

ENZYMATIC FORMATION OF INDOLE-3-CARBOXALDEHYDE FROM INDOLE-3-ACETIC ACID^{1,2}

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It was first suggested by Tang and Bonner (14) that indole-3-carboxaldehyde is the product of the oxidative degradation of indole-3-acetic acid (IAA) by crude enzyme preparations from etiolated pea seedlings. Using the more active bean root enzyme, Wagenknecht and Burris (15) attempted without success to obtain the 2,4-dinitrophenylhydrazone of the aldehyde. On the basis of chromatographic data, Racusen (9) reports that the pea enzyme forms small amounts of the aldehyde. More recently, Wagenknecht (personal communication) has made similar observations with the bean root system. Ray and Thimann (10) and Ray (11) have recently reported that the enzyme from *Omphalia flavida* is unable to form the aldehyde. The recent work of Maclachlan and Waygood (6) likewise has left unsettled the nature of the end product.

Manning and Galston (7) have reported the formation in the pea system of two products having high R_f values and giving qualitative color tests which indicate a similarity to our products # 4 and # 5 (see below). They were unable to demonstrate the formation of indole-3-carboxaldehyde, and also ruled out *ortho*-formamidoacetophenone, *ortho*-aminoacetophenone, and 4-hydroxyquinoline as major end products, although they believe that the indole ring is ruptured.

Chromatographic separation and spectrophotometric examination of the products of the IAA oxidase-peroxidase system from *Lupinus albus* L. (13) gave no indication that the aldehyde was formed; likewise, the results obtained when the chromatograms were tested with 2,4-dinitrophenylhydrazine hydrochloride, modified Salkowski (3), and Ehrlich reagent suggested the absence of the aldehyde. In view of the conflicting results, attempts were made to supplement the purified IAA oxidase with the auxiliary enzyme systems presumed to be present in the crude enzyme preparations employed in the early investigations. The present report is concerned with the coupling of the oxidase system to the cytochrome oxidase system. Under these conditions the formation of indole-3-carboxaldehyde as a major reaction product was observed.

Subsequent reexamination of the reaction products from the oxidase-peroxidase system, particularly those freshly prepared from etiolated lupine hypocotyls, indicated the normal formation of the aldehyde in small amounts. Therefore, in order to obtain data on

the oxidation mechanism, indole-3-carboxylic acid and a number of substituted indole-3-acetic acid derivatives were tested both with the oxidase-peroxidase system alone and with the added cytochrome system. It is of interest to note that since this paper was submitted, Jones and Taylor (5) have reported the isolation of both indole-3-carboxaldehyde and indole-3-carboxylic acid from cabbage extracts.

MATERIALS AND METHODS

Preparation and purification of the IAA oxidase from *Lupinus albus* L. has been previously described (13). The course of the oxidation of IAA was followed with the Warburg manometric apparatus under the conditions outlined earlier (13) except where 0.4 M tris-(hydroxymethyl)-aminomethane buffer at pH 7.2 was used in an attempt to approach the pH optimum of the cytochrome oxidase system and still maintain IAA oxidase activity. Under these conditions a small amount of phosphate, 4 micromoles per flask, was added for optimum IAA oxidase activity. The normal 3-ml reaction mixture was enzyme; 1 ml 0.4 M orthophosphate buffer, pH 6.3; 0.3 micromole $MnCl_2$; 0.3 micromole sodium-2,4-dichlorophenolate; (in the main compartment) 0.1 ml M KOH with a standard wick in the center well; and 30 micromoles of sodium indole-3-acetate in the side arm. When the cytochrome system was to be coupled to the oxidase, cytochrome oxidase was added to each flask in the form of 0.2 ml of a 5% suspension of homogenized rat liver, together with cytochrome c, usually 2×10^{-5} M, and 0.3 micromoles of Al^{+++} .

Ascending chromatography on Whatman 3-mm filter paper was carried out with a solvent mixture consisting of 28% NH_4OH , water, and isopropyl alcohol in proportions ranging from 1:1:6 to 1:1:12, depending on whether it was desired to obtain greater resolution of the more acidic or the neutral fraction of the products. The reaction products to be discussed have been designated by numbers 1 to 5 in order of increasing R_f value (13). This has been done in preference to R_f designations alone because the relative positions of the components were retained in the various solvent mixtures used. The products (13) which appeared important in this study were the apparently large # 2 (R_f 0.6) group of non-indolyl fluorescent compounds and the # 4 and # 5 (R_f 0.8 and 0.9), presumably polymeric indolyl, products which bracket the aldehyde. The # 1 (R_f 0.45) indolyl component (13) was not encountered in this study—presumably because the reaction with crude enzyme was not permitted to go to exhaustion.

In some experiments, the substrate was IAA, labeled with C^{14} either in the methylene (12) or in the carboxyl position (13). In order to obtain suffi-

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cient activity on the chromatograms to permit reasonable counting time and accuracy, it was found necessary to label the substrate in each flask with approximately 30 microcuries of the methylene-labeled IAA or 3 microcuries of carboxyl-labeled IAA. C^{14} in the reaction products was measured by counting the chromatograms directly. Sixth-tenths of a milliliter of the enzyme reaction mixture was applied to a 4-inch segment of the chromatograph sheet. After chromatographic separation, sections 6 cm wide were counted by advancing the strip 1 cm at a time through the masked chamber of a modified PCC-10 proportional counter (Nuclear Measurements Corp., Indianapolis, Ind.).

Qualitative survey of the products on the chromatograms from all of the Warburg experiments was made by ultraviolet examination (Mineralight SL 2537) and by swabbing the strips with Ehrlich's reagent, 2,4-dinitrophenylhydrazine hydrochloride, and modified Salkowski reagent (3). The sensitivity of the ultraviolet scanning was greatly enhanced by illuminating the chromatograms from the back. Quantities of the indole-3-carboxaldehyde which were normally undiscernible were thus rendered detectable.

Fractionation and colorimetric determination of indole-3-carboxaldehyde and other indole products in the reaction mixture was carried out by the following procedure. One ml of the reaction mixture from the Warburg flask was added to 9 ml of buffer containing 7.5% NaHCO_3 and 2.5% Na_2CO_3 , and extracted with three 15-ml portions of peroxide-free ether. IAA was estimated in the aqueous phase with the modified Salkowski reagent (3) after the bulk of the dissolved ether had evaporated. Although the inadequacies of the Salkowski method have been well documented by previous investigators (8), the procedure was used in an attempt to obtain an evaluation of the residual IAA and the IAA equivalents of some of the products. It had been hoped that the Salkowski data along with the aldehyde and oxygen uptake values would permit an approximation of the reaction balance.

For the determination of indole-3-carboxaldehyde, the ether phase was evaporated to dryness in an air stream and the residue was taken up in 5 ml of absolute ethanol. A suitable aliquot of the ethanol solution was made up to 5 ml with ethanol, and to this was added 0.5 ml of 2,4-dinitrophenylhydrazine hydrochloride reagent (100 mg of 2,4-dinitrophenylhydrazine hydrochloride in 100 ml of 6N HCl). The colored solution was read in the Klett photoelectric colorimeter, using the No. 54 filter. Color development is instantaneous; the color is stable for at least an hour and apparently is unaffected by the presence of other indolyl compounds. The standard curve for indole-3-carboxaldehyde has a range of 0 to 200 micrograms over 340 Klett units. Recovery values in samples added to the Warburg reaction mixture substantiated the reliability of the extraction method.

Purification of the indole-3-carboxaldehyde for identification was carried out by strip chromatography of the fraction extractable from the reaction mixture

by ether at pH 10. The aldehyde fraction was eluted with acetone, concentrated, and the chromatography repeated. A small amount was taken up in ethanol for the ultraviolet absorption measurement; another aliquot was mixed with powdered KBr and a disk was prepared for infrared spectrophotometry. The invaluable help of Dr. J. H. Pomeroy and Mrs. C. A. Craig in this phase of the work is gratefully acknowledged.

Since indole-3-carboxaldehyde does not react appreciably with modified Salkowski reagent, the latter was used to determine the non-aldehyde, Salkowski-positive, indolyl compounds soluble in ether at pH 10 (the # 4 and # 5 products on the chromatograms), and a survey was made by comparison with the IAA standard curve.

The author wishes to acknowledge the provision of the substituted IAAs so generously made available for this investigation. Na-indole-3-glycolate was supplied by Dr. Marvin D. Armstrong of the University of Utah. Ethylindole-3-glyoxylate was obtained from Dr. Bruce Stowe of Harvard University; the ester was hydrolyzed by the method of Elks et al (1). Ethylindole-3-isobutyrate was provided by Dr. H. Erdtman and Dr. Å. Jönsson of the Tekniska Högskolan, Stockholm, Sweden; the free acid was prepared by their method (2). Samples of 1-methyl-IAA, 2-methyl-IAA, and 2-carboxy-IAA were obtained from Dr. Sidney Fox of the University of Florida. Indole-3-carboxylic acid was supplied by Dr. Norman E. Good of the Science Service Laboratory, London, Ontario, Canada. Dr. K. Mann of the Upjohn Laboratories, Kalamazoo, Michigan, provided

TABLE I
RESPONSE OF IAA OXIDASE TO THE CYTOCHROME SYSTEM

IAA- OXIDASE PREPA- RATION	CYTO- CHROME C	OXYGEN UPTAKE*	IAA USED	PRODUCT # 4**	INDOLE-3- CARBOX- ALDEHYDE
<i>ml</i>	<i>moles</i>	<i>micro- moles</i>	<i>micro- moles</i>	<i>micro- moles</i>	<i>micro- moles</i>
0.0	1×10^{-4}	-0.49	0.9	0.02	0.21
0.5	1×10^{-4}	0.22	0.9	0.1	0.62
"	5×10^{-5}	2.9	6.35	0.34	2.69
"	1×10^{-5}	9.8	14.7	0.79	4.0
"	5×10^{-6}	11.2	15.8	1.03	3.52
"	1×10^{-6}	14.3	18.9	1.17	2.28
"	0×0	13.9	16.6	1.05	0.83
1.0	1×10^{-4}	-0.31	1.43	0.07	0.62
"	5×10^{-5}	2.2	6.0	0.34	2.28
"	1×10^{-5}	14.5	20.0	1.35	4.35
"	5×10^{-6}	17.5	21.7	1.7	4.14
"	1×10^{-6}	18.0	22.1	1.66	3.2
"	0×0	17.3	21.1	1.58	1.79

* These values were obtained after 90 min at 30° C. The amounts of reaction products listed were obtained from 3.0 ml reaction mixture. Flasks containing cytochrome c also contained 0.2 ml of 5% rat liver homogenate.

** The estimation of reaction product #4 is described under Materials and Methods.

the 5-hydroxy-IAA. The indole-3-carboxaldehyde was obtained from Dow Chemical Company, Midland, Michigan.

RESULTS AND DISCUSSION

Indole-3-carboxaldehyde has for a long time figured prominently as a possible reaction product of IAA oxidation. The data on substrate utilization (14) and the respiratory quotients observed (15) were suggestive in this respect. Only recently (9) has this product been detected in small amounts. Earlier studies of the author (13) showed that with the enzyme from *Lupinus albus*, regardless of its state of purity, significant amounts of the aldehyde were not accumulated. Therefore, in order to maintain the role of the aldehyde as an intermediate prod-

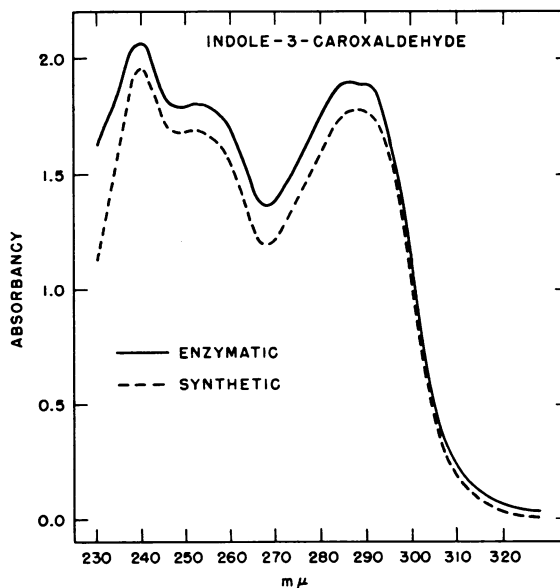


FIG. 2. The ultraviolet absorption spectrum of synthetic indole-3-carboxaldehyde and of material isolated from enzyme reaction mixture, in ethanol.

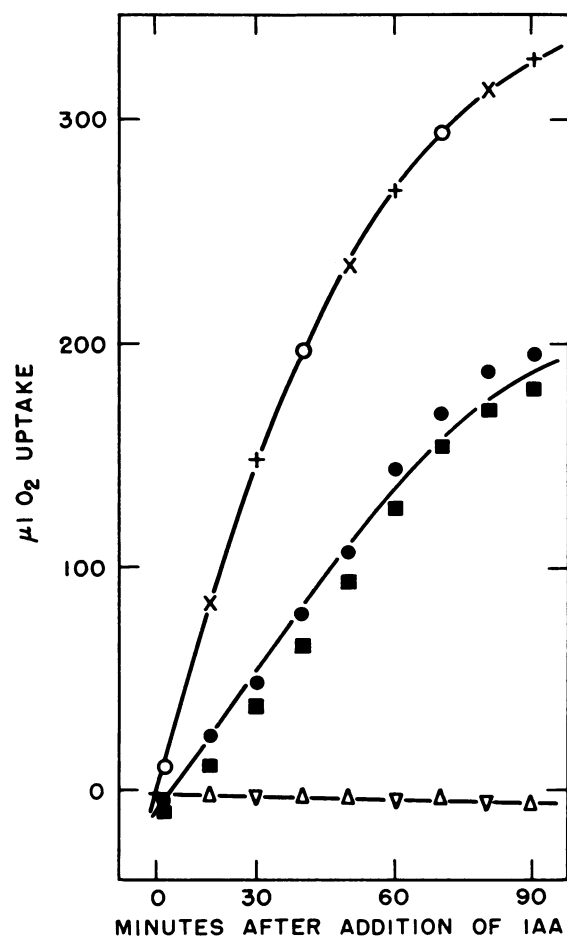


FIG. 1. Effect of cytochrome system on oxygen uptake with a dialyzed oxidase preparation, Mn^{++} , and 2,4-dichlorophenol. ○, reaction mixture without added ingredients; ×, cytochrome oxidase; ●, Al^{+++} ; △, cytochrome c; ■, cytochrome c + cytochrome oxidase; ●, cytochrome c + cytochrome oxidase + Al^{+++} ; ∇, cytochrome system minus IAA oxidase, or IAA oxidase plus the cytochrome system minus 2,4-dichlorophenol.

uct of IAA-oxidation, one must assume either its rapid further oxidation in the reaction sequence or that it is not formed in quantity under the experimental conditions. However, the first hypothesis cannot be substantiated since the oxidase obtained from lupine is incapable of utilizing indole-3-carboxaldehyde as a substrate.

In the present experiments it was found that, if the right concentration ratios of oxidase and cytochrome c are selected, a 50% depression in the oxygen uptake is observed as is nearly the case in figure 1. Before the concentration response data in table I were established, this restriction of the oxygen uptake was thought to indicate a complete shift in the metabolic pattern. However, the relationship between oxygen uptake and cytochrome c (table I) may be conservatively interpreted to suggest that cytochrome c is inhibitory up to a concentration of 10^{-6} M, at which level it is slightly stimulatory. The aldehyde response is parallel to the oxygen uptake in part, but the highest level of aldehyde is reached at 10^{-5} M cytochrome c, and the most efficient conversion of IAA to aldehyde is at 5×10^{-5} M cytochrome c. At this level, with 0.5 ml of enzyme, approximately 6 moles of IAA were oxidized by three moles of oxygen to give three moles of aldehyde. This molar ratio would be significant if the Salkowski assay could be assumed to be reliable; however, the 1-ml enzyme experiment (table I) and the C^{14} data (fig 4 and 5) suggest that this is not the case. It is attractive to suggest that the cytochrome system is competing as the terminal oxidase by diverting hydrogen from the peroxidase portion of the oxidase-peroxidase system so that a reducible intermediate is not regenerated.

The relation of the formation of the # 4 product

to the aldehyde and the cytochrome system is more clearly shown in the methylene- C^{14} labeled IAA experiments than the data in table I indicate.

In figure 2 the ultraviolet absorption spectrum of synthetic indole-3-carboxaldehyde is compared with that of the material obtained from the enzyme experiment, and the corresponding infrared absorption spectra are shown in figure 3. The present identification and isolation in quantity of indole-3-carboxaldehyde after action of IAA oxidase in presence of cytochrome c and cytochrome oxidase shows that the usual reaction sequence can be diverted from its main course. The relative significance of this part in the metabolism of IAA by plants remains to be established (5).

Two possible intermediate precursors of the aldehyde were tested with crude and purified enzyme, in the absence and in the presence of the cytochrome c system. Indole-3-glyoxylic acid was totally inert even with the crude enzyme, since there was no oxygen uptake or product formation as shown by the chromatograms, while indole-3-glycolic acid (table II) gave the aldehyde in substantial amounts independent of the cytochrome system. Neither acid shows any oxygen uptake with the cytochrome system in the absence of the oxidase, although a trace of the aldehyde could be detected on the chromatograms from the reaction of indole-3-glycolic acid and the cytochrome system. Recently Greenburg and Galston (4) have observed the instability of indole-3-glycolic acid and have suggested a role for it in the formation of auxin. Indole-3-glyoxylic acid should presumably be the intermediate between indole-3-glycolic acid and the aldehyde; the fact that it was not utilized by IAA oxidase suggests that decarboxylation occurs simultaneously with dehydrogenation.

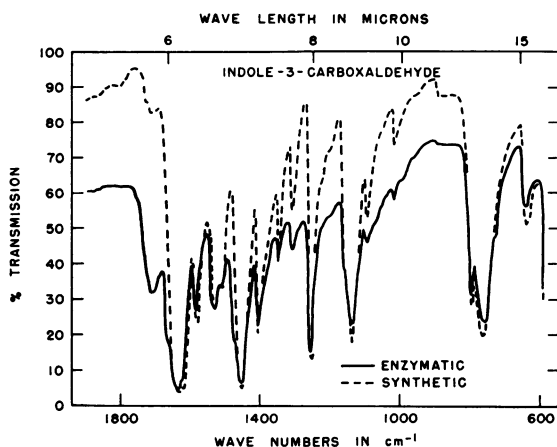


FIG. 3. Infrared spectra of synthetic and enzymatic indole-3-carboxaldehyde; the KBr disc technique was used. In the region not shown in the figure, the spectra of the two compounds were essentially identical.

To shed light on the primary site of oxidation, three substituted IAAs were studied with enzyme preparations of different degrees of purity. The results with crude and with highly purified IAA oxidase are given in table II. Despite the substitution in the side chain, indole-3-isobutyric acid was found to be a better substrate than IAA in that it supported a higher oxygen uptake, and, in the case of the electrophoretically homogeneous enzyme, the chromatograms showed no residual substrate. Chromatograms of the reaction mixture from the crude enzyme showed residual substrate. Similar oxygen uptake data have been reported by Ray and Thimann (10) with the enzyme from *Omphalia flavida*.

TABLE II
RESPONSE OF SUBSTITUTED INDOLE-3-ACETIC ACID TO THE OXIDASE FROM *L. ALBUS*

SUBSTRATE	CYTOCHROME SYSTEM (2×10^{-5} M CYTOCHROME c)	CRUDE OXIDASE FROM ETIOLATED LUPINE HYPOCOTYLS				ELECTROPHORETICALLY HOMOGENOUS OXIDASE			
		UPTAKE * OXYGEN	IAA USED **	PRODUCT # 4 †	ALDE- HYDE ‡	OXYGEN UPTAKE *	IAA USED **	PRODUCT # 4 †	ALDE- HYDE ‡
IAA	-	10.6	16	0.69	0.21	15.2	22	1.06	1.03
	+	11.4	16	0.69	3.93	11	16.3	0.67	5.17
Indole-3-glycolic acid	-	6.5	...	0.085	16.1	9.25	...	0.17	24.9
	+	7.2	...	0.12	18.2	8.84	...	0.17	25.9
Indole-3-isobutyric acid	-	16.1	...	1.08	0.21	20.9	...	1.89	1.03
	+	19	...	1.73	0.21	20.3	...	2.91	1.45
1-Methylindole-3-acetic acid	-	11.4	...	0.65	0.41	6.83	...	0.64	0.85
	+	12	...	0.67	3.86	8.0	...	0.69	5.59
2-Carboxyindole-3-acetic acid	-	10	...	0.1	0.0	0.0	...	0.0	0.0
	+	4.3	...	0.0	0.0	0.0	...	0.0	0.0

All values are expressed in micromoles.

* These values were obtained after 90 min at 30° C. The initial substrate concentration was 30 micromoles.

** Salkowski test with IAA as the reference standard.

† The analysis of product # 4 and of indole-3-carboxaldehyde is described in the text.

‡ In the colorimetric determination, indole-3-carboxaldehyde was used as the reference standard. This restricts the accuracy of the results obtained with the IAA derivatives.

Considering the level of substrate utilized and the oxygen uptake, the amount of substance reacting with 2,4-dinitrophenylhydrazine was low; it is possible that an oxindole (11) causes the response. However, these data suggest that the alpha carbon of the side chain is not the primary site of oxidation. It appears rather that the ring system is the site of attack and that ring substitution should block the oxidation. Two compounds of this type were tested: 1-methylindole-3-acetic acid and 2-carboxylindole-3-acetic acid.

With the purified IAA oxidase, no oxygen uptake was observed with 2-carboxylindole-3-acetic acid as a substrate. The ability of the frozen crude enzyme preparations from 10-day-old etiolated hypocotyls, in contrast to the electrophoretically purified material, to metabolize the 2-carboxy-IAA constitutes the first evidence of a restriction in range introduced by purification of the system. This suggests that part of the changes in activity observed on aging and purification of the crude enzyme from both etiolated and non-etiolated tissue may be caused by the loss of some essential material. The dialyzed enzyme gave results similar to the electrophoretically purified enzyme prepara-

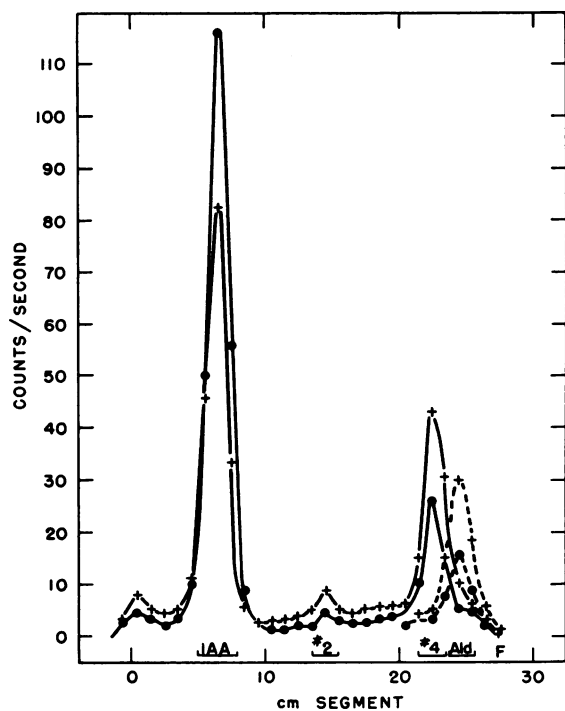


FIG. 4. Paper chromatography of the products from IAA-2-C¹⁴ obtained by enzymatic reaction in TRIS buffer at pH 7.2. The locations of residual IAA, products # 2, # 4, indole-3-carboxaldehyde, and of the solvent front are indicated. The reaction time was 30 minutes (●), or 90 minutes (+). The solid lines show the response to the IAA oxidase system alone, the broken lines the results with addition of the cytochrome c-cytochrome oxidase system (2×10^{-5} M cytochrome c).

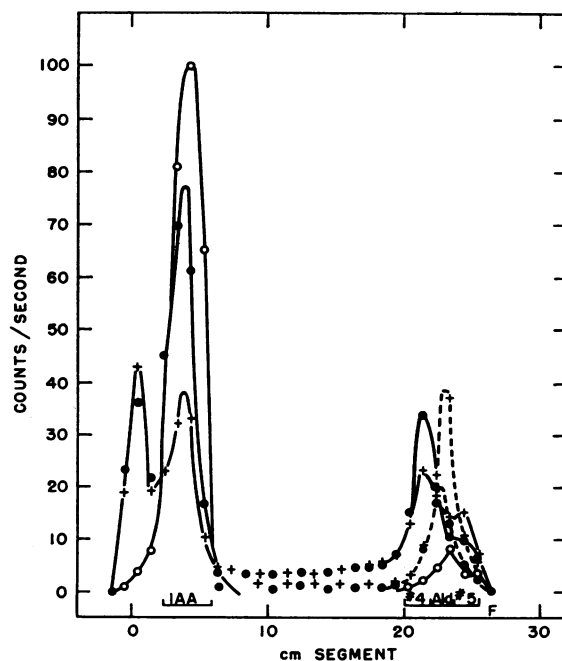


FIG. 5. Paper chromatography of the reaction products from IAA-2-C¹⁴ obtained in phosphate buffer, pH 6.3. The solid lines show the effect of the oxidase system, the broken lines indicate the results in presence of IAA oxidase and cytochrome system (2×10^{-5} cytochrome c). The reaction times were 30 minutes (●) and 90 minutes (+). Shaking of the IAA for 90 minutes (○) without enzyme proved that little spontaneous decomposition had taken place.

tion. In further experiments, none of the lupine enzyme preparations has been found capable of oxidizing 5-hydroxy-IAA, tryptophan, indole-3-acetonitrile, indole-3-carboxaldehyde, or indole-3-carboxylic acid.

Attempts were made to amplify and corroborate the data on the reaction by experiments with C¹⁴-labeled IAA, with the label either in the carboxyl group (IAA-1-C¹⁴) or in the methylene group (IAA-2-C¹⁴) of the side chain (12, 13).

The concentration of IAA-2-C¹⁴ declined during the course of the reaction, as measured by the decrease in its C¹⁴ counts, and increasing activity was recovered in the products. This correlation was observed with samples taken after 30-, 60-, and 90-minute reaction periods (see figs 4 and 5, in which the 60-minute values are omitted). Fig 4 shows the activity distribution found on the chromatograms when the oxidase used IAA-2-C¹⁴ as a substrate, in the presence and absence of the cytochrome system, in TRIS buffer at pH 7.2. Similarly, figure 5 shows the response to the same enzymes in phosphate buffer at pH 6.3. pH 6.3 is the optimum for the partially purified oxidase used, while the pH 7.2 TRIS buffer represents compromise with the optimum for cytochrome oxidase.

The effect of the cytochrome system (minus the

IAA oxidase) after 90 minutes is also shown in figure 5 by the presence of activity in the aldehyde and product # 5 areas of the chromatogram. The predominant shift in activity from the # 4 product to the aldehyde, which is found on the addition of the cytochrome system in both figures 4 and 5, is evidence of a major change in the reaction pathway.

The unstable and apparently complex reaction product # 4 (fig 4 and 5) may contain the oxindole described by Ray and Thimann (10). However, when this fraction was removed from the chromatograms and examined, the ultraviolet and infrared spectra were complex, and on rechromatography, three or four components were found, including one which appears to be an anthranilate. It is assumed that oxidation or decomposition, or both, occurred during manipulation and rechromatography.

The immobile fraction found below IAA on the chromatogram (fig 5) could be detected only by the tracer technique; it is neither fluorescent nor does it show indolyl reactions. Phosphate buffer at pH 6.3 favors its formation. The presence of the cytochrome-cytochrome oxidase system had little influence on its formation. Infrared spectra of this material indicated the absence of phenyl or indolyl groups; the only discernible functional groups were methylene and carboxyl groups. The aliphatic nature of this reaction product is thus indicated, and because of the immobility on chromatograms, a strongly acid character is suggested.

Experiments with carboxy- C^{14} -IAA yielded predominantly non-radioactive products; the C^{14} was recovered as $BaC^{14}O_3$ from the center well. An exception to this was the non-indolyl product # 2 formed in TRIS buffer (fig 4), which still carried C^{14} , whether carboxyl- or methylene-labeled IAA was used as a substrate. It is weakly acidic, shows green fluorescence, and is assumed to be aromatic. The buffer used for its formation is immaterial.

The present data show that catabolism of IAA may occur by more than one route. With respect to the primary site of action of IAA oxidase, the observations are in harmony with the results of other laboratories (7, 10, 11); the ring system is altered by the primary attack. Further work will be required to unravel the reaction sequence and identity of the products.

The author wishes to acknowledge the assistance of Mrs. Sara Berliner in carrying out many of these experiments.

SUMMARY

Indole-3-carboxaldehyde is a major reaction product of the action of IAA oxidase provided the system is coupled to the cytochrome-cytochrome oxidase system. Indole-3-glycolic acid yields indole-3-carboxaldehyde with equal efficiency both in the absence and

presence of cytochrome c. Experiments with substituted indole-3-acetic acids as substrate indicate that the ring system, not the side chain, is the primary site of oxidation. The two position of the indole ring is probably the primary site of dehydrogenation. The use of IAA-2- C^{14} as substrate made possible the detection of a reaction product which apparently is an aliphatic acid. The formation of this product is unaffected by the cytochrome system suggesting that it is the terminal oxidation product.

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