tion with acetic anhydride gave the acetyl derivative, m.p. 207–210°, no depression on mixed melting point with an authentic specimen of acetylpodophyllotoxin. Treatment with piperidine in boiling alcohol (footnote 4, procedure for epipicropodophyllin) gave picropodophyllin (m.p. 213–223°) in 75% yield; acetyl derivative, m.p. 211–214°, no mixed m.p. depression with an authentic specimen of acetylpicropodophyllin. The ultraviolet and infrared absorption spectra were identical with those of podophyllotoxin from podophyllin.[5]

**Savinin.**—Fractions D, E and F were combined (0.12% yield) and crystallized first from benzene then from absolute ethanol, yielding large, colorless, transparent prisms m.p. 146.4–148.4°, $[\alpha]^{21}D$ −87° (*c* 0.95, chloroform).

*Anal.* Calcd. for $C_{20}H_{14(16)}O_6$: C, 68.57 (68.16); H, 4.03 (4.57); mol. wt., 350.3 (352.3). Found: C, 68.63; H, 4.65; $OCH_3$, nil.; loss of weight on drying, nil.; mol. wt. (Signer, isopiestic), 360.

The Gaebel test[6] for the methylenedioxy group was positive. The ultraviolet absorption spectrum (Fig. 2) bore no resemblance to that of podophyllotoxin. In the infrared (Fig. 1), the absence of a band around 3450 cm.$^{-1}$ indicates the absence of a hydroxyl group, while bands at 1751 and 1651 cm.$^{-1}$ are strong evidence for the presence of an unsaturated lactone. Savinin was insoluble in cold and hot dilute aqueous sodium hydroxide and therefore has no phenolic or carboxylic acid function. Solubility in alcoholic alkali after short boiling, with formation of a water-soluble salt, indicated a lactone. Structural studies are in progress on this compound.

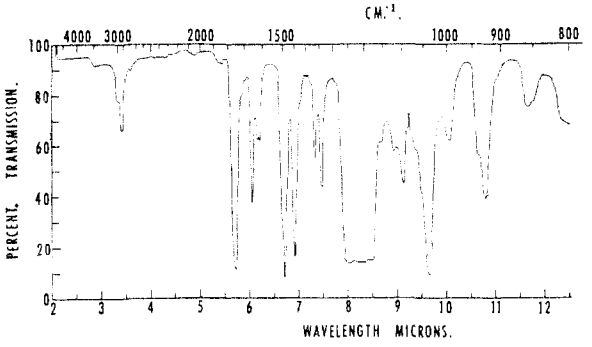


Fig. 1.—Infrared absorption spectrum of savinin in chloroform.

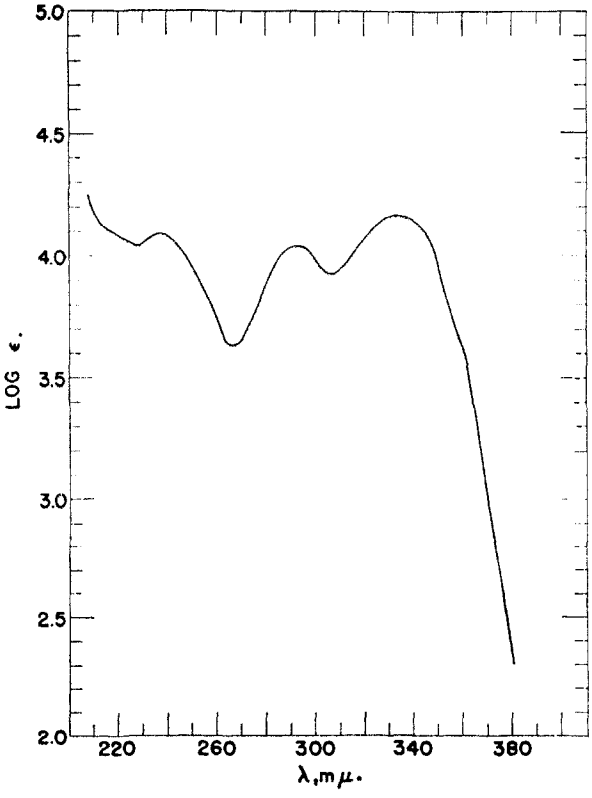


Fig. 2.—Ultraviolet absorption spectrum of savinin in 95% ethanol (based on mol. wt., 298.3).

(5) J. L. Hartwell and W. E. Detty, THIS JOURNAL, **72**, 246 (1950); A. W. Schrecker and J. L. Hartwell, *ibid.*, **74**, 5672 (1952).

(6) G. O. Gaebel, *Arch. Pharm.*, **248**, 225 (1910).

TABLE I

PODOPHYLLOTOXIN IN DIFFERENT *Juniperus* SPECIES

| Juniper | Source | Podophyllotoxin, % |
|---|---|---|
| Savin[a] | S. B. Penick and Co. | 0.20 |
| *J. virginiana*[b] | Bethesda, Maryland | .10 |
| *J. lucayana*[c] | Florida | .10 |
| *J. scopulorum*[d] | California | .17 |
| *J. sabina* var. *tamariscifolia*, male plant[d,e] | California | .14 |

[a] The dried needles of an evergreen stated by the supplier to be *J. sabina*. [b] Identified by Mr. E. C. Leonard, Smithsonian Institution, Washington, D.C. [c] Provided through the courtesy of Mr. R. A. Bonninghausen, Florida Board of Forestry, Tallahassee, Fla. [d] Provided through the courtesy of Dr. J. W. Duffield, Institute of Forest Genetics, Placerville, California. [e] The female plant, collected from the same source, yielded a new active substance instead of podophyllotoxin. These results will be reported in a later communication.

**Distribution of Biological Activity in Savin.**—This is summarized in Table II. The xylene mother liquor is being investigated for other active components.[7]

TABLE II

DISTRIBUTION OF TUMOR-DAMAGING ACTIVITY IN SAVIN

| Substance | Yield, % | MED (approx.),[a] μg./g. | Proportion of total activity,[b] % |
|---|---|---|---|
| Original savin | 100 | 400 | 100 |
| Podophyllotoxin | 0.20 | 2 | 40 |
| Savinin | 0.10 | >1000 | 0 |
| Xylene mother liquor | 2.3 | 25 | 37 |

[a] Minimum effective dose for a single subcutaneous injection in mice bearing implants of Sarcoma 37. [b] Yield × 400/MED. The failure of the last three items in this column to add up to 100% is due to accumulated losses in the fractionation and to large uncertainties in the values of MED.

(7) A series of crystalline waxes called "etholides," probably linear polyesters of ω-hydroxy fatty acids, have been isolated from *J. sabina* and other conifers [J. Bougault and L. Bourdier, *Compt. rend.*, **147**, 1311 (1908); *J. pharm. chim.*, **29**, 561 (1909)]. One of these, m.p. 67–69°, obtained by us from savin, had no activity against Sarcoma 37 in mice.

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## Biosynthesis of Carotene in Phycomyces

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RECEIVED SEPTEMBER 5, 1952

The effect of β-ionone on β-carotene production in *Phycomyces*[1] raises many questions as to the course of carotenoid biosynthesis. While it seems most probable that the β-ionone may be incorpo-

(1) G. Mackinney, T. Nakayama, C. D. Buss and C. O. Chichester, THIS JOURNAL, **74**, 3456 (1952).

TABLE I

EFFECT OF TIME ON CULTURE DEVELOPMENT

| Time in hr. | Sugar,[a] g./100 ml. A[c] | B | Nitrogen[b] % of original A | B | Dry matter in g. A | B | Pigment μg./g. A | B | $D_{370}:D_{450}$ A | B |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 2.62 | 2.62 | 100 | 100 | ... | ... | ... | .. | ... | ... |
| 42 | 2.30 | .. | 46.7 | 53.0 | 0.268 | 0.208 | 93 | 822 | 0.336 | 0.266 |
| 50 | 1.80 | 2.06 | 31.4 | 48.1 | .375 | .238 | 185 | 1200 | .181 | .182 |
| 65 | 1.55 | 1.74 | 26.9 | 31.4 | .439 | .392 | ... | 853 | .166 | .137 |
| 90 | 1.29 | 1.29 | 19.8 | 21.6 | .462 | .438 | 463 | 932 | .140 | .142 |
| 113 | 1.27 | 1.03 | 19.6 | 22.8 | .388 | .405 | 585 | 1170 | .126 | .123 |

[a] Alkaline ferricyanide–ceric sulfate. [b] Micro-Kjeldahl. [c] A, controls; B, ionone-treated.

rated directly into the carotene molecule, it is still an assumption. We observed in our previous report that the age of the culture was quite critical in determining the magnitude of the effect, so we have extended the duration of the experiments, determining dry weight production, utilization of sugar and nitrogen as well as production of pigment. The antioxidant role of diphenylamine has been investigated by Turian[2,3] and Goodwin.[4] It became therefore of interest to determine whether β-ionone could overcome the adverse effect of diphenylamine (DPA) on carotene production. Finally it seemed desirable to set down in detail the techniques whereby the effects have been observed.

## Methods

**Time of Treatments.**—The β-ionone and DPA may be added at the start, when the medium is inoculated, or after a given period to permit reasonable growth. Both procedures have been used.[5]

**Inoculation—1. By Spores.**—For spores, the *Phycomyces* is cultured on 10% wort–3% agar for 3 to 4 weeks, on a flat 1-pint bottle, surface *ca.* 14 × 9 cm. The heavy growth is shaken gently with 75 ml. of water, and each petri dish is inoculated with 2.5 ml. of the resultant suspension by pipet. To ensure uniform growth on the supported filter paper, we have used the spore culture inoculum.

**2. By Vegetative Transfer.**—The vegetative transfer requires a more solid support. For qualitative demonstration, we have transferred portions of young white vegetative growth to the center of an agar plate. A spreading colony develops rapidly, covering the plate in about 36 hours. Recently such transfers have been made on a weak gelatin medium with good results. The medium can be dissolved in warm water and the mycelium harvested on a buchner funnel. There appears to be no reason why this procedure should not be made quantitative, though the growth is obviously not of uniform age throughout the giant colony.

**Temperature.**—For convenience, after the initial incubation, the plates are normally exposed on a table at room temperature, unstacked. However, we have grown cultures at 25° and then retarded subsequent growth by holding them at 15° to study pigment formation.

**Method of Application.**—The DPA is added in alcoholic solution, 0.1 ml. per plate to give a final concentration of 1 in 33,000, occasionally also of 1 in 50,000.[5]

The β-ionone has been added in three ways. A spray in alcoholic solution from an atomizer has been found unsatisfactory owing to erratic and non-uniform growth. If a drop of the ionone is placed on the surface of the filter paper, growth will occur around the drop, and will be highly colored, so that growth inhibition is apparently purely a surface effect. We now place the ionone at four points just off the filter paper support. The petri dish is *ca.* 9 cm. in diameter and 7 cm. filter paper is used. A hanging drop of ionone on the under side of the lid is equally effective for vegetative transfers, but is not so effective for spore cultures. The reason for this may be that in the former case, pigment formation and growth are proceeding concurrently, whereas in the latter, growth has already taken place. Absorption may therefore take place *via* the vapor as well as from solution.

**Harvesting.**—In the present series of experiments, the tared filter papers with the washed growth are lyophilized to constant weight. The loss in weight of control papers is corrected for.

**Chromatography.**—In our cultures, we have invariably found a heavy β-carotene band, phytofluene and a small but clearly defined lycopene band. We doubt that our strain produces α-carotene, as the spectrum of the first colored eluate is unequivocally that of β-carotene. There is also on occasion, a colorless zone preceding the phytofluene with a blue fluorescence. Its absorption does not interfere with the 368 mμ maximum for phytofluene.

**Calculations.**—Since the colored component of our strain is 98–99% β-carotene, we have approximated its concentration routinely from the optical density at 450 mμ of the crude extract in petroleum ether, using an absorption coefficient of 250 l. per g. cm.

While we have chromatographed many samples, it has not been practicable to do so in all cases. We have been interested in lycopene and phytofluene fluctuations only insofar as they may have affected β-carotene. We have routinely measured the optical densities of the crude extracts at several wave lengths and we have used the ratios $D_{500}:D_{450}$ and $D_{370}:D_{450}$ as measures of lycopene and phytofluene, respectively, relative to β-carotene. The expediency of the procedure is justified on the ground of speed and also of accuracy so long as the β-carotene continues to represent 98% or more of the colored components.

**Sources of Ionone.**—Novoviol, beta (Fritzsche Bros.) redistilled was used in our early work. We have since used Ionone (100%:99% β, 1% α, Dodge and Olcott) with similar results.

## Experimental Results

**The Effect of Time on Carotene Production.**—During short periods, 5 to 23 hours, striking results were observed (1) when β-ionone was added to young cultures. We show in Table I results for cultures with and without β-ionone, added at the time of inoculation, for five time intervals. Sugar and nitrogen utilization are also given. The figures are based on five culture plates per sample. Growth and degree of pigmentation was very uniform and consistent within each set at the time of the first harvest, 42 hours. Previous experience had shown that a minimum of 35 to 40 hours was required for appreciable growth, and application of the ionone at the edge eliminated the erratic growth due to sprays. It is however difficult to maintain consistency in this type of experiment over long periods. Growth and sporulation as the cultures mature become more uneven. To avoid selection of plates for the periodic harvests, they were marked at random at the beginning.

From Table I, it is seen that for the untreated samples in the initial stages, the sugar and nitrogen are utilized somewhat more rapidly, and dry matter accumulates faster than for the treated samples. The pigment content for the latter averaged 995 μg. per g. dry matter, with a P.E. of 107, and the quantity of pigment per unit growth remains therefore constant from an early stage. This is not the case for the controls, where pigment production is secondary to growth

(2) G. Turian, *Helv. Chim. Acta*, **33**, 13, 1988 (1950).

(3) G. Turian and F. Haxo, *J. Bact.*, **63**, 690 (1952).

(4) T. W. Goodwin, *J. Biochem.*, **49**, Proc. xxiii (1951).

(5) In this paper, results are reported only for DPA added at the higher concentration after an initial period of 35–40 hours, favorable to growth. The effect of the ionone had to be determined at the most unfavorable level of DPA attainable, on cultures with good initial growth.

in the early stages and levels off at approximately one-half the concentration for the treated samples, in 90 to 100 hours.

**Diphenylamine and the Ionone Effect.**—Several experiments have been run to determine whether β-ionone could overcome the adverse effect of DPA on carotene production. Results vary with culture age and degree of maturity. A 36-hour culture (6 plates each) was treated with ionone, ionone + DPA, DPA and a fourth set served as control. Twelve hours later, yields of carotene were 38.7, 5.9, 1.1 and 4.1 μg., respectively, per culture set. A 74-hour culture, similarly treated, showed no such effect, averaging 36 μg. carotene per set, though a slight increase, *ca.* 25% was noted on a weight basis. However since both DPA and ionone influence normal culture development, a direct effect of the ionone can only be postulated after DPA treatment if there is an increase in the absolute amount of pigment synthesized *per culture*.

A series of runs were then made in which cultures were incubated for 40 hours, after which different treatments were applied. To eliminate possible differences in the rates of absorption of ionone and DPA, one was applied at stated time intervals after the other. At this stage, much trouble was encountered with the temperature controls. Only the results of Table II are strictly comparable. Those of Table III are comparable horizontally. The physiological ages of the cultures for the latter, at the time of ionone or DPA treatment are not identical, and this is reflected in differences in for example the response to the ionone alone. The DPA treatment, followed by ionone 3 hours later varies also. It seems clear that on a weight basis, the β-ionone overcomes in part the DPA effect. It never equals the ionone-treated samples alone, and it may or more often may not be as effective as the controls. It appears in general to be most effective with young cultures. This of course has been characteristic of the ionone treatment unaffected by the complication of DPA action.

TABLE II

EFFECT OF DPA AND IONONE

| Treatment | β-Carotene, μg./5 cultures | μg./g. |
|---|---|---|
| β-Ionone | 78.5 | 296 |
| Control | 49.5 | 197 |
| β-Ionone (DPA 3 hr. later) | 43.2 | 238 |
| β-Ionone (DPA 2 hr. later) | 35.4 | 173 |
| β-Ionone (DPA 1 hr. later) | 25.5 | 175 |
| β-Ionone (DPA 0 hr. later) | 17.7 | 128 |
| DPA (β-ionone 1 hr. later) | 17.3 | 111 |
| DPA (β-ionone 2 hr. later) | 16.6 | 126 |
| DPA (β-ionone 3 hr. later) | 16.2 | 113 |
| DPA | 14.4 | 77.4 |

TABLE III

PIGMENT PRODUCTION AND DPA-IONONE EFFECTS WITH TIME

| Time in hr.[c] | Control | | Ionone | | DPA Alone | | DPA + Ionone at once | | DPA + Ionone 3 hr. later | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1[a] | 2[b] | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| 0 | 2.54 | 141 | ... | .. | .. | ... | ... | ... | .. | ... |
| 12 | 36.5 | 185 | 100.5 | 543 | 17.6 | 115 | ... | ... | 67 | 438 |
| 48 | 189 | 401 | 229 | 627 | 29.1 | 98 | 51 | 175 | 64 | 192 |
| 96 | 284 | 710 | 446 | 1230 | 19 | 58 | 105 | 292 | 95 | 271 |

[a] Micrograms carotene per 5 cultures. [b] Micrograms per gram dry mycelium. [c] After addition of ionone, or of DPA alone.

## Miscellaneous

**Temperature.**—It had been thought from visual observation that a greater difference in carotenoid content would be found, between treated and control cultures, if held at 15° than when maintained at 25°. This however proved illusory. Regular 36-hour cultures were subdivided and held at 15° and 25° for an additional 24 hours, with and without ionone. Treated cultures contained 1565 and 2240 μg. of pigment per g., for 15° and 25°, respectively, compared with 119 and 144 for the controls. A second run confirmed a ratio of *ca.* 1.3 in the quantities of carotene produced at the two temperatures, regardless of treatment.

**Vitamin A.**—A slight yellowing of the culture was noted when vitamin A alcohol or acetate was added to an agar or gelatin medium. The yellowing, shown by chromatography to be due to β-carotene is however only observed when the vitamin A has been added to the medium prior to autoclaving. We steam distilled 0.5 g. of Crystalets (Chas. Pfizer and Co., Crystalline vitamin A acetate stabilized with gelatin and sugar) and obtained 250 ml. of distillate. This was ether-extracted, the extract evaporated and transferred to alcohol. Placed in the usual liquid medium, it enhanced carotene production, as shown by a marked yellowing of the culture during the next 12–24 hours.

## Discussion

We feel that any extensive discussion of the above phenomenon must await the outcome of labeled ionone studies. Citral is completely ineffective in promoting carotene synthesis. Whether β-cyclocitral might show a response is not known.

The most attractive hypothesis, essentially a speculation at this stage, is that either β-ionone or a large fragment of it including the ring structure, is incorporated into the carotene molecule.[6] If the probability of this is conceded, it would follow that carotene production in the mold is limited by the rate at which it can effect ring closure of the appropriate intermediate. This is supplied by β-ionone and apparently by breakdown products of vitamin A.

(6) Evidence reported at the Pacific Slope Biochemical Conference, Berkeley, October 11, 1952, indicates that with carbonyl-labeled ionone, the carotene is inactive. Also, ionone has no effect on production of carotene in a nitrogen atmosphere. Two possibilities therefore exist—that a β-ionone fragment is incorporated or that the effect is indirect. It may be added that no effect was obtained with methylheptenone.

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# β-Arylserine Ethyl Esters[1]

BY WALTER H. HARTUNG, T. T. DITTRICH AND YEN-TSAI CHANG[2]

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β-Arylserines are of interest because the 3,4-dihydroxy compound is the hypothetical precursor of the hormones elaborated by the suprarenal medula, *viz.*, norepinephrine and epinephrine; *e.g.*

$$3,4\text{-}(HO)_2C_6H_3\text{—}CHOH\text{—}\underset{\displaystyle NH_2}{\underset{|}{CH}}\text{—}COOH \longrightarrow 3,4\text{-}(HO)_2C_6H_3\text{—}CHOH\text{—}CH_2NH_2 + CO_2$$

Perhaps the non-catechol analogs may serve as models with which to examine the prospect of *in vivo* enzymatic decarboxylation to give rise to arylethanolamines, all of which would be expected to exhibit pressor properties.[3]

The following convenient reactions have been used to prepare the ethyl esters of phenyl- and *p*-chlorophenylserine.

(1) Number 13 on Amino Acids. For No. 9 see W. E. Weaver and W. H. Hartung, *J. Org. Chem.*, **15**, 741 (1950).

(2) Fellow American Foundation for Pharmaceutical Education, 1948–1951.

(3) K. H. Beyer, *Advances in Chemistry*, Ser. No. 2, 37 (1950).