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The Levels of Soluble Nucleotides in Wheat Aleurone Tissue[1]

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G. G. COLLINS, C. F. JENNER, AND L. G. PALEG
Department of Plant Physiology, Waite Agricultural Research Institute, The University of Adelaide, Glen Osmond, South Australia

ABSTRACT

The content of soluble nucleotides in aleurone layers isolated from mature wheat (*Triticum aestivum* var. Olympic) grain was investigated. The most abundant nucleotides were adenosine triphosphate, uridine triphosphate, and uridine diphosphoglucose. Smaller amounts of guanosine triphosphate, cytidine triphosphate, adenosine diphosphate, and nicotinamide adenine dinucleotide were also identified. The levels of some of these nucleotides were increased after incubation of the tissue under certain conditions.

Nucleotide levels were measured at intervals during incubation of aleurone layers in water. The changes observed are discussed in relation to a response by the tissue to wounding.

Gibberellic acid applied to the aleurone layer of mature cereal grain in the absence of the embryo stimulates the activity and secretion of a number of hydrolytic enzymes (10, 20, 22). A lag phase of some 6 to 8 hr occurs between the addition of GA_3 and the first detectable increase in enzyme activity (9, 12).

Investigators of the mode of action of GA_3 have paid close attention to its effect on nucleic acid metabolism (8, 25, 32). The results are not unequivocal, however, and it was desirable to investigate other aspects of metabolism which could be influenced by the hormone. The soluble nucleotides, in addition to their function as precursors for nucleic acid synthesis, participate in many different biochemical reactions, and it was expected that a study of the effect of GA_3 on these compounds would reveal facets of hormone action not previously known.

Phillips and Paleg (23) reported a method by which up to 20 g of wheat aleurone can be prepared, relatively free of endosperm, in about 60 min. This tissue responds to exogenous GA_3 in a fashion apparently identical to that of both embryo-free half-seeds and hand-peeled aleurone layers. The results reported here indicate the levels of soluble nucleotides in this tissue and the effect on these compounds of the technique used to isolate the tissue. The companion paper describes the effects of GA_3 on the metabolism of the nucleotides (12).

MATERIALS AND METHODS

Mature wheat grain (var. Olympic) was stored over a saturated solution of $CaCl_2 \cdot 2H_2O$ (19); the seeds, before use, were bisected transversely; and the embryo halves were discarded.

The following solvents were redistilled before use: triethylamine, isobutyric acid, ethanol, diethyl ether (over $FeSO_4$ and CaO) and 1-propanol (over KOH). Water was distilled twice and deionized. All other chemicals and solvents used were of reagent grade.

All equipment and water was sterilized before use by autoclaving at 121 C and 6.8 kg pressure for 20 min. A sterilizing solution for the half-seeds was prepared by suspending 5 g CaOCl in 100 ml water, shaking the suspension for 10 min, and then filtering. The half-seeds were soaked in the filtrate for 2 hr, rinsed 10 times in water, and then allowed to imbibe water for 24 hr at 30 C.

After imbibition, the half-seeds were aseptically transferred to a sterile glass jar; then a weighted polyethylene bottle (sterilized by immersion in 70% ethanol) was inserted in the jar. The jar was capped and rotated at 50 rpm for 30 min (23) with periodic additions of water. The suspension was filtered, and the tissue was returned to the jar and rolled for a further 7 to 8 min. After further filtration and thorough rinsing, the tissue was drained on absorbent paper and weighed out for use. Phillips and Paleg (23) found that bacterial and fungal colonies were almost completely absent even 36 hr after isolation of the tissue. Since most incubations used here were 24 hr or less, no further precautions were taken.

The technique used to extract and separate the soluble nucleotides was modified from procedures reported by Cole and Ross (11), Isherwood and Barrett (13), and Jenner (15). Treated tissue was transferred to a cold room, and all operations, unless otherwise stated, were carried out at 4 C. The tissue was first rinsed in ice-cold water and drained on absorbent paper, and then it was disintegrated with an Ultra-Turrax in 5% (w/v) trichloroacetic acid containing 0.15% (w/v) 8-hydroxyquinoline. Each extract was left to stand for 15 min, and an equal volume of a solution of chloroform-isoamyl alcohol (24:10, v/v) was then added (29). After vigorous shaking, the mixture was centrifuged at 4000 rpm for 5 min. The upper aqueous layer was transferred to twice its volume of diethyl ether, shaken, and allowed to settle, and the aqueous layer was again extracted with twice its volume of ether. The cell debris at the interface was washed three times with small portions of water; the washings were passed through the ether and finally added to the bulk of the extract.

To remove much of the non-nucleotide material, we passed the acid extract through columns of Dowex AG50 W-X8 resin, 200 to 400 mesh in the H^+ form, followed by DEAE-cellulose (Whatman DE-11) in the HCO^-_3 form. The eluate from the Dowex columns was first adjusted to pH 4.6 with 2 M NH_4OH and then extracted with an equal volume of ether before being passed through the cellulose.

Finally, the nucleotides and other phosphate esters were eluted from the cellulose with 0.5 M triethylammonium bi-

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carbonate at pH 7.4 (26). The effluent was evaporated to dryness *in vacuo* at a temperature not exceeding 30 C and applied as a thin band to Whatman No. 3MM paper previously washed with 0.1 M oxalic acid followed by 2 M acetic acid. The paper was developed in 1-propanol-ammonia-water-0.2 M EDTA (72.5:0.1:27.3:0.1, v/v) adjusted to pH 7 with acetic acid.

After development, the UV[2]-absorbing areas were located, cut from the paper, and soaked briefly in absolute ethanol to remove excess salt. The strips were then eluted with water, and the eluate was dried *in vacuo*. Further separation was achieved by paper chromatography (Whatman No. 1) in two dimensions using isobutyric acid-ammonia-water (57:4:39, v/v) followed by 95% ethanol-1 M ammonium acetate, pH 7.5, 10 mM with respect to EDTA (7:3, v/v).

Nucleotides were located by UV light, cut from the paper, and eluted with 0.01 N HCl for a minimum of 4 hr. Three blank areas of about the same size as those of the nucleotides were removed from each chromatogram and similarly eluted. The amount of each nucleotide was estimated by means of its extinction coefficient from the corrected absorbance at 260 nm. The extinction coefficients used (24) were ($\times 10^{-3}$): ATP, 14.3; ADP, 14.5; CTP, 6.1; UTP, 9.9; UDP-sugars, 9.9; and GTP, 11.8.

Identification of Nucleotides. The absorption spectra in 0.01 N HCl were determined in a recording spectrophotometer and compared with the spectra published in P-L Biochemicals Circular OR-10 (24). R_F values were compared against authentic nucleotides after paper chromatography with the following solvent systems: (*a*) isobutyric acid-ammonia-water (66:1:33, v/v) (24); (*b*) isobutyric acid-ammonia-water (57:4:39, v/v) (24); (*c*) 95% ethanol-1 M ammonium acetate, pH 7.5, 10 mM in EDTA (7:3, v/v) (24); (*d*) 95% ethanol-1 M ammonium acetate, pH 3.8, 10 mM in EDTA (7:3, v/v) (24); (*e*) 1-butanol-acetic acid-water (5:2:3, v/v) (31); (*f*) electrophoresis for 60 min in 50 mM tris-citrate buffer (pH 4.8) at 30 v/cm (15).

Cochromatography was carried out by adding mixtures of authentic nucleotides to aleurone tissue before extraction. The extracts were separated by two-dimensional chromatography with solvents *b* and *c*, and *b* and *f* described above.

Hydrolysis of nucleotides was effected with either 98% formic acid in sealed tubes held at 175 C for 30 min (33) or 1 N HCl at 100 C for 60 min (1). The hydrolysis products were chromatographed on Whatman No. 1 paper with 95% isopropanol-concentrated HCl (65:17.4, v/v, made to 100 ml with water) as solvent.

UV-absorbing compounds, indistinguishable from UDP-glucose, were hydrolyzed in 0.01 N HCl (pH 2) at 100 C for 15 min (6). The hydrolysate was passed through Amberlite IR-120 (H^+) and IR-4B (OH^-) resins; the effluent was taken to dryness and chromatographed on Whatman No. 1 paper in ethyl acetate-pyridine-water (10:4:3, v/v). With the aid of markers of authentic sugars, the sugar-containing areas were cut from the paper, eluted with water, and estimated by the anthrone method (15).

Starch Determinations on Aleurone Tissue. After extraction of the tissue with trichloroacetic acid, the residue was washed in ethanol, air-dried, and finely ground with a mortar and pestle. In some cases the determination was made on oven-dried aleurone, which was then ground in a homogenizer.

Samples were first boiled in water and then dispersed in $HClO_4$ and precipitated with iodine (27). The starch-iodine complex was decomposed, and the starch was dissolved in 0.5 N NaOH (18) and estimated by the anthrone method (17).

RESULTS

Residual Starch Adhering to Isolated Tissue. Some starchy endosperm remains attached to the isolated aleurone tissue. This residual contamination was determined as being 56% of the total dry weight after 30 min of the isolation procedure and 15% after a further 7 to 8 min (average of duplicate results).

The Identity of UV-absorbing Compounds. Nine major UV-absorbing areas were discerned after two-dimensional chromatography. These were eluted with 0.01 N HCl (pH 2), and their absorbance was measured at wavelengths of 260, 296, and 320 nm in 1-cm quartz cuvettes. Two of the major components had a ratio of absorbance at 260 to 296 nm of 0.88 to 0.96, a value too high to be characteristic of nucleotides. The absorption spectra of the other seven compounds were determined with a Unicam SP 800 recording spectrophotometer. These data, together with chromatographic mobilities and the characteristics of the products of hydrolysis, were sufficient to identify the UV-absorbing areas as ADP, ATP, CTP, GTP, UTP, UDP-sugars, and NAD.

Six or seven additional UV-absorbing areas were also detected, but these were too weakly absorbing to be measured accurately. The small amounts of these minor components did not change in response to the treatments subsequently applied to the tissue. The following nucleotides were not detected: AMP, UMP, CMP, GMP, ADP-glucose, NADP, and CDP. NAD was present, but poor recovery from the cation exchange resin precluded accurate measurement. This effect has also been reported elsewhere (14).

One substance, which moved to R_F 0.47 in isobutyric acid-ammonia-water and R_{AMP} 1.27 in ethanol-ammonium acetate, gave a light blue fluorescence under UV light. Other fluorescent material was confined mainly to the origin of the chromatograms.

Traces of adenine were found in hydrolysates from CTP, and of cytosine in hydrolysates from ATP, indicating that these compounds were not completely separated by the two-dimensional system of chromatography used. For samples of ATP contaminated with CTP the absorbance values at 296 nm were about 5% of the values at 260 nm. As pure ATP has no extinction at 296 nm, and at 296 and 260 nm the extinction values of CTP are almost equal, it was assumed that the degree of contamination of ATP by CTP amounted to about 5%. No correction was made for this in the levels of either nucleotide.

Material resembling UDP-glucose was subjected to mild acid hydrolysis, followed by paper chromatography of the neutral fraction. Four reducing substances were separated, with mobilities identical to glucose, fructose, galactose, and xylose. The molar ratio of these substances was 1.00:0.44:0.44:0.38, respectively, and the molar ratio of UDP to total reducing substance was 1.00:0.94. The recovery of reducing compounds was 90%. As attempts to resolve these substances in solvents containing borate (7) were not successful, they were measured as a single fraction called "UDP-sugars."

A minor UV-absorbing component which appeared at R_F 0.32 in isobutyric acid-ammonia-water and R_{AMP} 2.08 in ethanol-ammonium acetate was also subjected to mild acid hydrolysis. Chromatography of the neutral fraction revealed reducing substances with mobilities identical to glucose and fructose. These were present in the molar ratio of 1.0:3.2, respectively. A compound corresponding to UMP was identified after hydrolysis of this component in 1 N HCl. The molar proportion

[2] Abbreviation: UV: ultraviolet.

of UDP to total reducing substance was 1.0:2.5, respectively. The identity of this component was not further investigated.

Authentic nucleotides corresponding to those identified in the tissue were added to aleurone tissue which was then extracted for soluble nucleotides. The final chromatogram was compared to one derived from tissue without such addition, and the pattern of UV-absorbing areas was found to be identical.

The Recovery of Authentic Nucleotides. An aliquot of a mixture of authentic nucleotides was added to one of two otherwise identical batches of aleurone tissue. Both lots of tissue were then extracted for soluble nucleotides. Another aliquot of the authentic mixture, equal to that added to the tissue, was developed in the two-dimensional system of paper chromatography used for the tissue extracts. The UV-absorbing compounds corresponding to the added nucleotides were eluted from the three papers and measured. The percentage recoveries, calculated by difference, were as follows: ATP, 96; ADP, 98; AMP, 95; UTP, 96; UDP, 84; UMP, 93; UDP-sugars, 85; CTP, 92; CDP, 96; GTP, 86; GDP, 82; GMP, 96.

No corrections, on the basis of the recovery data, have been made on subsequent measurements.

The Level of Nucleotides in Residual Starch. Since about 15% of the dry matter of isolated aleurone layers consisted of starch, it was necessary to determine how much nucleotide the starchy material contained.

Table I. *Content of Soluble Nucleotides in Freshly Isolated Wheat Aleurone*

Values in parentheses are the standard deviations of the mean (mean of 18 determinations).

Nucleotide	Amount in Aleurone
	nmoles/g dry wt
ADP	20 (±1.9)
ATP	218 (±5.5)
UTP	72 (±3.5)
GTP	34 (±1.9)
CTP	34 (±1.4)
UDP-sugars	204 (±9.8)

Two samples of tissue were prepared, equal in fresh weight but with starch contents of 54 and 28% of the total dry weight. These were analyzed for levels of nucleotides. The results showed that the sample with the greater amount of starch had the smaller amount of nucleotide. For calculation of the weight of aleurone in each sample, the assumption was made that starch accounts for 80.9% of the total weight of the endosperm (30). The estimated weight of endosperm was subtracted from the total weight of the sample, and the amounts of each nucleotide were expressed on the basis of the weight of aleurone (including testa-pericarp). From these data it was concluded that the vast majority, if not all, of the nucleotides are derived from the aleurone and essentially none from the adhering endosperm. No ADP-glucose was detected in this experiment.

The Level of Nucleotides in Aleurone Tissue. Portions of aleurone tissue weighing 4 g fresh weight were extracted for soluble nucleotides by the method described. The results are shown in Table I and are the means of 18 separate determinations. Since the dry weight was calculated consistently to be 25% of the fresh weight, the values correspond approximately to nanomoles per g dry weight of tissue.

Of the nucleotides measured, 88% contain the bases adenine and uracil. ATP and UDP-sugars are the most abundant individual components and together account for 72% of the total. The molar proportions of the four nucleoside triphosphates, ATP, UTP, GTP, and CTP, are 1.00:0.33:0.16:0.16, respectively, and the molar ratio of ATP to ADP is approximately 11:1. No AMP was detected at any stage in the experiments.

Changes in Composition of Nucleotides during Incubation of Tissue. Aleurone tissue was isolated and weighed into portions of 4 g fresh weight. One of these portions was extracted immediately for soluble nucleotides, and the other was incubated with 10 ml of water in a 9-cm Petri dish at 30 C and then also extracted. The results (Table II) show that the levels of most of the nucleotides change during the course of the incubation. During the first 1 to 2 hr, the amounts of UDP-sugars and ATP fall, while those of ADP, UTP, GTP, and CTP rise. Except for CTP, the values return to within 12% of the control level by the 24th hr.

It is apparent that modifications in the nucleotide pool are initiated during the first few hours of incubation of aleurone tissue in water. These fluctuations could represent a wounding

Table II. *Changes in Levels of Soluble Nucleotides during Incubation of Wheat Aleurone in Water*

A separate sample of aleurone was prepared for each period of incubation. This was divided into two equal portions, one of these being used to determine the initial nucleotide content of the sample and the other to measure the levels after incubation for the times indicated. The results are the averages of three separate determinations.

Nucleotide	Sample	Time of Incubation					
		1 hr	2 hr	3 hr	6 hr	15 hr	24 hr
		nmoles nucleotide/4 g fresh wt					
ATP	Initial	209	246	209	227	205	214
	Incubated	214 (102)[1]	225 (91)	192 (92)	229 (101)	211 (103)	208 (97)
ADP	Initial	20	18	27	17	19	21
	Incubated	21 (105)	27 (150)	32 (118)	20 (118)	18 (95)	18 (86)
UDP-sugars	Initial	219	254	181	173	203	196
	Incubated	183 (84)	178 (70)	157 (87)	200 (116)	206 (101)	218 (111)
UTP	Initial	80	93	64	59	60	75
	Incubated	100 (125)	121 (130)	82 (128)	83 (141)	86 (143)	66 (88)
GTP	Initial	30	30	34	42	33	33
	Incubated	35 (117)	30 (100)	30 (88)	42 (100)	31 (94)	37 (112)
CTP	Initial	28	35	29	41	33	37
	Incubated	37 (132)	37 (106)	30 (103)	45 (110)	48 (145)	54 (146)

[1] Percentage of initial level.

response of the tissue and consequently were investigated in greater detail.

The Response of Soluble Nucleotides to Isolation of Tissue. Aleurone tissue was prepared from half-seeds previously imbibed for periods of 24, 26, and 30 hr. Some tissue was immediately extracted for nucleotides, and the remainder was incubated in water for 2, 4, or 6 hr and then also extracted. Changes in the levels of ATP, UTP, and UDP-sugars were followed and are shown in Figure 1. In general, the changes observed over the 6 hr of incubation were similar for each time of imbibition of half-seeds and also corresponded to those shown in Table II. It was concluded that the technique used to prepare the aleurone was, in itself, causing changes in the amounts of nucleotides and that the changes were responses to the wounding of the tissue during isolation.

Assay of the Ambient Solution for the Presence of Nucleotides or Bases. The possibility was examined that the fall in the levels of some nucleotides shortly after tissue isolation was due to leakage into the ambient solution.

The ambient solutions from two of the experiments described in the previous section were removed from around the tissue, and an equal volume of ice-cold 0.4 N $HClO_4$ was added. After 15 min at 4 C, the extracts were neutralized with 0.33 N KOH and left at 4 C for several hours. $KClO_4$ and acid-insoluble material were then removed by centrifugation, and the supernatants were examined by chromatography.

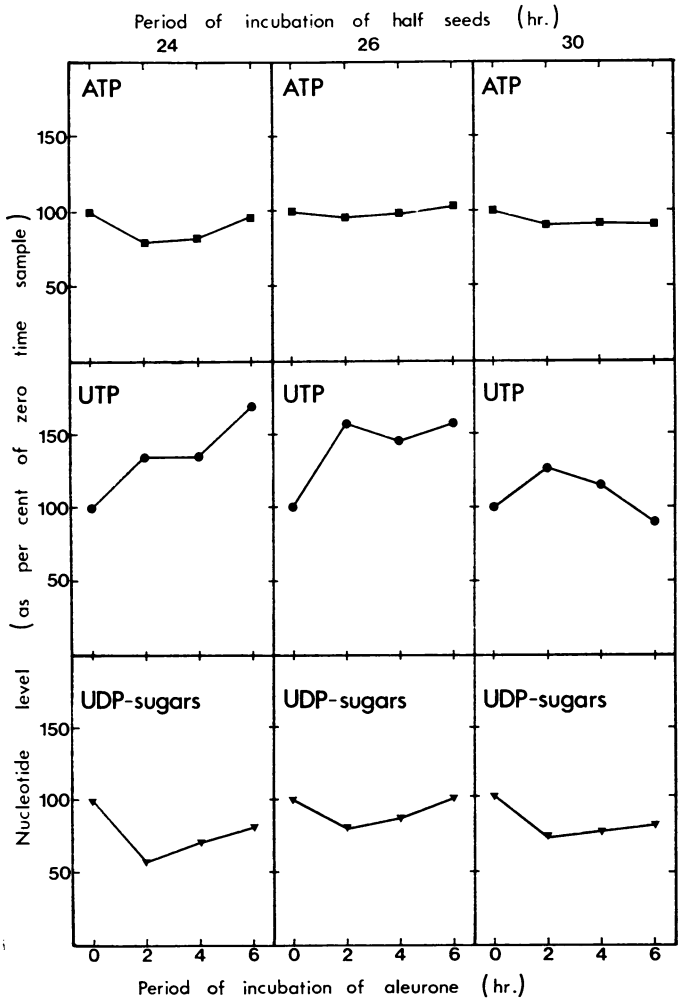

FIG. 1. Changes in the levels of ATP, UTP, and UDP-sugars extracted from wheat aleurone cultured in water. Four lots of aleurone layers (4 g each) were prepared from the distal halves of wheat seeds previously imbibed for 24, 26, or 30 hr. The tissue was extracted for nucleotides immediately or incubated in 10 ml water at 30 C for a further 2, 4, or 6 hr before extraction. Nucleotide levels are plotted as a percentage of the values recorded at the time of isolation.

Table III. *Effect of Inorganic Phosphate on Nucleotide Levels*

Wheat aleurone (4 g fresh wt) was incubated in water or dilute solutions of KH_2PO_4 for 7 hr, and the nucleotide content was then determined. In one set of treatments, the tissue was incubated in Petri dishes in 10 ml of solution, while in the other it was gently shaken in 250-ml flasks containing 30 ml of solution.

Nucleotide	Petri Dish		Shaken	
	Water[1]	0.25 mM KH_2PO_4[2]	Water[2]	1 mM KH_2PO_4[3]
	nmoles nucleotide/4 g fresh wt			
ADP	21 (117)[4]	18 (100)	20 (111)	18 (100)
ATP	227 (100)	254 (112)	294 (130)	328 (145)
CTP	53 (100)	70 (132)	121 (228)	132 (249)
GTP	36 (100)	46 (128)	54 (150)	58 (161)
UTP	129 (100)	189 (146)	219 (170)	209 (162)
UDP-sugars	196 (110)	194 (109)	178 (100)	199 (112)

[1] From three experiments.
[2] From two experiments.
[3] From one experiment.
[4] Percentage of lowest level.

The samples derived from aleurone of half-seeds imbibed for 24 hr were divided into two portions, one of which was developed in isopropanol-HCl-water (170:41:39, v/v) and the other in isobutyric acid-ammonia-water (57:4:39, v/v). Samples derived from the aleurone of half-seeds imbibed for 26 hr were developed in 95% ethanol-1 M ammonium acetate, pH 7.5, 10 mM with respect to EDTA (7:3, v/v). Areas corresponding to authentic bases and nucleotides were eluted from the papers and measured for UV absorbance. Those areas corresponding to guanine were eluted with 1.6 N HCl, other bases with 0.1 N HCl, and nucleotides with 0.01 N HCl (31).

It was found that only small amounts of compounds resembling nucleotides could be detected in the ambient solution. The largest quantities were found after 2 hr of incubation, and no nucleotides could be detected after 4 or 6 hr. Although some UV-absorbing material was detected after chromatography with the isobutyric acid solvent, none was found after development of the 26-hr sample with ethanol-ammonium acetate. The amounts of ATP and UDP-sugars lost from the tissue were not of the same order as those found in the ambient solution.

It was concluded, therefore, that the decline in the levels of nucleotides could not be ascribed to the leakage of the nucleotides themselves. This work, however, does not rule out the possibility that the nucleotides were degraded to their corresponding bases in the ambient solution and that these, in turn, were metabolized to non-UV-absorbing compounds. Such a process seems unlikely.

Factors Affecting the Amounts of Nucleotides. Certain treatments were found to alter the amounts of some nucleotides, especially the nucleoside triphosphates. In these experiments the prepared aleurone layers were incubated for 7 hr either in dilute solutions of KH_2PO_4 or under conditions of constant agitation or a combination of both factors. The results were compared with those obtained from tissue incubated without agitation in water and are shown in Table III.

The results for each treatment were derived from different experiments and may simply reflect differences in batches of tissue. However, where P_i was added to the external medium, the levels of some nucleotides, especially the triphosphates, were increased. Furthermore, in almost all cases the most pronounced effect was on the amount of CTP, while the levels of ADP and UDP-sugars remained unaltered.

DISCUSSION

A technique has been described for the extraction and separation of soluble nucleotides from isolated aleurone layers of wheat, and the recovery of these compounds was greater than 80 to 90%. Derivatives of adenine and uracil together accounted for almost 90% of the nucleotides measured. This finding is similar to data obtained for wheat plants (3), corn roots (11), yeast (21), *Spirodela oligorrhiza* (4), immature wheat grain (15), phloem exudate (5), and *Chlorella pyrenoidosa* (28).

Individual nucleotide levels differ somewhat from those found in immature wheat grain by Jenner (15). Twenty days after anthesis, the amounts of ATP, UTP, and UDP-glucose in immature grain were 2.8-, 5.2-, and 6.8-fold higher, respectively, than those found in mature aleurone (per g fresh weight), yet derivatives of cytosine and guanine in the former were either absent or too low to be measured. In addition, the developing grain contained a significant quantity of ADP-glucose which appeared to be correlated with starch synthesis and which was not found in either the aleurone or endosperm of mature grain. Furthermore, the nucleotide content of the endosperm of the developing grain was higher than that of the testa-pericarp fraction (which included aleurone and embryo), while in the mature grain no nucleotides could be detected in the endosperm. All these differences could reflect a change in metabolism from starch synthesis to starch degradation.

Both the addition of dilute solutions of KH_2PO_4 to the incubation medium and agitation of the tissue induced increases in the levels of nucleotides, especially those of ATP, UTP, CTP, and GTP. A phosphate deficiency has been reported to cause a fall in the level of phosphate esters in *Spirodela* (4). However, in contrast to the results obtained with aleurone tissue, in *Spirodela* the relative proportion of the esters remained constant. It has been observed that the level of ATP in red blood cells is increased significantly when additions of inorganic phosphate are made to normal blood (16).

The effect of agitation could be attributed to more efficient aeration. However, it can be seen from Table III that while the level of ATP increased by 30%, that of CTP increased by more than 120%. Either the response of CTP to agitation of the tissue is different from that of the other nucleotides, or some factor other than aeration is contributing to the increase in amount of CTP. No conclusions can be made regarding changes in rates of metabolism of the nucleotides affected.

Fluctuations in the nucleotide content of aleurone during its incubation in water were found to be closely associated with the method used to isolate the tissue from the starchy endosperm. Compared to most other plant parts, the cell walls of the aleurone layer are exceptionally thick. Nevertheless, damage occurs during preparation, and it was concluded that this damage provided the stimulus for the modification in nucleotide levels.

Bagi and Farkas (2) demonstrated that ribonuclease activity in tobacco leaves increased after the leaves were mechanically damaged. A similar increase has been reported to occur in barley aleurone layers after separation from the starchy endosperm (10). It seems probable, therefore, that some of the changes which occurred in the nucleotide levels of wheat aleurone during the 6 hr after its separation from the starchy endosperm may have resulted from degradation and resynthesis of part or all of the RNA complement. Since the levels approach their original values after about 6 hr, it appears that the integrity of the tissue is regained. Thus, in the following paper, which describes the effect of GA_3 on the metabolism of the soluble nucleotides, isolated aleurone layers were preincubated in water for 6 hr before treatment with hormone.

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