and victorin strongly indicate that both may have a similar mode of action. Susceptible tissues which have previously been exposed to victorin fail to respond to the addition of DNP. If victorin has an effect similar to that of DNP, it is probable that the rate-limiting phosphate acceptor systems are by-passed.

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31 March 1958

Crystallization of Chlorophylls

It has been pointed out that the successful crystallization of the chlorophyll pigments depends on high purity and the presence of water (*1*). We wish to report (*2*) the observation that the precipitation of the chlorophylls from highly impure extracts in organic solvents by washing with water constitutes a coprecipitation of *crystalline chlorophyll a* with amorphous chlorophyll *b*. This observation was made during a systematic spectrophotometric survey of each fraction obtained during the preparation of microcrystalline chlorophylls according to the method of Jacobs *et al.* (*1*). A sample of the petroleum ether extract of the pigments (see below) showed a pronounced absorption band with a peak at 745 mμ. According to Jacobs and Holt (*3*), this absorption band (corrected for scattering) is associated with a microcrystalline suspension of chlorophyll *a*.

The following example provides a generalized description of our procedure. Four pounds of fresh spinach was blended with acetone and filtered through a pad of Hyflo Super Cel on Whatman No. 1 filter paper in a large Büchner funnel. About 600 ml of solution passed through the filter before chlorophyll appeared. This solution, containing some of the yellow pigments and acetone soluble lipids, was discarded. After further acetone extraction of the gross content of the chlorophylls, the pigments were transferred in a separatory funnel to a single 500-ml portion of Skelly solvent F by successive treatment of 1-liter portions of the acetone extract with 2.5-liter portions of distilled water. Crystallization of chlorophyll *a* was already apparent in the petroleum ether after the second transfer from acetone (dashed curve of part A, Fig. 1). The solid curve of part A, Fig. 1, was obtained after all the pigments had been transferred to the petroleum ether. The dashed curve represents the spectrum of a 1:7.5 dilution with petroleum ether, while the solid curve represents the spectrum of a 1:30 dilution. The pigments were precipitated in the centrifuge and washed several times with fresh petroleum ether.

The separation of chlorophylls *a* and *b* was achieved at this point by the chromatographic procedure utilized by Jacobs *et al.* (*1*). The crystallization of the individual chlorophylls was accomplished in a manner somewhat similar to that used by these authors. The isopropyl alcohol–pentane solution of chlorophyll obtained as the effluent from the chromatographic column was thoroughly washed with water. During this procedure, microcrystals of chlorophyll *a* appeared as shown by the absorption spectrum (part B, Fig. 1). Chlorophyll *b* was removed from the sucrose adsorbent with acetone. This was followed by transfer of the chlorophyll *b* to petroleum ether by addition of water. Thorough water washing of the petroleum ether layer was continued until microcrystalline chlorophyll *b* appeared as shown by the absorption spectrum (part B, Fig. 1). Collection of the crystals was considerably simplified by the use of a model L Spinco ultracentrifuge (20,000 *g* for up to 30 minutes).

It should be remarked that all operations were carried out in a cold room at 4°C. Chromatography of the pigments at room temperature resulted in obvious color changes while the pigments were still on the sucrose adsorbent.

In addition to the spectrophotometric studies presented here, other physical studies of these crystals have been carried out (*4*). G. Donney of the Geophysical Laboratory of the Carnegie Institution of Washington took several x-ray powder diagrams of our crystalline preparations (*5*). It was found that the powder diagrams of the mixture of chlorophylls *a* and *b* precipitated from petroleum ether were identical with the powder diagrams of the pure chlorophyll *a* crystals. Such an observation has also been made with artificial mixtures of chlorophylls *a* and *b* (*6*). Since the spectrum of the redissolved precipitate shows the presence of chlorophyll *b*, this indicates that chlorophyll *b* in an amorphous form coprecipitates with the microcrystalline chlorophyll *a*. The less likely possibility that a mixed crystal with practically unchanged parameters is formed is not excluded by these considerations.

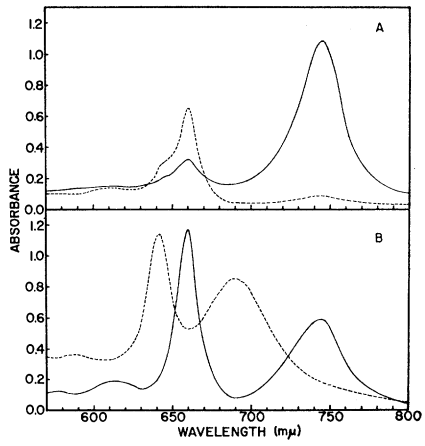

Fig. 1. Absorption spectra of precipitated chlorophylls in petroleum ether. *A*, Spectra of mixed chlorophylls at two stages of crystallization (see text). *B*, Spectra of pure chlorophyll *a* (solid curve) and chlorophyll *b* (dashed curve) showing absorption bands for both dissolved and microcrystalline chlorophylls. The spectra were obtained with a Cary model 14 spectrophotometer with a 1-cm cell; they have not been corrected for scattering.

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15 April 1958

Inhibition of Adrenal Steroid 11-Oxygenation in the Dog

Inhibition of adrenal cortical secretion by a direct action on steroid biosynthesis has been described following administration of amphenone B [3,3-di(*p*-aminophenyl)butanone-2 dihydrochloride], but limitations imposed by the toxic effects of this substance have led to the search for other inhibitory agents. Recently, the

compound 2-methyl-1,2-bis(3-pyridyl)-1-propanone (SU 4885) (*1*), has been synthesized by Allen and Bencze (*2*). This report is concerned with the action of this drug on adrenal cortical secretion in the dog.

Normal dogs were given an intravenous infusion of ACTH, and adrenal venous blood was obtained at timed intervals according to the method of Nelson and Hume (*3*). The plasma was extracted with chloroform and subjected to paper chromatography, according to the technique of Bush (*4*) and Zaffaroni (*5*). Identification was carried out by chromatographic and spectrophotometric means. In each case the free steroid ran on the Bush ("B 3" and "B 5") and Zaffaroni (toluene–propylene glycol) systems at identical rates with known steroid standards. Acetylation of each of these unknown compounds also resulted in acetates that moved at identical rates with known acetates of each. The presence of 17,21-dihydroxyketones was indicated by a positive Porter-Silber reaction (*6*) in the appropriate instances. Sulfuric acid spectra were in good agreement with those of appropriate standard compounds. All steroids isolated from adrenal venous blood gave, in addition, maximal absorption in ultraviolet light at 238 to 240 mμ, and the positive soda-fluorescence reaction of Bush (*4*) for Δ^4,3-ketones. Pregnane-3α,17α,21-triol-20-one (tetrahydro-S) was identified in the urine of a patient receiving the drug by negative reactions for Δ^4,-3-ketones, positive Porter-Silber and blue tetrazolium reactions, identical running rates both of the free and acetylated compound with those of authentic tetrahydro S and sulfuric acid spectra with absorption peaks at 315 and 410 mμ. Quantitative estimation was carried out by elution of the steroids off paper and application of the blue tetrazolium and Porter-Silber reactions. Total 17-hydroxycorticosteroids in blood were determined by the method of Nelson and Samuels (*7*).

A single intravenous injection of 75 mg of SU 4885 bitartrate per kilogram caused a decrease in adrenal venous 17-hydroxycorticosteroids to 10 percent of the initial value within 10 minutes followed by a return to 75 percent within 3 hours. The most notable effect, however, was seen when the individual steroids were identified. Figure 1*a* shows that Δ^4-pregnene-11β,17α,21-triol-3,-20-dione (cortisol) was reduced from an initial value of 6.0 μg/min to unmeasurable levels within 20 minutes and was only 1.2 μg/min 5 hours later. Simultaneously, Δ^4-pregnene-17α,21-diol-3,20-dione (Reichstein's compound S) appeared and rose steadily from previously undetectable levels to 5.6 μg/min at 5 hours, thereby accounting for the greater part of the 17-hydroxycorticoid secretion

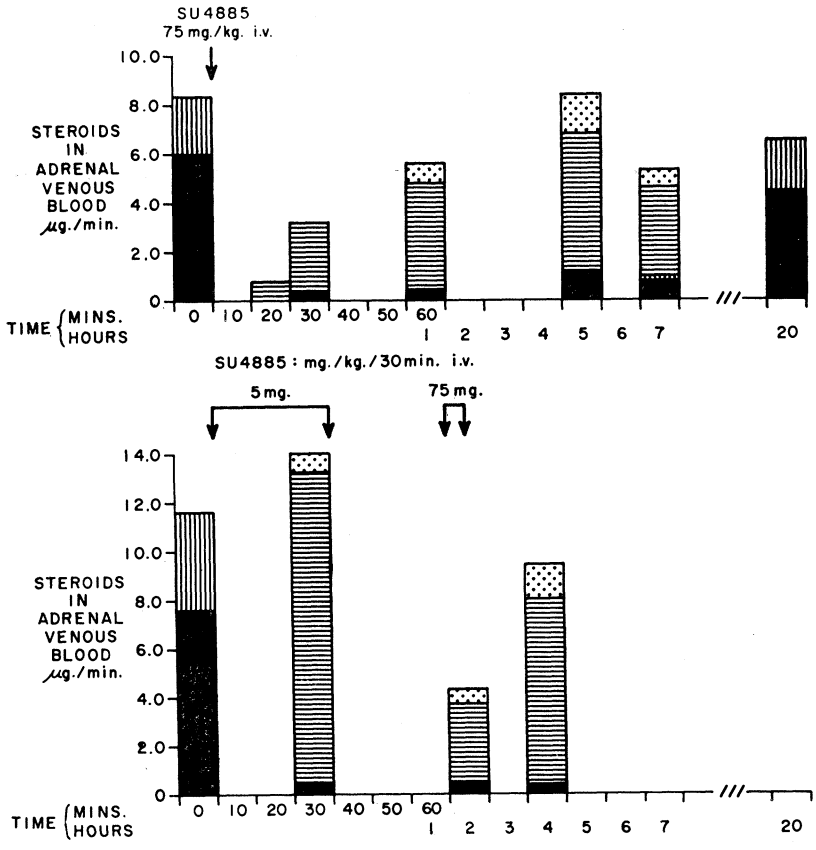

Fig. 1. (*a*) Effect of single injection of SU-4885 (75 mg/kg) on steroids in adrenal venous blood of a 19.3-kg dog. (*b*) Effect of 30-minute infusions of SU-4885 (5 mg and 75 mg/kg) on a 23-kg dog. Solid areas, cortisol; vertical lines, corticosterone; horizontal lines, 11-deoxycortisol (compound S); dotted areas, deoxycorticosterone.

at this time. Δ^4-Pregnene-11β,21-diol-3,-20-dione (corticosterone), which was present together with cortisol in the untreated animal, disappeared, and Δ^4-pregnene-21-ol,3,20-dione (deoxycorticosterone) was identified. The inhibition of 11-hydroxysteroids lasted for approximately 8 hours, and 20 hours after the injection, cortisol had returned to its initial value.

In further experiments the infusion of only 5 mg of the drug per kilogram over a period of 30 minutes produced no fall in total 17-hydroxycorticosteroids and, in fact, a rise was observed, but Fig. 1*b* shows that cortisol was reduced from 7.5 μg/min to 0.5 μg/min with the appearance of 12.5 μg of compound S. In contrast with these findings, amphenone hydrochloride, when injected in a dosage equivalent to 75 mg of SU 4885-bitartrate per kilogram, produced a temporary decrease in cortisol and corticosterone, both of which returned to 50 percent of the initial values in 2 hours, but without the appearance of 11-deoxy compounds, indicating that amphenone had no selective action on 11-oxygenation.

Preliminary clinical studies made on a patient suffering from Cushing's syndrome indicate that SU 4885 may have an action in man similar to that in the dog. The administration of the drug to this patient was followed by the appearance of large amounts of tetrahydro-S in the urine and was accompanied by a decreased excretion of tetrahydrocortisone and tetrahydrocortisol.

These experimental results demonstrate that SU 4885, in large doses, causes a generalized suppression of adrenal steroid biosynthesis which is followed by a more prolonged period during which 11-oxygenation is selectively inhibited. In smaller doses, the effect on total steroid secretion is not seen, but a profound inhibition of 11-oxygenation occurs, indicating that this particular enzyme system is especially sensitive to the drug. Similar results with this substance have recently been obtained by another group of investigators (*8*).

It is to be anticipated that a study of the relationship between the chemical structure of this substance and its mode of action may lead to the elaboration of further compounds capable of inhibiting adrenal enzyme systems (*9*).

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n-Butyl 5-Chloro-2-pyrimidoxyacetate—a Plant Growth Regulator Analog

Since the first demonstration of plant growth stimulation by substituted phenoxyacetic acids (*1*), much attention has been directed toward this series of compounds in an attempt to correlate the position and type of substituent with observed growth promotion. The results of the many investigations aimed at the elucidation of the mechanism of growth-regulator action have been resolved into three general theories.

One of these theories (*2*) supposes that there is a chemical reaction between the regulator and appropriate groups, probably nucleophilic, on some plant substrate, resulting in the formation of new convalent bonds. It has been shown that the most likely point of attack on the ring is at the positions *ortho* to the ether oxygen. Another theory (*3*) gives major importance to the shape of the regulator molecule and the specificity of its "fit" onto some receptor within the plant. In this case, the benzene nucleus with its substituents acts as a whole at the locus or point of attachment, and chemical reactions at the ring are considered unlikely. The third and most recent theory, unlike the other two, is not concerned with the relations of the regulator with an "active site," but suggests that the growth-regulating activity of a compound is primarily associated with its ability to chelate metal ions, such as Mg^{++} (*4*).

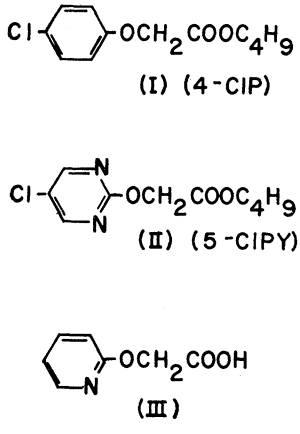

Fig. 1. Structural formulas.

In order to make a comparative test of these theories, we have prepared the butyl ester of 5-chloro-2-pyrimidoxyacetic acid (Fig. 1, II) (*5*). This compound is almost identical with the very active growth regulator, butyl *p*-chlorophenoxyacetate (Fig. 1, I) in its size, shape, ring planarity, molecular weight, halogen and ester reactivity, and also in those physical constants which were measured. However, the pyrimidine offers no possibility of nucleophilic reaction in the positions *ortho* to the ether oxygen (positions 1 and 3), and an aromatic type of substitution has never been observed to occur in positions 4 or 6 of the pyrimidine nucleus.

The plant growth regulatory activity of compounds I and II (Fig. 1) was measured by use of the oat coleoptile, wheat coleoptile, oat first-internode, and slit-pea curvature bioassays. In each case, the phenoxy ester was shown to be a highly active growth stimulator, while the pyrimidoxy ester was completely inactive.

An example of this difference in activity is shown in Fig. 2. For these tests, 4-mm sections of first internodes of dark-grown oat seedlings were rotated at *p*H 5.0 in citrate-phosphate buffer containing 2 percent sucrose and the compound under investigation at concentrations of 10^{-3} to $10^{-7}M$ (*6*). After about 20 hours, the oat sections were removed, their length was measured, and the data were subjected to statistical analysis. Results similar to those presented in Fig. 1 were obtained at *p*H 7.5 in 0.067*M* phosphate buffer, but were less clearly defined because of a diminished difference in growth between the treated sections and controls under alkaline conditions (*6*).

Consideration of the structure of compound II (Fig. 1) shows that it should be a chelating agent in the ways proposed for indole-3-acetic ester and for derivatives of phenoxyacetic acid. In addition, chelation could occur through the two nitrogens as it does in similar

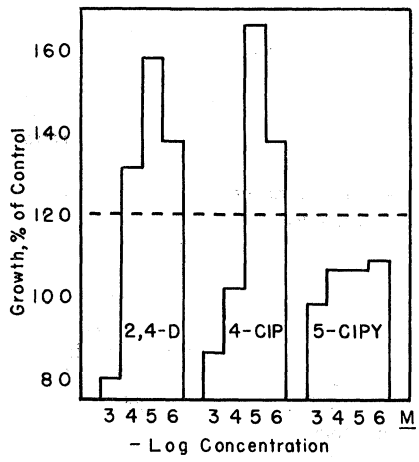

Fig. 2. Growth of oat first-internode sections at *p*H 5.0. Values above the broken line represent statistically significant promotion.

ureas and pyrimidines. Regarding the specificity of its "fit" onto a receptor, the molecule is spatially almost indistinguishable from that of compound I (Fig. 1) and while the pyrimidine ring would be expected to be more hydrophilic than the benzene ring, the recent descriptions of the high degree of plant growth stimulation by 2-pyridoxyacetic acid (Fig. 1, III) (*7*) indicate that lipophilic character may not be as important as previously suggested. Consequently, the inactivity of compound II (Fig. 1) does not substantiate two of the current theories, and it leads us to conclude that the *ortho* positions may indeed bear an important relation to biological activity in the phenoxy acid series (*8*).

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