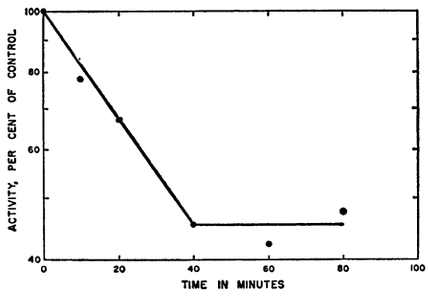

Fig. 1. Heat stability of acid phosphatase in 1.0*M* acetate, *p*H 5.2, at 50°C.

stable than the other at *p*H 5.2. After heating for 100 minutes at 50°C and centrifuging to remove the precipitate, 53 percent of the activity with *p*-nitrophenylphosphate and 55 percent of the protein remained. On the basis of these analyses and an examination of the heat stability curves for the point of interception of the slopes of the two phases of the curves, it is concluded that the heat-labile portion comprised about half of the acid phosphatase preparation.

An examination of the enzymes by polyacrylamide gel electrophoresis was carried out; there was only one enzymatically active zone when the enzymes were prepared in tris buffer (Fig. 2). After the purified preparation was dialyzed overnight against 2 liters of 1.0*M* acetate buffer at *p*H 5.2, two zones were detected on the gels. After dialysis to remove tris buffer, the formation of two isozymes in acetate is readily reversible. Only one band could be detected when the same preparation was again dialyzed against tris buffer at *p*H 7.5, but two bands were always found when tris buffer was replaced by acetate (*p*H 5.2). When the enzyme was heated in acetate buffer for 10 minutes at 50°C the new acid phosphatase could not be detected.

When acid phosphatase was centrifuged in an analytical ultracentrifuge, only one boundary was apparent, regardless of which buffer was used. Thus the new acid phosphatase does not appear to be produced by aggregation. The sedimentation coefficient ($s_{20,w}$) was 4.7 in tris buffer (*p*H 7.5), but 2.5 in acetate buffer (*p*H 5.2).

The results may suggest an expansion of the molecule at the lower *p*H similar to that which was reported for serum albumin (*5*). Both phosphatases seem to have the same mass, so that their separation on polyacrylamide gels reflects either the acquisition of an increased positive charge by the heat-labile protein when in acetate buffer or a change in conformation, possibly brought about by the lower *p*H or altered buffer composition. Both inorganic and organic salts are important in controlling protein conformation (see *6*).

Apparently the two phosphatases are isozymes and are not simply interconvertible forms of the same enzyme. Once heated, additional amounts of the labile acid phosphatase are not produced.

RICHARD C. STAPLES
WILLIAM J. MCCARTHY
Boyce Thompson Institute, Yonkers, New York
MARK A. STAHMANN
Department of Biochemistry, University of Wisconsin, Madison

References and Notes

1. T. Ikawa, K. Nisizawa, T. Miwa, *Nature* **203**, 939 (1964); J. J. Blum, *J. Cell Biol.* **24**, 223 (1965).
2. P. H. Williams and R. C. Staples, *Contrib. Boyce Thompson Inst.* **22**, 269 (1964).
3. A. Torriani, *Biochim. Biophys. Acta* **38**, 460 (1960).
4. AOAC, *Official Methods of Analysis* (Assoc. of Official Agricultural Chemists, Washington, D.C., ed. 8, 1955).
5. C. Tanford, *Physical Chemistry of Macromolecules* (Wiley, New York, 1961), pp. 383–384.
6. P. H. von Hippel and K.-Y. Wong, *Science* **145**, 577 (1964).
7. Supported in part by NSF research grant No. GB-2275 to R.C.S.

30 June 1965

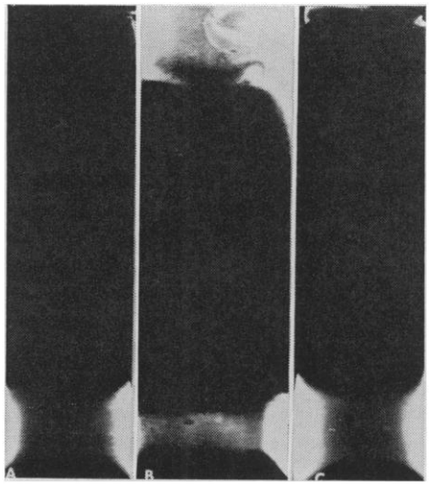

Fig. 2. Photographs of polyacrylamide gels stained with Gomori stain (*3*) using β-glycerophosphate. Phosphatase was heated in appropriate buffer on a water bath at 50°C for 100 minutes, centrifuged to remove precipitate, and placed on the gels for electrophoresis. Anode is at the bottom. (*A*) Enzyme in 0.1*M* tris, *p*H 7.5. (*B*) Enzyme in 1.0*M* acetate, *p*H 5.2, not heated. Note second band at top. (*C*) Enzyme in 1.0*M* acetate, *p*H 5.2, heated.

Inhibitory Oxidation Products of Indole-3-Acetic Acid: Enzymic Formation and Detoxification by Pea Seedlings

Abstract. *Extracts of etiolated pea seedlings oxidize indole-3-acetic acid, a plant auxin, to 3-hydroxymethyloxindole. At physiological* p*H this compound is dehydrated to 3-methyleneoxindole, a highly reactive sulfhydryl reagent and inhibitor of cell growth. 3-Methlyeneoxindole is in turn detoxified by an enzymatic, triphosphopyridine nucleotide–linked reduction to 3-methyloxindole, a nontoxic compound. These enzymatic conversions may be responsible for some of the responses to auxin, particularly sensitivity to its inhibitory effects on growth.*

A search for the biochemical basis for the action of indole-3-acetic acid, a plant auxin, with *Escherichia coli* as a model organism, revealed that both photooxidation of indole-3-acetic acid by riboflavin and its enzymatic oxidation by horseradish peroxidase yield a potent inhibitor of the growth of microorganisms as well as of higher plants (*1*). Although the earliest detectable product of the oxidations, 3-hydroxymethyloxindole, has no toxic effect of its own, it is rapidly dehydrated to 3-methyleneoxindole, a potent sulfhydryl reagent and inhibitor of sulfhydryl enzymes. The bacteriostatic effect of 3-methyleneoxindole is transitory as it is reduced to 3-methyloxindole, a nontoxicant, by a bacterial enzyme utilizing the reduced form of diphosphopyridine nucleotide as the reductant (*2*). Reactions similar to those of the model system (Fig. 1), if found in plants, could maintain precise adjustment of the intracellular level of the highly active 3-methyleneoxindole, and might therefore be involved in the regulatory action of indole-3-acetic acid. We now describe such reactions in extracts of pea seedlings.

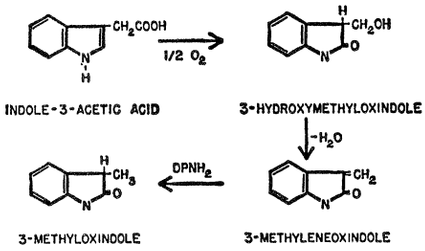

Fig. 1. Formation and reduction of 3-methyleneoxindole.

Extracts of 7-day-old etiolated seedlings (roots and stems separately) were prepared by homogenization in a blender. Clarified by centrifugation, the extracts were dialyzed against distilled water for 18 hours. Oxidation of indole-3-acetic acid was estimated by following its disappearance by a colorimetric method (*3*) from reaction mixtures containing 10 m*M* potassium phosphate buffer (*p*H 6.2), 1 m*M* $MnSO_4$, 0.001 m*M* 2,4-dichlorophenol, 0.3 m*M* indole-3-acetic acid, and extracts of roots (12 mg of protein) or of stems (10 mg of protein). The mixtures were incubated for 45 minutes at 22°C. Similar amounts of indole-3-acetic acid, 14 μmole and 12 μmole, respectively, were oxidized by the extracts of the roots and of the stems.

Production of bacteriostatic compounds, 3-hydroxymethyloxindole or 3-methyleneoxindole or both (*4*), accounted for 19 percent of the indole-3-acetic acid oxidized by the root extract, but for only 11 percent in the case of the stem extract. Of the 2.7 μmole of the inhibitor produced by the root preparation, 1.4 μmole were recovered and identified as 3-hydroxymethyloxindole after extraction with ether and paper chromatography (see *1*).

Identification is based on comparison of the biological, chemical, and physical properties of the compound produced by pea seedlings with those reported for 3-hydroxymethyloxindole (*1, 2, 5*). An ultraviolet-absorbing compound with bacteriostatic action had an R_F on paper chromatography, with isopropanol-water (5 : 95) as the solvent, identical with that of 3-hydroxymethyloxindole; on elution from the chromatogram with water, it was found to have the ultraviolet-absorption spectrum of 3-hydroxymethyloxindole (Fig. 1*A*). Like the spectrum of 3-hydroxymethyloxindole, the spectrum of the compound produced by extracts of pea seedlings changed slowly at *p*H 5.0 (Fig. 2*B*) and rapidly at *p*H 8.5 (Fig. 2*C*) to that of 3-methyleneoxindole; the latter reacted characteristically with a sulfhydryl compound, as indicated by the considerably decreased and changed absorption spectrum (Fig. 2*D*).

A small amount of 3-methyleneoxindole was also detected as a product of oxidation of indole-3-acetic acid; however, the exact quantity was not measurable because this compound forms an inactive polymer of unknown composition during the concentration (by evaporation of solvent) necessary for analysis by paper chromatography. This formation of polymer could account for the discrepancy between the amount of inhibitor that is detectable by bioassay and the amount of 3-hydroxymethyloxindole that was recovered.

Oxidation of reduced triphosphopyridine nucleotide (TPNH) by extracts of pea seedlings is accelerated more than twofold by 3-methyleneoxindole. Diphosphopyridine nucleotide is also oxidized, but only one-fifth as rapidly. Both roots and stems contain this activity, which is also found in commercial dried peas and in pea flour. This observation suggested that the extracts catalyzed a TPNH-linked reduction of 3-methyleneoxindole to 3-methyloxindole, a nontoxicant.

In a test of this possibility, 0.9 μmole of 3-methyloxindole was recovered after an extract of roots (240 mg

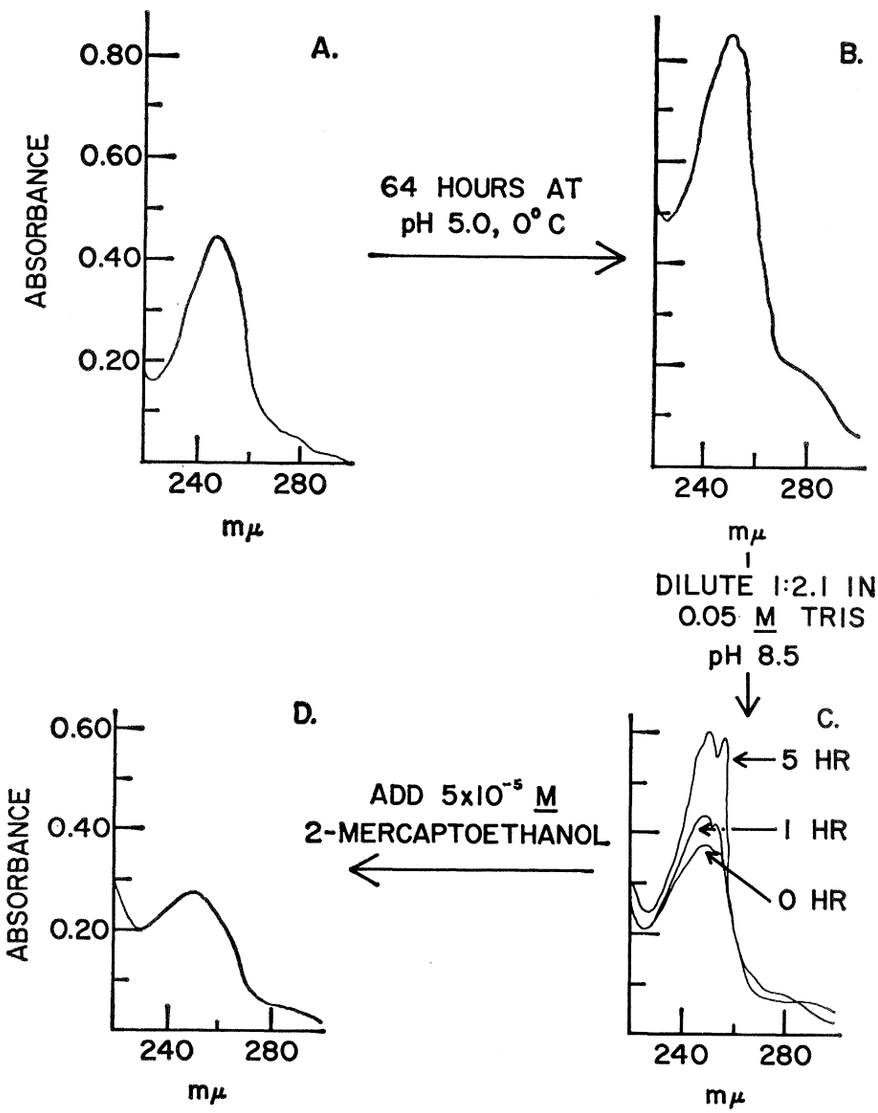

Fig. 2. Spectra and reactions of an inhibitor and its derivatives produced by extracts of pea seedlings.

of protein) was incubated for 10 minutes at 22°C with 33 m*M* potassium phosphate buffer (*p*H 6.5), a system generating TPNH (0.0133 m*M* triphosphopyridine nucleotide, 0.67 m*M* DL-isocitrate, and 1 mg of isocitric dehydrogenase), and 0.0133 m*M* 3-methyleneoxindole (*6*) in a volume of 150 ml.

A closely similar result was obtained with an extract of the stems. In these experiments, only traces of the 3-methyleneoxindole remained unchanged, although only one-half of it had been reduced to 3-methyloxindole. Because 3-methyloxindole is not metabolized by these extracts, the rest of the 3-methyleneoxindole must have undergone other reactions in addition to reduction of its exocyclic double bond. These reactions might well include addition to sulfhydryl groups in the extract and polymerization during the evaporation of ether extracts for chromatography.

Plant tissues known to be subject to the effects of indole-3-acetic acid on growth thus contain an enzyme that can convert the acid to a potent inhibitor of cell growh, 3-methyleneoxindole, and another enzyme that converts this inhibitor to a nontoxic compound. These enzymes may mediate certain of the responses to indole-3-acetic acid, especially inhibition, and local changes in the activities of either enzyme in vivo could play an important role in auxin-dependent tropisms (*7*).

C. C. STILL
CAROL C. OLIVIER
H. S. MOYED

Department of Microbiology,
School of Medicine,
University of Southern California,
Los Angeles 90033

References and Notes

1. T. T. Fukuyama and H. S. Moyed, *J. Biol. Chem.* **239**, 2392 (1964).
2. C. C. Still, T. T. Fukuyama, H. S. Moyed, *ibid.* **240**, 2612 (1965).
3. Y. W. Yang and J. Bonner, *Arch. Biochem. Biophys.* **13**, 11 (1947).
4. Bacteriostatic compounds were estimated by tube dilution assays with *Escherichia coli* as the test organism. This procedure does not distinguish 3-methyleneoxindole from 3-hydroxymethyloxindole because the latter is converted to the former rapidly and nonenzymatically in the medium used for the assay.
5. R. L. Hinman and J. Lang, *Biochemistry* **4**, 144 (1965).
6. We thank R. L. Hinman for gifts of 3-methyloxindole and 3-bromooxindole-3-acetic acid, which yields 3-methyleneoxindole when dissolved in water.
7. Assisted by the Hastings Foundation Fund (University of Southern California School of Medicine) and by grants from NIH (GM-11269) and NSF (GB-3608).

10 May 1965

Actinomycin D and Hydrocortisone: Intracellular Binding in Rat Liver

Abstract. *DNA was found to be the major intracellular binding site for labeled actinomycin D administered in vivo. In analogous studies, hydrocortisone did not bind to nuclear structures. Furthermore, following the administration of actinomycin D, a highly positive correlation was found between the RNA content of the nucleus and the ability of the nucleus to synthesize RNA.*

Actinomycin D has been extensively used in studies of the metabolism of living cells (*1*). Interpretations of the results of these experiments are frequently based on the implicit assumptions that the primary cellular effect of actinomycin D is the inhibition of RNA synthesis, and that this inhibition is the specific result of binding of the antibiotic to DNA (*2*, *3*). These assumptions are justified usually by reference to other work showing that actinomycin D causes (i) marked inhibition of uptake of radioactively labeled RNA precursors into RNA in vivo (*4*) and (ii) marked inhibition of DNA-dependent RNA polymerase activity in vitro (*5*). However, it is apparent that the in vivo effects may be caused by properties of this drug which are not related to DNA binding. For example, the inhibition of uptake of RNA precursors into RNA could result from inhibition of transport of exogenous precursors to the intracellular sites of RNA synthesis. The in vitro activity of the drug in the RNA polymerase system primed by DNA (*5*) and the demonstrated binding of actinomycin D to DNA in vitro (*6*) do not exclude the possibility that, in vivo, the antibiotic may bind to subcellular sites other than DNA, and may interfere with cellular processes other than RNA synthesis. The availability of radioactive actinomycin D enabled us to investigate the binding of actinomycin D in vivo and thereby to evaluate certain current assumptions regarding the mechanism of action of the drug.

In a parallel study we investigated the binding of hydrocortisone to subcellular fractions of rat liver, since others have suggested that the steroid hormones are genotropes (*7*) and have shown that actinomycin inhibits the action of steroids in vivo (*1*).

Actinomycin D-H^3 [specific activity (S.A.), 250 μc/μmole], prepared biosynthetically from *Streptomyces antibioticus* cells grown in the presence of methyl-labeled methionine (*8*), was the gift of Herbert Weissbach (NIH) and Edward Katz (Georgetown University). Unlabeled crystalline actinomycin D was obtained from the Merck Institute for Therapeutic Research (courtesy of Clement A. Stone); its concentration was calculated from its extinction at 440 mμ by using a molar extinction coefficient of 2.48×10^4 (*8*). Hydrocortisone-1,2-H^3 (S.A. 12.6 c/mmole) and cytidine-H^3, uniformly labeled (S.A. 3.68 c/mmole), were purchased from New England Nuclear Corp. Cytidine-H^3-5′-triphosphate (S.A. 1.1 c/mmole) was purchased from Schwarz Bio Research Inc. Calf thymus DNA was obtained from Worthington Biochemical Corp. All rats used were 4- to 5-week-old males of the Osborne-Mendel strain weighing 75 to 100 g, and had food freely available unless otherwise specified. Radioactivity was measured by counting either in a toluene scintillator containing 5 g of 2,5-diphenyloxazole and 0.1 g of 1,4-di(5-phenyl-2-oxazolyl)-benzene per liter or in the scintillator described by Bray (*9*). All counts were corrected to an efficiency of 20 percent (actual efficiencies ranged from 1 to 35 percent).

Rats were injected intraperitoneally with either actinomycin or hydrocortisone. After an appropriate interval, they were killed and their livers removed for subcellular fractionation (*10*). The tissue was gently homogenized in solution SM (0.32*M* sucrose, 2 m*M* $MgCl_2$, 1 m*M* potassium phosphate, *p*H 6.8), and the crude nuclear sediment obtained by centrifugation at 850*g* for 10 minutes was washed twice with solution SM. The crude nuclear sediment was purified by suspension in a final concentration of 2.1*M* sucrose (containing 1 m*M* $MgCl_2$ and 1 m*M* potassium phosphate buffer, *p*H 6.8) and centrifugation at 52,000*g* for 90 minutes. This step yielded a purified nuclear pellet and a floating fraction containing nuclear contaminants (microsomes, mitochondria, fragmented cells, whole cells, and a few nuclei). In some experiments (rats Nos. 1, 2, and 3), the purified nuclei were lysed by gentle homogenization with a loose