FACTORS AFFECTING GROWTH OF TOBACCO CALLUS TISSUE AND ITS INCORPORATION OF TYROSINE [1, 2]

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The elaboration of specific compounds for specific types of differentiation has been suggested to explain the production of roots, shoots, and flowers by plants. The search for compounds directly or indirectly influencing morphogenesis has produced only a few well defined substances such as indoleacetic acid, the gibberellins, and the kinins. Experiments using these compounds in tissue cultures have revealed first that the effect of the compound is not only dependent on its concentration in the medium, but also is dependent on the concentrations of other compounds present (15, 19, 22), and second that these growth regulating compounds influence a wide variety of anatomical (19), physiological (15), and biochemical (6, 16, 17, 18) responses of the cultured tissue. One of the most interesting systems described (19) is that in which the production of buds or shoots by tobacco callus tissue in culture is influenced by the concentration of the four factors: indoleacetic acid, kinetin, phosphate, and tyrosine. The present paper reports an investigation of the growth of tobacco callus cultures and of the fate of C^{14}-labeled tyrosine in this system.

METHODS

TISSUE CULTURES: Callus tissue was obtained from Dr. F. Skoog. Cultures were maintained on the modified White's basal medium described by Skoog and Tsui (20) supplemented by increasing the KH_2PO_4 to 62.5 mg/l and by adding indoleacetic acid (2.0 mg/l) and kinetin (0.2 mg/l). The medium was adjusted to pH 5.4, then autoclaved at 120° C for 15 minutes. Cultures were maintained in a liquid medium in 20 × 150 mm Pyrex tubes. Each tube contained 15 ml of medium, and was closed by an aluminum cap. One piece of tissue in each tube was supported at the liquid surface (8) by a folded 90 mm circle of Whatman's No. 42 filter paper.

When solidified media were desired, 1 % agar was incorporated into the liquid media; 50 ml portions were used in 125 ml Erlenmeyer flasks. The flasks were closed with cotton plugs; three pieces of callus tissue were placed on the agar in each flask.

EFFECTS OF NUTRIENTS AND GROWTH FACTORS ON TISSUE: These experiments were a randomized complete block with ten replicates. Concentrations of tyrosine, indoleacetic acid, kinetin, and phosphate were varied. The change in fresh weight was followed by weighing each piece aseptically during the course of the experiment. Infection was judged only by visual inspections. The appearance of buds and shoots during the experiment also was recorded. At the termination, the total nitrogen in the tissue pieces of five of the ten replicates was determined; the tissue pieces were digested (9) and ammonia in aliquots of the final distillate was estimated by the method of Moore and Stein (14).

FRACTIONATION OF TISSUE: Tissue pieces were blotted dry, weighed, and homogenized in 3 to 10 volumes of chosen buffer with a TenBroeck homogenizer. The fraction designated "cell walls" was recovered either by filtration through a fritted disk Pyrex funnel (porosity M, Corning Glass Works) or by centrifuging at 1,000 × G for 10 minutes. After cell walls were washed two or three times with the homogenizing medium the washings were combined with the supernatant.

In experiments where cell walls were extracted with solvents, the cell wall residue was recovered and washed with the solvent in the manner described for the cell walls.

The fraction designated "soluble protein" was prepared from combined washings and supernatant from the cell walls preparation by adding three volumes of ethanol and heating the solution to boiling. After cooling, the denatured soluble protein was collected by centrifugation at 5 to 10,000 × G for 15 minutes; the pellet then was washed two or three times in the centrifuge tube with 75 % ethanol.

The combined alcoholic supernatant and washings were concentrated in a climbing film vacuum evaporator to give the fraction designated soluble amino acids. When desired, this fraction was desalted by the method of Buchanan (7) using columns containing 5 ml of settled Dowex 50 X4 (H^+ form).

HYDROLYSIS OF FRACTIONS: To liberate the amino acids, fractions were hydrolyzed in redistilled constant boiling hydrochloric acid (approximately 1 ml/g fresh tissue) in sealed tubes at 120° C for 6 hours. Excess acid was removed by evaporating in a vacuum desiccator over flake NaOH; the humin was removed from samples for quantitative analysis by filtering aqueous extracts of the hydrolysate through fritted disk funnels (porosity F, Corning Glass Works). Filtrate and washings were combined and evaporated to dryness.

Samples of the soluble amino acids for quantitative analysis were hydrolyzed at 120° C for 15 minutes in 2 *N* hydrochloric acid to convert glutamine and as-

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paragine quantitatively into ammonia and the corresponding dicarboxylic amino acids.

PAPER CHROMATOGRAPHY: Samples on Whatman's No. 1 chromatography paper were subjected to descending chromatography using *n*-butanol-acetic acid-water (4:1:5 v/v) (24) in the first dimension and followed by phenol-buffer (5:1 w/v; the aqueous buffer consisted of 6.3 % sodium citrate, 3.7 % KH_2PO_4, and 0.5 % ascorbic acid) (3). The amino acids were revealed by spraying the air dried papers with a solution of 0.5 g ninhydrin in 250 ml *n*-butanol and 12.5 ml 2 *N* acetic acid when the phenol solvent was used, and with 0.16 g ninhydrin in a solution of 0.16 ml collidine in 100 ml *n*-butanol saturated with water if only the butanol-acetic acid solvent was used.

The hydrolysates were co-chromatographed with authentic samples of amino acids to establish the identity of the compounds revealed.

QUANTITATIVE ANALYSIS OF AMINO ACIDS: The method of Moore, Spackman, and Stein (13) was used with minor modification. Dowex 50 X8 (−400 mesh) was used in the 150 cm column and gave satisfactory resolution of tyrosine and phenylalanine only when operated at 50° C with the pH 3.18 buffer until 320 ml of effluent had been collected, and then at 60° C and pH 4.25 until a further 360 ml of effluent had been collected. The 15 cm columns were operated as described (13) for the estimation of basic amino acids in protein hydrolysates and the 50 cm column as described by Spackman, Stein, and Moore (21) for the chromatography of the soluble amino acids. The effluent was collected in 2.0 ml fractions; 1.0 ml samples were analyzed for amino acids by the method of Moore and Stein (14). The combined color yield and recovery from the columns for each amino acid was estimated using the standard mixture of amino acids prepared by Spinco Division, Beckman Instruments Inc., supplemented by samples of other amino acids (California Corporation for Biochemical Research) where necessary.

Hydroxyproline, which does not resolve completely from aspartic acid when chromatographed as described above, was estimated on the combined fractions from the aspartic acid region by re-chromatography at 30° instead of 50° C; the resolution is virtually complete at 30°. Hydroxyproline gives a yellow color with ninhydrin and was estimated by measuring optical densities at 440 mμ, whereas the optical density of the blue color from aspartic acid was measured at 570 mμ. Corrections in color yield were made for any small areas of overlap between peaks.

After chromatography of aliquots of the soluble amino acids on 150 cm columns, the remainders of the fractions from each peak were combined and desalted (7). Paper chromatography with authentic specimens validated the peaks assigned the identification of aspartic acid, threonine, serine, glutamic acid, glycine, alanine, and tyrosine.

DETERMINATION OF RADIOACTIVITY: All samples counted were prepared in the 0.2 *N* buffers used to elute the 150 cm columns. Aliquots of these solutions were mixed with an equal volume of 2 % gelatin in 0.3 *N* (NaOH) and 1.0 ml of the resulting solution was dried on a copper planchet 1.0 inch in diameter. All samples were counted with the same counter for a minimum period of five minutes, and the results were expressed as counts/minute/ml corrected for coincidence loss where necessary and for background.

The specific activity of the amino acids was obtained by plotting the concentration of amino acid in each of the effluent fractions against the radioactivity in counts/minute/ml for the corresponding fraction. The slope, and the standard deviation, of the best line through the resulting points was calculated by the method of least squares assuming that concentration is the more accurate estimate. The slope of the best line represents the specific activity of the amino acid and the units used herein are counts/minute (corrected)/μmole of amino acid.

PREPARATION OF TYROSINE-C^{14} (BIOSYNTHESIZED): Chlorella pyrenoidosa was grown in a medium (McBride, L. private communication, 1958) containing/1: NH_4NO_3, 2.0 g; $MgSO_4 \cdot 7\ H_2O$, 0.308 g; KH_2PO_4, 1.96 g; K_2HPO_4, 3.43 g; $CaCl_2 \cdot 2\ H_2O$, 0.073 g; $KHCO_3$, 1.25 g. Iron was provided in the ferric form at 5 ppm and chelated by EDTA; the micro elements were provided by 0.5 ml/l of Hoagland's Solution A5 (Higher plant strength diluted 1/25).

The phosphates were adjusted to pH 6.2, and the remaining nutrients without $KHCO_3$ were autoclaved separately at 120° C for 30 minutes. The $KHCO_3$ was saturated with CO_2, filtered through a Morton bacterial filter and added aseptically to the medium.

To biosynthesize tyrosine, the algae were grown in four liters of medium containing 2.5 mc C^{14}/1 as $KHCO_3$. Growth was allowed to continue until complete, and the algae were collected by centrifugation, and hydrolyzed with acid. The hydrolysate was chromatographed on Dowex 1 X10 (50 × 2 cm column) (2, 10) and eluted with 0.1 *N* acetic acid. Fractions from 82 to 112 ml of effluent contained tyrosine and yielded 9.3 mg of radiochemically pure tyrosine, as shown by paper chromatography. Tyrosine was chromatographed on Dowex 50 X8 (150 × 1 cm); its specific activity was $106.5 \pm 2.8 \times 10^3$ cpm/μM.

INCORPORATION OF TYROSINE-C^{14} INTO CULTURED TISSUE: The medium used in all the experiments with radioactive tyrosine had the same composition as that in which the tissue had been maintained except for the addition of tyrosine at 100 mg/l.

When uptake of tyrosine by cultured tissue was to be followed, slices of callus were cut and put into a sterile medium containing DL-tyrosine-2-C^{14}. The suspensions were shaken at room temperature, sampled aseptically at intervals, and the residual radioactivity was measured under the standardized conditions.

Biosynthesized tyrosine was used when the distribution of radioactivity among fractions of the tissue

was studied. For this experiment two 125 ml respirometer vessels with alkali wells were used. Each vessel contained 25 ml of medium, about 8 g of tissue, and tyrosine yielding 2.2×10^6 cpm; they were shaken for 24 hours at a temperature of 24 to 26° C. The respired carbon dioxide was trapped in 0.5 ml of 2 *N* sodium hydroxide in the well. The sodium hydroxide recovered at the end of the experiment was made to a standard volume, and counted.

After shaking for 24 hours, the medium from each flask was sampled aseptically. It then was diluted 1/10 with medium and 0.5 ml plated on tryptone-agar medium and incubated. No colonies appeared on any of the plates. The tissue recovered by filtration was fractionated. Radioactivity of each of the fractions was determined.

Results

Growth of Cultured Tissues: Two experiments employed various concentrations of phosphate, indoleacetic acid, kinetin, and tyrosine in the modified White's basal medium. Concentrations (mg/l) and times of weighing tissues were:

	Exp. XV	Exp. XVIII
KH_2PO_4	62.5, 250.0	250.0
Indoleacetic acid	1.0, 2.0	1.0, 2.0, 4.0
Kinetin	0.2, 0.4, 1.0	0.2, 1.0
Tyrosine	0, 200.0	0, 25.0, 100.0
Times of weighing (weeks)	0, 2, 4, 8	0, 2, 6

Summarized results for experiment XV are given in table I and for experiment XVIII are given in table II. Table III summarizes the two experiments.

Table I

Experiment XV. Growth of Tobacco Callus Tissue in Culture*

		$T_{0.0}$		$T_{200.0}$	
		$P_{62.5}$	$P_{250.0}$	$P_{62.5}$	$P_{250.0}$
$K_{0.2}$	$I_{1.0}$	2.38	2.83	1.96	1.80
	$I_{2.0}$	2.64	2.47	1.91	2.00
$K_{0.4}$	$I_{1.0}$	2.18	2.30	1.93	1.65
	$I_{2.0}$	1.85	2.14	1.98	1.95
$K_{1.0}$	$I_{1.0}$	1.87	1.91	1.79	1.80
	$I_{2.0}$	1.64	1.86	1.65	1.90

T = tyrosine; P = KH_2PO_4; subscript = mg/l; K = kinetin; I = indoleacetic acid.

Coefficient of variation 18 %.

*Ratio of final to initial weight in each treatment after 2 weeks of growth. (Average of 10 replicates).

Treatments giving differences significant at 1 % were kinetin, tyrosine, and kinetin-tyrosine interaction; and at 5 % was the indoleacetic acid-tyrosine interaction.

At 4 weeks, tyrosine-phosphate and indoleacetic acid-tyrosine-phosphate interactions were significant at 5 %, and after 8 weeks of culture the former had become significant at 1 %.

Table II

Experiment XVIII. Growth of Tobacco Callus Tissue in Culture*

		$T_{0.0}$	$T_{25.0}$	$T_{100.0}$
$I_{1.0}$	$K_{0.2}$	2.08	2.26	2.21
	$K_{1.0}$	1.96	2.16	2.31
$I_{2.0}$	$K_{0.2}$	1.94	2.24	2.34
	$K_{1.0}$	1.67	2.00	2.17
$I_{4.0}$	$K_{0.2}$	1.94	1.85	2.12
	$K_{1.0}$	1.72	1.78	2.13

Symbols as in table I. Coefficient of variation 14 %.

* Ratio of final to initial weight in each treatment after 2 weeks growth. (Average of 10 replicates).

Treatments giving differences significant at 1 % were indoleacetic acid, tyrosine, and kinetin; and none were significant at 5 %.

The effect of indoleacetic acid could only be demonstrated between 2.0 mg/l and 4.0 mg/l (significant at 1 %). After 6 weeks growth, the interactions kinetin-tyrosine and kinetin-tyrosine-indoleacetic acid gave differences significant at 5 %.

Table IV summarizes the results of total nitrogen determinations on the tissue pieces from experiment XV after 11 weeks of culture.

Detailed analysis of variance showed that the absolute growth of the tissue in experiment XV was different from that in experiment XVIII. Analysis also revealed, however, (table III) that there was no significant difference due to an interaction between the variable experiment and the levels of indoleacetic acid or the levels of kinetin used. This shows that, although the growth of the tissue differed in the two experiments, the relative effects of the two levels of each growth substance were the same in each case. Therefore, the results of the two experiments can be combined to give the following conclusions:

I. Kinetin at concentrations above 0.2 mg/l suppresses the fresh weight gain of the tissue.

II. Indoleacetic acid gives the maximum increase in fresh weight at concentrations between 1.0 and 2.0 mg/l, whereas 4.0 mg/l is definitely inhibitory.

III. At concentrations up to 100 mg/l, tyrosine increases the fresh weight gain of the tissue, but at 200 mg/l the amino acid inhibits the weight increase.

IV. Whether 62.5 or 250 mg/l of KH_2PO_4 is supplied has little influence on the fresh weight of the tissue.

Although variation in levels of the growth hormones and tyrosine produced marked effects on the growth of the tissue in terms of fresh weight and total nitrogen, the number of tissue pieces in any one treatment which produced buds or shoots never exceeded two.

The production of buds or shoots by cultured tissue was compared in the presence and absence of 1 % agar. White's basal medium was supplemented with 250 mg/l KH_2PO_4 and 1.0 mg/l indoleacetic acid. Combinations of two levels of kinetin (0.2 and 1.0

TABLE III

COMPARISON OF GROWTH OF TOBACCO CALLUS TISSUE IN TWO EXPERIMENTS*

		$I_{1.0}$	$I_{2.0}$
Experiment XV	$K_{0.2}$	2.83	2.47
	$K_{1.0}$	1.91	1.86
Experiment XVIII	$K_{0.2}$	2.08	1.94
	$K_{1.0}$	1.96	1.67

*Ratio of final to initial weight in each treatment after 2 weeks growth. (Average of 10 replicates).

Symbols as in table I. Medium—White's basal medium + KH_2PO_4 250 mg/l and no tyrosine.

Treatments giving differences significant at 1 % was kinetin; and at 5 % were indoleacetic acid and "experiment".

There were no significant differences in the interactions of the three variables.

mg/l) and two levels of tyrosine (0.0 and 200.0 mg/l) were used; cultures were maintained in darkness. After 63 days, no tissue grown in the absence of tyrosine had produced buds or shoots. Tissue grown in liquid medium containing tyrosine had not produced buds or shoots. Tissue pieces cultured on 1 % agar however, had produced buds or shoots. On 0.2 mg/l kinetin and 200 mg/l tyrosine, 1/15 of the tissue pieces produced buds or shoots in 45 days and 5/15 in 63 days, whereas with 1.0 mg/l kinetin and 200 mg/l tyrosine 11/15 of the tissue pieces had produced buds or shoots in 45 days and 15/15 in 63 days.

FRACTIONATION OF TISSUE: Fractionation of callus tissue and determination of the nitrogen in each fraction gave the following distribution: cell walls, 27 to 42 %; soluble protein, 11 to 21 %; soluble amino acids, 44 to 56 % of the total nitrogen. Amount of nitrogen in the cell wall fraction was influenced little with homogenization in the following media: water, 50 % ethanol, sodium borate (1.1 g/l) at pH 9.2 (12), M/15 phosphate at pH 6.00, 7.00, or 7.95, or 20 % glucose at pH 7.0. Likewise, the cell-wall nitrogen was affected little with any of the above solvents or with M/15 phosphate at pH 7.0 containing 10^{-3} M EDTA, 90 % phenol, 6 M urea, formamide, 0.1 M EDTA, 0.1 *N* sodium hydroxide, or 0.1 *N* hydrochloric acid at room temperature, or formamide at 100° C for 1 hour. When the cell walls were heated, however, at 100° C for 1 hour with 0.1 *N* NaOH or 90 % formic acid, then part of the wall material dissolved and the amount of nitrogen bound to the treated cell walls decreased. When the time of heating was doubled, there was no marked decrease in the amount of nitrogen remaining bound to the cell walls.

The amount of material extracted from the cell walls with 90 % formic acid at 100° C for an hour decreased markedly with successive treatments, as demonstrated by hydrolysis of the extracts and separation of the liberated amino acids by paper chromatography. The removal of nitrogenous material from the cell wall by formic acid was not due to liberation of free amino acids.

The nitrogen content and yield of residue after extraction of cell walls with 0.1 *N* NaOH or 90 % formic acid at 100° C for 1 hour suggested that the formic acid had extracted more non-nitrogenous material from the cell walls than had NaOH. This extraction of non-nitrogenous material could not be reduced by the ethanol precipitation procedure of Albanese (1).

When formic acid extracted cell walls which contained 18 mg N/g, were extracted with 0.1 *N* NaOH at 100° C for 1 hour, the nitrogen content decreased to 14.5 mg N/g dry weight. This residue contained hydroxyproline, as did a precipitate obtained by adjusting the NaOH extract to pH 4.5 with acetic acid, adding three volumes of ethanol and boiling. The supernatant after removal of this precipitate contained 43 % of the total nitrogen in the formic acid extracted cell walls used. This may be explained by hydrolysis of protein, but the small decrease in the nitrogen content of the formic acid extracted cell walls by NaOH extraction requires that a non-specific solubilization of the cell walls has also taken place. In most of the subsequent experiments, formic acid was used to extract cell walls. These extractions gave four fractions from the tissue; soluble amino acids, soluble protein, formic acid soluble cell wall material and cell wall residue.

The presence of amino acids in hydrolysates of the cell walls, formic acid soluble cell wall material and the cell wall residue was demonstrated by paper chromatography and the results are given in table V. Hydrolysates of the cell walls and the cell wall residue gave spots which co-chromatographed with hydroxyproline, whereas this spot was absent from chromatograms of the hydrolyzed formic acid soluble cell wall material.

Cell walls were extracted at 100° C for 1 hour with 0.1 *N* sodium hydroxide and then washed free

TABLE IV

TOTAL NITROGEN IN TOBACCO CALLUS TISSUE CULTURES GROWN FOR ELEVEN WEEKS*

		T_0		T_{100}	
		$P_{62.5}$	P_{250}	$P_{62.5}$	P_{250}
$K_{0.2}$	$I_{1.0}$	2.38	3.19	3.48	2.98
	$I_{2.0}$	3.36	3.48	3.52	4.36
$K_{0.4}$	$I_{1.0}$	2.67	3.49	3.17	1.92
	$I_{2.0}$	2.43	2.70	3.64	3.27
$K_{1.0}$	$I_{1.0}$	1.80	2.59	2.41	2.55
	$I_{2.0}$	1.81	2.01	3.40	2.78

* Average of five replicates; values corrected to an initial weight of 100 mg. Data as mg N. Tissues were those used in experiment XV, table I.

Symbols as in table I. Coefficient of variation 36 %.

Treatments giving differences significant at 1 % was kinetin; and at 5 % were tyrosine and indoleacetic acid-tyrosine interaction.

TABLE V

AMINO ACIDS OBSERVED ON PAPER CHROMATOGRAMS OF SOLUBLE AMINO ACID EXTRACT AND HYDROLYSATES FROM VARIOUS FRACTIONS OF CALLUS CELLS

AMINO ACID	CELL WALLS	CELL WALL MATERIAL SOLUBLE IN FORMIC ACID	CELL WALL RESIDUE	SOLUBLE PROTEINS	SOLUBLE AMINO ACIDS*
Alanine	+	+	+	+	+
β-Alanine					+
γ-Aminobutyric ac.					+
Arginine	+	+	+	+	+
Aspartic ac.	+	+	+	+	+
Asparagine					+
Glutamic ac.	+	+	+	+	+
Glutamine					+
Glycine	+	+	+	+	+
Hydroxyproline	+		+		
Lysine					+
Ornithine					+
Proline	+	+	+		
Serine	+	+	+	+	+
Threonine	+	+	+	+	+
Tyrosine	+	+	+	+	+**
Leucine + isoleucine + phenylalanine	+	+	+	+	
Lysine + histidine + ornithine	+	+	+	+	
Valine + methionine	+	+	+	+	

* Desalted by the method of Buchanan (7); unlike the other fractions, this material was not hydrolyzed.
** Observed when tyrosine was supplied in the medium.

of alkali with water. The residue was autoclaved in water at 120° C overnight and the nitrogen in the extract and the residue estimated. The extract contained 0.83 mg and the residue 6.4 mg nitrogen.

CELL WALLS OF TOBACCO PITH CELLS: Pith tissue was removed from stems of tobacco plants (var. Wisconsin No. 38) as described by Bryan and Newcomb (6). The cell walls from this material and from callus tissue cultured for 4 weeks were isolated in M/15 phosphate at pH 7.0 and washed six times with the medium. The nitrogen in the cell walls expressed as a percentage of the total nitrogen was 2.3 % for the pith and 30 % for the callus tissue. When the cell walls were isolated by washing three times with the phosphate buffer used for the homogenization and then three times with 0.1 *N* NaOH at room temperatures, the nitrogen content was 1.7 % for the pith and 21 % for the callus tissue.

INCORPORATION OF RADIOACTIVE TYROSINE INTO TISSUE: When the uptake of radioactive tyrosine

TABLE VI

DISTRIBUTION OF RADIOACTIVITY BETWEEN FRACTIONS OF TOBACCO TISSUE*

	EXPERIMENT 1		EXPERIMENT 2	
Tissue weight (mg)	8,120		740	
Initial radioactivity (total cpm × 10^{-4})	220		220	
Fraction	*cpm* × 10^{-4}	*% Initial*	*cpm* × 10^{-4}	*% Initial*
Respired carbon dioxide	8.2	3.7	7.6	3.5
External medium	9.0	4.1	10.7	4.9
Soluble amino acids	101	45	102	45
Soluble proteins (by difference)	10	4.5	15.5	7.0
Formic acid soluble cell wall material	23	10.5	23	10.4
Cell wall residue	38.0	17.3	19	8.7
Total	189	85	178	80

* Cultured for 24 hours in the presence of labeled tyrosine and a complete growth medium.

was followed, it was found that the radioactivity in the medium described a curve of decreasing slope, so that approximately two-thirds of the radioactivity had disappeared after 24 hours. Therefore, slices of tissue were kept in the tissue culture medium with biosynthesized tyrosine for 24 hours and then fractionated. The results of the measurements of radioactivity in the fractions from the duplicate experiments are shown in table VI.

The external medium and the soluble amino acids were desalted according to Buchanan (7); their C^{14} distribution is shown in part A of table VII. The soluble proteins, formic acid soluble cell wall material, and a portion of the cell wall residue were hydrolyzed in constant boiling hydrochloric acid for 6 hours at 120° C. The radioactivity and free amino acids were estimated after removal of the excess acid and of the humin; these results are shown in part B of table VII.

The radioactivity and quantity of each amino acid in the hydrolysates of the soluble amino acids, soluble proteins, formic acid soluble cell wall material, and the cell wall residue are shown in tables VIII and IX. The only identified amino acid which was radioactive was tyrosine, although in the soluble amino acid fraction a second small peak of radioactivity appeared between glycine and alanine. The elution curve of the radioactivity of this compound was not exactly coincident with the elution curve for the alanine and the identity of the compound was not pursued.

Discussion

In the experiments described here concerning the growth of the tobacco callus tissue in culture, the interaction of several growth factors in influencing the growth of the tissue was demonstrated. The growth of the tissue either in terms of fresh weight or total nitrogen was influenced not only by the concentration of kinetin and tyrosine in the medium, but also by the combined effects of tyrosine with indoleacetic acid, with kinetin and with phosphate. The results of these experiments differ importantly from those described by Skoog and Miller (19), in that the production of buds or shoots was negligible. It was shown that growth of the callus on a medium containing 1 % agar led to the extensive production of buds or shoots by all callus pieces, whereas tissue pieces supported in the meniscus of the same medium in the absence of agar produced very few buds or shoots. No reason was found for this difference, and our studies on the incorporation of tyrosine into tissues were made with liquid media.

The first step in investigating tyrosine metabolism was to measure the specific activity of all the amino acids both free and bound in the tissue after feeding C^{14}-labeled tyrosine. The known partial destruction of amino acids when proteins are hydrolyzed in the presence of carbohydrate and the possibility that tyrosine would act as a precursor of the lignin of the cell wall (4) led to attempts to separate the proteinaceous material of the cell from insoluble materials which contained no protein. A variety of solvents failed to yield a nitrogen free insoluble residue. The final fractionation procedure used gave, in addition to the soluble amino acids, three protein-containing fractions which differed in their solubility properties. These have been called soluble proteins, formic acid soluble cell wall material, and the cell wall residue.

After acid hydrolysis and separation by ion exchange chromatography (13, 21), the quantity and radioactivity of every amino acid in each of the hy-

Table VII

Distribution of C^{14} Among Cell Fractions and Hydrolysates of Fractions Recovered from Tobacco Callus Tissue Supplied C^{14}-Labeled Tyrosine

	Experiment 1			Experiment 2		
	cpm × 10^{-4}	Recovery of C^{14}	μM*	cpm × 10^{-4}	Recovery of C^{14}	μM*
A. C^{14} in fractions separated on Dowex 50 (H^+)						
External medium						
Material not retained	3.0	33**		3.1	29**	
Amino acids	5.2	58	1.3	5.6	52	1.2
Recovery	8.2	91		8.7	81	
Soluble amino acids						
Material not retained	3.6	3.6		3.6	3.6	
Material displaced prior to amino acids	11.6	11.5		10.8	10.6	
Amino acids after hydrolysis	67.9	67.2	70	64.0	62.7	54
Recovery	82.1	82.0		78.4	76.9	
B. C^{14} in hydrolysates from cell fractions						
Solube proteins	2.3	23***	49	2.8	17***	49
Formic acid soluble cell wall material	12.4	54	243	12.2	53	290
Cell wall residue†	7.2	19	71	6.7	35	59

* μm of α-amino nitrogen determined by ninhydron (leucine standard).

** Recovery in each fraction as percentage of total radioactivity added to column.

*** Recovery as percentage C^{14} in each fraction relative to the C^{14} of entire tissue.

† Corrected to total cell wall residue.

TABLE VIII

RADIOACTIVITY OF TYROSINE IN FRACTIONS FROM TISSUES SUPPLIED C^{14}-LABELED TYROSINE*

	EXPERIMENT 1			EXPERIMENT 2		
	CPM/μMOLE × 10^{-4}	CPM-TOTAL × 10^{-4}	%**	CPM/μMOLE × 10^{-4}	CPM-TOTAL × 10^{-4}	%**
Original tyrosine	106.5 ± 2.8	220		106.5 ± 2.8	220	
Soluble amino acid-tyrosine	91.0 ± 11.9	32.1	32	118 ± 3.8	46	45
peak near alanine		0.34	0.34		0.41	0.40
Soluble protein-tyrosine	11.3 ± 0.88	1.2	12	11.1 ± 0.73	0.86	5.4
Formic acid soluble cell wall material-tyrosine	9.64 ± 0.24	3.7	16	10.0 ± 0.82	4.5	22
Cell wall residue-tyrosine	2.00 ± 0.21	0.45	1.2	2.10 ± 0.23	0.35	1.8
Total		38			52	
Total (% original cpm)		17			24	

* Standard deviations calculated by the method of least squares.
** % = cpm in amino acid as a percentage of the cpm in the fraction from which it was isolated.

drolyzed tissue fractions was estimated. Two of the fractions, the soluble proteins and the formic acid soluble cell wall material, differed little from each other in their amino acid composition (table IX) and rate of incorporation of tyrosine (table VIII). The cell wall residue appeared to represent a distinct entity, for it differed markedly from the other two proteinaceous fractions not only in having a lower rate of incorporation of tyrosine, but also in its amino acid composition and exclusive possession of hydroxyproline. The localization of hydroxyproline in this fraction is of interest, as Steward, Thompson, and Pollard (23) concluded that high levels of hydroxyproline in plant tissues are associated with high levels of metabolic activity. The contrasting high levels of hydroxyproline and the low rate of incorporation of

TABLE IX

AMINO ACID COMPOSITION OF TISSUE AND ITS FRACTIONS AFTER 24 HOURS IN PRESENCE OF TYROSINE-C^{14} (BIOSYNTHESIZED)**

	TISSUE AMINO ACIDS		SOLUBLE AMINO ACIDS		SOLUBLE PROTEIN		FORMIC ACID SOL.		CELL WALL RESIDUE	
	FREE	BOUND	1	2	1	2	1	2	1	2
Hydroxy proline	...	1.97	...	...	...	...	...	...	18.1	26.8
Aspartic acid	0.63*	3.27	8.2*	7.4*	8.6	8.5	7.5	7.5	4.9	4.6
Threonine	0.13	2.10	1.7	1.5	5.0	5.1	4.2	4.3	5.4	5.2
Serine	0.27	2.84	3.4	3.4	6.1	6.0	5.5	5.4	9.2	8.8
Glutamic acid	2.45*	3.66	31.6*	28.9*	9.9	10.1	8.6	8.8	3.8	3.7
Proline	0.02	2.83	0.5	...	5.8	4.8	6.3	6.4	6.1	6.7
Glycine	0.19	3.09	2.2	2.5	8.3	7.9	7.0	7.1	4.8	4.2
Alanine	0.39	2.95	4.9	4.7	8.6	8.1	6.8	6.8	4.0	3.6
Valine	0.11	2.65	1.2	1.7	7.0	7.3	5.3	5.3	6.5	6.2
Methionine	0.05	0.23	...	1.4	1.2	...	0.7	0.4	0.4	0.5
iso-Leucine	0.18	1.62	1.8	2.6	4.4	4.7	3.6	3.8	2.0	2.2
Leucine	0.09	2.70	1.3	0.9	7.7	7.6	6.1	6.5	3.3	3.2
Tyrosine	0.47	0.95	5.1	6.5	2.2	2.6	1.8	1.9	2.9	2.4
Phenylalanine	0.03	1.25	0.5	0.4	3.9	3.4	3.0	2.9	1.4	1.1
β-Alanine	0.11	...	1.8	1.1	...	...	...	...	...	...
Lysine	0.09	3.14	1.1	1.1	5.7	5.4	6.4	5.9	9.1	9.9
Histidine	...	0.87	...	...	1.4	1.4	1.7	1.9	2.3	2.7
Ammonia	2.23*	8.39	26.5*	28.3*	10.8	13.8	22.2	21.1	13.9	6.7
Arginine	...	1.48	...	...	3.4	3.5	3.4	3.7	2.1	1.4
γ-Amino butyric acid	0.67	...	8.4	8.0	...	...	...	...	...	...
% Recovery of ninhydrin color on chromatography			96-100	102-106	90	78	86	81	78	70

* These values represent the sum of the free compound and that present as the amide.
** Free and bound amino acids are expressed in μm/g fresh weight (average of exp. 1 and 2). Amino acids of the fractions are expressed as moles % of the amino acids recovered.

tyrosine into the cell wall residue suggests that their generalization cannot be extended to the protein fractions of tissues.

The protein fractions which were obtained in these experiments have different metabolic activities as indicated by the rates of replacement of tyrosine. These rates suggest that the most active fraction is the soluble protein, followed by the formic acid soluble cell wall material, and that the least active is the cell wall residue. Together the last two fractions, when isolated as cell walls from callus tissue, contained approximately 25 % of the total nitrogen, whereas cell walls from tobacco pith, which is regarded as nonproliferating, contained only 2 % of the total nitrogen. This difference between rapidly growing and nonproliferating tissues suggests that the cell walls fraction is important under conditions of high metabolic activity. Its primary function is not necessarily in intermediary metabolism but may be either in a storage or a structural role, for although the soluble proteins had the highest rate of replacement, they accumulated only 0.019 μM of tyrosine, while the combined formic acid soluble cell wall material and cell wall residue accumulated 0.065 μM of radioactive tyrosine in 24 hours.

In the preceding discussion the fate of tyrosine has been considered only in terms of its incorporation into proteins. However, in the analysis of the cell wall residue, the recovery of radioactivity in the liberated tyrosine (table VIII) was only 1.2 to 1.8 % of the radioactivity of the whole fraction. The recovery of radioactivity was 98 to 98.5 % when an authentic sample of radioactive tyrosine was chromatographed. The small percentage of the radioactivity of the fraction which is present as intact tyrosine residues would be consistent with the incorporation of tyrosine into the lignin of the cell wall. The incorporation of tyrosine into lignin has been observed by Brown and Neish (4), and it also has been shown that *p*-hydroxyphenylpyruvic acid is a precursor of the tyrosine in the cell wall protein, and simultaneously of the lignin of the cell wall (5) We hope to be able to report later our examination of the lignin of the cell wall residue and the extent to which tyrosine is incorporated.

The localization of hydroxyproline in the cell wall residue is of interest, for its presence in a protein has been thought to be characteristic of the collagen group of structural proteins (11), although some members of the keratin group contain hydroxyproline (3). Evidence against a similarity of the cell wall residue protein to collagen is its low levels of glycine and alanine, the presence of aromatic amino acids in the protein, and the fact that the residue cannot be converted into a soluble gelatin either in alkali at 100° C or in water at 120° C (11).

The amino acid composition of the cell wall residue differed from that of the other fractions in having lower levels of the dicarboxylic amino acids, glycine, alanine, valine, *iso*-leucine, leucine, and arginine, and higher levels of serine and lysine. In addition to these differences the molar ratio of tyrosine to phenylalanine in the cell wall residue was greater than one, whereas it was less than one in the other protein-containing fractions.

When the amino acid compositions of the three fractions are considered as a whole, the only outstanding features are the absence of cystine and the low and variable amounts of methionine present.

The specific activity data show that 12.5 % of the tyrosine in the soluble protein was replaced in 24 hours. Corresponding figures for the replacement of tyrosine in the formic acid soluble cell wall material and the cell wall residue were 10.5 % and 2.2 %, respectively. The specific activity of the free tyrosine shows that the tyrosine originally present was mixed with approximately six times as much added radioactive tyrosine during the course of the experiment. This large uptake of tyrosine by the tissue is also indicated by the low level of radioactivity in the external medium after 24 hours, and by the fact that the concentration of tyrosine in the tissue (0.43-0.51 μM/g fresh wt) is very close to the original concentration (0.55 μM/ml) in the medium. The radioactive tyrosine was provided to the tissue at such a high level that the amino acid pool of the tissue was overloaded, and thus the quantitative results presented here represent a response to an abnormally high level of tyrosine in the tissue. Qualitatively, the lack of appearance of carbon 14 in any amino acid but tyrosine shows that tyrosine is not an intermediate but an end product in amino acid biosynthesis.

Summary

The effect of varied concentrations of indoleacetic acid, kinetin, phosphate, and tyrosine on the growth and differentiation of tobacco callus tissue cultured in a liquid medium has been studied. The growth rate was influenced not only by the concentration, but also by the relative levels of each substance.

It was observed that callus tissue produced buds and shoots when grown on a medium containing agar, and it produced only callus when supported at the surface of a liquid medium of the same composition.

After fractionation of the callus tissue, 25 to 40 % of the total cell nitrogen remained associated with the insoluble cell wall fraction. This fraction was separated further by extraction with formic acid into two fractions which differed in amino acid composition, rate of incorporation of tyrosine, and in the presence or absence of hydroxyproline. The significance of these results is discussed in terms of tissue growth.

The incorporation of C^{14}-labeled tyrosine into the tissue was readily demonstrated during a 24 hour period of incubation. In this time, 12.5 % of the tyrosine in the soluble protein fraction had been replaced, 10.5 % in the formic acid soluble cell wall material and 2.2 % in the cell wall residue. No other amino acid became radioactive after the tissue had been cultured in the presence of C^{14}-labeled tyrosine for 24 hours.

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