Dormancy Associated with Repression of Genetic Activity [1, 2]

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Dormancy of buds and seeds is a well known and common phenomenon in the plant world. It is of interest because it has not yet become understandable. The dormant tissue has available to it by definition all of the environmental conditions of temperature, water supply, nutrition, etc., required for growth. The dormant tissue is alive, it respires, and in some cases grows very slowly in size (10). Yet a dormant tissue lies idle; it does not grow normally. There is within the dormant tissue some factor or mechanism which restricts growth and cell multiplication.

Many physical and chemical factors are known which possess the ability to end dormancy in one or another tissue or organ. These factors include, for example, proper photoperiod, treatment with the appropriate temperature, application of gibberellic acid, application of ethylene chlorohydrin, application of potassium thiocyanate, or thiourea, and in some cases, the mere passage of time. It is not immediately obvious that these dormancy-breaking factors possess any characteristic in common. Nonetheless, it is probable that there must be some point at which the mechanisms by which the several factors act upon dormancy merge, and play upon one or a few common facets of cell function.

If we view the problem of dormancy within the framework of molecular biology, a hypothesis immediately suggests itself, namely, that in the dormant cell the genetic material is completely, or nearly completely, repressed. According to this hypothesis, the genetic material would be unable to express itself in the form of production of the messenger RNA which is essential to enzyme synthesis and hence to growth and metabolism. The present paper constitutes a first approximation analysis of whether the hypothesis of gene repression as the cause of dormancy is a tenable one. The method consists firstly in comparison of the rates of RNA synthesis by dormant and awakening buds, and secondly in comparison of the abilities of the chromatin of such buds to support DNA-dependent RNA synthesis.

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Materials and Methods

The dormant buds of potato tubers formed the experimental material for the present investigation. Potato tubers exhibit a rest period of 2 or more months after harvest. During this period the buds of the tuber will not grow, even if they are placed in a physically favorable environment. The dormancy of potato tuber buds may, however, be broken at any time by application of ethylene chlorohydrin (4, 5). Bud growth commences 10 to 17 days after the beginning of a 3-day treatment with ethylene chlorohydrin (8).

Freshly harvested potato tubers were immediately stored at 4°. Under these conditions, complete dormancy is maintained for a period of several months. Tuber samples sufficient to supply 30 to 200 buds per treatment were removed from cold storage at the beginning of each experiment on in vivo incorporation of precursor into RNA, and tuber samples sufficient to supply approximately 5 g fresh weight of bud tissue removed from cold storage at the beginning of each experiment on chromatin isolation.

Treatment for the breaking of dormancy was carried out in closed containers containing ethylene chlorohydrin (The Matheson Co., Inc.) in a concentration of 2 ml per kg tubers (8). The ethylene chlorohydrin was contained on a piece of cotton which in turn was contained in a petri dish in the bottom of the container. After an ethylene chlorohydrin treatment period of 3 days, the tubers were removed to the open air for the remainder of the period before final harvest. In the present experiments, visible growth of potato buds commenced approximately 4 days after the end of ethylene chlorohydrin treatment, or 7 days after its commencement. Control buds were similarly harvested from nontreated tubers. The buds of nontreated tubers remained dormant over the 2-month period of cold storage during which the several lots of tubers used were observed.

Incubation of Excised Potato Buds in Uridine-2-C^{14}. Rate of RNA synthesis by potato buds was determined with excised buds incubated with the metabolite under investigation. Buds were excised from the tuber with minimal amount of contaminating adjacent nonbud tissue. Such contamination as did occur did not contribute importantly to the results since the nonbud (tuber) tissue is very inactive in

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RNA synthesis (see below). Each sample was incubated in a petri dish containing 5 ml of H_2O and 3 μg of penicillin as well as 0.2 μmole of uridine-2-C^{14}, specific activity 24 μc per μmole. Incubation was for 2.5 hours at 24°. In certain incubations, as outlined below, a pretreatment of the buds for 2 hours with 6 mg actinomycin D per ml preceded the incubation in uridine-2-C^{14}.

Extraction and Determination of RNA and DNA. At the end of the incubation period the buds were first washed to free them of uridine-2-C^{14} containing solution, and next ground in a glass homogenizer. The samples were depigmented by washing twice in cold methanol. They were then washed one time in cold acetic acid methanol, 3 times in cold 5 % trichloroacetic acid, and twice in ethanol-ether (1: 1 mixture). This washing procedure freed the ground tissue of small-molecule labeled metabolites. The tissue was next treated for hydrolysis of RNA and DNA by the general methods of Schmidt and Tannhauser as outlined by Ts'o and Sato (11). The washed homogenate was incubated for 17 hours in 0.3 N KOH at 37°. This treatment hydrolyzed the RNA to 2',3' ribonucleotides. The hydrolyzed solution was then made 5 % in perchloric acid and the precipitate of potassium perchlorate and DNA centrifuged off. On the supernatant fraction the content of ribonucleotides was determined by optical density at 260 mμ and by colorimetric orcinol reaction. Radioactivity of the ribonucleotide solution was determined on aliquots plated on planchets and counted in a Nuclear Chicago D-47 gas flow counting system. DNA was determined in the potassium perchlorate precipitate by hydrolysis at 100° for 10 minutes in 0.5 N perchloric acid. The resulting hydrolysate was then neutralized with KOH, the potassium perchlorate centrifuged off, and the deoxyribonucleotide content of the supernatant fraction determined by optical density at 260 mμ and by the diphenylamine reaction of Burton (2). Radioactivity was determined as in the case of the RNA ribonucleotides.

Isolation of Chromatin. Chromatin was isolated from dormant and awakened potato buds by the general methods of Huang and Bonner (6). The excised buds were ground with sand in a mortar at 4° in a grinding medium consisting of sucrose, 0.25 M, tris pH 8 0.05 M, $MgCl_2$ 0.001 M, and β-mercaptoethanol, 0.001 M. The ground material was filtered successively through cheese cloth and miracloth to remove cell debris, and centrifuged at 10,000 × *g* for 30 minutes to pellet the chromatin. The resulting pellet was successively washed by resuspension and repelleting in grinding medium, and twice with Tris, pH 8, 0.05 M. The recovery of DNA in the so purified chromatin amounted to 35 to 40 % of that present in the tissue.

Chromosomally Supported RNA Synthesis. The effectiveness of the isolated chromatin in support of DNA-dependent RNA synthesis was determined according to the general methods of Bonner, Huang, and Gilden (1). For this purpose use was made of a standard reaction mixture (1) for conduct of DNA-dependent RNA synthesis, namely per 0.34 ml: 0.1 μmole each of GTP, CTP, and UTP, 10 μmole Tris buffer, pH 8.0, 0.1 μmole C^{14} ATP (1 μc/μmole), 1 μmole $MgCl_2$, 0.25 μmole $MnCl_2$, 3 μmole β-mercaptoethanol. This reaction mixture was fortified with 10 to 20 μg of *E. coli* RNA polymerase purified by method of Chamberlin and Berg (3) to the stage of their fraction 3. To the polymerase-containing reaction mixture was added 50 μg DNA, either as pure DNA or as chromatin of the desired variety and the whole was then incubated at 37° for 10 minutes. The reaction mixture was stopped by rapid filtration on a Schleicher and Schull Type B-6 filter followed by 4 washings with cold 5 % trichloroacetic acid. The dried filters were then counted in a Nuclear Chicago D-47 gas flow counting system.

Results

The data of figure 1 concern a typical experiment in which buds pretreated for 3 days with ethylene

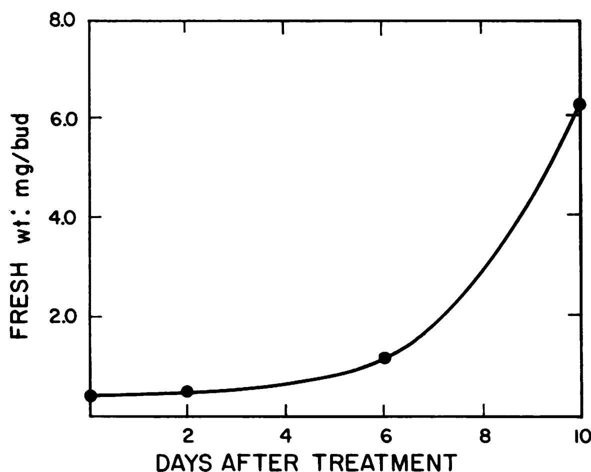

FIG. 1A. Fresh weight of the buds of potato tubers at varying times after a 3-day pretreatment with ethylene chlorohydrin.

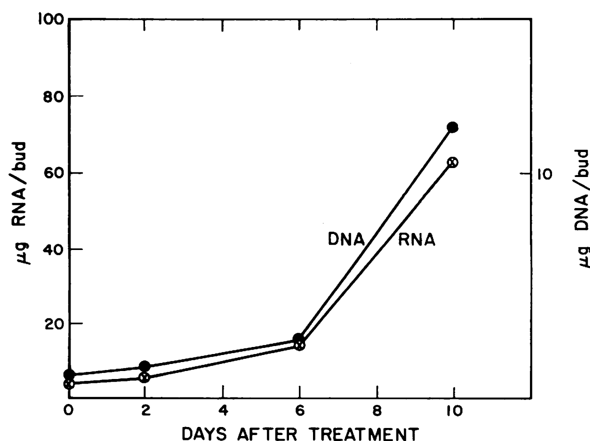

FIG. 1B. RNA and DNA content of buds of potato tubers at varying times after 3-day pretreatment with ethylene chlorohydrin.

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chlorohydrin were harvested at various periods after the end of such treatment. Growth of buds as followed by increase in fresh weight increased by 2.5-fold above the initial weight by the sixth day after treatment, and rapid growth occurred by the tenth day after treatment. Changes in RNA content parallel, although they are slightly more dramatic than those in fresh weight, and start at an earlier time, namely are detectable within 2 days after treatment. DNA content, a measure in this instance of cell number, parallels growth in fresh weight. We may conclude then, that as a result of ethylene chlorohydrin treatment, the buds of previously dormant potato tubers acquire the ability to increase not only in volume but also in cell number as measured by DNA content, and in RNA content.

The data of figure 2 concern the ability of buds harvested at different times after ethylene chlorohydrin treatment to synthesize RNA and DNA. Synthesis is in this instance measured by incorporation of uridine-2-C^{14} into the 2 different kinds of nucleic acid. It is apparent from the data of figure 2 that the buds of dormant potato tubers possess an exceedingly limited ability to incorporate uridine into RNA or DNA, and are in fact almost totally devoid of this ability. Rate of RNA synthesis doubles within 2 days after the end of ethylene chlorohydrin treatment and is 130-fold the dormant level by 10 days after treatment. Alterations in rate of DNA synthesis are equally evident. This rate increases markedly over the first 2 days after ethylene chlorohydrin treatment, increases by 20-fold over the 6-day period and by 130-fold over a 10-day period, as is also shown in figure 2. The amount of RNA synthesis as measured by uridine-2-C^{14} incorporation into RNA, per unit of bud DNA, provides a measure of RNA

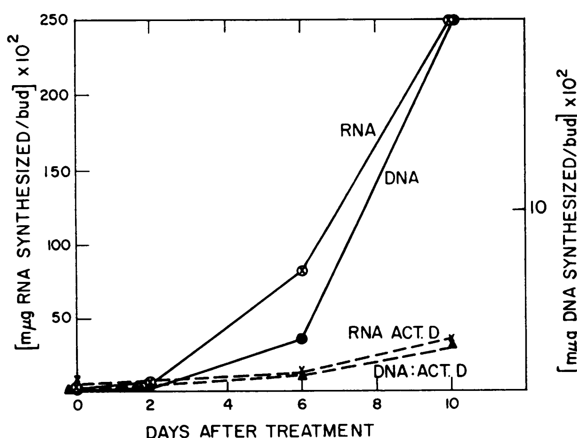

FIG. 2. Rate of incorporation of the carbon of uridine-2-C^{14} into RNA and DNA by buds of potato tubers at varying times after 3-day pretreatment with ethylene chlorohydrin. In the 2 lower curves of figure 2, the buds were incubated in solution containing not only uridine-2-C^{14}, but also actinomycin D.

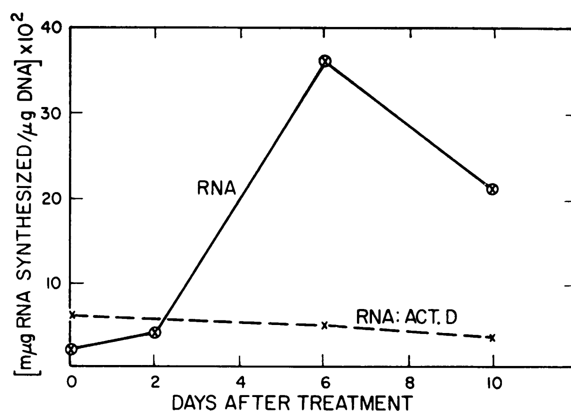

FIG. 3. Rate of RNA synthesis in buds of potato tubers at varying times after a 3-day pretreatment with ethylene chlorohydrin, and on the basis of RNA synthesized per unit DNA per unit time. In the lower curve the buds were subjected to treatment with actinomycin D.

production per cell. This also increases as a result of ethylene chlorohydrin treatment (fig 3).

The production of RNA by nondormant potato buds is inhibited by actinomycin D pretreatment (fig 2). It is clear therefore that the RNA production by such buds is of the DNA-dependent variety (7, 9).

The experiments outlined above establish that dormant potato buds synthesize RNA at a rate which is exceedingly small compared to that in growing, nondormant buds. They do not, of course, establish the basis of this difference. The experiments outlined below establish that the block to RNA synthesis in dormant buds lies at the level of the repression of chromosomal activity.

It is already established that chromatin may be isolated from the tissues of plant material with the genetic control machinery intact (1). It has further been established that the DNA of the derepressed genes of chromatin is available for transcription by added exogenous RNA polymerase (1). The rationale of the present experiments is then that of isolation of chromatin, and determination of the extent of derepression of such chromatin by determination of the ability of chromatin to support DNA-dependent RNA synthesis in the presence of added exogenous RNA polymerase. The data of table I concern a typical experiment. For this experiment one set of tubers was first treated for 3 days with ethylene chlorohydrin, and then left to grow at 25° for 10 days. Three days before the expiration of the 10-day period, 2 further sets of tubers were removed from cold storage, and one treated for 3 days with ethylene chlorohydrin. The third set remained at 25° in a container similar to that used for the ethylene chlorohydrin treatment, but without ethylene chlorohydrin. This third set then serves as the dormant, untreated control. From each set 5 to 10 g fresh weight of buds were removed and chromatin prepared

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Table I. *Effectiveness of Chromatin of Dormant and of Non-dormant Potato Buds in the Support of DNA-dependent RNA Synthesis by Exogenous RNA Polymerase*
For composition of reaction mixture see Materials and Methods.

50 μg of DNA supplied to system as:	RNA synthesized μμmole AMP incorp per 10 min
Potato DNA (deproteinized)	3370*
Chromatin of potato tuber	0
Chromatin of dormant buds	122
Chromatin of buds from tubers at end of 3-day treatment with ethylene chlorohydrin	1412
Chromatin of buds from tubers 10 days after 3-day treatment with ethylene chlorohydrin	1538

* Incorporation due to polymerase alone (150 μμmole) subtracted.

as outlined under Materials and Methods. It may be remarked that roughly 25 kg of potatoes yield about 5 g fresh weight of dormant potato buds. The ability of each kind of chromatin thus obtained to support DNA-dependent RNA synthesis in the presence of added exogenous *E. coli* RNA polymerase was then determined. The data of table I show that potato DNA is highly effective in the support of DNA-dependent RNA synthesis, as would be expected. Chromatin from the potato tuber itself is totally ineffective in this function. Chromatin from dormant potato buds is almost, but not quite completely, inactive in the support of DNA-dependent RNA synthesis, less than one-thirtieth as effective as deproteinized potato DNA. The effect of ethylene chlorohydrin treatment upon the chromatin of potato buds is dramatic. Chromatin isolated from buds harvested at the end of the 3-day treatment with ethylene chlorohydrin is more than 10-fold more effective in the support of DNA-dependent RNA synthesis than is the chromatin of dormant buds. Only a further small increase in ability of bud chromatin to support DNA-dependent RNA synthesis takes place during the succeeding 10 days after the 3-day ethylene chlorohydrin treatment.

Discussion

Dormant potato buds possess a very limited capability for the conduct of both DNA-dependent RNA synthesis and of DNA replication. Treatment with ethylene chlorohydrin, a classical compound for the breaking of dormancy, removes the strictures to both RNA and DNA synthesis. The RNA synthesized by potato buds after ethylene chlorohydrin treatment is made by DNA-dependent RNA synthesis since it is actinomycin D-inhibitable in vivo. The RNA synthesized by the potato buds represents therefore transcription of the genetic material of the potato genome. Why do dormant potato buds not make RNA in quantity? The block to RNA synthesis could in principle be attributable to any one of a great number of defects, as for example, lack of RNA polymerase, limited availability of one or all of the requisite riboside triphosphates, etc. Among the possible causes of lack of RNA synthesis in dormant potato buds is the possibility that the genetic material of dormant potato buds is repressed. This is shown to be in fact the case. Chromatin isolated from dormant potato buds and incubated in a complete reaction mixture containing all of the requisites for the conduct of DNA-dependent RNA synthesis, namely riboside triphosphates, RNA polymerase, etc., is little effective in the conduct of RNA synthesis. Chromatin isolated from potato buds which have been caused to become nondormant by ethylene chlorohydrin treatment is highly active in the conduct of DNA-dependent RNA synthesis. We may say, therefore, that the genome of dormant potato buds is very largely repressed. Whether such repression is the sole cause of dormancy remains to be established.

Summary

The buds of dormant potato tubers incorporate uridine-2-C^{14} into RNA and DNA at a highly limited rate. Rate of such synthesis is markedly increased after pretreatment of the tubers with ethylene chlorohydrin, a treatment which also breaks dormancy.

RNA synthesis by the buds of nondormant potato tubers is inhibited by actinomycin D, and is hence of the DNA-dependent type.

Chromatin of the buds of dormant potato tubers is almost totally incapable of the support of DNA-dependent RNA synthesis by added exogenous RNA polymerase. The chromatin of nondormant buds of potato tubers (in which dormancy has been broken by treatment with ethylene chlorohydrin) is highly effective in the support of DNA-dependent RNA synthesis by added exogenous RNA polymerase.

It is concluded that the genetic material of the buds of dormant potato tubers is largely in a repressed state, and that the breaking of dormancy is accompanied by derepression of the genetic material.

Acknowledgments

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C^{14} Amino Acid Incorporation by Spinach Chloroplast Preparations [1, 2, 3]

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The chloroplast fraction from higher plants has been reported to incorporate amino acids in vitro (1, 2, 4, 7, 8, 9). We have investigated incorporation by spinach (*Spinacia oleracea*) chloroplast fractions and as yet have been unable to demonstrate clearly that the chloroplasts themselves are responsible for the observed incorporation. A severe and major problem appears to be the prevalence of contamination by bacteria.

A typical time course for incorporation of C^{14}-L-leucine, C^{14}-L-phenylalanine or a mixture of C^{14}-L-amino acids by a chloroplast preparation usually continues for at least 8 hours. Frequently the rate increases after the first 3 or 4 hours. Addition of the other 19 amino acids and amides does not enhance but actually inhibits incorporation of any single amino acid. Evidence that the incorporated amino acid is probably in a peptide linkage includes transfer of the originally labeled amino acid from a trichloracetic acid insoluble to soluble form by either acid hydrolysis or papain or trypsin digestion of the isolated labeled protein. Incorporation is inhibited 75 % or more by 10^{-3} M of either chloramphenicol, streptomycin, puromycin, or arsenate. It is not inhibited by Zephiran chloride (1: 5000), penicillin (500 units/ml), ribonuclease (1 mg/ml), deoxyribonuclease (1 mg/ml), or fluoride (10^{-3} M). Incorporation over this long time period is not dependent upon or increased by adding ATP, an ATP generating system, or various substrates such as glucose, malate, ascorbate, or glycolate.

The reaction appears to require O_2 since incubation under N_2 gives a very large inhibition (table I). The enhancement by light (1, 9) can only be consistently reproduced under conditions of low O_2 tension. Photophosphorylation does not seem to be the basis of the light effect because uncouplers (propylamine) had no effect on the light stimulation. On the other hand substrate amounts of TPN do enhance the effectiveness of light (table I) and in other experiments this effect was not shared by TPNH or by catalytic amounts of TPN. The inhibitor of O_2 evolution, *p*-chlorophenyl-1, 1-dimethylurea (CMU), eliminates the light effect, as does the addition of an O_2 trapping system (glucose and glucose oxidase). It seems certain that stimulation by light under these conditions is likely to be simply another manifestation of the O_2 requirement. The pH optimum for incorporation is very broad, running from pH 4.5 to 8.0. Finally, in agreement with previous work (9) a concentration greater than 40 μmoles leucine per milliliter is necessary to achieve the maximum rate of leucine incorporation.

Since many of the unusual characteristics of the chloroplast fraction incorporation system could be explained if microbial contamination were a serious problem, chloroplast preparations were plated out on

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