The Isolation and Characterization of Two Phototoxic Furanocoumarins (Psoralens) from Diseased Celery

LESTER D. SCHEEL, VERNON B. PERONE, ROBERT L. LARKIN, AND RICHARD E. KUPEL

From the U. S. Department of Health, Education, and Welfare, Public Health Service, Division of Occupational Health, Cincinnati 2, Ohio

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When domestic celery develops "pink-rot" disease, caused by the fungus *Sclerotinia sclerotiorum*, it is capable of producing phototoxic lesions on the exposed skin of celery harvesters who handle it. Two compounds were isolated from diseased celery and were shown to have a high order of biologic activity as determined by the bioassay method. These compounds were identified through their melting points, carbon-hydrogen determinations, and infrared and ultraviolet spectrographic analyses in comparison with known synthetic compounds, and were shown to be 4,5′,8-trimethylpsoralen and 8-methoxypsoralen.

In the harvesting of domestic celery a blistering cutaneous disorder has been observed (Birmingham *et al.*, 1961) which affects the exposed skin of the people working in the fields. The observed skin changes have long been correlated with the occurrence in the plants of a condition called "pink-rot" (Henry, 1933). This plant disease has been shown to be caused by the growth of a fungus, *Sclerotinia sclerotiorum*, on the celery stalks, usually starting near the soil line. By biological assay Perone *et al.* (1963) have shown that only the diseased portions of the plants contain products which, when placed on the skin and exposed to sunlight or ultraviolet radiation of 3200–3700 A with a peak at 3650 A, produce the blistering type of lesion characteristically seen in celery harvesters.

This paper will describe the procedure used to isolate and characterize two phototoxic psoralens from diseased celery tissue. The compounds found were 8-methoxypsoralen and 4,5′,8-trimethylpsoralen.

METHODS

Twelve crates of diseased celery were obtained from growers in Michigan during July, 1961. The celery heads and stalks were trimmed to save only the diseased portions for study material. The 15 kg of diseased material obtained was homogenized in a Waring Blendor to a slurry consistency and frozen. The frozen material was lyophilized and stored at room temperature for extraction.

The 1.5 kg of dry material was placed in large separatory funnels and extracted twice with 6 liters of petroleum ether. The 12 liters of extract were concentrated by evaporation at room temperature to a volume of 500 ml and placed in the refrigerator at 4° for several days. A mixture of 2.735 g of yellowish-brown and greenish-white crystals formed and was filtered from the mother liquors. Bioassay of the crystals indicated a very high activity.

The two crystalline forms were separated by fractional crystallization. The mixed crystals were dissolved in 200 ml of hot absolute methanol, and 0.625 g of yellowish-brown crystals (F-1) separated on cooling. These were filtered off, and 350 ml of water was added to the mother liquors. On standing in the refrigerator 0.855 g of greenish-white crystals (F-2) separated out and were filtered off. The melting point of the F-1 crystals was 225–228° and of the F-2 crystals 136–138°. This wide divergence of melting points indicated the presence of at least two compounds. Bioassay indicated that F-1 and F-2 were both active.

Purification and Characterization of F-1 as 4,5′,8-Trimethylpsoralen.—The F-1 crystals were recrystallized from absolute methyl alcohol and the melting point of the isolated crystals was sharpened to 226–228°. An authentic sample of 4,5′,8-trimethylpsoralen furnished by the Paul B. Elder Co. had a melting point of 227–228°. The melting point of the F-1 crystals mixed with the synthetic trimethylpsoralen crystals was 226–228°.

To confirm the identity of the F-1 crystals with 4,5′,8-trimethylpsoralen the ultraviolet spectra in ethyl alcohol were compared and found to be identical (Fig. 1). The molecular extinction coefficients were calculated using the equation $\epsilon = a/LC$ where a = absorbance, L = the length of the light path in cm, and C = the concentration of the absorbing solution in moles/liter. The molecular extinction coefficients of the F-1 sample and the 4,5′,8-trimethylpsoralen were found to be identical at 250 mμ: $\epsilon_{250} = 2.81 \times 10^4$ mole^{-1} cm^{-1}.

Using the KBr pellet technique for infrared spectra, we compared the F-1 crystals with the standard 4,5′,8-trimethylpsoralen (Fig. 2). Examination of these spectra further substantiated the similarity of the F-1 crystals and the 4,5′,8-trimethylpsoralen. Since it was not possible to prove the methyl group definitely by infrared analysis the carbon hydrogen analysis was performed to prove further the composition of the F-1 crystals. The results shown in Figure 1 leave little doubt that the F-1 crystals isolated are actually 4,5′,8-trimethylpsoralen. Since this compound has not been isolated as a natural product previously, the biologic activity of the isolated compound was tested on the rabbit skin and was found to be identical with the activity of the synthetic sample (0.1 μg/sq in. = 1+ reaction) (Perone *et al.*, 1963).

Purification and Characterization of F-2 as 8-Methoxypsoralen.—The ultraviolet spectrum of the F-2 crystals (Fig. 3) dissolved in ethyl alcohol was found to be similar to the spectrum reported (Stanley *et al.*, 1957) for 8-methoxypsoralen except that the shoulder at 262 mμ was masked. After decoloration of the crystals with carbon and recrystallization from water the ultraviolet absorption spectrum of the F-2 crystals became exactly like the spectrum reported for 8-methoxypsoralen. The purification also raised the melting point to 145–148°, which is in agreement with that reported in the literature. To confirm the similarity of the F-2 crystals to 8-methoxypsoralen a sample of 8-methoxypsoralen was obtained from the Paul B. Elder Co. and the ultraviolet and infrared spectra were determined for both the F-2 crystals and the 8-methoxypsoralen. These spectra are identical as shown in Figures 3 and 4. The extinction coefficients at 250 mμ were found to be the same ($\epsilon_{250} = 3.03 \times 10^4$ mole^{-1} cm^{-1}).

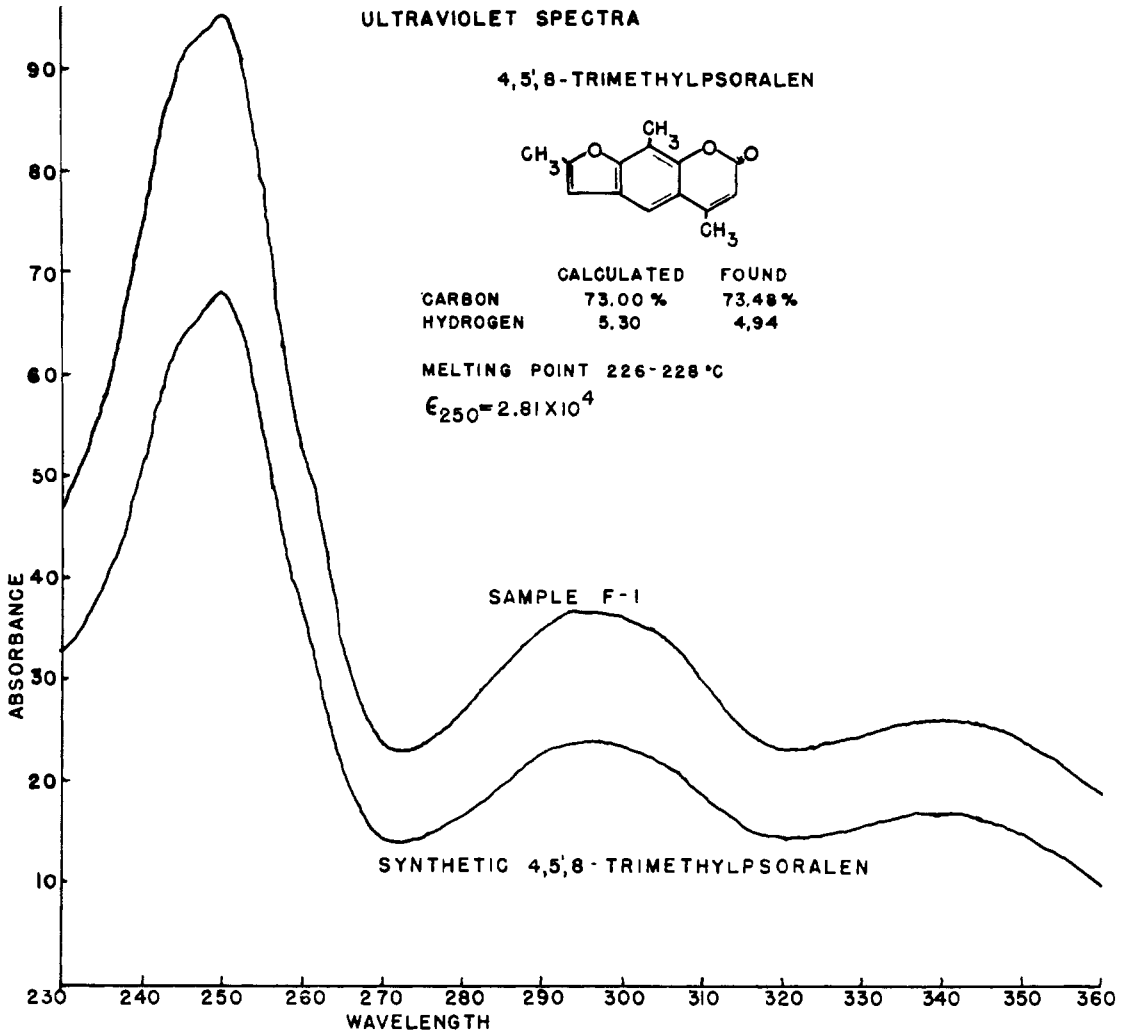

FIG. 1.—Ultraviolet spectra of F-1 crystals isolated from diseased celery and 4,5′,8-trimethylpsoralen in ethyl alcohol.

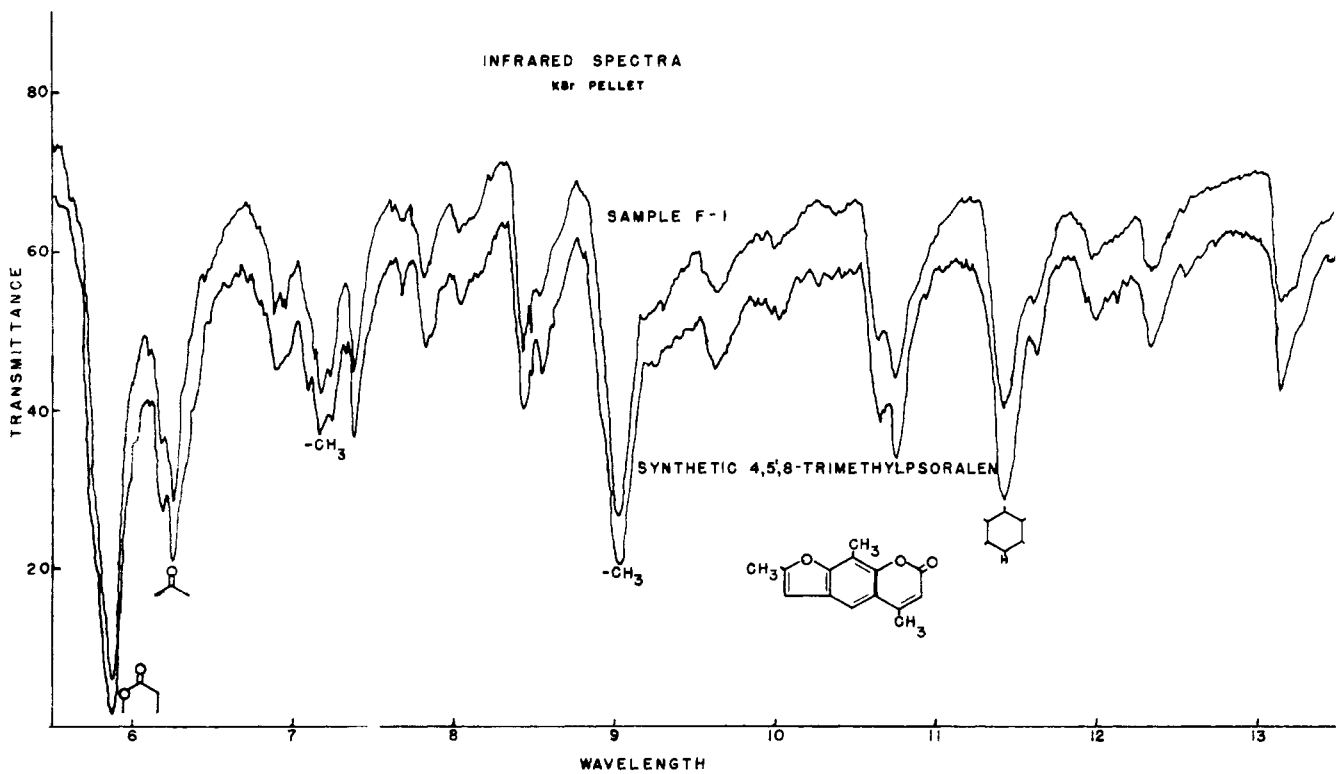

FIG. 2.—Infrared spectra of F-1 crystals isolated from diseased celery and 4,5′,8-trimethylpsoralen by the KBr pellet technique.

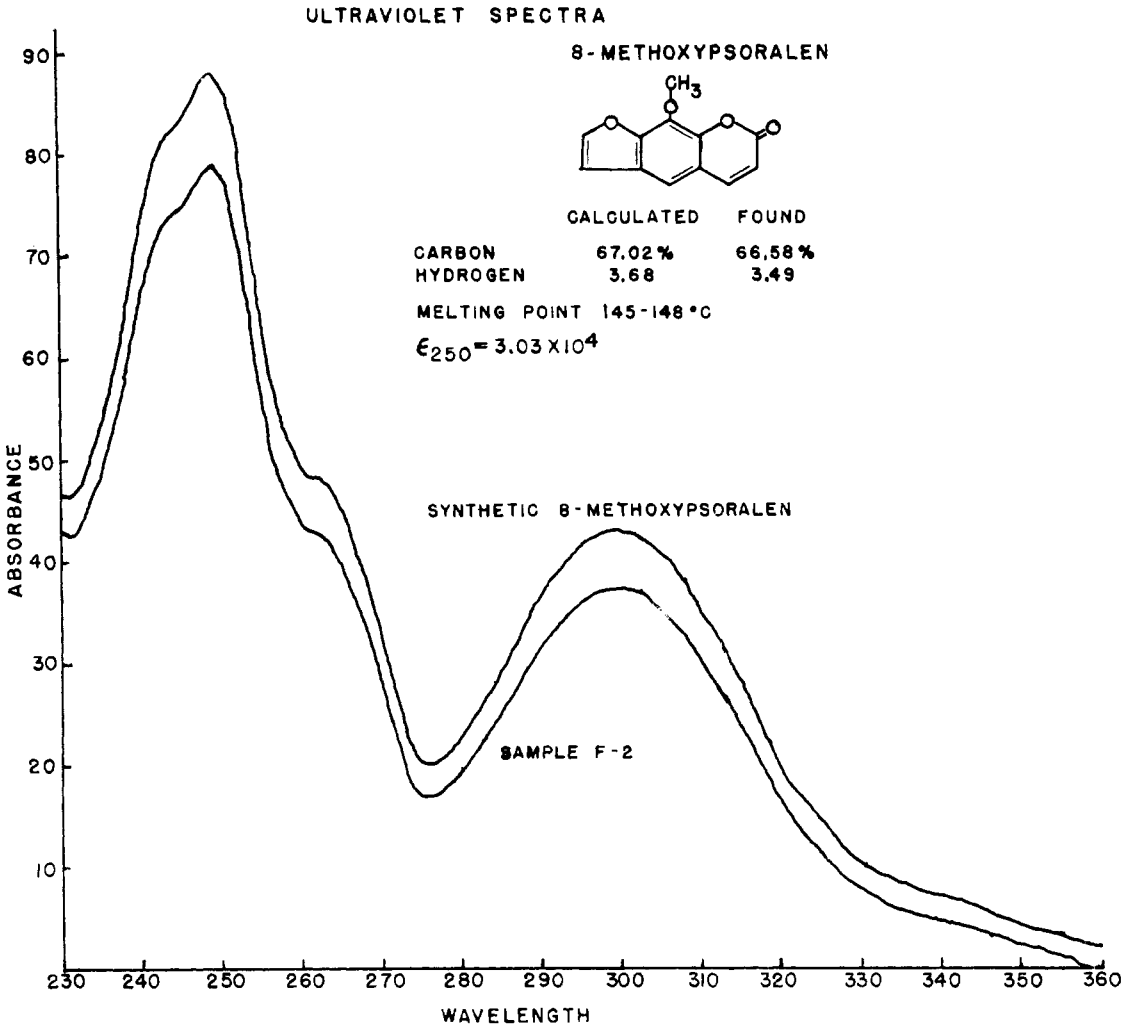

FIG. 3.—The ultraviolet spectra of F-2 crystals isolated from diseased celery and 8-methoxypsoralen in ethyl alcohol.

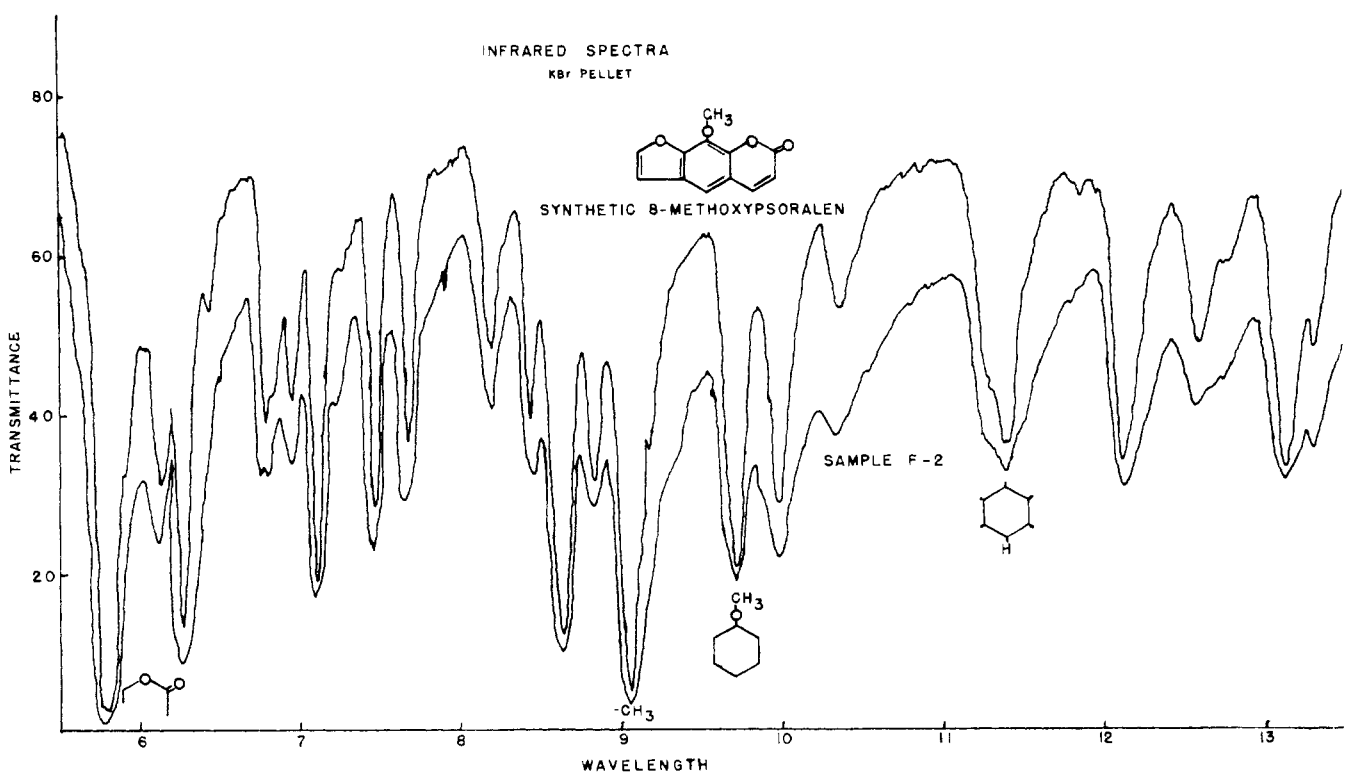

FIG. 4.—The infrared spectra of the F-2 crystals isolated from diseased celery and 8-methoxypsoralen by the KBr pellet technique.

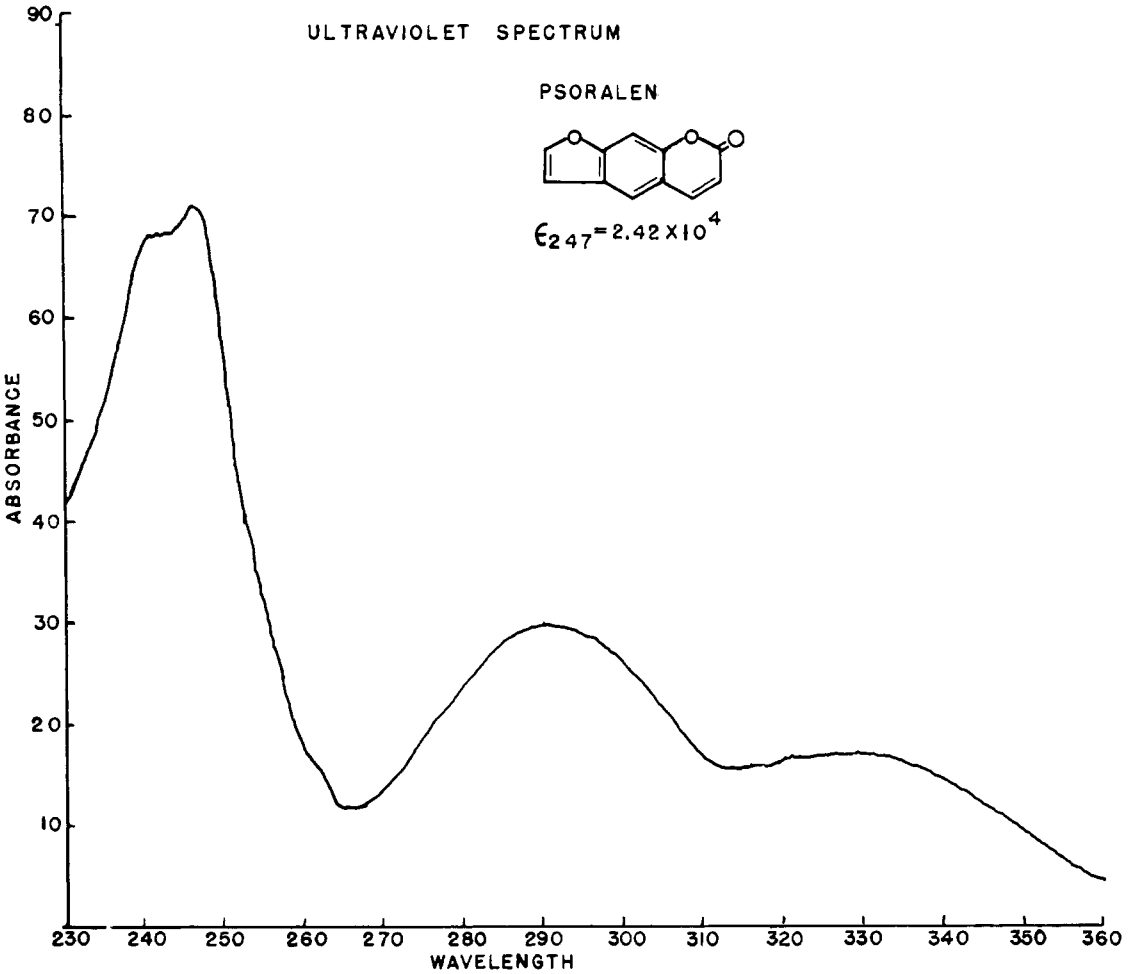

FIG. 5.—The ultraviolet spectrum of psoralen.

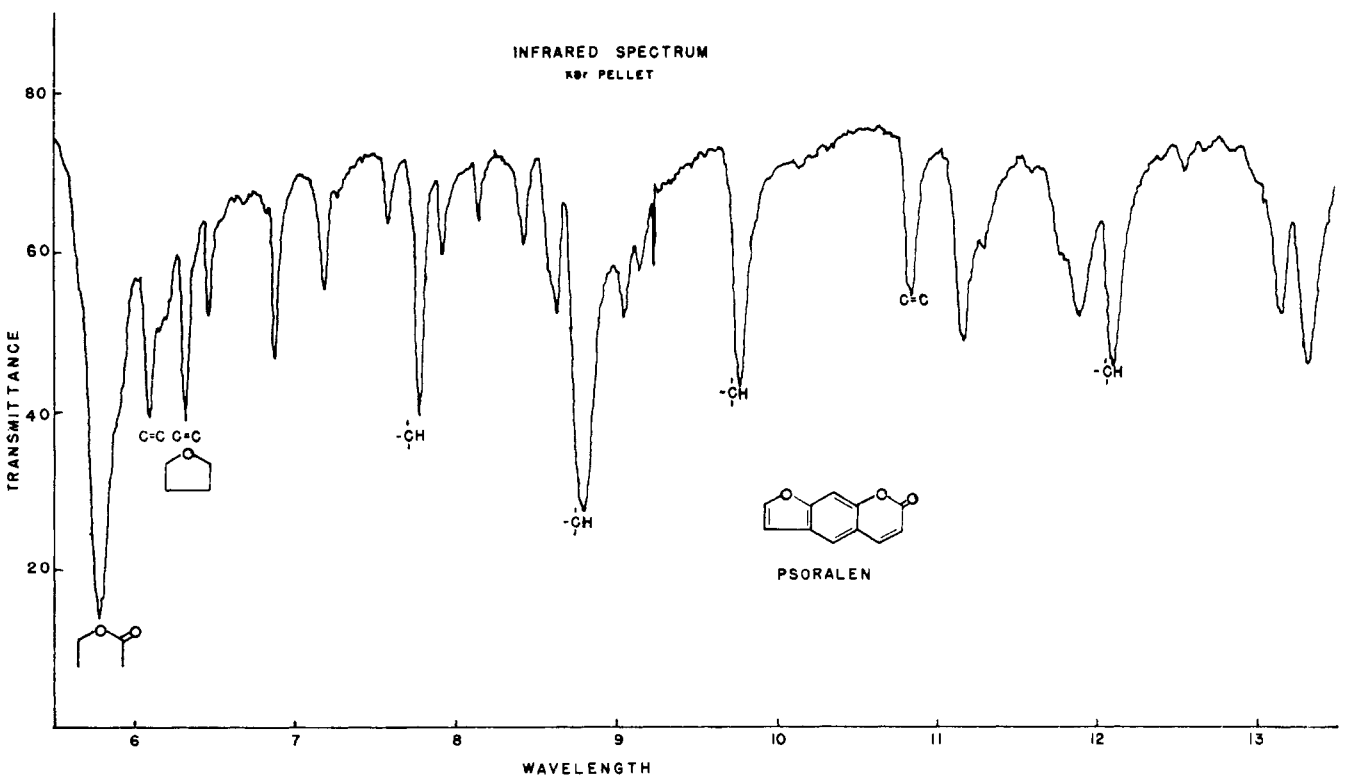

FIG. 6.—The infrared spectrum of psoralen.

The melting points and mixed melting points of the F-2 crystals and the authentic sample were all in the range of 145–148°.

As shown in Figure 3 the carbon and hydrogen values found agree with the calculated values for 8-methoxypsoralen in both samples.

To prove further the identity of the F-2 crystals and 8-methoxypsoralen the two samples were bioassayed on the same rabbit with the Woods Light used to activate the samples. The F-2 crystals were shown to have the same biologic activity (1.5 μg/sq in. = 1+ reaction) (Perone *et al.*, 1963) as the 8-methoxypsoralen.

The above evidence is considered proof that the F-2 crystals are 8-methoxypsoralen.

Since the psoralen nucleus is known to be a highly active phototoxic chemical (Pathak and Fitzpatrick, 1959) it was necessary to prove that the F-1 and F-2 crystals are different from this compound. To do this the ultraviolet and infrared spectra were used (Fig. 5, 6) and the extinction coefficient was calculated as shown in Figure 5. The spectra were not the same as those for either 4,5′,8-trimethylpsoralen or 8-methoxypsoralen, and the molecular extinction coefficient (ϵ_{247} mμ = 2.42 $\times$ 10^4 mole^{-1} cm^{-1}) was not the same as for the other compounds. This evidence further substantiates the identification of the isolated compounds.

The bioassay of psoralen and synthetic 4,5′,8-trimethylpsoralen showed that both these compounds have the same biologic activity (0.1 μg/sq in. = 1+ reaction). However, the ultraviolet and infrared spectra of psoralen are different from those of the F-1 crystals and synthetic, 4,5′,8-trimethylpsoralen. The presence of strong $-CH_3$ absorbance bands at 715 and 905 μ in the synthetic 4,5′,8-trimethylpsoralen and the F-1 crystals proves that the F-1 crystals are not psoralen.

Discussion

The availability of a semiquantitative bioassay was of primary importance in the isolation and characterization described. The photoactivation necessary to elicit the toxic response made the assay quite specific. In addition, the structure and activity studies of Pathak and Fitzpatrick provided significant guides for the limits of phototoxic activity within the coumarin family of compounds.

In this work the infrared and ultraviolet spectra of the crude ether extracts indicated that the coumarins may be present in diseased celery extracts, and the approach used successfully demonstrated that extraction with nonpolar solvents completely removed the active ingredients from the dried plant tissue. The use of conditions thereafter that would prevent opening the lactone ring was necessary to achieve purification of the isolated products. From the spectral and analytical data presented it is judged that neither of the isolated compounds is psoralen itself.

4,5′,8-Trimethylpsoralen has been synthesized (Gupta and Seshadri, 1953) but has not been identified as a natural product until the present time. Its occurrence as a product of the *Sclerotinia sclerotiorum* growth on the celery plant remains an isolated observation. This laboratory would appreciate any communication from others who may have observed phototoxic effects from *Sclerotinia sclerotiorum* growing on other plant species. This type of information is essential to an understanding of the symbiotic relationship between celery and *Sclerotinia sclerotiorum*.

In similar extraction attempts using normal healthy celery tissue no identifiable psoralen products have been obtained. However, if the isolated mold is seeded on normal celery and allowed to grow at room temperature, active extracts are obtained.

The identification of 8-methoxypsoralen as one of the active products in diseased celery extends the observed occurrence of this naturally occurring psoralen (Dean, 1952) to the mold family as either a constituent of the organism or a metabolic product resulting from the symbiosis between the mold and the celery plant.

Detailed analysis of the infrared spectra given in this paper will be presented at a later date and is currently being worked out at this laboratory.

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