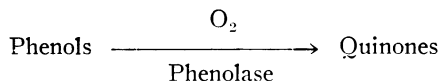


# FORMATION OF AUXIN FROM TRYPTOPHAN THROUGH ACTION OF POLYPHENOLS<sup>1, 2</sup>

S. A. GORDON & L. G. PALEG<sup>3</sup>

DIVISION OF BIOLOGICAL & MEDICAL RESEARCH, ARGONNE NATIONAL LABORATORY, ARGONNE, ILLINOIS

Plant preparations that convert tryptophan to indoleacetic acid (IAA) characteristically have low reaction yields. Since such preparations sometimes also contain IAA-oxidase activity, inhibition of the oxidase might raise the yields of IAA. Accordingly, polyphenolic inhibitors (1, 11, 19, 33, 40) were added to incubation mixtures containing tryptophan and a seedling enzyme preparation that was able to convert tryptophan to IAA. More than tenfold increases in auxin yield resulted. However, the presence of auxin in enzymeless controls, plus experiments on the recovery of added IAA, indicated that the enhancements observed might be caused by a polyphenolic oxidation of tryptophan to IAA. We shall present evidence that the sequence



Quinone + Tryptophan  $\rightarrow$  Indolepyruvic acid  $\rightarrow$  IAA  
occurs in plant material, and describe several of its characteristics.

## METHODS

**ENZYME PREPARATION:** Mung bean seedlings (*Phaseolus aureus*, Roxb.), 7 to 8 days old, were the usual source of enzyme. The plants were grown in gravel periodically flushed with tap water. An 18-hour day at 25 C was alternated with a 6-hour night at 18 C. Illumination came from a mixture of 4500°K-fluorescent tubes and tungsten lamps in a wattage ratio of 5:1. This supplied an irradiance of 2,500 ft-c at plant level.

Lyophilized cell-free extracts were used unless otherwise noted. Leaves and buds, 2½ kg, in 500 g portions, were frozen in liquid N<sub>2</sub>. A cold-room was used for subsequent operation. One of the frozen portions was dispersed in 0.2 M tris(hydroxymethyl)aminomethane buffer, pH 7.2, with a colloid mill. The dispersion was clarified by centrifugation through sharkskin paper in a basket head. Thereupon an additional 500 g of tissue was dispersed in the

filtrate and recentrifuged. The same procedure was repeated until all of the 2.5 kg were processed. Each filtrate was kept above pH 6.8 by adding N NaOH as required. About 900 ml of the final filtrate were centrifuged 4 hours at 44,000  $\times g$  to yield a supernatant fraction containing 42 mg/ml of solids and 1.2 mg/ml of nitrogen precipitated by trichloroacetic acid (TCA). The total volume was adjusted to pH 7.2, lyophilized, and stored in vacuo over P<sub>2</sub>O<sub>5</sub> at 2 C. For use, the dry powder was dissolved in distilled water at a concentration of 42 mg per ml.

Fresh enzyme preparations were made by freezing tissues in liquid N<sub>2</sub> and grinding them in a mortar with twice their weight of phosphate buffer, 0.1 M, pH 7.2. The slurry was filtered through nylon cloth and centrifuged at 105,000  $\times g$  for 30 minutes. The supernatant liquid was adjusted to pH 7.2 and used immediately; 3 ml were added to each incubation flask.

**INCUBATION & EXTRACTION PROCEDURE:** Unless otherwise noted, the incubation mixture consisted of 0.02 M L-tryptophan, 42 mg of enzyme, 0.03 M tris buffer, and catechol at 0.01 M in a total volume of 10 ml. Solutions of the various components were adjusted to pH 7.2 just before use. The tryptophan (Mann) was previously extracted with diethyl ether for 1 day in a Soxhlet apparatus to remove ether-soluble indolyl impurities.

Incubation mixtures, in 25-ml Erlenmeyer flasks, were placed in a Dubnoff shaker at 30 C and agitated in the dark at a frequency of one cycle per second for 1 hour. A zero-time sample, taken immediately after mixture of the components, was used as a base for the zero-order reaction rates represented by the data. Reactions were halted by rapidly bringing the contents of a flask to boiling point with a bunsen flame. On cooling, the pH was adjusted to 9.2 with NaOH. Neutral and basic fractions were removed with diethyl ether, as was the acid fraction after adjusting the aqueous phase to pH 3 with HCl. Suspended water was precipitated from the ether with the acid fraction by storage at -20 C for several hours. The ether was then decanted and removed from the extract by gentle boiling over a steam bath.

All of the diethyl ether used was purified by distillation from aqueous CaO-FeSO<sub>4</sub> (28). Phenolic solutions were freshly prepared. Commercial reagents were used throughout with the exception of 3-nitrocatechol, which was first purified by sublimation in vacuo, mp 59-69 C.

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<sup>3</sup> Present address: Waite Agricultural Research Institute, Adelaide, South Australia.

**AUXIN ASSAY:** The acid fraction was taken up in a small quantity of methanol previously purified by distillation from Zn-KOH, and a portion was resolved by paper chromatography (cf. 16 for details). Following ascending chromatography in *n*-butanol: 28% ammonia: water, 10:1:1 v/v/v, IAA, as indicated by parallel marker strips, was eluted with methanol and estimated colorimetrically with the iron-perchloric acid reagent (ibid). Less than 2  $\mu\text{g}$  IAA in heterogeneous extracts cannot be assayed satisfactorily by the above procedure. The term "tr." is used in the data to indicate that a faint trace of color was seen at the  $R_f$  of IAA on marker chromatograms. No IAA was detected in the various appropriate controls of tryptophan alone.

**INDOLE-3-ACETALDEHYDE & INDOLE-3-PYRUVIC ACID:** Indoleacetaldehyde was synthesized following the procedure of Gray (20), and stored as the bisulfite-addition complex. Free indoleacetaldehyde was liberated from an aqueous solution of the bisulfite complex by adding  $\text{Na}_2\text{CO}_3$ , and extracted with ether. The aldehyde was transferred to added water by evaporation of the ether.

Indolepyruvic acid was synthesized by condensing indole-3-aldehyde with hippuric acid to form the acetylated benzoyl azlactone (mp 207–209 C uncor); the azlactone was then hydrolyzed to indolepyruvate (5). The elementary composition was, in percentage: C-65.28 (65.01 theoretical); H-4.66 (4.47); O-23.03, by difference, (23.62); N-7.03 (6.89). Its melting point, with decomposition, was 212.0 to 215.0 C, uncor, when introduced at 200 C, and 211.5 to 214.0 C, uncor, when introduced at 205 C. The *p*-nitrophenylhydrazone melted at 153 to 154° uncor (cf. 10), with darkening and decomposition.

## RESULTS

The influence of catechol on the yield of IAA from tryptophan is shown in table I. IAA is formed from tryptophan by the action of catechol without enzyme. However, all four plant preparations produced

TABLE II  
RECOVERY OF IAA ADDED TO ENZYME & CATECHOL MIXTURES

IAA ADDED, $\mu\text{g}$	% RECOVERY		
	CATECHOL	ENZYME	CATECHOL + ENZYME
500	86	88	81
100	66	84	70
20	35	35	25

more auxin in the presence of the phenol. The latter results are not inconsistent with a suppression of IAA inactivation by catechol.

To test this explanation, the effect of catechol on the recovery of added IAA was determined. Synthetic IAA, recrystallized as the sodium salt, was added to incubation mixtures. These were assayed for IAA after a 1-hr incubation period. The recoveries obtained are given in table II. The loss of IAA with catechol alone was about the same as that with enzyme alone. Mixture of the catechol and enzyme did not increase the recovery of IAA; actually, a somewhat lower recovery was observed. These results indicate that exposure to enzyme and to catechol caused a significant loss of IAA, and that the enhanced yields of IAA in the presence of catechol (table I) were not caused by an inhibition of an IAA-oxidase action.

An alternative hypothesis appeared compatible with the above data: catechol alone can degrade the amino acid to IAA, a degradation that is enhanced in the presence of the plant enzymes. In the following experiments this hypothesis is elaborated.

**EFFECT OF VARIOUS PHENOLS:** The activities of several mono- and dihydric phenols in the enzymatic and non-enzymatic conversion of tryptophan to IAA are given in table III. Catechol was the only phenol tested that formed significant amounts of IAA without enzymatic participation. The enzyme was re-

TABLE I  
EFFECT OF CATECHOL ON CONVERSION OF TRYPTOPHAN TO IAA BY SEVERAL PLANT PREPARATIONS

	MUNG BEAN		MUNG BEAN		WITHOUT ENZYME	
	-C***	+C***	-C	+C	-C	+C
$\mu\text{g}$ IAA/hr	2.0*	31*	0**	14**	0	10-11
$\mu\text{g}$ IAA/hr/mg N	2.0	31	...	...	...	...
$\mu\text{g}$ IAA/hr	1.6†	29†	2.8†	15†	tr.†	36†
$\mu\text{g}$ IAA/hr/mg N	0.7	13	2.2	12	...	22

\* Lyophilized.

\*\* Lyophilized, boiled 10 minutes.

\*\*\* -C, without catechol; +C, with catechol.

† Non-lyophilized. *Avena sativa*: etiolated coleoptile and primary leaf, age 72 hours. *Helianthus annuus*: terminal leaves and buds from 1-month-old green plants.

TABLE III  
TRYPTOPHAN CONVERSION TO IAA IN PRESENCE  
OF VARIOUS PHENOLS

ADDEND	CONC. M	IAA, % YIELD RELATIVE TO CATECHOL	
		- ENZYME	+ ENZYME
Catechol	10 <sup>-2</sup>	12 $\mu\text{g}=100$	32 $\mu\text{g}=100$
3-Nitrocatechol	10 <sup>-2</sup>	tr.	47
Protocatechuic acid	10 <sup>-2</sup>	0	150
Caffeic acid	10 <sup>-3</sup>	tr.	77
Chlorogenic acid	10 <sup>-2</sup>	tr.	100
Tyrosine	10 <sup>-3</sup>	0	0
Dihydroxyphenylalanine	10 <sup>-3</sup>	0	12
Resorcinol	10 <sup>-2</sup>	0	0
Pyrogallol	10 <sup>-2</sup>	...	32
Phenol	10 <sup>-2</sup>	0	73
<i>o</i> -Nitrophenol	10 <sup>-3</sup>	0	0
<i>o</i> -Cresol	10 <sup>-2</sup>	0	4
<i>m</i> -Cresol	10 <sup>-2</sup>	0	29
<i>p</i> -Cresol	10 <sup>-2</sup>	0	98

quired for the activity of the other phenols, and increased the yield from catechol. The ortho-dihydroxy structure seems to be required for appreciable activity, since resorcinol, tyrosine, *o*-nitrophenol and *o*-cresol were ineffective; these compounds also have significantly lower oxidation rates than their *o*-dihydroxy analogs (22, 26). The activity of phenol, pyrogallol, and *m*- and *p*-cresol probably derive from their ready conversion by phenolase containing systems to the vicinal dihydroxy analogs (9, 30).

Quite active in producing IAA were the *para*-substituted protocatechuic, caffeic and chlorogenic acids, *p*-cresol, and, to a lesser extent, dihydroxyphenylalanine. Their effectiveness indicates that the nature of the *para* substituent is not highly specific, a characteristic consistent with the inference that a *p*-substitution stabilizes the *o*-quinoid structure through repeated oxidations and reduction (39). Apparently the substituent can involve either a carbon or a nitrogen linkage.

**INHIBITORS OF CATECHOL-MEDIATED AUXIN FORMATION:** Known inhibitors of phenolic reactions were then tested for their effect on auxin formation (table IV). The reducing agents were highly effective

TABLE IV  
EFFECT OF REDUCING & CHELATING  
AGENTS ON IAA YIELD

ADDEND	CONC. M	$\mu\text{g}$ IAA/hr	
		- ENZYME	+ ENZYME
None	...	12	30
Ascorbic acid	10 <sup>-2</sup>	0	3.6
Cysteine	10 <sup>-2</sup>	0	0
Diethyldithiocarbamate	10 <sup>-2</sup>	2.6	5.1
Ethylenediamine-tetraacetate*	10 <sup>-2</sup>	2.8	20
<i>o</i> -Phenanthroline	10 <sup>-3</sup>	4.8	24
$\alpha,\alpha$ -Dipyridyl	10 <sup>-3</sup>	12	25
Azide*	10 <sup>-1</sup>	12	36

\* Catechol concentration  $5 \times 10^{-3}$  M; 10<sup>-2</sup> M in all others.

tive in lowering the yield; ascorbic acid and cysteine, which would be expected to act as oxidation inhibitors and to reduce quinones, gave virtually complete inhibition of both the enzymatic and non-enzymatic formation of IAA. The chelating compounds reacting preferentially with iron caused a 15 to 30% inhibition, though inhibition by phenanthroline in the system without enzyme, where dipyrindyl has no effect, suggests that this class of compounds might act in part as competitive reductants. The copper reagent, diethyldithiocarbamate, caused an 85% reduction in the amount of IAA produced with the enzyme. Interestingly, azide, which apparently inhibits both tyrosinase (24) and laccase (25), had no effect on the yield with or without the enzyme even at a concentration of 10<sup>-1</sup> M. Tryptophan probably was responsible for the lack of inhibition, since azide did retard the initial rate of catechol oxidation by the enzyme when tryptophan was omitted. (Solutions of catechol & enzyme darkened rapidly, but not in the presence of azide.)

TABLE V  
EFFECT OF NITROPHENOLS ON FORMATION OF IAA

ADDEND	CONC. M	RELATIVE YIELD (%) ENZYME + TRYPTOPHAN	
		+	+
		CATECHOL*	PHENOL*
None	...	30 $\mu\text{g}=100$	22 $\mu\text{g}=100$
<i>o</i> -Nitrophenol	10 <sup>-4</sup>	100	...
	10 <sup>-3</sup>	130**	110
	10 <sup>-2</sup>	150	130
<i>p</i> -Nitrophenol	10 <sup>-4</sup>	110	90
	10 <sup>-3</sup>	95	120
	10 <sup>-2</sup>	110	100
2,4-Dinitrophenol	10 <sup>-4</sup>	110	100
	10 <sup>-3</sup>	92	85
	10 <sup>-2</sup>	58	31
3-Nitrocatechol	10 <sup>-2</sup>	110	...

\* 10<sup>-2</sup> M

\*\* Catechol concentration, 10<sup>-3</sup> M.

**EFFECT OF NITROPHENOLS:** Mono-nitrophenols inhibit neither the catechol- nor the phenol-mediated formation of IAA. The data in table V indicate that when significant differences are observed, enhancement rather than inhibition of the reaction occurs. As with the lack of azide inhibition, the explanation may lie in the presence of tryptophan. The higher concentrations of 2,4-dinitrophenol significantly decreased the amount of IAA obtained with both catechol and phenol. Though 3-nitrocatechol could partially replace catechol function in the formation of IAA (table III), the presence of nitrocatechol and catechol in equimolar concentrations yielded the same amount of IAA as did catechol alone.

**TYROSINASE:** From the preceding experiments it appeared that a polyphenolase action was involved in the conversion of tryptophan to IAA. The results

TABLE VI  
IAA FORMATION WITH TYROSINASE\*

	IAA/ $\mu$ g/hr
Tyrosinase + catechol	0
Tyrosinase + tryptophan	0
Catechol + tryptophan	6
Tyrosinase + catechol + tryptophan	25

\* Medium: L-tryptophan 0.02 M, catechol  $10^{-2}$  M, tris buffer 0.025 M, mushroom tyrosinase (Worthington) 0.25 catecholase units, total volume 10 ml, pH 6.5.

obtained when mushroom tyrosinase was used as the enzyme (table VI) support this view. Tryptophan and tyrosinase produced no IAA. When catechol was added, tyrosinase was able to reproduce the action of the mung bean enzyme.

**ACTION OF QUINONES:** Since the degradation of tryptophan to IAA is an oxidative reaction, the non-enzymatic formation of IAA by catechol suggested that a first step might involve dehydrogenation of the phenol. Preflushing of the solutions with  $N_2$  gas (passed through Cu turnings at 800 C & water scrubbed) caused a 90% inhibition of the non-enzymatic formation of IAA and a 70% inhibition of the enzymatic reaction with the mung bean preparation. This indicated an oxidative reaction with direct participation of  $O_2$ . Since the *o*-quinone is considered to be the product of catecholase action (30), a reaction requiring oxygen (ibid), the activity of *o*-benzoquinone was examined.

Benzoquinone was synthesized by the silver-oxide oxidation of catechol, using the method described by Tarpley (38). Freshly prepared ethereal solutions were shaken with 0.02 M aqueous solutions of tryptophan, pH 7.2. A reddish pigment (irreversibly decolorized by sulfuric acid) quickly appeared in the aqueous phase. After 1 hour, with occasional vigorous agitation, the acid fraction was separated from the aqueous layer and assayed for IAA. Nine  $\mu$ g were found.

**INDOLEACETALDEHYDE:** Tryptophan conversion to IAA in plant material is considered to go through either indolepyruvic acid or tryptamine to indoleacetaldehyde, the immediate precursor of the auxin (12, 29). Furthermore, oxidation of tryptophan by dicarbonyls such as isatin has been used as a source of indoleacetaldehyde (2, 27). The Strecker degradation of an  $\alpha$ -amino acid by dicarbonyls (32, 34), a catalytic oxidation by quinones to the cyanohydrin, and the novel variant proposed by Spenser et al. (37) leading to an unstable carbinolamine, also suggest the intermediate formation of indoleacetaldehyde. The likelihood that the aldehyde participated in the polyphenol-mediated conversion of tryptophan to IAA was, therefore, explored.

The participation of tryptamine in the reactions between tryptophan and IAA would increase the probability of indoleacetaldehyde as an intermediate, inas-

much as the aldehyde is the first product of amine-oxidase action. Tryptamine  $\cdot$  HCl, 50 mg, was incubated in the usual manner with catechol and catechol plus mung bean enzyme. No IAA was obtained from either medium. Thus, if tryptophan is decarboxylated to tryptamine by the catechol system, subsequent conversion of the amine to IAA apparently does not take place.

As a more direct approach, about 200  $\mu$ g of indoleacetaldehyde was incubated with enzyme, with catechol, and with catechol plus enzyme. Though control flasks of aldehyde alone yielded no IAA, 6  $\mu$ g of IAA were produced by adding just the enzyme. No auxin was found in the extracts of the mixtures containing catechol with or without enzyme. Evidently a component of the crude enzyme was able to oxidize the aldehyde to IAA, though the aldehyde is not so oxidized in the presence of the catechol system. Catechol without enzyme definitely did not convert indoleacetaldehyde to IAA, though the auxin is formed from tryptophan under similar conditions.

Further evidence that indoleacetaldehyde is not an intermediate in the phenol-mediated tryptophan to IAA reaction is provided by the effect of the aldehyde-reagent dimedon. The tryptophan-catechol reaction was carried out with the presence of 1.5 mg dimedon in a 10-ml volume. Control flasks without dimedon produced 11.2 mg IAA per hour, whereas those with dimedon yielded 12.6. Since dimedon is presumed to be specific for aldehydes (41) and reacts with indoleacetaldehyde (8, 17), a free aldehyde does not appear to be involved.

**INDOLEPYRUVIC ACID:** Oxidation of tryptophan to IAA via the keto-acid was then considered, since tryptamine and indoleacetaldehyde were evidently not intermediates. Indolepyruvate seemed particularly appropriate in view of its spontaneous breakdown in solution (4, 6, 17, 23) in part to IAA, an instability that is compatible with the formation of auxin by tryptophan and catechol without enzyme.

Following the procedure of Kaper and Veldstra (23), the acid fractions of tryptophan-catechol incubation mixtures (1 hr) were taken up in methanol and chromatographed in ascending isopropanol-ammonia-water 10:1:1. The developed strips were sprayed with a solution containing 5%  $HClO_4$ -0.05 M  $FeCl_3$  in a 50:1 ratio. Extracts of control incubations with synthetic indolepyruvate and of incubation blanks without tryptophan or catechol were also chromatographed. Five of the decomposition spots characteristic of indolepyruvic acid were found in the sprayed chromatograms of the extracts of the tryptophan-catechol reaction. They correspond to the spots A, D, IAA, H, and IAld described by Kaper and Veldstra. This indicates that indolepyruvic acid is formed during the phenolic degradation of tryptophan.

To evaluate this conclusion, we determined whether labeled indolepyruvate was formed when  $C^{14}$ -labeled tryptophan was incorporated into the medium. The incubation mixture consisted of 0.02 M D,L-tryptophan-3- $C^{14}$  (New England Nuclear Corp.,

TABLE VII  
FORMATION OF IAA BY ACTION OF THE PROLYL-CATECHOL COLOR COMPLEX UPON TRYPTOPHAN

CATECHOL	PROLINE	ENZYME	FERRICYANIDE	TRYPTOPHAN*	INCUBATION TIME, min	IAA, $\mu\text{g}$
+	+	-	-	-	60	0
+	+	+	-	-	60	0
+	+	-	+	-	15	0
+	+	-	+	-	60	0
+	+	-	+	+	30	5
+	+	-	+	+	60	8
+	+	+	-	+	30	8
+	+	+	-	+	60	12

\* Tryptophan was added 15 minutes after the preceding components were combined. Phosphate buffer 0.05 M, pH 7.8, 30 C; catechol 0.005 M, proline 0.02 M, enzyme 42 mg,  $\text{K}_3\text{Fe}(\text{CN})_6$  0.02 M, tryptophan 0.02 M.

5.82 mC/mm), catechol, and enzyme. After an incubation period of 1 hour, the acid and neutral fractions in ether were concentrated in vacuo and taken up in 95% ethanol. This was added directly to a  $25 \times 1$  cm column of Solka-Floc cellulose powder which previously had been washed with dilute ammonium hydroxide, dilute hydrochloric acid, water, ethanol, and ether. The column was developed with a solvent mixture consisting of water-saturated butanol:95% ethanol (9:1) to which was added 0.2 ml of concentrated HCl per 100 ml of solution. A parallel column was run with 10 mg of indolepyruvate; each 1 ml of eluate was tested with ammoniacal silver nitrate (Tollens' reagent) to indicate the position of the keto-acid. (Crystallization occurred in several eluates of the marker column that were refrigerated overnight.) The eluate from the column that had the incubation extract was reduced in vacuo to a volume of about 0.5 ml. Indolepyruvate, 20 mg, was added and the mixture was taken up in 95% ethanol. Water was slowly added until a cloudiness appeared and then 15 mg of *p*-nitrophenylhydrazine dissolved in glacial acetic acid was added. The mixture was refrigerated overnight to crystallize the *p*-nitrophenylhydrazone. This was successively recrystallized from hot alcohol three additional times. The specific activity of each batch of crystals, as measured by a windowless flow counter, was, respectively, 380, 280, 240, and 260 counts per minute per mg. These results corroborate the conclusion that indolepyruvate is formed during the phenol-mediated conversion of tryptophan to IAA.

#### ACTIVITY OF PROLYL-CATECHOL COLOR-COMPLEX:

The formation of color complexes by the action of the catechol-oxidase system on amino acids has been investigated in some detail. Under suitable conditions, the complex formed spontaneously by catechol and an amino acid is identical with that formed enzymatically, and is able to deaminate a number of primary amino acids (3, 21, 22, 39).

Catechol was therefore incubated with a 4-mol excess of ferricyanide in the presence of proline at pH 7.8, conditions that rapidly oxidize the catechol (3, 22). A reddish-violet pigment formed quickly.

As is indicated in table VII, no IAA could be detected either at 15 or 60 minutes, with or without enzyme, in the absence of tryptophan. (Similar results were observed with glycine in place of proline.) However, the color complex formed from proline and catechol by either ferricyanide or the enzyme converted subsequently added tryptophan to IAA. Of the two oxidation systems, the enzyme was more effective.

**OPTIMUM REACTION CONDITIONS:** The yields of IAA obtained with the catechol-tryptophan system by varying the incubation conditions are given in the succeeding tables and figure. In these experiments another lyophilized mung bean enzyme preparation of lower specific activity was used. It was prepared in a similar fashion (cf. Methods) from a cell-free supernatant fraction containing 36 mg of solids and 1.1 mg of TCA-precipitable nitrogen per ml. Except as noted, the customary incubation and assay procedures were used.

In table VIII it is indicated that a catechol concentration of  $10^{-2}$  M yields relatively large amounts of IAA. The optimum concentration of catechol for the medium used is probably somewhat higher; however, the large amount of concomitantly formed oxidation products interfered with the assay for IAA.

As described under Methods, the pH of an incu-

TABLE VIII  
EFFECT OF CATECHOL CONCENTRATION ON YIELD OF IAA

CATECHOL CONC. M	IAA $\mu\text{g/hr}$	
	- ENZYME	+ ENZYME
$10^{-1}$	tr.*	14*
$10^{-1}$	tr.*	19*
$5 \times 10^{-2}$	12	18
$5 \times 10^{-2}$	12	15
$10^{-2}$	10	15
$10^{-2}$	11	14
$10^{-3}$	3	6
$10^{-3}$	3	5
$10^{-4}$	0	0
$10^{-5}$	0	0

\* Values unreliable because of excessive catechol oxidation products on chromatogram.

TABLE IX

EFFECT OF POST-INCUBATION pH ON APPARENT YIELDS OF IAA FROM CATECHOL &amp; TRYPTOPHAN

pH*	IAA, $\mu\text{g}$	
	- ENZYME	+ ENZYME
7.5	tr.	13.3
8.0	tr.	15.1
9.2	7.5	17.8

\* pH During incubation was 7.2.

bation mixture was adjusted to 9.2 after enzyme inactivation for removing basic and neutral fractions. We found that this pH adjustment contributed to the yield of IAA from the tryptophan-catechol reaction, both with and without the enzyme present. This effect, illustrated by table IX, is probably caused by an increase in the spontaneous oxidation rate of catechol as the hydrogen ion concentration decreases (3). The table indicates that the contribution of the enzyme over the spontaneous rate could be enhanced at pH < 9. This enhancement of the enzyme contribution is also suggested by the curves in figure 1, which show the effect of varying the pH of the incubation medium.

Figure 1 indicates that the spontaneous oxidation yield of IAA is maximal at  $\sim$  pH 9.5, whereas the optimum for the enzymatic system is  $\sim$  8.5. With-

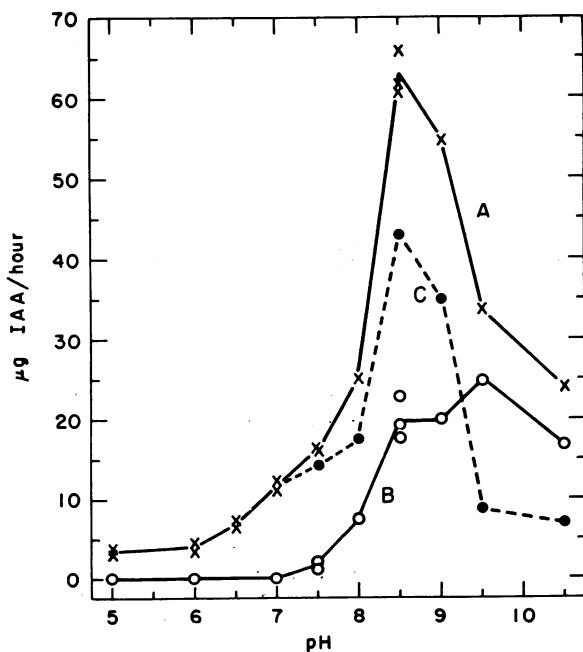


FIG. 1. The influence of pH of the incubation medium on the yield of IAA. All components preadjusted to indicated pH with HCl or NaOH, and similarly adjusted to pH 8.2 before the fractionation sequence. A, with enzyme; B, without enzyme; C, A minus B.

out the enzyme, no IAA was found under the conditions of pH 5 to 7. Since the most alkaline solutions darkened quite rapidly, the drop in yields therein might be attributed to competitive humin formation. The amount of IAA formed from tryptophan by alkaline decomposition (18) would be insignificant relative to the amounts of IAA dealt with here.

## DISCUSSION

The action of a phenol-phenolase system upon tryptophan is a new reaction sequence for the production of auxin (31). Its characteristics are consistent with currently proposed mechanisms for the oxidative deamination of amino acids by the phenolase complex (cf. 9, 30, 39). These mechanisms hold that oxidation of a vicinal dihydroxy phenol to the corresponding *o*-quinone is catalyzed by phenolase. The quinone condenses spontaneously with amino acids to form a *p*-substituted reddish pigment of the amino-quinone class. This pigment reacts again with free amino acid to form the *o*-quinonimine as a second condensation product. The quinonimine spontaneously rearranges, hydrolyzes, and is reoxidized, presumably to the primary pigment; in this process, ammonia and a carbonyl compound are liberated.

The observations in the present study may be considered in terms of the above framework.

A. We have shown that enzyme preparations from the mung bean seedlings, as well as the oat and sunflower, form comparatively large amounts of auxin from tryptophan in the presence of catechol.

B. Other *o*-dihydroxy phenols, or compounds readily converted to that structure, can replace catechol, with similar orders of activity. The nature of the *p*-substituent appears to be relatively non-specific.

C. The catechol system is inhibited by reducing and chelating agents; the copper-reagent diethyldithiocarbamate is particularly effective.

D. Mushroom tyrosinase can substitute for the mung bean enzyme in the tryptophan-catechol system; a polyphenolase action of the mung enzyme may be inferred. Using auxin formation as a criterion, the pH optimum with the mung bean enzyme is 8.5.

E. A polyphenolase action of the enzyme would use  $\text{O}_2$  as an acceptor and form a quinone as the first product. We find that the action of the mung bean enzyme-catechol system is inhibited in a  $\text{N}_2$  atmosphere and that the formation of IAA from tryptophan can also be accomplished by contact of the amino acid with ethereal solutions of *o*-benzoquinone.

F. An analogous model can be constituted with other amino acids that are inactive per se in producing auxin. For example, catechol reacts with proline in the presence of ferricyanide as an oxidant. The complex so produced forms IAA upon the subsequent addition of tryptophan.

G. Our data indicate that indolepyruvic acid is formed by the reaction of phenols with tryptophan, and that indolepyruvate, not tryptamine or indoleacetaldehyde, is the intermediate in the phenol-medi-

ated conversion of tryptophan to IAA. This conclusion is compatible with the formation of a carbonyl product on breakdown of the quinonimine-amino acid complex. The non-enzymatic formation of IAA here observed is also compatible with the decomposition characteristics of indolepyruvate, which degrades spontaneously to IAA.

We believe that the experiments described permit the conclusion that tryptophan is oxidatively deaminated (22) to indolepyruvate by oxidized dihydric phenols; the keto-acid then breaks down spontaneously to IAA. The chief action of the mung bean enzyme is that of a phenolase, i.e., catalysis of the primary oxidation of phenols to the *o*-quinoid form, though the enzyme may also channel or enhance the conversion of indolepyruvate to IAA (cf. 15).

The auxin produced in these phenolic reactions with tryptophan has not been rigorously shown to be IAA. We have assumed that it is IAA because the substance: A: behaves as an organic acid in aqueous ether solvent systems; B: is an auxin by both *Avena* section and curvature tests; C: forms a chromophore with the iron-perchloric acid reagent that has the same absorption spectrum as the chromophore formed by IAA; D: has the same migration rate as IAA on chromatography with *n*-butanol: ammonia: water, 10:1:1 ( $R_f$  0.35) and acetic acid (0.1N): methanol: *n*-butanol, 98:1:1 ( $R_f$  0.60), using both colorimetric and biological criteria of distribution on the chromatogram.

Does the interaction between phenols and tryptophan represent the usual pathway of auxin biogenesis? The distribution of biological activity on chromatograms of the acid fraction from the reaction of tryptophan and the mung bean enzyme is qualitatively similar to the distribution obtained from the reaction of tryptophan, catechol, and enzyme. However, we feel that the above pathway may not be followed in *normal* tissue on the basis of the following two considerations.

First, there are indications that the phenolases of plants are associated with cellular particulates (7). Yet none of the essentially intact cell organelles produce IAA from tryptophan in the absence of catechol (14): such activity is a soluble component of the cytoplasm. Though the preceding can hardly be considered convincing evidence, the phenols and phenol oxidase in intact cells are very probably spatially separated. The tryptophan-phenolase-phenol reaction could thus be considered as a latent, potentially operative, mechanism for auxin formation in the plant. Its components normally would be compartmentalized *in vivo* and so prevented from interaction at significant rates. The lysis which occurs after wounding of plant tissue, wherein activation of the phenolase complex takes place, may then produce abnormal quantities of growth hormone via the phenol-tryptophan reaction. Callus and gall formation could be histogenic consequences of such hyperauxiny, and there would be no need to postulate the function of a specific wound hormone such as traumatin in the morphogenesis of wound regeneration.

Second, normal auxin biogenesis is highly sensitive *in vivo* to X- and gamma-radiation (13,35). This radiosensitivity is rapidly manifest in three ways: as a reduced level of the free hormone, as alterations in morphological phenomena dependent upon hormonal concentration, and as a reduction in activity of the soluble enzyme converting tryptophan to auxin. In current experiments we have found that the phenolase-mediated conversion of tryptophan to IAA is unimpaired in preparations obtained from seedlings immediately after exposure to single X-ray doses of 5 kr.

Since the phenolase complex probably is activated by the disruptions in cellular integrity accompanying chronic high-energy irradiation, a consequent hyperauxiny could well be the basis for the hyperplasia and neoplasms that are commonly observed (36) in such irradiated plants.

#### SUMMARY

Phenols, under conditions leading to their oxidation, react with tryptophan to form the auxin indoleacetic acid. The action is catalyzed by polyphenolase enzymes of mung bean, oat, and sunflower, and occurs spontaneously at alkaline pH. Various *ortho*-dihydric phenols, or phenols readily oxidized to that form, are effective. The reaction is inhibited by replacement of air with  $N_2$ , and by reducing and chelating agents. It can be duplicated by mushroom tyrosinase, by exposure of tryptophan to ethereal solutions of *o*-benzoquinone, or by adding tryptophan to a prolyl-catechol pigment formed via either ferricyanide or enzymatic oxidation. It is shown experimentally that indolepyruvic acid and not tryptamine or indoleacetaldehyde is the probable intermediate in the conversion of tryptophan to indoleacetic acid by the enzyme system. These observations are consistent with current concepts of oxidative deamination of amino acids by quinonimines. The pertinence of this new pathway of auxin biosynthesis is discussed in regard to normal auxin biogenesis, wound regeneration, and radiation-induced hyperplasia.

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