CHANGES IN CELLULAR CONSTITUENTS DURING CALLUS FORMATION IN POTATO TUBER TISSUE CULTURED *IN VITRO*

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Introduction

Since the process of callus formation in explants provides an excellent material for a study of metabolic regulation in the growth and differentiation of plant cells, it is a primary importance to elucidate the physiological and biochemical events during callus formation in explants excised from potato tubers. Though it is known that the process of callus formation in potato explants involves two phases of cell growth, *i. e.*, cell division and cell expansion, the relation between the respiratory shift and the pattern of cell growth or other materially important processes during callus formation has been partially considered[1,2,3,7]. Exogenous addition of α-naphthaleneacetic acid (NAA, 1.6×10^{-6} M) is essential to callus initiation in explants cultured *in vitro* after a lag phase extending over the first three days where DNA synthesis and cell division are predominated[2,7,8,13].

The respiratory rate is increased to 2 to 3 folds immediately after inoculation[2]. This rise is also revealed in explants regardless of whether or not they are cultured in the nutrient medium. The question of the dependence of auxin-induced growth on auxin independent metabolic changes in storage tissues has been partially resolved[15]. In such effect of auxin on the growth of potato explants is the result of the induced respiration after explant preparation. Although the potato explants are unusually rich in reserved starch in parenchyme cell, the growth of explants is not only based upon storage materials but also largely dependent upon the nutrients provided by the medium, and metabolized in explants into storage pool, into cell structural materials, and to provide energy for the growth and differentiation of explants.

Changes in the cellular constituents of explants during callus development have been investigated as a basis for the study of the mechanisms by which

cellular materials are synthesized and transformed to differentiating cells. The purpose of this paper is to describe such change of pattern of starch degradation and assimilation of major cell components and to discover further its potential uses in cellular metabolism and development.

Materials and Methods

Plant materials

The tubers of *Solanum tuberosum* L, cv. Irish Cobbler, were grown in the field of Faculty of Agriculture, the Hokkaido University, Sapporo, and after harvest were scrubbed clean to cure wound periderm formation at room temperature for a week then stored in a dark room at 4°C.

Explant preparation

Tubers warmed up to room temperature were immersed for 30 min in 2% solution of Antiformin (5% sodium hypochlorite ingredient) with a drop of Tween #20. Columns of parenchyme tissue were obtained by using a cork borer (11 mm diam.) along the long-axis of pith of each tuber and these were further sterilized for 60 min in 5% solution of Antiformin (actually 0.25% hypochlorite) in a glass container with lid, then transferred to a clean sterile cabinet. After removing both ends further bores were made along these columns with a steriled cork bore (7 mm diam.) to isolate potato rods inside bore. These rods were cut into discs (as explants) of 1 mm thickness by the aid of special slicer with adjustable spacer. These discs were washed in sterile distilled water and blotted dry with filter papers which had been subjected to heat sterilization at 150°C for 2 hrs. Four disc shaped explants were inoculated to a 100 ml Erlenmeyer flask containing 30 ml of solidified medium. The flasks were sealed with aluminium foil and placed in a culture room at 25°C. in the dark or as described in the result section. A care was taken to eliminate the contamination of phytopathogenic bacteria inside potato pith.

Modified White's medium

The medium was a modified White's medium of that used by OKAZAWA *et al.*[14] Basal medium composition is as follows;

A) Major mineral components		B) Minor mineral components	
$MgSO_4$	360 mg/l	$MnSO_4$	4.5 mg/l
$Ca(NO_3)_2$	200	$ZnSO_4$	1.5
Na_2SO_4	200	H_3BO_4	1.5
KNO_3	80	KI	0.75
KCl	65	Na_2MoO_4	0.025
NaH_2PO_4	16.5	$CoCl_2$	0.025

C) FeEDTA 20 mg/l

D) Vitamins, sucrose and amino acids

myo-Inositol	100 mg/l	Sucrose	20 g/l
Nicotinic acid	5	Casamino acid	2
Thiamine HCl	1.0	Bacto-agar	8
Pyridoxine HCl	1.0	pH adjusted to 5.6 with	
Ca Pantothenate	0.1	0.1 M HCl or NaOH before	
Biotin	0.02	addition of agar.	

The medium was made up with glass distilled water. Stock solution of 10 times concentrated White's mineral salts and stock solution of 20 times concentrated organic constituents were used for medium preparation. Fe-EDTA was also made a stock from $FeSO_4$ chelating with EDTA. Callus was induced on this basal medium containing 0.3 mg/l of α-naphthaleneacetic acid (NAA 1.6 μM) or as described in the text. All media were sterilized by autoclaving at 120°C and 1.2 kg/cm^2 for 10 min.

Culture conditions

Four explants were inoculated onto 30 ml of nutrient medium in a 100 ml Erlenmeyer's flask being autoclaved and solidified. All operations were carried out in sterile cabinet in inoculation room using sterile instruments. Media used were: 1) control medium (basal medium without NAA) and 2) auxin medium (basal medium with NAA). The cultures were incubated in a dark room at 25°C. Explants cultured in control medium and auxin medium are described as control culture and auxin culture, respectively. Samples were harvested at appropriate intervals on the course of 3 weeks' culture for analysis described below.

Measurement of fresh and dry weight

The explants taken from the culture medium were carefully washed with distilled water, and blotted dry with filter paper. The fresh weight was measured immediately after these operations. The dry weight was measured after the explants had been dried in an oven at 120°C for 2 hrs and later at 80°C for further 12 hrs.

Determination of protein content

The dried samples were ground with 80% ethanol in a mortar and centrifuged at 5,000 × g for 5 min. This procedure was repeated three times. Then the pooled supernatants were discarded and the residues were suspended in ether-acetone mixture (2 : 1, v/v) and warmed upto boiling temperature. The lipid extraction was repeated twice. The resulting residues were heated

to remove ether-acetone, and the dried powder was obtained. The protein-nitrogen content in this powder was determined by nesslerization after digestion in concentrated H_2SO_4.

Determination of nucleic acid content

The extraction and estimation of nucleic acid was carried out by the method of OGUR and ROSEN[11] with minor modification[10]. The explants under methanol were ground in a mortar and centrifuged to remove the supernatant. The residue was reextracted with boiling 80% ethanol, then with a series of solvents, absolute ethanol, ether and acetone. The resulting powder was extracted first with 1 M-perchloric acid at 2°C and suspended with 0.3 M KOH at 37°C and cold 1.2 M perchloric acid overnight. The protein content of the KOH hydrolysate was determined by the method of LOWRY *et al.*[9] using bovine serum albumin as a standard. The RNA content of the perchloric acid supernatant was determined from optical density reading at 260 nm and 300 nm. DNA was isolated further by heating the residue in 1.2 M perchloric acid. Correction was made with protein and phosphorus content of the nucleic acid fractions.

Fractionation of Carbohydrates

70% ethanol-soluble materials. About 300 mg of frozen sample was ground in a mortar with pestle, and extracted three times with 70% ethanol. After centrifugation the extracted material was dried. The extractives were freeze-dried, to determine mono-, and oligosaccharides.

Ethanol-ether-acetone-soluble materials. The dried residue was further extracted successively with absolute ethanol, ethanol-ether and ether-acetone, respectively, at 70°C. This solvent fraction was allowed to evaporate for determination of sugar-lipids.

Cold water-soluble materials. The dried powder was suspended in ice cold water and extracted oligosaccharide and amylose.

Amylolysate. The insoluble materials were suspended in 0.05 M acetate buffer, pH 4.8, heated to boil for 5 min, and were treated with α-amylase for 3 hrs at 36°C. The hydrolysate was heated to terminate enzyme reaction and cooled to extract the degradation product two times with 70% ethanol.

Pectic substance. To the remainder of the already extracted materials was added 0.2 M ammonium oxalate, 5 ml of pH 4.5, and the mixture was shaken in boiling water bath for 90 min. After cooled and centrifuged the solution was dialyzed overnight against water. Ethanol was added to dialyzed solution until its concentration was over 90%, and the precipitate that formed was allowed to settle for 24 hrs. The separated precipitate was pectic substance.

Holocellulose and lignin. The pectin-extracted material was suspended in 5 ml of water in a glass-stoppered tube, in an water bath at 75°C for 2 hrs. Two drops of glacial acetic acid and 45 mg of $NaClO_2$ were added and after 1 hr these additions were repeated. The holocellulose was washed with water and dried. Klason lignin was extracted from H_2SO_4 treated material with water thoroughly repeating wash and resuspension.

Hemicellulose. The holocellulose was fractionated by successive extractions with 5 and 24% (W/V) KOH solution for a total of 12 hrs at 36°C. The residue was centrifuged and thoroughly washed with water by suspension and centrifugation. The alkaline extracts and washings were combined and neutralyzed at 4°C with cold acetic acid. The solution was dialyzed for 24 hrs against running water followed by distilled water, and the hemicellulose was precipitated in the same manners in ethanol.

α-Cellulose. The residual α-cellulose was washed with water, acetone and ether, dried and weighed. The yield of α-cellulose was corrected for the amount of ash which is contained. α-Cellulose was digested with cellulase prepared from "Cellulase ONOZUKA". The degree of crystalline of cellulose was determined by hydrolyzing the residual cellulose with 6 M HCl at 90°C. This procedure was repeated several times and plotted the percent hydrolysis.

Determination of carbohydrate content. For conventional chemical analysis of starch and wall components, two procedures were used: the gravimetric procedure comprising glass-filter trapped dried material (Whatman GFC, 24 mm diam.), at 100°C to a constant weight, and the colorimetric procedure of 0.2% anthrone positive compounds, in 75% H_2SO_4 at 100°C for 12 min., at 680 nm.

Procedures for metabolism experiment.

Experiments for the metabolism of uniformly labeled glucose and glycine by the explants were carried out in a Warburg respiratory apparatus at 25°C. The explants after harvest were washed with distilled water, blotted dry with filter paper. An aliquot of the explants was placed in a weighing bottle for the determination of fresh and dried weight. Each Warburg flask contained 1.0 g of fresh explant in 2.0 ml of 0.05 M potassium phosphate buffer, pH 5.6, with 3 μC glucose-U-^{14}C or glycine-U-^{14}C in the main compartment and 0.3 ml of 5 N NaOH in the center well. The uniformly labeled glucose (261 mC/mM) and glycine (108 mC/mM) were purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England. After an incubation period of 2 hours, the explants were collected, rinsed with 0.05 M phosphate nonradioactive buffer, pH 5.6, and immediately crushed in a mortar. The water soluble materials were extracted with 80% methanol

and further separated by means of ion exchange resins into neutral, basic and acidic fractions. The slurry was centrifuged and the precipitate was again extracted by successive suspension and decantation for removal of the materials soluble in methanol followed by acetone-diethyl ether (2 : 1, v/v). The resulting dry powder was suspended in 2 ml of 5% chilled perchloric acid (PCA) and centrifuged for 5 min to extract acid soluble materials. In one experiment with glucose-U-^{14}C, the pellets were suspended in 0.05 M acetate buffer pH 5.0, and subjected to α-amylase hydrolysis for 2 hrs at 37°C. After the extraction of the hydrolysates, the residues were incubated with 1 N KOH at 37°C overnight. In this fraction, alkaline soluble polysaccharide (hemi-cellulose) and protein were removed from the residual cell wall. In the other experiment with glycine-U-^{14}C, the nucleic acids were extracted from the precipitates by exposing to 2 ml of 0.5 N PCA at 90°C for 30 min. (DNA) following the extraction by 2 N chilled PCA (RNA). The proteins were removed by incubating with 4 ml of 0.3 N NaOH for overnight at 37°C, and precipitated by 10% TCA. An aliquot of these fractions was spread over a scintillation liquid to assay for the radioactivity in a scintillation spectrometer[5] Total homogenate and $Ba^{14}CO_3$ were corrected the radioactivities.

Results

Growth of potato explants.

The effect of the application of auxin (1.6×10^{-6} M NAA) on the course

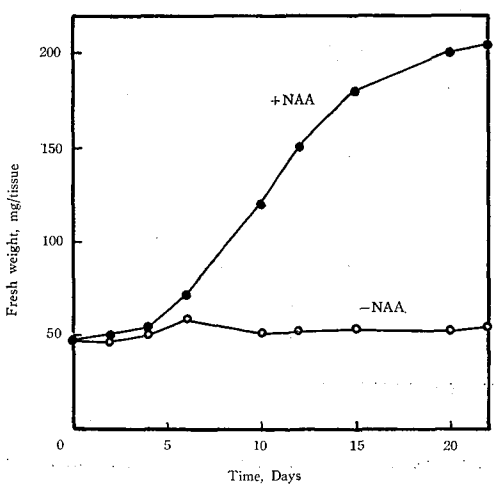

Fig. 1. Growth of explant cultured in nutrient medium supplemented with or without NAA, 1.6×10^{-6} M.

of callus development in terms of fresh weight increase in explant is shown by the data of Fig. 1. in which the fresh weight per explant are plotted against time. It is significant that potato explants in auxin medium showed an active growth and a striking quadruple in yield was obtained at the end of 3-weeks' culture. The curves in the figure indicate an initial lag phase of 5 days after inoculation followed by a linear increase in fresh weight when explants were cultured in auxin medium. Whereas explants in basal medium did not show any sign of growth throughout the 3 weeks period of culture.

An attempt has been made to estimate the dry weight increase which is a real measurement of growth and development of cultured explants. Since the explants excised from potato tubers are rich in starch grains in cells, the degradation of the storage products is one of the main interests in the study of biochemical changes during callus development. Nevertheless, an increase was observed in explants cultured in auxin medium while explants in basal medium did not show any significant change in dry matters.

It is well investigated that the NAA has marked effect on the water uptake by potato tuber tissue[15]. The moisture content was continuously increased in explants cultured in auxin medium during the first 2 days and secondly during the day 5-8 of culture. While a slightly less significant increase in moisture content was observed in explants of control culture.

Freshly cut slices from potato tubers were contained approximate 89% of moisture while that of potato callus was 95% or more.

Accumulation of DNA, RNA and Proten

Significant increase in DNA multiplication in auxin culture was an obvious indication of cell division and relatively prolonged interphase of DNA multiplication was observed as cell expansion (Fig. 2). The maximum rate of growth was attained during the interphase of DNA multiplication of days 8-12.

RNA was increased right after the explants were inoculated on the nutrient medium and followed by increase in protein in a similar extent (Fig. 3, 4). The increase in RNA and protein in an indication of growth and development in explant cultured in auxin medium.

Fractionation of Polysaccharides

Figure 5 shows the degradation of starch and the accumulation of cell wall materials in the explants cultured in auxin-and basal medium. The relatively greater deposition of hemicellulose and cellulose was observed in the explants in control culture, but slightly lesser extent in auxin culture

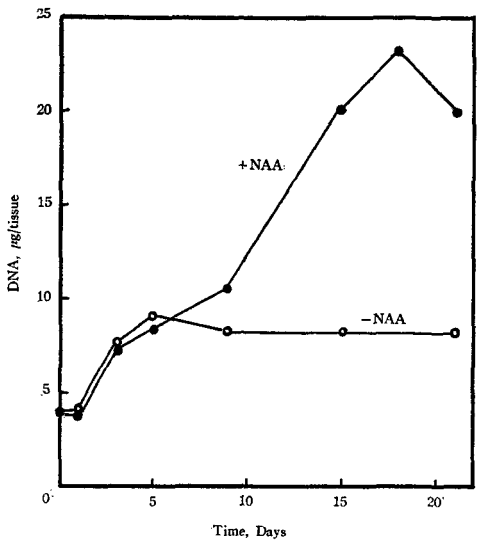

Fig. 2. Changes in DNA content of potato tissue cultures.

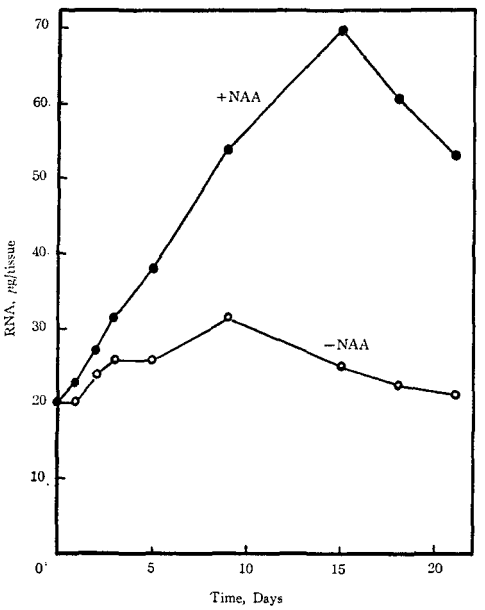

Fig. 3. Change in RNA content of potato tissue cultures.

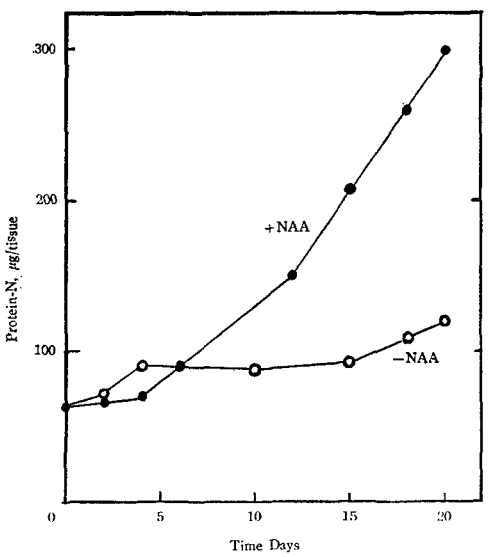

Fig. 4. Changes in protein-N content of potato tissue cultures.

in the early 2 days. However, the deposition of cell wall materials in auxin culture was found to correlate well with the growth of callus and with the corresponding degradation of starch in explants. The more significant correlation was the increases in cellulose and DNA contents, both of which were intrinsic materials in plant cells inherently involved in cell division and expansion.

To assess possible differences between the patterns of changes in carbohydrate metabolism in explants, the amounts of two main polysaccharide components of cells, reserve starch grains and wall polysaccharide, and the relative incorporation of glucose into these polysaccharides were determined by special attension.

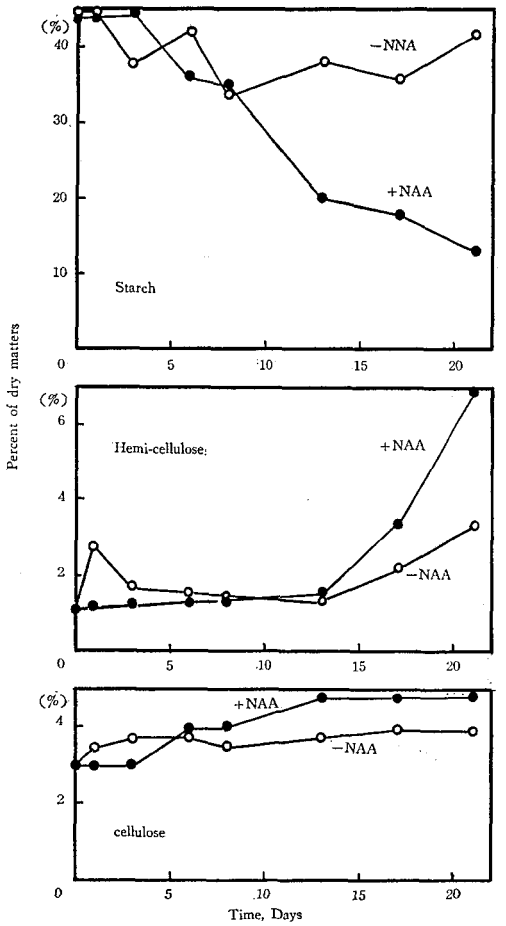

Fig. 5. Changes in percentage composition of starch and cellwall materials in potato tissue cultures.

Glucose uptake

Glucose uptake was measured by summing up the radioactivity each of isolated fraction from the explant after a 2-hour incubation period of uniformly labeled glucose ($3\,\mu C/10^{-4}$ M/10 explants). The explants of potato tissue slices on the medium with or without auxin took up glucose from the incubation medium at about the same rate after the cultures were initiated, while on the course of callus development the explants on the auxin

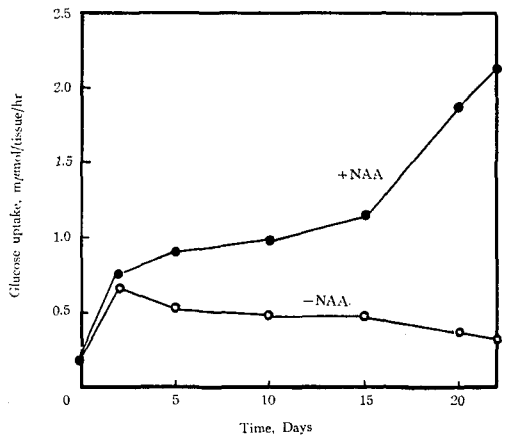

Fig. 6. Change in rate of glucose uptake by potato tissue bultures.

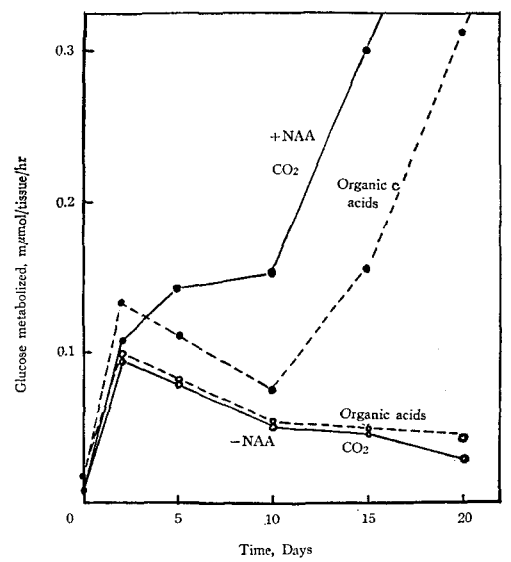

Fig. 7. Changes in rate of glucose catabolism by potato tissue cultures.

medium utilized more glucose than those of the quiescent tissue on the auxin-free medium (Fig. 6). The metabolic activation of the explants by the aging after slicing the parenchymatous tissue of potato tubers ovbiously has a marked effect on the increased rate of glucose uptake, since there appears to be no great difference of glucose utilization between the explants on the nutrient medium with or without auxin during the initial lag phase of cultures. Approximately 50% of the total label taken up was remained in the neutral fraction after the extraction by aqueous methanol followed by the separation of acidic and basic fractions by means of ion exchange resins. Fig. 7 illustrates the recovery of $^{14}CO_2$ and of water soluble organic acids and amino acids from the explants. The developing tissue produced more $^{14}CO_2$ over the level of soluble acids while the quiescent tissue gradually reduced rate both of $^{14}CO_2$ evolution and soluble acid accumulation after on-set of the metabolic activation by slicing.

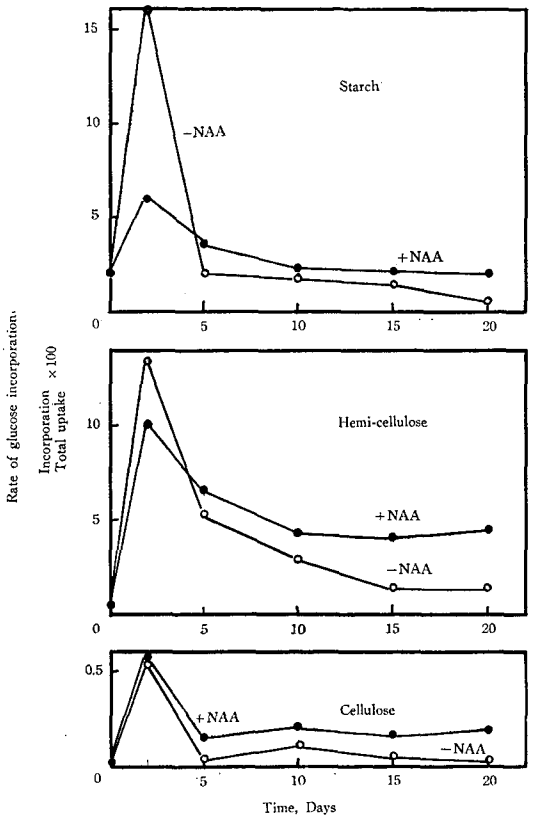

Fig. 8. Changes in rate of glucose incorporation into starch, and cellwall materials in potato tissue cultures.

The insoluble fraction.

The insoluble fractions are mainly representing the glucose incorporation into the skeletal structure of the cellular constituents. The labeling patterns observed in the insoluble fractions shown in Fig. 8. are very similar to each other. There were considerable incorporation of glucose into the polysaccharide fractions, especially into hemicellulose fraction on the second day after initiating cultures of the explants. On the other hand, the greater exchange of glucose to starch occurred in the explant incubated in the basal medium, and there appears to have a consistent incorporation of the label into the hemicellulose, protein and cellulose fractions from the explant cultured on the auxin medium. It is evident that an early rise in polysaccharide synthesis may be due to the formation of wound periderm of the tissue surfaces after slicing of the potato tuber, and later, these polysaccharides

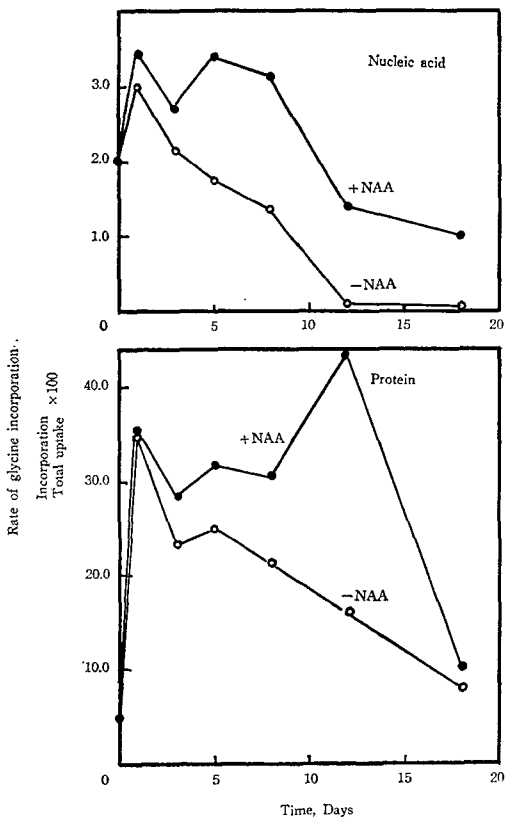

Fig. 9. Changes in rate of glycine incorporation into nucleic acid and protein in potato tissue cultures.

are deposited on the cell wall during the callus development in the tissue in which newly divided cells showed the greater utilization of glucose from the incubating solution.

Incorporation of glycine into nucleic acid and protein.

The study was undertaken to investigate further the effect of auxin on synthesis of nucleic acid and protein in the explants cultured on the auxin and auxin-free media on the course of callus development. The results indicate in fig. 5 that the auxin induced glycine incorporation into protein and nucleic acid fractions. The explants on the auxin-free medium also gave rise a high rate of incorporation into nucleic acid and protein immediately after the initiation of cultures. In fig. 9, it is again indicated that the metabolic activation of the explants was also occurred in the tissues whether they were incubated with or without auxin. However, the responses of auxin on these sythetic activities in the explants were prolonged depending upon the presence of auxin in the nutrient medium where the changes in rate of the protein synthesis following the nucleic acid synthesis were correlated in time.

Discussion

Culture of explants of potato tubers induces a series of cytological and metabolic events which within few days lead to redediffernentiation (callus formation) of explants. These responses are similar to general in wound periderm formation, commonly referred to as induced metabolic activation[4]. The time course of the respiratory activation of metabolic systems in explants during callus formation has been extensively studied[1,2,7,8]. Hormonal control of callus formation was also found to require a transcription of genetic information followed by translation to de novo synthesis of proteins[13,16]. Little attension has been paid to the correlation between metabolic activation and changes in cytological constituents. Investigations of the changes in the composition of the explants during callus development can be used as a basis for the study of the mechanism by which the cellular materials are synthesized during callus development. The formation of callus in the explants has been studied in this way, and the estimation of cellular constituents of whole explants compared at different stages of growth gives a clear indication of the change occurring in explant during callus development.

Because of the high and variable amounts of starch in potato explants, the starch content was determined during callus formation. When sugar content increases, the sugar is formed from starch; also when sugar is decreased this compound in part converted to starch. Even sucrose can be

absorbed from the medium into internal cytosol compartment of cells, starch in explants is degraded in auxin culture but not in control culture in which sucrose are inverted to hexoses. As in other plant tissues the dissolution of starch to sugars also appeared to be retarded in potato explants in the absence of auxin. This observation would be explained if starch is degraded by phosphorylase to G-1-P which is then engaged to metabolic pathway, the rate of disappearance of starch would be retarded by the lack of auxin in medium. This evidence that starch is degraded to sugar in auxin culture was confirmed for potato explants. The accumulations of C^{14}-glucose to form starch attributed to metabolism of the formation and degradation of starch in both cultures are extensively engaged to metabolic activation system and auxin induced system.

The metabolic activation by aging after slicing the storage organ of potato tuber obviously has a marked effect on the increased rate of glucose uptake in the explants cultured *in vitro*. Although there appears to be no great difference between the explants cultured on the nutrient medium with or without auxin for an initial period of 2 days, the explants on the auxin medium utilized more glucose for catabolic reactions than those on the basal medium. Since growth and development of the explants *in vitro* were mainly based on the utilization of carbohydrate and amino acid in the nutrient medium, the rise of glucose uptake and utilization was resulted from the metabolites more available to the synthesis of cell structural materials and to provide energy for the growth activities of the explants.

It appears, therefore, that the greater growth rate and concomitant biosynthetic activities of the tissue on the course of callus development in this study could be explained on the basis of an increased rate of glucose metabolism, but such an explanation could show nothing about the underlying mechanisms for the auxin-induced callus development in the explants.

The synthesis of both DNA, RNA and protein has been shown to be induced in potato tissue by slicing[17]. In biological views, if the differences in the explants cultured *in vitro* are caused by new synthesis of enzymes brounght about by hormonal or other regurating factors, the nucleic acids might be expected to play an active role. Furthermore, plant hormones are increasingly considered to act on nucleic acid metabolism[16].

The quiescent tissue cultured on the basal medium is characterized by loss of synthetic activity of nucleic acid and of protein after the metabolic activation by slicing. Although it is not known the cause and effect relationship that an addition of auxin to the nutient medium gives rise to callus development in the explants, the content of protein and the growth of explant

on the course of cultural period and changes in the rate of protein synthesis following nucleic acid synthesis are correlated in time.

The sterile cultures of explants isolated from potato tubers have been shown to grow for a long time on the nutrient medium with additions of auxin and kinetin[8]. Although kinetin was found to be indispensable for the continuous growth and callus proliferation, but freshly cut explants did not require an additional kinetin for the initiation of callus development in explants. Therefore, it has been suggested that a certain level of native cytokinin(s) in the potato tuber may serve as a stimulus for the initiation of callus development in response to auxin action[6]. Furthermore, the relationship of the metabolic activation by slicing discussed here to the activation of the natural cytokinin directed system is not at all clear.

Summary

The changes in cellular constituents during callus formation were studied by using explants excised from the tubers of *Solanum tuberosum, L.* aseptically cultured *in vitro,* as a model system of differentiation.

The stack of data obtained shows that the growth and development of this model system occurs in two phases. The first, which may be characterized as a dedifferentiation phase (callus initiation), occupies the first five days, and the second, which may be called differentiation phase (callus development), extends over the rest of culture period. The evidence of the two phases is shown strikingly by the metabolism data. When explants are excised from potato tubers the intrinsic metabolism and respiration are low, and the immediate effect of transfer to the nutrient medium is an activation of metabolism which extends over the first five days.

The formation and degradation of starch in explants are extensively engaged with metabolism which supports directly the synthesis of cellwall materials and indirectly the respiratory system even though sucrose can be utilized from the medium. These seem to play an important role in the synthesis of cellular constituents for the callus proliferation.

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