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Cytokinin Regulation of Protein Synthesis during the Early Stages of Shoot Formation from Cultured Tobacco Cells

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During tobacco (*Nicotiana tabacum* var. Bright Yellow) cell culture cytokinins can induce and redifferentiate shoots or roots from cells cultured *in vitro*.

Relationships between the synthesis of certain proteins and the disappearance of other proteins during the early stage of shoot formation, under the specific actions of cytokinins, were sought using DEAE cellulose column chromatography, polyacrylamide gel electrophoresis and immunological methods.

Our results show that cytokinins cause the induction of certain characteristic proteins, when leaves begin to form from cultured cells *in vitro*. During shoot formation some proteins synthesized for cell proliferation are repressed. These changes in protein synthesis caused by cytokinins in cultured tobacco cells are discussed in regard to morphological observations.

INTRODUCTION

Cytokinins appear to regulate many different actions in plant, for example cell division, enlargement, and differentiation.[1] However, conclusive evidence for specific regulatory functions has yet to be reported. In some studies of cytokinin actions, the investigation has been made into their influence on protein synthesis. For example, Jouanneau *et al.*[2,3] reported that kinetin promotes protein synthesis in tobacco cell suspensions and that at least one specific difference in protein pattern could be observed before the first cell division occurred. During senescence, it has been reported that cytokinins affect the nucleic acid and protein metabolism.[4~7] In relation to protein metabolism, other cytokinin actions on the expansion of cotyledons[8] and on dormant duckweed[9] have been reported.

During cell culture cytokinins can induce the formation of shoots or roots from cultured tobacco cells[10] by a mechanism as yet not understood. This paper reports cytokinin action on protein synthesis as it is related to organ formation (redifferentiation) in cultured tobacco cells *in vitro*.

MATERIALS AND METHODS

Chemicals. 2, 4–D (2, 4-dichlorophenoxyacetic acid) was purchased fromTokyo Kasei Kogyo Co., Tokyo; Kinetin (6-furfurylaminopurine) from Sigma Chemicals Co., St. Louis: Polyclar AT from the General Aniline and Film Corp., Dyestuff and Chemical Division, New York and incomplete adjuvant from Itaron Laboratory,

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Tokyo. Zeatin (6-[*trans*-4-hydroxy-3-methyl-but-2-enyl amino] purine) was provided by Dr. Koshimizu, the Department of Food Science and Technology, Kyoto University. L-[U－^{14}C] leucine (270 mCi/mmol) was obtained from the Radiochemical Center, Amersham, England.

Cell culture. Tobacco (*Nicotiana tabacum* var. Bright Yellow) callus (Strain T5)[11] and Linsmaier and Skoog basic medium[12] were used in these experiments. For shoot formation cultured cells were inoculated in a medium containing 5×10^{-5}M zeatin (zeatin callus) or 10^{-5}M kinetin (kinetin callus). For the control cells were inoculated on a medium containing 10^{-6}M 2, 4–D (2, 4–D callus). All cell cultures were incubated at 25°C in the dark. To get growth curves about 50 mg of callus was inoculated in a test tube containing 10 ml of agar medium and the fresh weight was measured.

DNA, RNA and protein determination. DNA and RNA were extracted according to the method of Schneider,[13] except that the acid insoluble material was defatted by heating it at 50°C in ethanol-ether (1: 1. v/v) for 15 min before hot trichloroacetic acid (TCA) hydrolysis. DNA content was determined by the diphenylamine method as described by Burton.[14] RNA was determined by the orcinol method as described by Mejbaum.[15] Soluble protein was extracted by homogenizing callus in a glass homogenizer containing 0.1 M phosphate buffer, pH 7.5, centrifuging the homogenate at 10,000 g for 15 min and collecting the supernatant. Protein content was determined by the method described by Lowry *et al.*[16]

^{14}C-leucine incorporation and DEAE cellulose column chromatography. Callus, incubated with ^{14}C-leucine, was homogenized with 0.1 M phosphate buffer (pH 7.5) in a chilled mortar, then the homogenate was centrifuged at 10,000 g for 15 min. The supernatant was dialysed against 0.01 M phosphate buffer (pH 7.5) overnight. The extract was applied to a DEAE cellulose column and eluted with a combination of linear and step gradients of NaCl. Radioactivity was determined as follows. An equal volume of 10% TCA was added to part of the fractionated solution and the precipitate was collected on a glass fiber paper (Whatman GF/C) and washed with 5% TCA, ethanol and ether. Using a toluene base scintillator, the radioactivity was counted with a Beckmann LS–100 type liquid scintillation spectrometer.

Disc electrophoresis. Polyacrylamide gel electrophoresis of the fractionated protein was performed according to the method of Davis.[17]

Preparation of antigen and antiserum. Callus or tobacco leaves grown in a green house for 3 months, was homogenized in a chilled mortar with 1/5 its weight of Polyclar AT and an equal volume of 0.05 M phosphate buffer (pH 7.5) containing 1% Na-ascorbate. The pH was adjusted to 7.5 with 1 N NaOH. The homogenate was squeezed through gauze, then centrifuged at 10,000 g for 30 min. Protein was precipitated by saturating the mixture with ammonium sulfate. The precipitate was collected by low speed centrifugation, after which it was dissolved in a small amount of and dialysed against 0.05 M phosphate buffer (pH 7.5). All procedures were carried out at 4°C. The protein solution (antigen) was stored in a freezer after the addition of 12.5% glucose.[18] Two male rabbits were immunized with antigen from 15 day-old 2, 4–D callus and two others with antigen from tobacco leaf. Five doses of the same antigen (the first three, 20 mg as protein and the last two, 10 mg) in incomplete Freund

adjuvant were administered intraperitoneally at 1 week intervals. Blood was taken from the carotid artery 1 week after the last injection. Sera were prepared by the usual method.[19]

Antibody-antigen reactions. Antibody-antigen reactions were studied using the double diffusion method in agar gel as described by Ouchterlony.[20]

RESULTS AND DISCUSSION

Although 2, 4–D callus, Strain T5, grows well in the absence of cytokinins, it will only form shoots when transferred to a medium with 5×10^{-5}M zeatin as the cytokinin. Figure 1 shows that over a period of 20 days 2, 4–D callus increased in fresh weight more than zeatin callus did. In zeatin callus, the growth rate was slower and reached a plateau 15 days after inoculation, whereas the 2, 4–D callus grew logarithmically for more than 20 days.

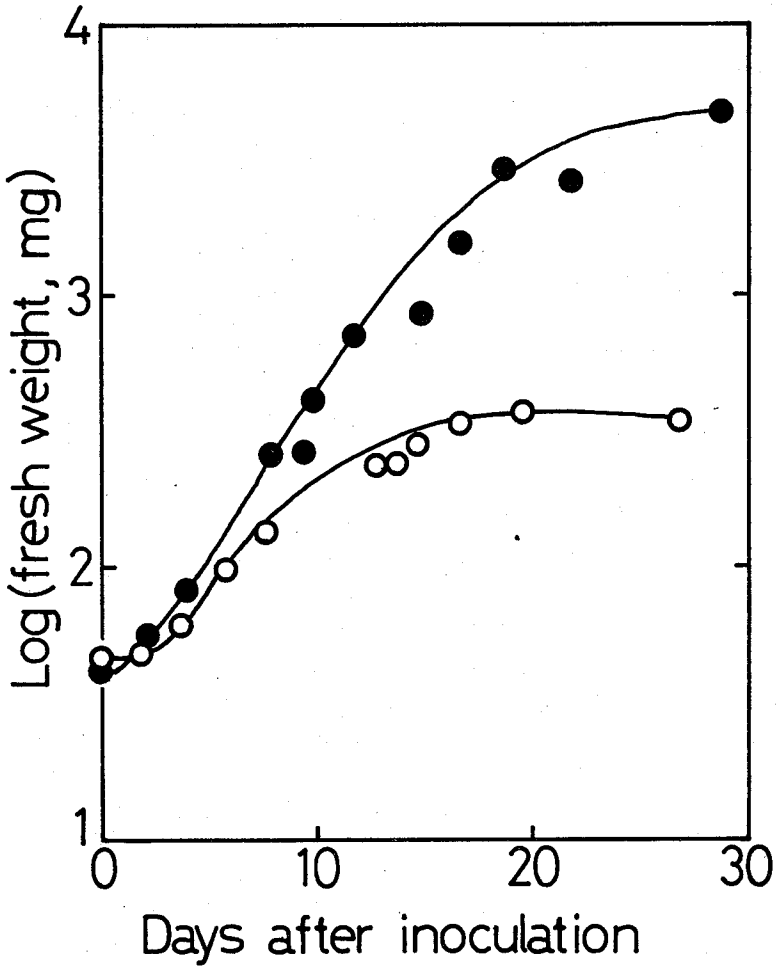

Fig. 1. Growth curves for tobacco cells cultured on a medium containing 10^{-6} M 2, 4–D (—●—)and 5×10^{-5} M zeatin (—○—). The average fresh weight of 3 replicales is given in the figure.

In many cases, callus requires auxins and an additional low concentration of cytokinin promotes cell growth.[10,21] In contrast our results show a high concentration (5×10^{-5}M) of zeatin inhibits callus growth. Nudel *et al*[22] also reported the inhibitory effects of a high concentration of kinetin on growth, as well as the synthetic activity of other cell components in tobacco callus.

About 1 week after inoculation tracheid-like cells, lignified cells, and cell organization were observed in zeatin callus, but only 6 to 8 weeks after transfer was the shoot visible. In some reports on tobacco[23] and soybean[24] kinetin was also shown to promote tracheid-like cell production.

Table I shows that in zeatin callus RNA and protein concentration per gram fresh weight began to increase 5 to 6 days after inoculation, then 15 days later DNA increased, whereas the contents per fresh weight in 2, 4–D callus remained constant.

Table I. Contents of DNA, RNA and Protein.

	Days			
	0	6		15
DNA (mg/g fresh wt)				
2, 4–D (A)	0.038	0.065		0.056
Zeatin (B)	—	0.052		0.104
B/A	1.00	0.80		1.86
RNA (mg/g fresh wt)				
2, 4–D (C)	2.80	2.72		3.08
Zeatin (D)	—	4.11		4.14
D/C	1.00	1.51		1.34
	Days			
	0	5	10	16
Buffer soluble protein (mg/g fresh wt)				
2, 4–D (E)	1.34	1.54	1.35	1.33
Zeatin (F)	—	1.83	2.28	1.98
F/E	1.00	1.19	1.69	1.48

Incorporation of ^{14}C-leucine into proteins and DEAE cellulose column chromatography of these proteins were investigated and the results shown in Figs. 2 and 3. In Fig. 2, protein peaks for zeatin callus were eluted at a higher concentration of NaCl than were those for 2, 4–D callus. The radioactivity of ^{14}C-leucine, incorporated into protein showed a relatively high level. A remarkable and characteristic peak at the concentration of 0.3 M NaCl for zeatin callus (B) was obtained as compared to that for 2, 4–D callus (A). The eluent with 0.1 N NaOH shows a high absorption but much less radioactivity. Phenolics seem to cause the high U. V. absorption at 260 and 280 nm.

To compare protein patterns at different stages, ^{14}C-leucine was incorporated and DEAE cellulose column chromatography was performed. As shown in Fig. 3, patterns for the total protein and the radioactivity incorporated into 2 day-old zeatin callus are

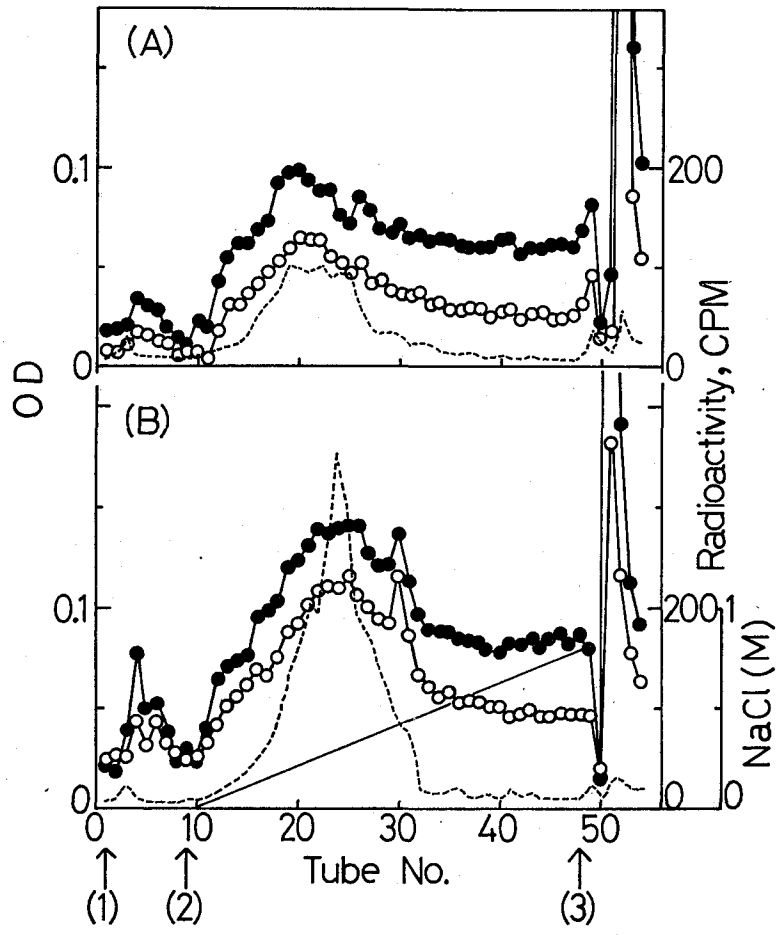

Fig. 2. DEAE cellulose column chromatography after the incorporation of ^{14}C-leucine into protein fractions of tobacco calluses cultured for 15 days with 10^{-6} M 2,4–D (A) and with 5×10^{-5} M zeatin (B). Five grams of 2, 4–D callus and 2 g of zeatin callus were separately incubated in 5 ml of medium containing 0.625 μCi of ^{14}C-leucine for 5 hr, then the protein solution was prepared by the procedures described in "Methods". DEAE cellulose column: (1×7 cm) 3.8 mg (A) and 8.9 mg (B) proteins were applied and eluted with: (1) 20 ml of 0.01 M phosphate buffer (pH 7.5), (2) 120 ml of a 0 to 0.8 M gradient of NaCl in 0.01 M phosphate buffer (pH 7.5), (3) 20 ml of 0.1 N NaOH. (—●—) shows the optical density at 260 nm, (—○—) that at 280 nm and (·····) radioactivity.

not much different from those for the 2 day-old 2, 4–D callus (A and B in Fig. 3). However, 15 day-old zeatin callus showed quite different patterns from the 15 day-old 2, 4–D callus for both total protein and incorporated radioactivity in protein (C and D in Fig. 3). A 15 day culture period on a medium containing zeatin enhanced the synthesis of certain characteristic proteins (fraction 3 on C and D in Fig. 3) which are specific to young leaves of the tobacco plant (E in Fig. 3). Even the protein pattern of incorporated ^{14}C-leucine showed the specific peak at fraction 3 in Fig. 3 (D), the same as in Fig. 3 (E). This means that in zeatin callus protein synthesis differs from

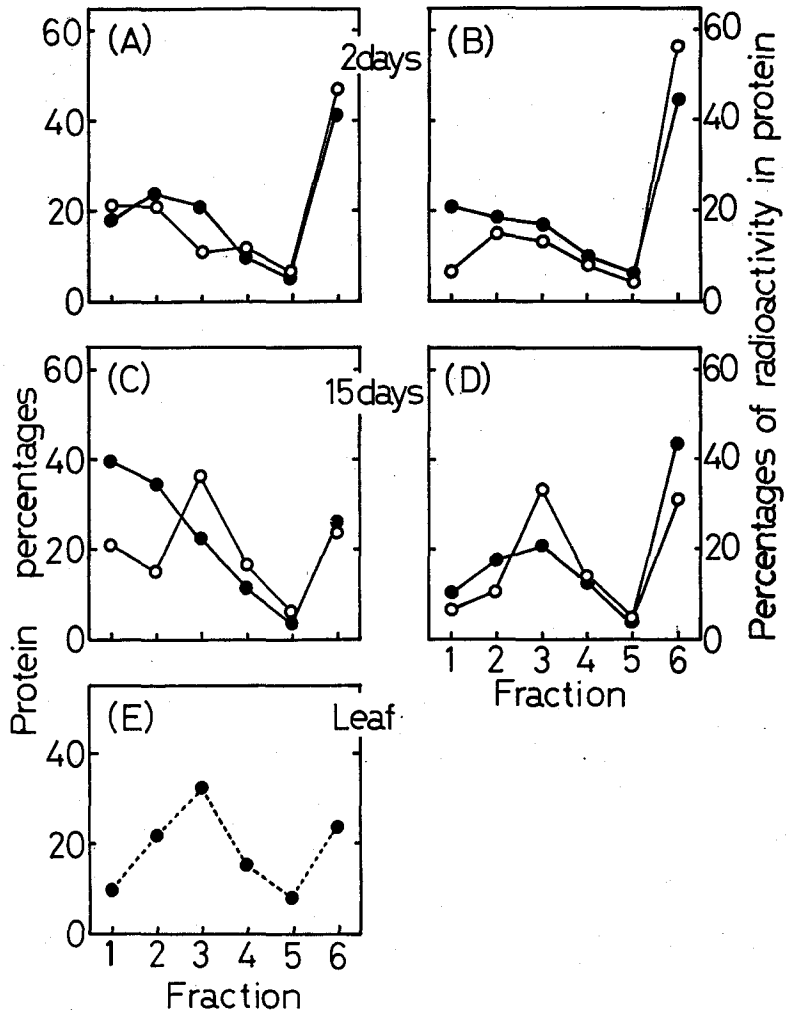

Fig. 3. DEAE cellulose column chromatography. (A) and (C) show the percent of total protein in each protein fraction (omitting fraction 1) in 2 day-old and 15 day-old cultured cells. (—●—) indicates 2, 4–D callus and (—○—) zeatin callus. (B) and (D) show the percent of radioactivity incorporated into proteins (omitting fraction 1) at the 2nd and 15th day after the beginning of cell culture. Symbols are the same as above. (E) shows the percent of total protein in each protein fraction in a young leaf (···●···).

A few grams of 2, 4–D callus and zeatin callus, which had been cultured for 2 or 15 days were separately incubated in 5 ml of medium containing 0.1 μCi of ^{14}C-leucine for 2 hr. Extracts were prepared by the procedures described in "Methods". The phosphate buffer soluble fraction from two grams of tobacco young leaf was extracted using the same methods. The extract was applied to a column (1×5 cm) in 0.01M phosphate buffer (pH 7.5) and eluted with the following solutions: 0.01M phosphatebuffer (pH 7.5) (fraction 1), 0.1 M NaCl in 0.01 M phosphate buffer (pH 7.5) (fraction2), 0.2 M NaCl in the same buffer (fraction 3), 0.3 M NaCl in the same buffer (fraction 4), 0.5 M NaCl in the same buffer (fraction 5), and 0.2 N NaOH (fraction 6). Protein content was determined by the method of Lowry *et al.*[17]

that in 2, 4–D callus, and that zeatin callus protein content becomes similar to that of leaves after about 15 days of culture. Therefore, we investigated, in detail, the differences in protein patterns between 15 day-old 2, 4–D callus and 15 day-old zeatin callus using electrophoresis.

Protein fractions from 15 day-old 2, 4–D callus and 15 day-old zeatin callus obtained from DEAE cellulose column chromatography (Fig. 3) were electrophoresed with polyacrylamide gel. As shown in Fig. 4 slight discrepancied were detected between 2, 4–D and zeatin callus. In zeatin callus 3 bands, (b), (c), and (d) appeared in F1, F3, and F4, which were not found in 2, 4–D callus. Band (a) was found only in 2, 4–D callus. Fractions 5 and 6, which were eluted with 0.5 M NaCl and 0.2 N NaOH, respectively, were not well mapped by electrophoresis. Jouanneau *et al.*[2,3] reported the enhancement of ^{14}C-leucine incorporation by cytokinin in tobacco callus and, using disc electrophoresis, that at least one specific band appeared after the first generation. Our results showed that at least 4 different bands were found in tobacco callus

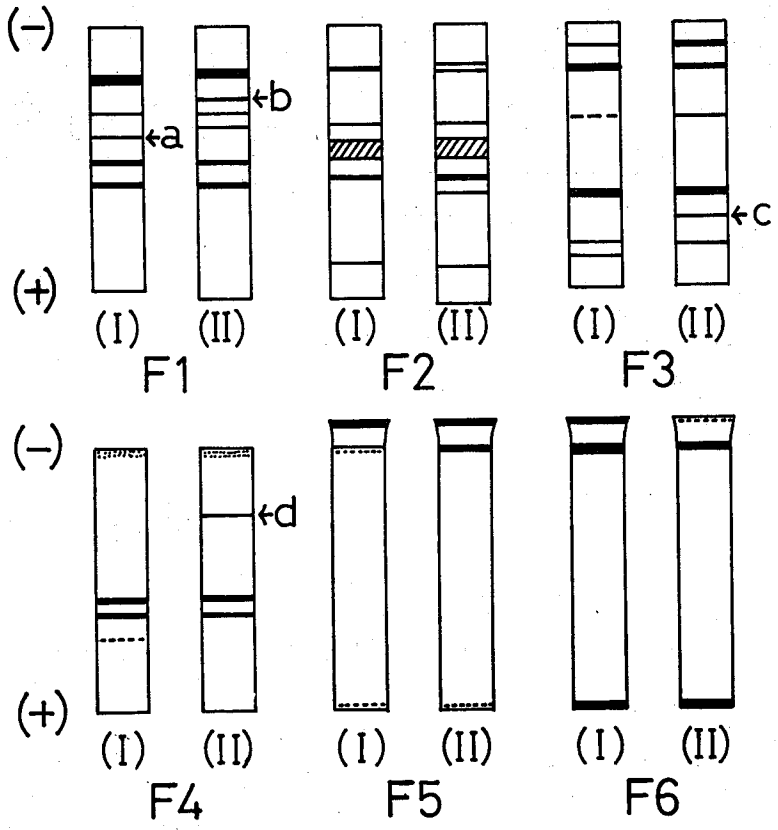

Fig. 4. Polyacrylamide gel electrophoresis of proteins fractionated in Fig. 3. Fl, 2, 3, 4, 5, and 6 correspond to fraction 1, 2, 3, 4, 5, and 6 in (C) of Fig. 3. F5 was neutralized with 0.2 N HCl before electrophoresis. Samples containing protein, ranging from 50 to 150 μg (except F5 which contained 10 to 20 μg of protein) were layered on gels. After electrophoresis the gel was stained with 1% amido black 10 B, then destained with 7% acetic acid. (I) is from 2, 4–D callus and (II) from zeatin callus.

treated with and without zeatin for 15 days. The characteristic synthesized proteins in zeatin callus (C and D in Fig. 3) which occurred in quantitative amounts, did not give quantitative amounts as the very specific protein bands at F3 in Fig. 4. However, band (c) is a characteristic protein band in F3 from zeatin callus and band (c), as well as bands (b) and (d), is believed to contain proteins which are significantly involved in regenerating leaves from cultured cells.

To confirm and determine qualitatively the protein changes due to cytokinins, which accompany shoot formation in cultured cells, we used immunological techniques. In these experiments, we used kinetin instead of zeatin as the cytokinin.

To obtain antigen from kinetin callus, we inoculated callus on a medium containing 10^{-5}M kinetin and cultured them for 5, 10, 15, and 32 days. Kinetin callus cultured for 32 days produced no visible organogenesis; though the callus, itself, became harder

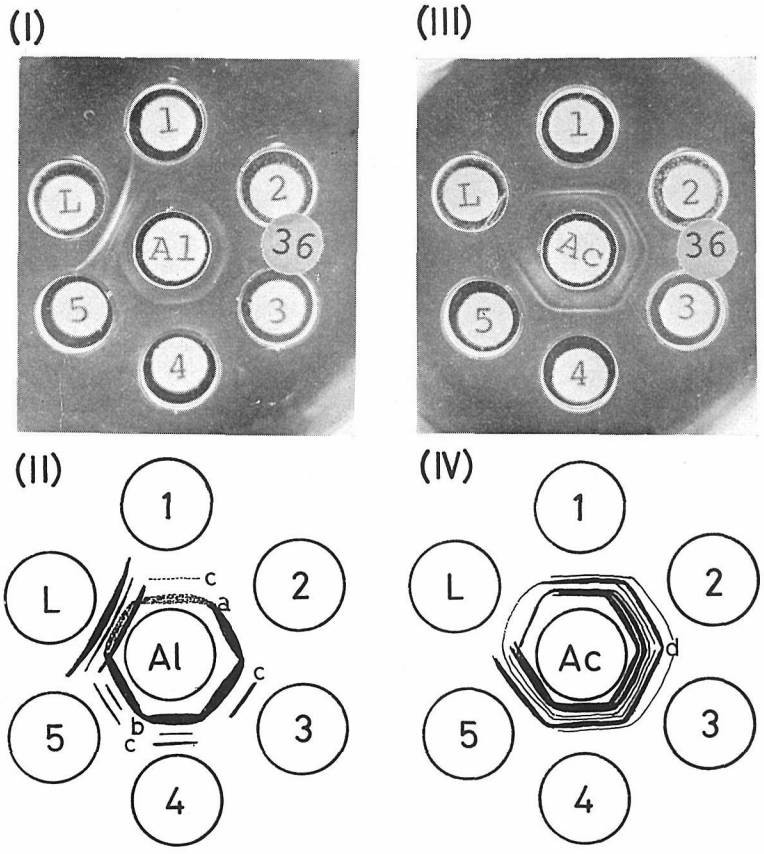

Fig. 5. (I) and (III), photographs: (II) and (IV), diagramatic representation of immunodiffusion patterns observed under the microscope. Fifty μl of antiserum was put into the central hole and 150 μg (as protein) of antigen into the outer holes. Agar phates were incubated for 36 hr at 25°C.
[Ac]; anti 2, 4–D callus serum, [Al]; anti leaf serum, [1]; antigen of 2, 4–D callus cultured for 15 days, [2]; antigen of kinetin callus cultured for 5 days, [3]; kinetin callus cultured for 10 days, [4]; kinetin callus cultured for 15 days, [5]; kinetin callus cultured for 32 days, [L]; antigen of tobacco leaves grown in a green house.

and more compact. Figure 5 shows results obtained with the double diffusion method and indicates an antigenic difference between 2, 4–D and kinetin callus. In kinetin callus, Fig. 5 (I) and (II), a specific precipitin line (b) appeared between [Al] and [4] and [5], which was not observed in 2, 4–D callus. The precipitin line (a) between [Al] and antigens of calluses were observed, but there was no line [Al] and [1] 12 hours after the beginning of diffusion (not shown in the figure). Line (a) between [Al] and [5] was the strongest, while line (a) between [Al] and [2] was the weakest at that time. As in Fig. 5 (III) and (IV), antigens of kinetin calluses were similar to those of 2, 4–D calluses. However, line (d) disappeared between [Ac] and [5]. These results show that some types of proteins specific to the 2, 4–D callus do not exist in the kinetin callus, and that new proteins, similar to leaf proteins, are synthesized. Furthermore, 2, 4–D and kinetin calluses retain some common protein constituents which seem differ considerably from those of the tobacco leaf.

These results also indicate that 2, 4–D and kinetin calluses retain common protein constituents. However, it appears that kinetin callus is synthesizing some characteristic proteins which cause the formation of, or become part of the leaves.

In the cultured tobacco cell line, Strain T5, these results show that cytokinins cause the induction of certain characteristic proteins, which begin to form leaves from cultured cells *in vitro*. Simultaneously, other protein syntheses are repressed by cytokinins for shoot formation. However, we do not yet know now cytokinins act and regulate the protein syntheses for organogenesis in cultured cells.

"Cell differentiation is based almost certainly on the regulation of gene activity".[25] If some genes are activated, RNA and protein syntheses may, subsequently, be induced or repressed. By adding a high concentration of cytokinin to cultured dedifferentiated cells, genes characteristic to a specific organ may be activated, and a factor specific to constantly dividing cells may be inactivated. The results of gene activation and inactivation would then appear in the protein constituents. Our results, showing that cytokinins induced the formation of some characteristic proteins and repressed the formation of others, are consistent with this hypothesis.

Evidence that cytokinin affects RNA synthesis has been reported by Roychoudhury *et al.*[26] and Matthysse *et al.*[27] Roychoudhury *et al.* reported that kinetin stimulates the synthesis and release of nuclear RNA in coconuts. Matthysse *et al.* showed that kinetin enhances the rate of RNA synthesis by isolated nuclei of tobacco and soybean calluses and by pea buds.

It would thus seem that cytokinins reacts with certain cell components to regulate gene activity, which is followed by the promotion of RNA synthesis and the effects on protein metabolism shown in our results. Thereafter, organ formation should occur morphologically. However, from our results on callus induction (dedifferentiation) from differentiated tissue by auxins,[28~31] the actions of cytokinins for redifferentiation seem to have conjugated metabolic relationships with auxin action. Dedifferentiation by auxins is the reverse reaction to redifferentiation by cytokinins.

We are investigating the triggering action of cytokinins for redifferentiation at present.

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REFERENCES

(1) F. Skoog and D. J. Armstrong, *Ann. Rev. Plant Physiol.*, **21**, 359 (1970).
(2) J. P. Jouanneau and C. Pésud-Leonöl, *Physiol. Plant.*, **20**, 834 (1967).
(3) J. P. Jouanneau, *ibid.*, **23**, 232 (1970).
(4) D. J. Osborne, *Plant Physiol.*, **37**, 595 (1962).
(5) G. J. VonAbrams and H. K. Pratt, *ibid.*, **43**, 1271 (1968).
(6) R. K. Atkin and B. I. S. Srivastava, *Physiol. Plant.*, **23**, 304 (1970).
(7) C. Martin and K. V. Thimann, *Plant physiol.*, **49**, 64 (1972).
(8) A. H. G. C. Rijven and V. Parkash, *ibid.*, **47**, 59 (1971).
(9) P. J. A. McCombs and R. K. Ralph, *Biochem. J.*, **129**, 403 (1972).
(10) F. Skoog and C. O. Miller, Symp. Soc. Exp. Biol., **11**, 118 (1957).
(11) Y. Yamada, K. Kiso, J. Sekiya and T. Yasuda, *Agr. Biol. Chem.*, **36**, 1055 (1971).
(12) E. M. Linsmaier and F. Skoog, *Physiol. Plant.*, **18**, 100 (1965).
(13) W. C. Schneider, *J. Biol. Chem.*, **161**, 293 (1945).
(14) K. Burton, *Biochem. J.*, **62**, 315 (1956).
(15) W. Mejbaum, *Z. Physiol. Chem.*, **258**, 117 (1939).
(16) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
(17) B. J. Davis, *Ann. N. Y. Acad. Sci.*, **121**, 404 (1964).
(18) R. Heitefuss, D. J. Buchanan-Davidson and M. A. Stahmann, *Arch. Biochem. Biophys.*, **85**, 200 (1959).
(19) D. H. Cambell, J. S. Garvey, N. E. Cremer and D. H. Sussdorf, "Methods in Immunology", W. A. Benjamin, New York, 1963, p. 22.
(20) O. Ouchterlony, *Acta Pathol. Microbiol. Scand.*, **32**, 231 (1953).
(21) J. P. Helgeson, S. M. Kruger and C. D. Upper, *Plant. Physiol.*, **44**, 193 (1969).
(22) U. Nudel and E. S. Bamberger, *ibid.*, **47**, 400 (1971).
(23) L. Bergmann, *Plant.*, **62**, 221 (1964).
(24) D. E. Fosket and J. G. Torrey, *Plant Physiol.*, **44**, 871 (1969).
(25) R. J. Britten and E. H. Davidson, *Science*, **165**, 349 (1969).
(26) R. Roychoudhury, A. Datta and S. P. Sen, *Biochim. Biophys. Acta*, **107**, 346 (1965).
(27) A. G. Matthysse and M. Abrams, *Biochim. Biophys. Acta*, **199**, 511 (1970).
(28) T. Yasuda dnd Y. Yamada, *Biochem. Biophys. Res. Comm.*, **40**, 649 (1970).
(29) Y. Yamada, T. Yasuda, M. Koge and J. Sekiya. *Collogues internationaux C. N. R. S.*, **193**, 137 (1971).
(30) Y. Yamada, and T. Yasuda, *Biochem. Biophys. Res. Comm.*, **43**, 488 (1971).
(31) T. Yasuda dnd Y. Yamada, *FEBS Lett.*, **18**, 115 (1971).