# The Filipin Complex*

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ABSTRACT: Thin-layer and column chromatography show that the antifungal antibiotic filipin contains a minimum of eight filipin-like pentaenes. Three of the components, which seem to constitute 96% of the complex, were crystallized after partition chromatography on siliceous earth and adsorption chromatography on silica gel. Characterization data on the components indicate close chemical similarity.

The isolation of the crystalline antifungal antibiotic filipin was described by Whitfield *et al.* (1955). Structure studies have been reported by Berkoz and Djerassi (1959), Dhar *et al.* (1960, 1964), Djerassi *et al.* (1961), Ceder and Ryhage (1964), and Golding *et al.* (1964).

Recent advances particularly in thin-layer chromatography and partition chromatography have prompted us to investigate the homogeneity of the filipin used in these studies. Thin-layer chromatography on silica gel yielded the first evidence that filipin was a mixture, while subsequent partition chromatography revealed it was composed of at least eight components. Therefore, we will refer to the filipin described in the literature as the filipin complex. The chromatographic methods for isolating the three major components of the filipin complex as well as their characterization are the subjects of this paper.

## Materials and Methods

*Thin-Layer Chromatography.* Thin-layer chromatography plates (10 × 20 cm) were prepared from 60 g of silica gel $HF_{254}$ (E. Merck, AG-Darmstadt, Germany) suspended in 60 ml of 0.2 M $KH_2PO_4$ and 60 ml of 0.2 M $Na_2HPO_4$. They were air dried and then activated at 130° for 2 hr. The 0.3-mm thick plates were developed with normal saturation in methylene chloride–methanol (85:15). The filipins were visualized by their fluorescence under 366-mμ light and quantitated by densitometric measurement.

*Partition Chromatography.* The solvent systems were prepared from dimethylformamide, water, ethyl acetate, and cyclohexane. The solvents were mixed and the two phases were separated. Proportions were as given in Table I. Siliceous earth, either Dicalite 436 or 4200 (Great Lakes Carbon Corp.), was slurried in upper phase and mixed with lower phase (0.4 ml/g). The mixture was poured into a glass tube and packed by gravity with flowing upper phase until the bed height was constant. The liquid was then drained to about 0.5 in. above the bed.

The filipin was dissolved in lower phase (approximately 5 ml/g of sample), and mixed with siliceous earth (2 g/ml of lower phase) and enough upper phase to make the mixture fluid. This sample mixture was carefully poured onto the top of the prepared column bed, and the liquid was drained to the level of the sample layer. After adding fresh upper phase, the column was developed at a flow rate of approximately 0.1–0.2 ml/min per g of column bed. The filipins were detected as they emerged from the column by their characteristic pentaene ultraviolet absorption spectra. They were subsequently quantitated by absorption at ~354 mμ (methanol) and labeled by their thin-layer chromatography behavior. The filipins were isolated from the appropriate fractions by precipitation with water and the precipitate was collected by filtration with filter aid. The filipins were leached from the filter cake with methyl ethyl ketone–methanol (1:1), the solution was filtered, and the filtrate was concentrated to dryness *in vacuo*.

*Silica Gel Chromatography.* Silica gel for chromatography (7734, E. Merck, AG) was mixed with a buffer solution containing $KH_2PO_4$ and $Na_2HPO_4$ (27.2 and 28.4 g, respectively, per kg of gel). The gel was heated gradually to remove the unbound water, then activated at a gel temperature of 130° for 2 hr. It was then mixed with methylene chloride and methanol (95:5) and poured into a glass column.

The filipin was dissolved in methanol and mixed with prepared silica gel (approximately 8 g of gel/g of filipin). The solvent was removed by evaporation at room temperature, and the dried mixture was sifted into a layer of solvent on top of the column bed. The column was developed at a flow rate of approximately 35 ml/min per kg of bed with methylene chloride–methanol (90:10).

## Experimental Section and Results

Crystalline filipin complex obtained by the procedure of Whitfield *et al.* was the starting material for the following experiments.

*Designation of the Filipin Components.* Figure 1 shows the thin-layer chromatography behavior of crystalline filipin complex. The components were

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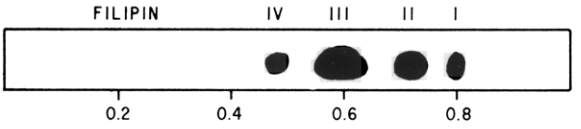


FIGURE 1: Thin-layer chromatography of crystalline filipin complex.

designated according to their decreasing mobilities as filipin II ($R_F$ 0.7), III ($R_F$ 0.6), and IV ($R_F$ 0.5). The material having an $R_F$ of 0.8 was initially designated as filipin I. This was later shown to be a mixture and therefore will be referred to as filipin I complex. Densitometric measurements indicated that the relative ratios of the several components remains essentially unchanged when these antibiotics are observed by fluorescence emission, sulfuric acid charring, or permanganate–periodate spray reagent. The filipin complex contains approximately 4, 25, 53, and 18% of filipin I complex, II, III, and IV, respectively (Figure 2).

*Separation of the Filipins by Partition Chromatography.* An example of the separation of a typical crystalline filipin complex by partition chromatography is shown in Figure 3. Filipin complex (300 mg) was chromatographed on 20 g of Dicalite 436 with solvent system A. The partially purified filipins II, III, and IV used as starting materials below were obtained in this manner.

*Isolation of Filipin I Complex.* Crystalline filipin complex (7.5 g), containing approximately 885 mg of filipin I complex by ultraviolet and thin-layer chromatography analysis, was chromatographed on 2000 g of Dicalite 436 with solvent system B. The fractions containing filipin I complex were combined and mixed with 10 g of filter aid and 0.16 volume of water. After standing 30 min, the precipitated filipin I complex was collected by filtration, washed with water, dissolved in 760 ml of methyl ethyl ketone and methanol (1:1), and concentrated *in vacuo* to dryness. The dry residue was dissolved in 400 ml of 1-propanol. The solution was clarified by filtration, concentrated *in vacuo* to a volume of 60 ml, and cooled for 4 hr at 0°. The crystalline filipin complex was removed by filtration and dried in a high vacuum to constant weight (331 mg).

TABLE I

| System | Dimethyl-formamide | Water | Ethyl Acetate | Cyclo-hexane |
|---|---|---|---|---|
| A | 4 | 0.4 | 10 | 20 |
| B | 4 | 0.4 | 10 | 22 |
| C | 4 | 0.4 | 10 | 30 |
| D | 4 | 0.4 | 10 | 24 |
| E | 4 | 0.4 | 10 | 16 |

*Resolution of Filipin I Complex.* Further chromatography of filipin I complex on Dicalite 436 with solvent system C, followed by ultraviolet analysis of the collected fractions revealed the presence of at least five partially separated components, all having the typical filipin-like ultraviolet spectra. The characterization of each of these components is not yet complete. The characterization data for filipin I complex are included in this report (Table II) for comparison with filipins II–IV.

*Isolation of Filipin II.* Partially purified filipin II (5 g) was rechromatographed on 200 g of Dicalite 4200 with solvent system A. The fractions containing filipin II were combined and mixed with filter aid and 0.5 volume of water, and refrigerated overnight. After filtration, the filter cake was leached with 1140 ml of methyl ethyl ketone–methanol (1:1). The residue was again chromatographed on Dicalite 4200 with

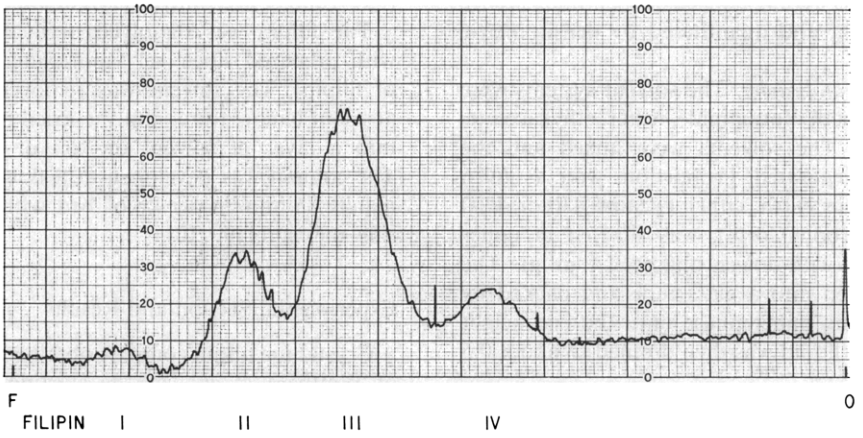


FIGURE 2: Densitometric measurement of fluorescence emission from the filipin components after their separation by thin-layer chromatography (light source, 366-mμ wavelength.)

TABLE II: Properties of Filipins I (Complex), II, III, and IV.

| Property | I Complex | II | III | IV |
|---|---|---|---|---|
| Elementary analysis (%)[a] | | | | |
| Carbon | 66.97–67.24 | 63.69–63.93 | 61.01–62.14 | 61.82–61.94 |
| Hydrogen | 9.29—9.54 | 9.06—9.09 | 8.74–9.03 | 8.81—9.04 |
| Oxygen (direct) | 23.52–23.85 | 25.71–25.82 | 27.40–27.50 | 27.66 |
| $[\alpha]_D^{25}$ (*c* 0.8%, DMF) (deg) | −330 | −237 | −245 | −251 |
| Melting point, °C (dec) | 200–205 | 181–195 | 163–180 | 204 |
| Ultraviolet Absorption Max (MeOH) λ (mμ) | *a* | *a* | *a* | *a* |
| 238 (sh) | 6.34 | 6.90 | 5.78 | 6.00 |
| 243 | 6.72 | 7.09 | 6.22 | 6.65 |
| 293 (sh) | 17.10 | 18.37 | 17.44 | 17.53 |
| 308 | 44.38 | 42.08 | 41.28 | 42.83 |
| 321 | 95.39 | 85.33 | 85.10 | 86.04 |
| 337 | 157.64 | 135.37 | 136.81 | 136.74 |
| 354 | 153.70 | 132.65 | 134.27 | 130.37 |

[a] The elementary analyses given are ranges obtained on several samples of each filipin; DMF, dimethylformamide.

solvent system D. The fractions containing filipin II were diluted with 0.5 volume of water to induce crystallization. Recrystallization from 1-propanol afforded 1.26 g of filipin II.

Thin-layer chromatography and further partition chromatography showed that this preparation was a single component. The characterization data are tabulated in Table II. The infrared spectrum in mineral oil mull is given in Figure 4 and the nuclear magnetic resonance spectrum in deuterated dimethylformamide is shown in Figure 5 (chemical shifts expressed from tetramethylsilane).

*Isolation of Filipin III.* Partially purified filipin III (2 g) was rechromatographed on 200 g of Dicalite 4200 with solvent system E. The filipin III was crystallized by addition of 0.16 volume of water, collected by filtration with filter aid, and washed with water. The cake was leached with 500 ml of methyl ethyl ketone–methanol (1:1), and the solvent was removed by distillation *in vacuo*. The dry residue was crystallized from 1-propanol to afford 1.08 g of filipin III, which by thin-layer chromatography and further partition chromatography was a single component. The characterization data are tabulated in Table II. The infrared spectrum is essentially indistinguishable from that of filipin II. The nuclear magnetic resonance spectrum is shown in Figure 6.

*Isolation of Filipin IV.* A filipin III–IV mixture (16 g) was chromatographed on a column of silica gel (3.5 kg). The fractions enriched in filipin IV, which emerged from the column after filipin III, were concentrated *in vacuo* to dryness to afford 1.67 g of material. This was rechromatographed on silica gel (350 g), and the further enriched filipin IV was concentrated again to dryness *in vacuo* (1.24 g). It was then chromatographed on 200 g of Dicalite 4200, with solvent system E. The filipin IV was crystallized by addition of 0.16 volume of water, collected by filtration with filter aid, and washed with water. The cake was leached with 1000 ml of methyl ethyl ketone–methanol (1:1) and the solvent was removed by distillation *in vacuo* (820 mg).

This preparation was finally chromatographed on 200 g of Dicalite 4200 with the same solvent system. The filipin IV was crystallized by addition of 0.16 volume of water, collected by filtration with filter aid, and washed with water. The cake was leached with methanol, and the solvent was removed by distillation *in vacuo* (609 mg). Since filipin IV crystallizes only with great difficulty and with degradation, no purification

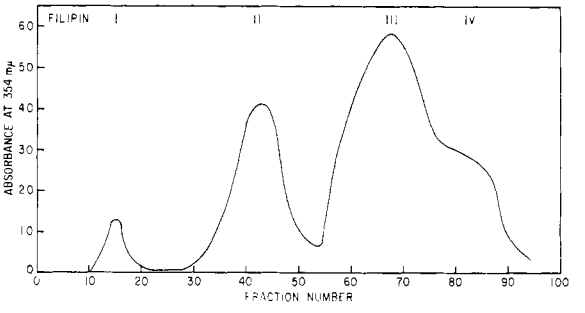


FIGURE 3: Separation of crystalline filipin complex by partition chromatography.

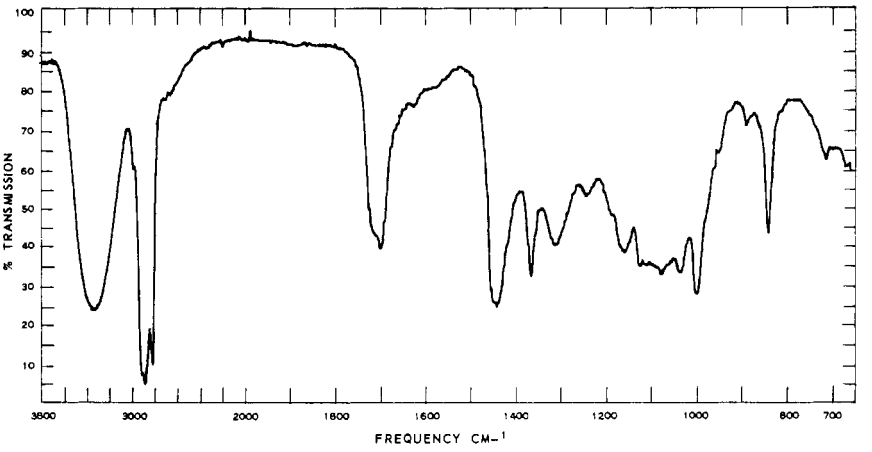


FIGURE 4: Infrared absorption spectrum of filipin II in nujol mull.

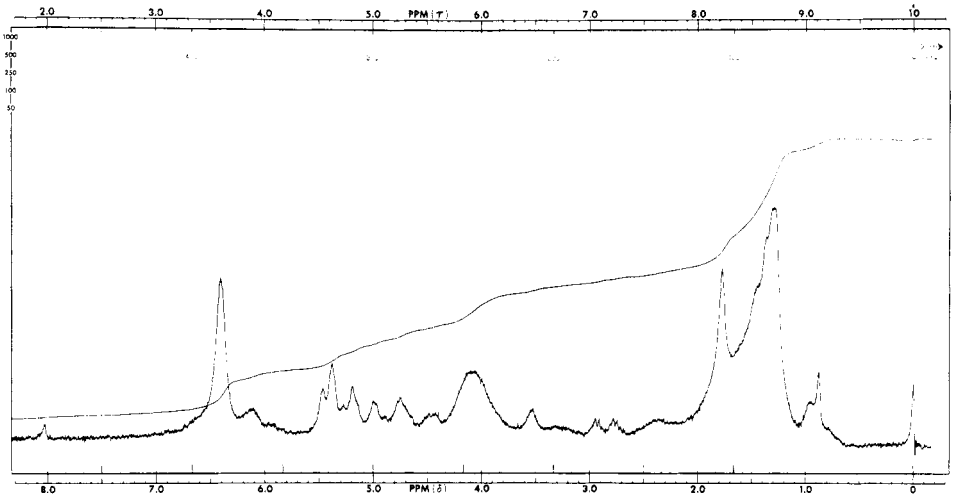


FIGURE 5: Nuclear magnetic resonance spectrum of filipin II in deuterated dimethylformamide.

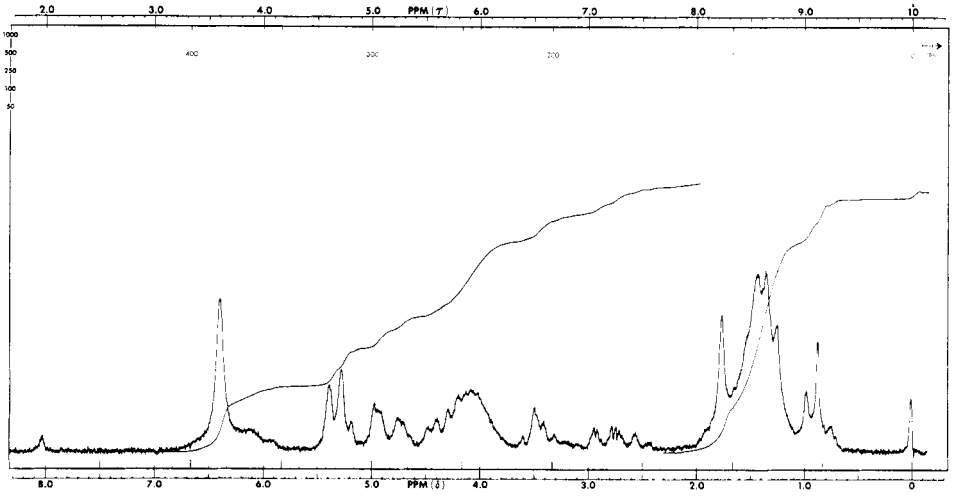


FIGURE 6: Nuclear magnetic resonance spectrum of filipin III in deuterated dimethylformamide.

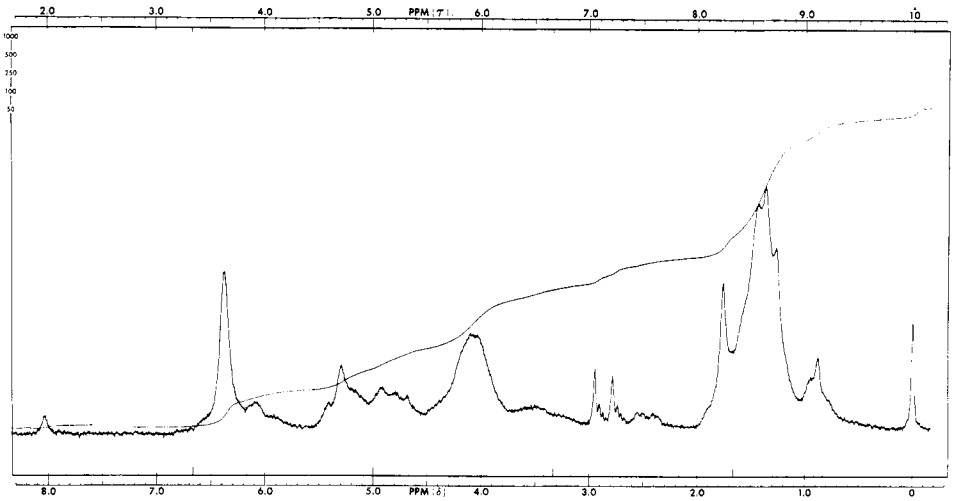


FIGURE 7: Nuclear magnetic resonance spectrum of filipin IV in deuterated dimethylformamide.

TABLE III: Antifungal Spectrum of Filipin Complex, I (Complex), II, III, and IV.

| Test Organisms | Minimum Inhibitory Concentration (mcg/ml) | | | | |
|---|---|---|---|---|---|
| | Crystalline Complex | I Complex | II | III | IV |
| *Nocardia asteroides* | >1000 | >1000 | >1000 | >1000 | >1000 |
| *Blastomyces dermatitidis* | 1 | 10 | 1 | 1 | 10 |
| *Coccidioides immitis* | 1 | 100 | 1 | 10 | 10 |
| *Geotrichum*-sp. | 10 | >1000 | 10 | 10 | 100 |
| *Hormodendrum compactum* | 10 | >1000 | 10 | 10 | 10 |
| *Phialophora verrucosa* | 10 | >1000 | 10 | 10 | |
| *Cryptococcus neoformans* | 1 | 100 | 1 | 1 | 10 |
| *Histoplasma capsulatum* | 10 | 100 | 1 | 1 | 100 |
| *Sporotrichum schenckii* | 10 | >1000 | 10 | 10 | 100 |
| *Monosporium apiospermum* | 10 | >1000 | 10 | 10 | 100 |
| *Trichophyton rubrum* | 10 | >1000 | 10 | 10 | 100 |
| *Microsporum canis* | 10 | >1000 | 10 | 10 | |
| *Trichophyton interdigitale* | 10 | >1000 | 10 | 10 | 100 |
| *Candida albicans* abbott | 10 | >1000 | 10 | 10 | 10 |
| *Trichophyton violaceum* | 10 | >1000 | 10 | 10 | |
| *Trichophyton asteroides* | 10 | >1000 | 100 | 10 | 10 |
| *Trichophyton mentagrophytes* UC 4797 | 10 | >1000 | 100 | 10 | 10 |
| *Trichophyton mentagrophytes* UC 4860 | 10 | >1000 | 10 | 10 | 10 |

by a final crystallization was attempted. Thin-layer chromatography and further partition chromatography indicated this filipin IV preparation was a single component. The characterization data are tabulated in Table II. The infrared spectrum is essentially indistinguishable from filipin II. The nuclear magnetic resonance spectrum is shown in Figure 7.

## Discussion

The crystalline antifungal antibiotic filipin previously described in the literature as a single entity was found to be a mixture of at least eight components all of which possess the typical filipin pentaene chromophore. We have succeeded in isolating three of these components in crystalline form. These three, which we labeled filipins II, III, and IV, comprised approximately 96% of the original filipin complex. The other five were isolated as a crystalline mixture that is called filipin I complex. It is interesting to note that thin-layer chromatography analyses of the mother liquors after the isolation of the crystalline filipin complex

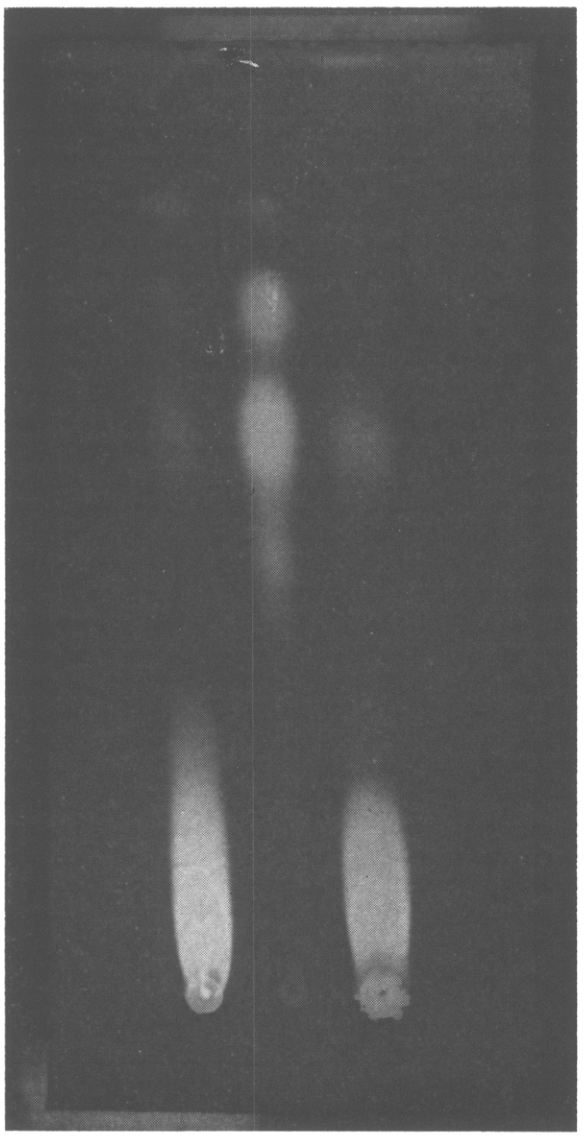

FIGURE 8: Thin-layer chromatography of fungichromin (left, 500 μg), crystalline filipin complex (center, 20 μg), and lagosin (right, 700 μg).

indicate the presence of at least seven more filipin-like components, a total of at least 15 components present in the fermentation broths.

The characterization data shown in Table II should be considered in view of the disparate structures propounded by Djerassi, Ceder, Dhar, Golding, and their coworkers. The analytical data suggest that the filipins differ from each other in molecular size.

Little reliance is placed on the data on filipin I complex since they reflect the contribution of at least five components. However, the joint contribution of this complex (about 4%) and that of filipin II (about 25%) would have a significant effect on the elemental analyses of crystalline filipin complex. Whether these differences between the filipins are of carbon chain length or oxygen content, or a combination of both cannot be deduced from the data available. Although not successful in determining individual molecular weights of the parent filipins by mass spectrometry, we will report elsewhere on molecular weights and fragmentations of the pertrimethylsilyl ethers.

Because of the interesting pentaene relationships between the filipins, fungichromin (Cope *et al.*, 1962) and lagosin (Dhar *et al.*, 1960, 1964), preparations of these antibiotics were compared by thin-layer chromatography (Figure 8), with fluorescence emission as the method of visualization. The fungichromin preparation (supplied by Sharp and Dohme, Division of Merck & Co.) was found to be approximately 65% pentaene by ultraviolet absorption and the lagosin (supplied by Glaxo Laboratories) 36% pentaene. The major pentaene components of the lagosin and fungichromin preparations were inseparable in the system used for resolving the filipins as well as in more polar systems such as methylene chloride–methanol (8:2 and 7.5:2.5). Interestingly though, the fungichromin preparation contained components possessing a filipin-like pentaene chromophore which chromatographed like filipins I, II, and III, while the lagosin preparation contained only trace amounts of pentaene materials which chromatographed like filipins I and II but a significant amount of a filipin-like pentaene which chromatographed like filipin III.

Finally, since the filipin complex is an antibiotic which has been widely investigated for its antifungal activity, a comparison of the antifungal activities of the various filipins is presented in Table III. It can be seen that the activity of the complex is a reflection of the predominant amounts of filipins II and III which it contains.

## Acknowledgments

We thank Mr. D. R. Wait for his valuable assistance, Miss A. Dietz for the antifungal evaluations, Dr. T. F. Brodasky for densitometric analyses, and the members of the Physical and Analytical Chemistry Unit for microanalyses and spectral data.

## Added in Proof

Preliminary mass spectrometry data show the molecular weight of the pertrimethyl ether derivatives of hydrogenated filipins II, III, IV to be identical (E. J. Hessler and M. F. Grostic, personal communication).

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# Analytical Studies on Nuclear Ribonucleic Acid Using Polyacrylamide Gel Electrophoresis*

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ABSTRACT: Rat liver nuclear and cytoplasmic ribonucleic acids (RNAs) have been analyzed by electrophoresis on polyacrylamide and mixed polyacrylamide–agarose gels. Many species of nuclear RNA were found which are not detected in the cytoplasm. On the lower molecular weight side, the nucleus contained, in addition to a distinct 18S RNA species, several species with mobilities somewhat slower than 4S transfer ribonucleic acid (tRNA) and without detectable amino acid acceptor activity. On the high molecular weight side, there were several species of nuclear RNA, with mobilities slower than 30S RNA, which labeled rapidly, unlike cytoplasmic RNA. Newly synthesized 30S RNA and 18 S were found to appear in the cytoplasm at the same time, in contrast to results of studies by others. These results also suggested that "35S" and "45S" nuclear RNAs, as defined by sucrose gradient analysis, were not single species, but rather are composed of many components of different molecular weights. This highly complex nature of nuclear RNA makes it difficult to define precursor–product relationships between nuclear and cytoplasmic RNAs. However, these studies indicate that polyacrylamide gel electrophoresis offers a more discriminating technique for the study of nuclear RNA function and metabolism.

In a previous paper, we have described the usefulness of polyacrylamide gels for the analytical electrophoretic fractionation of cRNA from mammalian tissues (Peacock and Dingman, 1967). Recently, Loening (1967) and Loening and Ingle (1967) have also presented similar studies on the RNA from a variety of tissues. Because of the importance of the cell nucleus in the synthesis and maturation of cRNA (for reviews, see Harris, 1963a; Prescott, 1964; Perry, 1967) and because of the extensive evidence for the existence of species of RNA in the nucleus that are not found in the cytoplasm (Hiatt, 1962; Sporn and Dingman, 1963; Shearer and McCarthy, 1967), we have sought, in the present study, to use the high resolving power of gel electrophoresis to reexamine some of the physical and metabolic properties of nuclear RNA (nRNA).

## Experimental Section

*Materials.* Pancreatic ribonuclease A (salt free, lyophilized) and pancreatic deoxyribonuclease I (electrophoretically purified, RNase free) were the products of Worthington Biochemical Corp. Sarkosyl NL-97 (sodium lauroyl sarcosinate) was a gift of the Geigy Chemical Corp. [6-$^{14}$C]Orotic acid hydrate (4–5 mc/mmole), [$^{3}$H]cytidine (5–6 c/mmole), and [$^{14}$C]amino acid mixture were purchased from New England Nuclear Corp.

Rats were Fischer males weighing 120–250 g. *In vivo* labeling of rat liver RNA was accomplished by intraperitoneal injection of aqueous solutions of the appropriate isotope at specified times prior to removing the tissue. All animals were fed *ad lib.*

*Methods.* ISOLATION OF SUBCELLULAR FRACTIONS. The methods used for the removal and homogenization of the tissue have been previously described (Peacock and Dingman, 1967). Purified liver nuclei were obtained from crude nuclear pellets that had been twice washed with solution A by methods described previously (Sporn and Dingman, 1966). In some cases, extraction of purified nuclei with Triton X-100 was performed by suspending the purified nuclei homogeneously in a solution which was 0.32 M sucrose, 0.001 M $MgCl_2$, 0.001 M potassium phosphate (pH 6.5), and contained 0.3% Triton X-100 (Lazarus and Sporn, 1967) followed by centrifugation. The extraction was repeated once more prior to isolation of nRNA.

ISOLATION OF RNA. cRNA was isolated as described

* From the Chemistry Branch, National Cancer Institute National Institutes of Health, Bethesda, Maryland 20014
*Received September 25, 1967.*