THE CHEMISTRY AND BIOLOGICAL ACTIVITY OF EPIRODIN, A HEPTAENE ANTIBIOTIC FROM EPICOCCUM NIGRUM

CAROL JEANNE MCGRATTAN

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THE CHEMISTRY AND BIOLOGICAL ACTIVITY OF EPIRODIN,
A HEPTAENE ANTIBIOTIC FROM EPICOCUM NIGRUM

BY

CAROL J. MCGRATTAN
B.A. Salem State College, 1972

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# TABLE OF CONTENTS

LIST OF TABLES ................................................................. ix
LIST OF FIGURES ............................................................. xi
ABSTRACT ........................................................................... xv

I. INTRODUCTION ................................................................. 1
   A. Metabolic Products ....................................................... 1
   B. Epicoccum nigrum and its Metabolites ......................... 2
   C. Macrolide Antibiotics .................................................. 10

II. MATERIALS AND METHODS ............................................ 20
   A. General Techniques ................................................... 20
      1. Amino Acid Analysis ................................................. 20
      2. Elemental Analysis .................................................. 20
      3. Fluorometry ............................................................. 20
      4. Gas-Liquid Chromatography ..................................... 20
      5. Infrared Spectroscopy .............................................. 20
      6. Mass Spectrometry .................................................. 20
      7. Nuclear Magnetic Resonance Spectroscopy ................. 21
      8. UV-Visible Spectrophotometry .................................. 21
      9. Thin Layer Chromatography ..................................... 21
   B. Biological Assays ....................................................... 24
      1. Bacillus Assay ......................................................... 24
      2. Brine Shrimp Assay ................................................ 25
      3. Chlorella Assay ....................................................... 26
   C. Organism and Stock Culturing ....................................... 27
   D. Procedure for Growth of E. nigrum and Isolation of Humic Acid ........................................... 28
1. Amino Acid Analysis and Phenol Determination of Humic Acid ................................................. 29

E. Procedure for Growth of Epicoccum nigrum and Isolation of Epirodit ................................................ 31

F. Stability of Epirodit .............................................. 33

G. Characterization of Epirodit: Classification Tests ................................................................. 35

1. Bromine in Carbon Tetrachloride ..................... 35

2. Hydroxylamine Hydrochloride - Ferric Chloride Reaction ............................................................ 35

3. Periodic Acid ................................................... 35

4. Potassium Permanganate Solution ..................... 35

5. Tollen's Reagent ................................................ 36

H. Spectroscopic Studies on Epirodit .............................. 36

1. Infrared Spectrum ................................................ 36

2. Mass Spectrum .................................................... 36

3. Nuclear Magnetic Resonance ................................ 37

4. UV-Visible Absorption Spectra ................................ 37

I. Chemical Methods Used in the Analysis of Epirodit ......................................................... 38

1. Acetylation of Epirodit Mix .............................. 38

2. Acid Hydrolysis ................................................ 39

3. Bromination of Epirodit ...................................... 40

4. Hydrogenation of Epirodit .................................. 41

5. Neutralization Equivalent .................................. 41

6. Periodate Oxidation .......................................... 43

7. Periodate Oxidation of Epirodit Following Saponification ......................................................... 44

8. Potassium Permanganate Oxidation of Epirodit ................................................................. 45

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9. Saponification of Epirodin.................. 46
10. Sodium Borohydride Reduction of Epirodin. 46

J. Biological Activity of Epirodin............. 47
1. Antibacterial Activity of Epirodin........ 47
2. Interaction of Epirodin with Sterols...... 49
3. Inhibition of Yeast by Epirodin......... 51
4. Antifungal Activity of Epirodin.......... 53
5. Effect of Epirodin on the Fruit Fly...... 54
6. Epirodin and Hemolysis.................... 55

III. RESULTS AND DISCUSSION..................... 61
A. Growth of Epicoccum nigrum............... 61
B. Isolation and Analysis of Humic Acid...... 62
C. Extraction and Isolation of the Epirodins.. 71
D. Stability of Epirodin...................... 80
E. Characterization of Epirodin: Classification Tests.......................... 85
   1. Solubility Behavior...................... 85
   2. Bromine in Carbon Tetrachloride....... 89
   3. Hydroxylamine Hydrochloride - Ferric Chloride Reaction.................. 89
   4. Periodic Acid Test...................... 90
   5. Potassium Permanganate Solution....... 90
   6. Tollen's Reagent....................... 90
F. Spectroscopic Studies on Epirodin......... 91
   1. Infrared Analysis........................ 91
   2. Mass Spectrum............................ 95
   3. Nuclear Magnetic Resonance............. 100
   4. UV-Visible Absorption Spectrum........ 105
LIST OF TABLES

1. Concentration of epirodoxin in bacterial assay........ 47
2. Preparation of sterol-epirodoxin solutions......... 49
3. Composition of YNDB medium................................. 51
4. Preparation of epirodoxin solutions for erythrocyte assays................................. 55
5. Normal physiological levels of serum constituents 58
6. Elemental analysis of humic acid....................... 63
7. Amino acid analysis of humic acid....................... 65
8. Rₜ values for the epirodoxins in various solvent systems................................. 76
9. Activity of epirodoxin mix against B. subtilis and C. pyrenoidosa................................. 77
10. Stability of epirodoxin mix against light........... 81
11. Stability of epirodoxin in various solvents........ 84
12. Stability of epirodoxin at various pH levels...... 86
13. Assignments for the absorption bands observed in the infrared spectrum of epirodoxin mix........ 94
14. Effect of riboflavin and visible light on the absorbance of epirodoxin in 0.05 M phosphate buffer, pH 6.9.................................................. 127
15. NMR peak assignments for acetylated epirodoxin mix based on the NMR spectrum of acetylated filipin................................. 133
16. Acetylation of epirodoxin mix............................. 140
17. Elemental analysis data on epirodoxin mix........... 164
18. Uptake of H₂ by epirodoxin mix in glacial acetic acid................................. 166
19. Determination of the neutralization equivalent of epirodoxin mix................................. 168
20. Rₜ values of aliphatic acids developed in ethyl acetatet:2.5% ammonia (95:5)................................. 181
21. Spray reactions and $R_p$ values of various acids... 182  
22. $R_T$ values of dicarboxylic acids......................... 184  
23. Determination of the neutralization equivalent of saponified epirodi mix......................... 190  
24. Absorbance readings of a 10 ug/ml solution of epirodi mix in solutions containing varying concentrations of sterol......................... 208  
25. Fluorescence of a 10 ug/ml solution of epirodi with increasing amounts of cholesterol........... 214  
26. Effect of epirodi on the growth of *S. cerevisiae* as measured by absorbance at 530 nm........ 216  
27. Effect of epirodi added sixteen hours prior to inoculation of *S. cerevisiae* as measured by absorbance at 530 nm......................... 219  
28. Effect of ergosterol on the ability of epirodi to inhibit the growth of *S. cerevisiae* as measured by absorbance at 530 nm......................... 223  
29. Extent of hemolytic action of epirodi mix by absorbance readings at 550 nm......................... 231  
30. Effect of erythrocyte concentration on hemolysis by epirodi......................... 235  
31. Effect of serum on hemolysis by epirodi at 80 ug/ml.......................... 241  
32. Ability of cholesterol at 50 ug/ml to inhibit hemolysis by epirodi mix......................... 244  
33. Effect of an osmotic stabilizer, sucrose, on hemolysis by epirodi mix......................... 248
LIST OF FIGURES

1. Flavipin................................................................. 4
2. Orcinol, resorcinol.................................................. 6
3. Orsellenic acid, p-hydroxycinnamic acid...................... 7
4. Leucomycin $A_1$, Erythromycin $A_1$......................... 12
5. Tetrins $A$ and $B$, nystatin.................................. 15
6. Eurocinolides $A$ and $B$, filipin, mycoticins $A$ and $B$. 16
7. Dermestatin $A$ and $B$........................................... 17
8. Candidin, amphotericin $B$........................................ 19
9. Hydrogenation apparatus......................................... 42
10. TLC of ether-soluble portion of hydrolyzed humic acid. System of Martin and Haider.................. 68
11. TLC of ether-soluble portion of hydrolyzed humic acid. System of Smith et al. .................. 70
12. TLC of various fractions in the isolation procedure of the epirodis........................................ 73
13. Dose-response curve for the activity of epirodis mix against $B. subtilis$ and $C. pyrenoidosa$...... 79
14. Effect of constant exposure to fluorescent light on the stability of epirodis mix....................... 83
15. Effect of pH on the stability of epirodis mix... 88
16. Infrared spectrum of epirodis mix............................ 93
17. TLC of epirodis mix and the trimethylsilyl ether derivative of epirodis mix............................... 97
18. Lower portion of the mass spectrum of the trimethylsilyl ether derivative of epirodis mix. 99
19. Graph of Log $m/e$ vs. Log (distance from $m/e = 28$ for $N_2$)....................................................... 102
20. Nuclear magnetic resonance spectrum of epirodis mix................................................................. 104
<table>
<thead>
<tr>
<th>No.</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>38.</td>
<td>Ultraviolet spectrum of brominated epirodin mix</td>
<td>160</td>
</tr>
<tr>
<td>39.</td>
<td>Infrared spectrum of brominated epirodin mix</td>
<td>163</td>
</tr>
<tr>
<td>40.</td>
<td>UV-visible absorption spectrum of the 2,4-dinitrophenylhydrazone derivative of periodate-treated epirodin mix</td>
<td>173</td>
</tr>
<tr>
<td>41.</td>
<td>UV-visible absorption spectrum of 2,4-dinitrophenylhydrazine</td>
<td>175</td>
</tr>
<tr>
<td>42.</td>
<td>Infrared spectrum of the 2,4-dinitrophenylhydrazone of periodate-treated epirodin mix</td>
<td>177</td>
</tr>
<tr>
<td>43.</td>
<td>GLC elution patterns for the standard dicarboxylic acids and the unknown oxidation product from epirodin mix</td>
<td>186</td>
</tr>
<tr>
<td>44.</td>
<td>GLC elution pattern for the mixed sample of standard dicarboxylic acids and the unknown oxidation product from epirodin mix</td>
<td>188</td>
</tr>
<tr>
<td>45.</td>
<td>Visible absorption spectrum of saponified epirodin mix</td>
<td>192</td>
</tr>
<tr>
<td>46.</td>
<td>TLC of epirodin mix and saponified epirodin mix</td>
<td>195</td>
</tr>
<tr>
<td>47.</td>
<td>UV-visible absorption spectrum of NaBH₄-reduced epirodin mix</td>
<td>198</td>
</tr>
<tr>
<td>48.</td>
<td>Proposed working structure for the epirodin molecule</td>
<td>203</td>
</tr>
<tr>
<td>49.</td>
<td>Effect of increasing amount of sterol on the absorbance of epirodin mix</td>
<td>211</td>
</tr>
<tr>
<td>50.</td>
<td>Effect of increasing amounts of sterol on the visible absorption spectrum of epirodin mix</td>
<td>213</td>
</tr>
<tr>
<td>51.</td>
<td>Effect of epirodin mix on the growth of <em>S. cerevisiae</em></td>
<td>218</td>
</tr>
<tr>
<td>52.</td>
<td>Determination of stability of epirodin mix for the duration of the <em>S. cerevisiae</em> assay</td>
<td>221</td>
</tr>
<tr>
<td>53.</td>
<td>Ability of ergosterol to reverse the inhibition on growth of <em>S. cerevisiae</em> by epirodin</td>
<td>225</td>
</tr>
<tr>
<td>54.</td>
<td>Effect of epirodin mix on the hemolysis of human erythrocytes</td>
<td>233</td>
</tr>
<tr>
<td>55.</td>
<td>Effect of human erythrocyte concentration on hemolysis by epirodin mix at 80 ug/ml</td>
<td>237</td>
</tr>
</tbody>
</table>
56. Percentage of erythrocytes hemolyzed with an increasing cell/epirodin ratio................. 239
57. Effect of serum on hemolysis by epirodin mix at 80 ug/ml........................................ 243
58. Ability of cholesterol to inhibit hemolysis by epirodin.............................................. 246
59. Effect of sucrose on hemolysis by epirodin mix.. 250
60. Effect of time on hemolysis by epirodin mix..... 254
A study was made of the humic acids and the epirodin pigments produced by the fungus Epicoccum nigrum. Epirodin mix was shown to be biologically active against Artemia salina and Bacillus subtilis, and to a lesser extent, against Chlorella pyrenoidosa. Spectroscopic analyses revealed much information concerning the physical and chemical nature of the epirodins. UV-visible absorption studies and quantitative hydrogenation showed the chromophore to be a lactone carbonyl in conjugation with a conjugated heptaene function. By isomerization in the presence of iodine and light, this chain was shown to exist in an all-trans configuration. The individual epirodins contained the same chromophore. Infrared analyses indicated the presence of hydroxyl groups as well as methyl, methylene, and carbonyl functions. Mass spectrometry of the trimethylsilyl ether determined the epirodins to have a high molecular weight of about 953 and about 8-10 hydroxyl groups. Chemical studies showed them to be sensitive to periodate, indicating the presence of 1,2-glycol groups, and permanganate. Azelaic and suberic acids were isolated from permanganate oxidation. Bromine was readily taken up. Two free acid functions were found to be present with a third becoming apparent upon saponification. Epirodin mix was capable of being acetylated, reinforcing the presence of a number of hydroxyl functions. From the data obtained, a working structure for the epirodin mole-
cule was proposed.

Investigations were made concerning the nature of the biological activity of epirodin mix. Fungi were slightly sensitive to the toxin while against yeasts, it was more effective. The inhibition against yeasts could be reversed by addition of sterol. Epirodin mix was found to be capable of lysing human erythrocytes. Hemolysis could be completely reversed by addition of serum, or partially reversed by addition of sterol or of $\gamma$-globulin. The mode of action of epirodin mix along with its chemical properties suggested that it was related to the polyene macrolide antibiotics.

Examination of the humic acids showed them to contain numerous amino acids and phenolic compounds. They were inactive against A. *salina*, B. *subtilis*, and C. *pyrenoidosa*. They did not appear to be related to the epirodin pigments in any way.
I. INTRODUCTION

A. Metabolic Products.

Molecules synthesized in the fungal cell, as in most cell systems, can be classified into one of two broad groups: the primary metabolites and the secondary metabolites. Primary metabolites comprise those molecules that are found in practically all cells and serve as functional constituents. They include compounds such as nucleic acids, proteins, polysaccharides, and lipids. Secondary metabolites, on the other hand, can be detected only in certain species. $\beta$-carotene, for example, is found in many species of fungi; however, it is by no means universally distributed. And while the effect of primary metabolites on a system is characteristic, the role of secondary metabolites is usually dubious.

There are several additional distinctions between the two groups of metabolites. The primary metabolites are essential to the life of the cell; the secondary metabolites are not. The latter's formation depends on the content of the medium to a great extent. Also, secondary metabolites are produced from a few key intermediates of primary metabolism, and they are produced when a substrate other than carbon becomes limited. This suggests that secondary metabolism provides a pathway for the removal of intermediates that would otherwise accumulate. Thus it enables the primary processes leading to these intermediates to remain operational during times of stress (2). The work in
this thesis concerns the pigmented secondary metabolites of *Epicoccum nigrum*.

**B. Epicoccum nigrum and Its Metabolites.**

The mold genus *Epicoccum* first appeared in the literature in 1816 with a description by Link. He described it as having "Compact, round stroma with spread, subglobulose sporidia. The sporidia fall down from it dispersed, and they do not hide themselves as in *Dermosporium*, nor do they fall down in thick layers as in *Tuberculariae*." (3). However, Link's description did not include any information concerning spore type or color, or how the spores were distributed. Therefore, there was some confusion regarding its location on the taxonomic scale, and many different strains of *Epicoccum* were isolated. But in 1959 Schol-Schwarz demonstrated that all seventy strains of the genus *Epicoccum* could be united into a single species, *Epicoccum nigrum* Link (4). He also identified a second member of the genus, *Epicoccum andropogonis* Cesati. According to the presently accepted classification, these two molds constitute the section Dictyosporae of the family Tuberculariaceae dematicae of the Order Moniliales of the Fungi Imperfecti (1).

*Epicoccum nigrum* is a highly pigmented mold, ranging in color from red to orange-brown depending on the age of the mycelium. In 1903, van Iterson (5) was the first to acknowledge the red pigmentation on filter paper caused by the mycelium of *Epicoccum nigrum*. In 1908, Lindner (6) studied
alcoholic extracts of the red mycelium and described the change of mycelial color from red to brown-red in aging cultures. But it was not until 1912, when Naumann began his work, that the pigments were studied to any great extent (7,8). He studied the effects of salts, carbohydrates, various nitrogen sources, osmotic pressure, light, temperature, and several gases on the production of pigmentation. He found that the fungus produced more red pigment in the darkness than in the light. He demonstrated the presence of a purple-red pigment in young mycelia which was soluble in methanol, ethanol, and water. In acidic solution Naumann's pigment was yellow; however, upon addition of alkali, it resumed its red color. Naumann also observed the transition from the purple-red color to brown if the pigment was exposed to air. He was later able to show that this oxidized pigment was identical to that found in aging mycelium, and it was no longer responsive to changes in pH.

Research on the pigments of Epicoccum nigrum was apparently discontinued for a period of time. However, it reappears in the literature in 1951 with the work of Moreau and Moreau (9). They cultivated the organism on maize and obtained a yellow culture fluid from a brown-red mycelium. Upon addition of sulfuric acid, a fine red precipitate was observed. But if more acid were added, the precipitate disappeared. However, if ammonia was added, it turned a bright lemon yellow. Thus Moreau and Moreau described the pigment as being yellow in alkali and red in acid, the exact opposite
of what Naumann had observed.

Schol-Schwarz (4) also carried out several experiments on the pigments of the red colored mycelium. He found the color to be yellow in acid, red in weak ammonia, but golden yellow in excess alkali. Thus it was becoming more and more evident that there was more than one pigment being produced by Epicoccum nigrum, and that the various groups of workers were most probably working with quite different compounds.

Meanwhile Bamford, Norris, and Ward (10) had begun growing Epicoccum nigrum in shaking liquid cultures. The organism was forming a dark red mycelium with the culture fluid being the same color. From this fluid was isolated a yellow pigment which they characterized as flavipin (3,4,5-tri-hydroxy-6-methylphthalaldehyde) (Fig. 1).

![Flavipin molecule](image)

**Figure 1. Flavipin.**

Flavipin had first been isolated in 1956 by Raistrick and Rudman (11) from Aspergillus flavipes and Aspergillus terreus. In studying these organisms, Raistrick and Rudman had found that the production of flavipin was markedly influenced by cultural conditions, especially by the composition of the culture medium. For example, they initially isolated flavipin from Aspergillus flavipes grown in Raulin-Thom solution.
where it was obtained in good yield. But if the organism were cultured in Czapek-Dox medium, there was no flavipin obtained.

While following the procedure of Bamford et al. (10), Poppen (1) was unable to demonstrate any production of flavipin in Epicoccum nigrum. Burge (12), who was working with the same UNH strain of Epicoccum nigrum used in this thesis, also had difficulty in obtaining the pigment. Yet he found that if he used mycelium already growing in liquid culture as inoculum, flavipin was indeed produced.

Bamford et al. (10) found flavipin to have considerable biological activity. At pH 3.5 and a concentration of 12.5 μg/ml, it almost completely prevented the germination of Botrytis allii conidia. However, the activity was quickly lost if the pH was maintained at 4.5 or above. A low antibacterial activity of flavipin was observed which was attributed to the pH of the medium. Eka (13) also noted the fungistatic action of his pigment at pH levels less than 5.0. He found that at concentrations of 5% (w/v), the growth of Botrytis allii was inhibited to 82%, whereas inhibition to Epicoccum nigrum was only 1%. However, at sufficiently low levels of flavipin such as 0.3%, Eka found that the growth of both organisms was slightly stimulated: 0.1% for Botrytis allii and 0.6% for Epicoccum nigrum. Burge (12) was able to show some activity in the flavipin he isolated from the UNH strain of Epicoccum nigrum. He found it to have minimal activity against Bacillus megaterium spores as compared with
other bacterial inhibitors, but it was still somewhat active at concentrations of 0.5 mg/ml. And at this same concentration, it was shown to have considerable phytotoxic activity against both Chlorella pyrenoidosa and Chlorella vulgaris.

As was mentioned previously, Foppen (1) was unable to detect any flavipin in his culture broth. Instead he had a red water-soluble pigment which was unstable in ethanol, methanol, and acetone. Like the pigment of Naumann (7,8), it was yellow in acid and red at pH 7. He noted that if acetone or ethanol were added to the red culture broth in volumes as low as 5% (v/v), a fibrous red floating coagulate was formed. Such a phenomenon was known to be characteristic for proteins, so Foppen began investigating the possibility that the pigment might be some sort of a polypeptide.

From 1967 to 1969, Martin, Haider, and Richards (14,15,16) published a series of reports on the "humic acids" produced by the fungus Epicoccum nigrum. They showed that this darkly colored pigment consisted mainly of polymerized amino acids and phenols, such as orcinol and resorcinol (Fig. 2)

\[
\begin{align*}
\text{orcinol} & : \quad \text{OH} \\
& : \quad \text{CH}_3 \quad \text{OH}
\end{align*}
\]

\[
\begin{align*}
\text{resorcinol} & : \quad \text{OH} \\
& : \quad \text{OH}
\end{align*}
\]

Figure 2.

which were released during the microbial degradation of plant phenolic polymers in the soil. In addition, Epicoccum nigrum

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could synthesize orsellenic acid and p-hydroxycinnamic acid (Fig. 3) from non-aromatic substances. These two basic mole-
cules could then be altered by oxidation of methyl groups and side chains, by decarboxylation, and by introduction of additional hydroxyl groups. The humic polymers were seen to vary considerably with respect to the relative percentages of specific structural units as well as to the molecular weight. However, the "humic acids" all had similar chemical properties due to the numerous carboxyl and phenolic groups on the molecule. Molecular weight determinations gave a range of 5,000-200,000 daltons.

By comparing his red water-soluble pigment with the "humic acids", Foppen (1) was able to show that they were the same type of compound. Martin and Haider (14) had shown that the production and variation of the polymers depended not only on the fungus, but also on the media employed. Foppen demonstrated that the red pigment was obtained only in media containing yeast autolysate. While Martin and Haider (15) had shown the molecular weight to range from 5,000 to 200,000 daltons, Foppen obtained a value of 4400 daltons. Burge (12)
calculated a value of 4200 daltons for the "humic acids" produced by the UNH strain of *Epicoccum nigrum*. In this thesis the phenols and amino acids of the UNH strain are studied. Burge (12) found the "humic acid" devoid of biological activity against *Bacillus megaterium*. In this work, activity against several alternate systems is examined.

Both Eka (17) and Foppen (1) have made studies concerning the lipid content of *Epicoccum nigrum*. Fatty acids were examined and found to be consistent with the composition of other fungal cells. Carotenes seen included \(\beta\)- and \(\gamma\)-carotene. Also found were torularhodin and rhodoxanthin which had never been isolated from a fungal system. The principle sterol component was ergosterol.

Apart from flavipin, the "humic acids", and the carotenes, there appeared to be no other identified pigments produced by *Epicoccum nigrum*. However, while working with the organism, Burge (12) noted two closely related orange pigments, quite different from those already described. They possessed considerable activity against *Bacillus megaterium* and a lesser amount of activity against *Chlorella pyrenoidosa*. He assigned to the compounds the names of epirodin A and epirodin B\textsubscript{mix}. Burge looked into some of their spectral properties and at the stability of the compounds at various pH values. Epirodin A was shown to have a molecular weight in the range of 750 ± 100 daltons. Further study into the biological activity of the pigment revealed it to be capable
of inhibiting the DNA-dependent RNA polymerase of *Eschericia coli*. Furthermore, it was found that it inhibited RNA synthesis by binding directly to the RNA polymerase and not to the DNA. Prior to this discovery, only the ansamycin antibiotics such as the rifamycins, streptovaricins, tolypomycins, and geldamycins, and the antibiotic streptolydigin were known to inhibit RNA polymerase. The ansamycins inhibit the initiation of RNA synthesis while streptolydigin is effective both against chain initiation and chain elongation. The latter appeared to be the mechanism for epirodin A action. However, none of the classes of these antibiotics appeared to be chemically related. Epirodin represented the first DNA-dependent RNA polymerase inhibitor to be isolated from the Fungi Imperfecti, the others having all been isolated from members of the Actinomycetes.

Work into the nature of epirodin was continued by Buckley (18). He devised a simpler scheme for the isolation of the compound, and was able to demonstrate the presence of yet another substance which, although unpigmented, possessed biological activity. However, he focused his research on the epirodin. He determined the epirodins to be fairly strong acids by their solubility in 0.1 M NaHCO₃. A positive reaction to the ferricyanide-ferric chloride reaction suggested a phenolic system. This information, along with the ultraviolet spectrum, indicated that epirodin might be derived from some sort of a quinone-type system. While a negative leucomethylene blue test ruled out the possibility of epirodin
being a benzoquinone or a naphthaquinone, the NMR, infrared, and ultraviolet spectra appeared consistent for an anthraquinone system. Buckley also noted a pH effect in the UV spectrum of epirodin known to occur in hydroxyanthraquinones. These observations prompted him to propose that epirodin might therefore belong to the family of anthraquinone-derived antibiotics. Two hydroxyanthraquinones, daunomycin (19) and luteoskyrin (20), had already been shown to inhibit DNA-directed RNA synthesis. Luteoskyrin, which is also a yellow pigment, had proven to be a potent inhibitor of \textit{E. coli} RNA polymerase. Thus the hydroxyanthraquinone nucleus for epirodin appeared to be an attractive structure which should be examined in greater detail.

In this thesis the investigation of epirodin is continued. Further studies on the pigment expose a broader spectrum of biological activity than was previously realized. Also the behavior of the pigment under various conditions as well as its chemical structure are probed. As a result of these experiments, it is shown that epirodin cannot be a hydroxyanthraquinone as suggested by Buckley. Instead, the author proposes that epirodin be listed in the class of compounds referred to as the "macrolide antibiotics".

C. Macrolide Antibiotics.

As a class the macrolide antibiotics are characterized by the possession of a macrocyclic ring of carbon atoms closed
by lactonization. Rings containing less than twelve carbons are unstable and thus not seen in nature. Those macrolides which have fourteen or sixteen-membered rings share many similar properties and are referred to simply as the "macrolide antibiotics". Those compounds with larger rings consisting of twenty to thirty carbon atoms also have many features in common including a conjugated double bond system. Thus they are known as the "polyene macrolide antibiotics".

The simple macrolides are represented by compounds such as leucomycin A₁ and erythromycin A₁ (Fig. 4). Both have sixteen-membered rings and two sugar moieties attached as side chains. Like most other macrolides, these antibiotics are produced by Streptomyces species, and are highly active against gram-positive bacteria and gram-negative cocci, but far less active against gram-negative bacilli (21). Ōmura (22) has shown that the antimicrobial activity of the macrolides involves binding of the antibiotics with the ribosomes and inhibition of protein synthesis.

The macrolides are all highly crystalline and are soluble in most organic solvents; however, they are insoluble in water and highly non-polar solvents such as hexane. Many have a UV spectrum, but no compound isolated as yet has been shown to possess a visible spectrum.

The "polyene macrolide" group also has been characterized regarding both its chemical properties and its antibiotic spectrum. All of the compounds purified thus far have had fairly high molecular weights ranging from 700 to 1300 daltons. They
Leucomycin A₁

Erythromycin A₁

Figure 4.
tend to be poorly soluble in water and in non- and medium-polar organic solvents, fairly soluble in very polar organic solvents such as pyridine and dimethylsulfoxide, and extremely soluble in ethanol and methanol. Some of the polyene macrolide antibiotics are soluble in acidic or basic media; however, this depends on the presence of either acidic or of basic nitrogen functions. However, in such solutions, the antibiotics tend to be less stable. "Heat and light also cause rapid deterioration, and sensitivity to these factors increases with increasing length of the conjugated polyene chain." (23)

"Biologically, all of the polyene antibiotics have in common a very pronounced activity against yeasts and fungi, but no significant antibacterial properties." (23) Three of them, nystatin, amphotericin B, and trichomycin, are commonly used in the medical treatment of fungal infections. Deep mycoses resulting from Blastomyces, Candida, Cryptococcus, and Histoplasma have all been treated successfully.

The mode of action of the polyene antibiotics was virtually unknown until the early 1960's. However, since then, both Kinsky (24) and Lampen (25) have shown that in organisms sensitive to polyenes, these substances are apparently bound to sterols in the cell membrane. The combination of the antibiotic with the cell results in distortion and malfunction of the membrane thereby allowing essential metabolites to leak out (26,27). "Other effects such as inhibition of glycolysis, respiration, and cell death must be regarded as secondary.
Metabolic activities in cell-free systems are virtually unaffected by the polyenes. Bacteria, intact and as protoplasts, do not take up polyenes and are unaffected by the antibiotics." (28).

In addition to their ability to inhibit the growth and sporulation of yeast and other fungi (29), some of the polyene antibiotics are known to have antiprotozoal activity as well (30). Because of their affinity for sterols, they have been used clinically in the control of serum cholesterol levels (31) and prostate malfunction (32) in dogs. They also display larvicidal and chemosterilant activity in some insects, apparently by blocking the uptake of dietary cholesterol (33).

The polyene antibiotics can usually be identified by their characteristic ultraviolet and visible absorption spectra. They are most easily classified on the basis of the length of the conjugated double bond chain as well as whether or not they contain a carbohydrate moiety. When present, the carbohydrate is most often nitrogen-containing mycosamine (28).

MM-8, which was isolated in 1965, was the first triene to be discovered. Resistaphylin and protopicin are other representative compounds of the triene class of antibiotics. Members of this class are unique among the polyenes in that they possess great activity against bacteria and little activity against yeasts and fungi. Included in the tetraene group are the tetrins A and B, and nystatin, the latter of which is widely used as an antibiotic (Fig. 5).

Within the pentaene antibiotics class, there are three
Figure 5.
Eurocinolide $A: R = \text{CH}_3$
Eurocinolide $B: R = \text{H}$

Filipin

Mycoticin $A: R = \text{H}$
Mycoticin $B: R = \text{CH}_3$

Figure 6.
Dermostatin A: $R=\text{CH}_3$

Dermostatin B: $R=\text{CH}_2\text{CH}_3$

Figure 7.
subgroups (Fig. 6). The eurocinolides A and B are both con-
ventional pentaënes. However, filipin, fungichromin (lagosin),
and chainin are methylpentaënes instead. There are deviations
observed in both the UV absorption spectrum and the infrared
spectrum of the methylpentaënes when compared to the spectra
of normal pentaënes. A third subgroup consists of those pen-
taënes that are conjugated with the lactone group such as the
mycoticins A and B.

The hexaënes have been studied very little, and only
dermostatin has been elucidated structurally. It is rather
unique in that it has a ketone function conjugated with the
hexaëne chromophore (Fig. 7).

The heptaënes, like the pentaënes, vary quite extensively
among themselves. Candidin and amphotericin B represent one
group where there is no aromatic moiety (Fig. 8). Others such
as aureofungin and candidicidin have a side chain of p-amino-
acetophenone. There is also a third group where the aromatic
side chain is N-methylated as in candidimycin.

It is apparent then that both the macrolide antibiotics
and the polyene macrolide antibiotics, while sharing certain
basic chemical and structural properties among themselves,
have distinct differences. Epirodin has been found to possess
many of the properties of these two groups of antibiotics.
Thus this thesis is presented with experimental evidence that
epirodin should indeed be considered a member of the macro-
lide antibiotics, specifically, the polyene macrolide anti-
biotics.
Figure 8.

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II. MATERIALS AND METHODS.

A. General Techniques.

1. Amino Acid Analysis. Amino acid analyses were obtained by Dan Kerchensteiner of the Department of Biochemistry on a Beckman Amino Acid Analyzer, Model 120C.

2. Elemental Analysis. Carbon, hydrogen, nitrogen analyses were performed on a F and M Model 185 instrument by Dee Cardin (Chemistry Department, UNH).

3. Fluorometry. Fluorescence determinations were made on a Farrand spectrofluorometer, Model MK-1.

4. Gas-Liquid Chromatography. A Barbar-Colman Model 5000 Gas Chromatograph equipped with a hydrogen flame ionization detection system was used. Analyses were done on a 6 ft X 4 mm (I.D.) glass U-tube column packed with 80/90 mesh 12% diethylene glycol succinate on Anachrom A (Waters Associates, Inc., Framingham, Mass.). Retention times (R_T) were calculated from the point of injection to the midpoint of the eluted chromatogram peak of the respective compounds.

5. Infrared Spectroscopy. IR spectra were obtained on a Perkin Elmer Spectrophotometer, Model 710 A.

6. Mass Spectrometry. Mass spectra were performed by Michael Pazden of the Chemistry Department of UNH. The instrument used was a Hitachi-Perkin Elmer mass spectrometer, Model
21

7. **Nuclear Magnetic Resonance Spectroscopy.** NMR spectra were obtained by Susan Hathaway (Chemistry Department, UNH) using a Jeol MH 100 NMR spectrometer. Samples were run in various solvents at a frequency of 100 MHz.

8. **UV-Visible Spectrophotometry.** For complete absorption spectra, a Beckman DB-G Grating Spectrophotometer with an attached 10 inch recorder was used. When readings at specific wavelengths were necessary, a Beckman DU-2 Spectrophotometer was used.

2. **Thin Layer Chromatography (TLC).** Precoated 20 X 20 cm silica gel plates (EM Reagents) were used. They were activated at 105° for 30 minutes and stored in a desiccator prior to use. The plates were developed in unlined, covered, rectangular glass TLC chambers with the solvents indicated. \( R_F \) values were calculated as:

\[
R_F = \frac{\text{Distance from origin to spot center}}{\text{Distance from origin to solvent front}}
\]

The spots were detected either by direct visualization, under a UV light, or with one of the spray reagents described below,

a. **Aniline-diphenylamine-phosphoric acid (34).** The spray reagent was prepared by dissolving 2 g diphenylamine, 2 ml aniline, and 10 ml 85% phosphoric acid in 100 ml acetone. The plates were sprayed and heated at 105° for 5-10 minutes. Various color reactions are given by reducing sugars.

b. **Benzidine-trichloroacetic acid (34).** The reagent was
prepared by dissolving 0.5 g benzidine in 10 ml acetic acid, adding 10 ml 40% (w/v) aqueous trichloroacetic acid, and diluting the mixture to 100 ml with ethanol. The plate was sprayed and heated to 105° for 5-10 minutes. The appearance of dark red-brown spots is indicative of sugars.

g. Bromocresol green (34). Bromocresol green indicator (0.04 g) was dissolved in 100 ml ethanol, and 0.1 N sodium hydroxide was added dropwise until a blue color just appeared. When used as a spray reagent for the detection of acids, acidic compounds appear yellow while the background stains blue.

d. Charring with sulfuric acid. Silica plates were sprayed with concentrated sulfuric acid and placed in a 105° oven for 5-10 minutes. The presence of darkly colored spots indicates the presence of organic material.

e. Ehrlich reagent (34). One g 4-dimethylaminobenzaldehyde was dissolved in 25 ml concentrated hydrochloric acid and 75 ml methanol. The plate was sprayed and warmed. Amines appear as red spots on a white background.

f. Glucostat reagent (Worthington Co.). One mg glucose oxidase, 0.5 mg peroxidase, and 20 mg o-dianisidine - di HCl were diluted to 100 ml with distilled water. After lightly spraying the silica plate, the appearance of purplish spots indicates the presence of glucose.

g. Morgan-Elson reagent (34). This reagent is specific for amino sugars and consists of two separate solutions. Reagent I was prepared by taking 0.5 ml of a mixture of 5 ml 50% aqueous potassium hydroxide and 20 ml ethanol and dissolving

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it in 10 ml of a solution of 0.5 ml acetylacetone in 50 ml n-butanol. Each solution had to be fresh, and they were mixed together just before use. For Reagent II, 1 g 4-dimethylamino-benzaldehyde was dissolved in 30 ml ethanol, and 30 ml concentrated hydrochloric acid were added. The chromatogram was first sprayed with Reagent I and heated at 105° for 5 minutes. The plate was then sprayed with Reagent II and heated 5 minutes at 105°. Amino sugars appear as red spots.

h. Ninhydrin (34). Ninhydrin (0.3 g) was dissolved in 100 ml n-butanol, and 3 ml of acetic acid were added. The plate was sprayed and heated at 105°, giving spots for amino acids, amines, and amino sugars.

i. Pauly reagent. The following stock solutions were prepared:

(1) Sulfanilic acid........0.9% in 0.12 M HCl
(2) Sodium Carbonate.....10.0%
(3) Sodium nitrite........4.5%

The reagent was prepared just prior to use. To 100 ml of sulfanilic acid were added 10 ml NaNO₂. The solution was mixed, and 110 ml Na₂CO₃ were added. The resulting solution was sprayed on the plate very lightly. The development of spots ranging in color from yellow to red to brown is indicative of phenols, amines, and heterocyclic compounds which can couple with the diazotized sulfanilic acid (34).

i. Periodate-benzidine reagent. The chromatogram was sprayed with 0.1% sodium metaperiodate and allowed to react 5-10 minutes. It was then sprayed with a solution of benzi-
dine prepared by dissolving 0.5 g in 20 ml glacial acetic acid and 80 ml ethanol. White spots on a blue background are indicative of 1,2 glycols and \( \alpha \)-hydroxy-ketones, -aldehydes, and -acids.

**k. Schweppe reagent (34).** Ten percent aqueous glucose and 10% ethanolic aniline solutions were prepared. Twenty ml of each were mixed and diluted to 100 ml with n-butanol. The plates were sprayed and heated to 105\(^{\circ}\) for 5-10 minutes. Compounds containing carboxylic acid groups appear as pale brown spots on a white background.

**l. Vanillin-sulfuric acid (34).** The reagent consisted of a solution of 1 g vanillin in 100 ml concentrated sulfuric acid. Following generous application of the spray, the plate was heated to 105\(^{\circ}\) for 5 minutes. Spots ranging in color from yellow to red to purple indicate the presence of phenols and higher alcohols.

**B. Biological Assays.**

There were several assays used throughout this research to monitor the toxicity of various compounds.

**1. Bacillus Assay (18).** Medium was prepared consisting of the following:

1. Tryptone (Difco) .......... 0.50 g
2. Yeast extract (Difco) ... 0.25 g
3. Glucose ...................... 0.10 g
4. Agar (Difco) .................. 1.00 g
5. Distilled water ............. 100 ml

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The pH was adjusted to 6.2 and the medium was autoclaved for 17 minutes. A spore suspension was prepared containing 1 ml of Bacillus subtilis spore suspension (Difco) in 99 ml sterilized distilled water, yielding a final concentration of 10^7 spores/ml. Three ml of this suspension was then used to inoculate 100 ml of medium. The medium was incubated at 80°C for 20 minutes in order to activate the spores.

Levels of biological activity were determined by the paper disc method as described by Jayaraman et al. (35). The samples to be tested were dissolved in either water, ethanol, methanol, or dimethyl sulfoxide. Twenty ul of the test solution was absorbed by each 6.3 mm sterile paper disc (Difco). The plates were incubated in the dark at room temperature for 18 hours. Toxicities were measured by the size of the zone of inhibition around the paper disc.

2. Brine Shrimp Assay. The brine shrimp assay based on the method of Harwig and Scott (36) was also used to estimate toxicities. The shrimp, Artemia salina L., were obtained as eggs (Carolina Biological Supply Co.) and hatched in 1% sodium chloride at room temperature. After 24 hours, when the larvae began emerging, the unhatched eggs and the larvae were transferred to brine shrimp medium (BSM) composed of the following:

(1) NaCl........................................30.0 g
(2) CaCl₂·2H₂O..........................0.3 g
(3) MgSO₄·7H₂O..........................0.5 g
(4) MgCl₂·6H₂O..........................1.5 g
(5) KCl.................................0.8 g
(6) MgBr₂·6H₂O.........................0.1 g
(7) Glycine............................6.0 g
(8) Distilled water.............1000 ml

The pH was adjusted to 6.5, and the medium was autoclaved and allowed to cool.

Screening was done using a paper disc method. Sterile paper discs (6.3 mm) (Difco) to which had been applied 20 μl of the solution to be tested were placed in the wells of porcelain spot plates. The discs were left until the solvent, either ethanol, methanol, or water, had evaporated. Then 0.5 ml of BSM containing 20-60 larvae was added. The wells were covered with a plate of glass so as to prevent evaporation. The larvae were incubated at 37°C for 18 hours. At the end of this period, the number of dead shrimp was tallied. Toxicities were expressed as percentages of the number of organisms that had died over the total present in a given well:

\[
\text{Toxicity} = \frac{\text{Number of dead larvae}}{\text{Total number of larvae}} \times 100
\]

2. Chlorella Assay (18). Buffered agar plates were prepared with medium consisting of the following:

(1) KNO₃.................................1.00 g
(2) KH₂PO₄...............................2.50 g
(3) K₂HPO₄...............................2.50 g
(4) MgSO₄·7H₂O.........................0.25 g
(5) Glucose...........................10.0 g
(6) Trace element solution........0.4 ml
(7) Distilled water.............1000 ml

The medium was divided into two 500 ml portions, one of which was made 2% in agar. The two portions were autoclaved separately, cooled to approximately 50°C and divided into 50 ml aliquots. Five ml of a liquid culture of Chlorella pyrenoidosa (UNH strain) prepared in the same medium were then added to the non-agar portions. This was mixed with an equal part of the 2% agar portion, and plates were poured with the resulting seeded 1% agar medium.

Levels of activity were determined by the paper disc method as described by Jayaraman et al. (35). The samples to be tested were dissolved in either ethanol, methanol, or water. Twenty ul of test solution were applied to each 6.3 mm disc (Difco). The plates were incubated at room temperature under fluorescent lighting for 24 hours.

C. Organism and Stock Culturing.

The strain of Epicoccum nigrum used in this thesis was that of Burge (12) who had isolated it from a moldy seed sample. The organism was maintained on slants consisting of malt extract agar. The composition was as follows:

(1) Malt extract (Difco)...........20 g
(2) Peptone........................1 g
(3) Glucose..........................20 g
(4) Agar (Difco).....................20 g
(5) Distilled water..............1000 ml
The medium was divided into 10 ml aliquots, placed in test tubes plugged with cotton, and autoclaved for 17 minutes. The tubes were allowed to cool in an inclined position. They were inoculated with mycelia of *E. nigrum*. The new cultures were left to grow for 5 days at room temperature and in the dark. They were then stored in the refrigerator at 4°C.

D. Procedure for Growth of *E. nigrum* and Isolation of Humic Acid.

For humic acid determinations, the fungus was grown in liquid media. Either malt extract medium containing:

1. Malt extract (Difco)...........20 g
2. Peptone (Difco)...............1 g
3. Glucose......................20 g
4. Distilled water............1000 ml

or yeast autolysate medium made up of:

1. Yeast extract (Difco).........5 g
2. Glucose.........................10 g
3. Distilled water..............1000 ml

was used to culture the organism. Five ml aliquots were dispensed into screw cap test tubes, and 100 ml aliquots into fifteen 500 ml Erlenmeyer flasks, all of which were then sterilized. The test tubes were inoculated with mycelia from stock slants and allowed to grow for 5 days at room temperature in the dark. The resulting cultures were then used as inocula for the media in the Erlenmeyer flasks. The flasks
were incubated under constant lighting at 25° in a rotary shaker (Psycrotherm, Model G27, New Brunswick Scientific Co., New Brunswick, N.J.) running at 220 rpm for either 5 or 21 days. At the end of the growth periods, the cultures were filtered through Whatman No. 1 filter paper. The filtrate for each set of cultures was pooled and taken almost to dryness in vacuo. The residue was dissolved in 50 mM Tris buffer, pH 7.5. The resulting solution was dialyzed 3 times against 2000 ml of the same buffer for 12 hour periods. The material remaining in the dialysis tubing was concentrated and placed on a Sephadex G-25 (Sigma Chemical Co.) column and eluted with the same 50 mM Tris buffer. The brown band was collected and taken to dryness in vacuo. The residue was dissolved in a minimum volume of distilled water and dialyzed twice against 6000 ml aliquots of distilled water for 12 hours. The non-dialyzable material was then used to obtain the elemental analyses, amino acid analyses, and phenol content determinations reported in this thesis. Also, biological assays were conducted using this material.

1. Amino Acid Analysis and Phenol Determination of Humic Acid. Before the humic acid could be examined for amino acid and phenol content, it was necessary that the samples be hydrolyzed. Samples (25 mg) were transferred to hydrolysis tubes to which were added 2 ml constant boiling HCl and 1 drop of 0.5 N hydrazine. The tubes were evacuated, sealed, and placed in a 105° oven for 24 hours. They were then removed from the oven, allowed to cool, and taken to dryness by opening the tubes.
and placing them in a vacuum desiccator along with beakers separately containing 50 g NaOH pellets and 50 ml concentrated H₂SO₄. The desiccator was evacuated, and the samples were left to sit for 24 hours.

For amino acid analyses, 3-4 mg samples of the hydrolysis residue were taken up in sodium acetate buffer, pH 5.25, and injected for resolution of the basic amino acids. For acidic components, sodium acetate buffer, pH 3.25, was used, and for neutrals, pH 4.30 buffer.

For examination of the phenol content of the humic acids, the same hydrolyzed samples were used. The residue was washed 3 times with ethyl ether, 25 ml each time. The extracts were combined, dried, and dissolved in ethanol to give a concentration of 5.0 mg/ml. Twenty ul samples were then applied to silica TLC plates and developed in a two-dimensional solvent system described by Haider and Martin (14). Solvent I consisted of chloroform:acetic acid (8:2), and Solvent II of dibutyl ether:acetic acid (10:1). Plates were sprayed with vanillin-sulfuric acid. R_P values were compared with standards, and the phenols were identified by both R_P and color reaction to the spray. As a confirmation, a second two solvent system was used. Solvent I consisted of ethyl ether:acetic acid:petroleum ether (100:3:97) developed to a height of 9 cm while Solvent II was composed of ethyl ether:petroleum ether (3:97) developed to 15 cm. Again R_P values and color reactions were compared with standards.
E. Procedure for Growth of Epicoccum nigrum and Isolation of Epirodin.

Cultures for the extraction of epirodin were prepared by pouring 200 ml of the same malt extract medium used for stock culturing the fungus into 2.5 liter low form culture flasks (wide base). Ten flasks were used at one time. Each flask was then autoclaved and inoculated with 5 ml of previously sterile distilled water containing the washings from a slant of *E. nigrum*. The cultures were incubated for 14 days at room temperature in the dark.

The steps in the isolation procedure are outlined in Scheme 1. After the 14 day incubation period, each culture flask was extracted overnight at room temperature with 500 ml of 95% ethanol. The extracts were decanted and the cultures reextracted overnight with a second 500 ml portion of ethanol. The combined ethanol extracts (10 liters) were suction filtered through a Buchner funnel and concentrated to dryness in vacuo. The dry residue was extracted with 300 ml 95% ethanol. The ethanol-soluble material, Fraction I, was filtered and applied to an alumina column. The column was prepared by pouring 120 g of alumina (Fisher Scientific, 80-200 mesh) into the dry column (14 X 3.8 cm) and then washing the support with 500 ml 95% ethanol. The crude sample dissolved in ethanol was applied to the column and eluted in a batch-wise manner. Fraction II consisted of 2000 ml 95% methanol, all of which was discarded. The column was then eluted with 1000 ml 80% methanol (Fraction
Scheme 1. Extraction of Epicoccum nigrum Cultures and Isolation of Epirodin. I.

Epicoccum nigrum (10 culture flasks)

95% Ethanol (10 liters)

Ethanol soluble Ethanol insoluble

Conc. to dryness

Residue

Ethanol extraction (300 ml)

Ethanol soluble Residue

Fraction I

Alumina column 95% Methanol (2 liters)

80% Methanol (1 liter)

Fraction III Fraction II (discard)

Silica column CHCl₃:CH₃OH (7:3)

Epirodin mix
III) which was concentrated to dryness in vacuo. The residue was extracted with 10 ml absolute methanol and the methanol extract dried in vacuo. The methanol-soluble material was then taken up in a minimum volume of methanol (1-2 ml) and applied to a silicic acid column (24 X 2.2 cm). The column was prepared from 50 g of silicic acid (Fisher Scientific) activated at 105°C for an hour, cooled, and slurried with chloroform:methanol (7:3). Prior to sample application, the column was washed with 300 ml solvent. The sample was then eluted with the same solvent. The intense orange band containing pure epirobin mix was collected. This fraction was used for all qualitative and quantitative chemical analyses, as well as for most of the spectroscopic studies including NMR and IR. The individual epirobins were resolved by TLC and used for several UV-visible absorption studies.

As the work on epirobin was nearing completion, an alternative shorter procedure was devised for the isolation of the compound. The tedious and time-consuming columns were eliminated. The isolation involved simply washing the crude E. nigrum extract with a series of solvents as shown in Scheme 2. The procedure uses only 1000 ml crude extract as opposed to the 10 liters used previously, but the yield of epirobin mix is greater.

F. Stability of Epirobin.

The stability of epirobin was determined by following either its biological activity or its absorbance at 429 nm.
Scheme 2. Extraction of Epicoccum nigrum Cultures and Isolation of Epirodin. II.

Epicoccum nigrum (1 culture flask)

- Ethanol extraction (1000 ml)
  - Ethanol soluble
    - Concentrated to dryness
  - Ethanol insoluble
    - Residue
      - Methanol extraction (100 ml, 25 ml, 25 ml)
        - Methanol soluble
          - Residue
            - 0.1 N HCl extraction (50 ml, 25 ml)
              - Residue
                - HCl soluble
                  - Acetone extraction (10 ml, 10 ml, 5 ml)
                    - Epirodin Mix
                      - Residue
G. Characterization of Epirodin: Classification Tests.

1. **Bromine in Carbon Tetrachloride Solution.** Approximately 20 mg of epirodin mix were dissolved in 2 ml of methanol. A 5% solution of bromine in CCl₄ was then added dropwise until the bromine color persisted.

2. **Hydroxylamine-Ferric Chloride Reaction (37).** Approximately 5 mg of epirodin in 1.0 ml 3.5 N NaOH was mixed with 1.0 ml 2 M hydroxylamine-HCl. After standing 2 minutes, 1.0 ml 33% (v/v) HCl in water and 1.0 ml of FeCl₃ solution (0.37 M FeCl₃ in 0.1 N HCl) were added to the reaction mixture. A positive reaction consisted of the production of a purple color. A positive reaction to this test indicates the presence of either an ester, lactone, or aldehyde group.

3. **Periodic Acid (38).** The reagent was prepared by dissolving 0.5 g of meta-periodic acid in 100 ml of distilled water. To 2 ml of this reagent was added one drop of concentrated nitric acid; the test tube was shaken. Approximately 10 mg of epirodin mix were then added; the mixture was shaken 10-15 seconds; and 2 drops of 5% aqueous silver nitrate were added. The instantaneous formation of a white precipitate of silver iodate constituted a positive reaction. Periodic acid selectively oxidizes 1,2-glycols, α-hydroxy aldehydes, α-hydroxy ketones, and α-hydroxy acids.

4. **Potassium Permanganate Solution (38).** To about 25 mg of epirodin mix in 2 ml of ethanol was added dropwise a 2%
solution of potassium permanganate until the purple color persisted. Compounds having either ethylenic or acetylenic linkages are capable of decolorizing a solution of potassium permanganate.

5. **Tollen's Reagent** (39). Two ml of 5% aqueous silver nitrate were placed in a test tube along with 1 drop of 10% sodium hydroxide solution. A 2% ammonia solution was then added dropwise with constant shaking until the silver oxide precipitate just dissolved. About 25 mg of epirodin were tested with this reagent. A positive reaction, indicated by the precipitation of metallic silver, is given by aldehydes as well as by acyloins, certain phenols, and other reducing agents.

H. **Spectroscopic Studies on Epirodin.**

1. **Infrared Spectrum.** A sample of epirodin was smeared onto a NaCl disc, and the IR spectrum was recorded using air as the reference.

2. **Mass Spectrum.** In order to obtain a mass spectrum, it was necessary that a more volatile derivative of epirodin be prepared. The trimethylsilyl ethers were prepared in the following manner: to 9.8 mg epirodin mix in a 5 ml round-bottom flask was added one vial of "Tri-Sil" (Pierce Chemical Co.). The solution was allowed to sit for 1 hour and was concentrated almost to dryness in vacuo. It was transferred to a 50 ml
round-bottom flask and 25 ml CCl₄ were added. The solution was gravity filtered to remove any solid material and concentrated to dryness in vacuo. The residue was extracted twice with 25 ml portions of hexane. The extracts were combined and taken to dryness in vacuo, yielding the trimethylsilyl derivative. TLC was done to insure that the product did not contain a large amount of unreacted epirodin mix.

The mass spectrum was run, and m/e values were compared to a reference, Tris (perfluorohexyl)-s-triazine. By using a plot of log m/e versus log distance from m/e = 28 on the spectrum, m/e values higher than the reference compound could be extrapolated.

3. **Nuclear Magnetic Resonance.** An NMR spectrum was obtained on a solution of epirodin mix in DMSO-d₆.

4. **UV-Visible Absorption Spectra.**

a. The UV-visible spectrum. Individual epirodins were separated by TLC in isopropanol:methanol (1:1). The spots were scraped from the plate; the silica was eluted with methanol; and the spectra of the individual components were obtained.

b. Effect of solvent and pH. Spectra were obtained on 10 µg/ml solutions of epirodin in distilled water, 0.05 N HCl, 0.05 N NaOH, and 0.05 N HCl in ethanol.

c. Photosensitivity in the presence of I₂ catalyst. A series of 10 µg/ml solutions of epirodin mix in ethanol was prepared and then made 2 µg/ml in I₂ (40). Spectra were run.
after exposure to light for periods of 0, 5, 10, 15, 30, 45, and 60 minutes. The effect of I₂ catalysis with petroleum ether as solvent was also noted.

d. Photoinactivation due to flavins. To determine whether or not epirodin was affected by flavins, solutions containing epirodin mix at 10 µg/ml and riboflavin at 0.01 umole/ml in 0.05 potassium phosphate buffer, pH 6.9, were illuminated for 5, 10, 15, 20, 30, 45, and 60 minutes, and absorbance was measured at 429 nm (41,42).

I. Chemical Methods Used in the Analysis of Epirodin.

1. Acetylation of Epirodin Mix. The acetylating reagent consisted of a solution of acetic anhydride in pyridine, 1:4 (v/v). Conducting the reaction in pyridine prevents acetic acid from interfering with the acetylation, thus allowing the reaction to proceed more smoothly (39).

a. Qualitative acetylation. To 198 mg of epirodin mix was added 20 ml of acetylating reagent. The solution was left to sit for 24 hours, and the excess acetic anhydride was then hydrolyzed with 50 ml distilled water. The solution was transferred to a separatory funnel, extracted with 25 ml of chloroform, and the aqueous layer was discarded. The chloroform layer was washed with 100 ml water 5 times to remove as much acetic acid and pyridine as possible. The chloroform layer was then transferred to a 50 ml round-bottom flask and concentrated in vacuo to remove the chloroform, leaving only the
residue and traces of pyridine. Water was used to wash the residue and the solid was redissolved in a minimum volume of chloroform. The product was crystallized by addition of hexane to the solution.

b. Quantitative acetylation. For quantitative acetylation, epirocin mix was weighed into 100 ml round-bottom flasks. Exactly 5.00 ml acetylating reagent was added to each flask, and the flasks were swirled until the epirocin was completely into solution. The flasks were then left to sit for 24 hours. Ten ml water were added to hydrolyze the excess reagent, and the solutions were transferred to 400 ml beakers. The amounts of acetic acid thus formed were then determined by titration with 0.5 N NaOH. The solutions were mixed continuously with a magnetic stirrer, and the neutral point was determined with a pH meter. A blank to determine the total amount of acetic anhydride available for acetylation was run by placing 5.00 ml of acetylating reagent in a round-bottom flask and treating it the same way as the epirocin solution. From the data obtained, the molar equivalent weight per hydroxyl group was calculated according to the following equation:

\[
\text{molar eq. wt.} = \frac{\text{mg of sample}}{\text{(ml blank-ml test)}(N \text{ NaOH})}
\]

2. Acid Hydrolysis. Epirocin mix (90.94 mg) was dissolved in a minimum amount of methanol (about 2 ml). HCl (100 ml 1N) was added and the solution refluxed for 3 hours. After the solution had cooled, it was decanted out of the flask leaving behind a dark gummy solid. The aqueous solution
was extracted twice with 100 ml ether causing a solid to precipitate at the interface. Thus there were four fractions present: the ether and aqueous layers, the solid precipitate, and the gummy solid remaining in the reaction flask.

The gummy solid was dissolved in 1 N NaOH and the solution was taken to dryness in vacuo. The residue was extracted with methanol until the extracts remained colorless. The solution was then taken to dryness in vacuo.

The solid material which had appeared at the interface of the aqueous and ether phases was dissolved in methanol and the solution taken to dryness in vacuo. The silver nitrate test for alkyl halides (43) was done by warming the unknown solid in an alcoholic solution of AgNO₃ (1 g in 30 ml ethanol). Formation of a precipitate that is insoluble in dilute nitric acid is indicative of an alkyl halogen compound.

The aqueous phase was concentrated in vacuo, and applied to a TLC plate and developed.

The ether layer was concentrated in vacuo. A portion of the sample was applied to a silica gel column (1 X 6 cm). The silica was activated at 105° for 30 minutes, slurried with benzene, and the column was poured. The column was eluted with benzene and fractions were collected and applied to a TLC plate; those with the same spots were pooled.

3. Bromination of Epirodin. The bromination of epirodin was carried out in the dark so as to prevent free radical substitution and encourage addition across double bonds. To 65 mg of epirodin mix in a 100 ml round-bottom flask was added
70 ml glacial acetic acid and 10 ml bromine. The flask was wrapped in foil and allowed to sit for 4 hours. The solution was concentrated to a thick oil in vacuo. The residue was taken up in 20 ml methanol and then dried in vacuo 3 times. The resulting solid was recrystallized from 95% ethanol and dried for 24 hours in a vacuum desiccator.

4. Hydrogenation of Epirodin. Hydrogenation of epirodin mix was carried out in a catalytic hydrogenation apparatus as shown in Fig. 9. The precision of this type of set-up is 1-2% (44). The Pt₂O catalyst (100 mg) was placed in the reaction vessel, 10 ml glacial acetic acid was added, and H₂ was bubbled through until the platinum was completely reduced.

Epirodin mix (108.28 mg) was dissolved in a minimum amount of glacial acetic acid and added to the reaction flask. The amount of H₂ uptake was recorded as a function of time. The total volume of hydrogen consumed after 18 hours was then converted to STP and used to determine the number of double bonds per molecule of epirodin. The solution of hydrogenated epirodin was neutralized with dilute NaOH and extracted with 50 ml ethyl ether 3 times. The extracts were combined and taken to dryness in vacuo.

5. Neutralization Equivalent (38). Samples ranging from 1.3-5.6 mg were weighed into 50 ml round-bottom flasks. Each was dissolved in 10.00 ml 95% ethanol and titrated with standard NaOH (0.01008 N). A blank consisting of 10.00 ml ethanol was also titrated. One drop 0.1% aqueous bromthymol blue solu-
Figure 9. Hydrogenation Apparatus.
tion was added to each flask as indicator. The endpoint was taken as the appearance of a blue color in the previously epirodin-colored solution. The neutralization equivalent was calculated as follows:

\[
\text{Neutralization equivalent} = \frac{\text{Weight (mg) of sample} \times 1000}{(\text{ml sample} - \text{ml blank})(N)}
\]

6. Periodate Oxidation. Epirodin mix (107 mg) was weighed into a 250 ml round-bottom flask and dissolved in 105 ml 95% ethanol. Three 25.00 ml aliquots were pipetted into 400 ml beakers. Periodic acid reagent (50.00 ml) was then added (1.50 g sodium meta-periodate in 25 ml water diluted to 500 ml with glacial acetic acid). Each solution was mixed, covered with a watch glass, and allowed to sit at room temperature for 30 minutes. Potassium iodide reagent (20.00 ml) was added (30 g in 200 ml water). After the solutions had stood for a few minutes, 100 ml water were added to each one. They were then titrated with 0.1 N sodium thiosulfate to the disappearance of the iodine color. Two ml of starch indicator (5% aqueous solution) were added, and the titrations were continued to the disappearance of the starch-iodine color.

A blank was run by adding KI solution to the periodate reagent before adding any epirodin. Thus there was no available periodate for the epirodin to react with, and the blank value could be determined.

The molar equivalent weight was determined using the following equation:

\[
\text{Molar equivalent weight} = \frac{2(\text{weight (mg) of epirodin sample})}{(\text{ml blank} - \text{ml test})(N \text{ Na}_2\text{S}_2\text{O}_3)}
\]
The titrated solutions were pooled and taken to dryness in vacuo. Fifty ml water were added to the residue, most of which dissolved. The remaining solid was dissolved in 50 ml ether, and both solutions were transferred to a separatory funnel. The two phases were mixed, and then the ether layer was removed. The aqueous layer was extracted twice more with 50 ml aliquots of ether. The extracts were combined and taken to dryness in vacuo, giving 66 mg of product.

A 2,4-dinitrophenylhydrazone derivative was then prepared following the procedure of Shrinér et al. (38). The 2,4-dinitrophenylhydrazone solution was made by adding 2 ml concentrated sulfuric acid to 0.4 g 2,4-dinitrophenylhydrazone in a 25 ml Erlenmeyer flask. Three ml distilled water were added dropwise with swirling. Once solution was complete, 10 ml 95% ethanol were added, thus forming the reagent. Periodate-treated epirodin mix (66 mg) was dissolved in 2.5 ml ethanol and 2 ml of the 2,4-dinitrophenylhydrazone reagent were added. The precipitated derivative was recrystallized from ethanol and water.

2. Periodate Oxidation of Epirodin Following Saponification. The procedure used for periodation with prior base treatment was based on that of Cope et al. (45). To 306 mg epirodin mix in a round-bottom flask was added 30 ml distilled water. Sixty mg of solid NaOH were added giving approximate concentrations of 10 mg/ml epirodin and 2 mg/ml NaOH. The solution was refluxed for 1 hour, cooled to room temperature, and diluted with 250 ml ethanol. Aliquots of this solution
were then removed for quantitative periodate determinations.

To 25.00 ml of the above solution was added 50.00 ml of the periodate reagent, and titrations were performed as described in the preceding section. The molar equivalent weight was calculated according to the same equation.

8. Potassium Permanganate Oxidation of Epirodin. Epirodin mix (128 mg) was dissolved in 250 ml boiling water. The reaction flask was maintained at 75-85°C, and the solution was stirred continuously. Solid potassium permanganate was added slowly until the purple color started lingering a few moments before disappearing. At this point a dilute KMnO₄ solution was prepared and added dropwise until the purple color persisted. The solution was left heating and stirring for 1 hour to insure oxidation of any epirodin that might be remaining. The solution was gravity filtered to remove the manganese dioxide which had formed. The precipitate was discarded leaving a clear purple filtrate shown to be strongly basic when tested with pH paper. Three N HCl was added dropwise to reduce the remaining permanganate. The solution was filtered a second time leaving a clear, colorless, acidic solution. The filtrate was extracted once with 150 ml ether and twice with 75 ml portions. The extracts were combined and concentrated in vacuo. Products of the reaction were examined by both TLC and gas-liquid chromatography (GLC). For TLC the products were examined in acetone-acetic acid-water (8.5:1.5:0.5), followed by spraying with bromocresol green.

For GLC the free acids were first converted into methyl
esters. This was accomplished by dissolving the product residue in 5 ml redistilled methanol. With constant stirring, BF₃ gas was passed through the solution for 5 minutes, followed by a 10 minute reflux. The methanol was removed in vacuo, and the esterified products were dissolved in methylene chloride for injection into the gas chromatograph. The column temperature was maintained at 200°C while pressures of H₂, air, and N₂ were set at 20, 40, and 10 lbs., respectively.

2. Saponification of Epirodin. A 100 mg sample of epirodin mix was dissolved in 50 ml 3 N KOH in 50% methanol. The solution was refluxed for 3 hours, cooled, and acidified with concentrated HCl. It was then extracted with 50 ml ether twice, and the extracts were combined and taken to dryness in vacuo. The residue was stored for 48 hours in a vacuum desiccator. Samples (1.0-1.5 mg) were weighed out, dissolved in 10.00 ml 95% ethanol, and the neutralization equivalents were determined as previously described.

10. Sodium Borohydride Reduction of Epirodin. The procedure for borohydride reduction of epirodin was taken from Fieser and Fieser (46). Forty mg epirodin were dissolved in 100 ml 85% tetrahydrofuran (THF) in water. Twelve g NaOH were added so as to make the solution 3 N. One g NaBH₄ was then added. The solution was then refluxed for 20 hours with continuous stirring so as to prevent the formation of two layers. The solution was cooled, and the layers were allowed to separate. An aliquot of the top (THF) layer was removed and taken
to dryness in vacuo. The residue was washed with ether, and the washings were combined and taken to dryness in vacuo.

J. Biological Activity of Epirodin.

1. Antibacterial Activity of Epirodin. The following assay system was devised to determine whether epirodin is bacteriostatic or bacteriocidal. Liquid medium consisting of 30 g of Trypticase Soy Broth (BBL) in 1000 ml distilled water was prepared. The solution was divided into four 250 ml aliquots, two of which were made 1% in agar. The media was autoclaved for 17 minutes. The two liquid portions were each inoculated with 1 ml of a 10^7 spore suspension of B. subtilis, and the spores were activated at 80° for 20 minutes. The cultures were then placed in a shaking water bath at 37° and allowed to grow until they were quite cloudy in appearance.

A solution of epirodin mix in ethanol was prepared at a concentration of 50 mg/ml. From it a series of dilutions was prepared with the concentrations shown in Table 1. Twenty ul of each solution were soaked onto 6.3 mm paper discs (Difco) which were then placed in sterile test tubes, and the solvent was allowed to evaporate. One ml of sterile liquid medium was then added to each tube. Thus the concentration in Tube #1 was 1 mg/ml while in Tube #10, it was 2 X 10^{-3} mg/ml. One loopful of the B. subtilis culture was added to each tube. The tubes were transferred to a 37° shaking water bath where the cultures were allowed to grow for 4 hours. Those tubes
**TABLE 1.**

**Concentration of Epirodin in Bacterial Assay.**

<table>
<thead>
<tr>
<th>Tube #</th>
<th>Concentration of Epirodin in Solution</th>
<th>Concentration of Epirodin in Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50 mg/ml</td>
<td>1.00 mg/ml</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>0.50</td>
</tr>
<tr>
<td>3</td>
<td>12.5</td>
<td>0.25</td>
</tr>
<tr>
<td>4</td>
<td>6.25</td>
<td>0.125</td>
</tr>
<tr>
<td>5</td>
<td>3.12</td>
<td>0.063</td>
</tr>
<tr>
<td>6</td>
<td>1.56</td>
<td>0.032</td>
</tr>
<tr>
<td>7</td>
<td>0.78</td>
<td>0.016</td>
</tr>
<tr>
<td>8</td>
<td>0.39</td>
<td>0.008</td>
</tr>
<tr>
<td>9</td>
<td>0.20</td>
<td>0.004</td>
</tr>
<tr>
<td>10</td>
<td>0.10</td>
<td>0.002</td>
</tr>
<tr>
<td>Control</td>
<td>0.00</td>
<td>0.000</td>
</tr>
</tbody>
</table>
which had become cloudy at the end of this period were assumed to have too low a level of epirodir for it to exert any inhibitory influence on the bacterial growth. Tubes exhibiting no growth were said to contain a bacteriostatic level of epirodir.

To determine the bacteriocidal level of epirodir, the tubes containing bacteriostatic levels were checked for ability to grow. These cultures were streaked onto the agar-containing medium which had been poured into Petri dishes. Those plates exhibiting growth reflected bacteriostatic levels of epirodir; plates containing no growth indicated bacteriocidal levels.

2. Interaction of Epirodir with Sterols. For studying the binding of polyene antibiotics with sterols, Bittman and Fischkoff (47) recommend dissolving the sterol component and antibiotic in dimethylformamide (DMF) and doing the experiments in 1 mM Tris-HCl-10 mM NaCl at pH 7.4. Also, the concentrations of the stock solutions should be such that the final concentrations of DMF in the spectral solution is less than 1%. Thus stock solutions of epirodir mix and the various sterols were prepared at concentrations of 5.0 mg/ml in DMF. Solutions containing various ratios of sterol to epirodir were prepared as shown in Table 2. The indicated amounts were mixed and diluted to 10 ml with the 1 mM Tris-HCl-10 mM NaCl buffer. The solutions were allowed to sit 30 minutes, and the absorption spectra were run. Blanks containing the appropriate amounts of sterol and DMF were used. The effect of sterols on the absorption spectrum was examined with cholesterol (Fisher), ergosterol (Calbiochem), stigmasterol (Calbiochem), and sitosterol
TABLE 2.
Preparation of Sterol-Epirodin Solutions.

<table>
<thead>
<tr>
<th>Sterol:Epirodin</th>
<th>ul Epirodin*</th>
<th>ul Sterol*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5:1</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>4:1</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>3:1</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>2:1</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>1:1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

*Five mg/ml in DMP
(Calbiochem). The interaction of epirodin mix and various sterols in ethanolic and methanolic solvent systems was also examined by the same method.

For fluorescence studies, only the interaction of cholesterol with epirodin was examined. The solutions were prepared in the same manner as those for the absorption spectral studies. Using the spectrofluorometer, readings were taken at 397 nm, the emission maximum of epirodin mix.

3. Inhibition of Yeast by Epirodin. The ability of epirodin to inhibit the growth of the yeast *Saccharomyces cerevisiae* was studied based on the method of Gottlieb et al. (29). Two liters of yeast nitrogen dextrose base medium (YNDB) (48) were prepared. Its composition is shown in Table 3. The organism was maintained as stock cultures on 2% agar slants of the above medium. Inocula for the yeast study were prepared by placing a stab of a *S. cerevisiae* colony into a 16 mm test tube containing 5 ml sterile medium. The organism was grown for 24 hours in a rotary shaker at 26°C. A loopful of the resulting culture was transferred to an identical tube and grown for 24 hours. This was again repeated to stabilize the inoculum.

The yeast assay was done in 25 ml Erlenmeyer flasks, each containing 10 ml sterile YNDB medium. A stock solution of epirodin mix in methanol was made at a concentration of 2.5 mg/ml. Volumes of 100, 60, 40, 20, 10, and 5 ul were added to the flasks so as to give final concentrations of 25, 15, 10, 5, 2.5, and 1.25 ug/ml epirodin. A control flask containing 100 ul of methanol but no epirodin was also run. To each flask was added
**TABLE 3.**

**Composition of YNDB Medium.**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>10 g</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>10 g</td>
</tr>
<tr>
<td>Histidine hydrochloride</td>
<td>20 mg</td>
</tr>
<tr>
<td>Methionine</td>
<td>40 mg</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>40 mg</td>
</tr>
<tr>
<td>Biotin</td>
<td>4 ug</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td>800 ug</td>
</tr>
<tr>
<td>Folic acid</td>
<td>4 ug</td>
</tr>
<tr>
<td>Inositol</td>
<td>4 mg</td>
</tr>
<tr>
<td>Niacin</td>
<td>800 ug</td>
</tr>
<tr>
<td>p-Aminobenzoic acid</td>
<td>400 ug</td>
</tr>
<tr>
<td>Pyridoxine hydrochloride</td>
<td>800 ug</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>400 ug</td>
</tr>
<tr>
<td>Thiamine hydrochloride</td>
<td>800 ug</td>
</tr>
<tr>
<td>Boric acid</td>
<td>1 mg</td>
</tr>
<tr>
<td>Copper sulfate·5 $H_2O$</td>
<td>80 ug</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>200 ug</td>
</tr>
<tr>
<td>Ferric chloride·6 $H_2O$</td>
<td>400 ug</td>
</tr>
<tr>
<td>Manganese sulfate·$H_2O$</td>
<td>800 ug</td>
</tr>
<tr>
<td>Sodium molybdate·2$H_2O$</td>
<td>400 ug</td>
</tr>
<tr>
<td>Zinc sulfate·7 $H_2O$</td>
<td>800 ug</td>
</tr>
<tr>
<td>Potassium phosphate monobasic</td>
<td>2 g</td>
</tr>
<tr>
<td>Magnesium sulfate·7 $H_2O$</td>
<td>1 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>200 mg</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>200 mg</td>
</tr>
<tr>
<td>Distilled water</td>
<td>2000 ml</td>
</tr>
</tbody>
</table>
1.0 ml of the stabilized yeast culture. Growth was measured by absorbance readings of the culture at 530 nm taken at 2 hour intervals for 16 hours. The instrument was set with a blank consisting of uninoculated medium. For the duration of the assay, the flasks were in a rotary shaker set at 26° and 100 rpm. The entire procedure was done in the dark so as to prevent degradation of epirodirn. To determine whether or not epirodirn maintained its level of activity for the duration of the experiment, a duplicate set of flasks was grown where the epirodirn was added 16 hours prior to the inoculum. The flasks were placed in the shaker under identical conditions and growth of *S. cerevisiae* was measured as before.

To determine the effects of sterol, a series of concentrations of epirodirn were assayed, but each flask also contained ergosterol at 50 pg/ml. This was done by adding 100 ul of a 5 mg/ml stock solution in methanol to each flask. Growth of the organism was monitored at 2 hour intervals as was previously described.

4. Antifungal Activity of Epirodirn. The experimental procedure followed to detect antifungal activity by epirodirn was based on that of Gottlieb *et al.* (29). Agar plates with the antibiotic incorporated into the medium were streaked with the appropriate organism and observed periodically for signs of growth.

Czapek-Dox agar (BBL) was the medium used. Fifty g were dissolved in 1000 ml distilled water and the solution was boiled for 1 minute, and then autoclaved. Aliquots of the agar
were made either 5, 25, or 50 ug/ml in epirodon, and plates were poured. Controls were also run containing the same medium but no epirodon. The plates were streaked with either Epicoccum nigrum, Aspergillus flavus, Rhizopus nigricans, or Fusarium oxysporum. Each organism at each concentration as well as all of the controls were run in triplicate. The plates were observed for growth at 2, 3, and 6 days.

5. Effect of Epirodon on the Fruit Fly. Drosophila melanogaster medium was prepared containing the following:

(1) Glucose ..................... 18 g
(2) Agar (Difco) ................. 20 g
(3) Yeast extract (Difco) ...... 20 g
(4) Cornmeal ..................... 90 g
(5) Sodium propionate ........... 5 g
(6) Distilled water .............. 1000 ml

Everything but the cornmeal was mixed together and heated until solution was complete. The cornmeal was added and the mixture boiled for 1 minute. Two ml aliquots were added to sterile 16 mm test tubes which were plugged with cotton. Into each of 15 tubes was placed one male fruit fly and one virgin female. The pairs were left for 4 days and then removed. The number of adults hatched per tube was counted, and means and standard deviations were calculated.

To determine if epirodon could affect D. melanogaster development, the assay was repeated with the medium containing 1000 ppm epirodon mix. The number of adults hatched was eval-
uated and compared to the results for the controls.

6. Epirodin and Hemolysis. The ability of epirodin to cause hemolysis of erythrocytes was investigated following the basic procedure of Kinsky et al. (42,49,50). Fresh human blood was obtained and allowed to coagulate. The specimen was centrifuged and the serum removed. The remaining clot was washed with isotonic saline (0.154 M NaCl) to remove as many intact red cells as possible. These cells were then washed 4 times with saline to remove any other blood components that might be present. The cells were then diluted with saline so that when 0.50 ml of this washed cell suspension was added to 4.50 ml water, the absorbance of the resulting solution at 550 nm was 0.510.

A stock solution of epirodin mix was prepared at a concentration of 2 mg/ml in DMF. The stock was diluted as shown in Table 4 so that 0.25 ml of each concentration could be added. Thus each tube contained an identical amount of DMF. In addition to 0.25 ml of the appropriate epirodin solution, each assay tube contained 0.50 ml erythrocyte suspension and 4.25 ml saline. The tubes were incubated in a water bath at 37° for 2 hours, centrifuged, and absorbance readings were recorded on the supernatant at 550 nm. Each tube was blanked with a solution containing 4.75 ml saline and 0.25 ml of the appropriate epirodin solution. A control was also run containing 4.25 ml saline, 0.50 ml red cell suspension, and 0.25 ml DMF to see if the solvent alone could rupture the cells.
### TABLE 4.

**Preparation of Epirodin Solutions for Erythrocyte Assays.**

<table>
<thead>
<tr>
<th>Mls Stock Epirodin*</th>
<th>Mls DMF</th>
<th>Concentration of Epirodin</th>
<th>Concentration in Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>9.0</td>
<td>0.2 mg/ml</td>
<td>10 ug/ml</td>
</tr>
<tr>
<td>2.0</td>
<td>8.0</td>
<td>0.4</td>
<td>20</td>
</tr>
<tr>
<td>3.0</td>
<td>7.0</td>
<td>0.6</td>
<td>30</td>
</tr>
<tr>
<td>4.0</td>
<td>6.0</td>
<td>0.8</td>
<td>40</td>
</tr>
<tr>
<td>5.0</td>
<td>5.0</td>
<td>1.0</td>
<td>50</td>
</tr>
<tr>
<td>6.0</td>
<td>4.0</td>
<td>1.2</td>
<td>60</td>
</tr>
<tr>
<td>7.0</td>
<td>3.0</td>
<td>1.4</td>
<td>70</td>
</tr>
<tr>
<td>8.0</td>
<td>2.0</td>
<td>1.6</td>
<td>80</td>
</tr>
<tr>
<td>9.0</td>
<td>1.0</td>
<td>1.8</td>
<td>90</td>
</tr>
<tr>
<td>10.0</td>
<td>0.0</td>
<td>2.0</td>
<td>100</td>
</tr>
</tbody>
</table>

*2 mg/ml in DMF

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To determine whether the epirodi/erythrocyte ratio, and not the absolute concentration of the antibiotic, determines the extent of hemolysis, an alternate assay was set up where the amount of epirodi remained constant while the erythrocyte concentration varied. Each assay tube contained 0.25 ml of the 1.6 mg/ml epirodi solution to give a final concentration of 80 ug/ml. To the tubes was then added 0.25, 0.35, 0.45, 0.55, 0.60, 0.70, 0.80, 0.90, 1.00, and 2.00 ml of erythrocyte suspension. Isotonic saline was added so that the total volume of each tube was 5.00 ml. The tubes were incubated at 37° for 2 hours, centrifuged, and absorbance readings at 550 nm were obtained. A blank containing epirodi at 80 ug/ml was used.

To determine whether or not serum could inhibit hemolysis by epirodi, an assay was set up where each tube contained a constant amount of epirodi and erythrocytes, but increasing amounts of serum. Thus to each of 14 test tubes was added 0.25 ml 1.6 mg/ml epirodi to give a final concentration of 80 ug/ml, and also 0.50 ml of erythrocyte suspension. To the first five tubes were added 0.25, 0.20, 0.15, 0.10, and 0.05 ml serum to give concentrations that were 5%, 4%, 3%, 2%, and 1%, respectively, of the total volumes. A 20% solution of serum in saline was then prepared and 0.190, 0.125, 0.100, 0.063, and 0.025 ml aliquots were added to the next five assay tubes to give concentrations of 0.75%, 0.50%, 0.40%, 0.25%, and 0.10% serum. Then a 0.1% solution of serum in saline was prepared and 2.5, 0.5, 0.25, and 0.05 ml aliquots were added to
the remaining four tubes to give concentrations of 0.05\%, 0.01\%, 0.005\%, and 0.001\% serum. Each assay tube was then diluted to 5.0 ml with saline. Again, the tubes were incubated, centrifuged, and absorbance readings were obtained with an 80 \mu g/ml epirodisin blank.

The ability of cholesterol to inhibit the lytic effect of epirodisin on erythrocytes was evaluated in an assay where each tube contained equal amounts of red cells and cholesterol but increasing amounts of epirodisin. Thus each tube contained 0.50 ml erythrocyte suspension, 50 ul of a 5 mg/ml cholesterol solution in DMP, and 0.25 ml of the epirodisin solutions described in Table 4. The tubes were diluted to 5.0 ml, incubated, centrifuged, and measured as for the original assay using the same blanks.

To identify other serum components that might be able to inhibit hemolysis by epirodisin, assays were conducted where each constituent would be present at its normal physiological level. Table 5 shows the normal ranges as stated by Davidsohn and Henry (51) as well as the concentrations at which they were tested. The appropriate amount of each was weighed into a test tube and 0.50 ml of erythrocyte suspension, 0.25 ml of 1.8 mg/ml epirodisin, and 4.25 ml saline were added. Following a 2 hour incubation period at 37\degree, the extent of hemolysis was determined.

The effect of an osmotic stabilizer in the assay system was also investigated. The series of epirodisin solutions described in Table 4 was used giving concentrations ranging from 10 to 100 \mu g/ml. To each tube was added 0.50 ml erythrocyte

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### TABLE 5.

**Normal Physiological Levels of Serum Constituents.**

<table>
<thead>
<tr>
<th>Component</th>
<th>Normal Range</th>
<th>Assay Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>32-57 mg/ml</td>
<td>50 mg/ml</td>
</tr>
<tr>
<td>γ-Globulin</td>
<td>0.5-2.0 mg/ml</td>
<td>1.0 mg/ml</td>
</tr>
<tr>
<td>Urea</td>
<td>.12-.44 mg/ml</td>
<td>0.50 mg/ml</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.6-0.9 mg/ml</td>
<td>1.0 mg/ml</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>1.5-3.8 mg/ml</td>
<td>4.0 mg/ml</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>0.3-0.8 meq/l</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>.06 meq/l</td>
</tr>
<tr>
<td></td>
<td></td>
<td>.24 meq/l</td>
</tr>
<tr>
<td></td>
<td></td>
<td>.24 meq/l</td>
</tr>
</tbody>
</table>
suspension, and instead of 4.25 ml isotonic saline, an equal volume of 0.25 M sucrose was used. The assay was conducted in the same manner with the two hour incubation period.

The kinetics of the hemolyzing process were also briefly examined. Tubes containing epirodin at 10, 40, 50, and 60 ug/ml were set up with 0.50 ml erythrocyte suspension, and 4.25 ml saline in each one. Six tubes of each concentration were prepared so that they could be incubated for different lengths of time. Time zero was taken to be at addition of the red cells, and the tubes were incubated for 5, 10, 20, 40, 60, or 120 minutes. At the appropriate times, they were removed from the 37° water bath, immediately centrifuged, and the absorbance at 550 nm was recorded with the appropriate blanks.

The ability of epirodin whose chromophore has been destroyed by illumination with visible light in the presence of flavins to cause lysis was also checked. Thus to 0.25 ml of a 1.6 mg/ml solution of epirodin mix were added 4.25 ml saline and 0.08 moles riboflavin. The solution was exposed to light for two hours to permit destruction of the polyene chromophore. One half ml of erythrocyte suspension was added, and the solution was incubated for 2 hours at 37°. Hemolysis was measured as in the previous assays.
III. RESULTS AND DISCUSSION.

A. Growth of Epicoccum nigrum.

*E. nigrum* grew consistently well on each of the different media used. Solid malt extract medium was used for stock culturing the organism as well as for the isolation of epirordin. When cultured on this medium, the entire surface became covered by a white mycelium within several days, and small areas of yellow-orange pigmentation began to appear. By the end of the first week, pigment had diffused throughout the medium, and areas of older mycelial growth began to turn brown. After about ten days, the entire mycelium was a brownish-orange color, and small areas of young white mycelium were starting to develop on top of it. At about this same time, small dark red-orange pigmented beads were becoming apparent on the surface. By the end of the two week growth period, there were numerous beads present, and the entire surface was a deep red-brown. At this point the cultures were extracted for the appropriate compounds.

*E. nigrum* was also grown in shaking liquid cultures for humic acid analyses. Yeast extract and malt extract media were used. Growth of the organism was similar in both media. On the day following inoculation, furry white balls of mycelium could be seen. By three days the cultures were already pigmented a yellowish-brown in the yeast extract medium and more of an orange-brown color in the malt extract.
medium. As the cultures aged, the colors darkened steadily, and the viscosity of the solutions continually increased. By the end of the growth periods, both sets of cultures had become a dark brown and were of a porridge-like consistency.

B. Isolation and Analysis of Humic Acid.

Three sets of cultures were grown for humic acid analysis, with each set containing 15 individual culture flasks. One set contained malt extract medium and was grown for 5 days while a second identical set was maintained for 21 days. The third set contained yeast extract medium and was grown for 5 days. Each set was treated identically for the isolation of the humic acids.

The initial step involved separation of the mycelia from the culture medium. This was accomplished with great difficulty as the medium would set to a gel upon standing, especially for the set of cultures which had been grown for 21 days. Because of this, as the cultures were poured into a buchner funnel, they were continually diluted with distilled water. After being freed of mycelial fragments, the culture broths of each set were combined and concentrated almost to dryness in vacuo. The concentrates were dark brown and very slimy. Each was dissolved in a minimum volume of 50 mM Tris-HCl buffer, pH 7.5 (approximately 25 ml). This material was transferred to dialysis tubing (2 in. X 6 in.).
and dialyzed 3 times against 2000 ml aliquots of the buffer for 12 hour periods. After the first dialysis, the buffers had turned a dark orange and were very cloudy. The second buffer aliquots were a clear dark yellow, while the third were pale yellow and clear. The material remaining in the dialysis tubing was treated and purified as described in the previous section.

None of the humic acid isolates was found to possess any biological activity at 10 mg/ml against either C. pyrenoidosa, B. subtilis, or A. salina.

An elemental analysis was done on a sample of each humic acid isolate grown under the various cultural conditions. Results are presented in Table 6 along with those of previous workers. It is apparent that the elemental composition of the humic acids was not consistent and may have depended on the cultural conditions. That the percentage of carbon dropped for the malt extract culture grown for 21 days as compared to that grown for 5 days might indicate that the humic acid in the older cultures is composed of more highly oxidized substances. However, in examining the CHN determinations reported by Foppen (1), Martin et al. (14), and Burge (12) which were done on samples isolated from cultures grown in the same yeast extract medium as was the sample reported in this work, the similarity in the 3 day product of Foppen (1) to the 22 day product of Martin et al. (14) would indicate that culture time is not an important factor. Thus there does not appear to be any obvious correlation between
<table>
<thead>
<tr>
<th></th>
<th>Carbon</th>
<th>Hydrogen</th>
<th>Nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malt Extract Medium (5 days)</td>
<td>40.28</td>
<td>7.02</td>
<td>6.15</td>
</tr>
<tr>
<td>Malt Extract Medium (21 days)</td>
<td>35.80</td>
<td>6.70</td>
<td>7.16</td>
</tr>
<tr>
<td>Yeast Extract Medium (5 days)</td>
<td>33.19</td>
<td>7.31</td>
<td>10.54</td>
</tr>
<tr>
<td>Foppen (1) (3 days)</td>
<td>54.9</td>
<td>5.8</td>
<td>6.3</td>
</tr>
<tr>
<td>Martin et al. (14) (22 days)</td>
<td>53-59</td>
<td>5.1</td>
<td>6-8.5</td>
</tr>
<tr>
<td>Burge (12) (5 days)</td>
<td>41.6</td>
<td>5.9</td>
<td>5.6</td>
</tr>
</tbody>
</table>

*a Average of duplicate determinations.
the results obtained by the different workers. The carbon content of 33.19% obtained in this work is significantly lower than those reported previously and the nitrogen content of 10.54% is higher. The latter suggests a greater relative percentage of amino acids being incorporated into the humic acid as compared to the amount of phenolic material.

Following hydrolysis of the humic acid samples, amino acid analyses were obtained. Results are presented in Table 7 along with those of Foppen (1). By comparing the different values, it is seen that the amino acid content did not change much if a culture was grown for 5 days or 21 days, as long as the medium was the same. It is only for valine and alanine that there were any significant differences. However, if an alternative nutritive source such as yeast extract was used, the content was very dissimilar. Different amino acids as well as different ratios were seen to occur. Foppen (1), using the same yeast extract medium, observed fewer amino acids and different relative amounts.

Phenol composition was examined using the same hydrolyzed samples used for the amino acid determinations. Haider and Martin (15) reported the isolation of phenols from ethyl ether extracts of nutrient broth in which E. nigrum was growing. However, all attempts with this procedure were futile. Troublesome emulsions were repeatedly obtained which, after finally separating, could not be shown to contain any phenols. In their paper these authors had also reported that the formation of humic acid was associated with the dis-
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Malt Extract (5 days)</th>
<th>Malt Extract (21 days)</th>
<th>Yeast Extract</th>
<th>Foppen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Arginine</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Cystine</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Glycine</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td>5</td>
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<tr>
<td>Histidine</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Leucine</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Lysine</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Methionine</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Proline</td>
<td>9</td>
<td>8</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Serine</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Threonine</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Valine</td>
<td>0</td>
<td>3</td>
<td>7</td>
<td>1</td>
</tr>
</tbody>
</table>

*Relative molar amounts for each preparation.*
appearance of phenols. Thus ether extracts of aqueous acid-
ic solutions of hydrolyzed samples of humic acid were exam-
ined for phenols. Tentative identifications were made using
two-dimensional TLC (Fig. 10). By comparison with standards,
spot 3 corresponded to 3,5-dihydroxybenzoic acid, 5 to resor-
cinol, 6 to orcinol, 7 to 2,4-dihydroxytoluene, 9 to 2,4-
dihydroxybenzoic acid, and 11 to orsellinic acid. By com-
parison with the results of Martin and Haider (15), 1 was
tentatively identified as 2,4,6-trihydroxybenzoic acid, 2 as
phloroglucinol, 4 as protocatechuic acid, 8 as 2,4,5-trihy-
droxytoluene, 10 as cresorsellinic acid, and 12 as 1,3-dihy-
droxy-5,6-dimethylbenzene.

An alternate set of solvents was used to identify the
phenols and produced the pattern shown in Fig. 11. In this
system, the phenols had $R_P$ values which were very close to
each other making it difficult to compare them with the stan-
dards. However, the various color reactions to the vanillin-
sulfuric acid spray reagent helped considerably in the iden-
tification. 3,5-Dihydroxybenzoic acid produced a bright blue
spot while the 2,4 counterpart turned purple. Orcinol was a
yellowish-purple and resorcinol, a bright red-pink. The iden-
tification of spots corresponding to 5 and 7 was only ten-
tative as they were so close to the other phenolic compounds.

In their study of phenols produced by E. nigrum, Haider
and Martin (15) reported each of the phenols found in this
work. In addition, they were able to identify $p$-hydroxy-
cinnamic acid, $p$-hydroxybenzoic acid, 5-methylpyrogallol,
Figure 10. TLC of Ether-Soluble Portion of Hydrolyzed Humic Acid. System of Martin and Haider (15). Sprayed with Vanillin-Sulfuric Acid. Spots with Solid Lines were Identified by Comparison with Standards.
Figure 11. TLC of Ether-Soluble Portion of Hydrolyzed Humic Acid. 2: 3,5-Dihydroxybenzoic Acid, 3: 2,4-Dihydroxybenzoic Acid, 4: Orcinol, 5: Orsellenic Acid, 6: Resorcinol, 7: 2,4-Dihydroxytoluene. Solvent System of Smith et al. (52,53). Sprayed with Vanillin-Sulfuric Acid.
and methyl phloroglucinol, among others. Thus it is seen
that the phenols in the humic acid produced by the UNH strain
of *E. nigrum* are consistent with those reported to be pre-
sent by other workers. Similar results were obtained with
each of the three humic acid samples, indicating that the
composition of the media and the length of the growth per-
iod are not significantly reflected in the qualitative phe-
nol content.

C. *Extraction and Isolation of the Epirodins.*

Extraction of *E. nigrum* cultures with ethanol gave a
crude extract which was dark, reddish-brown in color and bio-
logically active against *B. subtilis*, *C. pyrenoidosa*, and
*A. salina*. Thin layer chromatography of the crude extract
indicated the presence of several yellow pigments and nu-
merous fluorescent compounds (Fig. 12). The crude ethanol
extracts were taken to dryness *in vacuo*, generally yielding
15-30 g of solid material for 10 culture flasks.

The residue was washed with 300 ml 95% ethanol, giving
a dark, reddish-orange solution. The insoluble material
was discarded, and the 300 ml ethanol-soluble portion was
applied to an alumina column. Material eluted with the ini-
tial developing solvent, 95% methanol, was pale yellow and
was discarded. The eluate with the second solvent, 80%
methanol, was a dark yellow (Fraction II). Thin layer chro-
matography of this fraction gave the pattern indicated by
Figure 12. TLC of Various Fractions in the Isolation Procedure of the Epirodins. 1: Crude Ethanol Extract; 2: Fraction II, 3: Epirodin Mix (10 ul of a 1.0 mg/ml solution). Solvent System Consisting of Acetone: Methanol (1:1). Solid Lined Areas Indicate Pigmented Spots; Slashed Lines Represent Streaking; Dotted Lines Indicate Fluorescent Spots.
No. 2 in Fig. 12. The solution gave a positive reaction to glucostat spray reagent, indicating the presence of glucose. Fraction II was taken to dryness in vacuo, and the residue dissolved in a minimum amount of methanol, usually about 5 ml, and applied to a silicic acid column. TLC of this band gave a negative reaction when sprayed with glucostat showing the glucose to have been removed. Two yellow-orange spots were visible with $R_p$ values of 0.76 and 0.42 on plates by EM Reagents. On Eastman plates spots with $R_p$ values of 0.22 and 0.48 are obtained using the same solvents. Each spot had a small amount of fluorescent material trailing it. This fraction was designated as epirodin mix. The individual spots were identified as epirodin T for the top spot and epirodin L for the lower spot.

After working with the above chromatographic procedure to the isolation of epirodin mix for some time, a much simpler procedure was devised by which greater yields could be isolated in a much shorter time period. Whereas the procedure using columns takes about 10 days to complete, the alternate method can be done in a single day. In a typical isolation using the older procedure, from 19.0 g of crude extract could be isolated 540 mg of epirodin mix, or a yield of 2.9%. With the alternate procedure, from 4.50 g of crude extract was isolated 531 mg epirodin mix, or a yield of 11.8%. TLC and the UV absorption spectrum showed the purity of this product to be comparable to that from the older isolation procedure. This four-fold increase in yield along
with the reduction in isolation time makes the new isolation procedure highly advantageous.

Separation of the individual epirodins was accomplished by TLC. Each spot was scraped off the plate and eluted with methanol; the solutions were centrifuged, and the supernatants were taken to dryness in vacuo to give the individual compounds. $R_p$ values of the epirodins were examined in a variety of solvent systems. Results are presented in Table 8. The highest degree of resolution and the least amount of tailing was obtained with an isopropanol:methanol (1:1) solvent system.

Against A. salina, epirodin mix was found to be 100% lethal at a level of 1.0 mg/ml. When the concentration was dropped to 0.5 mg/ml, only 38% of the organisms died. The activity of epirodin against B. subtilis and C. pyrenoidosa was checked at a variety of concentrations ranging from 1-15 mg/ml. As can be seen in Table 9 and Fig. 13, the bacterium was more susceptible than the alga, and the toxicity increased almost linearly with concentration. The activity of epirodin mix against B. subtilis was slightly lower than that reported by Buckley (18) who found that a 1.0 mg/ml solution of epirodin mix produced zones of inhibition of 4.5 mm, while 3 mm zones were obtained in this work. Buckley also showed epirodin T to result in no inhibition of C. pyrenoidosa at 1.0 mg/ml while epirodin L was capable of producing a 2 mm zone. His results are consistent with those obtained in this work where a zone of 1-2 mm was produced.
### TABLE 8.

**RP Values for the Epirodines in Various Solvent Systems.**

<table>
<thead>
<tr>
<th>Solvent System</th>
<th>RP Epirobin T</th>
<th>RP Epirobin L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol:ethanol (8:2)</td>
<td>0.88</td>
<td>0.79</td>
</tr>
<tr>
<td>Methanol:acetone (1:1)</td>
<td>0.92</td>
<td>0.85</td>
</tr>
<tr>
<td>Methanol:acetone:benzene (4:4:1)</td>
<td>0.88</td>
<td>0.71</td>
</tr>
<tr>
<td>Methanol:acetone:benzene (2:2:1)</td>
<td>0.83</td>
<td>0.45</td>
</tr>
<tr>
<td>Methanol:acetone:benzene (1:2:2)</td>
<td>0.28</td>
<td>0.13</td>
</tr>
<tr>
<td>Methanol:acetone:hexane (2:2:1)</td>
<td>0.87</td>
<td>0.63</td>
</tr>
<tr>
<td>Methanol:acetone:toluene (2:2:1)</td>
<td>0.81</td>
<td>0.44</td>
</tr>
<tr>
<td>Methanol:acetone:carbon tetrachloride (2:2:1)</td>
<td>0.88</td>
<td>0.56</td>
</tr>
<tr>
<td>Methanol:acetone:chloroform (2:2:1)</td>
<td>0.87</td>
<td>0.47</td>
</tr>
<tr>
<td>Ethanol:acetone:benzene (2:2:1)</td>
<td>0.56</td>
<td>0.10</td>
</tr>
<tr>
<td>Isopropanol:ethanol (1:1)</td>
<td>0.76</td>
<td>0.42</td>
</tr>
<tr>
<td>Isopropanol:ethanol:benzene (2:2:1)</td>
<td>0.65</td>
<td>0.22</td>
</tr>
<tr>
<td>n-Propanol:acetone:pyridine (1:1:2)</td>
<td>0.47</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*Ten ul of a 1.0 mg/ml solution of epirodin mix in methanol applied in each system. 20 X 20 cm silica gel plates activated at 105° for 30 minutes, spotted, and developed to a height of 15 cm.*

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**TABLE 9.**

*Activity of Epirodin Mix Against *B. subtilis* and *C. pyrenoidosa.*

<table>
<thead>
<tr>
<th>Concentration of Epirodin (mg/ml)</th>
<th>Activity (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>B. subtilis</em></td>
</tr>
<tr>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>15</td>
<td>12</td>
</tr>
</tbody>
</table>

*aTotal diameter of inhibition zone less disc diameter.*

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Figure 13. Dose - Response Curve for the Activity of Epirodin Mix Against A, *B. subtilis*, and B, *C. pyrenoidosa*.
by a 5.0 mg/ml solution of epirodin mix against the alga.

D. Stability of Epirodin.

The effects of several physical conditions on the stability of epirodin were studied. The effect of constant exposure to light for various periods of time was examined by following both its biological activity and its absorbance at 429 nm. The absorbance of a 50 μg/ml solution in ethanol was measured on a daily basis while the activity of a 10 mg/ml solution in methanol was tested periodically against B. subtilis. Results are presented in Table 10 and illustrated in Fig. 14. Observations showed that as the color intensity decreased, so did the toxicity. The slopes of the two curves are very similar indicating that the activity of epirodin is likely to be at least partially associated with the chromophore. Filipin, which has a pentaene chromophore, is also known to lose its biological activity upon prolonged exposure to light (54). Tingstad and Garrett (55) correlated this loss in activity with the decay of the ultraviolet absorption due to the chromophore. Rickards et al. (56) were also able to demonstrate a similar loss of activity with decreased absorbance with lagosin, another pentaene compound.

The effect of solvent on the stability of epirodin mix was monitored by following biological activity against B. subtilis of 10 mg/ml solutions of epirodin mix in ethanol, methanol, and water. As can be seen in Table 11, the toxi-
### TABLE 10.

**Stability of Epirodin Mix Against Light.**

<table>
<thead>
<tr>
<th>Length of Exposure (days)</th>
<th>Absorbance (429 nm) (50 μg/ml)</th>
<th>Activity (mm) (10 mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.19</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>1.14</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>1.00</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>0.99</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>0.88</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>0.52</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>0.43</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>0.35</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>0.30</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>0.20</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>0.18</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>0.17</td>
<td>-</td>
</tr>
</tbody>
</table>

*a* Constant exposure to fluorescent lighting (30w) at a distance of two feet.
Figure 14. Effect of Constant Exposure to Fluorescent Light on the Stability of Epirodin Mix as Determined by the Absorbance at 429 nm (●) and by the Activity against *B. subtilis* (○).
### TABLE 11.

**Stability of Epirodin in Various Solvents.**

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Activity of 10 mg/ml Solutions (mm)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methanol</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>17</td>
<td>9</td>
</tr>
</tbody>
</table>

\(^a\)Total diameter of inhibition zone less disc diameter.
city remained fairly constant in methanol but steadily decreased in both ethanol and water.

Absorbance measurements were used to measure stability at various pH levels. Absorbance readings at 429 nm of 20 μg/ml solutions of epirodin mix in water, 0.01 N HCl, and 0.01 N NaOH were taken over a 37 day interval. Results are presented in Table 12 and Fig. 15. It appears that both high and low pH values produced a larger initial decrease in absorbance as compared with water. However, within one week, the rates of decrease were stabilized to the point where they were comparable in all three systems. Also worth noting are the differences in initial absorbance readings for each pH. The epirodin mix is highly colored in alkaline media while being much less so in acid.

**E. Characterization of Epirodin: Classification Tests.**

1. **Solubility Behavior.** Epirodin mix was found to be soluble only to a very small extent in water, virtually insoluble in acid, but highly soluble in base. In non-polar or slightly polar organic solvents such as benzene, carbon tetrachloride, and chloroform, epirodin mix was insoluble. In ethyl acetate, it was slightly soluble, whereas in acetone and ethanol, it was very soluble, and in methanol, the most soluble. It appears that a portion of the epirodin molecule must be highly polar due to its solubility in polar organic solvents. Yet a second portion must be non-polar to account
**TABLE 12.**

**Stability of Epirodin at Various pH Levels.**

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Absorbance at 429 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.01 N HCl</td>
</tr>
<tr>
<td>0</td>
<td>0.384</td>
</tr>
<tr>
<td>1</td>
<td>0.333</td>
</tr>
<tr>
<td>7</td>
<td>0.192</td>
</tr>
<tr>
<td>14</td>
<td>0.172</td>
</tr>
<tr>
<td>22</td>
<td>0.132</td>
</tr>
<tr>
<td>29</td>
<td>0.102</td>
</tr>
<tr>
<td>37</td>
<td>0.000</td>
</tr>
</tbody>
</table>
Figure 15. Effect of pH on the Stability of Epirodin Mix as Measured by the Absorbance at 429 nm. Curve A, in 0.1 N HCl; B, in Distilled Water; C, in 0.1 N NaOH.
for its low solubility in water. The high solubility observed in aqueous solutions at high pH compared to the low solubility at low pH levels indicates the presence of an acidic function. Epirodin mix was also soluble in dilute (5%, w/v) sodium bicarbonate solution. According to Shrinker et al. (38), only carboxylic acids, sulfonic acids, and sulfinic acids are soluble in this solvent. Thus the type of acid function which epirodin may have is limited.

Oroshnik and Mebane (23) reported similar solubility behavior for the polyene antifungal antibiotics. "As a group they show very poor solubility in the common organic solvents and in water but can be dissolved to a very reasonable extent in very polar solvents like pyridine, dimethylformamide, and dimethylsulfoxide. Some, containing basic nitrogen functions or acidic groups, can be dissolved in aqueous acid or alkaline media, respectively." (23).

2. Bromine in Carbon Tetrachloride Solution. As epirodin is insoluble in CCl₄, a sample of mix was dissolved in methanol, and a solution of Br₂ in CCl₄ was added dropwise. The disappearance of the bromine color along with the lack of HBr production indicated the presence of an olefinic or acetylenic linkage in epirodin.

3. Hydroxylamine Hydrochloride–Ferric Chloride Reaction. Epirodin mix was found to give a positive response to this test. This indicated that epirodin must contain either an ester, a lactone, or an aldehyde group. However, failure to
obtain a positive reaction to 2,4-dinitrophenylhydrazine suggested that there were no aldehyde or ketone groups.

4. Periodic Acid Test. Epirodin mix was capable of reacting with periodic acid. This indicated the presence of one of the following: 1,2 glycol, $\alpha$-hydroxyaldehyde, $\alpha$-hydroxyketone, or an $\alpha$-hydroxy acid. Since the negative 2,4-dinitrophenylhydrazine reaction precluded the presence of both the aldehyde and ketone groups, epirodin must be either a 1,2 glycol or an $\alpha$-hydroxy acid.

5. Potassium Permanganate Solution. Addition of epirodin to a dilute (1%, w/v) potassium permanganate solution resulted in immediate decolorization of the permanganate color. This along with the decolorization of Br$_2$ confirmed the presence of ethylenic or acetylenic linkages.

6. Tollen's Reagent. Epirodin was found to be capable of reducing Tollen's reagent, ammoniacal silver nitrate. This indicated that epirodin is a reducing agent, but no functional group is specified. However, it is known (39) that polyhydroxy compounds will reduce silver ions in a solution containing silver-ammonia complex to metallic silver. This suggests that epirodin might be a polyhydroxy compound. As has been shown repeatedly (28), the polyene macrolide antibiotics contain many hydroxyl groups per molecule. For example, amphotericin B has eight while DJ400B contains eleven of them.
F. Spectroscopic Studies on Epirodin.

1. Infrared Analysis. An IR spectrum was obtained on a solid sample of epirodin mix. The spectrum is shown in Fig. 16 and band assignments are presented in Table 13. The presence of alcohol is indicated by both the broad O-H stretching band at 3400-3200 cm\(^{-1}\) and the C-OH stretching bands at 1060 and 1030 cm\(^{-1}\). Symmetric and asymmetric stretching of methyl and methylene groups are reflected in the peaks at 2960, 2925, and 2850 cm\(^{-1}\). The bending vibrations of the methyl groups appear at 1460 and 1375 cm\(^{-1}\), and \((\text{CH}_2)_n\) rocking results in the band at 725 cm\(^{-1}\). The strong peak at 1720 cm\(^{-1}\) is indicative of a carbonyl function, specifically as an ester carbonyl in conjugation with an alkene. The somewhat broad band at 1610-1570 cm\(^{-1}\) reflects the C=C stretching of a polyene while that at 950 cm\(^{-1}\) reflects the CH bending of this group. Those peaks at 1260 and 1110 cm\(^{-1}\) result from asymmetric and symmetric C-O-C stretching of an ester group.

Thus the IR spectrum indicates that epirodin mix contains methyl and methylene groups, several conjugated unsaturated functions, one of which is conjugated with an ester, and several alcoholic groups. This is consistent with the interpretation of the spectrum obtained by Buckley (18) using a KBr pellet. He reported that unsaturation, the presence of an -OH and possibly a C=O were indicated by the spectrum.
Figure 16. Infrared Spectrum of Epirodin Mix.
**TABLE 13.**

**Assignments for the Absorption Bands Observed in the Infrared Spectrum of Epirodin Mix.**

<table>
<thead>
<tr>
<th>Absorption Band (cm⁻¹)</th>
<th>Assignment</th>
<th>Functional Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>3400-3200</td>
<td>O-H stretching</td>
<td>polymeric, intermolecular H-bonded alcohols</td>
</tr>
<tr>
<td>2960</td>
<td>as CH₃</td>
<td></td>
</tr>
<tr>
<td>2925</td>
<td>as CH₂</td>
<td></td>
</tr>
<tr>
<td>2850</td>
<td>s CH₂</td>
<td></td>
</tr>
<tr>
<td>1720</td>
<td>C=O</td>
<td>-C=O-O=O-</td>
</tr>
<tr>
<td>1610-1570</td>
<td>C=C</td>
<td>polyene</td>
</tr>
<tr>
<td>1470</td>
<td>CH₂ scissor</td>
<td></td>
</tr>
<tr>
<td>1460</td>
<td>as CH₃</td>
<td></td>
</tr>
<tr>
<td>1375</td>
<td>s CH₃</td>
<td></td>
</tr>
<tr>
<td>1260</td>
<td>as C-O-C</td>
<td>ester</td>
</tr>
<tr>
<td>1110</td>
<td>s C-O-C</td>
<td>ester</td>
</tr>
<tr>
<td>1060,1030</td>
<td>C-O stretching</td>
<td>alcohols</td>
</tr>
<tr>
<td>950</td>
<td>CH (out-of-plane)</td>
<td>polyene</td>
</tr>
<tr>
<td>725</td>
<td>(CH₂)ₙ rocking</td>
<td></td>
</tr>
</tbody>
</table>
2. **Mass Spectrum.** Epirodin was found to be non-volatile in the mass spectrometer so the trimethylsilyl (TMS) ether derivative was prepared. Thin layer chromatography of the derivative along with a sample of epirodin mix is illustrated in Fig. 17. In the solvent system used, isopropanol-methanol (1:1), the derivative had a greater $R_F$ value, and chromatographed as a single spot rather than as two spots like epirodin mix. There were also two faint spots corresponding to the epirodins. These reflect a small amount of unreacted material.

The mass spectrum of the TMS derivative was obtained with the inlet temperature set at $200^\circ$ and the chamber heater at $250^\circ$. The background pressure was $8 \times 10^{-7}$ mm. Upon introduction of the sample, the pressure rose to $2 \times 10^{-6}$ mm. A large number of peaks was obtained, most of which were at lower mass values. The lower portion of the spectrum is shown in Fig. 18.

In 1964 Golding *et al.* (57) presented a paper describing the application of TMS ether derivatives for the determination of polyols by mass spectrometry. In it they state: "The high mass regions of the spectra......are dominated by groups of isotope peaks, separated by multiples of 90 mass units from the molecular ion and corresponding to successive eliminations of neutral trimethylsilanol units." This was not observed in the spectrum of the TMS ether derivative of epirodin mix.

The high mass peaks for the unknown were assigned values
Figure 17. TLC of Epirodin Mix (1) and the Trimethylsilyl Ether Derivative of Epirodin Mix (2). Solvent System of Isopropanol:Methanol (1:1).
Figure 18. Lower Portion of the Mass Spectrum of the Trimethylsilyl Ether Derivative of Epirodin Mix.
by comparison with a reference compound, Tris-(perfluorooctyl)-s-triazine (PCR, Inc., Research Chemical Division, Gainesville, Fla.) The dominant peaks in the spectrum of the reference compound were measured with respect to the distance from m/e of 28 for N₂. Thus m/e=146 corresponded to 3.1 cm, m/e=295 to 7.0 cm, and m/e=1108 to 34.5 cm. The values were plotted on log-log paper (Fig. 19), and the graph was used to calculate m/e values for the epirodin derivative.

The highest mass peak was 48.5 cm from N₂ corresponding to m/e=1600. The next farthest peak was at 21.4 cm indicating m/e=800. It would seem then that m/e=1600 represents the intact TMS-ether derivative of epirodin. Once the trimethylsilyl groups started coming off, they seemed to do so very rapidly and the peaks corresponding to the successive eliminations were not observed. The next peak at 800 probably corresponds to epirodin mix without any of the TMS-OH groups.

Using these values, the number of -OH groups on epirodin and the molecular weight of the compound were estimated. If x is the number of -OH groups on epirodin, then 1600-90x = 800 (where 90 is the weight of a neutral trimethylsilanol unit), and x = 9. Thus there appear to be nine hydroxyl groups on epirodin. If 800 is the weight of epirodin without these groups, then the molecular weight of epirodin would be 800+9(17) = 953 g.

2. Nuclear Magnetic Resonance. An NMR spectrum of epirodin mix in DMSO-d₆ is shown in Fig. 20. Other than the
Figure 19. Graph of Log m/e vs. Log (Distance from m/e = 28 for N\textsubscript{2}). Closed circles denote values for the reference compound and open circles, the TMS-ether derivative of epirodin mix.
Figure 20. Nuclear Magnetic Resonance Spectrum of Epirodin Mix.
broad peak at \( \delta = 3.3-3.4 \), the spectrum is identical to that obtained by Buckley (18). The difference is probably due to water in the solvent used. DMSO is very hydroscopic while the CD\(_3\)OD used by Buckley is not. In repeated runs in DMSO-d\(_6\), the size of this peak varied with relation to the other peaks indicating that it was indeed due to contamination.

The NMR spectrum of epirudin mix is difficult to interpret. The triplet at \( \delta = 0.96 \) ppm on the surface indicates methyl protons of -CH\(_2\)CH\(_3\) groupings. However, it may be due to a chance combination of doublets due to -CH-CH\(_2\) groups. The peak at \( \delta = 1.32 \) ppm is assigned to aliphatic hydrogens, and that at \( \delta = 2.58 \) ppm to the DMSO solvent. The doublet at \( \delta = 4.29 \) ppm indicates hydroxyl protons. The singlet at \( \delta = 8.7 \) ppm cannot be explained as it reflects aromatic hydrogens which the UV spectrum shows to be absent. The rounded multiplet at \( \delta = 6.45 \) ppm indicates olefinic protons. It does not appear that the difficult to interpret peaks are due to solvent contamination since similar ones were obtained by Buckley (18) using a different solvent.

4. **UV-Visible Absorption Spectrum.** The UV-visible absorption spectrum of a 10 ug/ml solution of epiprodin mix in 95% ethanol from 750 to 220 nm is shown in Fig. 21. There was a single broad peak at 429 nm with shoulders on either side, and a smaller peak at 217 nm. The same spectrum was obtained by both Buckley (18) and Burge (12) in their work with epiprodin.

The IR spectrum had shown that epiprodin most likely did
Figure 21. UV-Visible Absorption Spectrum of Epirodin Mix.

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not contain any aromatic moiety. This was confirmed in the UV by the transparent region from 180 to 290 where benzene and its polynuclear compounds such as anthracene and naphthalene usually absorb strongly.

Epirodin absorbs at a relatively long wavelength. The presence of unsaturation shown by the characterization tests suggests that this may form part of the epirodin chromophore. The presence of unsaturation in a molecule does cause absorption to occur at longer wavelengths. If the unsaturation is in the form of double bonds and they are conjugated, the bathochromic shift increases as an additive function. As Hamilton-Miller (28) discusses, if the absorption maxima are shifted enough, the compounds involved will be visibly colored as can be seen by inspecting various polyene compounds. Triene molecules are colorless or a very pale yellow; tetraenes such as the polyene antibiotics, nystatin and pimaricin, are pale yellow; pentaenes like filipin are a definite yellow; and heptaenes like amphotericin B are definitely orange. A polyene structure for the chromophore of epirodin would be consistent with the characterization tests for the compound. The compound is bright orange, and was shown to be unsaturated by both Br$_2$ addition and KMnO$_4$ oxidation. Such a proposed structure is in marked contrast to the hydroxyquinone structure suggested by Buckley (18). He attributed the strong visible absorption of the epirodins to impurities. As will be demonstrated later, hydrogenation of epirodin resulted in destruction of the chromophore. This presents ad-
ditional solid evidence that unsaturation plays a major role in the chromophore structure of epirodin.

Oroshnik and Mebane (23) report that the UV spectra of polyene antibiotics all have the same shape, and that they differ only in wavelength. It is also a characteristic that the main absorption band is resolved into four or five sharp peaks. Representative spectra are illustrated in Fig 22. The pentaene fungichromin and the heptaene candidin have spectra that are virtually identical in shape. However, their maxima differ by a shift of 60 nm for each peak. Oroshnik and Mebane (23) go on to say in their discussion that a carbonyl group in conjugation with a polyene system always "degrades" the spectral fine structure. In polyene ketones or short-chain polyene acids and esters, the fine structure is completely obliterated. In polyene acids or esters containing five or more double bonds, the degradation is less drastic. Epirodin was shown to contain an ester function by its positive reaction to the hydroxylamine hydrochloride - ferric chloride test. Thus it appears that the chromophore of epirodin is consistent with an ester or lactone function in conjugation with a polyene system. Such a chromophore would not produce the series of sharp peaks characteristic of the usual polyenes. Instead, the broad band with shoulders on either side such as is observed with epirodin would be expected.

According to Nielsen (58) the basic absorption maxima value for acids and esters with \( \alpha \) and \( \beta \) substituents is
Figure 22. Representative Spectra of Polyene Antibiotics: Tetraenes, Pentaenes, Hexaenes, and Heptaenes (23).
Representative ultraviolet spectra of polyene antifungal antibiotics: tetraenes and pentaenes (Corrected to 95% ethanol except for nystatin and fungichromin; see Tables 1, 2, 4, 5).

Fig. 1. Representative ultraviolet spectra of polyene antifungal antibiotics: heptaenes and heptaenes. (Corrected to 95% ethanol; see Tables 4, 5, 9, 10.)

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217 nm in ethanolic solution. Woodward (59, 60) reports that the wavelength is shifted 30 nm higher for each -ene in conjugation. If the chromophore of epirodin is indeed an ester or lactone in conjugation with a polyene system, then the 429 - 217 nm or 212 nm shift in maximum can be attributed to a polyene segment. At 30 nm per double bond, epirodin would contain 212/30 or 7.06 double bonds in conjugation.

The extinction coefficient of epirodin was calculated using a 10 ug/ml solution of epirodin mix in ethanol. (Fig. 21). By Beer's Law, $A = abc$ where $A$ is the absorbance; $a$, the absorptivity; $b$, the path length in cm; and $c$, the concentration in g/liter. For a 10 ug/ml solution, the absorbance was 0.505, giving a value of $5.05 \times 10^1$ for $a$. The molar absorptivity, $\varepsilon = a(MW)$, was then calculated using the molecular weight of 953 g obtained by mass spectroscopy. In this manner it was calculated to be $4.812 \times 10^4$ or $\log \varepsilon = 4.682$. Regarding the molar absorptivity of the polyene antibiotics, Hamilton-Miller (28) states that regression analyses of observed mean $\varepsilon$ values show that $\varepsilon = N \times 21,000$, where $N$ is the number of double bonds. For seven bonds, it works out to $1.47 \times 10^5$ with $\log \varepsilon = 5.168$. However, they report that when the chromophoric polyene system is conjugated with a lactone carbonyl, there is a substantial decrease in $\varepsilon$. This is consistent with the proposed chromophore for epirodin.

g. UV-Visible Spectra of the Individual Epirodins. The individual epirodins were resolved by TLC on silicic acid plates with isopropanol:methanol (1:1). The epirodins were

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eluted off the silica, dried in vacuo, and redissolved in ethanol to give the spectra shown in Fig. 23. The two compounds appear to have the same chromophore. Considerably more of the lower \( R_p \) compound was present than the higher one. The same distribution had been observed by Buckley (18).

b. Effect of Solvent and pH. Spectra were obtained on solutions of epirodin mix in acidic, basic, and neutral media (Fig. 24). In alkaline solution the same spectrum was obtained as in 95% ethanol. In distilled water, the spectrum remained essentially unchanged, but the intensity decreased slightly. In 0.1 N aqueous HCl the spectrum is almost completely degenerated. The peak flattened out and shifted to a slightly shorter wavelength (400 nm) and the intensity was markedly decreased.

Oroshnik and Mebane (23) report that this "spectral degradation" is characteristic of polyene antifungal antibiotics. It has been observed in the spectra of trichomycin and candidicidin as well as candidin and amphotericin B. They state that it is "not a change in the chromophore but in its physical environment; the polyene molecules, inadequately solvated, are associating with each other in clusters of two or more, tending toward micelle formation or colloidal dispersion". Addition of a more powerful solvent such as alcohol restores the normal spectrum. The spectrum of epirodin mix in 0.05 N HCl in 50% ethanol is shown in Fig. 25. It is completely regenerated. This proves that it is different physical conditions that are responsible for the changes observed.
Figure 23. UV-Visible Absorption Spectra of the Individual Epirodins. 1, Epirodin L; 2, Epirodin T.
Figure 24. UV-Visible Absorption Spectra of Epirodin Mix at Various pH Levels. 1, in 0.1 N NaOH; 2, in Distilled Water; 3, in 0.1 N HCl.

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Figure 25. UV-Visible Absorption Spectra of a 10 µg/ml Solution of Epirodin Mix Under Acidic Conditions, 1, in 0.05 N Aqueous HCl; 2, in 0.05 N HCl in 50% Ethanol.
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in the spectrum, and not the pH per se. Buckley (18) had proposed that the differences in the spectra in acidic and in basic aqueous media were due to differences in ionization states of a hydroxyanthraquinone structure in the epirodin molecule. This probably is not the case since the spectrum of epirodin is not degraded in ethanolic acidic solution. Instead, it appears that, as is the case for the polyene antibiotics, the degradation is simply the result of decreased solubility.

2. Photosensitivity in the Presence of I₂ Catalyst. Photosensitivity depends both on structure and configuration. For conjugated systems in solutions exposed to intense sunlight, photostereoisomerization usually competes with irreversible side reactions in which partial cleavage to colorless (fluorescent) compounds occurs. The stereoisomerization process usually involves cis-trans interconversions. These processes are spontaneous at room temperature. Although typically slow, these rates can be increased by changing the solvent, temperature, or other physical factors.

One of the most effective means of producing trans-cis rearrangements is by I₂ catalysis in the light. The conversion is rapid, and the formation of a mixture of cis-trans isomers from an all-trans compound can be seen visually by the decrease in color intensity. Zechmeister (40) reports that it is characteristic of carotenoids and other conjugated double bond systems, that upon exposure to fluorescent light in the presence of an I₂ catalyst, trans-cis rearrange-
ments occur resulting in shifts to lower wavelengths. He says that polyenes can be readily characterized simply by recording the UV-visible spectrum before and after catalysis by iodine.

Iodine catalysis is influenced by several physical factors among which are the pigment/iodine ratio and the mode and duration of illumination. Thus a series of epirodin mix and iodine solutions containing 10 ug/ml and 2 ug/ml, respectively, in 95% ethanol were exposed to light under identical conditions but for varying exposure times. Immediately after the illumination period, the spectra were recorded (Fig. 26). According to Zechmeister (40), "during an all-trans to cis rearrangement, the spectral curve is altered as follows in the visible region: the extinction value and the degree of fine structure decreases while the maxima migrate to shorter wavelengths". Such was the case observed with epirodin mix. Addition of I₂ with no exposure to light caused no change in in the absorption spectrum. However, as the duration of light exposure increased, the bands shifted toward shorter wavelengths while becoming broader and less intense. At 45 and 60 minutes the same spectra were obtained indicating that the trans-cis stereoisomers were in equilibrium with each other. Also, the fact that no peaks appeared at longer wavelengths than were present before stereoisomerization indicated that epirodin mix exists in an all-trans configuration in its natural state.

The I₂-catalyzed stereoisomerization of epirodin was also
Figure 26. Visible Absorption Spectra of Epirodin Mix Exposed to Fluorescent Light in the Presence of I$_2$ Catalyst for Various Time Intervals. 1, No Exposure to Light; 2, 5 Minute Exposure; 3, 15 Minutes; 4, 30 Minutes; 5, 60 Minutes. In Ethanolic Solution.
examined using petroleum ether as solvent. The extent of trans-cis rearrangement was far less (Fig. 27) indicating the all-trans configuration to be favored in this solvent.

d. Photosensitivity Due to Flavins. Posthuma et al. (41) have shown that illumination of the polyene antibiotic pimaricin with visible light in the presence of flavins results in destruction of the polyene chromophore. Kinsky et al. (42) reported a similar finding concerning filipin, another polyene compound. Thus the ability of riboflavin to destroy the polyene chromophore of epirodin was investigated.

Solutions of epirodin and riboflavin were exposed to light for different periods of time. It was found that the longer the epirodin solution was exposed to light, the less intense its absorption became. Absorbance measurements at 429 nm are given in Table 14. A plot of the absorbance vs. time is shown in Fig. 28. Like pimaricin and filipin, epirodin was not affected by flavin without exposure to light. Light alone caused some decrease in absorbance but not to the extent that occurred when riboflavin was present.

G. Chemical Methods Used in the Analysis of Epirodin Mix.

1. Acetylation of Epirodin Mix. Acetylation involves replacement of the hydrogen on an alcoholic hydroxyl group with acetyl (CH₃CO) according to the following equation:

\[ R-OH + (CH₃CO)₂⁻ \longrightarrow CH₃COOR + CH₃COOH \]

Pyridine is used as solvent because it combines with acetic...
Figure 27. Visible Absorption Spectra of Epirodin Mix Exposed to Fluorescent Light in the Presence of I₂ Catalyst Using Petroleum Ether as Solvent. 1, No I₂; 2, I₂ and Exposure to Light for 15, 30, or 60 Minutes.
**TABLE 14.**

**Effect of Riboflavin and Visible Light on the Absorbance of Epirocin at 10 ug/ml in 0.05 M Phosphate Buffer, pH 6.9.**

<table>
<thead>
<tr>
<th>Time of Exposure (min.)</th>
<th>Riboflavin</th>
<th>Light</th>
<th>Riboflavin and Light</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.418</td>
<td>0.418</td>
<td>0.417</td>
</tr>
<tr>
<td>5</td>
<td>0.418</td>
<td>0.415</td>
<td>0.381</td>
</tr>
<tr>
<td>10</td>
<td>0.418</td>
<td>0.413</td>
<td>0.360</td>
</tr>
<tr>
<td>15</td>
<td>0.418</td>
<td>0.409</td>
<td>0.338</td>
</tr>
<tr>
<td>20</td>
<td>0.418</td>
<td>0.408</td>
<td>0.312</td>
</tr>
<tr>
<td>30</td>
<td>0.417</td>
<td>0.408</td>
<td>0.294</td>
</tr>
<tr>
<td>45</td>
<td>0.417</td>
<td>0.407</td>
<td>0.220</td>
</tr>
</tbody>
</table>

Riboflavin at 2 ug/ml.
Figure 28. Effect of Riboflavin and Visible Light on the Absorbance of Epirodin. A: Riboflavin, B: Light Exposure, C: Riboflavin and Light Exposure.
acid as it is formed, driving the reaction forward (39). Similar reaction conditions have been used for the acetylation of polyenes such as chainin (61) and the mycoticins A and B (62). The acetylation of epirodin proceeded quite smoothly. The product was found to have a different solubility profile than epirodin mix. While epirodin is insoluble in organic solvents such as chloroform and ethyl ether, the acetylated counterpart was very soluble.

Several spectroscopic studies were done on acetylated epirodin, including NMR, UV-visible absorption, and infrared absorption. The NMR spectrum is shown in Fig. 29. It was not possible to obtain any sharp peaks. Series of complex multiplets were obtained instead. Tentative assignments are presented in Table 15 along with the peak positions of acetylated filipin (63). The acetate methyl groups appear to have similar chemical shifts. It looks as if acetylated epirodin, like filipin, has some kind of side chain because of the peaks attributable to methyl and methylene groups. Also present is a diffuse band at $\delta = 4.20$ ppm which is assigned to the olefinic protons of the polyene chromophore and the protons of the carbon atoms to which the acetate groups are attached. Acetylated flavofungin (64) and acetylated mycoticin (62), two other polyene compounds, also have similar NMR spectra. Their acetoxy protons have the same shift as in filipin and epirodin, as have the olefinic protons. It is interesting to note that in the NMR spectra of all three acetylated compounds as well as epirodin, the peaks never appear as concise
Figure 29. Nuclear Magnetic Resonance Spectrum of Acetylated Epirodin Mix.
TABLE 15.

NMR Peak Assignments for Acetylated Epirodin Mix Based on the NMR Spectrum of Acetylated Filipin.

<table>
<thead>
<tr>
<th>Peak Assignment</th>
<th>Acetylated Filipin</th>
<th>Acetylated Epirodin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₂, CH₃ groups of C₅H₁₁ side chain</td>
<td>0.86</td>
<td>0.95</td>
</tr>
<tr>
<td>Methyl group attached to doubly bonded carbon</td>
<td>1.83</td>
<td>-</td>
</tr>
<tr>
<td>Acetate methyl groups</td>
<td>2.01</td>
<td>2.05</td>
</tr>
<tr>
<td>Olefinic protons and protons on same carbon atoms as acetate groups</td>
<td>4.23-6.81</td>
<td>4.20</td>
</tr>
</tbody>
</table>
singlets, doublets, or triplets. Instead, they are diffuse and complex multiplets. For example, the 12 $\text{C-CH}_3$ protons of mycoticin A appear as a single broad band with no resolution at all.

The UV-visible absorption spectrum of a 10 µg/ml solution of acetylated epirodin in 95% ethanol is shown in Fig. 30. The peak is shaped in an identical manner to epirodin itself, indicating that acetylation did not affect the chromophore. The extinction coefficient was decreased in accordance with the increased weight of the acetylated epirodin molecule.

The infrared spectrum of a KBr pellet of acetylated epirodin is shown in Fig. 31. Notably absent is the broad band at 3400-3200 cm$^{-1}$ caused by alcoholic O-H stretching. The carbonyl band is also changed. In epirodin mix a sharp peak at 1720 cm$^{-1}$ was present which was assigned to $-\text{C=C-CO-}$.

In the acetylated compound the band is broader and extends to higher frequencies. This is due to the presence of acetate ester which appears at 1735 cm$^{-1}$. Another difference between the two spectra is the symmetric and asymmetric C-O-C stretching frequencies of the ester. Because of the different types of esters in acetylated epirodin, the peaks overlap, and one sees the broad band centered at 1210 cm$^{-1}$.

a. Quantitative acetylation. It is possible to quantitate the acetylation of a hydroxyl group in two separate ways. The first method involves hydrolyzing the acetyl derivative by saponification followed by determination of the neutral-
Figure 30. UV-Visible Absorption Spectrum of Acetylated Epirodin Mix.
Figure 31. Infrared Spectrum of Acetylated Epirodin Mix.
ization equivalent. In the second method, the excess acetylating reagent is hydrolyzed with water,

$$(\text{CH}_3\text{CO})_2 + \text{HOH} \rightarrow 2\text{CH}_3\text{COOH}$$

and the amount of acetic acid formed is determined. Mehlenbacher (65) recommends the latter procedure because the saponification method is more prone to error. A small error in titration results in a large error in calculation, making it necessary to do many determinations. When using hydrolysis of excess reagent as a means of quantitation and pyridine as solvent, Mehlenbacher says that the acetylation proceeds to 99.3%.

The data for the acetylation of epirodin mix are shown in Table 16. The number of hydroxyl groups was calculated according to the following equation:

$$\frac{(\text{ml blank}\text{-ml test})(0.5)(\text{MW epirodin}) - 2}{(\text{mg of sample})} = \zeta$$

A molecular weight of 953 g was used for epirodin. Since each mmole of hydroxyl groups removes one mmole of acetic acid, the difference in mmoles of base should give the number of mmoles of hydroxyl groups. This figure divided by the number of mmoles of epirodin mix should give the number of hydroxyl groups per molecule of epirodin. However, since epirodin itself contains two free acid groups as will be shown later (Neutralisation Equivalent), the actual number of hydroxyl groups should be 2 less than this value. Two has been subtracted to give the values indicated in Table 16. The average number of hydroxyl groups on each molecule
### TABLE 16.

**Acetylation of Epirodin Mix.**

<table>
<thead>
<tr>
<th>Sample Weight (mg)</th>
<th>ML for Titration$^a$</th>
<th>Blank-Test</th>
<th>No. of Hydroxyls</th>
</tr>
</thead>
<tbody>
<tr>
<td>blank</td>
<td>34.25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>21.02</td>
<td>33.50</td>
<td>0.75</td>
<td>15</td>
</tr>
<tr>
<td>19.91</td>
<td>33.28</td>
<td>0.87</td>
<td>18</td>
</tr>
<tr>
<td>20.25</td>
<td>33.33</td>
<td>0.92</td>
<td>21</td>
</tr>
</tbody>
</table>

$^a$ 0.5 N NaOH

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worked out to 18. Berkoz and Djerassi (66) indicate that the acetylation process is not always quantitative for poly­
gen with large amounts of hydroxyl groups, and that the error involved is often in the range of 10%. Mass spectrom­
etry had indicated that there were 9 hydroxyl groups on the epirodin molecule. There is definitely lack of agreement between the two techniques. The lack of precision in the chemical method suggests that this method may also lack some accuracy. Thus, while the number of hydroxyl groups determined by mass spectrometry was estimated by extrapolation, it is felt to be the more definite of the two tech­
niques. Therefore, it is assumed that epirodin contains 8-10 hydroxyl groups per molecule.

2. Acid Hydrolysis of Epirodin Mix. When epirodin mix was hydrolyzed in 1 N HCl, the hydrozylate was separated into four fractions: the ether layer, the aqueous layer, the solid precipitate, and the gummy solid remaining in the reaction flask. The gummy solid was examined first and was found to be soluble in methanol and base but insoluble un­
der acidic conditions. An IR spectrum was obtained by smear­ing the compound on a NaCl disc and is shown in Fig. 32. The lower spectrum is of epirodin mix on a NaCl disc. The two spectra appear to be of the same compound. This along with the similar solubility properties seems to indicate that the gummy solid remaining in the reaction flask after acid hydrolysis is simply unreacted epirodin.
Figure 32. Infrared Spectra of the Gummy Residue Produced During Acid Hydrolysis of Epirodin Mix (Upper Tracing) and of Epirodin Mix (Lower Tracing).

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The next fraction to be worked up was the solid precipitate which had appeared at the interface of the aqueous and ether layers during the extraction procedure. It was found to be far more soluble in pyridine than in methanol. A KBr pellet was prepared to obtain an IR spectrum (Fig. 33). Strong bands at 670 and 740 cm$^{-1}$ indicated the presence of a C-Cl bond. This was confirmed by a positive reaction to the silver nitrate test for alkyl halides (43). The bands at 1260, 1110, and 1060 cm$^{-1}$ either disappeared or were markedly decreased as compared to epirodin mix. These peaks had been assigned to symmetric and asymmetric C-O-C stretching of an ester function. This along with the appearance of the broad band from 2900-2600 cm$^{-1}$ indicated that the ester function of epirodin mix was acid hydrolyzed. The carbonyl band observed for epirodin was markedly decreased; however, the various polyene and methylene peaks remained intact as did the alcohol bands.

These results show that this fraction contained the epirodin mix that was hydrolyzed according to the following equation:

\[ RCOOR' + H_2O \xrightarrow{H^+} RCOOH + R'O_2H \]

The positive reaction of the silver nitrate test for alkyl halides showed that an additional side reaction was also occurring:

\[ -C=C- \xrightarrow{HX} -C-C- \]

The HCl was probably adding to the many unsaturated double bonds of epirodin. The hydrolyzed material could be recryst-
Figure 33. Infrared Spectrum of the Solid Precipitate Which Appeared During the Extraction of the Acid Hydrolysis Products of Epirodin Mix.
tallized from ethanol and had a melting point of 128-130°.

The aqueous layer from the extraction procedure was examined primarily for any sugars that might be present. Most of the polyene macrolide antibiotics contain either a sugar or an amino sugar moiety that is liberated upon acid hydrolysis of the parent molecule. Most commonly found is mycosamine which has been isolated from nystatin, pimari-
cin, candidin, and trichomycin (23). Thus the aqueous lay-
er was taken almost to dryness and spotted onto TLC plates. Glubostat reagent, Morgan-Elson reagent, aniline-diphenyl-
amine-phosphoric acid spray, Ehrlich reagent, and benzidine-
trichloroacetic spray, all of which are specific for either a sugar or an amino sugar, were unable to elicit a positive reaction. Although destruction of sugar may have taken place during acid hydrolysis, amino sugars are extremely stable to acidic conditions. Thus it may be concluded that epirodin mix contained no amino sugar moiety and probably no sugar moiety at all.

The ether layer was also concentrated in vacuo and ex-
amined by TLC in a solvent system consisting of benzene:
pyridine (99.5:0.5). After spraying the plate with vanillin-
sulfuric acid reagent, the pattern shown in Fig. 34 was ob-
tained. Two distinct spots were seen along with a faint spot just behind the solvent front and several smaller spots slightly above the origin. Numerous standard samples were tested in searching for a type of compound that would travel in a similar manner to the major spots. Those compounds...
Figure 34. TLC of Concentrated Ether Extract From Acid Hydrolysis Products of Epirodin Mix (1); 2: Vitamin K, 3: \( \alpha \)-Tocopherol, 4: 4-Methylclavicol. Developed in Benzene:Pyridine (99.5:0.5). Sprayed with Vanillin-Sulfuric Acid.
which fitted the requirement were ring systems with very long aliphatic side chains, among which were vitamin K, $\alpha$-tocopherol, and methylclavicol. Their TLC patterns are recorded in Fig. 34.

The concentrated ether layer was applied to a silicic acid column and eluted with benzene. Fractions were each examined by TLC to see which spot they contained. The largest spot in the TLC pattern, $R_f=0.382$, was the only one collected from the column in any significant amount. Thus those fractions containing this particular spot were pooled and concentrated in vacuo, yielding a very pale yellow oil.

Infrared spectra of the compound were obtained in both carbon tetrachloride and cyclohexane (Fig. 35,36). It is really not possible to make any solid conclusions from the spectral data. The unknown compound contains methyl and methylene groups. The ester function of epirodin did not appear to have been disrupted. There were still unsaturated functions present, some of which were conjugated. The hydroxyl groups seem to have disappeared as evidenced by the loss of the broad band at 3400-3200 cm$^{-1}$ seen in the spectrum of epirodin mix, and the appearance of C-Cl bands indicated the addition of HCl across double bonds.

As was just mentioned, the unknown compound was pale yellow in contrast to the brilliant orange-red color of epirodin. This change was also reflected in the UV-visible absorption spectrum. Thus while log e for epirodin mix at 429 nm was 4.682 for a 10 µg/ml solution, there was no ab-
Figure 35. Infrared Spectrum of the Compound Isolated from the Ether Layer in the Extraction of the Acid Hydrolysis Products of Epirodin Mix. In Carbon Tetrachloride.
Figure 36. Infrared Spectrum of the Compound Isolated From the Ether Layer in the Extraction of the Acid Hydrolysis Products of Epirodin Mix. In Cyclohexane.
sorption in the visible range for a 50 µg/ml solution of the unknown compound. However, there was absorption in the ultraviolet region which was not observed for epirodin mix (Fig. 37). For example, there was a small peak at 273 nm with a shoulder at 280 nm. Chromophores such as \((\text{C:C})_3\) and \((0:0)(\text{C:C})_2\) absorb at 273-273.5 nm while \((\text{C:C})_4\) absorbs at 280-280.5 nm. The peak at 212 nm may indicate a \((0:0)\) group while that at 234 nm may reflect a \((0:0)(\text{C:C})\) chromophore. Thus from the spectral data, it would seem that this ether layer contained epirodin to which HCl had added at unsaturated functions thereby destroying the chromophore. Epirodin itself is soluble in ether only to a very small extent. The increased solubility properties along with the loss of hydroxyl bands in the IR spectrum indicate that an additional side reaction also may have occurred:

\[
\text{R-OH} \xrightarrow{\text{HX}} \text{RX} + \text{H}_2\text{O}
\]

2. Bromination of Epirodin Mix. Sixty-five mg of epirodin mix had been used for the bromination procedure. From this were obtained 128 mg of product. Using a molecular weight of 953 g for epirodin as determined by mass spectrometry, the amount of Br\(_2\)/mole of epirodin was calculated. Sixty-five mg of epirodin is equivalent to \(6.82 \times 10^{-5}\) moles. The difference in final and initial weights is taken as the weight of bromine added and works out to \(7.88 \times 10^{-4}\) moles. Thus \(7.88 \times 10^{-4}\) moles of Br\(_2\) per \(6.82 \times 10^{-5}\) moles of epirodin gives a value of 11.5 moles of bromine atoms per mole.
Figure 37. Ultraviolet Spectrum of the Compound Isolated from the Ether Layer During the Extraction of the Acid Hydrolysis Products of Epirodin Mix.
of epirodin. This indicates that 5.75 moles of Br₂ were taken up per mole of epirodin. According to Polgar and Jungnickel (44), addition of halogen across olefinic linkages occurs very rapidly in the dark, while substitution proceeds very slowly. They also state that while addition of halogen across a single double bond is quantitative, it is not so for conjugated systems. The UV-visible spectrum of epirodin had indicated that seven double bonds in conjugation were present. Thus halogenation does not appear to have been complete.

Brominated epirodin could be crystallized and was obtained as a pale yellow-orange powder in contrast to epirodin mix which is orange-red and oily. The melting point of the brominated derivative, however, was not very sharp. The melting process started at 117° and was complete at 130°. This indicated that the bromination product was a mixture.

The toxicity of brominated epirodin mix was tested against B. subtilis, A. salina, and C. pyrenoidosa. It was found to be completely inactive at 10 mg/ml.

The spectrum of the brominated compound was examined in both the UV-visible and infrared regions. At a concentration of 1 mg/ml in ethanol, no visible absorption could be demonstrated. The UV absorption spectrum of a 100 μg/ml solution in 95% ethanol is shown in Fig. 38. There were several peaks and shoulders present. The primary peak corresponds to (O:CC)=C while the smaller ones show either (O:C), (C:C)₂, (C:C)₃, or (O:C)(C:C)₂. This indicates that des-
Figure 38. Ultraviolet Spectrum of Brominated Epirodin Mix.
struction of the chromophore at random points in the conjugated system probably occurred.

The infrared spectrum of brominated epirodin mix in a KBr pellet is shown in Fig. 39. It is virtually identical to that of epirodin mix. Since carbon-bromine stretching is reflected at 600-500 cm\(^{-1}\), which is beyond the range of the instrument, it would not show up. In the spectrum of epirodin, there was a band at 1610-1570 cm\(^{-1}\) which was assigned to C=C stretching of a polyene. Dienes absorb in this same range. Therefore, the random addition of bromine in the conjugated system makes it impossible to see any overall changes in the spectrum.

Elemental analysis of brominated epirodin, like that of epirodin, showed no nitrogen. There were however, differences in the relative amounts of carbon and hydrogen. While carbon accounts for 58.93% of the weight in epirodin, it constituted only 35.81% in its brominated counterpart. Likewise, the hydrogen dropped from 7.85% to 4.31%. This reflects the 5.75 moles of bromine or 920 g added which nearly doubled the molecular weight.

4. **Elemental Analysis of Epirodin Mix.** The results of C.H.N analysis on epirodin mix are presented in Table 17. Each determination was run in duplicate, and the data were averaged to give the results shown. Two separately isolated samples were used to insure that no gross contamination was present as a difference in the amount of contaminant would
Figure 39. Infrared Spectrum of Brominated Epirodin Mix.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Carbon</th>
<th>Hydrogen</th>
<th>Nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epirodistin Mix #1</td>
<td>58.93</td>
<td>7.85</td>
<td>0.00</td>
</tr>
<tr>
<td>Epirodistin Mix #2</td>
<td>57.55</td>
<td>7.75</td>
<td>0.85</td>
</tr>
<tr>
<td>Epirodistin B_{mix} (12)</td>
<td>44.13</td>
<td>5.49</td>
<td>0.75</td>
</tr>
<tr>
<td>Epirodistin Mix (18)</td>
<td>73.15</td>
<td>9.33</td>
<td>0.00</td>
</tr>
</tbody>
</table>

**TABLE 17.**

**Elemental Analysis Data on Epirodistin Mix.**
become apparent in a difference in the results. Also presented is the data obtained by Burge (12) who worked with the same compound. The differences observed between the two workers probably reflect the hydroscopic nature of epirodin. Using averaged figures of 58.24% for carbon and 7.80% for hydrogen, and a molecular weight of 953 g, it is possible to calculate the number of atoms per molecule of epirodin. Thus there are 550 g of carbon or 46 atoms, and 74 g of hydrogen or 74 atoms. Assuming the rest of the molecule is oxygen, there are 322 g or 21 atoms. Therefore the empirical formula for epirodin is calculated as being \( \text{C}_46\text{H}_{74}\text{O}_{21} \). In comparing these values with the CHN analysis of the brominated derivative, \( \text{C}_{46}\text{H}_{62}\text{O}_{21}\text{Br}_{12} \), a discrepancy is observed. The expected values would be 29.90% carbon, and 3.26% hydrogen, not the 35.81% carbon and 4.31% obtained.

5. **Hydrogenation of Epirodin Mix.** The hydrogenation of epirodin mix could be followed visually by the loss of color in the solution. While originally an intense orange-red, the solution gradually lost its color until it finally became colorless. The uptake of hydrogen was measured at regular intervals. The results are presented in Table 18. As can be seen, the hydrogenation proceeded rapidly in the first 20 minutes and then continued at a slower pace. After 18 hours, a total of 21.2 ml of the \( \text{H}_2 \) gas had been consumed. This figure, however, was obtained at 25° and 740 mm pressure, and thus must be converted to STP (0° and 760 mm).
<table>
<thead>
<tr>
<th>Time</th>
<th>Buret Reading (ml)</th>
<th>Ml Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 minutes</td>
<td>24.5</td>
<td>0.0</td>
</tr>
<tr>
<td>1</td>
<td>24.1</td>
<td>0.4</td>
</tr>
<tr>
<td>3</td>
<td>22.7</td>
<td>1.8</td>
</tr>
<tr>
<td>5</td>
<td>21.2</td>
<td>3.3</td>
</tr>
<tr>
<td>7</td>
<td>19.5</td>
<td>5.0</td>
</tr>
<tr>
<td>9</td>
<td>16.5</td>
<td>8.0</td>
</tr>
<tr>
<td>11</td>
<td>14.0</td>
<td>10.5</td>
</tr>
<tr>
<td>13</td>
<td>11.0</td>
<td>13.5</td>
</tr>
<tr>
<td>16</td>
<td>7.6</td>
<td>16.9</td>
</tr>
<tr>
<td>21</td>
<td>6.9</td>
<td>17.6</td>
</tr>
<tr>
<td>26</td>
<td>6.6</td>
<td>17.9</td>
</tr>
<tr>
<td>31</td>
<td>6.5</td>
<td>18.0</td>
</tr>
<tr>
<td>36</td>
<td>6.4</td>
<td>18.1</td>
</tr>
<tr>
<td>41</td>
<td>6.1</td>
<td>18.4</td>
</tr>
<tr>
<td>56</td>
<td>5.7</td>
<td>18.8</td>
</tr>
<tr>
<td>71</td>
<td>5.7</td>
<td>18.8</td>
</tr>
<tr>
<td>86</td>
<td>5.7</td>
<td>18.8</td>
</tr>
<tr>
<td>101</td>
<td>5.5</td>
<td>19.0</td>
</tr>
<tr>
<td>116</td>
<td>5.4</td>
<td>19.1</td>
</tr>
<tr>
<td>131</td>
<td>5.2</td>
<td>19.3</td>
</tr>
<tr>
<td>6 hours</td>
<td>4.2</td>
<td>20.3</td>
</tr>
<tr>
<td>18</td>
<td>3.3</td>
<td>21.2</td>
</tr>
</tbody>
</table>
While one mole of a gas occupies 22.4 liters at STP, it takes up 25.2 liters under the conditions of the experiment. Therefore, 21.2 ml/25.2 liters or $8.39 \times 10^{-4}$ moles of hydrogen added to 108.28 mg or $1.16 \times 10^{-4}$ moles of epirodin mix. From this data, it is calculated that epirodin contains $8.39 \times 10^{-4}/1.16 \times 10^{-4}$ or 7.23 double bonds.

Hydrogenated epirodin was colorless and had no visible absorption spectrum. The ultraviolet spectrum of a 10 ug/ml solution of the compound in ethanol had a single peak at 210 nm. This is near the short wavelength limit of the useful range of ethanol as solvent. In any case, the conjugated double bond system has been completely obliterated.

Hydrogenated epirodin mix showed no toxicity when tested against *B. subtilis*, *A. salina*, or *C. pyrenoidosa*.

6. Neutralization Equivalent. The neutralization equivalent data are presented in Table 19. The average value was calculated to be 458. Using a molecular weight of 953, these results showed that there are $953/458$ or 2.08 acid functions on each epirodin molecule. The solubility of epirodin mix in 5% sodium bicarbonate along with the lack of sulfur in the molecule indicate that they must be carboxylic acids.

7. Periodate Oxidation of Epirodin Mix. Periodate is capable of oxidizing compounds containing two or more =OH or =O groups attached to adjacent carbon atoms with cleavage of carbon-carbon bonds.
## TABLE 19.

**Determination of the Neutralization Equivalent of Epirodin Mix.**

<table>
<thead>
<tr>
<th>Sample Weight (mg)</th>
<th>Ml Blank&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ml Sample&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Neutralization Equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.60</td>
<td>0.05</td>
<td>1.35</td>
<td>431</td>
</tr>
<tr>
<td>1.63</td>
<td>0.05</td>
<td>0.42</td>
<td>440</td>
</tr>
<tr>
<td>2.22</td>
<td>0.05</td>
<td>0.54</td>
<td>453</td>
</tr>
<tr>
<td>1.34</td>
<td>0.05</td>
<td>0.34</td>
<td>463</td>
</tr>
<tr>
<td>2.07</td>
<td>0.05</td>
<td>0.48</td>
<td>482</td>
</tr>
<tr>
<td>1.30</td>
<td>0.05</td>
<td>0.32</td>
<td>481</td>
</tr>
</tbody>
</table>

<sup>a</sup> 0.01008 N NaOH
\[
\text{R-CHOH} + \text{HIO}_4 \rightarrow \text{RCHO} + \text{R'CHO} + \text{HIO}_3
\]

The excess \(\text{HIO}_4\) can be determined by then reducing it to \(\text{IO}_3^-\) by addition of excess KI under acidic conditions:

\[
\text{H}^+ + \text{IO}_4^- + 2\text{I}^- \rightarrow \text{I}_2 + \text{IO}_3^- + \text{OH}^-
\]

The amount of \(\text{I}_2\) thus formed can then be determined by titration with \(\text{Na}_2\text{S}_2\text{O}_3\) to the disappearance of the iodine color:

\[
\text{I}_2 + 2\text{S}_2\text{O}_3^- \rightarrow 2\text{I}^- + \text{S}_4\text{O}_6^-
\]

Identical aliquots containing 25.5 mg of epirodin were allowed to react with periodate. KI was added, and the \(\text{I}_2\) thus formed was titrated with thiosulfate. Readings of 52.10, 52.10, and 52.15 ml were obtained, giving an average of 52.12 ml of \(\text{Na}_2\text{S}_2\text{O}_3\). The total amount of periodate available was determined by adding KI prior to the addition of epirodin. Thus there was no \(\text{IO}_4^-\) left to oxidize the compound. When titrated with thiosulfate, a value of 53.45 ml was obtained for this solution. These data were then used to calculate the molar equivalent weight. The amount of thiosulfate used was:

\[
\text{meq. S}_2\text{O}_3^- = (\text{ml blank} - \text{ml test}) (N \text{ Na}_2\text{S}_2\text{O}_3) = (53.45 - 52.12)(0.1) = 0.133
\]

However, every \(\text{IO}_4^-\) that was reduced required 2 thiosulfate ions. Thus the 0.133 meq. of \(\text{S}_2\text{O}_3^-\) represented 0.133/2 or 6.65 \(\times\) 10\(^{-2}\) meq. of periodate. Since each aliquot of epirodin contained 25.5 mg, the molar equivalent weight was
Using a molecular weight of 953, each epirodin molecule contained 953/384 or 2.48 vicinal hydroxyl or \( \alpha \)-hydroxy functions. Since it was impossible to demonstrate the presence of any ketone groups, it is presumed that epirodin contains 2.48 \( \text{1,2-glycol} \) or \( \alpha \)-hydroxy acid groupings. Glycols are by no means uncommon among the polyene antibiotics. Cope and Johnson (45) showed fungichromin to be sensitive to periodate via a \( \text{1,2-glycol} \) while Borowski et al. (67) showed the same to be true of amphotericin B.

The products of the periodation were examined. An ether extract of the aqueous solutions used for titrations was taken to dryness in vacuo. Since 2.48 carbon-carbon bonds were shown to have been cleaved, epirodin must be in 2-3 fragments. A visible spectrum of the products retained the shape of the epirodin spectrum. Thus periodate did not cleave within the chromophore itself.

The products of any periodate oxidation contain aldehyde groups at the point of cleavage. Therefore, the epirodin ether-soluble fragments were treated with 2,4-dinitrophenylhydrazine to form the dinitrophenylhydrazone derivatives:

\[
\text{NO}_2 \quad \text{NO}_2 \quad \text{NO}_2 + \text{C}=\text{O} \rightarrow \text{C}=\text{NNH} \quad \text{NO}_2 + \text{H}_2\text{O}
\]

The precipitated derivative was recrystallized and examined. A melting point determination gave a value of 115-118\(^{\circ}\). The UV-visible absorption spectrum of a 50 \( \mu\text{g/ml} \) solution of the
derivative in ethanol is shown in Fig. 40, and the spectrum of a 10 μg/ml solution of 2,4-dinitrophenylhydrazine in ethanol is illustrated in Fig. 41. Both spectra contained the same absorption bands, and the chromophore of epirodin was not observed. This also suggests that the crystallized derivative does not contain the epirodin chromophore and is from a different part of the epirodin molecule.

The infrared spectrum is presented in Fig. 42. Notably absent was the carbonyl band which is so prominent in the spectrum of epirodin. The majority of the peaks are attributable to 2,4-dinitrophenylhydrazine. The only one which is not is the O-H stretching band at 3400-3200 cm⁻¹. Others such as those at 1610-1590 cm⁻¹ and 1510 cm⁻¹ reflect the phenyl nucleus, while those at 1520 and 1330 cm⁻¹ indicate symmetric and asymmetric stretching of an NO₂ group bonded to carbon in a phenyl ring.

Carbon, hydrogen, and nitrogen analyses of the 2,4-dinitrophenylhydrazone derivative showed the compound to be composed of 43.53% carbon, 18.00% nitrogen, and 4.08% hydrogen. The molecular weight of the compound was calculated on the basis of the percentage of nitrogen present. Epirodin itself contains no nitrogen. Assuming there is a 2,4-dinitrophenylhydrazone grouping present at each end of the epirodin fragment, the derivative must contain two groups or eight nitrogens. Eight nitrogens, each weighing 14, account for 112 or 18.00% of the molecular weight. Thus the molecular weight must be 623. Carbon constitutes 43.53%
Figure 40. UV-Visible Absorption Spectrum of the 2,4-Dinitrophenylhydrazone Derivative of Periodate-Treated Epirodin Mix.
Figure 41. UV-Visible Absorption Spectrum of 2,4-Dinitrophenylhydrazine.
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Figure 42. Infrared Spectrum of the 2,4-Dinitrophenylhydrazone of Periodate-Treated Epirodin Mix.

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of the weight, corresponding to 23 atoms, and H, 4.08% or 25 atoms. The remaining molecular weight is assigned to oxygen, giving a value of 34.39% or 13 atoms. The empirical formula of the derivative is thus calculated to be C_{23}H_{24-26}N_{8}O_{13}. The chemical structure of the 2,4-dinitrophenylhydrazone derivative would be:

![Chemical structure]

After eliminating the carbon, hydrogen, and nitrogen atoms of end groupings, there remain 9 carbon atoms, 15 hydrogen atoms, and 5 oxygen atoms for the R group. From the UV-visible spectrum, it is known that there are no conjugated double bond systems present. The IR showed no carbonyl groups whether they be ketones, acids, esters, or aldehydes. However, IR showed there are alcohols present, but these cannot be 1,2-glycols or they would have been cleaved by the periodate. Thus a proposed structure for the 2,4-dinitrophenylhydrazone derivative is as follows:

![Proposed structure]

Such a structure contains 23 carbons, 8 nitrogens, and 28 hydrogens, and is consistent with the observed IR and UV-visible spectra.

8. Periodate Oxidation Following Saponification. A sample of epirodin was saponified and diluted to a larger
volume from which aliquots corresponding to 24.6 mg of epi-
rodin were removed for titration with Na₂S₂O₃. The proce-
dure was identical to that used for epirodin itself. Read-
ings of 67.40 ml, 67.25 ml, 67.30 ml, and 67.38 ml were ob-
tained for the sample solutions giving an average of 67.33
ml. The value for the blank was 69.20 ml. Using the equa-
tion,

\[
\text{Molar equivalent weight} = \frac{2(\text{weight of sample in mg})}{(\text{ml blank-ml test}) (N \text{ Na}_2\text{S}_2\text{O}_3)} \]

\[
= \frac{2(24.6)}{(69.20-67.33)(0.1)}
\]

= 263

Using 953 g as the molecular weight, results indicated that
saponified epirodin contained 953/263 or 3.62 1,2-glycol or
α-hydroxy acid groupings per molecule. The unsaponified
compound was shown to contain 2.48 groupings. Thus sapon-
ification resulted in an increase of 1.14 sets of adjacent
carbon atoms bonded to hydroxyl groups. This suggests that
epirodin contains a masked glycol grouping which may be in-
volved in an ester or lactone which opens up on saponifica-
tion:

\[
\text{-C-O-C-C-} \quad \rightarrow \quad \text{-C-OH + CH}_2\text{-C-}
\]

Fungichromin exhibits a similar phenomenon (68). In neutral
solution, it consumes two molar equivalents of periodate
while three molar equivalents are consumed with prior base
treatment.

2. Potassium Permanganate Oxidation of Epirodin Mix.

Potassium permanganate is an oxidant for alkene functions

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(38), and reacts according to the following general equations:

1. \[2 \text{KMnO}_4 + H_2O \rightarrow 2 \text{KOH} + 2 \text{MnO}_2 + 3(0)\]

2. \[R\text{CH=CHR}^* + (0) \rightarrow R-\text{CH-CH}-R^* \xrightarrow{\Delta} R-C=O + O=C-R^*\]

Persistence of the purple color of \(\text{KMnO}_4\) is an indication that all double bonds have reacted, and that the compound is in its most highly oxidized state.

Epirodin mix was reacted with \(\text{KMnO}_4\), and the ether-soluble acidic components were examined. Straight and branched-chain aliphatic acid standards containing one to five carbon atoms could be resolved in a solvent system consisting of ethyl acetate:2.5% \(\text{NH}_4\text{OH}\) (95:5) (69) by thin layer chromatography on silica plates. Resolution was greatly improved when the solvent was aged 1-2 days. Spots were identified by spraying with bromocresol green reagent. \(R_F\) values of the various smaller monocarboxylic acids are shown in Table 20. When co-chromatographed with the standards, the oxidized product of epirodin travelled with the solvent front, indicating that it was less polar than the lower aliphatic, monocarboxylic acids. Attempts were made to identify the oxidation product by comparing its \(R_F\) value with other types of acidic compounds and using other systems and reagents. Data are presented in Table 21. The epirodin oxidation product was found to give a positive reaction with both Schweppes's reagent and bromocresol green. Those acids giving positive reactions to both sprays when
<table>
<thead>
<tr>
<th>Acid</th>
<th>$R_p$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formic</td>
<td>0.362</td>
</tr>
<tr>
<td>Acetic</td>
<td>0.656</td>
</tr>
<tr>
<td>Propionic</td>
<td>0.742</td>
</tr>
<tr>
<td>Butyric</td>
<td>0.776</td>
</tr>
<tr>
<td>Iso-butyric</td>
<td>0.783</td>
</tr>
<tr>
<td>Valeric</td>
<td>0.800</td>
</tr>
<tr>
<td>Iso-valeric</td>
<td>0.808</td>
</tr>
</tbody>
</table>

$R_p$ Values of Aliphatic Acids Developed in Ethyl Acetate:2.5% Ammonia (95:5).
<table>
<thead>
<tr>
<th>Acid</th>
<th>Spray I&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Spray II&lt;sup&gt;b&lt;/sup&gt;</th>
<th>System I&lt;sup&gt;a&lt;/sup&gt;</th>
<th>System II&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epirodin oxidation</td>
<td>+</td>
<td>+</td>
<td>0.810</td>
<td>0.980</td>
</tr>
<tr>
<td>product</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adipic</td>
<td>+</td>
<td>+</td>
<td>0.801</td>
<td>0.962</td>
</tr>
<tr>
<td>Citraconic</td>
<td>+</td>
<td>+</td>
<td>0.770</td>
<td>0.491</td>
</tr>
<tr>
<td>Citric</td>
<td>+</td>
<td>+</td>
<td>0.133</td>
<td></td>
</tr>
<tr>
<td>Deoxycholic</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Dihydroxymaleic</td>
<td>+</td>
<td>+</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Cit-epoxysuccinic</td>
<td>+</td>
<td>+</td>
<td>0.091</td>
<td></td>
</tr>
<tr>
<td>Trans-epoxysuccinic</td>
<td>+</td>
<td>+</td>
<td>0.089</td>
<td></td>
</tr>
<tr>
<td>Fumaric</td>
<td>+</td>
<td>+</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Glutaric</td>
<td>+</td>
<td>+</td>
<td>0.707</td>
<td>0.965</td>
</tr>
<tr>
<td>Glycolic</td>
<td>+</td>
<td>+</td>
<td>0.441</td>
<td></td>
</tr>
<tr>
<td>Glyoxylic&lt;sub&gt;α&lt;/sub&gt;</td>
<td>+</td>
<td>+</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>β-hydroxybutyric</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Itaconic</td>
<td>+</td>
<td>+</td>
<td>0.740</td>
<td>0.905</td>
</tr>
<tr>
<td>Lactic</td>
<td>+</td>
<td>+</td>
<td>0.487</td>
<td></td>
</tr>
<tr>
<td>Maleic</td>
<td>+</td>
<td>+</td>
<td>0.725</td>
<td>0.454</td>
</tr>
<tr>
<td>Malic</td>
<td>+</td>
<td>+</td>
<td>0.274</td>
<td></td>
</tr>
<tr>
<td>Malonic</td>
<td>+</td>
<td>+</td>
<td>0.618</td>
<td>0.755</td>
</tr>
<tr>
<td>Oxalic</td>
<td>+</td>
<td>+</td>
<td>0.010</td>
<td></td>
</tr>
<tr>
<td>Succinic</td>
<td>+</td>
<td>+</td>
<td>0.695</td>
<td>0.886</td>
</tr>
<tr>
<td>Tartaric</td>
<td>+</td>
<td>+</td>
<td>0.013</td>
<td></td>
</tr>
<tr>
<td>Usninc</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Vulpinic</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Schweppes reagent
<sup>b</sup>Bromocresol green
<sup>c</sup>Acetone: methanol (1:1)
<sup>d</sup>Acetone: acetic acid: water (85:10:5)
their spots were simply sprayed were developed in acetone: methanol (1:1) (System I). The unknown had an $R_P$ value of 0.810 in this system which upon comparison with the standards, permitted many of them to be eliminated. Subsequent TLC in acetone:acetic acid:water (85:10:5) (System II), where the $R_P$ of the unknown was 0.980, eliminated several additional compounds. Of those acids which remained as possibilities, it was noted that it most closely resembled a straight-chain, completely saturated, unsubstituted, dicarboxylic acid (e.g. adipic acid). However, the $R_P$ values did not allow a definite identification. The dicarboxylic acids were therefore identified using gas-liquid chromatography. Methyl esters were prepared of both the standards and the unknown. $R_T$ values of the standards were calculated and found to be as indicated in Table 22. Injection of the permanganate oxidation products of epirodin mix gave peaks with $R_T$ values that indicated the presence of suberic and azelaic acids. The elution pattern of both the standard acids and the unknowns are illustrated in Fig. 43. Identical volumes of solution were resolved at the same sensitivity settings so that the chromatograms would be comparable.

To insure that the dicarboxylic acid identification was correct, equal volumes of the reaction products and standards were mixed and injected at the same settings previously used. The standards appeared as they had before but with an increase in the $C_8$ and $C_9$ peaks (Fig. 44). The increase in peak height corresponded to the sum total of the peak

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<table>
<thead>
<tr>
<th>No. of Carbons</th>
<th>Acid</th>
<th>$R_T^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Malonic</td>
<td>1.00</td>
</tr>
<tr>
<td>4</td>
<td>Succinic</td>
<td>1.18</td>
</tr>
<tr>
<td>5</td>
<td>Glutaric</td>
<td>1.71</td>
</tr>
<tr>
<td>6</td>
<td>Adipic</td>
<td>2.36</td>
</tr>
<tr>
<td>7</td>
<td>Pimelic</td>
<td>3.12</td>
</tr>
<tr>
<td>8</td>
<td>Suberic</td>
<td>4.24</td>
</tr>
<tr>
<td>9</td>
<td>Azelaic</td>
<td>5.55</td>
</tr>
<tr>
<td>10</td>
<td>Sebacic</td>
<td>7.47</td>
</tr>
<tr>
<td>11</td>
<td>Undecanedioic</td>
<td>9.89</td>
</tr>
<tr>
<td>12</td>
<td>Dodecanedioic</td>
<td>13.05</td>
</tr>
</tbody>
</table>

*a Relative retention times based on malonic acid.*
Figure 43. GLC Elution Patterns for A, the Standard Dicarboxylic Acids, and B, the Unknown Oxidation Product from Epirodin Mix.
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Figure 44. GLC Elution Pattern for the Mixed Sample of Standard Dicarboxylic Acids and the Unknown Oxidation Product from Epirodin Mix.
heights observed with the individual solutions. The peak height for suberic acid in the standard mixture was 42 mm while the analogous peak of the oxidation mixture was 14 mm. The sum of these two peaks was 56 mm as compared to the height of 59 mm obtained when these two solutions were mixed. In the case of azelaic acid, the individual peak heights for the standard and the unknown were 36 mm and 28 mm, respectively. The sum was 64 mm as compared to the height of 66 mm observed for the two solutions together. Also, there was no skewing of the peaks observed when the solutions were mixed. These results were taken as being conclusive evidence that potassium permanganate oxidation of epirodin mix does indeed yield significant amounts of suberic and azelaic acids, and mostly the latter.

10. Saponification of Epirodin Mix. Epirodin mix was saponified and neutralization equivalents were determined. Data are presented in Table 23. The average equivalent weight was calculated to be 330. Using a molecular weight of 953 for epirodin, these results indicate that there are 953/330 or 2.89 acid groups per molecule of saponified epirodin. The unsaponified compound was shown to possess 2.08 acid functions. This suggests that epirodin contains two free acid groups and one ester function which can be hydrolyzed to yield a total of three free acid groups.

The visible spectrum of a 20 μg/ml solution of saponified epirodin mix in 95% ethanol is illustrated in Fig. 45.
**TABLE 23.**

_Determination of the Neutralization Equivalent of Saponified Epirodin Mix._

<table>
<thead>
<tr>
<th>Sample Weight (mg)</th>
<th>Ml Blank&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ml Sample&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Neutralization Equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.40</td>
<td>0.05</td>
<td>0.47</td>
<td>330</td>
</tr>
<tr>
<td>1.24</td>
<td>0.05</td>
<td>0.41</td>
<td>344</td>
</tr>
<tr>
<td>1.30</td>
<td>0.05</td>
<td>0.45</td>
<td>325</td>
</tr>
<tr>
<td>1.20</td>
<td>0.05</td>
<td>0.42</td>
<td>321</td>
</tr>
</tbody>
</table>

<sup>a</sup> 0.01008 N NaOH

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Figure 45. Visible Absorption Spectrum of Saponified Epicrodin Mix.
It is like that of the original compound indicating that saponification did not affect the chromophore.

Thin layer chromatography was used to determine whether or not basic hydrolysis resulted in fragmentation of the epirodin molecule. A chromatogram developed in acetone: methanol:benzene (3:3:2) is presented in Fig. 46. For each compound, 10 ul of a 1.0 mg/ml solution were applied. As always, epirodin mix appeared as two spots. The saponified epirodin mix contained trace amounts of the original compounds as seen by faint spots corresponding to those of epi-

rodin. However, there were two other spots with lower $R_p$ values and colors intensities comparable to epirodin mix. Charring with sulfuric acid produced no additional spots. Assuming the two spots of the basic hydrolysis product correspond to saponified versions of the two spots of epi-

rodin mix, epirodin must contain a lactone rather than an ester. Otherwise, the molecule would have been fragmented and additional spots should have been observed. Opening of a lactone, on the other hand, would result in the same num-

ber of spots, but with different $R_p$ values due to the change in the structure of the molecule. That epirodin is indeed a lactone was confirmed by allowing the saponified compound to remain in dilute HCl for 24 hours. TLC of the solution showed the $R_p$ values of the two spots to revert back to those of the original epirodin mix. The demonstration of epirodin as a lactone intensifies its relationship to the polyene macrolide antibiotics. The two things which they
Figure 46. TLC of Epirodin Mix (1) and Saponified Epirodin Mix (2). Developed in Acetone:Methanol:Benzene (3:3:2).
all have in common are the polyene chromophore and the lactone ring, both of which are present in epirodin.

11. Sodium Borohydride Reduction of Epirodin Mix. A sample of epirodin was reduced with NaBH₄ in tetrahydrofuran (THF). As the reaction proceeded, the color of the solution changed from a bright orange-red to more of a pale orange color. Upon completion of the reaction, an aliquot of the THF layer was taken to dryness in vacuo. The residue was extracted with ether, and the extract was dried and then re-dissolved in ethanol at a concentration of 75 μg/ml. The visible absorption spectrum of this solution is presented in Fig. 47. While epirodin produced a single, broad peak, its NaBH₄ reduced counterpart gave a series of much finer peaks. In addition, the absorption maximum was shifted to a shorter wavelength.

Under the experimental conditions used, borohydride is capable of reducing ester functions (46). The absorption maximum in the UV-visible spectrum of epirodin had indicated a chromophore of an ester (or lactone) group in conjugation with seven double bonds. The lack of fine structure had been attributed to a carbonyl in conjugation with the polyene system. According to Oroshnik (23), such a system always "degrades" the spectral fine structure, which in polyene ketones or short-chain acids is completely obliterated. In polyene acids or esters containing five or more conjugated double bonds, on the other hand, the degradation is less
Figure 47. UV-Visible Absorption Spectrum of NaBH₄-Reduced Epirodin Mix in Ethanol.
drastic. Upon reduction of the conjugated carbonyl group, the chromophore of epirodin presented itself as a series of 8-9 sharp peaks instead of the single broad peak with shoulders on either side.

Hamilton-Miller (28) also discusses the effect of conjugated carbonyl groups on the polyene spectrum, specifically as is found in the pentaene flavofungin and the hexaene dermostatin, both of which are polyene macrolide antibiotics. He reports that in these compounds, the usual type of polyene spectrum is absent. Instead, single peaks are observed, and they are at longer wavelengths than would be expected. This phenomenon he attributes to the chromophore:

\[ -\overset{\circ}{\gamma}(CH=CH)_{5} \text{ or } 6 \]

which virtually makes the pentaene into a hexaene and the hexaene into a heptaene. The spectra of two heptaenes, candidin and ascoconsin, in ethanol are presented in Fig. 22 (23). Absorption maxima occur at wavelengths virtually identical to those of reduced epirodin. For example, while the longest wavelength for candidin is 407 nm, that of reduced epirodin is 407 nm. As one progresses to shorter wavelengths, one sees peaks at 383 and 364 nm for candidin, and at 384 and 364 nm for reduced epirodin. These data indicate quite conclusively that the chromophore of epirodin does indeed consist of a lactone in conjugation with a seven double bond system.
12. **Structure of Epirodin.** From the results of the various spectroscopic and chemical methods used in the analysis of epirodin, a working structure for the epirodin molecule can be proposed. By mass spectrometry the molecular weight of the epirodin molecule was estimated at 953. The elemental analysis showed the molecule to be composed of 58.24% carbon, 7.80% hydrogen, and no nitrogen. Using sodium fusion and Beilstein's test, Buckley (18) showed no halogens or sulfur atoms to be present. Thus epirodin is composed only of carbon, hydrogen, and oxygen.

Spectroscopic studies were able to provide much information concerning the structure of epirodin. UV-visible absorption spectra were transparent in the regions where aromatic units absorb thereby indicating no aromatic moiety to be present. The broad shouldered peak at 429 nm was indicative of a \(-\text{O-}\overset{\circ}{\text{C}}-(\text{C}=\text{C})_{-}\) chromophore. Infrared spectra reinforced the absence of aromatic groups. They showed epirodin to contain hydroxyl, methyl, and methylene groups. In addition, a strong carbonyl band appeared. The wave number at which this group absorbed indicated it to be an ester in conjugation with a double bond. This information along with bands corresponding to \(-(\text{C}=\text{C})_{n}\) supported the structure of the UV-visible chromophore. The IR spectrum also indicated the absence of cis double bonds, and the iodine isomerization reactions indicated that the double bonds were trans.

Characterization tests on epirodin showed it to contain
no aldehyde or ketone functions. A positive hydroxylamine hydrochloride reaction indicated either an ester or a lactone grouping. Failure to cleave the epirodin molecule into two fragments following saponification eliminated the possibility of an ester. Epirodin, therefore, must contain a lactone function.

Hydroxyl groups were indicated by the infrared spectrum. Epirodin was shown to be sensitive to periodate. In quantitating this sensitivity, it was possible to demonstrate the presence of 2,48 -CH(OH)-CH(OH) - groupings. When the response of epirodin to periodate was checked following the saponification of epirodin, 3,62 groupings were indicated. This implies that there is a masked glycol involved in the lactone, -O-CH\textsubscript{2}-CH(OH) -. By derivatizing one of the fragments produced by periodate cleavage, its structure was determined to be:

\[
\text{H}_2\text{H} \quad \text{H} \quad \text{H} \\
\text{R-C-C-}(\text{CH}_2=\text{C})_4=\text{C-R'} \\
\text{OHOH} \quad \text{OH} \quad \text{OH}
\]

Determination of the neutralization equivalent showed epirodin to contain two free acid groups. The absence of sulfur along with the solubility in bicarbonate made it necessary that these be carboxylic acid groups. The isolation of the C\textsubscript{8} and C\textsubscript{9} dicarboxylic acids following permanganate oxidation indicated that one of the free acid functions must be at the end of a hydrocarbon chain. The other end of this side chain must be bonded to a carbon involved in a double bond. The second free acid group is probably bonded to a carbon which is adjacent to a hydroxyl-bonded carbon.
-CH-CH-, This would account for the lack of integers in 
OH COOH 
determining periodate sensitivity. Such a grouping is 
cleaved but not quantitatively, and would explain the 2.48 
and 3.62 which were obtained.

Combining these data, the author proposes the struc-
ture of epirodin to be similar to that shown in Fig. 48. The 
position of the masked glycol and the chromophore are cer-
tain. The carboxylic acid side chain is placed at the end 
of the chromophore; however, its position there is not def-
inite. All that is known is that it must be located such 
that it is extending out from the ring, and it must not in-
terfere with the all-trans configuration of epirodin. Also, 
based on the mechanism of inhibition by epirodin which will 
be discussed later, the conjugated system should be free and 
not masked by a long side chain in the middle of it. The 
second free acid group has been placed adjacent to the hy-
droxyl of the masked glycol because in all of the polyene 
antibiotics where there is such a group, it is at this lo-
cation. The hydroxylated chain is also placed in a position 
analagous to the other polyene compounds. Methyl groups are 
present as indicated by IR, but the number of them is not 
known. Nor is it possible to determine where they should 
be based on other compounds, because they do not seem to 
occur in any specific place. In any case, such a structure 
for the epirodin molecule would account for the data ob-
tained in this work.

The molecular weight has been estimated at 953 with an
Figure 48. Proposed Working Structure for the Epirodin Molecule.
empirical formula of C_{46}H_{74}O_{21}. The proposed structure accounts
for 816 and has the formula C_{43}H_{66}O_{14}. There remain 3 car-
bon atoms, 8 hydrogen atoms, and 7 oxygen atoms which should
be incorporated into the structure. Methyl groups are com-
monly found on the carbon skeletons of the polyene macro-
clide antibiotics, and their presence was suggested by the IR
spectrum. However, the number of groups and their location
was not determined. The discrepancy in the number of oxy-
gen atoms may reflect the hydroscopic nature of epirodin.

There is one anomaly, however, which cannot be over-
looked if epirodin is to be included in the polyene group
of antibiotics. That is the fact that all of the polyenes
encountered thus far have been isolated from members of the
Streptomycetaceae, namely, Streptomyces, Streptoverticillium,
and Chaine. The majority come from Streptomyces. Epirodin,
on the other hand, was isolated from Epicoccum nigrum, a
member of the Fungi Imperfecti. Thus the question might
arise as to whether or not such organisms with completely
different cell systems can produce the same type of com-pounds.
However, in discussing the biosynthesis of the polyenes,
Hamilton-Miller (28) states that "the structures of all the
polyenes so far elucidated are generally consistent with a
biosynthetic pathway involving condensation of acetate and
propionate units (the polyketide pathway)". As discussed by
Turner (70), the polyketide route is used mainly by fungi,
and to a lesser extent by bacteria and higher plants. In
any case, it leads almost exclusively to secondary metabo-
lites. He goes on to say "polyketides are the characteristic secondary metabolites of the Fungi Imperfecti." Therefore, since the polyenes are products of the polyketide pathway, and this pathway is characteristic of the class of fungi to which Epicoccum nigrum belongs, there is no reason why the same type of polyene compound could not be produced as a secondary metabolite by a fungus. Thus the proposed structure for epirodin is consistent with the biosynthetic capabilities of E. nigrum.

In studying the pigments of E. nigrum, Poppen (1) obtained data suggesting that some of the red pigments produced by the fungus were humic acids. However, investigation into the pigments of the UNH strain indicates that this is not the case. The humic acids have been shown to contain both phenols and humic acids. They are pigmented only to a slight degree and are a pale brown color. The intense orange-red of culture broth in which E. nigrum is growing is shown to be due to epirodin instead. This compound bears no resemblance whatsoever to the humic acids. While the humic acids have large extinction coefficients in the aromatic regions of the UV absorption spectrum, epirodin is transparent. Humic acid contains nitrogen, epirodin does not. Humic acid has no biological activity; epirodin has significant activity. Thus the results presented in this thesis show that the color of epirodin bears no relation to the presence of humic acids.
H. Nature of the Biological Activity of Epirodin Mix.

Generally speaking, the polyene macrolide antibiotics are inactive against *Schizomyces*, and active to various degrees against yeasts and dimorphic fungi, dermatophytes, and molds. Certain polyenes, however, like resistaphylin and dermostatin, do show significant activity against bacteria. Epirodin mix has been shown to inhibit the growth of *B. subtilis*, and to a lesser degree, *C. pyrenoidosa*. It has also been demonstrated to be lethal to *A. salina*. The following studies were performed in order to gain insight into the mechanism by which epirodin exerts its toxicity.

1. Antibacterial Activity of Epirodin. Cultures of *B. subtilis* containing various concentrations of epirodin were incubated for four hours. Tubes containing epirodin at 0.25 mg/ml, 0.50 mg/ml, and 1.00 mg/ml displayed no growth, indicating bacteriostatic levels of the compound. At concentrations of 0.125 mg/ml and below, epirodin did not inhibit bacterial growth. The culture media of the tubes containing 0.25, 0.50, and 1.00 mg/ml were then streaked onto solid media. Colonies appeared in less than 24 hours for all three concentrations. These results indicated that even at 1.00 mg/ml, epirodin is not bacteriocidal. Vazquez (71) reports that most antibacterial macrolide antibiotics (non-polyene), including spiramycin and carbomycin, are bacteriostatic. However, at concentrations 4-10 times the minimum
growth inhibitory concentration, these compounds are bacteriocidal. Epirodin could not be tested at greater concentrations because of difficulties encountered regarding its solubility.

2. Interaction of Epirodin with Sterols. In 1958, Gottlieb et al. (72) reported that addition of several sterols (e.g., cholesterol, ergosterol, stigmasterol, sitosterol) to culture medium could antagonize the growth inhibition by polyene antibiotics. Lampen et al. (73), while investigating the mechanism by which sterols nullified growth inhibition, obtained spectrophotometric evidence for complex formation between the antibiotics and the sterol. They found that incubation of the polyene with sterols in aqueous media had a pronounced effect on the spectra of the antibiotics which was distinguished mainly by a decrease in the extinction coefficient with no spectral shift. The effect of sterols on the spectrum of epirodin mix in aqueous medium was examined to see if epirodin might exert its toxicity by a mechanism similar to that of the polyene macrolide antibiotics.

Solutions of epirodin and sterol were prepared in varying proportions. Visible spectra were obtained, and it was seen that sterols did indeed cause a change to occur. Ergosterol caused the most dramatic effect while the effects of sitosterol and cholesterol were least pronounced. Stigmasterol had an intermediate effect. The absorbance readings of the epirodin solutions are presented in Table 24 and
### TABLE 24.

**Absorbance Readings of a 10 μg/ml Solution of Epirodin Mix in Solutions Containing Varying Concentrations of Sterol.**

<table>
<thead>
<tr>
<th>Sterol</th>
<th>10 μg/ml</th>
<th>20 μg/ml</th>
<th>30 μg/ml</th>
<th>40 μg/ml</th>
<th>50 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>0.405</td>
<td>0.400</td>
<td>0.360</td>
<td>0.340</td>
<td>0.330</td>
</tr>
<tr>
<td>Ergosterol</td>
<td>0.395</td>
<td>0.355</td>
<td>0.320</td>
<td>0.267</td>
<td>0.215</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>0.410</td>
<td>0.395</td>
<td>0.375</td>
<td>-</td>
<td>0.335</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>0.400</td>
<td>0.380</td>
<td>0.340</td>
<td>-</td>
<td>0.280</td>
</tr>
</tbody>
</table>

*aSolutions in 1 mM Tris-HCl - 10 mM NaCl, pH 7.4.*
graphed in Fig. 49. As can be seen, the absorbance decreased linearly with increasing amounts of sterol. The spectra of epirodirin with differing amounts of ergosterol are presented in Fig. 50 as a representative sample of the effect of sterols. As with the polyene antibiotics, no spectral shift was observed, only a decrease in the absorbancy. The absorbance of a control solution containing an equal amount of epirodirin but no sterol was 0.435. Thus at a sterol:epirodirin ratio of 5:1, cholesterol was able to reduce the absorbance of epirodirin to 76% of the original value, while ergosterol reduced it to 49.5%, sitosterol to 77%, and stigmasterol to 64.5%.

Using fluorescence techniques, it was shown that sterols are also capable of quenching the fluorescence of epirodirin. The fluorescence intensities of epirodirin with various amounts of cholesterol are shown in Table 25. Unlike the effect on absorbance, the decrease in fluorescence was not linearly proportional to the increasing amount of cholesterol. Instead, there was a rapid decrease with the initial addition of cholesterol to the solution. Then as more cholesterol was added, the effect became less marked.

Schroeder (74) has reported that, regarding the decrease in absorption and fluorescence of polyenes by cholesterol, no such phenomenon is observed if ethanol or methanol is used as the solvent. Such was the case with epirodirin. If methanol or ethanol were present at 50%, no interaction with sterols could be demonstrated. Thus it appears possible that the antibiotic mechanism of epirodirin might be the same as the poly-
Figure 49. Effect of Increasing Amounts of Sterol on the Absorbance of Epirodin Mix at 429 nm. A, Sitosterol; B, Cholesterol; C, Stigmasterol; D, Ergosterol.
Figure 50. Effect of Increasing Amounts of Sterol on the Visible Absorption Spectrum of Epirodin Mix.
1, 10 μg/ml Epirodin; 2, 10 μg/ml Epirodin and 20 μg/ml Ergosterol; 3, 10 μg/ml Epirodin and 50 μg/ml Ergosterol.
TABLE 25.

*Fluorescence of a 10 μg/ml Solution of Epirodin with Increasing Amounts of Cholesterol.*

<table>
<thead>
<tr>
<th>Concentration of Cholesterol (μg/ml)</th>
<th>Fluorescence Intensity (uamps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0445</td>
</tr>
<tr>
<td>5</td>
<td>0.0307</td>
</tr>
<tr>
<td>10</td>
<td>0.0277</td>
</tr>
<tr>
<td>20</td>
<td>0.0175</td>
</tr>
<tr>
<td>30</td>
<td>0.0122</td>
</tr>
<tr>
<td>40</td>
<td>0.0085</td>
</tr>
<tr>
<td>50</td>
<td>0.0065</td>
</tr>
</tbody>
</table>

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2. Inhibition of Yeasts by Epirodin Mix. The ability of epirodin to inhibit the growth of *Saccharomyces cerevisiae* was studied. Changes in the absorbance of the culture medium were measured and are presented in Table 26. Upon examination of the data, it is apparent that epirodin mix did indeed inhibit the growth of yeast. While at levels of 1.25 or 2.50 µg/ml the compound was virtually inactive, some inhibition of growth was seen at 2.5, 5.0, and 10.0 µg/ml. At concentrations of 15.0 or 25.0 µg/ml, the inhibition was more severe, cutting the rate of growth by almost 50%. The results are presented graphically in Fig. 51. It is interesting to note, however, that at none of the concentrations of epirodin tested was it able to completely inhibit the growth of the organism. Higher levels could not be tested because of difficulties in solubility.

Stability studies on epirodin had shown it to lose activity in aqueous media. Therefore, it was conceivable that, as the assay proceeded, the effective concentration of epirodin was decreasing. Therefore a duplicate set of flasks containing epirodin which had been added 16 hours (the length of an assay) prior to inoculation was run. The rates of growth at the various levels of epirodin are presented in Table 27. At the lower concentrations of epirodin, it was impossible to see any change. However, as seen in Fig. 52, at higher levels there seemed to be a slight increase in the rate of growth for those cultures where epirodin had been added 16 hours before the
### TABLE 26.

**Effect of Epirodin on the Growth of S. cerevisiae as Measured by Absorbance at 530 nm.**

<table>
<thead>
<tr>
<th>Time (Hrs.)</th>
<th>Concentration of Epirodin (ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>.000</td>
</tr>
<tr>
<td>4</td>
<td>.007</td>
</tr>
<tr>
<td>6</td>
<td>.035</td>
</tr>
<tr>
<td>8</td>
<td>.107</td>
</tr>
<tr>
<td>10</td>
<td>.265</td>
</tr>
<tr>
<td>12</td>
<td>.465</td>
</tr>
<tr>
<td>14</td>
<td>.755</td>
</tr>
<tr>
<td>16</td>
<td>.935</td>
</tr>
</tbody>
</table>

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Figure 51. Effect of Epirodin Mix on the Growth of *S. cerevisiae*. A, Control; B, 5.0 ug/ml; C, 15.0 ug/ml; D, 25.0 ug/ml.
TABLE 27.

Effect of Epirodin Added Sixteen Hours Prior to Inoculation of S. cerevisiae as Measured by Absorbance at 530 nm.

<table>
<thead>
<tr>
<th>Time (Hrs.)</th>
<th>Concentration of Epirodin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>.000</td>
</tr>
<tr>
<td>4</td>
<td>.011</td>
</tr>
<tr>
<td>6</td>
<td>.045</td>
</tr>
<tr>
<td>8</td>
<td>.140</td>
</tr>
<tr>
<td>12</td>
<td>.460</td>
</tr>
<tr>
<td>14</td>
<td>.730</td>
</tr>
<tr>
<td>16</td>
<td>.900</td>
</tr>
</tbody>
</table>
Figure 52. Determination of Stability of Epirodin Mix for the Duration of the *S. cerevisiae* assay. A, Control; B, 15.0 ug/ml Epirodin; C, 15.0 ug/ml Epirodin Added Sixteen Hours Before the Start of the Assay.
yeast inoculum. In his study of inhibition of *S. cerevisiae* by filipin, Gottlieb (29) was able to show a complete inhibition, and there was no change in absorbance for the duration of the assay. Results here indicate that such was not the case for epirodin at the concentrations tested. The compound retained the greatest part of its activity for the duration of the assay. Therefore, the growth of the organism cannot be attributed to destruction of epirodin, which would permit growth to occur.

The ability of sterol to reverse the inhibition of the growth of *S. cerevisiae* by epirodin was also investigated. Ergosterol was chosen because it is the primary sterol component of yeasts and other fungi. Fifty ug/ml concentrations were made in each flask, and the assay was conducted in the same manner as before. The changes in absorbance reflecting the increased growth of the organism are presented in Table 28. Results indicate that ergosterol did indeed reverse the inhibition by epirodin. This becomes more obvious when the data is shown graphically (Fig. 53). Curve B represents the decreased growth rate caused by epirodin as compared to the control curve, A. With addition of ergosterol to the medium, the rate of growth increased (Curve C) and became similar to that of the control culture containing no epirodin. Thus one sees Curves A and C as being identical for the first 10 hours, and then separating slightly in the later phases of the assay.

Gottlieb *et al.* (29) reported similar findings in their
TABLE 28.

Effect of Ergosterol on the Ability of Epirodin to
Inhibit the Growth of S. cerevisiae as Measured
by Absorbance at 530 nm.

<table>
<thead>
<tr>
<th>Time (Hrs.)</th>
<th>Concentration of Epirodin (ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>.000</td>
</tr>
<tr>
<td>4</td>
<td>.010</td>
</tr>
<tr>
<td>6</td>
<td>.045</td>
</tr>
<tr>
<td>8</td>
<td>.095</td>
</tr>
<tr>
<td>10</td>
<td>.205</td>
</tr>
<tr>
<td>12</td>
<td>.405</td>
</tr>
<tr>
<td>14</td>
<td>.690</td>
</tr>
<tr>
<td>16</td>
<td>.895</td>
</tr>
</tbody>
</table>
Figure 53. Ability of Ergosterol to Reverse the Inhibition of Growth of *S. cerevisiae* by Epirodin. A, Control; B, Epirodin at 15.0 ug/ml; C, Epirodin at 15.0 ug/ml and Ergosterol at 50 ug/ml.
study of the antagonism of cholesterol to filipin. Like ergosterol, cholesterol had no effect on the growth of the organism in the absence of the antibiotic. By increasing the concentration of sterol parallel to the increasing concentrations of filipin, these workers were able to show that the antagonism between the two compounds was not due to a simple competitive inhibition. This they based on the fact that the same ratios of filipin to cholesterol at different concentrations of the antibiotic did not allow equal growth of S. cerevisiae.

4. Antifungal Activity of Epirodin. As previously discussed, all of the polyene macrolide antibiotics encountered in nature thus far have been isolated from the Streptomycetaceae, soil-dwelling bacilli. All have been demonstrated to be significantly active against fungi, and are commonly referred to as "polyene antifungal antibiotics". In view of the fact that epirodin is a polyene compound, the question arises as to whether or not it, too, is toxic to fungi. It does not appear likely that E. nigrum would be producing a substance toxic to itself. To examine this possibility, the activity of epirodin against various fungi was investigated. E. nigrum was tested along with Fusarium oxysporum, another member of the Fungi Imperfecti. Two other organisms were also selected, each belonging to a different class. These were Rhizopus nigricans, a member of the Zygomycetes, and Aspergillus flavus which belongs to the Plectomycetes.
Agar plates with epirodin incorporated at various levels into the medium were prepared. The various fungi were streaked onto the plates which were then watched for signs of growth. Two days after being inoculated, *E. nigrum* was growing on each plate. The plates with 0.5 and 10 ug/ml appeared to be growing at identical rates; those with 20 ug/ml had a lesser amount of mycelia on the surface. By six days, all of the plates appeared to have reached the same level of growth. *F. oxysporum* handled the epirodin like the *E. nigrum*. After two days incubation, the organism was growing regardless of the level of epirodin. With 20 ug/ml, however, the rate seemed to be slightly slower. By six days, all of the plates had developed growth.

Unlike *E. nigrum*, *A. flavus* and *R. nigricans* were affected by the epirodin, and they each reacted in the same way. After two days, the control plates showed significant growth while those at 5 ug/ml epirodin were just beginning. Plates with 10 or 20 ug/ml were void of growth. However, by the sixth day, the control and 5 ug/ml plates were completely covered with mycelia, and the more concentrated plates were just beginning to develop.

Thus *F. oxysporum* and *E. nigrum*, the two members of the Deuteromycetes, were insensitive to the polyene. *A. flavus* and *R. nigricans*, on the other hand, were inhibited to a certain extent. The fact that they did grow after a period of time might be attributed in part to the decrease in effective concentration of epirodin with time. So it appears then that
all the polyenes are not necessarily as antifungal as is presently thought. It is a known fact that those which have been isolated from *Streptomyces* are potent antifungal agents, but the same cannot be said for epirodin. In one of his papers, Gottlieb et al. (29) examines the antifungal spectrum of filipin. He shows it to be potent against all classes of fungi, including *F. oxysporum* whose growth epirodin did not affect at 20 μg/ml. The inactivity of epirodin towards organisms like *E. nigrum* seems quite rational since an organism that produced a substance toxic to itself would be extinct shortly. Instead, it appears that the activity of epirodin is directed at organisms unlike itself, perhaps as a means of survival in a competitive environment.

5. Effect of Epirodin Mix on Drosophila. Gemrich (33) has demonstrated filipin to be mildly effective against *Musca domestica*, the common house fly. By binding sterols required in the diet, filipin demonstrated inhibition of growth and larvicidal activity. However, it was ineffective as a chemosterilant in the diet at 100 ppm. The ability of epirodin to interfere with growth and reproduction was studied using a similar assay but with the common fruit fly, *Drosophila melanogaster*, instead of the house fly.

Using the assay developed here, 15 pairs of adults on control media containing no epirodin produced 26, 23, 19, 38, 31, 29, 26, 41, 24, 33, 31, 33, 28, 40, and 24 offspring which survived to adulthood. It was calculated that, on the average, each pair can be expected to produce 29.73 adult flies. The
standard deviation for these controls is 6.2. In five replicate tubes, each containing epirodin at 1000 ppm, 27, 24, 31, 21, and 22 adult flies were produced per tube. The average was 25 which lies within one standard deviation of the control mean. Thus epirodin appears to be inactive against D. melanogaster at the concentrations tested.

In his study concerning M. domestica, Gemrich (33) found that of all the polyene antibiotics tested, only filipin had any activity. However, the degree of efficiency in controlling the fly population was well below that of available pesticides such as dieldrin and parathion. Thus epirodin, like the other polyene antibiotics, has very limited potential as an insecticide.

6. Epirodin and Hemolysis. Kinsky et al. (24,42,49,50) have studied in detail the hemolytic action of filipin and other polyene antibiotics. They have shown that the extent of lysis is dependent on the antibiotic/cell concentration ratio as well as on the absolute concentration of the antibiotic, and that hemolysis is inhibited by serum. In addition, they have shown that the hemolytic activity is associated with the chromophore since perhydrofilipin had approximately only 1/100 the hemolytic activity of the parent compound. Zondag, Posthuma, and Berends (41) have reported that illumination of the polyene antibiotic pimaricin with visible light in the presence of flavins caused the conjugated chromophore to be destroyed. Kinsky et al. (42) thus reported that treatment of filipin in this manner completely abolished

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its hemolytic action, thereby confirming that the polyene chromophore is responsible for biological activity. Epirodin mix was therefore subjected to a similar set of experiments as filipin to determine whether or not it behaved in an analogous manner.

A red blood cell suspension and a series of epirodin solutions were prepared. When increasing amounts of epirodin were added to a constant volume of erythrocytes, hemolysis was found to occur to greater extents as the concentration of epirodin increased. Thus the more cells that were lysed, the larger the amount of hemoglobin released and the greater the absorbance at 550 nm. The absorbance readings for the series of epirodin solutions are shown in Table 29 and illustrated in Fig. 54. As in the case of the polyene antibiotics tested by Kinsky (49), among which were filipin, amphotericin B, etruscomycin, candidin, and asconsin, hemolysis did not occur until a threshold concentration ratio of antibiotic per cell was reached. Like epirodin, in each case there was a sharp increase in the extent of lysis over a relatively narrow concentration range. The absorbance at 550 nm for complete hemolysis of the erythrocytes in water was 0.510. Using this figure, the percent hemolysis at each level of epirodin was calculated. At 10 μg/ml epirodin was able to lyse 8.6% of the cells. However, an equal volume of the dimethylformamide solvent resulted in 8.2% hemolysis. Thus no significant lysis occurred until 30 μg/ml, and it was virtually complete at 80 μg/ml.
### TABLE 29.

**Extent of Hemolytic Action of Epirodin Mix**

*by Absorbance Readings at 550 nm.*

<table>
<thead>
<tr>
<th>Concentration of Epirodin (ug/ml)</th>
<th>Absorbance</th>
<th>% Hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>.040</td>
<td>8.2</td>
</tr>
<tr>
<td>10</td>
<td>.044</td>
<td>8.6</td>
</tr>
<tr>
<td>20</td>
<td>.060</td>
<td>11.7</td>
</tr>
<tr>
<td>30</td>
<td>.175</td>
<td>34.4</td>
</tr>
<tr>
<td>40</td>
<td>.329</td>
<td>64.6</td>
</tr>
<tr>
<td>50</td>
<td>.380</td>
<td>74.5</td>
</tr>
<tr>
<td>60</td>
<td>.462</td>
<td>90.5</td>
</tr>
<tr>
<td>70</td>
<td>.480</td>
<td>94.2</td>
</tr>
<tr>
<td>80</td>
<td>.503</td>
<td>98.8</td>
</tr>
<tr>
<td>90</td>
<td>.511</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>.508</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure 54. Effect of Epirodin Mix on the Hemolysis of Human Erythrocytes.
The effect of a constant amount of epirodin on increasing amounts of red cells was examined to see if the antibiotic/cell ratio played any role in the extent of hemolysis observed. The absorbance readings at 550 nm with increasing volumes of red cell suspension are shown in Table 30. The amount of hemoglobin released was indeed found to be dependent on the number of erythrocytes present. As seen in Fig. 55, the amount of hemoglobin released initially increased as more erythrocytes were added; next, a plateau region occurred where adding more cells did not induce a greater extent of lysis. As the volume of red cells was increased even more, the amount of hemoglobin released decreased. By using the absorbance readings of 0.510 for complete hemolysis of 0.50 ml erythrocyte suspension in 4.5 ml water, the percent hemolysis occurring could be calculated. Thus for 0.25 ml erythrocytes one would expect a maximum of only 0.510/2 or 0.255. The observed absorbance was 0.250 indicating that hemolysis was complete. However, when 3.0 ml cell suspension was used, the maximum reading should have been 6 x 0.510 or 3.60. The observed reading was only 0.347 corresponding to 11% hemolysis. As seen in Fig. 56, the percent hemolysis steadily decreased as the erythrocyte concentration increased. Therefore, it can be concluded that for epirodin, like filipin, the antibiotic/cell ratio and not just the absolute concentration of epirodin is also a critical factor.

The above experiments were all done on erythrocytes washed extensively with isotonic saline. Kinsky (49) reported
TABLE 30. 

**Effect of Erythrocyte Concentration on Hemolysis by Epirodin.**

<table>
<thead>
<tr>
<th>Concentration of Cells (Ml added)</th>
<th>Absorbance (550 nm)</th>
<th>% Hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.250</td>
<td>94</td>
</tr>
<tr>
<td>0.50</td>
<td>0.494</td>
<td>97</td>
</tr>
<tr>
<td>0.75</td>
<td>0.564</td>
<td>74</td>
</tr>
<tr>
<td>0.80</td>
<td>0.568</td>
<td>70</td>
</tr>
<tr>
<td>0.90</td>
<td>0.550</td>
<td>60</td>
</tr>
<tr>
<td>1.00</td>
<td>0.542</td>
<td>53</td>
</tr>
<tr>
<td>1.10</td>
<td>0.488</td>
<td>44</td>
</tr>
<tr>
<td>1.25</td>
<td>0.483</td>
<td>38</td>
</tr>
<tr>
<td>1.50</td>
<td>0.474</td>
<td>31</td>
</tr>
<tr>
<td>1.75</td>
<td>0.424</td>
<td>24</td>
</tr>
<tr>
<td>2.00</td>
<td>0.390</td>
<td>15</td>
</tr>
<tr>
<td>3.00</td>
<td>0.347</td>
<td>11</td>
</tr>
</tbody>
</table>

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Figure 55. Effect of Human Erythrocyte Concentration on Hemolysis by Epirodin Mix at 80 µg/ml.
ABSORBANCE AT 550 NM

CONC. RBC'S (ML)

1.0

2.0

3.0

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Figure 56. Percentage of Erythrocytes Hemolyzed with an Increasing Cell/Epirodin Ratio.
that hemolysis by filipin rarely occurred if whole blood were used, and that serum inhibited lysis of red cells at very low concentrations. A similar phenomenon was observed regarding hemolysis by epirodin. Serum ranging from .001% to 5% of the total volume of the assay was tested for its ability to inhibit hemolysis. Results are presented in Table 31 and shown graphically in Fig. 57. At levels as low as 0.25%, serum was able to completely inhibit hemolysis. The percentage of cells lysed was 9.0% as compared to 8.2% for the control containing no epirodin. Only when the serum level was dropped to 0.01% of the volume was the lysis of cells complete.

Cholesterol and other sterols have been shown to interact with epirodin. Both UV-visible and fluorescence intensities of epirodin were decreased by addition of sterol, and ergosterol was capable of reversing the inhibition of yeast growth by epirodin. Therefore, it was necessary to determine whether or not it was the serum cholesterol which was causing the decreased hemolysis by epirodin. Thus an assay containing epirodin from 10-100 ug/ml and cholesterol at a constant value of 50 ug/ml was run. While reversal of hemolysis was by no means complete, fewer cells were hemolyzed as can be seen in Table 32 and Fig. 58. Thus, while epirodin at 80 ug/ml caused 99% hemolysis, with cholesterol at 50 ug/ml, only .225/.510 or 44% hemolysis was observed.

The concentration of cholesterol in the serum used to inhibit hemolysis by epirodin was 2.05 mg/ml as determined by the method of R.J. Henry (51). At 0.01% (v/v), this serum

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**TABLE 31.**

Effect of Serum on Hemolysis by
Epiprodin at 80 ug/ml.

<table>
<thead>
<tr>
<th>Concentration of Serum</th>
<th>Absorbance at 550 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>0.053</td>
</tr>
<tr>
<td>4.0</td>
<td>0.057</td>
</tr>
<tr>
<td>3.0</td>
<td>0.052</td>
</tr>
<tr>
<td>2.0</td>
<td>0.050</td>
</tr>
<tr>
<td>1.0</td>
<td>0.064</td>
</tr>
<tr>
<td>0.75</td>
<td>0.056</td>
</tr>
<tr>
<td>0.50</td>
<td>0.047</td>
</tr>
<tr>
<td>0.40</td>
<td>0.049</td>
</tr>
<tr>
<td>0.25</td>
<td>0.049</td>
</tr>
<tr>
<td>0.10</td>
<td>0.137</td>
</tr>
<tr>
<td>0.05</td>
<td>0.480</td>
</tr>
<tr>
<td>0.01</td>
<td>0.505</td>
</tr>
<tr>
<td>0.005</td>
<td>0.512</td>
</tr>
<tr>
<td>0.001</td>
<td>0.508</td>
</tr>
</tbody>
</table>

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Figure 57. Effect of Serum on Hemolysis by Epirodin Mix at 80 ug/ml.
Absorbance at 550 nm vs. % Serum (V/V)
<table>
<thead>
<tr>
<th>Concentration of Epirodin (ug/ml)</th>
<th>Absorbance (no cholesterol)</th>
<th>Absorbance (with cholesterol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>.040</td>
<td>.062</td>
</tr>
<tr>
<td>10</td>
<td>.044</td>
<td>.063</td>
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<tr>
<td>20</td>
<td>.060</td>
<td>.064</td>
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<tr>
<td>30</td>
<td>.175</td>
<td>.062</td>
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<tr>
<td>40</td>
<td>.329</td>
<td>.088</td>
</tr>
<tr>
<td>50</td>
<td>.380</td>
<td>.102</td>
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<tr>
<td>60</td>
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<td>.130</td>
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<tr>
<td>70</td>
<td>.480</td>
<td>.212</td>
</tr>
<tr>
<td>80</td>
<td>.503</td>
<td>.225</td>
</tr>
<tr>
<td>90</td>
<td>.511</td>
<td>.210</td>
</tr>
<tr>
<td>100</td>
<td>.508</td>
<td>.230</td>
</tr>
</tbody>
</table>
Figure 58. Ability of Cholesterol to Inhibit Hemolysis by Epirocin. A, Series of Epirocin Standards; B, Series of Epirocin Standards with Each Tube Containing 50 ug/ml Cholesterol.
was able to inhibit hemolysis by 99%. At 0.01%, 0.0005 ml of serum was used in the 5 ml assay. A cholesterol level of 2.05 mg/ml in the serum would give a concentration of 1.05 ug/ml cholesterol in the serum assay. At 50 ug/ml, cholesterol reduced hemolysis by epirocin at 80 ug/ml to 44% instead of the 99% hemolysis observed with no cholesterol. Thus it is apparent that some additional factor in serum is responsible for the reversal of hemolysis.

Other serum components were assayed for their ability to inhibit hemolysis by epirocin at their normal physiological levels. Urea, phosphatidyl serine, phosphatidyl inositol, glucose, and albumin were found to have no effect whatsoever on the lysis of erythrocytes. Absorbance readings at 550 nm were the same as for the 80 ug/ml standard. Fatty acids, on the other hand, seemed to increase the rate of lysis. At 80 ug/ml epirocin, fatty acid (0.24 meq/liter) decreased the time for complete lysis from 120 minutes to 60 minutes. With &b-globulin at 1.0 mg/ml, hemolysis decreased to 0.302/0.510 or 59% when epirocin was present at 80 ug/ml. Thus, of the serum components tested, only cholesterol and &b-globulin were able to reverse hemolysis to any extent.

Kinsky (49) has shown that hemolysis by nystatin and amphotericin B to be greatly reduced in the presence of an osmotic stabilizer such as sucrose. Conducting the epirocin assay in 0.25 M sucrose did not produce a similar result. As is seen in Table 33 and Fig. 59, the hemolysis was only reduced slightly. For nystatin and amphotericin B, Kinsky (49)
### TABLE 33.

**Effect of an Osmotic Stabilizer, Sucrose*, on Hemolysis by Epirodin Mix.**

<table>
<thead>
<tr>
<th>Concentration of Epirodin (ug/ml)</th>
<th>Absorbance at 550 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.032</td>
</tr>
<tr>
<td>20</td>
<td>0.030</td>
</tr>
<tr>
<td>30</td>
<td>0.039</td>
</tr>
<tr>
<td>40</td>
<td>0.214</td>
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<tr>
<td>50</td>
<td>0.318</td>
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<tr>
<td>60</td>
<td>0.393</td>
</tr>
<tr>
<td>70</td>
<td>0.453</td>
</tr>
<tr>
<td>80</td>
<td>0.490</td>
</tr>
<tr>
<td>90</td>
<td>0.508</td>
</tr>
<tr>
<td>100</td>
<td>0.513</td>
</tr>
</tbody>
</table>

* 0.25 M sucrose.
Figure 59. Effect of Sucrose on Hemolysis by Epirodin Mix.
A, in Isotonic Saline; B, in 0.25 M Sucrose.
found sucrose reduced the curve to a horizontal line.

The kinetics of the hemolyzing process caused by epiro-
din were briefly studied. Concentrations of epiro-
din used were 10, 40, 50, and 60 ug/ml. At 10 ug/ml, epiro-
din caused no hemolysis to occur during the two hour assay; at 40 and 50
ug/ml, increasing numbers of cells were ruptured; and at 60
ug/ml, the lysis was 90% complete. For each level of epi-
ro-din, hemolysis was measured at specific time intervals. Re-
sults are presented in Table 34 and Fig. 60. As the concen-
tration of epiro-din increased, so did the rate of lysis. Since
the amount of erythrocytes remained constant, the results sug-
gest that hemolysis by epiro-din is a first order reaction, de-
pending only on the antibiotic/cell ratio.

2. Mode of Action for Epiro-din. That the chromophore
of epiro-din might be its center of biological activity was
initially suspected during stability studies on the compound.
It was noted that the decrease in UV-visible absorption cor-
related with the decrease in activity. Later, it was dis-
covered that the chromophore consisted of a polyene chain in
conjugation with a lactone carbonyl. Chemical modifications,
such as hydrogenation and bromination, that resulted in des-
truction of the epiro-din chromophore, were also seen to abol-
ish the toxicity of epiro-din. Thus it appeared fairly certain
that the conjugated double bond system was the active portion
of the epiro-din molecule.

The mechanism of the polyene antibiotics was unknown
until around 1960. Then it was shown (75,76) that organisms
TABLE 34.

Effect of Time on Hemolysis by Epirodin Mix.

<table>
<thead>
<tr>
<th>Length of Incubation (minutes)</th>
<th>Concentration of Epirodin (ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>.042</td>
</tr>
<tr>
<td>10</td>
<td>.042</td>
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<tr>
<td>20</td>
<td>.044</td>
</tr>
<tr>
<td>40</td>
<td>.056</td>
</tr>
<tr>
<td>60</td>
<td>.043</td>
</tr>
<tr>
<td>120</td>
<td>.044</td>
</tr>
</tbody>
</table>
Figure 60. Effect of Time on Hemolysis by Epirodin. A, 10 ug/ml Epirodin; B, 40 ug/ml; C, 50 ug/ml; D, 60 ug/ml.
sensitive to polyenes bind these substances, probably to sterols in the cell membrane. Polyenes were shown to be capable of lysing red cells. Upon addition of cholesterol to the assay, lysis was reduced. Presumably, the polyenes bound to the extraneous cholesterol rather than that in the cell membrane, and thus the lysis was inhibited. Epirodin was shown to act in a similar manner. It readily lysed the erythrocytes, but addition of cholesterol reduced the extent of lysis.

The polyene antibiotics have been shown to be toxic to yeasts and fungi but not to bacteria. The absence of sterol in bacteria explains the reason why the compounds are not toxic to these organisms. There is no membrane component with which the polyene can bind. Dermostatin is the only hexaene known to possess any antibacterial activity. A few trienes are also active. It is interesting to note that dermostatin, like epirodin, has a carbonyl conjugated to the polyene system. Perhaps it is this carbonyl-polyene structure that is responsible for the antibacterii activity.

Epirodin mix has been shown to be toxic to yeasts and fungi, but not to the extent of the other polyene compounds. This information coupled with the antibacterial activity, suggests that epirodin may not behave in the same manner as the polyene antibiotics. Perhaps the lactone-polyene system results in enough of a change in polarity to cause the interaction between sterol and antibiotic to be less intense. Thus epirodin is effective against red cells but not against growing organisms such as yeasts and fungi.
IV. CONCLUSION

The UNH strain of *Epicoccum nigrum* was shown to produce humic acids with an amino acid and phenol content similar to that reported by other workers. They were found to be inactive against *A. salina*, *B. subtilis*, and *C. pyrenoidosa*.

Studies were made concerning the epirodin pigments which have been reported only in the UNH strain of the fungus. Epirodin mix was found to be highly soluble in very polar organic solvents, but insoluble in water. In less polar solvents, it was only slightly soluble, and in non-polar solvents, it was insoluble. Epirodin mix could not be dissolved in acidic media, but it was readily dissolved in basic media. Thus it was shown to contain both polar and non-polar functions. Solubility in dilute bicarbonate determined the epirodins to be fairly strong acids.

Thin layer chromatography of the epirodin pigment showed it to be composed of two compounds designated epirodin T for the higher *R_p* spot and epirodin L for the lower one. UV-visible studies were made on epirodin mix to determine the structure of the chromophore. Its *\( \lambda_{max} \)* suggested a series of seven conjugated double bonds; however, the shape of the spectrum was not the characteristic pattern for such a system. Following sodium borohydride reduction, the usual shape was observed. This indicated that the alkene chain was further conjugated to a carbonyl func-
tion. Photostereoisomerization of the molecule with I\textsubscript{2} catalysis showed that the chromophore existed in an all-
trans configuration. The two components of epirodin mix were found to contain the same chromophore.

Infrared analysis of epirodin mix showed it to contain hydroxyl and carbonyl functions as well as methyl and meth-
ylene groups. Mass spectrometry of the trimethylsilyl ether derivative of epirodin mix indicated a relatively high mo-
lecular weight for the parent compound. It was estimated at 953.

Standard qualitative organic methods were applied to epirodin to gain insight regarding its chemical nature. De-
colorization of bromine or permanganate solutions rein-
forced the existence of the conjugated alkene chromophore. A positive response to the hydroxylamine hydrochloride-ferric
chloride test showed a lactone or ester grouping to be present. A positive reaction to periodate indicated a 1,2-glycol or an \(\alpha\)-hydroxy acid. Further chemical studies were done, mostly of a quantitative nature. The failure of epirodin to fragment on saponification indicated that epirodin con-
tained a lactone and not an ester. In addition to the lactone, there were two free carboxylic acid groups. Eight to ten hydroxyl groups were present of which at least four were 1,2-glycols. There also appeared to be a third glycol involved in the lactone formation. Data suggested that there might also be an \(\alpha\)-hydroxy acid group. Hydrogen uptake by epirodin mix showed seven unsaturated functions thereby

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lending support to the proposed chromophore. Potassium permanganate oxidation of epirobin mix resulted in the isolation of two aliphatic, dicarboxylic acids, suberic and aze-
laic. From the data obtained, a working structure for the epirobin molecule was proposed.

Investigations were carried out regarding the biological activity of epirobin mix. In addition to its toxicity to \textit{B. subtilis} and \textit{A. salina}, epirobin was able to inhibit the growth of the yeast \textit{S. cerevisiae} and to cause lysis of human erythrocytes. Addition of sterol to the yeast assay resulted in almost complete reversal of inhibition. Sterol also retarded hemolysis, but it was not able to stop it completely. Human serum, on the other hand, was very effective in preventing hemolysis. Examination of several components of human serum showed only \(\gamma\)-globulin and cholesterol to be capable of reversing hemolysis to any extent.

From the chemical data obtained regarding the structure of epirobin mix and the behavior of the compound in the various biological assays, it appears that epirobin should be included among the polyene macrolide antibiotics. All of the members of this group of compounds discovered thus far have been isolated from soil-dwelling bacteria. Therefore, epirobin represents the first polyene macrolide antibiotic to be isolated from a fungal organism.


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60. Woodward, R.B.: Structure and absorption spectra. IV. Further observations on \( \alpha,\beta \) unsaturated ketones. J.
Am. Chem. Soc. 64, 76-77 (1942).


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