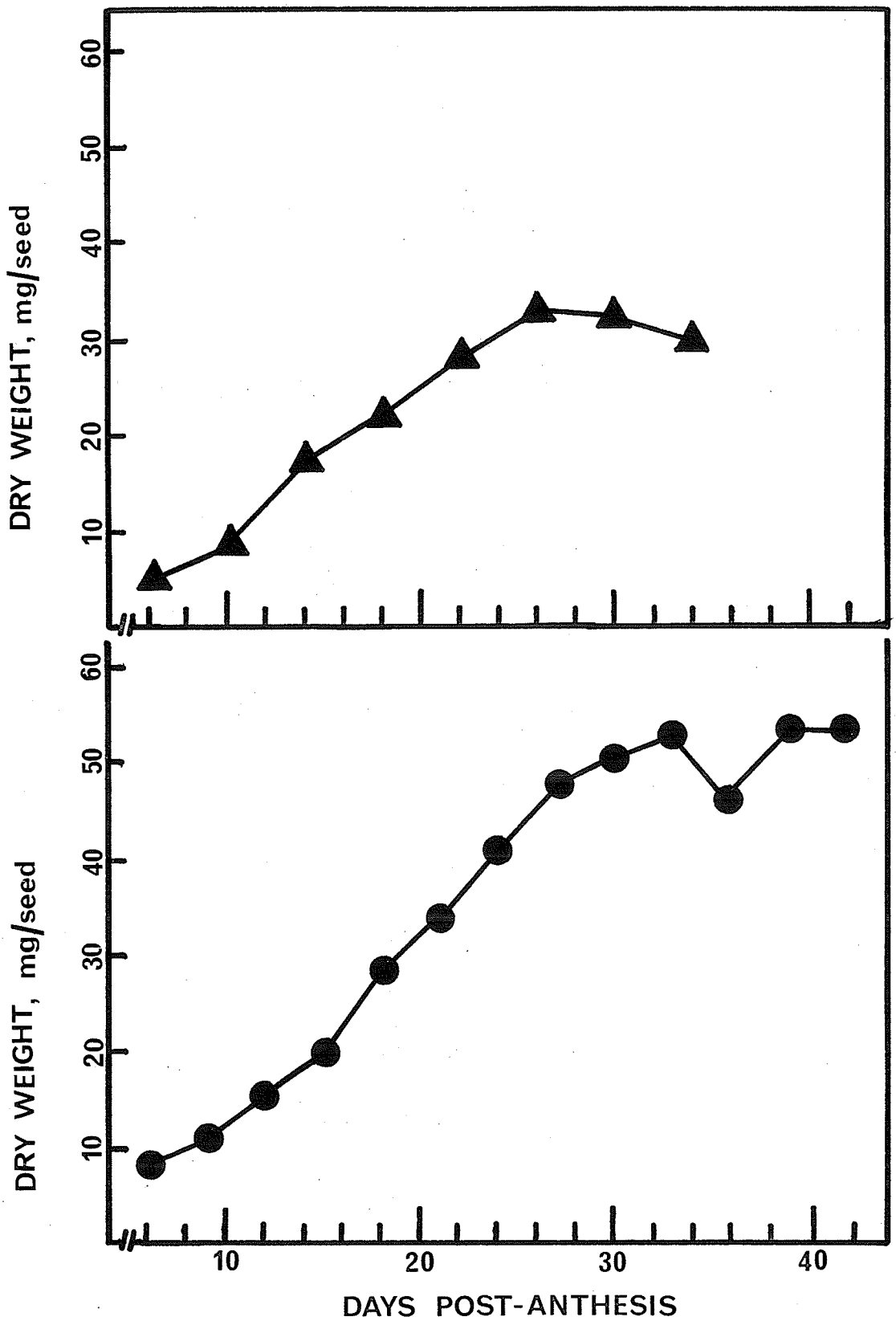

DRY WEIGHT, mg/seed
10
20
30
40
50
60
DRY WEIGHT, mg/seed
10
20
30
40
50
60
10
20
30
40
DAYS POST-ANTHESIS

FIGURE 15

Dry weight accumulation with kernel development for 6A250 triticale.

FIGURE 16

Dry weight accumulation with kernel development for Stewart 63 wheat.

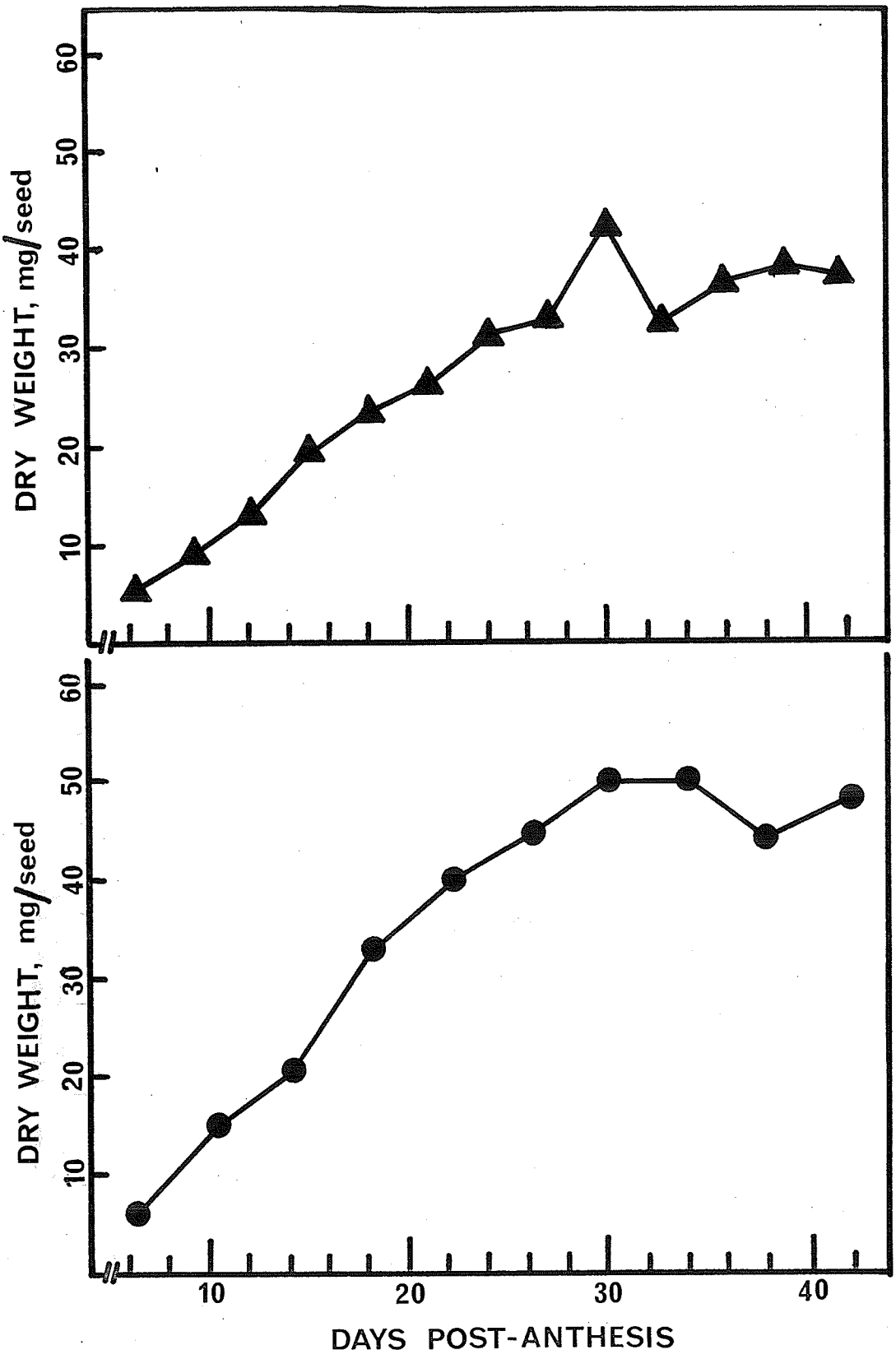

DRY WEIGHT, mg/seed
10
20
30
40
50
60
DRY WEIGHT, mg/seed
10
20
30
40
50
60
10
20
30
40
DAYS POST-ANTHESIS

FIGURE 17

Dry weight accumulation with kernel development for Prolific rye.

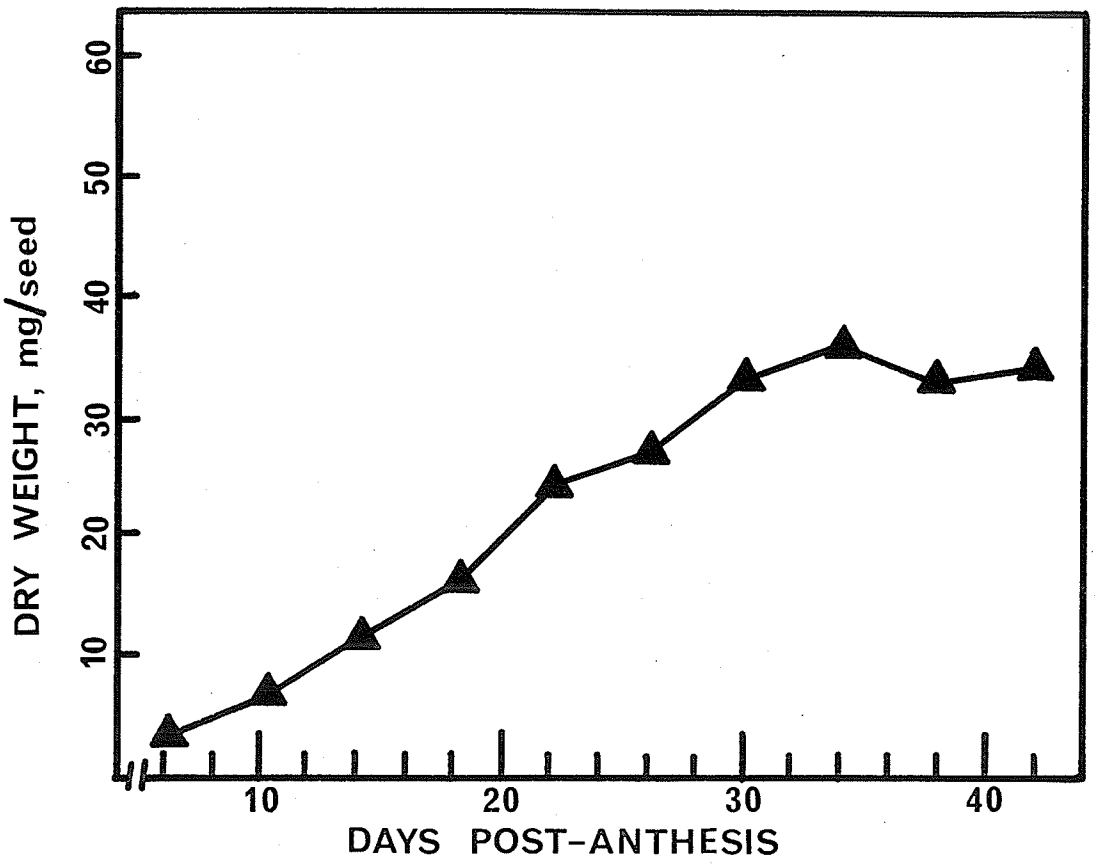

DRY WEIGHT, mg/seed
10
20
30
40
50
60
10
20
30
40
DAYS POST-ANTHESIS

FIGURE 18

Fresh weight change with kernel development for Manitou wheat.

FIGURE 19

Fresh weight change with kernel development for 6A190 triticale.

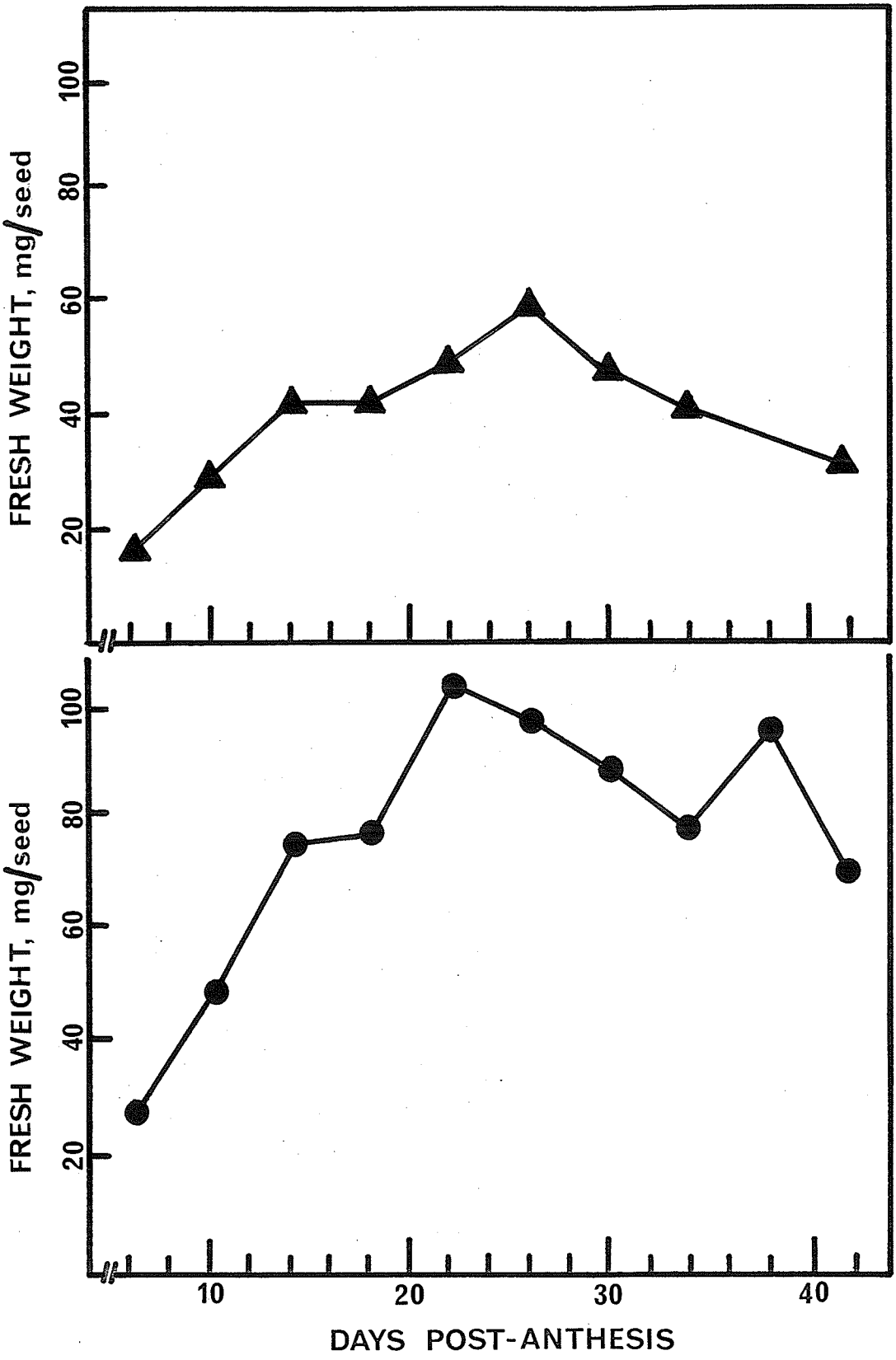

FRESH WEIGHT, mg/seed
20
40
60
80
100
FRESH WEIGHT, mg/seed
20
40
60
80
100
10
20
30
40
DAYS POST-ANTHESIS

FIGURE 20

Fresh weight change with kernel development for 6A250 triticale.

FIGURE 21

Fresh weight change with kernel development for Stewart 63 wheat.

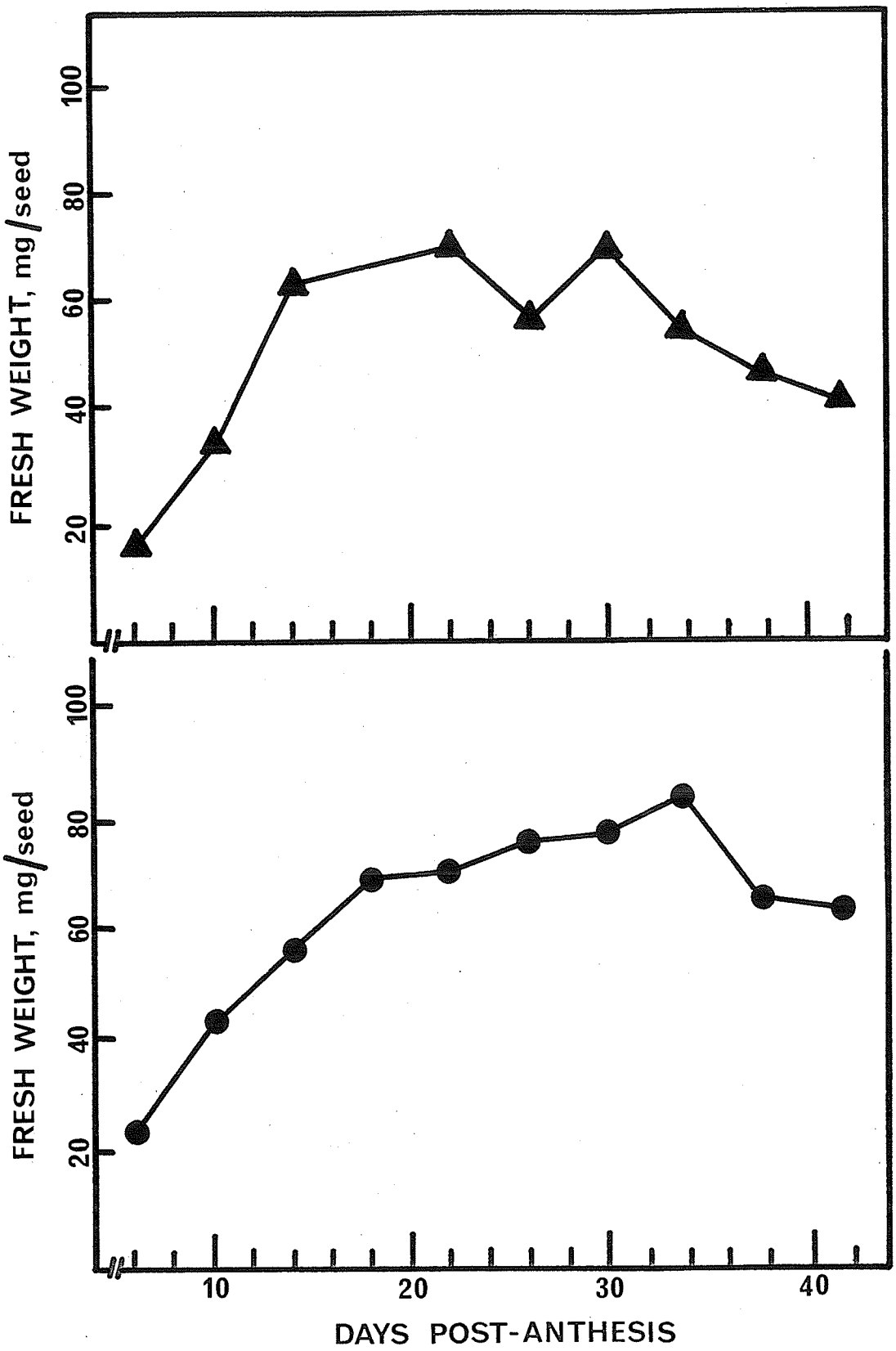
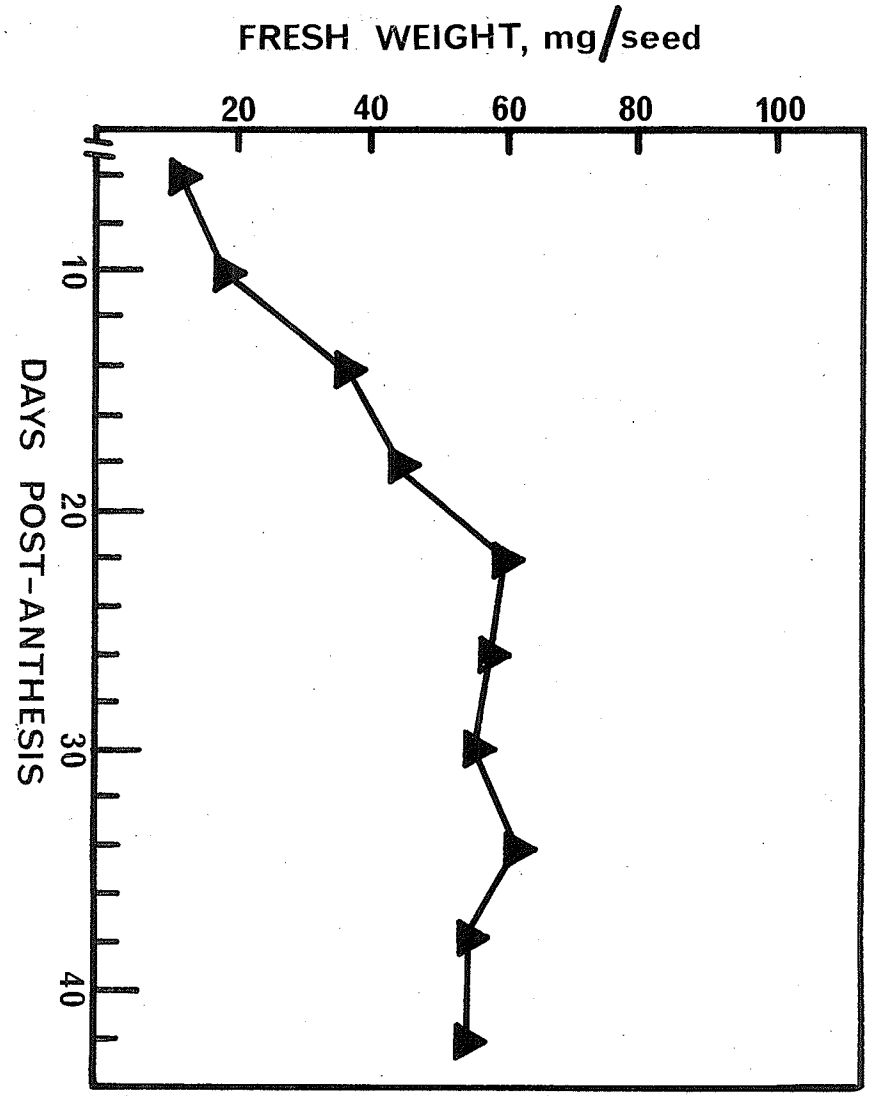

FRESH WEIGHT, mg/seed
100
80
60
40
20
FRESH WEIGHT, mg/seed
100
80
60
40
20
10
20
30
40
DAYS POST-ANTHESIS

FIGURE 22

Fresh weight change with kernel development for Prolific rye.


FRESH WEIGHT, mg/seed
20
40
60
80
100
10
20
30
40
DAYS POST-ANTHESIS

this relationship. If any correlation between dry weight gain and fresh weight exists, it does not operate uniformly in the cereals observed.

Soluble Protein

Up to 18 days post-anthesis, soluble protein levels increased rapidly in all of the cereals tested (Figs. 23-27). Stewart 63 and 6A190 displayed slight decreases in soluble protein at about 22 days, after which 6A190 increased to maximum at 38 days and Stewart 63 climbed to a maximum which lasted from 26 to 34 days post-anthesis. Both decreased after their respective maxima. Following the initial increases, Prolific and 6A250 also decreased in protein content (after 22 days post-anthesis) and subsequently increased to their maxima. Manitou displayed the same pattern, but the decrease occurred after 26 days. This initial maximum did not seem to be correlated with any of the other parameters tested. With maturation (42 days post-anthesis), all of the cereals showed either a levelling off or a decrease in soluble protein levels.

The maximum soluble protein levels (Figs. 23, 25, 27) were coincident with the cessation of dry matter accumulation in Manitou, 6A250, and Prolific (Figs. 13, 15, 17). Dry weight no longer increased after 30 days in Stewart 63 (Fig. 16) whereas the maximum protein level for this cereal occurred at 26 days (Fig. 26). 6A190 displayed the reverse situation. Dry weight gain (Fig. 14) levelled off at 33 days post-anthesis, but the protein level (Fig. 24) increased until 38 days. If the increases in soluble protein were due mostly to the synthesis of enzymic protein, it might be expected that the cessation of dry

FIGURE 23

Changes in soluble protein with kernel development for Manitou wheat.

FIGURE 24

Changes in soluble protein with kernel development for 6A190 triticale.

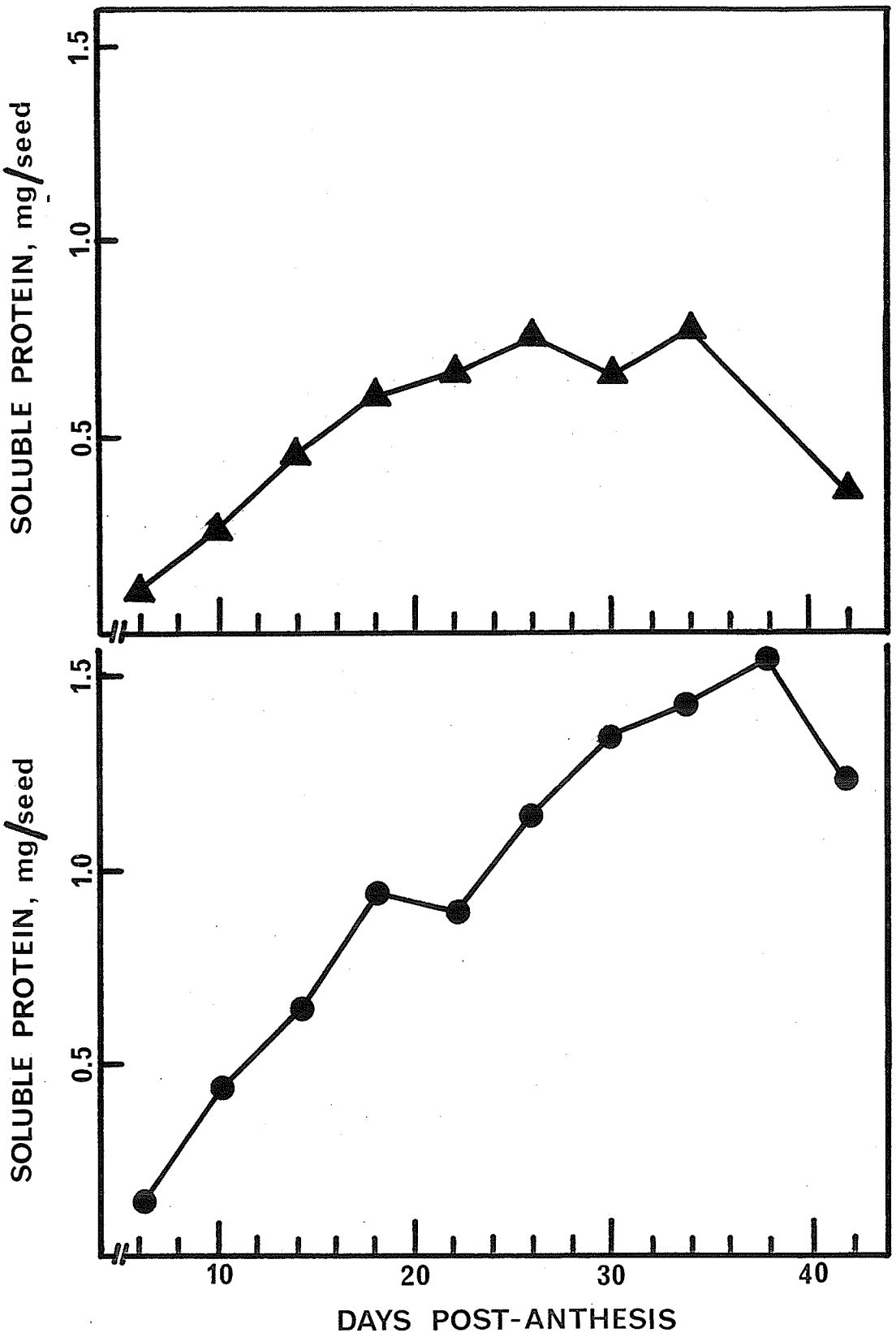

SOLUBLE PROTEIN, mg/seed
1.5
1.0
0.5
SOLUBLE PROTEIN, mg/seed
1.5
1.0
0.5
10
20
30
40
DAYS POST-ANTHESIS

FIGURE 25

Changes in soluble protein with kernel development for 6A250 triticale.

FIGURE 26

Changes in soluble protein with kernel development for Stewart 63 wheat.

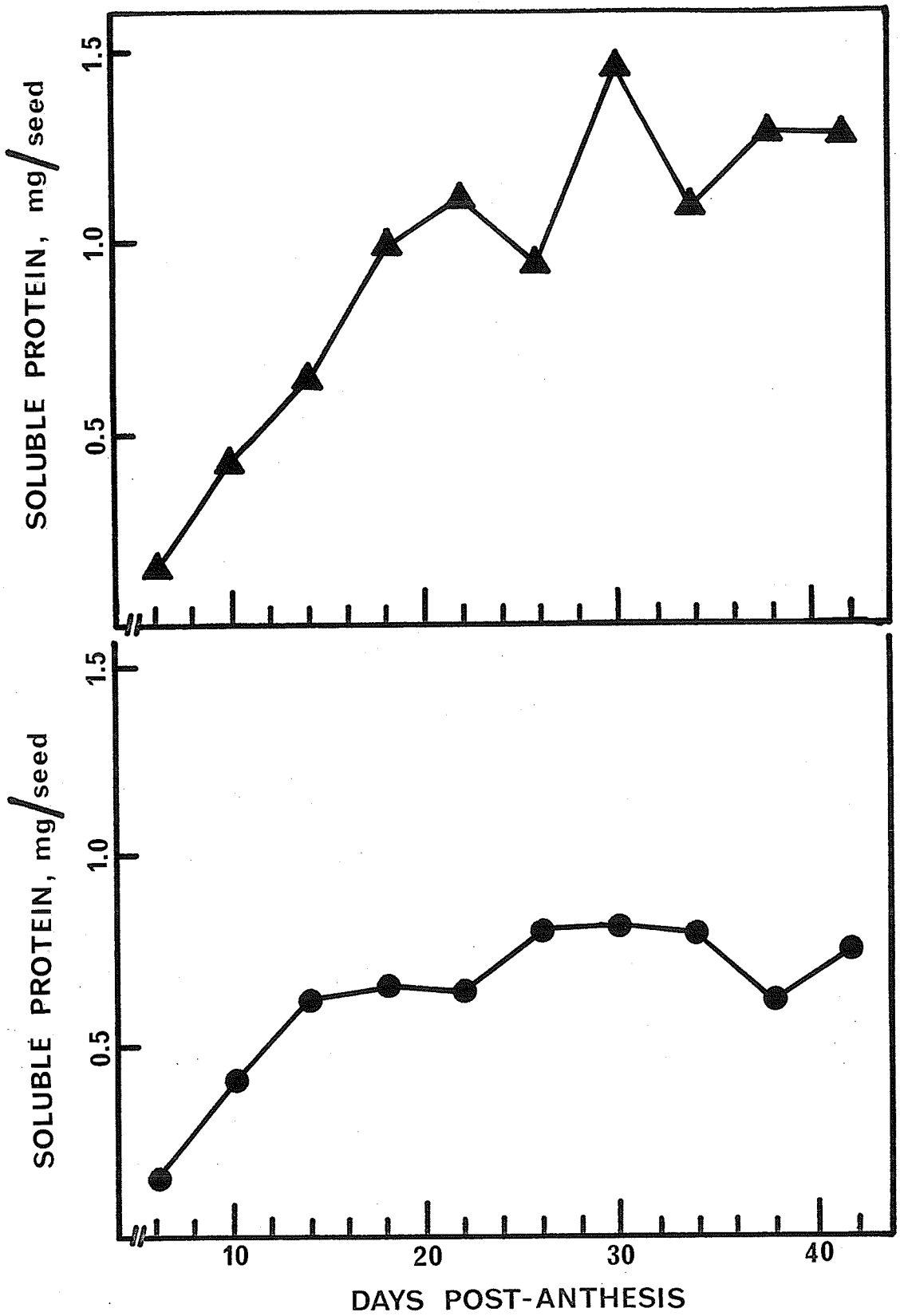
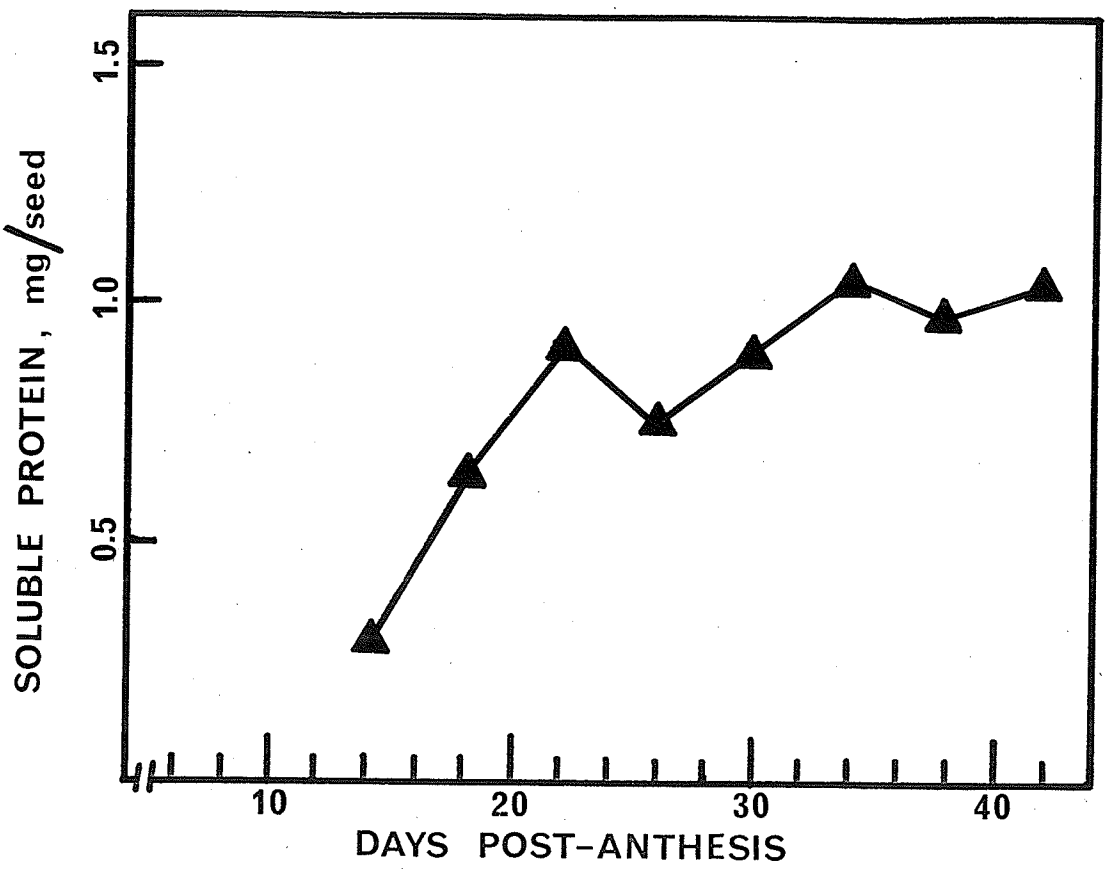

SOLUBLE PROTEIN, mg/seed
1.5
1.0
0.5
SOLUBLE PROTEIN, mg/seed
1.5
1.0
0.5
10
20
30
40
DAYS POST-ANTHESIS

FIGURE 27

Changes in soluble protein with kernel development for Prolific rye.


SOLUBLE PROTEIN, mg/seed
0.5
1.0
1.5
10
20
30
40
DAYS POST-ANTHESIS

matter accumulation would mark the end of protein synthesis as may be the case in Manitou, Prolific and 6A250. With 6A190, the apparent escalation in soluble protein after the dry weight has stopped increasing may reflect the production of enzymic protein α-amylase which is present at relatively high levels at maturity (Dedio *et al.*, 1975).

Sucrose Synthase and Sucrose

Sucrose synthase levels generally increased very rapidly during early kernel development in all of the cereals tested (Figs. 28A - 32A). By 14 days post-anthesis, the activities were near their maximum values, with the exception of 6A190. Sucrose synthase levels then remained at high levels for approximately 14 days until activities began to decline. This decrease was coincident with the termination of dry matter accumulation (Figs. 13-17). Also, the period of high sucrose synthase levels occurred at the time when the rate of dry weight increase was the greatest. Since Klassen (1970) had demonstrated the close relationship between starch accumulation and dry matter production in triticale, it seems likely from these results that sucrose synthase activity may have a significant role in the regulation of starch synthesis during kernel development.

As sucrose synthase activities climbed to high levels at 14 days post-anthesis, sucrose levels increased as well (Figs. 28B - 32B). However, the sucrose concentrations fell shortly after 10 days reflecting the increasing presence of sucrose synthase and the commencement of rapid dry matter accumulation. All of the cereals displayed a low for sucrose at 18 days post-anthesis followed by fluctuations in the levels. Except for the early development stages, sucrose levels

FIGURE 28A

Changes in hexokinase and sucrose synthase activity with kernel development for Manitou wheat.

FIGURE 28B

Changes in glucose and sucrose levels with kernel development for Manitou wheat.

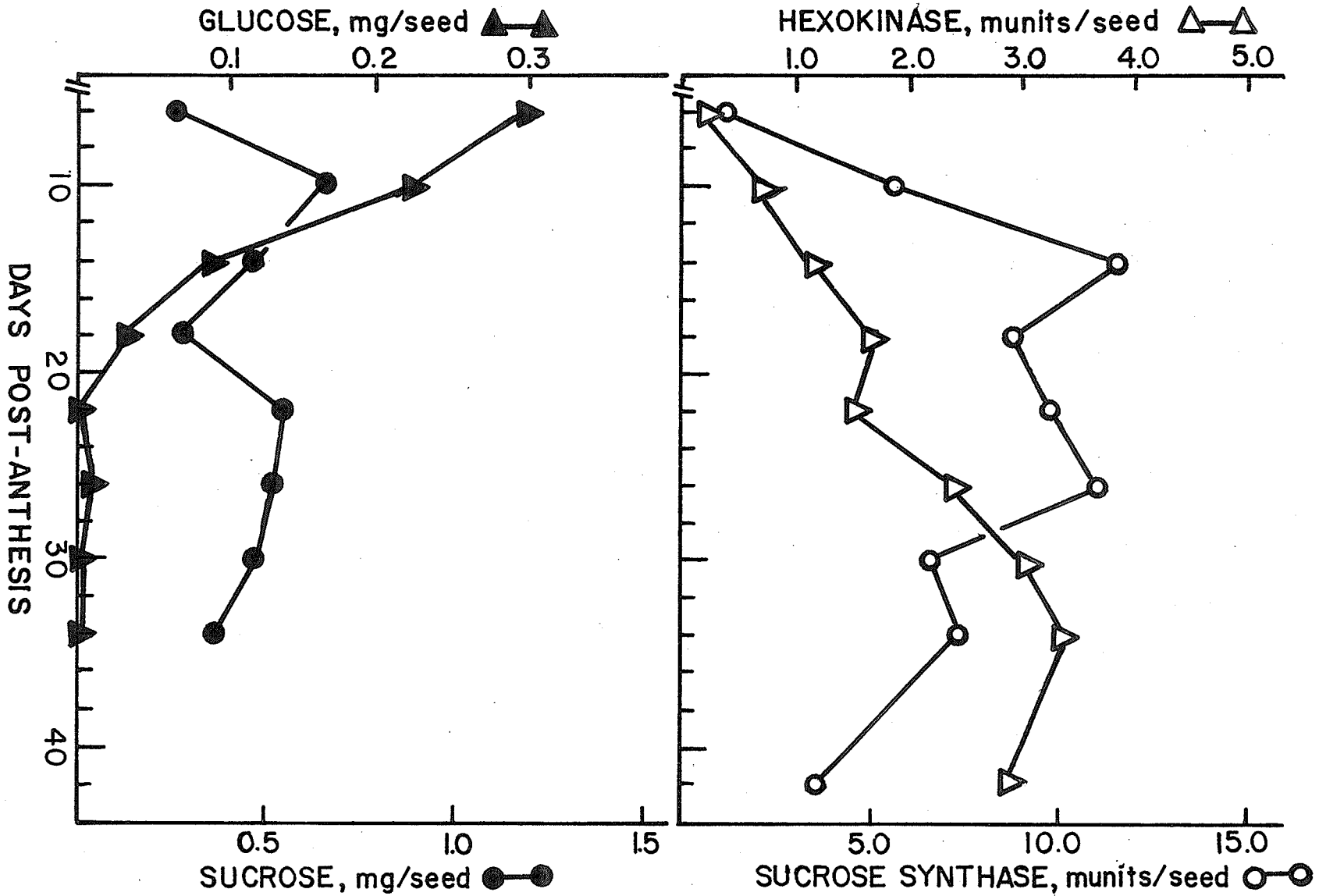

GLUCOSE, mg/seed
0.1
0.2
0.3
HEXOKINASE, munits/seed
1.0
2.0
3.0
4.0
5.0
DAYS POST-ANTHESIS
10
20
30
40
0.5
1.0
1.5
SUCROSE, mg/seed
5.0
10.0
15.0
SUCROSE SYNTHASE, munits/seed

FIGURE 29A

Changes in hexokinase and sucrose synthase activity with kernel development for 6A190 triticale.

FIGURE 29B

Changes in glucose and sucrose levels with kernel development for 6A190 triticale.

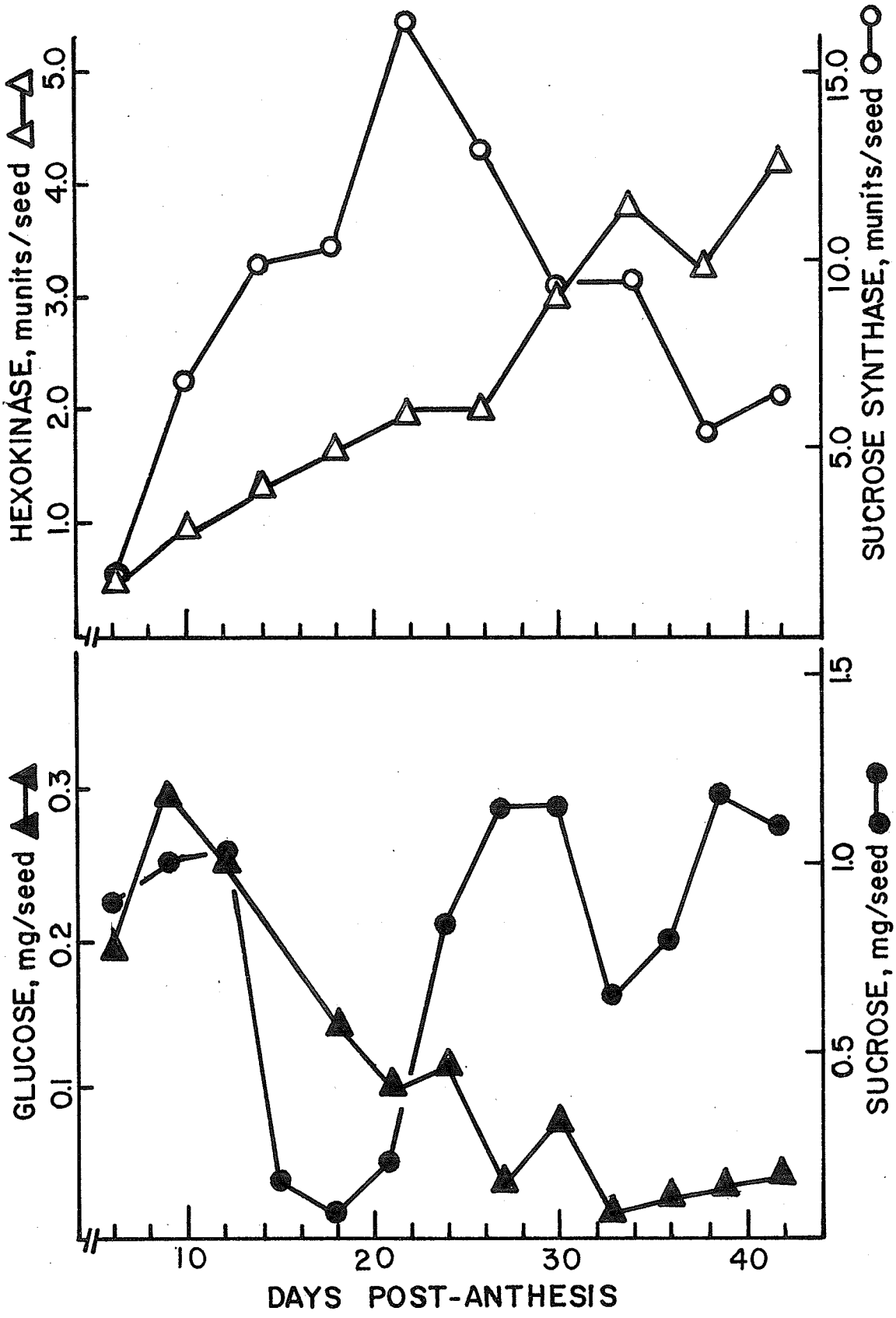

HEXOKINASE, munits/seed
SUCROSE SYNTHASE, munits/seed
GLUCOSE, mg/seed
SUCROSE, mg/seed
DAYS POST-ANTHESIS
1.0
2.0
3.0
4.0
5.0
5.0
10.0
15.0
0.1
0.2
0.3
0.5
1.0
1.5
10
20
30
40

FIGURE 30A

Changes in hexokinase and sucrose synthase activity with kernel development for 6A250 triticale.

FIGURE 30B

Changes in glucose and sucrose levels with kernel development for 6A250 triticale.

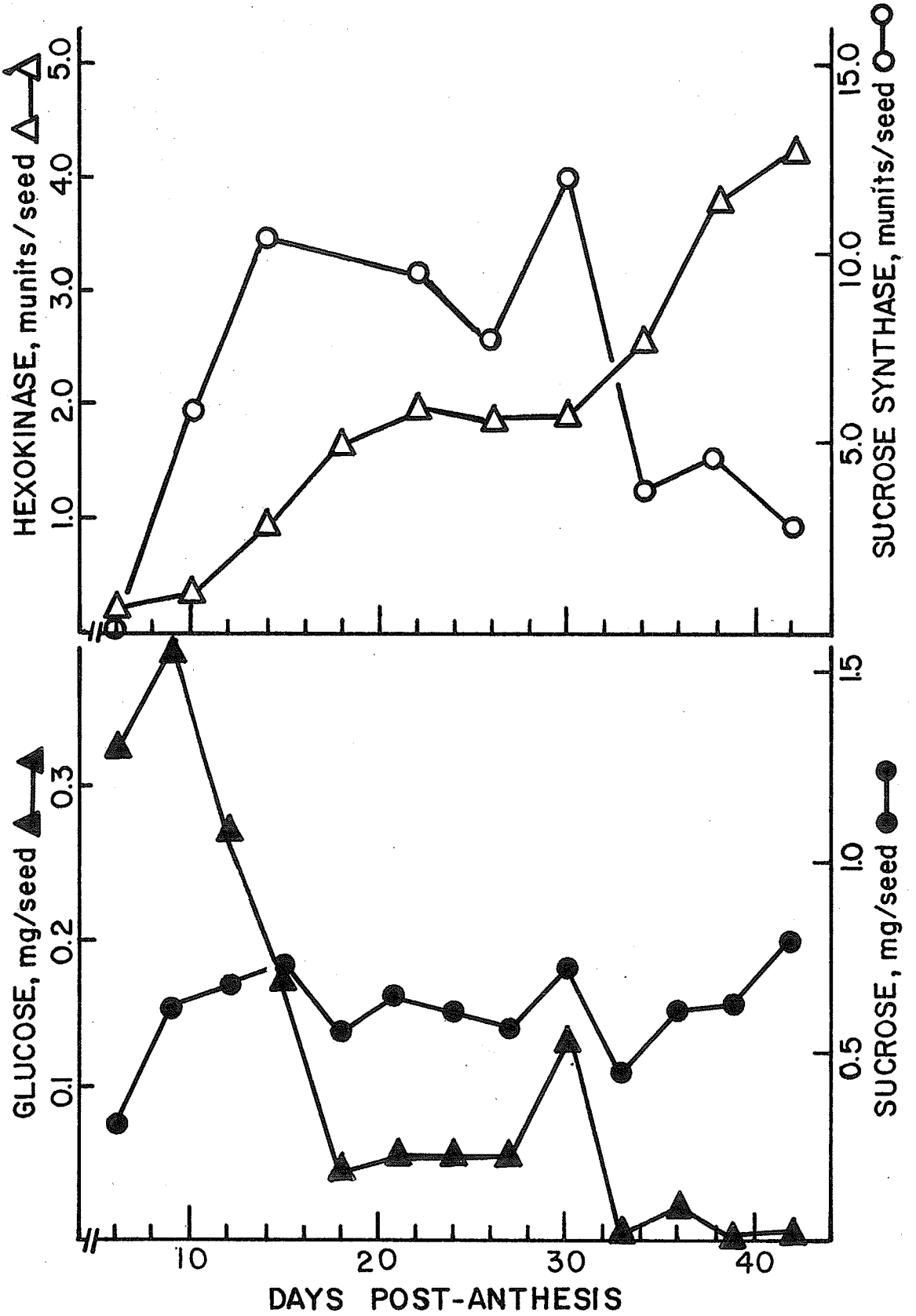

HEXOKINASE, munits/seed
SUCROSE SYNTHASE, munits/seed
GLUCOSE, mg/seed
SUCROSE, mg/seed
DAYS POST-ANTHESIS
1.0
2.0
3.0
4.0
5.0
5.0
10.0
15.0
0.1
0.2
0.3
0.5
1.0
1.5
10
20
30
40

FIGURE 31A

Changes in hexokinase and sucrose synthase activity with kernel development for Stewart 63 wheat.

FIGURE 31B

Changes in glucose and sucrose levels with kernel development for Stewart 63 wheat.

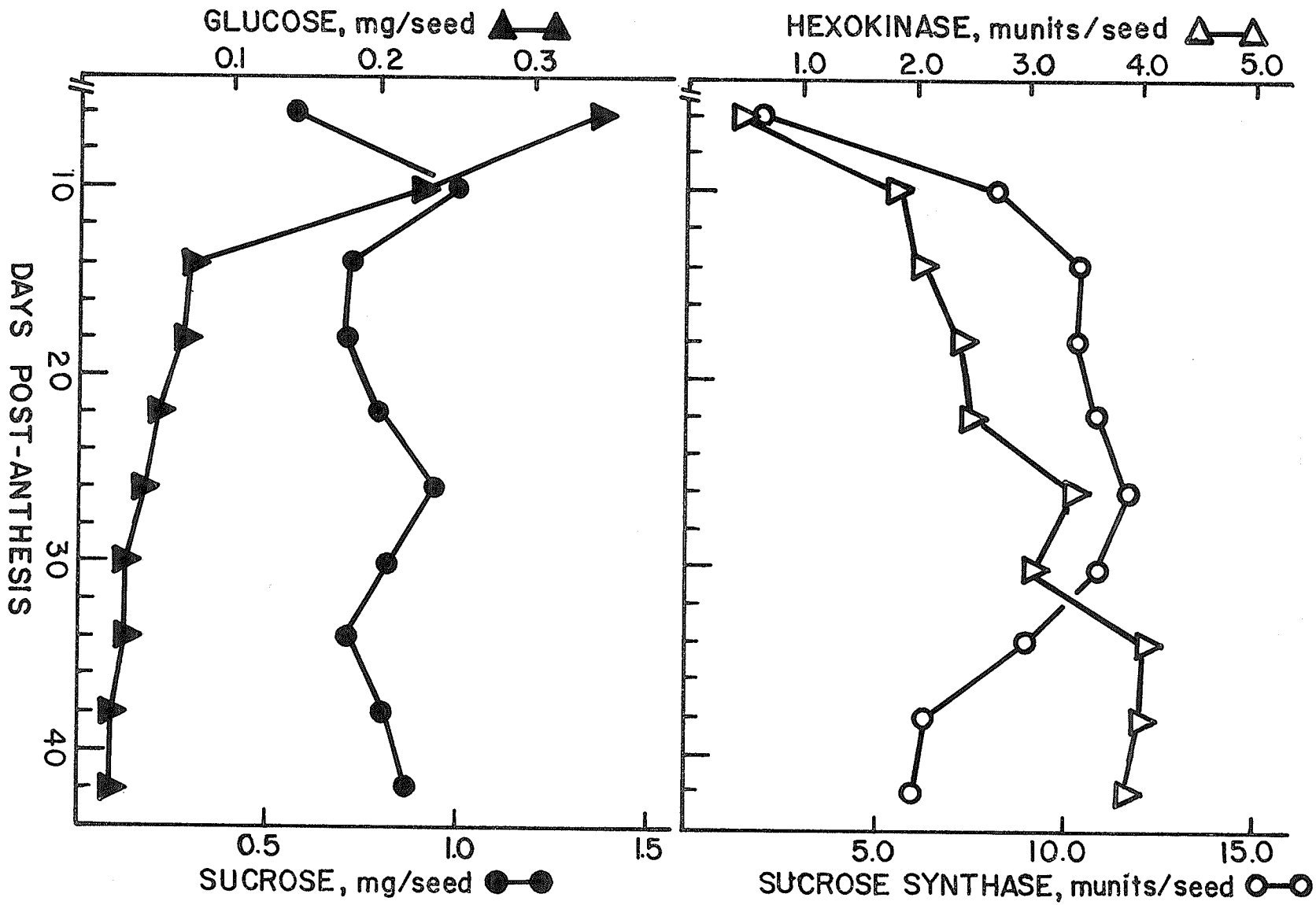
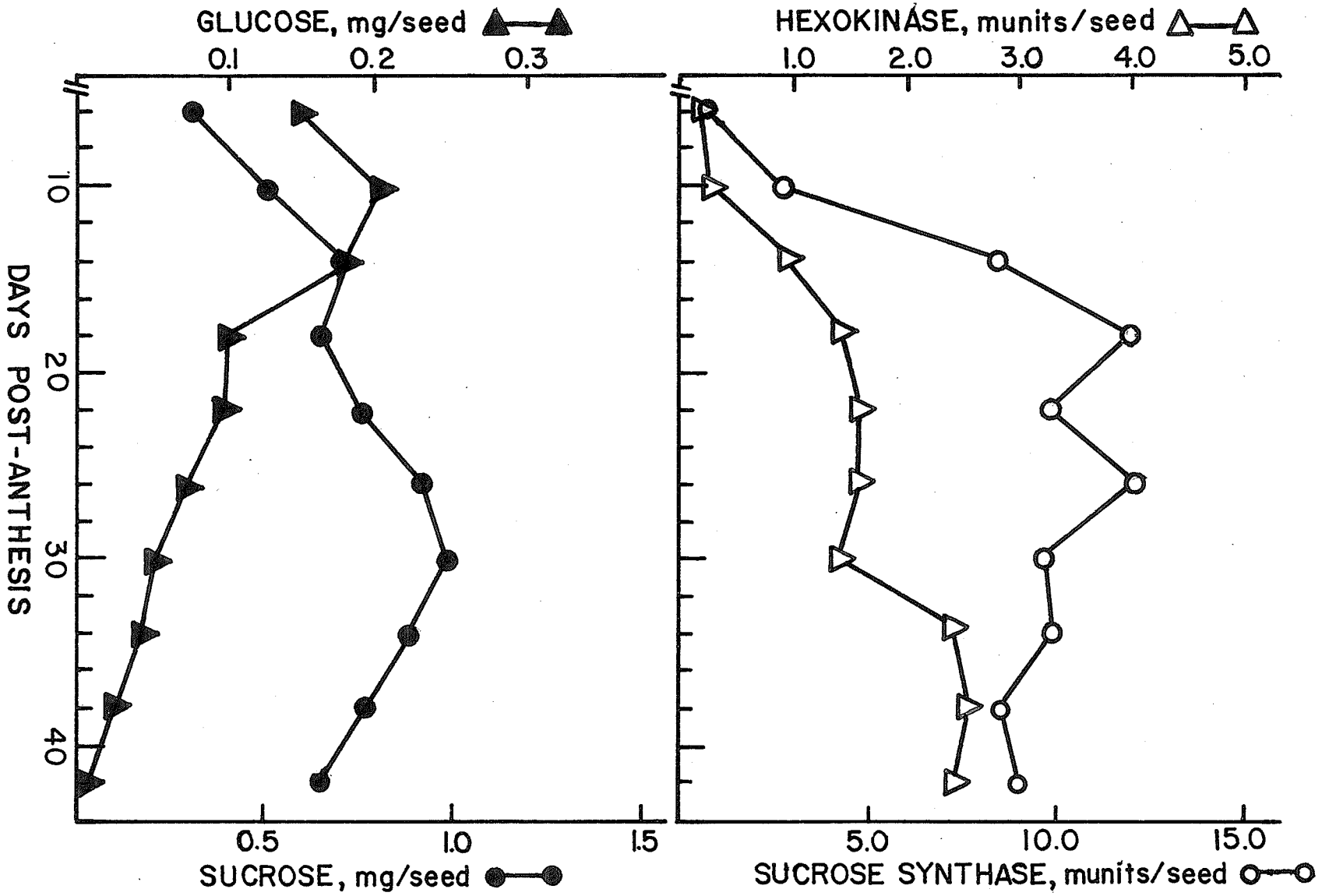

GLUCOSE, mg/seed
0.1
0.2
0.3
HEXOKINASE, munits/seed
1.0
2.0
3.0
4.0
5.0
DAYS POST-ANTHESIS
10
20
30
40
0.5
1.0
1.5
SUCROSE, mg/seed
5.0
10.0
15.0
SUCROSE SYNTHASE, munits/seed

FIGURE 32A

Changes in hexokinase and sucrose synthase activity with kernel development for Prolific rye.

FIGURE 32B

Changes in glucose and sucrose levels with kernel development for Prolific rye.


GLUCOSE, mg/seed
0.1
0.2
0.3
HEXOKINASE, munits/seed
1.0
2.0
3.0
4.0
5.0
DAYS POST-ANTHESIS
10
20
30
40
0.5
1.0
1.5
SUCROSE, mg/seed
5.0
10.0
15.0
SUCROSE SYNTHASE, munits/seed

remained at a fairly high level relative to glucose levels (Figs. 28B - 32B). The idea that an increased sink demand can increase photosynthate production at the source (Wardlaw and Moncur, 1976) may account for the constantly high sucrose levels in spite of increased sucrose synthase levels.

The one cereal which displayed different patterns in sucrose and sucrose synthase levels was 6A190. At 18 days, sucrose had dropped to 15 percent of the levels observed at 12 days post-anthesis (Fig. 29B). Sucrose levels increased beyond 18 days until at 26 days post-anthesis sucrose levels were higher than 12 days. From 18 to 22 days, sucrose synthase activity increased approximately 70 percent (Fig. 29A). Soluble protein levels actually decreased from 18 to 22 days (Fig. 24). Thus, if the increase in activity does represent newly synthesized sucrose synthase, the synthesis is probably occurring during a time when overall enzymic protein synthesis is not increasing.

During this 18 to 22 day period, the rate of dry matter accumulation was maximal (Fig. 14). Assuming that the dry matter increase is largely starch, it would appear that starch accumulation was not affected in this 18 to 22 day period. If the abnormal levels of sucrose and sucrose synthase activities in 6A190 are factors in the shrivelling of the kernel, they do not appear to exert their effect by a change in the rate of dry matter accumulation during this period.

Hexokinase and Glucose

The changes in hexokinase activity with seed development were similar in all the cereals tested (Figs. 28A - 32A). Initially, hexokinase activity increased at a moderate rate relative to sucrose synthase

activity. Following this initial rise in activity, the level of hexokinase either levelled off or decreased slightly. Between 22 and 30 days post-anthesis, hexokinase activities again increased in all of the cereals tested. After the second increase, Prolific rye, Stewart 63 and Manitou displayed a levelling off in activity with maturity (42 days) whereas 6A190 and 6A250 were still increasing in activity at 42 days. Thus, two periods of escalating activity with a stationary phase inbetween characterized the hexokinase patterns for these cereals.

Glucose levels, which were high at 6 to 8 days post-anthesis, declined rapidly during early kernel development (Figs. 28B - 32B). By 20 days post-anthesis glucose levels fell to approximately 50 percent of maximum values. As kernel development progressed beyond 20 days, glucose remained very low in the seeds. The level of glucose closely reflected the increased hexokinase activity whereas the sucrose levels were relatively high in most cultivars despite increases in sucrose synthase.

Neither glucose levels nor hexokinase activity during development appeared to correlate with dry weight accumulation. The initial increases of hexokinase activity were much slower than sucrose synthase, and the subsequent increases in hexokinase activity, especially the second increase, were not reflected by increases in dry weight. Rather, dry weight accumulation levelled off as hexokinase activity rose and then decreased slightly from the maximum. Possibly, hexokinase activity was required to degrade the remaining metabolites after starch synthesis ceased and hence, the resultant dry weight loss at maturity. Also, energy may have been needed for the final synthetic processes before the kernel completely matured. The second increase in hexokinase

directly corresponded with the decreases in sucrose synthase in all of the cereals observed. The results again suggest an increased role for hexokinase with the termination of starch synthesis.

The lack of correlation between hexokinase activity and starch production in these cereals is contrasted by the situation that exists in maize. Dent corn endosperm hexokinase rapidly increased from 10 to 22 days post-anthesis and subsequently decreased (Cox and Dickinson, 1973b). This 10 to 22 day range is a period of a rapid starch accumulation in maize. Since sucrose hydrolysis has been shown to be requisite for sugar movement into the endosperm of maize (Shannon, 1972), there is an obvious need for hexokinase before any starch synthesis can occur and hence, the correlation between hexokinase and starch production.

Sucrose hydrolysis does not seem to be required for translocation into the endosperm of wheat (Jenner, 1974). Thus, the cereals in this study may have utilized sucrose directly for starch synthesis via sucrose synthase and the subsequent synthetic enzymes. This would be consistent with the correlation observed between sucrose synthase and dry weight accumulation and the lack of correlation observed for hexokinase activity with kernel development.

Substrate Specificity of Crude Extract Hexokinase

Hexokinase from crude extracts of 30 day post-anthesis samples of the cereals observed was able to utilize either ^{14}C-glucose or ^{14}C-fructose as substrate (Table 7). In all cases except Prolific rye, a higher activity was obtained using ^{14}C-glucose.

TABLE 7. Rate of ^{14}C-glucose and ^{14}C-fructose utilization by 30 day post-anthesis crude extract hexokinase (munits/seed).

Substrate	Cultivar or line				
	Manitou	6A190	6A250	Stewart 63	Prolific
^{14}C-glucose	3.5	3.1	2.5	3.1	2.0
^{14}C-fructose	1.9	2.8	2.0	2.6	2.0

By 30 days post-anthesis, the hexokinase in the kernels had begun to increase, possibly to speed the utilization of metabolites left as starch synthesis began to shut down. The role of hexokinase at this point in kernel development might reflect itself in the preference of substrates. In other words, a greater use of glucose rather than fructose by crude extract hexokinase may be indicative of metabolic shifts occurring relative to seed formation. For instance, it may be that fructose is the more highly utilized substrate during early seed development due to the prevalence of sucrose synthase which catalyzes the conversion of sucrose to produce fructose and UDP glucose. As starch production falls, fructose may not be as abundant and glucose may become the predominant substrate. Although no evidence is available to substantiate any metabolic shift for the hexokinase, there was a clear preference for glucose by crude extract hexokinase during later seed development.

GENERAL DISCUSSION

The shrivelling phenomena associated with kernel maturation has been a major problem in the commercial utilization of triticale. For cultivar 6A190, it has been demonstrated that the starch content is lower per unit volume of kernel than smooth seeded cultivars (Hill *et al.*, 1974). A deficient supply of nutrients or a poor utilization of these compounds are two of a number of possible explanations that could account for the decreased starch content. This investigation has dealt with the conversion of sucrose and glucose to sugar phosphates, compounds that can be utilized for storage carbohydrate synthesis.

Metabolic differences were found during grain filling in the shrivelled triticale, 6A190, as compared to parental material, wheat, or a plump triticale. Sucrose levels dropped at 15 days post-anthesis to 5 percent of the maximum levels at 27 days in 6A190. An increase in sucrose synthase levels followed. The high for this enzyme in 6A190 appeared 7 days after the sucrose level dropped. Since sucrose synthase correlated well with dry weight accumulation in all of the cereals observed, the surge in sucrose synthase activity in 6A190 may be attributed to a response by the plant for increased starch synthesis. The low sucrose levels may modulate sucrose synthase, but the low concentration of sucrose might also be indicative of a low metabolic concentration throughout 6A190 at 15-21 days post-anthesis. Low concentrations of metabolites such as UDP glucose would more likely bring about increases in sucrose synthase. It seems doubtful, however, that

this metabolic aberration is responsible for shrivelling in 6A190 since dry weight accumulation was unchanged during this period (Fig. 14).

In all of the cereals observed, sucrose synthase levels remained at high levels during the most rapid period of dry weight increase. As dry weight accumulation began to fall off, the activities of sucrose synthase fell. Correlations between rapid starch formation and sucrose synthase have been observed in rice (Perez *et al.*, 1975), maize (Tsai *et al.*, 1970) and barley (Baxter and Duffus, 1973). These studies have suggested an important regulatory role for sucrose synthase in the regulation of starch synthesis in the developing grains. Likewise, the close association between sucrose synthase and dry weight accumulation in this study implicates the involvement of sucrose synthase in starch synthesis.

Patterns of hexokinase activity with kernel development in the cereals tested did not correlate with dry weight gain as did sucrose synthase. Initially, hexokinase activity rose more slowly than sucrose synthase. After a stationary phase where increases were slight or non-existent, the hexokinase activity rapidly increased once again as dry weight accumulation ceased and sucrose synthase activity fell. This pattern may be due to a greater energy requirement during the final stage of grain maturation. Thus, glycolysis might be stimulated. Barley endosperm extracts showed a similar pattern for hexokinase activity with kernel development up to 25 days post-anthesis (Baxter and Duffus, 1973).

Different patterns of hexokinase activity in the cereals of this investigation and in the studies conducted on maize (Tsai *et al.*, 1970 and Cox and Dickinson, 1973b) and rice (Perez *et al.*, 1975) suggest

different roles for hexokinase. In maize, sucrose hydrolysis was required for sucrose entry into the endosperm (Shannon, 1972). Presumably, hexokinase would be required to incorporate the hexoses into the metabolic stream. Accordingly, normal maize endosperm displayed a maximum midway (22 days post-anthesis) through the period of dry weight gain and decreased thereafter (Cox and Dickinson, 1973b). Present evidence (Jenner, 1974 and Sakri and Shannon, 1975) suggests that sucrose inversion is not necessary for transport into developing wheat endosperm. If sucrose in wheat, rye, or triticale could be utilized directly via sucrose synthase (without hydrolysis) to form the precursors of starch, then the requirement for hexokinase as far as starch synthesis was concerned might be far less than that for maize. This might explain the observed differences between the results observed in this study and those with maize for hexokinase activity with kernel development.

From this investigation, the cause of shrivelling in 6A190 cannot be attributed to abnormalities in either hexokinase or sucrose synthase activity with seed development. The shrivelled line, 6A190, displayed aberrations in sucrose levels and sucrose synthase activity during kernel filling, but since the rate of dry weight accumulation remained unchanged during this period, it is doubtful whether these deviations from the norm limited starch synthesis. The differences observed in sucrose and sucrose synthase may reflect a more general metabolic malfunction which seems to occur in 6A190.

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