Induction of Deoxyribonucleic Acid Synthesis in Potato Tuber Tissue by Cutting

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ABSTRACT

Incorporation of $^{32}$P-orthophosphate was found in the DNA fraction of aerobically incubated potato discs when examined by methylated albumin kieselguhr column chromatography. The estimation of DNA content of the disc was by a method developed for starchy tissues and showed that the incorporation of $^{32}$P was due to net synthesis of DNA. The DNA content of a disc rapidly increased after a lag period of about 12 hours. The increase continued during the entire test period although at a lower rate during the later period of aging. DNA synthesis was further examined by measuring the rate of incorporation of $^{3}$H-thymidine. The striking similarity which was found between changes in the rate of DNA accumulation and in the rate of $^{3}$H-thymidine incorporation indicates that the incorporation of $^{3}$H-thymidine actually represents the net synthesis of DNA. Although the experiments with microautoradiography revealed that DNA synthesis occurred exclusively in nuclei, no signs of cell division were detected by microscopic observation. DNA synthesis in potato discs was further examined by using inhibitors of protein and RNA synthesis and was sensitive to those inhibitors. The significance of the present results is discussed in relation to the role of wounding in the induction of DNA synthesis.

It is widely known that slicing plant storage tissues causes rapid changes in metabolism including the induction of various enzyme activities accompanied by RNA and protein synthesis (9, 18, 21, 23, 25). The enhancement of RNA synthesis and the role of the newly synthesized RNA have drawn wide attention and have been examined in detail (5, 6, 8, 11, 12, 16). Furthermore, the enhanced synthesis of ribosomes induced in carrot root and Jerusalem artichoke tuber by slicing and aging has been successfully exploited to investigate the formation of rRNA through the synthesis and selective cleavage of RNA precursors (17, 20).

In the course of a study of nucleic acid synthesis in potato discs during aerobic incubation using MAK1 column chromatography, we found that $^{32}$P-orthophosphate supplied to the aged discs was incorporated not only into RNA fractions but also into the DNA fraction. Although the radioactivity of $^{32}$P found in the DNA fraction in the effluent of a MAK column has been occasionally ascribed to $^{32}$P incorporated into RNA complexed with DNA (4), it was of interest to examine whether this apparent incorporation of $^{32}$P was due to net synthesis of DNA induced by slicing and aging of the tissue. The present report presents the results of experiments on induction of DNA synthesis by cutting and discusses the potential usefulness of the system for the study of regulatory mechanism of DNA synthesis in higher plants.

MATERIALS AND METHODS

Plant Material. Potato (Solanum tuberosum L. cv. Dan-shaku) tubers were purchased in large batches from a wholesale market in Sapporo, Hokkaido, and stored at 4 C. Tubers were sterilized by dipping in a solution of 0.1% (w/v) sodium hypochlorite for 30 min and were cut transversely into blocks about 30 mm in thickness. After being peeled, the tissue blocks were immersed in hypochlorite solution for another 10 min and washed with a large volume of sterilized water. Tissue cylinders, 12 mm in diameter, were obtained from parenchymatous tissue of the inner phloem with a cork borer. Discs, 1 mm thick, were prepared from the cylinders by cutting with a slicing device made of 20 razor blades arranged at 1-mm intervals, washed with five changes of sterilized water, and lightly blotted. All manipulations were carried out under aseptic conditions.

The discs were placed on double layers of filter paper moistened with 50 μg/ml chloramphenicol in a Petri dish and incubated at 25 C in the dark. They were transferred to a freshly prepared Petri dish every 2 days. Addition of chloramphenicol as a bacteriostatic agent had no effect on the DNA synthesis (Table II, experiment 1). Freshly cut discs and incubated discs will be called “fresh discs” and “aged discs,” respectively.

Incorporation of $^{32}$P. Fifteen discs, fresh or aged for 12 hr, were incubated for 3 hr at 25 C with 400 μc of $^{32}$P-orthophosphate (carrier-free) in 1 ml of 0.01 M acetate buffer at pH 5.5. After addition of 15 nonlabeled discs nucleic acids were extracted from the discs by a pyrophosphate-pheno1 method and were fractionated by MAK column chromatography as previously described (22).

Extraction and Quantitative Determination of DNA. Twenty discs (2.8 g fresh weight) were homogenized with 8 ml of cold 0.5 N PCA in a glass homogenizer. The homogenate was centrifuged, and the precipitate was washed three times with 0.5 N PCA. The supernatant fractions were combined and regarded as acid-soluble fraction. The acid-insoluble material was successively washed with ethanol, ethanol-ether (1:1), and ether and dried in air. The dry tissue powder was suspended in 4 ml of 0.1 M citrate buffer at pH 6.0, containing 2.5 mm CaCl$_2$, heated at 80 C for 5 min to dissolve starch, and cooled to 40 C. To the resulting paste were added 50 μg each of $\alpha$-amylase (3 × crystallized liquefying type $\alpha$-amylase of Bacillus subtilis, 1

Abbreviations: MAK: methylated albumin kieselguhr; PCA: perchloric acid.
Seikagaku Kogyo Co., Tokyo) and pancreatic RNase (Daiichi Pure Chemicals Co., Tokyo), and the mixture was incubated for 30 min at 40°C. The digested suspension was cooled in an ice bath and left to stand for 10 min after addition of 0.5 ml of 5 N PCA. The precipitate was separated by centrifugation and washed with 0.5 N PCA. The acid-soluble fraction obtained after enzymatic digestion was regarded as the RNA fraction. The precipitate was resuspended in 3 ml of 0.5 N PCA and kept in boiling water for 15 min. DNA thus hydrolyzed was estimated by Burton's diphenylamine reaction (3).

Incorporation of 3H-Thymidine. Duplicates of five discs were removed at appropriate intervals during aging and were incubated for 2 hr at 25°C in the dark with 1 μc of 6-3H-thymidine (5.0 c/m mole, Daiichi Pure Chemicals Co.) in 0.5 ml of sterile water containing 50 μg/ml chloramphenicol. The discs were washed by stirring for 30 min in 250 ml of an ice-cold 0.5 mM nonradioactive thymidine solution and processed for DNA extraction as described above. Aliquots of acid-soluble and DNA fractions were transferred to counting vials, and their radioactivities were measured in a Packard TriCarb scintillation spectrometer. Since the incorporation of 3H-thymidine into the other fractions (lipid, RNA, etc.) was negligible, the sum of 3H-thymidine in the acid-soluble and DNA fractions was regarded as total uptake.

Microautoradiography. Potato discs were aged in the presence of 3H-thymidine (50 μc/0.6 ml-9 discs) at 25°C in the dark. Chloramphenicol was added to the medium at a concentration of 50 μg/ml. The labeled discs were fixed with 5% glutaraldehyde in 0.1 M phosphate buffer at pH 6.8, then dehydrated by a series of ethanol and propyleneoxide, and finally embedded in Epon 812. Thin sections (approximately 1 μ in thickness) were prepared from the embedded tissues with a glass knife, placed on a glass slide, and covered with a photographic emulsion (Sakura NR-M2, Konishiroku Photo Ind. Co., Hino). After 2 weeks of exposure, the emulsion was developed, and the tissue sections were stained with azure B or methylene blue.

Experiments with Inhibitors. In order to examine the effects of various antibiotics and base and amino acid analogues on the development of activity of DNA synthesis in potato discs during aging, discs were aged for 24 hr in inhibitor solutions. Duplicates of four discs were sampled and incubated for another 2 hr with 0.5 ml of 1 μc 3H-thymidine solution in the presence of the same inhibitor as was employed during aging, and the incorporation of 3H-thymidine into DNA was measured.

RESULTS

Incorporation of 32P-Orthophosphate into Nucleic Acids. General profiles of nucleic acid synthesis in fresh and aged discs were examined by comparing the incorporation patterns of 32P-orthophosphate into various nucleic acid species. The results given in Figure 1 clearly show that 3P incorporation into tRNA, rRNA, and so called D-RNA (10) and the incorporation was significantly increased by aging for 12 hr. In addition, 3P incorporation was apparently found in the DNA fraction of discs aged for 12 hr (Fig. 1b) while none was found in the same fraction of fresh discs (Fig. 1a).

Change in the DNA Content of a Disc during Aging. We have further examined whether the apparent incorporation of 32P into the DNA fraction was due to net synthesis of DNA by measuring change in the DNA content of a disc. A method of nucleic acid extraction was developed for starchy tissues in this investigation (see “Materials and Methods”), and the yield of nucleic acids in the method was determined as follows. A preparation of 32P-labeled rRNA from tobacco leaves (260 cpm/μg RNA) or of 3H-labeled DNA from Escherichia coli (2.55 × 108 cpm/μg DNA) was added to the discs before homogenization, and the nucleic acids were extracted from the discs as described in “Materials and Methods.” The yield of nucleic acid was estimated by measuring radioactivity recovered in RNA or DNA fraction. As shown in Table I, the yield of DNA averaged 72.0 ± 4.5% in two experiments. Most RNA was removed by the enzymatic digestion, and the remainder in the DNA fraction was negligible.

The DNA content of a potato tuber disc was plotted against the period of aging in Figure 2. The content rapidly increased after a lag period of about 12 hr. The increase continued up to the end of observation (168 hr) although it slowed gradually. It is certain that net synthesis of DNA took place in the potato tuber discs during the aerobic incubation. Figure 2 also indicates that the rate of DNA accumulation was maximal after 24 hr of aging and was followed by a rapid decrease until 48 hr and a slower decrease thereafter.

Rate of Incorporation of 3H-Thymidine into DNA. DNA synthesis in discs during aging was further examined by measuring the rate of incorporation of 3H-thymidine into DNA. The results in Figure 3 show that the rate of incorporation in fresh discs was very low initially but started to increase rapidly after slicing and reached a maximum at 24 hr. Then the rate dropped sharply until 48 hr and continued to decrease gradually thereafter. The rate of total uptake of 3H-thymidine by discs showed a characteristic change with time of aging. The rate of uptake was high at the beginning of aging but decreased rapidly to nearly one-half of the initial value by 12 hr. During
Effects of Inhibitors

The effects of various inhibitors were examined to determine whether the development of activity of DNA synthesis was dependent upon protein and/or RNA synthesis which was activated by slicing and aging. A pyrimidine analogue and three amino acid analogues were tested first and proved to have significant inhibitory effects on the development of DNA synthesis, when administered to discs during first 24 hr of aging (Table II, experiment I). Inhibitory effects of these antibiotics, tested in experiment II, were particularly noticeable and added probability to the involvement of protein and RNA syntheses in the induction of DNA synthesis suggested by the results in experiment I. Results shown in Table II also indicate that all the chemicals used inhibited uptake of 3H-thymidine by discs to varying degrees, but in every case the degree of inhibition of DNA synthesis was significantly larger than that of thymidine uptake. Thus the inhibitory effect on DNA synthesis was not caused simply by blocking the uptake of 3H-thymidine.

**DISCUSSION**

DNA synthesis in excised storage tissues of higher plants has been investigated in relation to growth (1, 13) or callus formation (1, 19, 24), and the content of DNA in the tissues has been measured by standard methods adapted for non-storage tissues of higher plants or for animal tissues (2, 14). MAK column elution profiles of nucleic acids extracted from fresh and aged potato tuber slices which had been fed with 32P-orthophosphate suggested induction of DNA synthesis by aerobic incubation of the tissue. Accordingly, we tried to determine the DNA content by the established methods but were unsuccessful. A major problem was that potato tissue contained a large amount of starch which was acid-insoluble and interfered with hydrolysis of RNA by alkali, acid, or RNase. Furthermore, without removal of starch before extraction of DNA, it was impossible to apply the extraction with hot PCA because the treatment of acid-insoluble material resulted in a heavy starch paste. After several trials we exploited a procedure which included amylase and RNase treatment to remove the bulk of starch and RNA from the acid-insoluble materials of potato tissue. Even after the enzymatic digestion of starch and RNA, the residue proved to give a good and fairly reproducible yield of DNA by hydrolysis with hot PCA when Burton’s diphenylamine method was applied. The method may

the subsequent aging period the rate remained at a fairly constant value although a very slow decrease was observed.

**Cellular Localization of DNA Synthesis.** As shown in Figure 4, DNA synthesis occurred exclusively in nuclei. Labeled nuclei were generally scattered among the cells of a disc. An important observation was that no signs of cell division, such as mitotic figures or clusters of small cells, were detected by microscopic examination of about 500 cells each at 24, 36, 42, 48, and 72 hr of aging.

**Effects of Inhibitors of Protein and Nucleic Acid Synthesis.**

Table I. Recovery of Labeled Nucleic Acids Added during Extraction

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Labeled Material</th>
<th>Acid-soluble fraction</th>
<th>RNA fraction</th>
<th>DNA fraction</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>3H-DNA</td>
<td>5.5</td>
<td>18.6</td>
<td>67.4</td>
<td>91.5</td>
</tr>
<tr>
<td></td>
<td>3H-DNA</td>
<td>6.3</td>
<td>13.3</td>
<td>69.0</td>
<td>88.6</td>
</tr>
<tr>
<td></td>
<td>32P-RNA</td>
<td>3.6</td>
<td>87.9</td>
<td>5.4</td>
<td>96.9</td>
</tr>
<tr>
<td></td>
<td>32P-RNA</td>
<td>5.9</td>
<td>90.4</td>
<td>5.1</td>
<td>101.4</td>
</tr>
<tr>
<td>Mean recovery</td>
<td></td>
<td>87.2 ± 2.8</td>
<td>72.0 ± 4.5</td>
<td></td>
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</tr>
</tbody>
</table>

**Fig. 2.** Change in the DNA content of a disc during aging. DNA was extracted from 20 discs during aging as described in “Materials and Methods” and estimated by Burton’s diphenylamine reaction using calf thymus DNA as a standard. The points at 0 and 6 hr represent the values of quadruplicate experiments, and those of the rest represent the values of duplicate experiments (O). The rate of DNA accumulation was calculated by measuring slopes of the change in the content (O).

**Fig. 3.** Change in the rate of 3H-thymidine incorporation into DNA during aging. Discs were taken out at appropriate intervals during aging, incubated with 3H-thymidine for 2 hr, and processed for DNA extraction, and the radioactivity in the DNA fraction was measured (O). The sum of radioactivity in the acid-soluble and DNA fractions was plotted as total uptake (O). The points at each sampling time represent the values of duplicate experiments.
be applicable to other starchy tissues such as cereal seeds, leguminous seeds, or sweet potato roots. The present results strongly suggest that cutting induced net synthesis of DNA in potato tuber tissues with a lag period of less than 6 hr. Since the rate of thymidine incorporation into DNA in fresh discs was very small despite a relatively large uptake of thymidine, and since the incorporation rate continued to increase up to 24 hr despite a sharp decrease in uptake after 6 hr, the change in the rate of thymidine incorporation is not explained by the different activity of thymidine uptake of the discs. Furthermore, a striking similarity between changes in the rate of DNA accumulation (Fig. 2) and in the rate of thymidine incorporation into DNA (Fig. 3) shows that the incorporation of thymidine into the DNA fraction represents net synthesis of DNA found in the discs.

The fact that inhibitors with different inhibitory mechanisms, i.e., analogues and antibiotics, interfered with the development of activity of DNA synthesis in our experiments suggested that RNA and protein syntheses are involved in the process of the development; but we have investigated the effects of inhibitors applied continuously during the first 24 hr of aging, and the results do not rule out the possibility that the development of activity of DNA synthesis requires proteins synthesized only in a certain short period during aging. This problem is being currently investigated.

DNA synthesis so far reported in excised plant tissues is mostly followed by cell division. When auxin (IAA or 2,4-D) was supplied to excised Jerusalem artichoke tubers, DNA replication was induced, and the mitotic index also increased (1, 19, 24), although little DNA synthesis was detected in discs aged without auxin. Induction of DNA synthesis in Jerusalem artichoke tuber slices by auxin was recently confirmed by incorporation of labeled thymidine into the DNA fraction (Yasuda and Yamada, unpublished). On the other hand, DNA synthesis without cell division has been detected during incubation of excised tobacco pith in a nutrient medium without auxin (7). However, incubation of the pith tissue in the presence of auxin significantly enhanced DNA synthesis as well as cell division. Synthesis of nuclear DNA observed during aging of potato discs in the absence of any growth regulators was not accompanied by cell division, and it is clearly a response of the tissue to wounding as in the case of tobacco pith tissue. Since it is evident that callus is formed if excised potato tubers are incubated in a medium containing auxin, it is assumed that the added auxin modifies the regulatory mechanism of cell division to bring the cells into the meristematic state.

Table II. Effect of Inhibitors on the Development of Activity of DNA Synthesis

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Inhibitor</th>
<th>Incorporation into DNA</th>
<th>Total Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>None</td>
<td>20,450 cpm</td>
<td>48,000 cpm</td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol, 50 µg/ml</td>
<td>21,080 cpm</td>
<td>40,780 cpm</td>
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<td>L-Canavanine, 5 mm</td>
<td>10,120 cpm</td>
<td>34,370 cpm</td>
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<tr>
<td></td>
<td>DL-Ethionine, 10 mm</td>
<td>13,425 cpm</td>
<td>45,635 cpm</td>
</tr>
<tr>
<td></td>
<td>p-Fluorophenylalnine, 5 mm</td>
<td>11,430 cpm</td>
<td>32,230 cpm</td>
</tr>
<tr>
<td></td>
<td>5-Fluorouracil, 1 mm</td>
<td>7,220 cpm</td>
<td>27,440 cpm</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>14,530 cpm</td>
<td>37,015 cpm</td>
</tr>
<tr>
<td></td>
<td>Cycloheximide, 1 µg/ml</td>
<td>912 cpm</td>
<td>26,577 cpm</td>
</tr>
<tr>
<td></td>
<td>Puromycin, 100 µg/ml</td>
<td>5,120 cpm</td>
<td>19,665 cpm</td>
</tr>
<tr>
<td></td>
<td>Actinomycin D, 25 µg/ml</td>
<td>2,815 cpm</td>
<td>22,875 cpm</td>
</tr>
</tbody>
</table>

There is a possibility that endogenous auxin functions in the observed synthesis of DNA, but even if so, wounding must trigger the induction of DNA synthesis because DNA synthesis occurs only after the slicing. Cell proliferation was recently reported to be induced on the surface of potato tissue slices under a particular condition which did not include the addition of auxin (15).

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