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CALIFORNIA INSTITUTE OF TECHNOLOGY

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   Pasadena, California 91109

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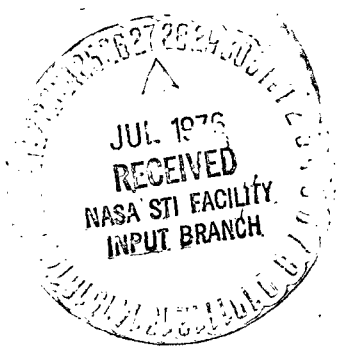


We have developed a highly sensitive fluorometric technique for the determination of biological and geo-organic compounds in ancient sediments and extraterrestrial samples. We have used this technique to establish chemical evidence for fossil pigments in an estraterrestrial sample. We have also developed a highly sensitive and specific fluorometric method for the determination of total primary amine nitrogen in soil samples.

1. Analysis of Murchison Meteorites

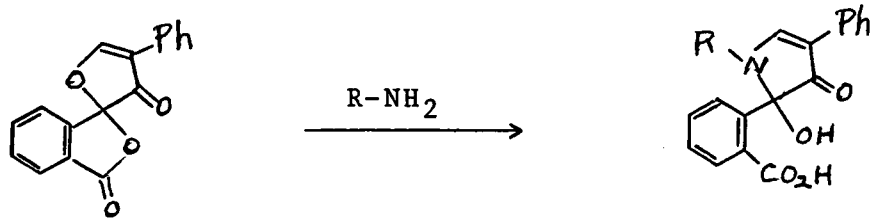
We have analyzed the organic solvent extracts of two pieces of Murchison meteorite. Dr. K. Kvenvolden of the U.S. Geological Survey has provided us with a 23 gram piece of the meteorites while a 13 gram piece of the meteorite was obtained from Dr. Roy Clarke, Jr. of the Smithsonian Institute. The analysis of the sample received from Dr. Kvenvolden indicated the presence of two grossly different types of pigments, a relatively polar pigment and a trace of non-polar compound. The analysis of the Smithsonian sample, on the other hand, indicated the pressence of only polar pigment. Although the polar pigment exhibits twin fluorescence peaks, one at 650 nm and the other at 720 nm, on excitation at 420 nm, the chemical properties differ from those of porphyrin compounds. The treatment of the sample with strong organic acid, for example, did not change its spectral characteristics. The total amount of the pigment extracted from the 23 gram piece was about one nanogram. We feel that it is significant to observe the spectrally similar polar pigments in the two different pieces of the meteorite and plan to publish the experimental data in

a respectable journal.

2. Fluorometric Method for the Determination of Total Primary Amines

The application of a novel reagent, fluorescamine, to the assay of primary amines has been investigated for the assay of primary amines in geological samples. The method can be used for an accurate and sensitive determination of primary amine in the ancient sediments as well as in the extraterrestrial samples.

Fluorescamine 4-phenylspiro[furan-2-(3H),1'-phthalan]-3,3'-dione (1) has properties which offer many advantages that are unique among existing amine detecting reagents. The reagent itself is nonfluorescent. This nonfluorescent compound of type 1 reacts with primary amines (R NH_2) to form pyrrolinones of type 2 which emit strong fluorescence around 475 to 490 nm with an excitation at 390 nm.



1 2

The fluorescamine reaction with primary amines is extremely rapid at alkaline pH and the fluorescent product is relatively stable. At pH 8-9 the average half-time for appearance of maximal fluorescence is measured in hundreds of milliseconds

at room temperature(1). Under the conditions of assay which are presently used there is a competing reaction of fluorescamine with water which leads to nonfluorescent degradation products. The reaction has been shown to proceed almost to completion with a large number of amino acids and peptides. In other words, one adds a nonfluorescent reagent to a solution containing an amino acid, peptide, or protein and within a fraction of a second maximal fluorescence, which is quite stable, is obtained. Very shortly thereafter the excess reagent is destroyed by reaction with water to yield nonfluorescent products.

Fluorescamine is generally added to the assay sample dissolved in water-miscible, nonhydroxylic solvent. Acetone and dioxane have been found particularly suitable, since they are commercially available in grades which are low in fluorogenic impurities. Other solvents which are useful include acetonitrile, dimethylsulfoxide, and tetrahydrofuran. Fluorescamine in acetone, acetonitrile, and dioxane is found to be stable for at least 2 to 10 weeks at room temperature. Hydroxylic solvents are unsuitable in most situations. Solvents such as methanol form additional products with fluorescamine (2,3).

Although there are many applications of fluorescamine to the assay of amino acids, peptides, proteins, and other primary amines, to obtain optimal utilization of this reagent, many details concerning the reactions still require further investigations. We have studied the effects of various parameters such as pH, temperature, organic solvent, and buffer

salts on the reactivity of fluorescamine. The test of the fluorescence method was also carried out with various geological samples.

Of those parameters which can be varied to maximize the extent of the fluorogenic reaction, a most important one is the pH of the medium. With most amines, adherence to a relatively narrow alkaline region is required (pH 8-9.5). If the pH is too low, the amine will be protonated and hence unavailable for reaction. Conversely, if the pH is too high, hydrolysis of the reagent will predominate over the fluorogenic reaction. Aliphatic amines and peptides generally require pH 8-8.5, while amino acids and proteins can preferentially be assayed at pH 8.5-9.5.

While the fluorogenic reaction is very sensitive to pH variations, the fluorescence intensities of the reaction products are much less affected by the pH. They are fairly constant between pH 4.5 and 10.5. In neutral or mildly alkaline medium, the fluorophors are stable at ambient temperatures for hours and in the dark even for days. In acidic solution, fluorescence rapidly deteriorates.

At a given pH, variation of temperature produced an apparent doubling in rate from 20°C to 50°C.

Hydrolysis of fluorescamine was found to be first order with respect to fluorescamine as evidenced by the linearity of logarithm fluorescence vs time. The rate of hydrolysis of fluorescamine increased with increasing pH, irrespective of

the organic solvent. Hydrolysis proceeded at less than half the rate in sodium phosphate compared to sodium borate buffer at pH 8.0, while a reduction in borate concentration from 0.10 M to 0.05 M at pH 9 resulted in a 5-fold increase in the rate of fluorescamine inactivation.

Since fluorescamine is rather quickly hydrolyzed in aqueous media, an excess of the reagent is required for the development of optimal fluorescence. Yet, at concentrations which are significantly higher than 1 μmole/ml of fluorescamine, fluorescence quenching is observed. While the nature of this quenching is not truly understood, it is believed to be due to a hydrolysis product of the reagent.

The fluorescence intensity is also affected by the amount of organic solvent which is present in the final assay sample.

Although the amino groups of proteins react readily with fluorescamine to give intense fluorescence, protein samples were usually hydrolyzed first either in alkaline or acidic solutions at an elevated temperature prior to their reaction with fluorescamine. A great advantage of hydrolysis, particularly in the analysis of protonacious substances in geological samples, is that some interfering materials can easily be separated from the protein materials or any primary amine compounds.

The test of the fluorescamine method for both Allende carbonaceous chondrites and Apollo 16 and 17 samples were

negative. The effects of geological samples on the fluorescamine reaction were studied by adding either Allende meteorites or organically cleaned reaction mixtures. Although there was a significant reduction in the fluorescence intensity of the reaction products of the sample, no obvious chemical quenching of the fluorescence was observed. The reaction with ammonia yields fluorescence which is about one-thousandth that obtained with amino acids.

3. Development of Three-Dimensional Fluorescence Spectroscopy

For complex spectra or mixtures of fluorescent compounds, the recording of a complete series of activation and fluorescence spectra is a rather tedious task. To simplify this data processing task, we have developed a device for the simultaneous recording of the activation and emission spectra. The technique plots the activation and emission wavelength on the abscissa and ordinate axes, respectively, with fluorescence intensity levels plotted by a series of isointensity contour similar to elevation levels on a topographic map. Typical data from such a fluorescence measurement technique is shown in the figure 1 and 2 for the fluorescence of benzo(a)pyrene (BP) which has been hydroxylated at four different positions. The fluorescence of 3-hydroxy- and 6-hydroxy BP's are shown in Fig. 1, both in methanol and an alkaline media. The conventional fluorescence spectra are also plotted, excitation spectra on vertical axis and the emission on horizontal axis. Although the chemical structures are closely related, the electronic spectra are quite distinct from each other both in neutral and in anionic

species. The difference is more obvious in three-dimensional presentation than the conventional one. The fluorescence properties of 7-OH- and 8-OH-BP's are shown in Fig. 2 in the same fashion. While the neutral species in methanol exhibit a slight difference, both in excitation and emission spectra, those of anionic species show a large difference in the three-dimensional display. Scattered light artifact appears as a narrow band diagonal to the abscissa and ordinate in this type of display.

It is evident that this method integrates all of the fluorescence spectral parameters in a given sample in such a way that, in effect, a "stereofingerprint" of fluorescent mixtures is presented in one plot. It thus delineates not only the activation and emission wavelengths, and emission intensities for each peak, but also the activation/emission spectral band stereoenvelopes. Accordingly, this method should permit unique characterization of even very closely related structures.

We know that most proteins contain tryptophan and all the protein contain tyrosine and that those aromatic amino acids strongly fluoresce even in intact protein. Thus, tryptophan-containing nonhistone shromosomal proteins floresce at 360 nm while histones which lact tryptophan fluoresce around 315 nm due to the tyrosine fluorescence. This is illustrated in Fig. 3 showing a typical fluorescence of both histone and nonhistone chromosomal proteins around 315 and 360 nm, respectively, with excitation in the range from 250 to 310 nm. An apparent fluorescence peak position generated by uncorrected mode of

fluorescent measurement is a function of both light source and the spectral response characteristics of photo detector. In the present study we have used a Xenon lamp as a light source and the gallium-arsenide photocathode tube which gives a rather flat response pattern from 300 to 800 nm region.

Fig. 4 illustrates a typical fluorescence of water suspension of microorganisms. Thus, Micrococcus lysodeikticus suspended in water a typical tyrosine-tryptophan fluorescence peaking around 340 nm with excitation in the range from 270 to 325 nm. This figure is a semilog plot in which the excitation and emission wavelengths are both displayed on a linear scale together with the fluorescence intensity on a log scale.

In Fig. 5, 80% methanol extract of halophilic photosynthetic bacteria is shown. Here we observe not only the fluorescence of chlorophylls but also that of free aromatic amino acids. The presence of more than one fluorescent compound in a mixture is readily apparent.

A wide variety of both biological and non-biological samples were examined by sequential extraction with organic solvents, hot water, weak base, and weak acid at elevated temperature followed by an examination of their three-dimensional fluorescence properties. This method permitted unique charactization of even very closely related chemical structures.

We feel that this work should also be published in an analytical journal since it displays the spectral data in a unique way to provide a large amount of information.

References

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2. Weigele, M., De Bernardo, S. L., and Leimgruber, W. (1973). Biochem. Biophys. Res. Commun. 50, 352.
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Fig. 1. Fluorograms of Hydroxybenzo(a)pyrenes

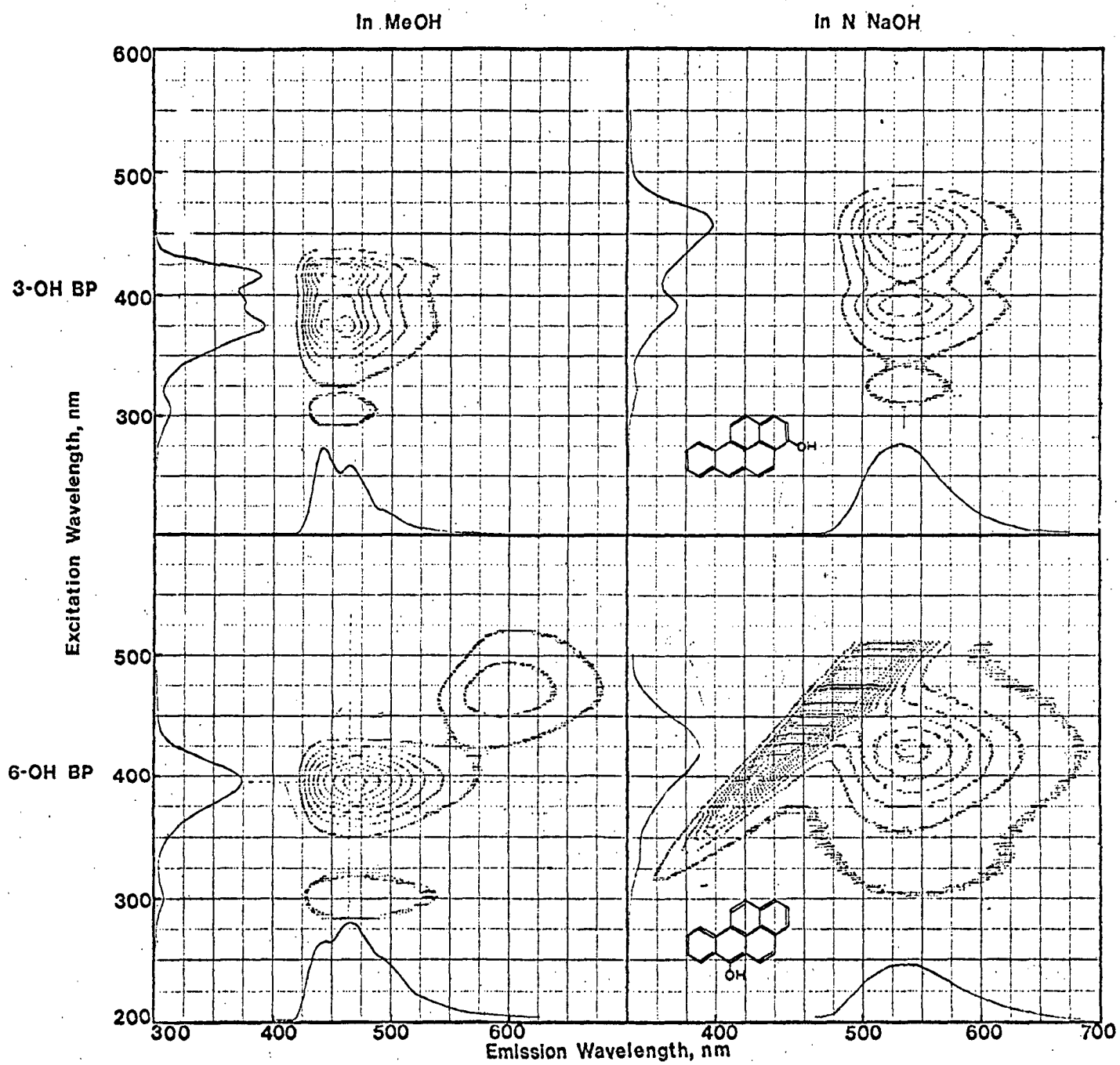


Fig. 2. Fluorograms of Benzo(a)pyrene Metabolites

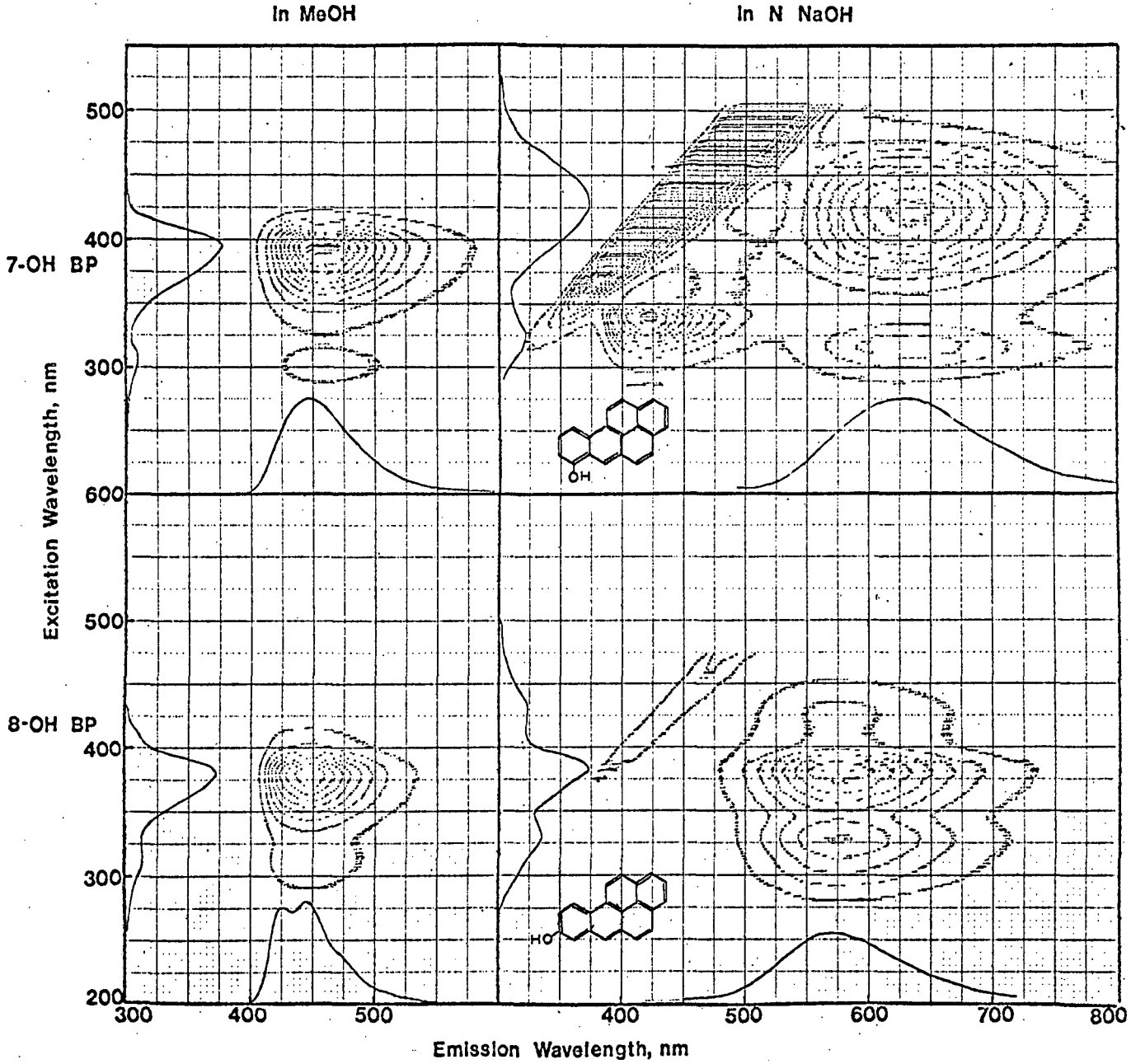


Fig. 3. **Fluorescence of Chromosomal Proteins**

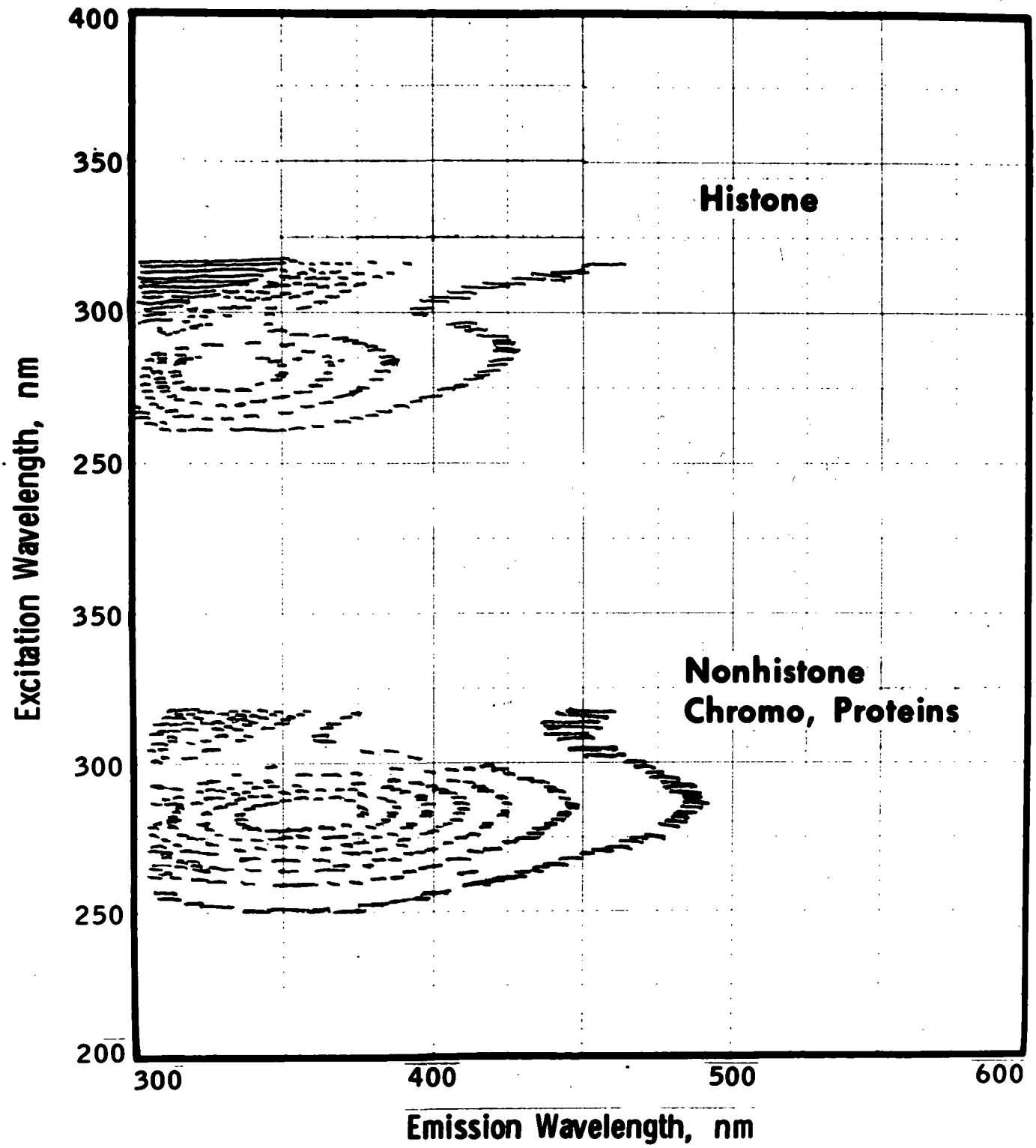
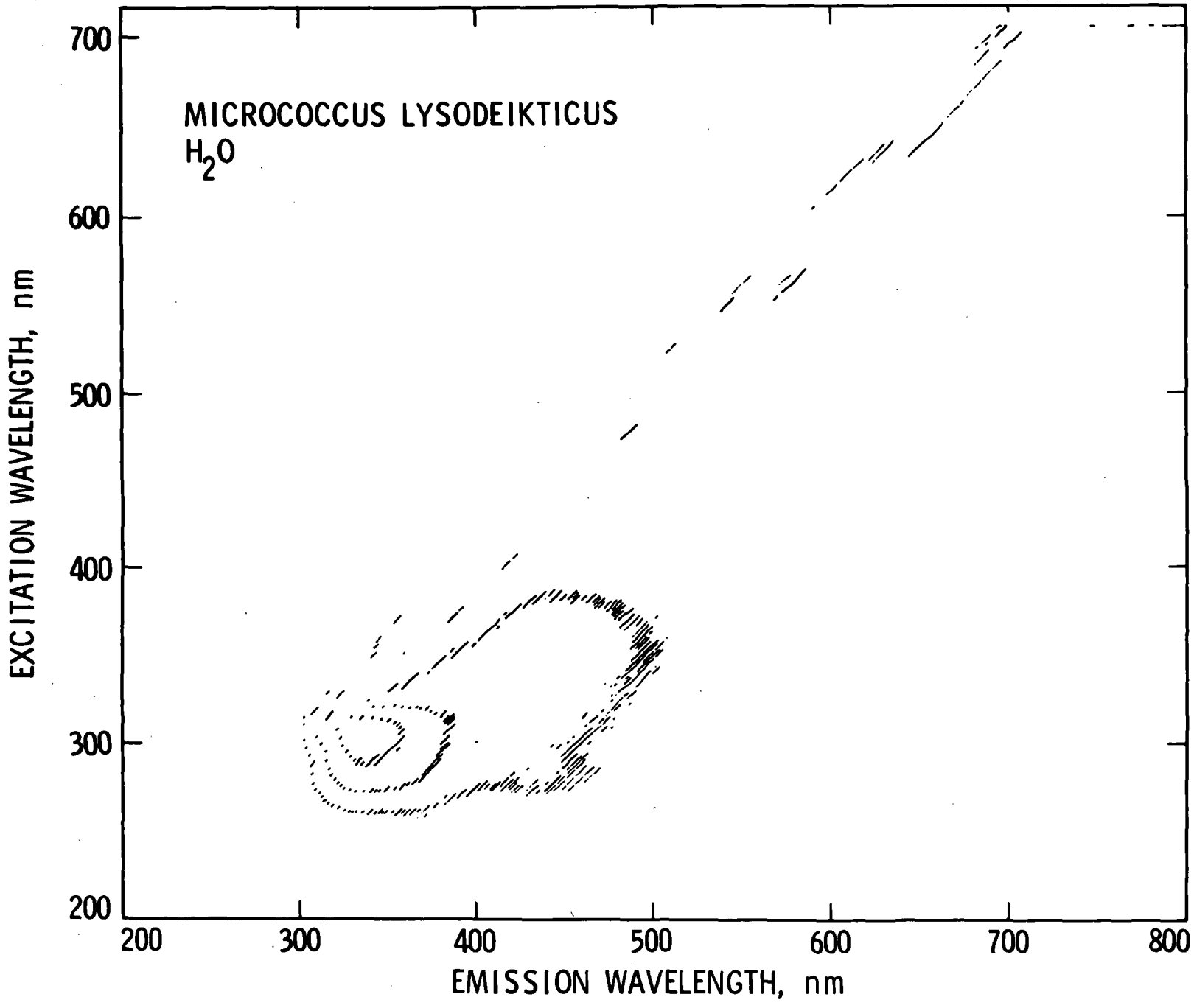


Fig. 4.


MICROCOCCUS LYSODEIKTICUS
H_2O
EXCITATION WAVELENGTH, nm
EMISSION WAVELENGTH, nm
200
300
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500
600
700
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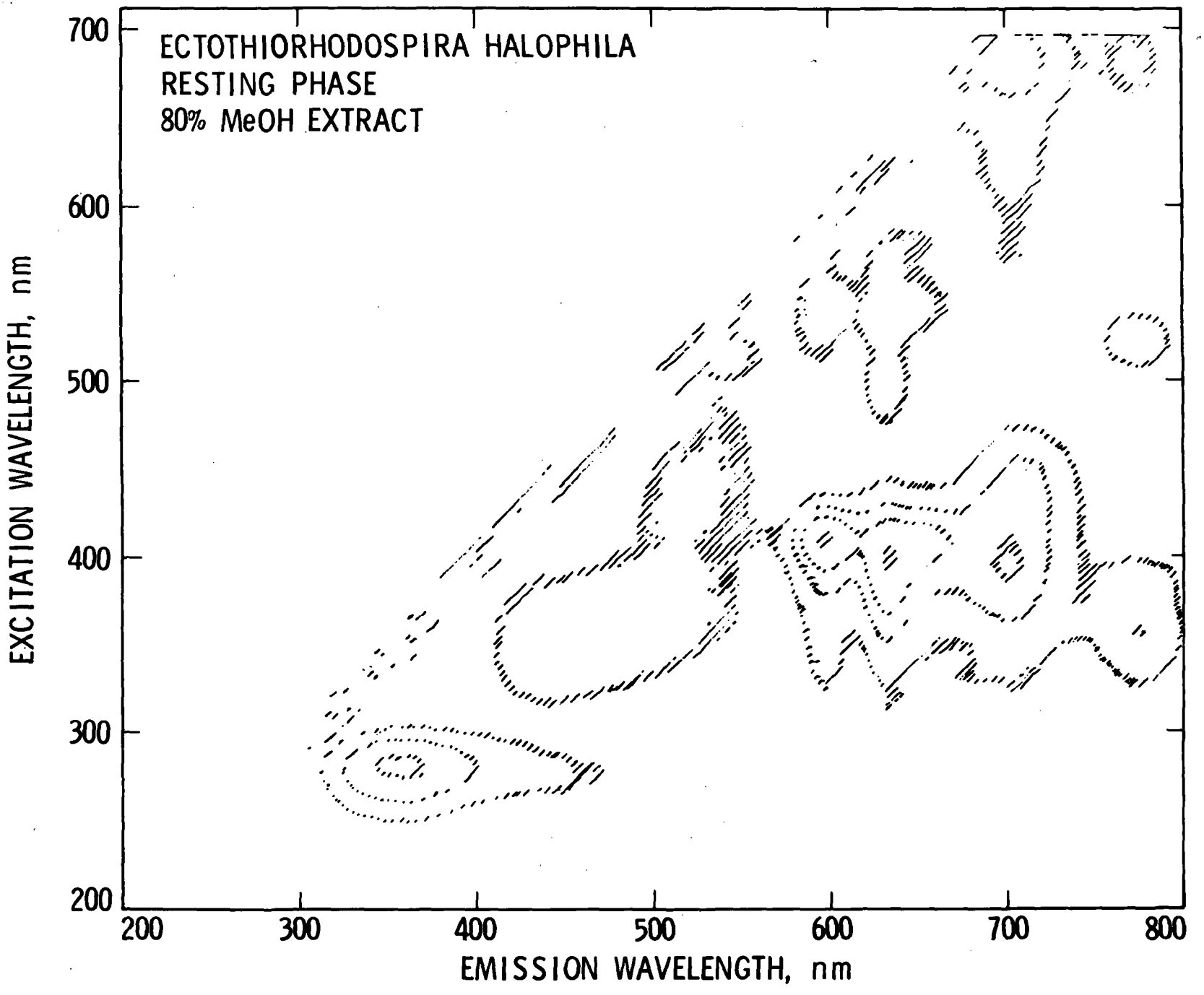
Fig. 5.


ECTOTHIORHODOSPIRA HALOPHILA
RESTING PHASE
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700
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