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Kinetin and Carbohydrate Metabolism in Chinese Cabbage

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ABSTRACT

The effects of kinetin on starch and sugar levels and on $^{14}CO_2$ and ^{32}P-orthophosphate labeling patterns of floated Chinese cabbage (*Brassica pekinensis*) leaf discs were investigated. Kinetin caused gross starch degradation. Neutral sugars were depressed by 30 to 40% in leaf tissue treated with kinetin for 24 hours. $^{14}CO_2$ labeling of leaf discs pretreated with kinetin for 24 hours showed increased radioactivity in chloroform-soluble material and most sugar phosphates, and a 35 to 40% decrease in radioactivity in the neutral sugars, glucose, sucrose, and fructose. Incorporation into ATP was increased by 40% by kinetin. ^{32}P-Orthophosphate uptake was inhibited 30% by kinetin. When corrected for uptake, kinetin stimulated incorporation into chloroform-soluble material but had little effect on other cell fractions. These results indicate that kinetin mobilizes starch reserves and increases the flow of sugars required for the synthesis of lipids and structural materials in floated discs.

The mechanism of action of the plant hormones, cytokinins, is at present unknown. Cytokinins have been shown to modify the incorporation of radioactive precursors into nucleic acids and proteins in excised leaf tissue (11). However, recent more critical studies (8, 14) suggest that these effects are due to suppression of protein degradation by cytokinins. Convincing evidence that cytokinins directly affect nucleic acid or protein synthesis in excised leaf tissue has not been obtained.

Many of the observed effects of cytokinins on nucleic acids and proteins could result indirectly from modification of cellular metabolism. For example, increased supply of energy or flow of metabolic intermediates could affect precursor pools and thus modify such processes as RNA or protein synthesis.

There is already some evidence to suggest that cytokinins affect carbohydrate metabolism in plants. Sugiura *et al.* (13) found that in detached leaves from *Nicotiana* species kinetin promoted net nucleic acid and protein synthesis only when internal sugar levels were sufficiently high. Boothby and Wright (4) showed that kinetin could replace external glucose in inducing maximal growth response in excised wheat coleoptiles. In kinetin-treated coleoptiles this growth response was associated with starch breakdown and an increase in sugars within the tissues. Kinetin also caused a large increase in reducing sugars released to the incubation medium when added to wheat endosperm. The authors concluded that kinetin increased starch degradation in wheat endosperm. Dennis *et al.* (6) reached a similar conclusion, examining starch levels in barley leaves with the electron microscope. Other indirect evidence suggests that cytokinins may increase glucose catabolism (10, 15).

We have previously shown (2) that cytokinins stimulate expansion of floated Chinese cabbage leaf discs while maintaining the levels of ribosomes and RNA in the discs. We report here effects of kinetin and sucrose on Chinese cabbage leaf disc expansion; on starch, sugar, and sugar phosphate levels; and on fixation and metabolism of $^{14}CO_2$ in floated leaf discs.

MATERIALS AND METHODS

PLANT GROWTH AND SAMPLING

Chinese cabbage (*Brassica pekinensis*, var. Wong Bok) plants were grown in a glasshouse at 25 C under natural light. Young rapidly expanding leaves 7 to 11 cm long from 4- to 6-week-old plants were routinely used. Discs (9-mm diameter) were removed from interveinal areas with a cork borer and floated on solutions made up in Vickery's solution (2). Equal numbers of discs from each half-leaf were randomly distributed between Petri dishes, and the dishes were incubated in a light room (600 lumens/sq ft) at 25 C under a 16-hr photoperiod. Following incubation the discs were washed with water, blotted dry, weighed and extracted.

STARCH AND SUGAR DETERMINATION

Starch. Leaf discs were ground with 4 ml of TM buffer (0.01 M $MgCl_2$, 0.01 M tris-HCl, pH 7.6). The homogenate was strained through fine cheesecloth. The mortar and cheesecloth were rinsed with 4 ml of TM buffer, and the total extract was centrifuged at 12,000*g* for 10 min. The pellet was extracted twice with TM buffer containing 2% Triton X-100 (to remove chlorophyll). After centrifugation the resulting pellet was suspended in 2 ml of TM buffer and incubated with pancreatic deoxyribonuclease I (10 μg) at 27 C for 25 min. The remaining insoluble material was washed twice with 95% ethanol. Starch in the residue was hydrolyzed with perchloric acid, and the resulting sugars were determined with anthrone (9).

Sugars. Neutral sugars from leaf tissue, purified by elution from DEAE-cellulose[1] and phosphocellulose (see below) were determined with anthrone (9).

NUCLEIC ACID EXTRACTION

Leaf discs were homogenized with 4 ml of 0.5% sodium dodecylsulfate, 0.5% disodium naphthalene-1,5'disulfonate, and 2 ml of 80% aqueous phenol containing 0.1% 8-hydroxyquinoline. Following centrifugation, nucleic acids were extracted from the aqueous layer and purified with cetyltrimethylammonium bromide (12). Purified nucleic acids were dissolved in TM buffer containing 5 mM EDTA, and their absorbance was estimated at 260 nm.

[1] Abbreviation: DEAE-: diethylaminoethyl.

RADIOACTIVE LABELING PROCEDURES

^{14}C-Carbon Dioxide. ^{14}C-Carbon dioxide was liberated from barium carbonate (10 mg; 55 μc; Radiochemical Centre, Amersham) with 6 M H_2SO_4 and drawn into a desiccator which contained floated leaf discs under partial vacuum. The vacuum was released as soon as CO_2 evolution ceased, and the leaf discs were allowed to photosynthesize under $^{14}CO_2$ in a light room (600 lumens/sq ft) at 25 C for 1 to 2 hr.

^{32}P-Orthophosphate. In other experiments floated leaf discs were treated with neutral (pH 7) carrier-free ^{32}P-orthophosphate (Radiochemical Centre, Amersham) in the light room for the required time.

EXTRACTION OF RADIOACTIVE TISSUE

Radioactive leaf discs were washed with a solution containing 0.01% sodium dodecylsulfate, 0.1 M NaH_2PO_4, then rinsed twice with distilled water. The discs were blotted dry, weighed, and extracted by one of the following procedures.

80% Ethanol Extraction. Fifty leaf discs (9-mm diameter) were homogenized with boiling 80% ethanol (5 ml, v/v) in a Kontes tissue grinder. The grinder was rinsed with 2 ml of 80% ethanol, and the homogenate was centrifuged at 2000g for 5 min. The pellet was re-extracted with 2 ml of 80% ethanol, and the combined extracts were stored at −15 C until chromatographed. The pellet was treated with 1 M HCl (5 ml) at 100 C for 48 hr under reflux. After centrifugation the residue was resuspended in water and filtered, and radioactivity on the filter was measured in a liquid scintillation spectrometer. Radioactivity in the other fractions was assessed by dissolving aliquots in hyamine hydroxide (1 M in methanol, 1 ml) and estimating radioactivity in a liquid scintillation spectrometer.

MCF Extractions. Leaf tissue was "killed" with 10 ml of MCF (methanol, chloroform, 7 M formic acid; 12:5:3, v/v) for 18 hr at −35 C and extracted according to the method of Bieleski and Young (3). The killed tissue was homogenized with 0.5 g of Hyflo Supercel in a glass Kontes grinder and filtered, the grinder was rinsed with 2 ml of MCF medium, and the chloroform and aqueous layers were separated by adding 3.6 ml of chloroform and 4.8 ml of water. The residue was re-extracted with 0.2 M formic acid in 20% methanol and filtered. The filtrate was combined with the aqueous phase and lyophilized. The residue from lyophilization was dissolved in TM buffer and passed through consecutive DEAE-cellulose and phosphocellulose columns. The wash from these columns was collected as the neutral fraction. The DEAE-cellulose was then eluted with 0.3 M triethylammonium bicarbonate to release adsorbed acidic compounds. The column fractions were lyophilized and dissolved in water, and the radioactivity in individual fractions was determined by liquid scintillation spectrometry. The residue following filtration was hydrolyzed with 5% trichloroacetic acid at 90 C for 40 min. The hydrolysate was filtered, and the trichloroacetic acid was evaporated from aliquots of the filtrate in order to assess radioactivity.

CHROMATOGRAPHY

80% Ethanol Extract. Aliquots (0.1–0.15 ml) were chromatographed in two dimensions in solvent I, 80% aqueous phenol (w/v), and solvent II, 1-butanol-propionic acid-water (1). Acid-washed Whatman No. 3MM paper was routinely used for chromatography. Chromatograms were radioautographed, and the radioactive spots were eluted with 20% ethanol and estimated in a liquid scintillation spectrometer with naphthalene-dioxane based scintillant (5).

MCF Extract. Aliquots (0.1–0.15 ml) of the acidic fraction of the MCF extracts were chromatographed in two dimensions in solvent III, 1-propanol-ammonia (sp.gr. 0.9)-water (6:3:1, v/v), and solvent IV, 1-propyl acetate-formic acid (90%)-water (11:5:3, v/v). All solvents contained 5 mM EDTA to improve resolution. Chromatograms were radioautographed, and the radioactive spots were eluted with 20% ethanol and estimated.

Table I. *Effects of Kinetin and Sucrose on Leaf Disc Expansion in Light and Dark at 25 C*

Sets of 40 leaf discs were floated for 3 days on solutions made up in Vickery's solution. Nucleic acids were extracted as described in "Materials and Methods." Kinetin was used at 5 μg/ml and sucrose at 1%. These concentrations gave near maximal effects on expansion.

Experiment	Treatment	Light		Dark	
		Fresh wt	Increase in fresh wt	Fresh wt	Increase in fresh wt
		g	%	*g*	%
A	Time zero	0.407		0.407	
	Control	0.528	29.8	0.447	9.8
	Kinetin	0.616	51.5	0.497	22.1
	Sucrose	0.689	69.3	0.665	63.4
	Kinetin + sucrose	0.924	126.0	0.750	84.7

	Treatment	Fresh wt	Total Nucleic Acid
		g	*mg*
B[1]	Time zero	0.420	0.56
	Control	0.615	0.73
	Kinetin	0.736	0.91
	Sucrose	0.760	0.76
	Kinetin + sucrose	1.003	0.91

[1] Leaf discs floated in the light.

RESULTS

EFFECTS OF KINETIN AND SUCROSE ON LEAF DISC EXPANSION IN LIGHT AND DARK

Chinese cabbage leaf discs floated on kinetin solutions in the light for 3 days show a 15 to 30% increase in fresh weight compared with untreated controls. Because this increase in fresh weight could have resulted from increased availability and utilization of internal carbohydrate following kinetin action, we first examined whether external supplies of sugars would substitute for kinetin and cause disc expansion.

The effects of kinetin, sucrose, and light on leaf disc expansion are shown in Table I. Kinetin stimulated leaf disc expansion in the light to a greater extent than in dark-floated discs. Leaf discs floated on sucrose in the light and dark expanded 69 and 63%, respectively. In the presence of sucrose, kinetin stimulated expansion more effectively in the light than in the dark. The combined effects of kinetin and sucrose on disc expansion in the light and dark were cumulative. Since kinetin stimulated fresh weight whether or not sucrose was present, kinetin action appeared to be additional to that of sucrose. Extraction of total nucleic acid from leaf discs floated on sucrose and kinetin solutions in the light confirmed that kinetin caused effects additional to those of sucrose. Although sucrose caused an increase in fresh weight of floated leaf discs, it did not alter the total nucleic acid content of the discs.

Table II. *Effects of Plant Growth Hormones on Expansion and Starch Content of Leaf Discs*

Growth conditions have been described (2). The large difference in starch content of the leaf discs between experiments A and B is due to different glasshouse daylight conditions. Forty leaf discs (A, 9-mm diameter; B, 11-mm diameter) were floated for 3 days on kinetin, GA_3, or IAA, each at 5 μg/ml. Starch determinations (see "Materials and Methods") are the average of duplicates.

Experiment	Treatment	Fresh wt	Increase in Fresh wt	Starch
		g	%	mg
A	Time zero	0.82		16.9
	Control	1.04	26.8	18.2
	Kinetin	1.38	68.3	13.2
B	Time zero	0.50		4.28
	Control	0.73	46.0	4.95
	Kinetin	0.92	84.2	2.92
	GA_3	0.83	66.0	2.12
	Kinetin + GA_3	1.06	112.0	2.61
	IAA	0.73	46.0	4.95

Kinetin caused increases in both fresh weight and total nucleic acid (20 and 25%, respectively) over 3 days (Table 1B). Kinetin produced the same increases in nucleic acid whether or not sucrose was present.

EFFECTS OF PLANT GROWTH HORMONES ON STARCH CONTENT OF LEAF DISCS

The effects of kinetin, GA_3, and IAA on expansion and starch content of floated leaf discs are shown in Table II. Both GA_3 and kinetin caused leaf disc expansion, their combined effects being cumulative. IAA was ineffective in the leaf disc expansion assay and had no effect on starch levels. Both GA_3 and kinetin enhanced net starch degradation; however, their combined effects on starch degradation were not cumulative. In the presence of sucrose, the kinetin effect on starch degradation was much reduced (Berridge, unpublished observation).

EFFECTS OF KINETIN ON PRODUCTS OF $^{14}CO_2$ ASSIMILATION

Tissue Extractions. To investigate further the effects of kinetin on carbohydrate metabolism, we followed carbon fixation and assimilation in leaf discs pretreated for 20 hr with kinetin and labeled for 2.5 hr with $^{14}CO_2$. The labeled tissue was extracted with boiling ethanol (see "Materials and Methods"). The total fixation of $^{14}CO_2$ was similar in both control and kinetin-treated leaf discs. Total ^{14}C radioactivity in 80% ethanol-soluble material was 13% lower in kinetin-treated than in control tissue. This was partially balanced by 13% greater ^{14}C radioactivity in the hot acid-soluble fraction from kinetin-treated discs compared with the control. Radioactivity associated with the residual pellet from kinetin-treated discs was twice that in the control, suggesting some redirection of metabolism to increased formation of structural cell components in the presence of kinetin. This was compatible with the increased fresh weight of kinetin-treated discs.

Chromatography of Ethanol Extracts. The 80% ethanol extracts were separated by two-dimensional chomatography (see "Materials and Methods"). Chromatograms were radioautographed to visualize the radioactive components present. A typical separation is shown in Figure 1A. Characterization of the individual radioactive spots by cochromatography of the extract with selected markers permitted identification of the major radioactive compounds and several of the minor components. Radioactivity recovered from the chromatograms as well resolved spots was 67.4 and 63.4% of the total radioactivity applied to control and kinetin chromatograms, respectively. Most of the remaining radioactivity occurred as a diffuse green-brown lipophilic area ("L" in Fig. 1A) running near the front in both solvents. Table III summarizes the effects of kinetin on the incorporation of $^{14}CO_2$ into the products of photosynthesis. The major kinetin effects were a 50% reduction in ^{14}C radioactivity in glucose and in spot 5, tentatively identified as a mixture of triose phosphates. In separate experiments, sucrose and aspartic acid contained less radioactivity in kinetin-pretreated discs. Kinetin also increased radioactivity in spots 12 and 15a and in succinic acid.

Two-dimensional chromatography of the 80% ethanol extract in an alternative solvent in the second dimension (1-butanol-acetic acid-water; 74:19:50, v/v) confirmed these results although resolution was less complete.

EFFECTS OF KINETIN ON $^{14}CO_2$ INCORPORATION INTO SUGARS AND SUGAR PHOSPHATES

Tissue Extraction. Since phosphate esters were poorly resolved in the above systems, the improved extraction and chromatographic procedures of Bieleski and Young (3) were subsequently used to isolate and fractionate sugar phosphates. Leaf discs treated for 24 hr with kinetin were exposed to $^{14}CO_2$ for 1.5 hr. The labeled tissue was extracted with MCF (see "Materials and Methods"). Kinetin caused a 25% stimulation of the radioactivity extracted into chloroform and a 35% decrease in radioactivity in the aqueous phase compared with control tissue. Much of this 35% decrease was evident in neutral compounds which were not retained by either DEAE-cellulose or phosphocellulose. Kinetin appeared to have little effect on the total radioactivity associated with material retained by DEAE-cellulose or on acid-soluble radioactivity in the residual pellet. In these experiments radioactivity of the final pellets was not assayed.

Chromatography of Phosphate Ester Fraction. Aliquots of the fraction eluted from DEAE-cellulose were chromatographed in solvents III and IV (see Fig. 1). The chromatograms were radioautographed, and the radioactive spots were eluted and estimated. Figure 1B shows a typical separation of ^{32}P-labeled phosphate esters by this procedure. Individual radioactive spots were identified by cochromatography with selected markers and by comparison with typical phosphate ester patterns (3). Table IV presents the effects of kinetin on $^{14}CO_2$-labeled phosphate esters. Radioactivity recovered from the chromatograms as phosphate esters was 27.8 and 33.0% of that applied to the control and kinetin chromatograms, respectively. The remainder washed from the chromatograms during chromatography as organic acids, amino acids, and other fast moving compounds. Kinetin increased the total ^{14}C radioactivity into ATP by 40%, and into most other observed phosphate esters by smaller amounts.

Chromatography of Neutral Fractions. Aliquots of the neutral fractions not retained by either DEAE-cellulose or phosphocellulose were chromatographed in solvents I and II. Chromatograms were radioautographed, and the radioactive spots were eluted and assessed for radioactivity. Radioactivity recovered represented 95% of that applied to both kinetin and control chromatograms. Table V summarizes the results and demonstrates that radioactivity associated with free sugars was dramatically reduced in the presence of kinetin.

Estimation of Total Free Sugars. To determine whether the

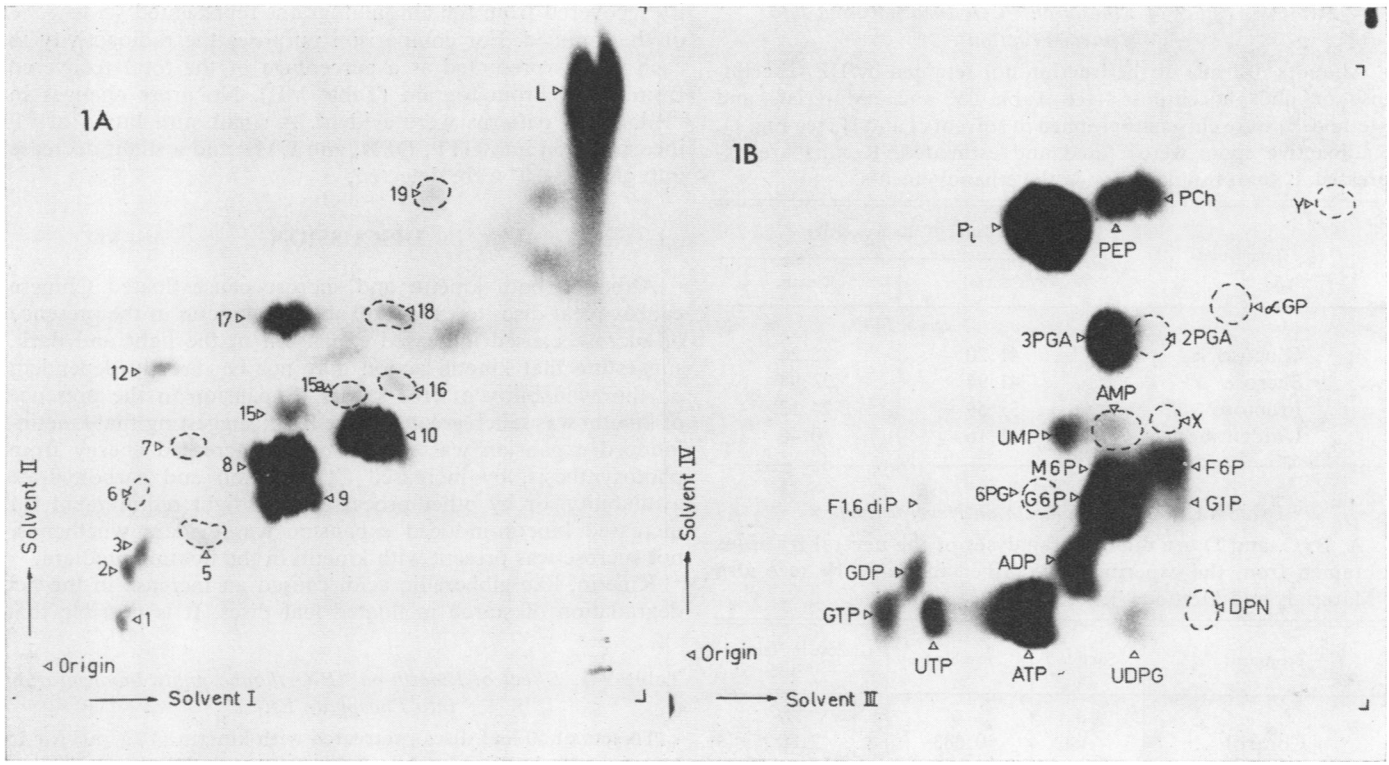

FIG. 1. Radioautographs of chromatograms of leaf extracts. A: 80% ethanol extract of $^{14}CO_2$-labeled leaf tissue; B: phosphate esters from ^{32}P-orthophosphate-labeled leaf tissue (see "Materials and Methods" for preparation of phosphate esters and description of solvents). PEP: Phosphoenolpyruvate; PCh: phosphatyl choline; 2PGA and 3PGA: 2- and 3-phosphoglyceric acid; M6P: mannose-6-P; F6P: fructose-6-P; G1P: glucose-1-P; G6P: glucose-6-P; 6PG: 6-phosphogluconic acid; F1,6diP: fructose-1,6-diP; UDPG: UDP-glucose; X: unknown.

Table III. *Kinetin Effects on Products of $^{14}CO_2$ Fixation*

Fifty leaf discs (9-mm diameter) were pretreated with kinetin (5 μg/ml) for 20 hr, then labeled with $^{14}CO_2$ for 2.5 hr. The radioactive tissue was extracted with boiling ethanol as described. An aliquot (0.14 ml) of the 80% ethanol extract was chromatographed in solvents I and II (see Fig. 1). Radioactive spots were eluted with 2 ml of 20% ethanol, and 0.1 ml was estimated. Spots 6, 15a, and 18 were not identified. Compounds in parentheses have tentative assignments.

Spot	Assignment	^{14}C Radioactivity	
		Control	Kinetin
		cpm, 0.1 ml	
1	Sugar diphosphate	490	550
2	Glucose 6-phosphate	620	600
3	Sugar phosphate	600	730
5	(Triose phosphate)	1,170	540
6	...	190	130
7	Aspartic acid	660	520
8	Sucrose (serine, glycine)	30,880	26,010
9	Glucose	24,180	12,540
10	Fructose (glutamine)	27,220	26,140
12	(Isocitrate)	260	510
15	Glutamic acid	1,080	990
15a	...	390	580
16	Alanine	490	510
17	Glycolic acid	2,680	2,660
18	...	1,610	1,630
19	Succinic acid	320	420

Table IV. *Effect of Kinetin on $^{14}CO_2$ Incorporation into Phosphate-Esters*

Fifty leaf discs (9-mm diameter) pretreated for 24 hr with kinetin (5 μg/ml) were exposed to $^{14}CO_2$ for 1.5 hr. The radioactive tissue was extracted with MCF at −35 C as described. One set of duplicate analyses was assayed. Aliquots (0.14 ml) of the fraction eluted from DEAE-cellulose (see "Materials and Methods") were chromatographed in solvents III and IV (Fig. 1B). Radioactive spots were eluted and estimated. Results are presented as total radioactivity in ethanol eluents.

Compound	Total ^{14}C Radioactivity in Phosphate Esters		^{14}C Radioactivity in Phosphate Esters	
	Control	Kinetin	Control	Kinetin
	cpm × 10^{-3}		*% of total phosphate esters recovered from chromatograms*	
ATP	3.88	5.46	2.9	3.5
ADP	2.90	3.50	2.2	2.3
UDP-glucose	2.60	2.92	1.9	1.9
Glucose-6-P	49.96	54.58	37.2	35.2
Mannose-6-P	10.36	12.36	7.7	8.0
Glucose-1-P	6.00	5.74	4.5	3.7
Fructose-6-P	18.18	21.84	13.5	14.1
3-Phosphoglycerate	16.78	18.32	12.5	11.8
Phosphatylcholine + phosphoenolpyruvate	19.58	26.58	14.5	17.1
Unknown	4.22	3.82	3.1	2.4

Table V. *Effect of Kinetin on $^{14}CO_2$ Incorporation into Neutral Fraction*

Aliquots (0.1 ml) of the fraction not retained by DEAE-cellulose or phosphocellulose (see Table IV and "Materials and Methods") were chromatographed in solvents I and II (see Fig. 1). Radioactive spots were eluted and estimated. Results are expressed as total radioactivity in the ethanol eluents.

Compound	Total ^{14}C Radioactivity	
	Control	Kinetin
	cpm × 10^{-4}	
Glucose	41.20	25.26
Sucrose	41.94	22.98
Fructose	37.58	24.12
Unidentified	1.16	0.46

Table VI. *Effect of Kinetin on Neutral Sugars*

A, B, C, and D are duplicate analyses of the neutral fractions obtained from the experiment described in Table IV (see also "Materials and Methods").

Treatment	Sample	Fresh wt	Glucose in Neutral Fraction
		g	*meq*
Control	A	0.683	2.80
	B	0.700	3.05
Kinetin	C	0.740	1.85
	D	0.746	2.00

decrease in radioactivity associated with neutral sugars was associated with a corresponding decrease in total sugar pools, the free sugars in the neutral fraction of the aqueous extracts were determined by the anthrone method (9). As shown in Table VI, free sugars were depressed by 35% in leaf tissue pretreated with kinetin. This closely corresponded to the 40% reduction in ^{14}C radioactivity in free sugars (*c.f.* Table V). Thus, although the total sugar pool decreases following kinetin treatment, the specific activity of this pool remains approximately constant. Time course experiments showed that the sugar pool initially drops in floated leaf discs. The recovery which occurs in untreated discs did not occur in kinetin-treated tissue. Leaf discs floated on sucrose had greatly elevated free sugars. However, kinetin depressed the free sugar content whether or not sucrose was present in the floating medium.

EFFECTS OF KINETIN ON ^{32}P-ORTHOPHOSPHATE INCORPORATION INTO TISSUE FRACTIONS AND PHOSPHATE ESTERS

Leaf discs floated on kinetin for 25 hr were treated with neutral (pH 7.0) carrier-free ^{32}P-orthophosphate for 90 min (see "Materials and Methods"). The discs were extracted and fractionated as described (see "Materials and Methods"). Kinetin-treated discs incorporated 30% less total ^{32}P. When corrected for decreased uptake, kinetin stimulated ^{32}P incorporation into the chloroform phase by about 35% but had no significant effect on gross ^{32}P incorporation into material in the aqueous phase or the pellet hydrolysate.

The aqueous phase was passed through DEAE-cellulose, and the fraction retained by DEAE-cellulose was eluted and chromatographed (see "Materials and Methods"). A typical autoradiograph of a chromatogram of the aqueous phase containing the phosphate esters is shown in Figure 1B. The individual radioactive compounds were eluted and estimated. Radioactivity recovered from the chromatograms represented 75 to 85% of that applied. For comparison purposes the radioactivity in each spot is presented as a percentage of the total recovered from each chromatogram (Table VII). No gross changes in ^{32}P labeling patterns were evident. A small stimulation of ^{32}P incorporation into GTP, DPN, and UMP and a slight decrease into glucose-6-P were detected.

DISCUSSION

Although both kinetin and sucrose cause floated Chinese cabbage leaf discs to expand (Table II), kinetin in the presence of sucrose caused increased expansion in the light and dark, suggesting that kinetin action may not be directly dependent on the availability of free sugars. Expansion in the presence of kinetin was much greater in the light, suggesting that kinetin-induced expansion was stimulated by increased energy from photosynthesis, by increased CO_2 fixation and carbohydrate availability, or by other processes. The light requirement for increased kinetin-induced expansion was evident whether or not sucrose was present with kinetin in the floating medium.

Kinetin, like gibberellic acid, caused an increase in the net degradation of starch in floated leaf discs. It is possible that

Table VII. *Effect of Kinetin on ^{32}P-Orthophosphate Incorporation into Phosphate Esters*

To sets of 50 leaf discs pretreated with kinetin (5 μg/ml) for 25 hr was added ^{32}P-orthophosphate (0.8 mc in 5 ml at pH 7.0). After 90 min the discs were washed and extracted as described (see "Materials and Methods"). Aliquots (0.1 ml) of the fraction eluted from DEAE-cellulose were chromatographed in solvents III and IV (see Fig. 1B). Radioactive spots were eluted and estimated. Since kinetin inhibited total ^{32}P uptake by 30%, the results are expressed as a percentage of total radioactivity recovered from the chromatograms. A′ and B′ are duplicates, as are C′ and D′.

Assignment	^{32}P Radioactivity			
	Control		Kinetin	
	A′	B′	C′	D′
	% of total recovered from chromatograms			
GTP	1.00	1.07	1.29	1.24
UTP	1.79	1.52	1.40	1.53
ATP	7.47	6.32	7.87	7.30
ADP	2.80	2.48	2.83	2.64
UDP-glucose	1.13	1.28	1.25	1.33
DPN	0.32	0.36	0.44	0.38
Fructose-1,6-diP	0.27	0.23	0.29	0.29
Glucose-diP (hexose-diP)	1.37	0.90	0.90	0.92
Glucose-6-P(Mannose-6-P)	11.77	10.49	11.25	11.08
Glucose-1-P	1.15	0.82	1.00	0.93
Fructose-6-P	1.93	1.92	1.84	1.77
AMP	1.18	0.88	1.01	1.15
UMP	0.86	1.02	1.07	1.16
Unknown	0.27	0.25	0.24	0.26
3-Phosphoglyceric acid	4.65	4.17	4.64	4.48
2-Phosphoglyceric acid	0.28	0.49	0.51	0.46
α-Glycerophosphate	0.23	0.26	0.23	0.26
Phosphoenolpyruvate	0.79	0.91	1.11	0.87
Phosphatyl choline	1.45	1.22	1.35	1.11
P_i	59.07	63.23	59.27	60.61
6-Phosphogluconic acid	0.06	0.06	0.07	0.06

these effects may occur by the induction of amylase, by a mechanism similar to that known to occur when barley aleurone tissue is treated with gibberellic acid (7). The effects of kinetin and gibberellic acid on starch were not additive, suggesting that both compounds may act through a single process. However the effects of kinetin and gibberellic acid on expansion were additive, suggesting independent modes of action to increase leaf disc expansion. Although kinetin increased the degradation of starch, there was a substantial decrease (30–40%) in soluble sugars in kinetin-treated discs after 24 hr (Table VI). Thus, the products of starch degradation did not accumulate as free sugars but were apparently utilized for the synthesis of cellular structural materials.

Kinetin did not affect total $^{14}CO_2$ incorporation into leaf discs floated on Vickery's solution for 24 hr. Fractionation of ethanol extracts from these discs showed that kinetin-treated discs had reduced levels of ^{14}C-glucose and ^{14}C-sucrose, indicating greater utilization of these sugars (Table III). These results were confirmed by more rigorous fractionation procedures which showed 35 to 40% decreases in radioactivity in glucose, sucrose, and fructose (Table V). The failure to observe such large decreases in radioactivity in sucrose and fructose in 80% ethanol extracts (Table III) was probably due to incomplete resolution during chromatography. Contaminating compounds would have been removed by the later extraction procedures. Total radioactivity eluted from DEAE-cellulose was unaffected by kinetin. However, the phosphate esters in this fraction from kinetin-treated tissue had 15% greater radioactivity than the controls. Total $^{14}CO_2$ incorporation into ATP was increased by 40% by kinetin.

Analysis of ^{32}P-orthophosphate incorporation into discs showed that, when corrected to equal total uptake, kinetin stimulated ^{32}P-orthophosphate incorporation into chloroform-soluble material but had very little effect on ^{32}P distribution between other fractions. Kinetin had little effect on the percentages of ^{32}P incorporated into the various phosphate esters. A small stimulation into GTP, DPN, and UMP and a slight decrease into fructose-6-P were observed in kinetin-treated tissue.

The results of ^{32}P-orthophosphate incorporation are difficult to interpret because of reduced total ^{32}P uptake in the presence of kinetin and the fact that in other experiments we have observed a large decrease in free P_i in discs floated with kinetin for 24 hr. Also the precise location of the P_i pools is not known, although it is likely that the vacuole is the main source of P_i. The results do suggest that kinetin causes increased synthesis of lipids required for membranes and for structural cell components.

Increased synthesis of ATP occurred during kinetin treatment (Table IV). The failure to observe increased phosphorylation of this compound following kinetin treatment (Table VII) could have been due to a change in the specific radioactivity of the P_i pool.

In view of the above data we can conclude that kinetin mobilizes starch reserves and increases the flow of sugars required for synthesis of lipids and structural materials in floated discs. However, neither $^{14}CO_2$ nor ^{32}P labeling of the tissue produced clear evidence for a specific kinetin effect on intermediary metabolism. If kinetin does act specifically to stimulate the utilization of carbohydrates, then the effect is not evident as an increase in the radioactive labeling of the intermediates of glycolysis. This may be because the pools of metabolic intermediates are small, fairly constant in size, and rapidly equilibrated. In these circumstances increased metabolism would cause increased radioisotope incorporation into intermediates only if the specific radioactivity of precursor pools was increased.

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