Plant Physiol. (1972) 49, 404-410

The Metabolism of Soluble Nucleotides in Wheat Aleurone Layers Treated with Gibberellic Acid[1]

Received for publication July 6, 1971

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ABSTRACT

The metabolism of soluble nucleotides was investigated in wheat (*Triticum aestivum* var. Olympic) aleurone layers treated with gibberellic acid. Whereas nucleotide levels were relatively unaffected by the treatment, a transient increase was observed in the incorporation of ^{32}P. The effect was maximal 30 to 60 minutes after gibberellic acid was administered, and by 180 minutes incorporation was lower than in the control. The greatest changes were detected in the nucleoside triphosphates, particularly in cytidine triphosphate. The findings are discussed in relation to the mode of action of gibberellic acid.

When barley aleurone layers are treated with gibberellic acid the activity of a number of hydrolytic enzymes is increased (24) and at least two of the enzymes are synthesised *de novo* (8, 12). The incorporation of ^{32}P into RNA of barley aleurone was reported to be enhanced by GA_3 after 30 min, and it was claimed that by 4 hr a new species of RNA was detected (4). Other results, however, argue against an effect of GA_3 on nucleic acid metabolism (24), and in addition there are conflicting data about the effect of actinomycin D on this tissue (19, 23, 28). There is, therefore, a need for additional information on the mode of action of GA_3 on the aleurone layer.

The soluble nucleotides participate both as cofactors in many diverse biochemical reactions and as precursors for the synthesis of nucleic acids. Thus, a study of the effect of GA_3 on the metabolism of these compounds could be expected to show aspects of the action of the hormone not readily detectable by other methods. In a previous paper (7), techniques were described for measuring the free nucleotides of wheat aleurone layers. This tissue responds to GA_3 in a fashion apparently identical to that of barley aleurone and, in contrast to the latter, can be easily prepared in sufficient quantity (22) to permit the measurement of the nucleotide content. This paper reports the effects of GA_3 on the metabolism of the nucleotides.

MATERIALS AND METHODS

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

GA_3 was purchased from Merck and Co., Inc., Rahway, New Jersey (97.4% GA_3); actinomycin D from Mann Research Laboratories, Inc., New York, New York; ^{32}P (carrier-free orthophosphate) from the Australian Atomic Energy Commission, Sydney, Australia.

GA_3 was sterilized before use by filtering through equipment obtained from the Millipore Filter Corporation, Bedford, Massachusetts (filter porosity, 0.22 μ). Glassware used in experiments involving ^{32}P was pretreated with Siliclad, a silicone-based preparation manufactured by Clay-Adams Inc., New York, New York.

Determination of α-Amylase. The method used was a modified version of the technique reported by Filner and Varner (8). Starch was prepared by boiling for 1 min a 0.15% suspension of native potato starch in 100 ml of 40 mM KH_2PO_4 in 1 mM calcium acetate. After cooling, the denatured amylopectin was removed by centrifugation, and the supernatant of crude amylose was used for the assay. I_2-KI indicator solution was made by dissolving 1.016 g KI and 0.1016 g I_2 in 100 ml of water. This was stored in the dark and diluted 10-fold with water prior to use.

After treatment, both the surrounding medium and the tissue were assayed for α-amylase activity. An aliquot of the medium was mixed with an equal volume of 10 mM calcium acetate, and the volume was then made up to a standard amount with 5 mM calcium acetate. The tissue was rinsed in ice-cold water, disintegrated with an Ultra Turrax high speed blender in 5 mM calcium acetate at 4 C, and centrifuged at 5000 rpm for 10 min; the supernatant was decanted. The crude enzyme preparations were then heated to 70 C for 20 min to inactivate β-amylase and centrifuged at 5000 rpm for 10 min.

In the assay procedure, an aliquot of the enzyme preparation was added to an equal volume of starch substrate; then at intervals 1 ml of this mixture was added to 1 ml of dilute I_2KI solution. After diluting to 5 ml with water, the absorbance was measured at 620 nm against a blank of I_2KI and water. All solutions were held at 30 C. Zero time samples were prepared by adding 0.5 ml of enzyme to 1 ml of I_2KI and then adding 0.5 ml of starch and diluting to 5 ml with water.

The data were expressed as percentages of the optical density of the zero time sample, and enzyme activity was calculated by taking the reciprocal of the time to reach 50% of the original color intensity (2). After correcting for dilutions, the results were expressed as Starch-Iodine-Color units per g fresh weight of tissue.

Estimation of Phosphate. Inorganic phosphate was precipitated from solution as the calcium salt (16) and estimated as vanadomolybdophosphate (20). Total phosphate was measured after digestion of samples with H_2SO_4 and H_2O_2 (1).

Preparation of Tissue and Separation of Nucleotides. The method used to isolate wheat aleurone from the starchy endosperm has been described in detail elsewhere (22).

[1] This research was supported by the Australian Wool Board through a Senior Postgraduate Scholarship awarded to G. G. C. and is from a thesis presented for a Ph.D. degree.

The procedure reported earlier (7) for the final separation of nucleotides by paper chromatography in two dimensions was found to be unsuitable for work involving ^{32}P, since P_i was not always completely separated from ATP. The substitution of 95% ethanol-1 M ammonium acetate, pH 3.8, 10 mM in EDTA (7:3, v/v) for the solvent with ammonium acetate at pH 7.5 was found to separate P_i and ATP completely in all cases. The cation exchange resin used previously was replaced by powdered cellulose (Whatman P-11). This allowed the quantitative recovery of NAD.

Previously (7), large changes in nucleotide levels were found to occur shortly after aleurone layers were isolated. Accordingly, unless stated otherwise, in this study the tissue was subjected to preincubation for 6 hr in water before use.

Detection of Radioactivity. Radioactive samples were dried on copper planchets and counted in a low background Nuclear-Chicago gas flow counter. Autoradiographs were prepared by fastening chromatograms to Kodak envelope-packed x-ray films. The films were developed in commercial reagents after exposure for several hours.

RESULTS

The Effect of GA_3 on the Production of α-Amylase. Immediately after isolation, samples of aleurone tissue were incubated in water or solutions of GA_3 (Fig. 1). The activities of α-amylase in the ambient solution and tissue are linearly related to the logarithm of the concentration of GA_3 up to about 10 μg/ml. Above 100 μg/ml the activity of the enzyme apparently declines. The ratio of the activity of α-amylase in the ambient solution to that in the tissue also increases as the level of GA_3 is raised from 1 ng/ml to 1 μg/ml, but above 1 μg/ml there is little further change. From these results, a concentration of 100 μg/ml was selected for all further experiments, since at that level it could be assumed that the hormone was not limiting.

Figure 2 illustrates the fact that, when GA_3 is added to freshly isolated aleurone, there is a lag period of about 8 hr before a stimulation is detected in the activity of α-amylase. This lag period is reduced to about 6 hr for tissue preincubated in water for 6 hr before GA_3 is introduced. Furthermore, the production of α-amylase is considerably greater in preincubated than in freshly isolated tissue.

In barley, extending the period of imbibition has been reported to shorten the induction period or increase the activity of α-amylase is response to GA_3 (17). Apparently, preincubating wheat aleurone and extending the period of imbibition of barley grain have similar effects on the responsiveness of aleurone tissue to applications of GA_3.

The Effect of Actinomycin D on α-Amylase Activity. It has been observed that actinomycin D inhibits the stimulation of α-amylase activity by GA_3 in barley aleurone if applied with the hormone, but is ineffective when added 7 hr later (28). Since the wheat aleurone tissue used here was preincubated in water for 6 hr, the effect of this treatment on the sensitivity of the tissue to actinomycin D was examined.

The production of α-amylase in both fresh and preincubated tissue was inhibited by actinomycin D, and the ratio of the amount of α-amylase in the ambient solution to that in the tissue fell consistently as the level of actinomycin D was increased. Regression analysis of the ratio of released to internal α-amylase against total α-amylase activity showed that the values obtained from adding actinomycin D to freshly isolated tissue were significantly different (1% level of significance) from those derived from preincubated tissue. However, the ratios at different concentrations of GA_3, derived from the data of Figure 1, and those obtained by adding actinomycin D to preincubated tissue were not statistically different.

The Effect of GA_3 on the Incorporation of ^{32}P into Nucleotides. A time course study of the effect of GA_3 on the amounts of nucleotides and the incorporation of ^{32}P into them was carried out, and the results are shown in Table I. With the

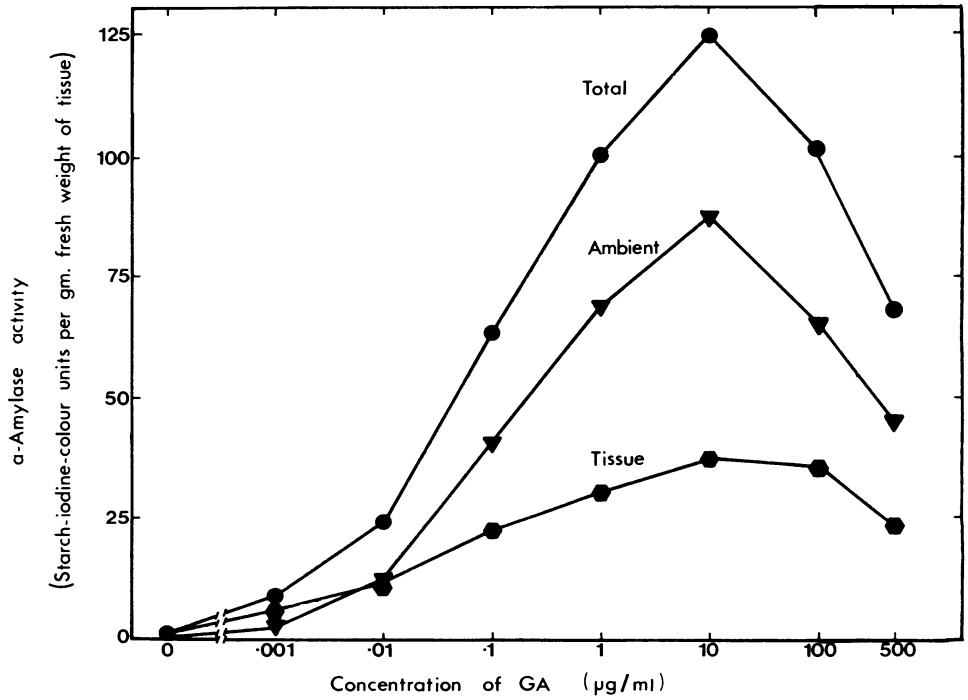

FIG. 1. α-Amylase production in response to GA_3. Wheat aleurone layers (2 g fresh weight) were isolated and incubated at 30 C in 7 ml of water or in solutions containing different concentrations of GA_3. The activity of α-amylase in both the tissue and the ambient solution was measured 24 hr later.

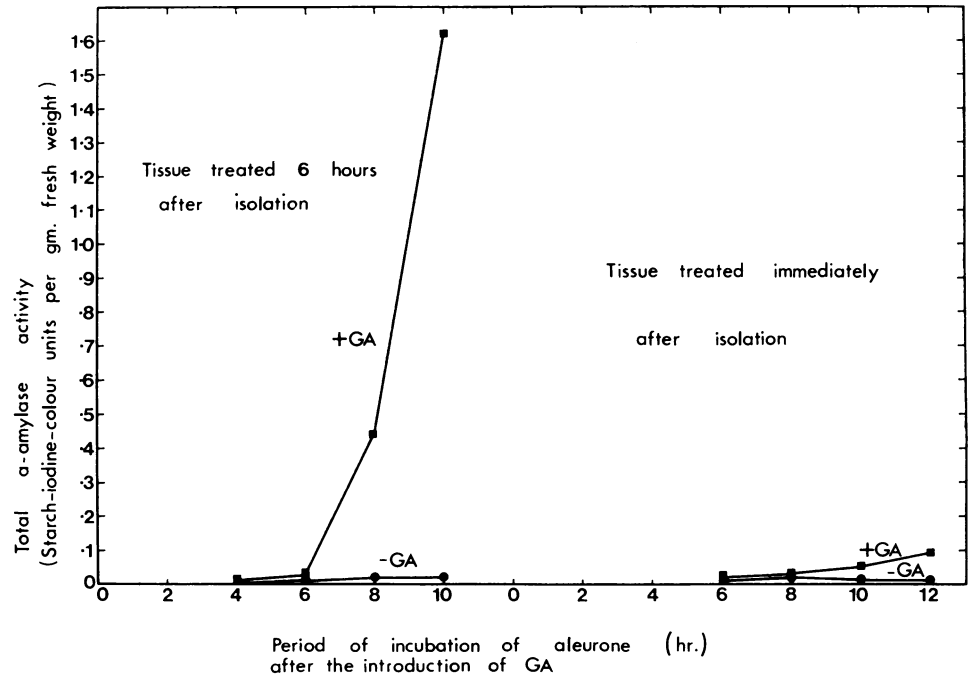

FIG. 2. The induction period for α-amylase induced by GA_3. Aleurone layers were cultured in water or GA_3 (100 μg/ml) immediately after isolation or after a further 6-hr preincubation in water. The ambient solution and the tissue were assayed for α-amylase at intervals up to 12 hr after the addition of GA_3.

exception of NAD and CTP, the levels of nucleotides (Table I[a]) were not significantly changed by the treatments applied to the tissue.

Although the total counts incorporated (Table I[b]) increased with extended times of treatment, a significant difference was recorded only between the mean values at 15 and 120 min (5% level). A similar trend was evident in the values for specific radioactivity (Table I[c]). The mean specific radioactivities of ATP and UTP were identical, and these two nucleotides were the most highly radioactive. The specific radioactivity of GTP was only 8% lower than that of ATP and UTP, yet the amount in the tissue was some 80% less. Moreover, in the absence of the hormone, CTP was more than twice as abundant as GTP, but was 30% less radioactive. The values for ADP and UDP-sugars were almost identical, and that of NAD was lowest of all.

GA_3 significantly affected the specific radioactivity of all the nucleotides, but, as the specific radioactivities of the nucleoside triphosphates displayed the greatest response to GA_3, the results for these compounds alone were transformed by adjusting them on the basis of the control values as 100. The adjusted means, together with the actual differences in specific radioactivity, are shown in Table II, where it is apparent that, after 15 min, GA_3 depressed the incorporation of ^{32}P into the triphosphates. After a further 15 min, GA_3 greatly increased it, and over the next 90 min the effect disappeared. The percentage effect of GA_3 on CTP was significantly greater than on the other three compounds, and the interaction of nucleotide with time is highly significant. For all four triphosphates, the absolute increase in specific radioactivity at 30 min was about the same. At 90 min, however, both the relative and absolute response of CTP to GA_3 was more evident. The results are consistent with a general increase in the phosphate metabolism of the nucleotides, accompanied by a specific effect on CTP. This is shown more clearly by the data in Table I: whereas the increase in specific radioactivity of ATP, UTP, and GTP at 30 min can be attributed to small reductions in their levels coupled with small increases in the number of counts incorporated, the increase in the specific radioactivity of CTP was due to greater radioactivity alone. On the other hand, at 90 min the effect of GA_3 on CTP can be ascribed almost wholly to a drop in the level of CTP. At the same time, the hormone had little effect on either the level or radioactivity of ATP and GTP and only slightly increased both level and radioactivity of UTP.

Aliquots of the radioactive solutions used in the previous experiment were counted and, since there were no significant differences between means, variations in the amounts of ^{32}P provided could not account for the differences recorded between times of incubation or for the effects of GA_3.

In another series of experiments, batches of tissue (4 g fresh weight) were incubated in 9-cm Petri dishes with 10 ml of solution. In the first of these, the medium after 6 hr preincubation was replenished with fresh solutions of water or GA_3 and the incubations continued for either 45 or 165 min. The medium was then replenished with solutions of phosphate (KH_2PO_4, 50 nM containing 50 μc ^{32}P) or phosphate and GA_3, and after a further 15 min the tissue was extracted for nucleotides. As with the previous experiment, only slight effects were noted on nucleotide levels. After 60 min the amounts of UDP-sugars, ADP, and UTP were depressed by GA_3 compared to the effects of the corresponding treatment with water, and these were the only significant effects on nucleotide concentrations observed. However, treatment with GA_3 for 60 min increased the over-all specific radioactivity by 26% (average of three separate values). The increases for ATP, UTP, GTP, and CTP were 15, 24, 24, and 35%, respectively. After a further 2 hr, the mean specific radioactivity of all nucleotides had fallen to a value 20% below the control.

In the second of these experiments, the ambient solutions were left relatively unchanged throughout the entire incubation. GA_3 was added as a highly concentrated solution (0.5 ml of 2 mg/ml), and ^{32}P was added directly from a microsyringe. At no time was the medium renewed. Values for specific radioactivity after 30 min of treatment (one experi-

Table I. *Time Course of the Effect of GA_3 on the Incorporation of ^{32}P into the Soluble Nucleotides of Wheat Aleurone*

Batches of tissue (4 g fresh wt) were incubated in flasks containing 30 ml of solution, shaken on a water bath at 30 C. After 6 hr preincubation in water, 7.5 ml of the ambient solution were removed from each flask and replaced with 7.5 ml of either water or GA_3 (final concentration, 100 μg/ml). The flasks were shaken for periods of up to 120 min. For the final 15 min of incubation, the ambient solutions were replaced completely by solutions of phosphate (50 nM KH_2PO_4 containing 50 μc ^{32}P) or phosphate and GA_3. The results are the means of two separate experiments.

(a) Nucleotide Levels

Nucleotide	Total Time of Incubation							
	15 min		30 min		90 min		120 min	
	−GA	+GA	−GA	+GA	−GA	+GA	−GA	+GA
	nmoles of nucleotide							
NAD	52	52	40	52	41	52	46	62
ADP	18	22	21	22	20	20	17	18
ATP	321	313	307	286	281	277	310	294
CTP	126	120	114	114	128	102	110	106
GTP	52	48	54	50	54	52	46	52
UTP	250	232	228	214	210	231	244	220
UDP-sugars	214	193	190	194	166	168	208	190
Means	147	140	136	133	128	129	134	134

(b) Total Counts Incorporated

Nucleotides	Total Time of Incubation							
	15 min		30 min		90 min		120 min	
	−GA	+GA	−GA	+GA	−GA	+GA	−GA	+GA
	cpm × 10^3							
NAD	0.8	0.7	0.5	0.9	0.7	0.8	0.6	0.9
ADP	2.7	2.7	2.8	3.4	3.2	2.9	3.1	3.2
ATP	85.4	81.0	96.3	105.8	100.7	102.2	119.8	111.4
CTP	19.9	18.2	18.5	25.4	25.0	24.4	27.2	25.5
GTP	12.8	10.6	15.5	16.9	17.8	17.2	17.0	18.4
UTP	66.3	59.3	71.4	77.4	74.4	86.0	93.2	84.9
UDP-sugars	30.2	25.4	29.0	29.4	28.6	31.2	41.2	35.2
Means[1]	36.2	32.9	38.9	43.0	41.6	44.0	50.2	46.4

(c) Specific Radioactivity

Nucleotide	Total Time of Incubation							
	15 min		30 min		90 min		120 min	
	−GA	+GA	−GA	+GA	−GA	+GA	−GA	+GA
	cpm/nmole							
NAD	15	14	13	18	17	15	12	14
ADP	151	124	133	160	165	150	190	168
ATP	265	260	314	375	361	372	380	372
CTP	160	151	166	228	200	246	246	240
GTP	246	228	287	342	320	330	366	372
UTP	264	257	312	372	362	380	376	374
UDP-sugars	140	132	156	156	172	186	194	185
Means[1]	204	192	228	272	264	277	292	285

[1] Excluding NAD.

Table II. *Changes in Specific Radioactivities of the Nucleoside Triphosphates*

Differences between the values with and without GA_3, derived from Table I, are shown below together with the effect of GA_3 expressed relative to the control = 100 (in parentheses). (Values in this table for the relative effect of GA_3 are calculated from the individual data rather than the means in Table I[c].) LSD for the relative effect of GA_3: 12 (5% level), 16 (1% level).

Nucleotide	Time of Incubation				Total
	15 min	30 min	90 min	120 min	
ATP	−5 (98)	61 (124)	11 (105)	−8 (98)	59
UTP	−7 (97)	60 (124)	18 (106)	−2 (98)	69
GTP	−18 (93)	55 (126)	10 (106)	6 (100)	53
CTP	−9 (95)	62 (138)	46 (127)	−6 (98)	93
Total	−39	238	85	−10	274

Table III. *Test of Significance of the Percentage Effect of GA_3 on the Specific Radioactivities of the Nucleoside Triphosphates*

The results obtained from 10 different experiments, covering incubation periods from 15 to 180 min, were pooled and analyzed for significance.

Nucleotide	Effect of GA_3	Value of Student's t	Level of Significance
	%		
ATP	101.5	0.383	NS[1]
UTP	102.0	0.485	NS
GTP	103.6	0.723	NS
CTP	112.6	2.099	5%

[1] Not significant.

ment only) were as follows: ATP, 440 (416); UTP, 484 (451); GTP, 384 (377); and CTP, 267 (303) cpm/nmole (values in parentheses are for treatment with the hormone). Thus, in spite of the absence of an effect of GA_3 under these conditions on ATP, UTP, and GTP, the specific radioactivity of CTP was again enhanced by the hormone, and this was a consistent finding in other experiments of 30 to 90 min duration. Only small effects on α-amylase production were detected with the different treatments.

In Tables III and IV, a total of 20 observations from 10 separate experiments have been pooled and statistically analyzed. The observations include incubation periods varying from 15 to 180 min. The mean percentage effects of GA_3 on the specific radioactivities of ATP, UTP, GTP, and CTP are shown in Table III, together with the values of Student's t test for the analysis of paired observations on the transformed data. Over a large range of treatments, GA_3 significantly enhanced the specific activity of CTP. Moreover, when the analysis is restricted to the values obtained over the range of incubations from 30 to 90 min, *i.e.*, the period of greatest effect, the level of significance for the effect of GA_3 on CTP increased to 0.1%.

In Table IV, the percentage effect of GA_3 on the nucleoside triphosphates is compared for pairs of compounds, and the value of Student's t test is computed for the mean difference derived from the 10 experiments. In addition, the correlation coefficients for the percentage effect of GA_3 on different combinations of the compounds are shown. It is apparent from the mean difference of the effect of GA_3 on different nucleotides that the metabolism of CTP is affected in a significantly different manner than that of ATP, UTP, and GTP. Also, ATP and UTP show almost identical trends. Furthermore, the correlations between the effects of GA_3 on ATP and CTP and on UTP and CTP are significantly lower than between ATP and UTP. The coefficient of determination is an estimate of the proportion of the sum of squares of the correlation coefficient which can be attributed to the interdependence of the sample populations. Thus, the assumption that ATP and CTP respond in like fashion to GA_3 accounts for only 35% of the total variance in the values for either of these nucleotides, whereas

Table IV. *Test of Significance of the Difference in Percentage Effect of GA_3 on Pairs of Nucleotides*

The percentage effect of GA_3 on the specific activities of ATP, UTP, GTP, and CTP was obtained from the pooled results of 10 different experiments. The mean difference of the effect of GA_3 on different pairs of the nucleotides was calculated, and the values were tested for significance. The correlation between the responses of the various pairs of nucleotides was also calculated.

Pairs of Nucleotides	Mean Effect of GA_3	Value of Student's t	Correlation Coefficient	Confidence Interval (95%)	Coefficient of Determination
	%				
ATP-GTP	2.2	0.873	0.859	0.673–0.943	73.8
GTP-UTP	1.9	0.795	0.770	0.498–0.904	59.3
ATP-UTP	0.5	0.388	0.947	0.871–0.979a[1]	89.7
ATP-CTP	11.0	2.258[2]	0.591	0.202–0.819b	34.9
GTP-CTP	9.3	2.104[2]	0.717	0.390–0.884	51.4
UTP-CTP	10.7	2.351[2]	0.654	0.298–0.850b	42.8

[1] a significantly different from b.
[2] Significance at the 5% level.

Table V. *The Effect of GA_3 on the Uptake of ^{32}P by Wheat Aleurone*

Batches of tissue (2 g fresh wt) were incubated in 9-cm Petri dishes in 7 ml of solution. After preincubation the tissue was treated with solutions of phosphate (KH_2PO_4, 0.05 mM containing 30 μc ^{32}P) or phosphate and GA_3 for the times indicated. The tissue was then rapidly washed in both 1 mM KH_2PO_4 and water and assayed for P_i and total acid-soluble phosphorus (average of two separate experiments). The experiment was carried out in two stages; the values for 5 and 10 min were obtained separately from those for 15 and 20 min.

Time	Level in Tissue		Radioactivity in Tissue		Specific Radioactivity	
	−GA_3	+GA_3	−GA_3	+GA_3	−GA_3	+GA_3
min	*nmoles × 10^{-3}/ g fresh wt*		*cpm × 10^{-3}/ g tissue*		*cpm/ nmole*	
	(a) P_i					
5	1.68	1.74	304.2	306.8	181	174
10	1.61	1.84	312.6	344.8	196	189
15	1.29	1.29	319.8	318.0	248	250
20	1.23	1.45	336.0	359.5	274	246
	(b) Total acid-soluble phosphorus					
5	24.9	27.5	397.8	392.0	16	14
10	28.7	29.0	420.8	444.8	15	15
15	23.9	26.4	352.0	343.5	15	13
20	24.2	25.9	386.8	402.0	16	16

the assumption that ATP and UTP respond similarly accounts for 90% of the total variance in either of these two compounds.

The Effect of GA_3 on the Uptake of ^{32}P by Isolated Aleurone. The finding that nucleotides were more highly radioactive in GA_3-treated aleurone could be attributed to a greater uptake of ^{32}P by the tissue, and so the influence of GA_3 on the influx of ^{32}P was investigated.

Uptake of ^{32}P was found to be a relatively slow process, since even after 90 min the specific radioactivity of the P_i in the ambient solution was 10- to 20-fold greater than that in the tissue. Moreover, uptake of radioactivity occurred in two phases, the first and most rapid of which was composed of ^{32}P which was more readily removed by washing the tissue with KH_2PO_4 than with water. The uptake of this component was considered to be due to adsorption, but attempts to remove it completely were successful only after extensive washing in nonradioactive P_i, by which time leakage from the tissue may have reached a significant level. Accordingly, in the experiment reported in Table V, the aleurone layers, after treatment, were placed in small porous holders and rapidly dipped three times into each of four 1-liter solutions of 1 mM KH_2PO_4 and four 1-liter portions of water. The results (Table V) showed that GA_3, up to 20 min after administration, had no effect on the uptake of ^{32}P. Similar results were obtained in another experiment of 60 min duration.

DISCUSSION

GA_3 increased the production of α-amylase by aleurone layers isolated from mature wheat grain, and the parameters of the response are similar to those reported for rice (18), barley (6, 19, 29), and wheat (22). The content of soluble nucleotides of wheat aleurone showed little response to treatment of the tissue with GA_3. However, significant changes occurred in the specific activities of some of the nucleotides, and these preceded the appearance of α-amylase by 4 to 5 hr. No effect of GA_3 was detected on the radioactivity of ADP. It seems likely, therefore, that the increase brought about by GA_3 in the specific radioactivity of ATP is the result of reactions involving the terminal phosphate only. The effect of the hormone was most evident on the specific radioactivities of ATP, UTP, GTP, and CTP. In particular, the effect on CTP appeared to be unique, in that it was greater and was detectable over a longer period than the effect on the other triphosphates.

The effect of GA_3 on the incorporation of ^{32}P into nucleotides was modified in experiments in which the medium was not renewed after the preincubation period. It seems likely that the release of P_i into the medium (generally amounting to a concentration of about 0.4 mM after 6 hr), and possibly the release of other substances as well, can obscure or lessen the incorporation of exogenous ^{32}P into nucleotides. Nevertheless, the presence of released P_i and possibly other substances does not prevent the production of α-amylase or eliminate the unique change in CTP metabolism after addition of the hormone.

The specific radioactivity of CTP in both the presence and absence of the hormone was generally 30 to 35% below that of ATP, UTP, and GTP, possibly indicating a slower rate of phosphate turnover in this compound. It could be postulated, therefore, that the greater effect of GA_3 on CTP was only apparent and resulted from the other triphosphates reaching isotopic equilibrium with the P_i in the tissue more rapidly than CTP. Thus, increases in the rate of their turnover in the presence of GA_3 would no longer be detectable. However, it can be seen from Table I(c) that the mean specific radioactivity of the nucleotides increases as the time of incubation is extended, although in each case ^{32}P was applied 15 min before the incubation was terminated. This result could be ascribed to an increasingly greater influx of ^{32}P into the tissue with time, but in an experiment designed to examine the uptake of radioactivity it was observed that a slow and almost constant rate was maintained from 5 to 60 min of incubation. Furthermore, when ^{32}P and GA_3 were administered simultaneously for 15 min (Table V), the specific radioactivity of the P_i in the tissue was almost identical (250 cpm/nmole) to the value obtained on another occasion when GA_3 was added for 60 min followed by ^{32}P for a further 15 min (238 cpm/nmole). Thus, the metabolism of the nucleotides themselves becomes increasingly more rapid with time, suggesting that they did not reach isotopic equilibrium with the P_i in the tissue during the experiment. In addition, in one experiment, GA_3 slightly depressed the specific radioactivities of ATP, UTP, and GTP after 30 min but increased the specific radioactivity of CTP by 14%.

Actinomycin D inhibited the GA_3-induced synthesis of α-amylase, suggesting that some or all of the effect of the hormone is on the synthesis of nucleic acids. It is clear, however, that actinomycin D affects other areas of metabolism in addition to its inhibitory effect on DNA-directed RNA synthesis. For example, carbohydrates can sometimes reverse the effects of actinomycin D (11), and inhibition of transcription occurs in reovirus which has no DNA (10). It has also been found that different species of RNA are inhibited by different concentrations of actinomycin D (5), and recently Pastan and Friedman (21) demonstrated that the antibiotic inhibits the incorporation of ^{32}P into phospholipids before an effect is detected on the synthesis of proteins.

No support for the effect of GA_3 being restricted to nucleic acid synthesis is found in the present work either. It is evident from Table II that, after treatment with GA_3 for 30 min, the absolute increases in the specific radioactivities of ATP, UTP, GTP, and CTP are approximately equal. However, if nucleic acid synthesis alone was stimulated by the hormone, the increases would be expected, instead, to be inversely proportional to the levels of the nucleotides (assuming approximately equimolar incorporation into RNA). These results, coupled with the unique effects of GA_3 on CTP metabolism and the decrease in the number of uracil-cytosine pairs in RNA extracted from dwarf pea seedlings treated with GA_3 (13), provide an alternative hypothesis for the mode of action of GA_3.

The importance of membranes of the endoplasmic reticulum and their attached ribosomes for protein synthesis has been discussed by Tata (27). There is not only a close correlation between membrane phospholipid synthesis and protein synthesis, but, in addition, the presence or absence of membranes may actually control the types of proteins synthesized. For example, Bulova and Burka (3) have found that unattached ribosomes synthesize different types of protein than those synthesized by ribosomes attached to membranes. In addition, Redman (25) and Ganoza and Williams (9) have shown that free ribosomes in mammalian cells synthesize intracellular protein, while membrane-bound ribosomes synthesize primarily extracellular protein. There seems little doubt that many of the enzymes that are induced by GA_3 in the aleurone layer have an extracellular destination, and one might thus conclude that their synthesis is determined not only by ribosomes and mRNA, but also by the provision of membranes.

The control of membrane synthesis was outlined by the initial work of Kennedy and Weiss (14), who indicated the relationship between cytidine nucleotides and phospholipid synthesis. Further studies have confirmed that, in addition to its role as a precursor in nucleic acid synthesis, phospholipid synthesis is a major function of CTP in cellular metabolism (15, 26). It seems reasonable to conclude that CTP plays an essential part in the specific synthesis of extracellular enzymes, *e.g.*,

α-amylase and proteinase, for which membranes are apparently indispensable. Thus, the results of the present work suggest that one of the earliest manifestations of the effect of GA_3 on mature wheat aleurone is an increase or alteration in membrane synthesis and that the new membranes so formed act as participants in the initiation of extracellular hydrolytic enzyme synthesis.

LITERATURE CITED

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