Plant Physiol. (1970) 45, 8-13

Regulation of Bud Rest in Tubers of Potato, *Solanum tuberosum* L.

VI. BIOCHEMICAL CHANGES INDUCED IN EXCISED POTATO BUDS BY GIBBERELLIC ACID[1, 2]

Received for publication, May 15, 1969

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ABSTRACT

The rest period of the potato tuber was studied in relation to certain biochemical changes that are induced by gibberellic acid (GA_3). The concentration of reducing sugars in excised plugs with buds treated with 10^{-4} M GA_3 decreased in the first 4 hours after treatment and then rapidly increased up to 70 hours. The pattern in control buds was similar, but the changes occurred more slowly. The response to GA_3 is temperature-dependent and is not limited to any particular tissue of the tuber. The concentration of reducing sugars in excised buds increased proportionally to the log of the concentration of GA_3 in a range from 10^{-8} to 10^{-4} M. At 10^{-3} M, GA_3 slightly inhibited production of reducing sugars. Malonate inhibits the initial decrease and the subsequent increase in reducing sugars in control buds, but not the increase induced by GA_3.

Total protein in buds was not influenced by 10^{-4} M GA_3 over a period of 40 hours, nor did activity of α-amylase increase significantly until 20 hours after beginning of treatment. Invertase activity was present initially and, in the presence of GA_3, increased after 20 hours. GA_3 had no effect on starch phosphorylase activity, which was always present and remained steady over the 20-hour test period.

In short term experiments the rate of protein synthesis and synthesis of specific protein fractions were not affected by 10^{-4} M GA_3, as measured by the incorporation of L-phenylalanine-U-^{14}C or by experiments with ^{14}C- and ^{3}H-labeled L-phenylalanine or L-leucine.

Certain chemical treatments, as well as wounding, can shorten or prolong the rest period of potato tubers (1, 3, 9, 13, 29, 35, 38, 40). Application of gibberellic acid promotes sprouting of intact tubers during tuber development or after they are harvested as marketable (23, 36, 38). Gibberellins occur naturally in potato tubers and increase in concentration at about the time that sprouting begins (31, 46).

Many workers have shown that GA_3[4] is involved in controlling the level of enzymes associated with carbohydrate metabolism (4, 19, 25, 32). There is mounting evidence that the hormone can induce synthesis of several hydrolytic enzymes (6, 14, 20, 49, 50). Working with *Avena fatua*, Simpson and Naylor (44) noted that the activity of maltase was higher in imbibed nondormant seeds than in imbibed dormant seeds. Gibberellin A_3 caused an increase in maltase in dormant seeds, with a resultant increase in sugar production. Moreover, whereas excised nondormant embryos would grow on a sugar-amino acid medium, dormant embryos would grow on this medium only if GA_3 was present (45). Thus in *A. fatua*, GA_3 appears to have dual functions—to induce in the aleurone layers synthesis of enzymes that hydrolyze carbohydrate reserves and to induce in the embryo enzymes that utilize the resulting substrates.

Development of cell wall invertase in tissue slices of *Helianthus tuberosa* in response to GA_3 was observed by Edelman and Hall (10, 11) and Bacon, MacDonald, and Knight (2). Palmer (34) reported similar results with root slices of *Beta vulgaris* L.

The expectation that GA_3 would promote protein synthesis in potato buds was heightened by the observation that GA_3 accelerates synthesis of RNA and DNA in excised buds from resting potatoes (39, 41, 49). We have shown (7) that reducing sugar content of potato buds increased shortly after excision, and the increase was accelerated by treatment with the hormone. It was therefore of interest to explore certain biochemical responses that occur in potato buds shortly after excision and treatment with GA_3.

MATERIALS AND METHODS

PLANT MATERIALS

White Rose potatoes were harvested 100 to 120 days after planting at which time they were in deep rest. The tubers were washed by hand and placed in the dark at 20°. They were used within 3 days after harvest and were cooled to 0° hr before an experiment was begun; thereafter all procedures associated with preparation of plugs were at this temperature. In most experiments plugs with an apical bud (eye) were excised with a potato "de-eyer" (internal diameter 0.8 cm) and cut to a length of 0.5 cm (35). In some experiments, 0.2 cm × 0.2 cm plugs with a bud were used. Such plugs with buds are hereafter referred to as "buds."

The buds were immersed for 5 min in a commercial preparation of sodium hypochlorite, rinsed five times with sterile distilled water, drained, and then transferred to sterilized Petri dishes containing two disks of 9-cm filter paper. Representative samples, treated in this manner, were found to be essentially free of bacteria. Ten buds were placed in each Petri dish. The treatment solutions were applied directly to each bud as a droplet (5 or 10 µl depending on the size of plug) with a microsyringe. After 30 min the filter paper was moistened with 1 ml of sterile distilled water,

[1] This investigation was supported in part by Research Grant GM-12885 from the United States Public Health Service.

[2] Submitted by M. D. C. to the Graduate Division, University of California, Davis, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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[4] Abbreviation: GA_3: gibberellic acid.

and the Petri dishes were placed in darkness at 20° except when temperature was the variable under study. Samples were removed for analysis at specific time intervals.

SUGAR ANALYSIS

Samples (10 plugs) were placed in 10 ml of boiling 95% ethanol for 15 min, then cooled and held for 24 hr. The sugars were extracted in a micro-Soxhlet apparatus and brought to 50-ml final volume with 80% ethanol. Total reducing sugars were determined on 1-ml aliquots of the ethanol extract by the methods of Nelson (30) and Somogyi (47). Paper chromatography was used to separate sucrose, glucose, and fructose from the original ethanol extracts which were dried and the residues dissolved in 1 ml of 80% ethanol. Sugars were detected by dipping the chromatographic strips in aniline phosphoric acid reagent and heating them at 110° for 15 min. Quantitative values were obtained by measuring the peak areas on a densitometer chart recording with a polar planimeter.

RESPIRATION

Buds (10 per sample) were placed in 125-ml Erlenmeyer flasks. A constant flow of air (190–195 ml/hr) was passed through each flask, so that the concentration of CO_2 did not exceed 0.5%. Effluent air was tested periodically by withdrawing a sample of air from the outlet tube with a microsyringe and injecting it into a gas chromatograph equipped with a 15.0-cm silica gel column and a thermal conductivity detector. Quantitative values were obtained by comparison with a standard gas mixture.

PROTEIN DETERMINATION

Samples (10 buds) were removed periodically from Petri dishes, weighed, frozen, and ground in a prechilled mortar with sand in 5 ml of ice-cold 1 M sodium chloride (pH 7.0). The suspension was centrifuged at 1000*g* for 30 min. Protein was determined by the Biuret method (15) using 2-ml portions of the supernatant fluid.

ENZYME ASSAYS

α-Amylase. Duplicate samples of 5 buds (0.5 cm × 0.5 cm) were ground in a prechilled mortar and 5 ml of ice-cold 0.01 M phosphate buffer (pH 6.8) plus 300 mg/liter potassium cyanide (9). The suspension was centrifuged at 10,000*g* for 30 min after which enzyme activity was determined in the supernatant fluid by the procedure of Paleg (33).

Invertase. Frozen, duplicate samples of 5 buds, which had been cut into 1-mm slices, were washed in several changes of distilled water. Enzyme activity was determined by the method of Edelman and Hall (12).

Phosphorylase. Duplicate samples of 10 buds each (0.2 cm × 0.2 cm) were ground in a prechilled mortar with enough cold distilled water to maintain a fresh weight to volume extract ratio of 1:10 (approximately 3.5 ml per sample). The suspension was centrifuged at 10,000*g* for 20 min. Enzyme activity was determined by the procedure of Madison (26), using citric acid buffer instead of maleic acid buffer.

INCORPORATION OF LABELED AMINO ACIDS

Solutions containing the following constituents were prepared in aqueous 0.05% Tween-20 in a final volume of 1 ml: 10 μc of L-phenylalanine-U-^{14}C (specific radioactivity 60 mc/mmole); 10 μc of L-leucine-U-^{14}C (specific radioactivity 200 mc/mmole); 25 μc of L-phenylalanine-^{3}H (specific radioactivity 2.5 c/mmole); 50 μc of L-leucine-^{3}H (specific radioactivity 2 c/mmole); 10 μc of L-phenylalanine-^{14}C + 9 × 10^{-5} M GA_3; 10 μc of L-leucine-^{14}C + 9 × 10^{-5} M GA_3.

Incorporation of L-Phenylalanine-U-^{14}C. Radioactivity in salt-soluble protein and salt-insoluble protein was determined in duplicate samples of 10 buds (0.2 cm × 0.2 cm) that had been treated for 0.5, 1, 2, 4, or 6 hr with L-phenylalanine-U-^{14}C or L-phenylalanine-U-^{14}C + GA_3. The samples were frozen in a 95% ethanol-Dry Ice bath and lyophilized, and the periderm was removed. The remaining dry tissue was ground to a fine powder in a large test tube and extracted two times with 2 ml of 1 M sodium chloride and three times with 2 ml of distilled water. The residue was resuspended and centrifuged each time, and the supernatant fluid was decanted. The combined supernatant fluids were frozen and dried by lyophilization. For zero time controls, duplicate samples were prepared in the same manner, but L-phenylalanine-U-^{14}C was added at the beginning of extraction.

For radioactivity determination, the residue was resuspended in distilled water and then filtered on Whatman No. 1 filter paper with several volumes of 95% ethanol. The disks were dropped into scintillation vials and dried at 80° for 12 hr. Then 5 ml of 2,5-bis-[2-(5-*tert*-butylbenzoxazolyl)]thiophene (0.4% in toluene) were added to each vial, and the samples were counted. The lyophilized protein extracts were dissolved in 1 ml of distilled water, and radioactivity was then determined on 0.1-ml samples by the method of Mans and Novelli (28). The residue and protein samples were counted in a liquid scintillation spectrometer.

RESULTS

CHANGES IN SUGARS

Time Course Studies. Treatment of excised buds with GA_3 resulted in a decrease in total reducing sugars during the first 4 hr, followed by a rapid increase which continued for the duration of the experiment (Fig. 1). The control (H_2O) responded similarly, but at a much slower rate. Plugs with periderm but without buds and plugs devoid of both responded in the same way to GA_3 as plugs with buds. Plugs with buds were most sensitive to GA_3 and plugs with periderm but without buds were least sensitive.

Effect of Concentration of GA_3. Total reducing sugars increased proportionally to the log of the concentration of GA_3, to a maximum of 10^{-4} M (Fig. 2). At a concentration of 10^{-3} M, GA_3 slightly inhibited production of reducing sugars. Changes in con-

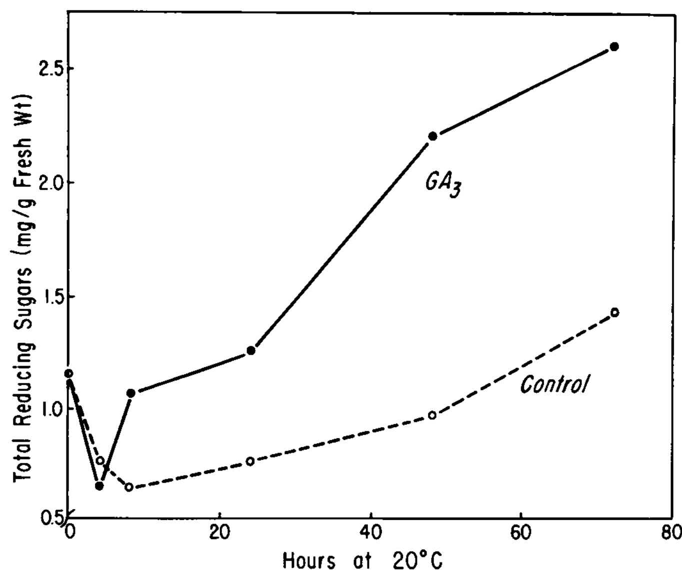

FIG. 1. Time course study of the effect of 10 μl of a solution of 10^{-4} M GA_3 (3.64 × 10^{-3} μg) on the production of reducing sugars in excised potato buds. Each point is the mean of two samples.

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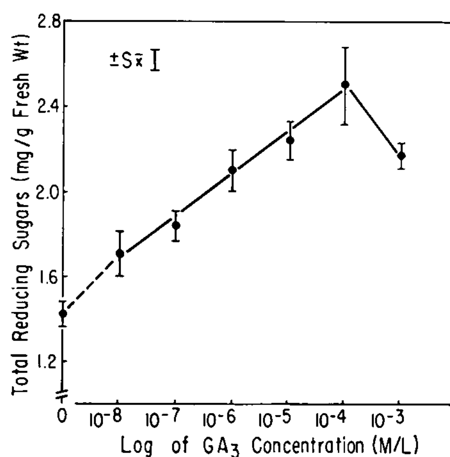

FIG. 2. (upper). Effect of concentration of GA_3 on reducing sugar content in excised potato buds incubated 48 hr at 20°. Each point is the mean of four samples.

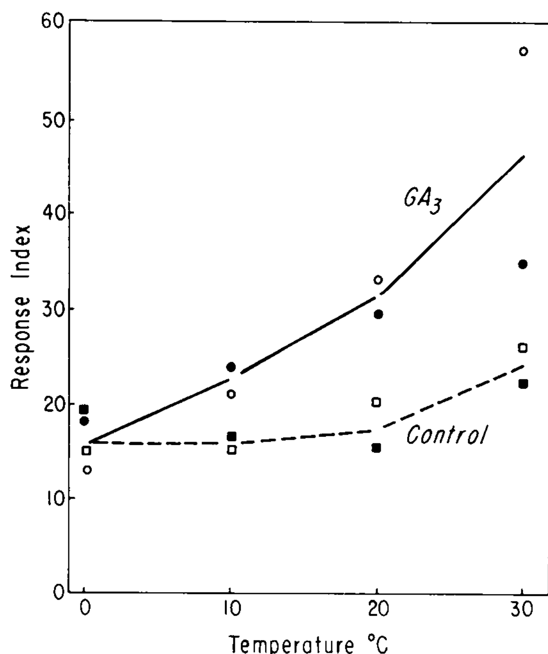

FIG. 3 (lower). The effect of GA_3 (10^{-4} M) on concentration of reducing sugars in excised potato buds at four temperatures. The response index (31) is a numerical value which represents the effect of GA_3 over a finite period of time. It is obtained by measuring the area under a time course curve with a planimeter and is expressed as a percentage of the total area of a rectangle bounded by the coordinate areas (24).

centration of glucose, fructose, and sucrose were followed over a 20-hr period after bud excision. Variability was sufficient to nullify treatment differences. However, glucose tended to decrease in the first 2 hr in both control and treated buds and GA_3 enhanced an increase in glucose in the next 3 hr. Concentration of glucose subsequently decreased and leveled off. Sucrose concentration increased slightly in the first 4 hr and then dropped off and remained steady over the next 16 hr. Fructose concentration did not fluctuate over the 20-hr treatment period, irrespective of treatment with GA_3.

Temperature. Time course experiments were run at 0°, 10°, 20°, and 30° with buds excised from freshly harvested tubers that had been stored for 1 or 7 days at 20° after harvest. Although the rates for production of reducing sugars varied with time, GA_3 treatment ultimately resulted in an increase in reducing sugar content (Fig. 3). Numerical values were obtained from time course curves for each temperature by the method of Lyon and Coffelt (24). The data clearly indicate that the response to GA_3 increased as temperature increased.

Respiration and Effect of Malonate. The respiratory rate of excised buds increased rapidly over the first 6 hr but leveled off after 10 hr (Fig. 4) whether or not the buds were treated with GA_3. The timing of the increase in respiration corresponds to the initial decrease in reducing sugars (see Fig. 1). Thus, if these two responses are related, treatment with malonate, which inhibits the development of respiration in tissue slices (21), could conceivably prevent the decrease in reducing sugars. As expected, malonate prevented the initial decrease but, surprisingly, had no effect on the subsequent increase in reducing sugars resulting from GA_3 treatment (Fig. 5).

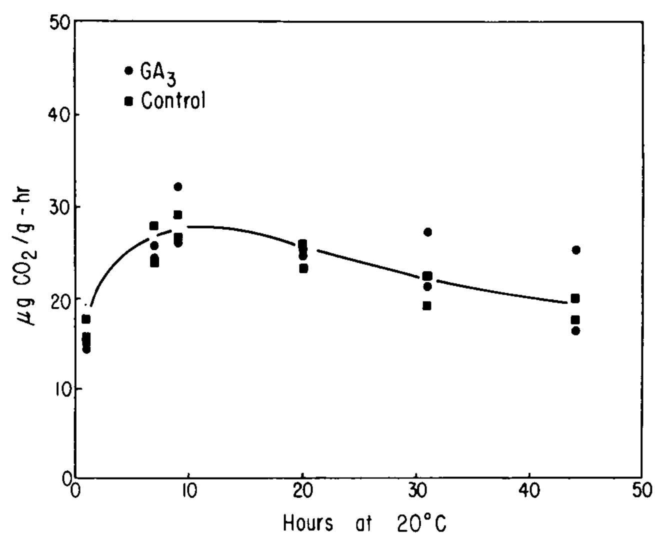

FIG. 4 (upper). Respiration of excised buds incubated in the presence or absence of GA_3 (10^{-4} M). Duplicate samples of each treatment are plotted.

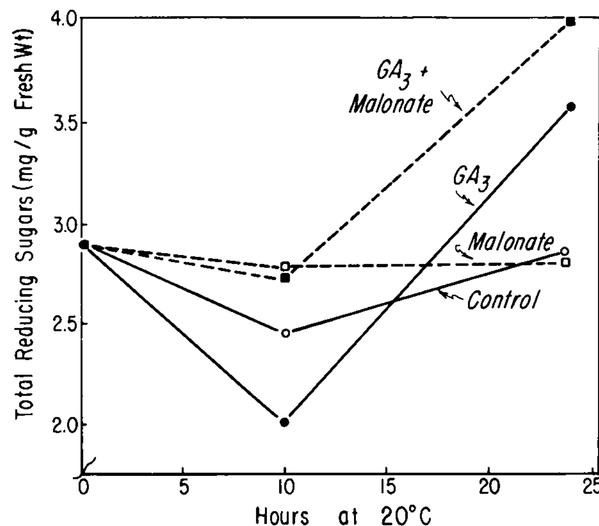

FIG. 5 (lower). Effect of GA_3 (10^{-4} M), malonate (10^{-2} M), and a mixture of the two on the reducing sugar concentration of excised buds. Each point is the mean of two samples.

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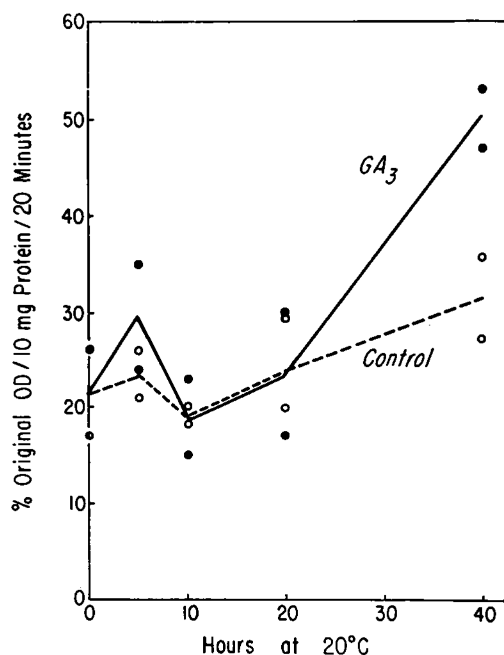

FIG. 6. Effect of GA_3 (10^{-4} M) on α-amylase activity of excised buds. Data for duplicate samples of each treatment are plotted.

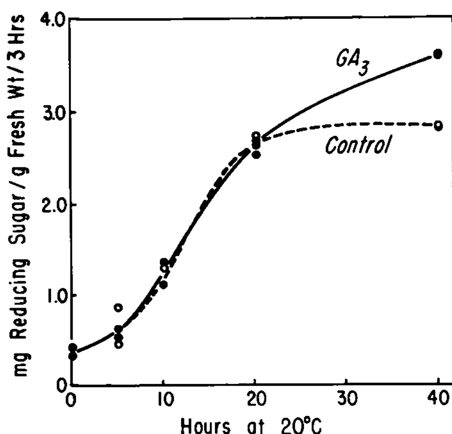

FIG. 7. Effect of GA_3 (10^{-4} M) on invertase activity of excised buds. Data for duplicate samples of each treatment are plotted.

CHANGE IN TOTAL PROTEIN AND HYDROLYTIC ENZYMES

After a slight increase in the first 2.5 hr, the total protein content of buds remained constant for the duration of the experiment. There was no change as a result of GA_3 treatment.

α-Amylase was present initially, and its activity remained unchanged during the first 20 hr, even in the presence of GA_3. However, at 40 hr a 66% increase in activity was noted in treated buds (Fig. 6). Invertase activity was low in freshly excised buds (Fig. 7). Compared with untreated buds, activity in GA_3-treated buds increased after 20 hr to a maximum in 40 hr. The increase resulting from treatment with GA_3 was about 30%. The activity of starch phosphorylase remained unchanged in both control and GA_3-treated samples.

INCORPORATION OF LABELED AMINO ACIDS

Since GA_3 had no effect on protein content or starch phosphorylase and its effect on α-amylase and invertase occurred much too late to account for the observed increase in reducing

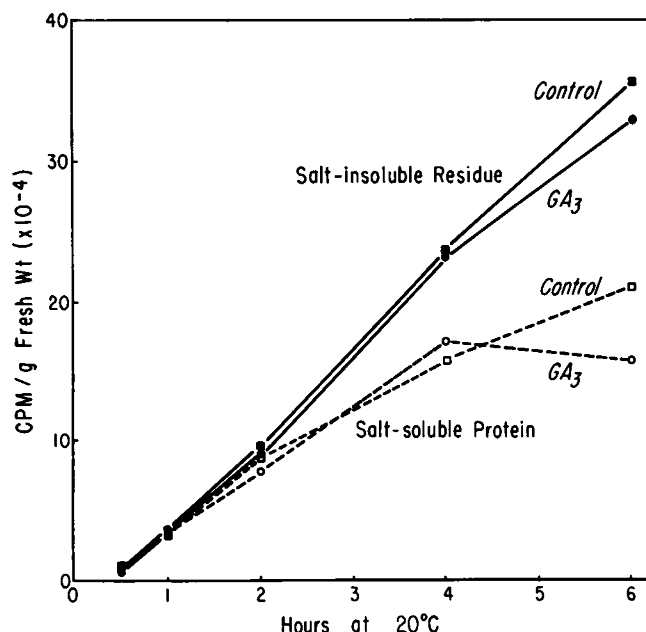

FIG. 8. Effect of GA_3 (10^{-4} M) on the incorporation of ^{14}C-L-phenylalanine into salt-soluble protein and salt-insoluble residues from excised buds. Each point is the mean of two samples.

sugars, experiments were run to determine if GA_3 affects synthesis of total protein or of specific protein fractions. After a slight lag, incorporation of L-phenylalanine-U-^{14}C into salt-soluble protein of the untreated buds was linear with time up to 6 hr (Fig. 8). In the presence of GA_3, the rate of incorporation fell off after 4 hr. Incorporation of L-phenylalanine-U-^{14}C into the salt-insoluble residue was linear over a 6-hr period, with or without GA_3. However, its rate of incorporation into the salt-insoluble residue was greater than the rate of incorporation into the salt-soluble protein.

In a more detailed study the effect of GA_3 on synthesis of specific proteins was studied by incubating buds in solutions containing L-phenylalanine-U-^{14}C + 9 × 10^{-5} M GA_3 or L-phenylalanine-U-^{3}H + water for 4 hr at 20°. The double labeled proteins were chromatographed on a DEAE-cellulose column using 1 N sodium chloride in 0.05 M phosphate buffer as developing solvent. The fluorescence was monitored continuously, and each fraction was counted in a liquid scintillation spectrometer after precipitation in the presence of crude DNA. Changes in the ^{3}H to ^{14}C ratios in the eluates were sought as an indication of an effect of GA_3 on protein synthesis. Similar experiments were run with bud incubation in L-leucine-U-^{3}H and -^{14}C for 2, 8, or 24 hr. GA_3 did not significantly alter the ^{3}H to ^{14}C ratio in any of these experiments.

DISCUSSION

Our studies indicate that GA_3 at very low dosage affects carbohydrate metabolism in potato plugs. Changes in content of reducing sugars similar to those measured in control plugs were observed in wounded potato tissues by Hopkins (18). The effect of GA_3 on the concentration of reducing sugars is of particular significance in view of the finding by Rappaport and Sachs (37) that wounding results in a marked increase in gibberellin concentration within 12 hr after wounding. This increase in gibberellins could account for the increases in reducing sugars detected in excised potato buds.

The increase in content of reducing sugars with increases in concentration of GA_3 after 48 hr (Fig. 2) could have been due to an increase in activity of α-amylase (Fig. 6) since this enzyme

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increased after 20 hr. Invertase may also have been involved (Fig. 7). The change in concentration of reducing sugars is not limited to any particular tissue since plugs free of buds also responded, although more slowly. Differences in penetration rate probably accounted for the timing of responses to GA_3. The release of reducing sugars due to GA_3 is probably not a primary factor in regulation of rest. Indeed, application of sugars to buds from resting tubers did not influence duration of the quiescent period (35). The release of sugars should be viewed as one consequence of gibberellin action leading to sprouting.

When potato tissue is wounded, there is a rapid increase in respiration which can be inhibited by malonate (21). Malonate-sensitive respiration indicates the participation of the tricarboxylic acid cycle (21, 22, 42). In the present study with excised (wounded) buds, malonate prevented the decrease in reducing sugars that occurs in the first 4 hr after excision; but it did not affect the subsequent increase in reducing sugars caused by GA_3 (Fig. 5). It is of interest that Romberger and Norton (42) observed that sucrose synthesis in potato tissue was independent of malonate-sensitive respiration. It might be argued that the malonate, which was supplied once at the beginning of the experiment, may have been metabolized in the first 10 hr, permitting the GA_3 to stimulate release of reducing sugars. However, it is clear from Figure 5 that the sugar concentration in the malonate control did not change significantly at any time during the course of the experiment. Activity of α-amylase and invertase in excised potato buds was increased by GA only after 20 hr (Figs. 6 and 7). The lag in enzyme production may have been due to the influence of β-inhibitor (of which abscisic acid is a major component) which occurs in large amounts in freshly harvested potatoes (17). Conceivably, there is an extended period before the inhibitor level decreases sufficiently to permit activity of GA_3. Shih and Rappaport (manuscript in the editor's hands) have shown that abscisic acid, which inhibits sprouting in potatoes (27), also inhibits RNA and DNA syntheses in buds, in both the presence and absence of GA_3. As expected (16), starch phosphorylase was present in relatively high amounts. Its activity was not affected by GA_3 treatment.

GA_3 did not influence the rate of protein synthesis in potato tubers until 4 hr after treatment, at which time the rate decreased (Fig. 8). A similar decrease in L-leucine-U-^{14}C incorporation into protein was observed by Varner, Ram Chandra, and Chrispeels (50) in experiments with the aleurone layer of barley. They concluded that this decrease was due, in part, to dilution of the labeled amino acids by the amino acids released from hydrolyzed protein, and, in part, to disappearance of labeled protein as a result of proteolysis. However, dilution of the label would have been primarily responsible for lesser incorporation in buds since protein is not secreted into the ambient solution.

Of interest was the relatively high incorporation of label into the salt-insoluble residue. Such incorporation indicates that either a high rate of protein synthesis was associated with the cell wall material or that L-phenylalanine was metabolized into phenolic polymers, such as lignin. The latter explanation is feasible since phenylalanine is a precursor of lignin synthesis (5) and potato slices can incorporate this amino acid into chlorogenic acid within 1 hr (48).

Fractionation of L-phenylalanine-^3H and -^{14}C and L-leucine-^3H and -^{14}C double-labeled proteins on DEAE-cellulose columns revealed no striking effect on protein content attributable to GA_3. The lack of a GA_3 effect indicates either that the hormone does not affect protein synthesis in the early hours of incubation, or that its effect is very specific and not detectable by the double labeling technique.

Click and Hackett (8), using protein synthesis inhibitors, concluded that most of the protein synthesized in potato tissue was related to the respiration increase in response to wounding. Sampson and Laties (43) concluded that the bulk of the RNA synthesized is of the ribosomal type. This massive synthesis could, conceivably, mask any specific effects of GA_3. Furthermore, in experiments with barley endosperm (49, 50), the radioactivity of the purified α-amylase fraction was very low. The result is significant, since the effect of GA_3 on incorporation of ^3H-precursors into RNA and DNA is limited to the bud apex and vascular tissue (39, 40), only a small fraction of the total tissue used. Therefore, despite our failure in these experiments to show any important difference in early protein synthesis in buds, it is premature to assume that none exists. On the other hand, other reasons for the effects of GA_3 (*e.g.*, membrane permeability) cannot be ruled out.

Acknowledgments—The authors wish to thank Drs. Mendel Mazelis and Roy Doi for their very helpful advice during the course of this research. Dr. Doi is acknowledged for aid in performing the double label experiments.

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