Krasnovsky, A. A. (1955), *Dokl. Akad. Nauk SSSR 103*, 283.
Krasnovsky, A. A. (1961), *Ann. Rev. Plant Physiol. 12*, 363.
Linschitz, H., and Rennert, J. (1952), *Nature 169*, 193.
Linschitz, H., and Sarkanen, K. (1958), *J. Am. Chem. Soc. 80*, 4826.
Livingston, R. (1955), *J. Am. Chem. Soc. 77*, 2179.
Livingston, R. (1960), *Quart. Rev. (London) 14*, 174.
Livingston, R., and Ryan, V. A. (1953), *J. Am. Chem. Soc. 75*, 2176.
Nieman, R. H., and Vennesland, B. (1959), *Plant Physiol. 34*, 255.
Porter, G. (1963), *Proc. Roy. Soc. (London), Ser. B: 157*, 293.
Rabinowitch, E., and Weiss, J. (1937), *Proc. Roy. Soc. (London), Ser. A: 162*, 251.
Seely, G. R. (1965), *J. Phys. Chem. 69* (in press).
Tollin, G., and Green, G. (1962), *Biochim. Biophys. Acta 60*, 524.
Tollin, G., and Green, G. (1963), *Biochim. Biophys. Acta 66*, 308.
Vernon, L. P. (1961a), *Acta Chem. Scand. 15*, 1645.
Vernon, L. P. (1961b), *Acta Chem. Scand. 15*, 1651.
Vernon, L. P. (1963), *in* Bacterial Photosynthesis, Gest, H., San Pietro, A., and Vernon, L. P., eds., Yellow Springs, Ohio, Antioch Press, p. 235.
Vernon, L. P., Shaw, E., Zaugg, W. S., and Ke, B. (1964), *Federation Proc. 23*, 227.
Vernon, L. P., Zaugg, W. S., and Shaw, E. (1963), *Natl. Acad. Sci.–Natl. Res. Council, Publ. 1145*, 509.
Zaugg, W. S. (1963), *Proc. Natl. Acad. Sci. U.S. 50*, 100.
Zaugg, W. S. (1964), *J. Biol. Chem. 239*, 3964.
Zaugg, W. S., Vernon, L. P., and Tirpak, A. (1964), *Proc. Natl. Acad. Sci. U.S. 51*, 232.
Zieger, G., and Witt, H. T. (1961), *Z. Physik. Chem. (Frankfurt) 28*, 273.
Zscheile, F. P., and Comar, C. L. (1941), *Botan. Gaz. 102*, 463.

Peroxidase-catalyzed Oxidation of Indole-3-acetic Acid*

R. L. Hinman and J. Lang

ABSTRACT: A thorough study of the products of the *in vitro* oxidation of indole-3-acetic acid (IAA), catalyzed by horseradish peroxidase in the absence of added hydrogen peroxide, has shown that at substrate concentrations of 2×10^{-4} M and below 3-methyleneoxindole is the end product and oxindole-3-carbinol is its precursor. To explain these products a reaction sequence for peroxidase-catalyzed oxidation of IAA is proposed in which the peroxidase functions as a one-electron oxidizing agent and an indolenine hydroperoxide is the first intermediate. The hydroperoxide is converted to oxindole-3-carbinol via an indolenine epoxide.

The oxidation of IAA is concentration dependent and at higher concentrations a neutral indole appears to be the principal product. Oxidation of indole-3-alkanoic acids by a variety of oxidizing agents shows that 3-alkylideneoxindoles are very common products of oxidation of 3-alkylindoles, but that several pathways are possible for the transformation.

The existence of enzyme systems in higher plants that catalyze the oxidative degradation of the plant-growth hormone indole-3-acetic acid (IAA)[1] has been recognized for many years, but the detailed pathway of auxin destruction has not been elucidated. The general status of the problem has been reviewed by Ray (1958).

On the other hand considerable progress has been made toward understanding the enzymes involved. These appear to be peroxidases (Ray, 1958, 1960) and this peroxidase-catalyzed oxidation of IAA is of particular interest as one of the few known cases in which oxygen is consumed and exogenous hydrogen peroxide is not required (Mason, 1957). In addition, it has recently been suggested that the oxidation may play a role in the growth-regulating functions of IAA (Kefford *et al.*, 1963).

In an effort to elucidate the reaction path, we have studied the behavior of IAA and a variety of related indolic materials in the presence of peroxidase from horseradish. A number of chemical models of the peroxidase system have also been designed to furnish additional information. In general, reactions have been followed and ultimate products have been identified where possible by ultraviolet absorption measurements. This method permits examination of unstable intermediates and products in the reaction mixture and minimizes the possibility of further reactions which may accompany isolation techniques (Ray, 1958).

* From the Union Carbide Research Institute, Union Carbide Corporation, Tarrytown, N.Y. *Received August 24, 1964.*

[1] Abbreviation used in this work: IAA, indole-3-acetic acid.

TABLE I: Reactions of Indoles with Oxidizing Agents.[a]

Indole	Peroxidase O_2	Peroxidase H_2O_2	*p*H 1 H_2O_2	*p*H 1 O_2	Fe^{+3} O_2	OX^- NCS NBS	$K_2S_2O_8$	NO_2^-	$h\nu/O_2$[b]
IAA	AO[c]	AO	AO	AO	AO[c]	AO	AO[d]	AO	AO
α-Methyl-IAA	AO[c]	AO	AO	AO	AO[e]				
α,α-Dimethyl-IAA	AO[e]		AO		NR (24)[c]				
1-Methyl-IAA	AO[f]		AO	AO					
2-Methyl-IAA	+[f]		+						
1,2-Dimethyl-IAA	VS		+						
2-Phenyl-IAA			+						
Ethyl indole-3-acetate	VS	OX			NR (24)				
Indole-3-propionic acid	NR (71)[c,g]		+[g]		NR (24)				
Indole-3-butyric acid	NR (71)[c]		AO[d]		NR (24)				
Skatole	NR (96)[c]	+	NR (5)		NR (24)				
3-Ethylindole			AO						
Tryptamine	NR (94)		AO						
Tryptophan	NR (94)		AO						

[a] All reactions carried out at spectrophotometric concentrations (10^{-4}–10^{-5} M indole) and product identification based on analysis of spectrum of reaction mixture. AO = 3-alkylideneoxindole; OX = oxindole or dioxindole; NCS = *N*-chlorosuccinimide; NBS = *N*-bromosuccinimide; + = reaction occurred but product was not identified; VS = very slow reaction to unidentified product; NR = no reaction for number of hours shown in parentheses. [b] With photosensitizer (Fukuyama and Moyed, 1964) or without (Ray and Curry, 1958). [c] At *p*H 3.5 (citrate) or 5.0 (acetate). [d] At *p*H 1 or 5.0. [e] At *p*H 3.5 (citrate); little or no reaction at *p*H 5.0. [f] Tried only at *p*H 5.0. [g] See Hinman and Frost (1961) for full description of spectra.

Peroxidase-catalyzed Oxidations in the Presence of Air—No Hydrogen Peroxide Added. Identification of Products. In solution the *ultimate* products of the peroxidase-catalyzed oxidation of IAA appear to be two: 3-methyleneoxindole and a neutral indole. A most important point is the discovery that the product composition is highly concentration dependent. At IAA concentrations of 2×10^{-4} M and below and peroxidase concentrations of 10^{-6}–10^{-8} M, the rapid enzymatic reaction was followed by slower nonenzymatic changes leading finally to 3-methyleneoxindole (compound VI, Chart I) as the principal product. At the lowest concentrations used a small amount of the indolic component was always present, and was recognized by the typical indolic shoulders at 277 and 285 mμ in the ultraviolet spectrum (Figure 1). This material was mistaken at first for residual IAA but it persisted long after the IAA had been consumed as shown by the Salkowski test, and, as discussed later, it was nonacidic. It appears from the published spectra that this substance was also a product from the action of the *Omphalia* peroxidase on IAA (Ray, 1956).

The proportion of the indolic component increased with increasing IAA concentration until at 10^{-2} M only indolic material was formed, even when the enzyme concentration was increased in proportion to the increase in substrate concentration (Figure 1).

In a preliminary account of this work (Hinman *et al.*, 1961) the nonindolic product was identified as 3-methyleneoxindole by the presence of its characteristic intense double peaks at λ_{max} 247 and 253 mμ (Figure 1) in the ultraviolet absorption spectra of the reaction mixtures. In subsequent work this previously unknown compound has been synthesized and characterized further by reactions typical of an α,β-unsaturated amide (Hinman and Bauman, 1964a).

The high reactivity of 3-methyleneoxindole, particularly toward dimerization or polymerization (Hinman and Bauman, 1964a), precluded its isolation from enzymatic reaction mixtures. Since 3-ethylideneoxindole is a more stable substance, α-methyl-IAA was used as a substrate in the peroxidase-catalyzed oxidation. 3-Ethylideneoxindole was identified as the principal product by the ultraviolet spectrum of the reaction mixture, and a small amount of this product was isolated and identified by comparison of its infrared spectrum with that of an authentic specimen.

The generality of the enzymatic reaction path in the presence of air is shown by the examples in Table I. 3-Isopropylideneoxindole was the principal product of the peroxidase-catalyzed oxidation of α,α-dimethylindole-3-acetic acid and 1-methyl-3-methyleneoxindole was formed from 1-methylindole-3-acetic acid. Oxidation of 2-methylindole-3-acetic acid was also catalyzed by peroxidase but, as would be expected, the product was not of the oxindole type (Figure 2). It was not characterized further. When both the 1- and 2- positions were substituted by methyls, reaction was very slow. In addition, the spectra of indole-3-propionic and -butyric acids, skatole, and ethyl indole-3-acetate were

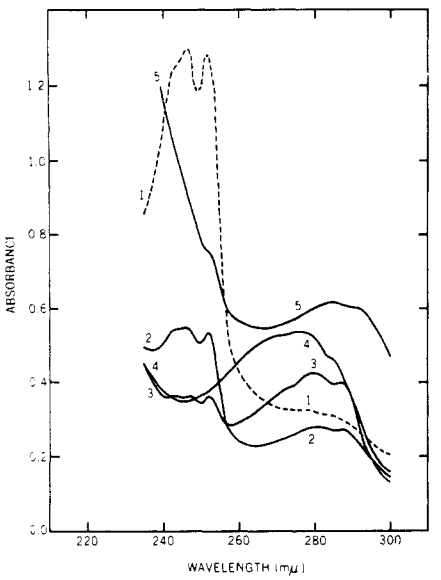


FIGURE 1: Effect of IAA and peroxidase concentration on product composition. All reactions carried out in 0.2 M acetate buffer (*p*H 5.0) for 24 hours at 25°. Curve 1, 2×10^{-4} M IAA + 10^{-7} M peroxidase; curve 2, 5×10^{-4} M IAA + 10^{-7} M peroxidase; curve 3, 10^{-3} M IAA + 10^{-7} M peroxidase; curve 4, 10^{-2} M IAA + 10^{-4} M peroxidase; curve 5, 5×10^{-4} M IAA + 10^{-6} M peroxidase. Above reaction nixtures diluted to what would be an initial IAA concentration of 10^{-4} M for recording spectra.

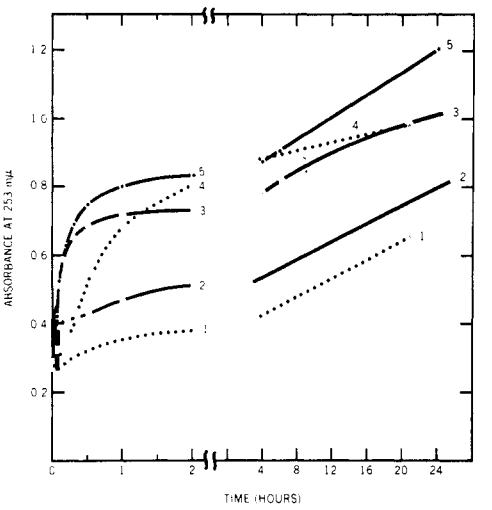


FIGURE 3: Rates of increase of ultraviolet absorption at 253 mμ of IAA and 1-methyl-IAA with hydrogen peroxide and oxygen as oxidants. Characteristic peaks of 3-alkylideneoxindole appeared in all cases well before the last recorded time. All initial indole concn = 10^{-4} M. Curve 1, 1-methyl-IAA in HCl-KCl buffer (*p*H 1), standing in air; curve 2, IAA + H_2O_2 (10^{-3} M) in HCl-KCl buffer (*p*H 1); curve 3, IAA + peroxidase (10^{-6} M), no added H_2O_2, in 0.05 M citrate buffer, *p*H 3.5; curve 4, 1-methyl-IAA + H_2O_2 (10^{-3} M) in HCl-KCl buffer (*p*H 1); curve 5, IAA + peroxidase (10^{-6} M) + H_2O_2 (2×10^{-4} M) in 0.05 M citrate buffer, *p*H 3.5.

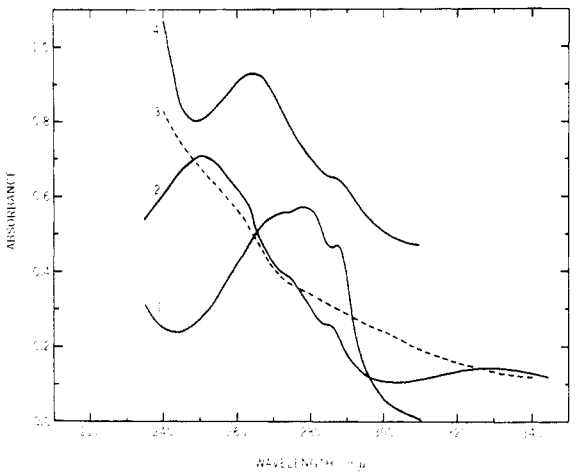


FIGURE 2: Ultraviolet absorption spectra of reaction products of peroxidase-catalyzed reactions of various indoles. All indole concentrations were 10^{-4} M prior to reaction. Curve 1, ethyl indole-3-acetate + peroxidase (10^{-6} M) in 0.05 M citrate buffer, essentially unchanged after 2 hours; curve 2, same as curve 1, 2 hours after addition of hydrogen peroxide (final concn = 2×10^{-4} M); curve 3, skatole + peroxidase (10^{-7} M) in 0.05 M citrate buffer (*p*H 3.5), 5 minutes after mixing; curve 4, 2-methyl IAA + peroxidase (10^{-7} M) in 0.2 M acetate buffer (*p*H 5) after 48 hours. (Absorbancy readings increased 0.4 unit.)

unchanged after many hours with peroxidase at *p*H 5, and in the first three cases at *p*H 3.5 where the enzyme exhibits higher activity (see below). (When hydrogen peroxide was added, all these compounds reacted rapidly.)

It should also be noted in Table I that conversion of indole-3-acetic acids to 3-alkylideneoxindoles is a very common oxidative pathway, not confined to enzyme-catalyzed reactions. Even exposure to air of acidified solutions of IAA and its α-methyl and 1-methyl derivatives caused this type of transformation to varying degrees (Figure 3).

While the above observations concern the *ultimate* products of the *in vitro* reactions, it is certain that 3-methyleneoxindole is formed at some stage after the enzymatic reaction and this may be true of the indolic product as well. For the former product this can be shown in a variety of ways. With IAA at 10^{-4} M and peroxidase at 10^{-7} M at *p*H 5, the IAA was consumed in about 1 hour. With peroxidase at 10^{-6} M no IAA remained after 6–8 minutes (Figure 4). In both cases, however, formation of 3-methyleneoxindole continued for upward of 24 hours (Figure 4). The rate of formation of 3-methyleneoxindole was greatly accelerated by heat. After completion of the enzymatic stage, conversion to 3-methyleneoxindole could be effected completely in 10–15 minutes at 100°. Cyanide was added before heating to ensure that the acceleration was not caused by increased enzyme activity. When cyanide was added

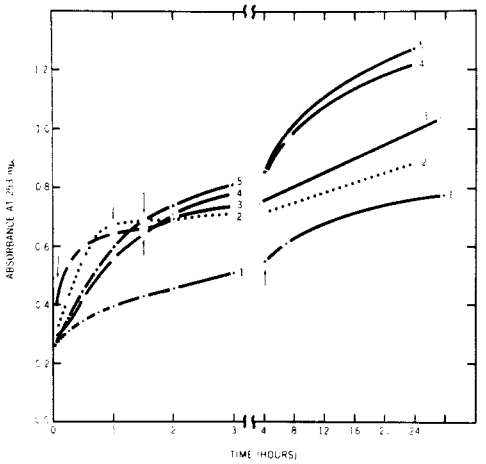


FIGURE 4: Rate of increase of ultraviolet absorption at 3-methyleneoxindole absorption max (253 mμ) during peroxidase-catalyzed IAA oxidation, as a function of buffer and enzyme concentration. Arrows indicate time of complete consumption of IAA (by Salkowski test). Initial IAA concn = 10^{-4} M in all cases. Curve 1, 5.8×10^{-8} M peroxidase (RZ = 3.0) in 0.005 M acetate buffer (pH 5.0); curve 2, 5.2×10^{-8} M peroxidase (RZ = 3.0) in 0.005 M citrate buffer (pH 3.7); curve 3, 10^{-6} M peroxidase (RZ = 0.64) in 0.2 M acetate buffer (pH 5.0); curve 4, 5.8×10^{-8} M peroxidase (RZ = 3.0) in 0.05 M acetate buffer (pH 5.0); curve 5, 10^{-7} M peroxidase (RZ = 0.64) in 0.2 M acetate buffer (pH 5.0).

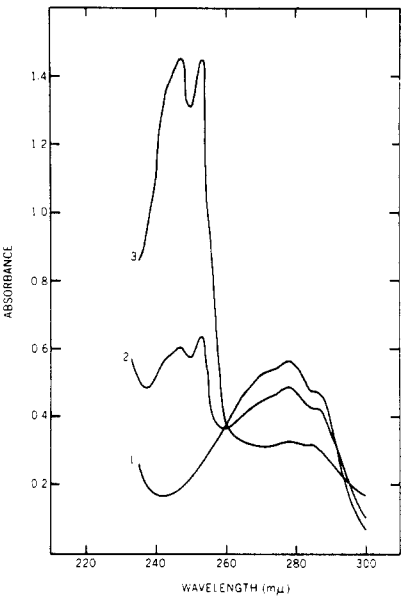


FIGURE 5: Relationship of extent of consumption of IAA in peroxidase-catalyzed oxidation to yield of 3-methyleneoxindole. Curve 1, IAA (10^{-4} M) in 0.2 M acetate buffer (pH 5.0); curve 2, IAA + peroxidase (10^{-7} M); 4×10^{-3} mmoles KCN added after 5 minutes and solution heated for 20 minutes in boiling-water bath; no further reaction occurred during 24 additional hours at room temperature; curve 3, same as 2 but KCN added after 62 minutes when all IAA had been consumed (by Salkowski test).

before completion of the enzymatic stage, the per cent conversion to methyleneoxindole was proportional to the per cent of IAA which had been consumed (Figure 5).

The last and perhaps most significant evidence is that 3-methyleneoxindole can be extracted from the aqueous reaction mixture with ether. The spectrum of the residual aqueous solution, which is similar to that of an oxindole, undergoes a gradual change to that of 3-methyleneoxindole, which can again be removed by extraction. This process can be repeated a number of times (Figure 6) and the same results were obtained when cyanide was added prior to the first extraction. Clearly, it is a nonenzymatic stage which is being observed.

Although 3-methyleneoxindole is the product of a postenzymatic step, it is the first product of the peroxidase-catalyzed oxidation of IAA to be unambiguously identified. As such it serves as the key for elucidating the complete reaction path including the enzymatic stage, where the closest correspondence between *in vivo* and model systems is likely to reside.

The identity of the precursor to 3-methyleneoxindole was suggested by the work of Fukuyama and Moyed (1964), who found that two principal products were formed in the nonenzymatic photosensitized oxidation of IAA in aqueous solution. The minor product was 3-methyleneoxindole, while the major one was a more polar substance with an oxindolic ultraviolet absorption spectrum, for which oxindole-3-carbinol (compound V) was suggested as a reasonable structure. The relationship of the two substances and the importance of the buffer strength to their interconversion was established by dissolving the more polar material in 0.2 M acetate buffer. Within 90 minutes the characteristic double maxima of 3-methyleneoxindole began to appear. Equilibrium was reached after 12 hours.

We have separated the products of the *peroxidase-catalyzed* oxidation of IAA using the chromatographic conditions employed by Fukuyama and Moyed (1964). Although a number of products were invariably present, spots corresponding to those of the photo-oxidation products had the same ultraviolet spectra after elution as those reported. When aliquots of the reaction mixture were removed periodically over a 24-hour period, chromatography revealed a steady decrease in the quantity of the more polar substance and an increase in 3-methyleneoxindole. Moreover, the ultraviolet spectrum of the more polar substance changed slowly in water, and more rapidly in 0.05 M acetate buffer (pH 5), to the spectrum of 3-methyleneoxindole. These changes correspond to those observed in the aqueous layer after ether extraction (Figure 6).

Since oxidation of higher concentrations of IAA follows a different pathway, a preparative-scale reaction on 204 mg of IAA was carried out by adding small portions to the reaction mixture after each

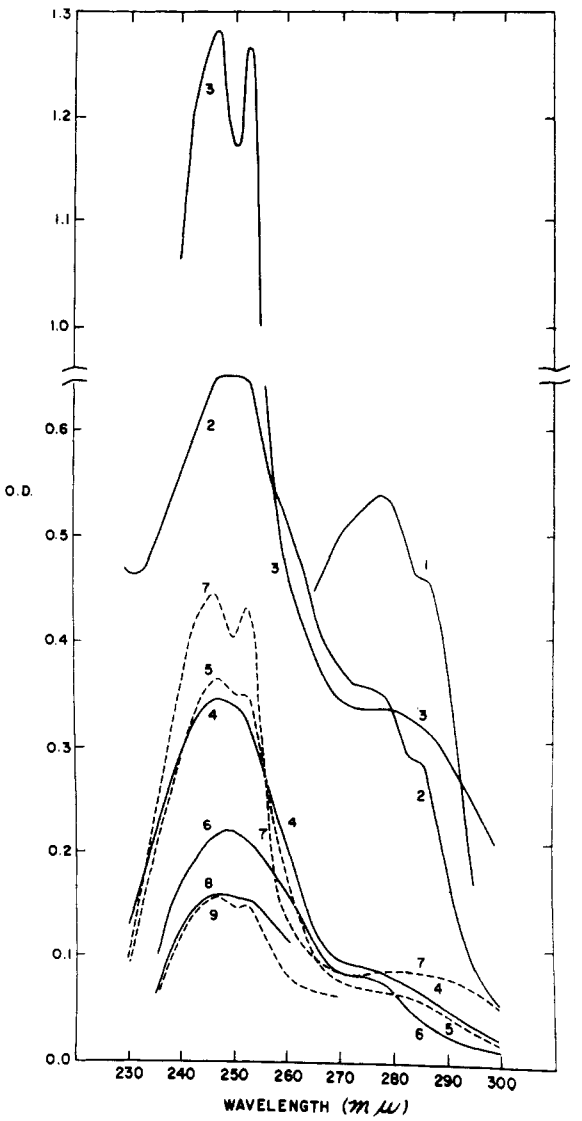


FIGURE 6: Spontaneous formation of 3-methyleneoxindole from the oxindolic product of peroxidase-catalyzed oxidation of IAA. Curve 1, IAA (10^{-4} M); curve 2, IAA (10^{-4} M) + peroxidase (RZ = 0.64, 10^{-7} M) after 76 minutes; curve 3, same as curve 2, after 23 hours; curve 4, water layer after extraction of solution 3 with ether; curve 5, solution 4 after 2.75 hours at room temperature; curve 6, water layer after extraction of solution 5 with ether; curve 7, solution 6 after standing 18.5 hours; curve 8, water layer after extraction of solution 7 with ether; curve 9, solution 8 after standing 3 days.

preceding quantity had been consumed. In this way 6 mg of a yellow solid was obtained which had the typical ultraviolet absorption spectrum of an oxindole. The intensities calculated on the assumption that the compound was pure oxindole-3-carbinol agreed very well with those reported for 3-methyloxindole and related compounds (Hinman and Bauman, 1964a). The infrared spectrum showed the typical >NH oxindole carbonyl bands and an additional band in the hydroxyl region at 2.94 μ. The two bands near 3 μ correspond very closely to those of 3-methyldioxindole (Hinman and Bauman, 1964a), but the latter is excluded by its different behavior on paper chromatograms. No evidence of a carboxyl carbonyl band was found. Although the NMR spectrum showed extraneous peaks at high fields, probably because of polymeric material, the remainder of the spectrum was completely consistent with the proposed structure.

These data show that the precursor to 3-methyleneoxindole is an oxindole more polar than 3-methyleneoxindole, that it bears an additional hydroxyl group, that it appears (from the NMR data) to have the 3-proton of an oxindole together with a larger number of protons on a carbon bearing an electron-withdrawing group, and that it is readily converted to 3-methyleneoxindole. The results leave little doubt that the precursor to 3-methyleneoxindole is oxindole-3-carbinol, especially in view of the fact that there is no major skeletal difference between IAA and 3-methyleneoxindole, only one carbon having been lost. Experiments with labeled IAA and IAA-oxidases from other sources have shown that the carboxyl carbon is lost and the 2-carbon and the methylene carbon are retained (Ray and Thimann, 1956; Stutz, 1958).

As the conjugate addition product of an α,β-unsaturated amide, oxindole-3-carbinol is a reasonable precursor to 3-methyleneoxindole. The slow conversion to the last compound is in accord with a slow approach to equilibrium. The effect of the acetate buffer concentration on the rate is explained by increased availability of acid to protonate the hydroxyl and/or of base to abstract the reactive 3-proton in a typical dehydration step. The extreme ease of elimination in this type of system has been noted previously; 1-methyl-3-(methoxymethylene)oxindole underwent loss of methanol even in the presence of mild catalysts such as barium carbonate (Wenkert *et al.*, 1953). The high lability accounts for the lack of success of previous attempts to synthesize oxindole-3-carbinol (Horner, 1941; Hellmann and Renz, 1951).

Proposed Pathway for Conversion of IAA to 3-Methyleneoxindole. Recognition of the importance of the buffer concentration to the rate of formation of 3-methyleneoxindole from its precursor has enabled us to relate our results with horseradish peroxidase to Ray's results with the *Omphalia* peroxidase. In earlier work (Hinman *et al.*, 1961) we found that methyleneoxindole formation was already evident after periods of time in which Ray's published spectra (1956) showed barely perceptible development beyond an oxindole stage. By reducing the citrate buffer concentration to the level used by Ray, we have found that the change of the spectra of the reaction mixture with time parallels Ray's results almost exactly, and that methyleneoxindole formation is very slow (Figure 7). The close correspondence of the ultraviolet absorption curves shows that Ray's compound B is oxindole-3-carbinol.

Ray has already pointed out (1956, 1960) in a very careful study the close similarity of the peroxidases

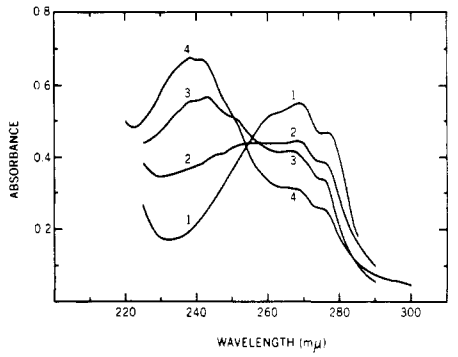


FIGURE 7: Effect of buffer composition and concentration on rate of IAA oxidation measured by absorption spectrum at end of first hour. All reactions contained 10^{-4} M IAA and peroxidase of RZ = 3.0. Curve 1, IAA in 0.2 M acetate buffer (pH 5); curve 2, IAA + peroxidase (5.8×10^{-8} M) in 0.005 M acetate buffer (pH 5.0); curve 3, IAA + peroxidase (5.8×10^{-8} M) in 0.05 M acetate buffer (pH 5.0); curve 4, IAA + peroxidase (5.2×10^{-8} M) in 0.005 M citrate buffer (pH 3.7).

from *Omphalia* and horseradish with respect to certain aspects of IAA oxidation. The identity of the chemical intermediates now confirms this point of view. We can therefore infer from Ray's work (1956) that in the horseradish peroxidase system carbon dioxide evolution parallels oxygen uptake and that the latter occurs in close conjunction with the enzymatic phase of the reaction.

Identification of the remaining steps in the oxidation of IAA is aided by recognition of the fact that *this reaction is one of the relatively rare examples of indole oxidation in which the hetero ring is not cleaved.* It is a very general rule that the first intermediate in the reaction of an indole with a wide variety of oxidizing agents is a β-hydroperoxy- or a β-hydroxyindolenine (Witkop and Patrick, 1951a; Ek *et al.*, 1952). In the next stage of oxidation two paths are available. Rearrangement of the hydroperoxide with addition to the indolenine double bond and concomitant ring opening is the more common route in both aqueous and nonaqueous media. The enzymatic cleavage of tryptophan (Knox and Mehler, 1950) has been formulated in this way (Ek *et al.*, 1952). Ring cleavage does not occur if addition of another group to the indolenine double bond can compete with rearrangement of the hydroperoxide. This group of reactions includes intramolecular addition of a pendant group such as hydroxyl (Witkop, 1950) or amino (Ek *et al.*, 1952), and, as in the case of indole itself, intermolecular dimerization as in indigo formation (Witkop and Patrick, 1951b). Ring opening is also avoided if the hydroperoxy group rearranges to a site other than the 2- position (Wasserman and Floyd, 1963).

Since ring cleavage of IAA does not take place, either the first step in the autoxidation is unique and occurs at the 2- position, or initial attack occurs as usual at the 3- position, but the common rearrangement and ring-opening process is deflected from its normal course by the unique structural character of the side chain. Acceptance of oxindole-3-carbinol as the precursor to 3-methyleneoxindole requires that the exocyclic double bond be formed after the carbonyl group. It is difficult to see how this could be accomplished if initial oxidation occurs at the 2- position. A normal first step is therefore preferred, and on this basis the pathway which best accommodates the known facts is shown in Chart I.

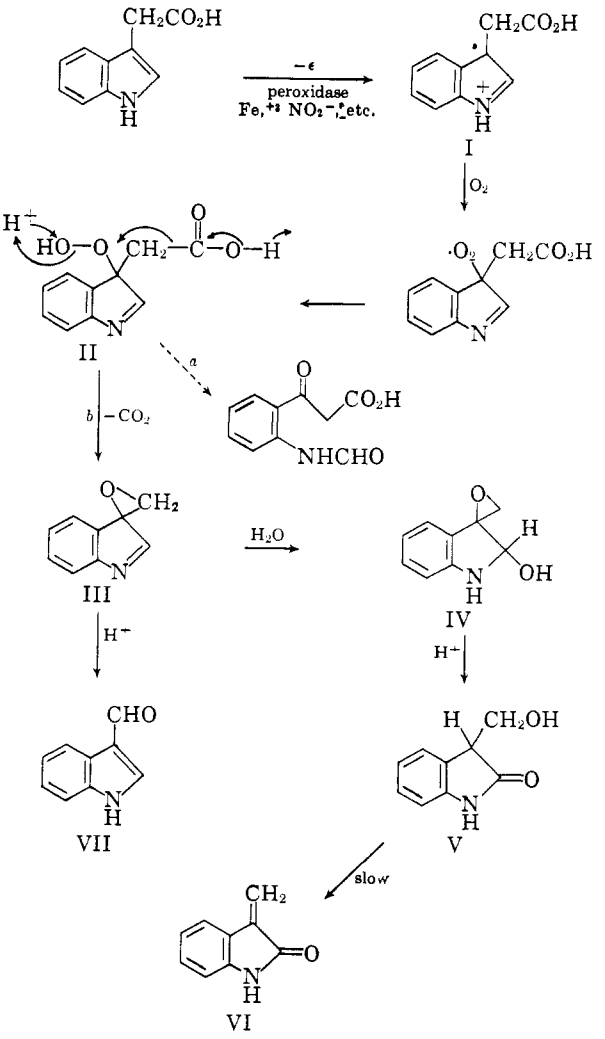
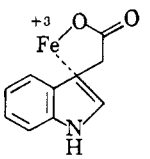


CHART I

The first step of this scheme is a one-electron oxidation of the indole ring in which Fe^{+3}, NO_2^-, and peroxidase all function in the same way to produce the free radical (I).[2] This is in keeping with the proposals of

[2] Suggestions that attack occurs by electron abstraction from the carboxylate anion (Waygood *et al.*, 1956) or by substitution of the side-chain methylene group are very improbable in view of the ease of oxidation of the indole nucleus. Against side-chain oxidation is the evidence of Ray (1956), Stutz (1958), and the present work that α,α-dimethylindole-3-acetic acid is also a good substrate for the reaction.

Ray (1960, 1962) and of Yamazaki and Souzu (1960). Oxygen uptake would of course be very rapid and might well occur while the radical was still bound to the enzyme. (Although Fe^{+3} is an effective catalyst for the autoxidation of IAA, it has little effect on other indoles, including other indole acids [Table I]. It is likely that chelation is required for electron abstraction and is very sensitive to the acid structure. If a chelate is formed, it undoubtedly has structure *i:*



with the metal ion residing between the two sites of highest electron density in the molecule [the carboxylate anion and the 3 position of the ring] and not, as has been suggested [Maclachlan and Waygood, 1956] between the anion and the ring nitrogen. The high electron density of the 3- position is well established [Hinman and Whipple, 1962]. In the absence of oxygen, electron transfer would still occur with formation of a radical such as I, which would probably decarboxylate. The observation that manganic ion effects decarboxylation of IAA in a nitrogen atmosphere [Maclachlan and Waygood, 1956] can be accounted for in this way, as can the protective effect of oxygen on the enzymatic activity of the *Omphalia* peroxidase [Ray, 1960]. The fact that mangani-Versene attacks indoles other than IAA whereas Fe^{3+} does not is probably a consequence of the fact that manganic ion is introduced in a chelated form of sufficiently high oxidation potential to attack the indole ring directly, whereas ferric ion requires chelation to permit electron transfer to take place.)

From the hydroperoxide II there are few plausible ways in which a hydroxyl group can be introduced on the side-chain carbon. Pathway *a* indicates the normal indole ring-opening reaction which is not observed in the enzymatic reaction, although it occurs in the mangani-Versene-catalyzed oxidation (Abramovitch and Ahmed, 1961). Pathway *b* shows the most reasonable steps by which the carbinol (compound V) can be formed. We believe that with a carboxyl group two carbons removed from an oxygen which may become electron deficient, *decarboxylation with concomitant epoxide formation is the predominant reaction which deflects the hydroperoxide rearrangement from its normal course.*[3]

Although no chemical precedent has been found for this type of epoxide formation, the ease with which the bond to the carboxyl group is donated to form a double bond when an incipient positive charge appears at the β-carbon as in the synthesis of 3-methyleneoxindole (Hinman and Bauman, 1964a) suggests that donation of this electron pair to the neighboring electron-deficient oxygen is a reasonable hypothesis. The final steps are then addition of water to the epoxide (compound III) and acid-catalyzed rearrangement of compound IV to the tautomeric carbinol.

To explain the observed uptake of oxygen and evolution of carbon dioxide in the enzymatic stage the proposed pathway requires rapid conversion of IAA to the epoxide (compound III). Intermediate A of Ray's (1956) study is then the epoxide (compound III), possibly mixed with its hydration product (compound IV), and B is oxindole-3-carbinol (compound V), probably mixed with some methyleneoxindole, as indicated by the dimples in the band head near 250 mμ.

A particularly attractive feature of this pathway is that by ring opening of epoxide III *prior* to hydration, an explanation is afforded for the formation of indole-3-aldehyde whose presence in peroxidase-catalyzed oxidations of IAA has been recorded from time to time (Racusen, 1955; Stutz, 1958; Abramovitch and Ahmed, 1961).

Other pathways have also been considered. As mentioned, an alternate scheme involving attack of oxygen at the 2- position of the cation-radical (I) can be excluded because the exocyclic double bond would be formed prior to the lactam carbonyl group. Stutz (1958) has proposed that initial attack occurs at the 2- position because of the failure of the oxidase from *Lupinus albus* L. to attack 2-carboxyindole-3-acetic acid. However, the electron-withdrawing carboxyl group would make electron abstraction difficult. Since 2-methylindole-3-acetic acid undergoes rapid reaction (Table I), it is apparent that this argument for attack at the 2- position is unfounded.

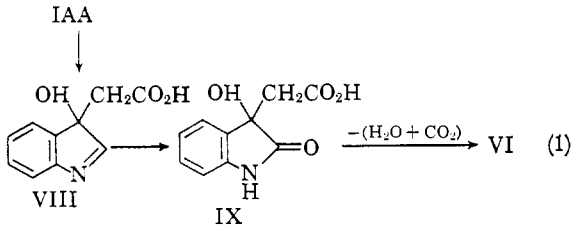
Although an oxidase from *Hygrophorus conicus* converts IAA to oxindole-3-acetic acid (Siehr, 1961), the last compound was unchanged in the presence of horseradish peroxidase with or without hydrogen peroxide present, and hence cannot be an intermediate in the reaction.

It also seemed possible that peroxidase might function as a hydroxylating agent (Ek *et al.*, 1952), forming 3-hydroxyindolenine-3-acetic acid (compound VIII). A reasonable series of steps can be formulated leading to dioxindole-3-acetic acid (compound IX), which would then undergo slow conversion to 3-methyleneoxindole[4] in the manner reported for the related β-hydroxy-β-phenylpropionic acid series (Noyce *et al.*, 1961). However this route, requiring loss of carbon dioxide in the last step rather than the first, fails to explain the presence of oxindole-3-carbinol as an intermediate, and is therefore excluded as the pathway of enzymatic oxidation.

It is possible, however, that this course is followed in the reactions of indoles with hydrogen peroxide both in acidic media and possibly with peroxidase as catalyst. The facile formation of 3-methyleneoxindole from IAA

[3] The authors are indebted to Professor H. H. Wasserman for suggesting the possibility of this pathway. Formation of an intermediate peroxylactone is unlikely in view of the stability reported for β-methyl-β-phenyl-β-peroxypropiolactone (Greene and Adam, 1964).

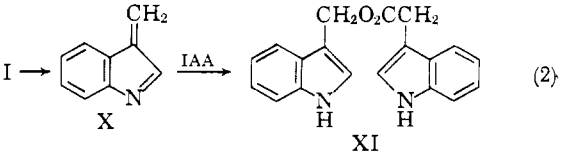
[4] Attempts to synthesize dioxindole-3-acetic acid for verification of this hypothetical reaction have been unsuccessful (Hinman and Bauman, 1964a).



under the former conditions (Figure 3) was the basis for an earlier proposal that this system could be used as a model for the peroxidase-catalyzed reaction (Hinman and Frost, 1961). Examination of a wider variety of structural types has since shown that the reaction is not as limited as the enzymatic reaction. Even 3-ethyl- and 3-*n*-propylindole yield products with spectra characteristic of the 3-alkylideneoxindole chromophore, though in these cases the reaction rate is much less than with IAA. The most reasonable common intermediate for all these cases is a dioxindole similar to compound IX. Since dioxindole-3-butyric acid is converted to a 3-alkylideneoxindole on standing in acidic media (Hinman and Bauman, 1964a), equation (1) is a plausible description of the pathway.

There thus appear to be several distinct paths by which the conversion of IAA to 3-methyleneoxindole can be accomplished. The present work establishes the outlines of three such routes. One-electron abstracting agents (Fe^{3+}, NO_2^-, peroxidase, $S_2O_8^{2-}$) may produce radical intermediates which react with oxygen, evolution of carbon dioxide occurring early in the reaction sequence. Reactions with acidic solutions of hydrogen peroxide probably pass through dioxindole-3-acetic acid, with decarboxylation in the last step. Finally, with halogenating agents (hypohalites, *N*-haloamides, and the like) the reaction undoubtedly involves an intermediate 3-halooxindole-3-acetic acid. In this last case the reaction sequence has been completely elucidated (Hinman and Bauman, 1964a,b).

Returning to the identification of the products of the IAA/peroxidase system, the nature of the indolic component of the reaction mixture from IAA has not been elucidated. It had an ultraviolet spectrum deceptively like that of IAA, but after extraction into ether could not be reextracted into aqueous sodium hydroxide. After standing for a short time in the presence of base, however, part of the material entered the aqueous phase, suggesting that hydrolysis to IAA may have occurred. A related observation is that mixtures of 10^{-2} M IAA and 10^{-4} M peroxidase after 22 hours at room temperature contained mainly indolic material, as determined by the ultraviolet spectrum of samples diluted 1:100 for measurement, whereas the same mixtures diluted after 3 hours and then allowed to stand for 19 hours more contained a substantial quantity of 3-methyleneoxindole. Isolation of the neutral indolic product was difficult, but a small quantity was obtained. It had an infrared spectrum resembling in gross features those of IAA and ethyl indole-3-acetate, but having a carbonyl band between those of the last two substances. From these few clues it seems possible that this product may be an ester (compound XI) of IAA and indole-3-carbinol, perhaps formed by addition of indole-3-acetate ion to 3-methyleneindolenine (compound X), which would be formed plausibly by decarboxylation of the ion-radical (I).



Effect of Variables on the Rate and Products of the Peroxidase-catalyzed Oxidation. In the course of this study a number of new characteristics of the peroxidase-catalyzed oxidation of indoles were discovered. In comparative studies of IAA and its side-chain methylated derivatives a remarkable *p*H dependency of the reaction was found. At *p*H 3.5 (citrate buffer) IAA, α-methyl IAA, and α,α-dimethyl IAA all reacted rapidly (Figure 8). At *p*H 5 (acetate buffer), on the other hand, IAA

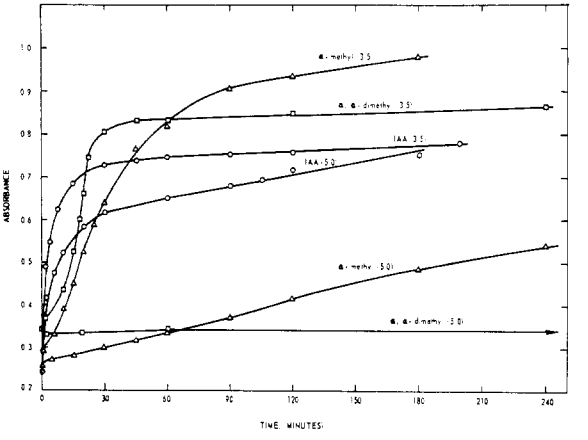


FIGURE 8: Rates of conversion of IAA and α-methyl derivatives to 3-alkylideneoxindoles. Indole concn = 10^{-4} M; peroxidase concn = 10^{-6} M; *RZ* = 0.64; *p*H shown in parentheses. Absorption measured at 253 mμ (IAA), 253.5 mμ (α-methyl IAA), and 258 mμ (α,α-dimethyl IAA).

reacted much more rapidly than α-methyl IAA and the α,α-dimethyl derivative showed *no* evidence of reaction. This unusual dependence of the *p*H-activity profile on the substrate structure may be related to changes in the enzyme structure necessary to accommodate the bulkier side chains of the substituted indoleacetic acids. Since the ionization constants of the acids are nearly identical, the effect cannot be explained on a basis of differing degrees of ionization. A related observation is that conversion of α-methyl IAA to 3-ethylideneoxindole was not catalyzed by ferric chloride at *p*H 5

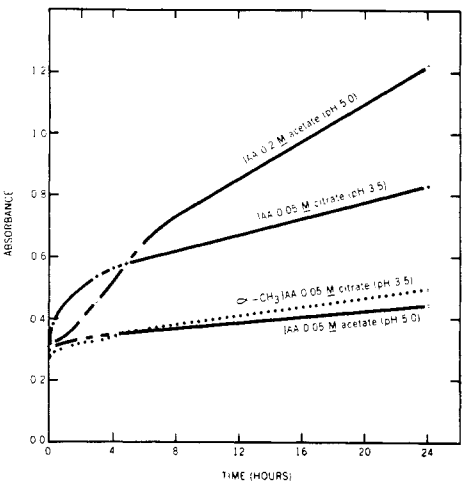


FIGURE 9: Rates of ferric chloride–catalyzed conversions of IAA and α-methyl IAA to 3-alkylideneoxindoles, measured by increase of optical density at 253 mμ. Final concentrations: indoles, 1.01×10^{-4} M; $FeCl_3$, 1.10×10^{-5} M for IAA reactions and 9.94×10^{-6} M for α-methyl IAA reactions. Characteristic peaks of 3-alkylideneoxindole appeared well before the end of each reaction.

but slow conversion did occur at *p*H 3.5 (Figure 9). (α,α-Dimethyl IAA was not attacked at either *p*H.)

Recognition of this structure-*p*H dependency led us to reexamine the activity of the enzyme toward indoles of somewhat different structure. At *p*H 3.5 the ethyl ester of IAA reacted at a slow but appreciable rate. No reaction occurred in the absence of the enzyme. Skatole did not react under these conditions.

Although under our conditions little reaction occurred in the absence of added hydrogen peroxide even at *p*H 3.5 unless the carboxyl was one carbon removed from the ring, attack on the indole ring became general when one equivalent of hydrogen peroxide was added (Table I). Even skatole was rapidly oxidized under these conditions, though the product mixture gave an ill-defined spectrum which could not be identified (Figure 2). Esters of IAA and α-methyl IAA were rapidly converted to products having the clearly defined ultraviolet spectra of oxindoles (Figure 2). Indole-3-ethanol, in which the position of the hydroxyl group relative to the ring is the same as that in IAA, was also attacked rapidly but the spectrum of the product mixture resembled the undefined trace of the skatole reaction mixture. In none of these examples did reaction occur in the absence of enzyme. Less than stoichiometric quantities of peroxide gave incomplete conversions.

In addition to these points it has been found that the course of the enzymatic oxidation of IAA is highly dependent on the concentration of all reaction components—substrate, enzyme, and buffer—as well as on the *p*H of the medium. The increased rate of formation of 3-methyleneoxindole from its precursor in buffer compared to water has been mentioned. However, the rate of this reaction is not strongly affected by variations in *p*H or buffer concentration (Figure 4). The effect of these variables is exerted primarily on the rate of the enzymatic stage of the reaction, and this in turn appears to affect the product composition, as determined by the absorption intensity of the 253-mμ peak of 3-methyleneoxindole, and the general shape of the absorption spectrum of the reaction mixture. Thus the rate of the enzymatic phase increases with buffer concentration (Figure 4, curves 2 and 4) and especially with *p*H (Figure 4, curves 1, 2, and 4). However, more rapid consumption of IAA is frequently associated with a decrease in the total quantity of 3-methyleneoxindole formed.

A decrease in the yield of 3-methyleneoxindole also accompanies the increased rate owing to an increase in enzyme concentration (Figure 4, curves 3 and 5). Apparently the formation in quantity of the first reaction intermediates enhances the rates of reactions which compete with formation of 3-methyleneoxindole. It should be noted from curves 4 and 5 of Figure 4 that an increase in buffer concentration can cause a rate increase sufficient to make up for a much less active enzyme preparation.

It is a particularly interesting point that the rates of IAA oxidations catalyzed by ferric chloride exhibit a similar response to *p*H and buffer concentration (Figure 9), illustrating again the utility of this system as a model for the enzymatic reaction. In contrast to the activity of hydrated ferric ion, the absorption spectra of solutions of hemin (5×10^{-5} M) and IAA (5×10^{-5} M) in 0.02 M acetate buffer (*p*H 4.93) showed no change over a 24-hour period with or without added hydrogen peroxide.

The change in reaction path with increasing substrate concentration has been discussed and is illustrated in Figure 1.[5] A change in product composition with enzyme concentration is also evident at the higher substrate concentrations (Figure 1, curve 5). With Japanese radish peroxidases a and b a 10-fold increase in enzyme concentration was accompanied by formation of an entirely different product, which may have been indole-3-aldehyde (Morita *et al.*, 1962). Previous workers in the field of IAA-oxidases have not recognized this point. A wide range of IAA concentrations has been used,[6] and since crude extracts of enzymes were employed, the enzyme concentration was also unknown. It is no wonder that there has been so little agreement on the nature of the products of the enzymatic reaction.

It is clear from these results that a reaction pathway can be identified only under highly specific conditions. This raises the question of the relevance of the pathway

[5] Oxidations catalyzed by ferric chloride and sodium nitrite behave similarly. At higher concentrations, such as those used by Tonhazy and Pelczar (1954), little change in the absorption spectra was noted.

[6] Examples of reported IAA concentrations include: 2.9×10^{-2} M (Manning and Galston, 1955); 10^{-2} M (Stutz, 1958); 2.2×10^{-3} M (Waygood *et al.*, 1956); 6×10^{-5} M (Ray and Thimann, 1956). Only in the last case are the concentrations in the range in which we have observed methyleneoxindole formation.

elucidated in the present work to the *in vivo* destruction of auxin. From the effect of substrate concentration, which exerts the biggest influence on product composition, an upper concentration limit can be placed on the reaction. Below 2×10^{-4} M the reaction path to oxindole-3-carbinol appears to remain fairly constant, although variations in rate and minor variations in product composition are still evident. This brings the reaction into the range of physiological importance. It raises some doubt about the relevance of the reported oxidations carried out at Warburg concentrations.

Below this concentration limit the evidence to date indicates that the oxidations of IAA catalyzed by horseradish peroxidase and by oxidases from other plant extracts all follow the same reaction path. A most convincing demonstration has been offered of the similarity of products and reaction pathway of IAA oxidation catalyzed by horseradish peroxidase, Japanese radish peroxidases a and b, and the peroxidase from *Omphalia* (Morita *et al.*, 1962). Although it is known that common peroxidases are composed of more than one active species (McCune, 1960), this work taken together with the generality of the chemical conversion of IAA to 3-methyleneoxindole suggests that the products and probably the pathway will be the same for most if not all peroxidases at physiological concentrations.

A further obstacle in the way of relating the *in vitro* experiments to the *in vivo* reactions is the heterogeneity of the plant cell where other enzymes or the cell wall (Siegel, 1956) may play a role in determining the reaction course. Stutz (1958) has shown that addition of other enzymes to an IAA-oxidase can divert the main reaction course. Although we have used peroxidases varying from *RZ* 0.64 to 3.0 in our studies and have found no difference in product composition, resolution of this question requires *in vivo* studies. Despite this limitation, however, the pathway proposed in the present work is the first complete and reasonably documented route to be put forward for auxin destruction, and requires first consideration in further studies in this area.

Experimental

1-Methylindole-3-acetic acid was prepared by the method of Snyder and Eliel (1948). *2-Phenylindole-3-acetic acid* was prepared by the method of Bauer and Andersag (1941). *2-Methylindole-3-acetic acid* was prepared by the reaction of 2-methylindole, formalin, and potassium cyanide in an ethanol-water mixture, followed by basic hydrolysis of the resulting nitrile, a method adapted from that of Bauer and Andersag (1941). The free acid, obtained in an over-all yield of 54% from 2-methylindole, melted at 194–198° decomp (reported mp 195–200 decomp [Pretka and Lindwall, 1954]). *1,2-Dimethylindole-3-acetic acid*, prepared by adaptation of the method used by Snyder and Eliel (1948) for 1-methylindole-3-acetic acid, melted at 171–173° decomp.

Anal. Calcd for $C_{12}H_{13}NO_2$: N, 6.89. Found: N, 6.42.

α-Methylindole-3-acetic acid, 3-ethyl- and 3-n-propylindole were gifts of Dr. Herbert Johnson, Union Carbide Chemicals Div., Union Carbide Corp., South Charleston, W. Va. *α,α-Dimethylindole-3-acetic acid* was prepared by the method of Erdtmann and Jonsson (1954). *Oxindole-3-acetic acid and 3-bromooxindole-3-acetic acid* were prepared by the method of Hinman and Bauman (1964b). *3-Methyleneoxindole* was prepared as described previously (Hinman and Bauman, 1964a).

Horseradish peroxidase was purchased from a number of suppliers in various states of purity. The rate of destruction of IAA was directly proportional to the purity of the peroxidase as measured by the *RZ* value (Table II). The product composition, as indicated by the ultraviolet absorption spectrum of the reaction mixture, was not affected significantly by the purity of the enzyme, although the total yield of methyleneoxindole was somewhat lower with the more active enzyme preparations.

Buffers. The following compositions are referred to as indicated. Other buffer concentrations were obtained by appropriate dilutions: *0.2 M acetate (pH 5.0)*, 71 ml of 0.2 M sodium acetate and 30 ml of 0.2 M acetic acid; *0.05 M citrate (pH 3.5)*, 47.5 ml of 0.1 M disodium citrate and 52.5 ml of 0.1 M hydrochloric acid; *0.067 M phosphate (pH 6.5)*, 70 ml of 0.067 M monopotassium phosphate and 30 ml of 0.067 M disodium phosphate.

Changes in ultraviolet absorption spectra of reaction mixtures were followed by carrying out the reactions in quartz cuvets of 1-cm light path, using a Beckman DK-2 recording spectrophotometer, equipped with a thermostated cell compartment (double thermospacers) maintained at 25.0 ± 0.1°.

Enzymatic Oxidations

(A) Using Atmospheric Oxygen (No Added Hydrogen Peroxide). These reactions were carried out as described previously (Hinman and Frost, 1961; Hinman *et al.*, 1961). Reaction mixtures in ultraviolet cuvets were left open to the air as a source of oxygen and were not stirred after the initial mixing of reactants. IAA concentrations were measured by the modified Salkowski test (Gordon and Weber, 1951). The time required for complete consumption of IAA also coincided with the time required for formation of an isosbestic point at 261 mμ in the ultraviolet spectra, as reported by Ray (1956). Isosbestic points also formed in this region of the spectra of the other indoleacetic acids which were unsubstituted in the 2- position; the time required to reach constant absorbancy at this point was used as a measure of the lifetime of the indole acid. Typical results with IAA and its derivatives with peroxidases of various degrees of purity are listed in Table II.

THE EFFECT OF *p*H on the rates of reaction is shown in Figure 5. In all cases reaction rates at *p*H 3.5 were greater than those at *p*H 5.0 in the enzymatic phase of the reaction. At *p*H 6.5 rates for the IAA reaction were still lower. At *p*H 5.0 the enzymatic phase of the reaction and the appearance of alkylideneoxindole varied with substitution in the order: 1-methyl- > IAA > α-methyl- >>> α,α-dimethyl (Figure 7). In the last case there

TABLE II: Effect of Enzyme Purity on Reaction Rate.

Indole Acid	Source of Peroxidase[a]	RZ[b]	Enzyme Concn (M)	pH	Buffer Concn (M)	Half-Life of Acid (min)[c]	Time for Complete Consumption of Acid (min)	Isosbestic Point (mμ)
IAA	NBC	0.64	10^{-6}	3.5	0.05		2[c,d]	262
IAA	NBC	0.64	10^{-6}	5.0	0.2	1	10[c,d]	262
IAA	NBC	0.64	10^{-6}	6.5	0.067	5	45[c,d]	262
IAA	W	0.97	10^{-6}	6.5	0.067	3.5	30[c,d]	262
IAA	NBC	0.64	10^{-7}	5.0	0.2	10	65[c]	
IAA	NBC	2.7[e]	10^{-7}	5.0	0.2	2	10[c]	
IAA	CBC	3.0	5×10^{-8}	5.0	0.05	11	70[c]	
α-Methyl	NBC	0.64	10^{-6}	3.5	0.05		<2[d]	263
α-Methyl	NBC	0.64	10^{-6}	5.0	0.2		45[d]	263.5
α,α-Dimethyl	NBC	0.64	10^{-6}	3.5	0.05		<2[d]	266
1-Methyl	NBC	0.64	2×10^{-7}	5.0	0.2		10[d]	266

[a] NBC = Nutritional Biochemicals Co.; W = Worthington; CBC = California Corp. for Biochemical Research. [b] *RZ* = OD at 403 mμ/OD at 275 mμ. [c] By Salkowski test. [d] Time required for absorption to become constant at isosbestic point. [e] Purified by chromatography on XE-64 ion-exchange resin and carboxymethyl-cellulose.

was no detectable reaction during a period of 23 hours. At *p*H 3.5 the relative order of rates was: IAA > α,α-dimethyl > α-methyl in the enzymatic phase of the reaction, but formation of the alkylideneoxindole was fastest from α,α-dimethylindole-3-acetic acid.

THE EFFECT OF THE SUBSTRATE CONCENTRATION on the course of the reaction is shown for IAA in Figure 1. Although there was an increase in absorption in the 235- to 270-mμ region at concentrations of 10^{-2} M, the basic shape of the indole absorption curve was unchanged and there was no evidence of the characteristic peaks at 247 and 253 mμ of 3-methyleneoxindole for periods up to 24 hours, even when the peroxidase concentration was raised to 10^{-4} M. (In this case reaction mixtures were diluted 1:100 for observation of ultraviolet spectra.) That the indolic material was not IAA was shown by extraction of the reaction mixture with ether. Although all of the indolic material went into the ether phase, most of it remained there when the ether was extracted with 0.1 M sodium hydroxide solution. When after 3 hours a reaction mixture, which contained principally neutral indole, was diluted to an IAA concentration of 10^{-4} M, formation of 3-methyleneoxindole ensued, but at a much slower rate than that in reaction mixtures originally made up to this concentration. The reduced rate of reaction may be an indication of enzyme inactivation or of slow conversion of the neutral indole to IAA which then reacts normally. An observation which may be related to the last point is that, in a typical experiment with 10^{-2} M IAA and 10^{-6} M peroxidase in 0.2 M acetate buffer, measured values of the Salkowski test decreased from 0.65 to 0.06 but rose again to 0.49 within 4 hours. In phosphate buffer at *p*H 6.5 the Salkowski values decreased from 0.45 to 0.39 and remained near the latter value during the remainder of the reaction time.

From these results it can be inferred that the neutral indole either gives the Salkowski test directly or is converted, perhaps by hydrolysis in the acidic medium, to IAA. Unlike conversion to 3-methyleneoxindole, formation of the neutral indole was not affected by buffer concentration. The same behavior was observed from 0.005 to 0.2 M in acetate, from 0.005 to 0.05 M in citrate, and in 0.067 M phosphate (*p*H 6.5).

The same effect of concentration on product composition was observed with α-methyl- and α,α-dimethylindole-3-acetic acid. In the last case the experiment was carried out at *p*H 3.5 in 0.05 M citrate buffer.

The possibility that the change in the reaction course at higher indole concentration was caused by lack of oxygen was ruled out by the fact that substantially the same product composition was observed when the reaction with IAA was carried out with oxygen bubbling through the ultraviolet cuvet.

In conversions of IAA to 3-methyleneoxindole, addition of small quantities of hydrogen peroxide or of manganous salts (typical concentrations IAA 10^{-4} M, peroxidase 10^{-7} M, manganous chloride 10^{-4} M), and 2,4-dichlorophenol appeared to increase the reaction rate in its early stages, but there was no apparent effect on the product composition, nor did addition of these materials seem to affect the dependence of product composition on substrate concentration. The over-all reaction rate was decreased appreciably by the presence of even 1% ethanol.

Under the usual conditions employed (Hinman, *et al.* 1961) conversion of IAA to 3-methyleneoxindole at 25° was essentially complete in 24 hours, although slight

increases in absorbancy were sometimes observed even up to 48 hours. The absorbancies at 253 mμ at the various time intervals given in Figure 4 were reproducible within 0.05 OD unit. Absorption of about the same intensity was attained by simply heating reaction mixtures for 10 minutes in a boiling-water bath, providing that heating was performed after completion of the enzymatic phase of the reaction. From the optical density at 253 mμ and the known ϵ_{max} of 3-methyleneoxindole at this wavelength (25,500) (Hinman and Bauman, 1964a), the concentration of the product was calculated as $\sim 5 \times 10^{-5}$ M or 50% conversion. This appeared to be the equilibrium mixture rather than complete conversion.

The enzymatic phase of the reaction could be inhibited by addition of potassium cyanide. A typical experiment is illustrated in Figure 5. Similar results could be obtained by flushing with nitrogen.

2-METHYLINDOLE-3-ACETIC ACID (10^{-4} M) was also attacked at *p*H 5 (peroxidase concentration 10^{-7} M), although reaction was slower than that of IAA. After 46.5 hours an absorption maximum at 264 mμ was observed together with typical indole peaks in the 280-mμ region (Figure 2). The absorption spectrum had the same characteristics as that obtained by the reaction of 2-methylindole-3-acetic acid with hydrogen peroxide at *p*H 1 (*vide infra*). 1,2-Dimethylindole-3-acetic acid was attacked, but very slowly, and the spectrum showed no well-defined maximum.

ISOLATION OF NEUTRAL INDOLES FROM CONCENTRATED SOLUTIONS OF IAA AND PEROXIDASE. A reaction mixture (250 ml) containing in final concentrations IAA (2×10^{-3} M), peroxidase (10^{-6} M), and acetate buffer (0.2 M) at *p*H 5 was allowed to stand at room temperature for 24 hours. The mixture, which contained a large amount of solid precipitate, was extracted with ether, and the ether extract was washed with 5% sodium bicarbonate followed by saturated sodium chloride solution, and finally was dried over sodium sulfate. The ultraviolet spectra of both the ether extract and the basic solution had characteristic indolic features. The ether layer was evaporated to dryness, and the brown residue was shaken with 5% sodium bicarbonate, then again extracted with ether. This extract was dried over sodium sulfate and evaporated, and the brown residue was dissolved in benzene and reprecipitated with *n*-hexane, yielding 6 mg of tan solid, mp 132–140° (decomp). The infrared spectrum (KBr) had the following principal bands (cm^{-1}): 3400, 1720, 1628, 1463, 1345, 1227, 1194, 1012, 743, 680. Ehrlich's reagent indicated that the material was an indole unsubstituted in the 2- position.

ENZYMATIC CONVERSION OF α-METHYLINDOLE-3-ACETIC ACID TO 3-ETHYLIDENEOXINDOLE. The reaction mixture was prepared by mixing 60 ml of an aqueous solution containing 0.135 g of α-methylindole-3-acetic acid and 1 ml of 1 M sodium hydroxide, 60 ml of an aqueous solution containing 150 mg of peroxidase, and 3 liters of 0.048 M citrate buffer, *p*H 3.5. The solution was stirred for 25 minutes, after which it was allowed to stand for 20 hours. The orange solution was then extracted with successive portions of ether until the aqueous solution was colorless. The combined ether extracts were washed successively with 5% sodium bicarbonate solution and saturated sodium chloride solution and were then dried over sodium sulfate. After 24 more hours the original reaction mixture was extracted again with ether. The ether extract was treated as before and the combined extracts were dried *in vacuo*. After 24 more hours the original reaction mixture was extracted with benzene. The extracts were treated as before and distilled almost to dryness *in vacuo*. The dark viscous oil which remained produced deep orange-brown crystals on standing. These were identified as 3-ethylideneoxindole by mp (136–140°), and by comparison of the ultraviolet and infrared spectra with those of an authentic sample.

The residue from the ether extracts was redissolved in an ether-benzene mixture and eluted from a column of Florisil using in order ether, benzene, and ethanol. 3-Ethylideneoxindole was detected in the ether eluent by its characteristic ultraviolet spectrum. The combined ether fractions were concentrated, and the yellow-orange needles which separated were removed by filtration and washed with ether. 3-Ethylideneoxindole, identified by its infrared and ultraviolet spectra, melted at 144–146°; reported mp 142° (Wenkert *et al.*, 1953).

PAPER CHROMATOGRAPHY OF IAA AND PEROXIDASE REACTION MIXTURES; SEPARATION AND IDENTIFICATION OF OXINDOLE-3-CARBINOL AND 3-METHYLENEOXINDOLE AS REACTION PRODUCTS. A reaction mixture composed of indole-3-acetic acid (1.05×10^{-4} M) and peroxidase (5.8×10^{-8} M) in 100 ml of 0.005 M acetate buffer (*p*H 5), containing 0.1% ethanol (used to dissolve the indoleacetic acid), was allowed to stand at room temperature for 24 hours. The reaction was followed by the Salkowski test, which showed that the IAA was consumed in about 4 hours (Figure 4). Aliquots of 25 ml were removed at 3.5, 7, and 24 hours and each was continuously extracted with ether for 3 hours. The dry concentrated ether extracts were chromatographed on Whatman No. 1 paper. The chromatograms were developed at 4° with a water–isopropyl alcohol system (95:5) as the developing solvent (Fukuyama and Moyed, 1964), using the descending technique. After drying at room temperature overnight, a number of absorbing spots and fluorescent spots appeared on the paper. Methyleneoxindole was identified as an absorbing spot under ultraviolet light with R_F 0.39–0.36 by comparison with an authentic marker run on each sheet. The absorption intensity of this spot increased from the first to the third extract. When this spot was cut out and eluted with 95% ethanol, the eluate had the spectrum of 3-methyleneoxindole. Another absorbing spot at R_F 0.67–0.64 was also eluted with water and its ultraviolet spectrum resembled that of an oxindole. After standing for 44 hours, the spectrum was partially converted to that of 3-methyleneoxindole. When the solution was mixed with acetate buffer to a final buffer concentration of 0.05 M, methyleneoxindole formation took place more rapidly.

Similar results were obtained in 5×10^{-2} M acetate buffer. The IAA was consumed much more rapidly (Figure 4). The peroxidase-catalyzed reaction was also carried out with similar concentrations of reactants but in 0.005 M citrate buffer at *p*H 3.7. These were the conditions used by Ray (1956) with the *Omphalia* peroxidase. The ultraviolet spectrum of this reaction mixture after 1 hour (Figure 7) was almost identical to that reported by Ray at the most extreme reaction time he described, about 90–105 minutes. In this reaction medium the peaks due to 3-methyleneoxindole developed very slowly after that period, and after 24 hours the intensity of absorption at 253 mμ was only about 75% of that in the stronger buffer solution. Removal of aliquots at 2, 6, and 24 hours and continuous ether extractions of these samples gave again on paper chromatography spots of both the 3-methyleneoxindole and oxindole-3-carbinol at R_F 0.42 and 0.72, respectively. 3-Methyleneoxindole was not detected in the extract taken at 2 hours.

ENZYMATIC CONVERSION OF INDOLEACETIC ACID TO OXINDOLE-3-CARBINOL; ISOLATION OF OXINDOLE-3-CARBINOL. A solution of 40 mg (0.23 mmole) of IAA in 2 ml of 95% ethanol was mixed with 1 liter of 0.05 M acetate buffer (*p*H 5) and 5 mg (10^{-4} mmoles) of peroxidase was added. The mixture was stirred magnetically for 7 hours, the rate of reaction being followed by means of Salkowski's reagent and ultraviolet spectra. At 45- to 75-minute intervals, when these tests showed that most of the IAA had been consumed, similar additions of IAA were made together with 1 mg of peroxidase. The final addition was made with 5 mg of peroxidase. In this way, a total of 204 mg of IAA (1.17 mmoles) and 13 mg of peroxidase (2.6×10^{-4} mmole) was added. After a total time of 7 hours the yellow cloudy mixture was split into four portions and frozen at $-23°$. Each portion was thawed as needed, filtered, and continuously extracted with ether for 3.5 hours. (Ultraviolet spectra showed that little reaction had taken place during the period of freezing.) The combined ether extracts were dried over sodium sulfate and concentrated *in vacuo* to a volume of about 5 ml. The presence of acetic acid was detected by its very strong odor. The extract was streaked on sheets of Whatman No. 3 paper and chromatographed in the manner described. The absorbing spot corresponding to the position associated with the carbinol was cut out and eluted with 100 ml of absolute ethanol. The ethanol was concentrated *in vacuo* to about 10 ml. The remainder was evaporated to dryness under a stream of nitrogen. The residual yellow solid weighed 6 mg. The ultraviolet spectrum of this material in 95% ethanol resembled that of an oxindole. Based on the assumption that it was a pure compound of mw 163, the following values were obtained: λ_{max} (ϵ_{max}): 280 sh (1250), 261 inflection (4360), 249 (6730), 245 inflection (6410). These values are those that would be expected of a substance such as 3-methyloxindole (Hinman and Bauman, 1964a). The infrared spectrum showed many resemblances to 3-methyldioxindole (Hinman and Bauman, 1964a) but also showed significant differences. The typical oxindole NH and carbonyl bands at 3.12, 5.84, and 5.90 μ, and an additional band in the hydroxyl region at 2.94 μ, showed that the compound was an oxindole with an additional hydroxyl group. The NMR spectrum in perdeuteriodimethyl sulfoxide showed three areas of absorption of diminishing intensity: at 2.76–3.18 τ in the aromatic region and at 6.12 and 6.60 τ, the last two in the regions identified respectively with methylene protons on a hydroxyl-bearing carbon, which also has two other electron-withdrawing groups on an adjacent carbon (Jackman, 1959), and with the ring protons at the 3- position of the oxindole (Hinman and Bauman, 1964a). No spin-spin splittings were observed because of the low concentration of material, but the intensity of the peak at 6.12 τ was greater than that at 6.60 τ, as would be expected for two hydrogens in the side-chain methylene group and one hydrogen at the 3- position of the ring. In these respects the NMR spectrum was entirely consistent with the proposed structure. However, other peaks at much higher fields (8.8–9.6 τ) were also present owing to highly shielded methylene or methyl groups probably present in dimeric or polymeric materials formed from oxindole-3-carbinol and/or 3-methyleneoxindole (Hinman and Bauman, 1964a). Dissolution of the oxindole-3-carbinol in any solvent was always accompanied by formation of insoluble material. In water the product identified as oxindole-3-carbinol underwent slow conversion to 3-methyleneoxindole, as measured by the change in the ultraviolet spectrum, and this transformation was much more rapid in buffer solutions.

(*B*) *Enzymatic Reactions with Added Hydrogen Peroxide.* These reactions were carried out as described previously (Hinman and Frost, 1961). All of the indole-3-acetic acid derivatives gave 3-alkylideneoxindoles by this method. Rates of formation of the 3-alkylideneoxindoles were sometimes (Figure 3), but not always, more rapid in the presence of peroxide. However, the increase in rate was not very large. On the other hand, compounds which did not react at all when peroxidase was functioning as an oxidase, i.e., skatole, ethyl indole-3-acetate, indole-3-ethanol, and related compounds, were all oxidized rapidly when stoichiometric quantities of hydrogen peroxide were present along with the peroxidase. In these cases, however, the product was not a 3-alkylideneoxindole. Esters of the indole-3-acetic acids yielded products which had the typical spectra of oxindoles with maxima near 250 mμ and an inflection or shoulder around 260–265 mμ. Allowing these materials to stand for longer periods or adding additional hydrogen peroxide did not change the spectra. On the other hand, skatole and indole-3-ethanol, both of which reacted extremely rapidly (complete conversion to a different spectrum taking only 2–3 minutes after mixing), yielded products with undefined ultraviolet spectra rising sharply below 275 mμ with a shoulder in the vicinity of 255–260 mμ (Figure 4).

Nonenzymatic Oxidations of Indole-3-alkanoic Acids in Acidic Media

With Air as the Source of Oxygen. When IAA, 1-methyl IAA, or α-methyl IAA were allowed to stand

in acidic solutions exposed to the atmosphere, the presence of 3-alkylideneoxindoles was soon detected by the characteristic changes in the ultraviolet absorption spectra. 1-Methyl IAA reacted especially rapidly and, after standing for 3 days, no residual indole remained. This reaction undoubtedly accounts for the instability in acidic media which has been observed by many workers. Reaction rate was a function of *p*H and alkylideneoxindole formation was noted even at *p*H 5, if solutions were allowed to stand for many days.

Oxidations of other indoles were also catalyzed by acid. For example, skatole, tryptophan, tryptamine, and the like all produced colored products when their solutions exposed to air were stirred in acid for short periods of time. However, if either the oxygen or the acid were excluded, no color change took place. It is well known that trimerization of indole (Hodson and Smith, 1957) and dimerization of indole derivatives (Hinman and Shull, 1961) are catalyzed by acids. However, the products are invariably white. Moreover, the products from simple autoxidation of indoles such as the indolenine hydroperoxides (Witkop and Patrick, 1951a) are also white. It follows from these experiments, therefore, that the familiar red tars and other products obtained by the action of acids on indoles and pyrroles are a result of the combined action of acid and oxygen. Since the rate of color formation is proportional to the acid concentration, it might be inferred that it is the indole conjugate acid which is undergoing oxidation, but this point has not been proved. Although the formation of 3-alkylideneoxindoles was observed at spectrophotometric concentrations, oxygen uptake and color formation also occurred at higher substrate concentrations.

With Hydrogen Peroxide Added. These reactions were carried out as described previously (Hinman and Frost, 1961). As shown in Table I, a wide variety of indoles was subjected to these reaction conditions. In almost all cases the formation of 3-alkylideneoxindole was detected. Reaction rates were quite rapid, although in the case of 3-ethyl- and 3-*n*-propylindole, formation of alkylideneoxindole was detected only after 19 hours. In the former case reaction was complete in about 8 days. Even 1,2-dimethylindole-3-acetic acid reacted under these conditions although the product could not be identified. The ultraviolet spectrum of 2-phenylindole-3-acetic acid changed fairly rapidly to what appeared to be, from the sharpness of the spectrum, a single product with maxima at 231, 260, and 302 mμ. Reaction was complete in about 4 hours and no further change took place over long periods thereafter. The product was not identified. The ultraviolet spectra of skatole and 2-phenylskatole were unchanged after exposure to these conditions for many hours.

Oxidations Catalyzed by Ferric Salts. When a solution of IAA (10^{-4} M) and ferric chloride (1.11×10^{-5} M) in 0.2 M acetate buffer (*p*H 5) was allowed to stand at room temperature, the formation of 3-methyleneoxindole was detected after a few hours by the characteristic ultraviolet absorption spectrum. Conversion to 3-methyleneoxindole was complete in about 24 hours. The rate of reaction increased with increasing buffer concentration and reaction occurred in citrate buffer at *p*H 3.5 as well as in acetate buffer (Figure 9). α-Methylindole-3-acetic acid reacted more slowly under these conditions, but no other indole examined showed evidence of reaction as shown in Table I. α,α-Dimethylindole-3-acetic acid was unchanged even at *p*H 3.5.

Oxidation of Indole-3-acetic Acid Catalyzed by Nitrite Ion. When a solution of IAA (10^{-4} M) and sodium nitrite (3.3×10^{-5} M) in acetate buffer at *p*H 5 was allowed to stand at room temperature, the characteristic peaks of 3-methyleneoxindole became visible in the ultraviolet absorption spectrum within 3 hours. Substantial conversion to 3-methyleneoxindole had occurred within a 24-hour period. At the higher concentrations used by Tonhazy and Pelczar (1954), namely, 3.3×10^{-3} M IAA and 10^{-3} M sodium nitrite, very little change in absorption occurred during this period. Similar behavior was observed with ferric chloride and concentrations of IAA above the spectrophotometric level. In both cases formation of precipitate showed that reaction was occurring.

Oxidation of IAA with Potassium Persulfate. Conversion of IAA (10^{-4} M) to 3-methyleneoxindole was evident within a very few minutes at *p*H 1 when potassium persulfate was present at 2×10^{-4} M concentration. Reaction was somewhat slower at *p*H 5.

Reactions of IAA with Positive Halogen Compounds. Reactions of IAA with *N*-chlorosuccinimide, *N*-bromosuccinimide, and sodium hypochlorite were carried out with equimolar portions of the reactants in a 2:1 acetic acid–water mixture at room temperature. Reactions were very fast, the indole absorption peaks disappearing within 60 seconds. The characteristic peaks of 3-methyleneoxindole at 253 mμ appeared rapidly, but the remainder of the spectrum including the peak of 247 mμ could not be discerned because of the acetate buffer absorption below 250 mμ. One experiment was carried out with sodium hypochlorite at *p*H 6. Reaction in this case was slow, but after 5 days the presence of a considerable amount of 3-methyleneoxindole was evident; the peaks at 248 and 253 mμ could be easily discerned.

Added in Proof

More recent observations show that IAA is very strongly absorbed by the polymeric products of 3-methyleneoxindole. In the absorbed form it may be taken for a neutral indole, perhaps also accounting for these observations.

References

Abramovitch, R. A., and Ahmed, K. S. (1961), *Nature 192*, 259.

Bauer, K., and Andersag, H. (1941), *Chem. Abstr. 35*, 18074; U. S. Pat. 2,222,344.

Ek, A., Kissman, H., Patrick, J. B., and Witkop, B. (1952), *Experientia 15*, 36.

Erdtmann, H., and Jonsson, A. (1954), *Acta Chem. Scand. 8*, 119.
Fukuyama, T. T., and Moyed, H. S. (1964), *J. Biol. Chem. 239*, 2392.
Gordon, S. A., and Weber, R. P. (1951), *Plant Physiol. 26*, 192.
Greene, F. D., and Adam, W. (1964), Abstracts of the 147th Meeting of the American Chemical Society, April 5, 1964, p. 30N; and private communication.
Hellmann, H., and Renz, E. (1951), *Chem. Ber. 84*, 901.
Hinman, R. L., and Bauman, C. (1964a), *J. Org. Chem. 29*, 2431.
Hinman, R. L., and Bauman, C. (1964b), *J. Org. Chem. 29*, 1206.
Hinman, R. L., Bauman, C., and Lang, J. (1961), *Biochem. Biophys. Res. Commun. 5*, 250.
Hinman, R. L., and Frost, P. (1961), *Plant Growth Regulation. Intern. Conf. 4th, Yonkers, N.Y.*, 205.
Hinman, R. L., and Shull, E. R. (1961), *J. Org. Chem. 26*, 2339.
Hinman, R. L., and Whipple, E. (1962), *J. Am. Chem. Soc. 84*, 7534.
Hodson, H. F., and Smith, G. F. (1957), *J. Chem. Soc.*, 3544.
Horner, L. (1941), *Ann. Chem. 548*, 117.
Jackman, L. M. (1959), Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry, New York, Pergamon, pp. 53–55.
Kefford, N. P., Kaur-Sawhney, R., and Galston, A. W. (1963), *Acta Chem. Scand. 17*, 5313.
Knox, W. E., and Mehler, A. H. (1950), *J. Biol. Chem. 187*, 419.
McCune, D. C. (1960), *Ann. N.Y. Acad. Sci. 94*, 723.
Maclachlan, G. A., and Waygood, E. R. (1956), *Physiol. Plantarum, 9*, 321.
Manning, D. T., and Galston, A. W. (1955), *Plant Physiol. 30*, 225.
Mason, H. S. (1957), *Advan. Enzymol. 19*, 79.
Morita, Y., Kameda, K., and Mizuno, M. (1962), *Agr. Biol. Chem. (Tokyo) 26*, 442.
Noyce, D. S., King, P. A., and Woo, G. L. (1961), *J. Org. Chem. 26*, 632.
Pretka, J. E., and Lindwall, H. G. (1954), *J. Org. Chem. 19*, 1080.
Racusen, D. (1955), *Arch. Biochem. Biophys. 58*, 508.
Ray, P. M. (1956), *Arch. Biochem. Biophys. 64*, 193.
Ray, P. M. (1958), *Ann. Rev. Plant Physiol. 9*, 81.
Ray, P. M. (1960), *Arch. Biochem. Biophys. 87*, 19.
Ray, P. M. (1962), *Arch. Biochem. Biophys. 96*, 199.
Ray, P. M., and Curry, G. M. (1958), *Nature 181*, 895.
Ray, P. M., and Thimann, K. V. (1956), *Arch. Biochem. Biophys. 64*, 175.
Siegel, S. (1956), *Quart. Rev. Biol. 31*, 1.
Siehr, D. J. (1961), *J. Am. Chem. Soc. 83*, 2401.
Snyder, H. R., and Eliel, E. L. (1948), *J. Am. Chem. Soc. 70*, 1703.
Stutz, R. E. (1958), *Plant Physiol. 33*, 207.
Tonhazy, N. E., and Pelczar, M. J. (1954), *Science 120*, 141.
Wasserman, H. H., and Floyd, M. B. (1963), *Tetrahedron Letters*, 2009.
Waygood, E. R., Oaks, A., and Maclachlan, G. A. (1956), *Can. J. Botany 34*, 905.
Wenkert, E., Bose, A. K., and Reid, T. L. (1953), *J. Am. Chem. Soc. 75*, 5514.
Witkop, B. (1950), *J. Am. Chem. Soc. 72*, 2311.
Witkop, B., and Patrick, J. B. (1951a), *J. Am. Chem. Soc. 73*, 2196.
Witkop, B., and Patrick, J. B. (1951b), *J. Am. Chem. Soc. 73*, 713.
Yamazaki, I., and Souzu, H. (1960), *Arch. Biochem. Biophys. 86*, 294.