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Comparison of Fluorescence and Photodynamic Activities of Whole Hematoporphyrin Derivative and Its Enriched Active Components [1,2]

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ABSTRACT—The in vivo biologic activities of the hematoporphyrin derivative (Photofrin) and the enriched, so-called "active fraction" (Photofrin II) were determined by measuring the necrosis produced in implanted tumors in DBA/2Ha mice exposed to various total doses of light (20-100 J/cm^2) after ip administration of 10 mg/kg standard doses of either Photofrin or Photofrin II. Total relative percentage increase in fluorescence in tumor tissue, as compared to fluorescence in control tissue, also was measured for both Photofrin and Photofrin II. In response to total light doses (630 nm) of 40-100 J/cm^2, mice that received Photofrin had comparable amounts of tumor necrosis to those mice that received Photofrin II. At doses of 40-60 J/cm^2, 80% tumor destruction resulted, and at 80-100 J/cm^2, tumor destruction was 100%. However, at a total light dose of 20 J/cm^2, the tumors that received Photofrin II exhibited 60-80% tumor necrosis, whereas those animals that received Photofrin had only small areas of patchy necrosis associated with signs of vascular thrombosis and hemorrhage into the surrounding perivascular stroma. A 25.2% total increase in maximal tissue fluorescence over that in controls was observed for animals that received Photofrin II, as compared to 13.9% for those animals that received Photofrin. It is concluded that the greater demonstrable efficacy of treatment with Photofrin II, as compared to treatment with Photofrin, is due to enrichment of those nonpolar hydrophobic components of the hematoporphyrin derivative mixture that are thought to be primarily responsible for the in vivo biologic activities.—JNCI 1985; 75:1135-1140.

Recently added to the armamentarium of the oncologist has been the development of PDT after sensitization with the porphyrin derivatives (*1, 2*). The basic concept for the use of PDT for malignant tumors is that certain molecules can function as photosensitizers. The presence of these photosensitizers in certain cells thus makes these cells vulnerable to light at the appropriate wavelength and intensity. The action of photosensitizers generally is to absorb photons of the appropriate wavelength sufficient to elevate the sensitizer to an excited state. The excited state of the photosensitizer subsequently results in the production of active intermediates, such as singlet oxygen (*3*). Uncertainty arises as to the exact targets of these excited intermediates responsible for cellular destruction, although damage to the nuclear material (*4*) and the cell membrane (*3*) have been reported.

Although many porphyrins are known to localize in malignant tumors, the hematoporphyrin derivative (HpD) has received the most attention since Dougherty et al. (*1*) reported the therapeutic effectiveness of PDT light combined with HpD in a wide variety of solid malignant tumors (*1-5*). The photosensitizing capacity of HpD appears to be due to its preferential retention in malignant tissues, as compared to normal tissues from which it is generally cleared for 24 hours. However, one should note that HpD also is retained for long periods of time in the reticuloendothelial system, especially the liver, kidney, and spleen (*6*). The exact mechanism for this preferential retention presently is unknown.

HpD is a complex mixture of porphyrins obtained from hematoporphyrin via an acetylation reaction followed by alkaline hydrolysis, which was first described by Lipson et al. (*7*). A great deal of recent work in the field has been devoted to the careful analysis of several of these different porphyrin components with respect to their chemical identity and their tumor-localizing and photosensitizing properties. In 1981, Dougherty et al. (*8*) described a gel filtration procedure designed to isolate the unknown structure thought to be the material primarily responsible for the photosensitizing activity of the HpD mixture, both in vitro and in vivo. In vitro and in vivo studies led Dougherty et al. to conclude that the active ingredient had twice the cytotoxic activity of that of the native HpD. Further studies in 1983 by Dougherty et al. (*9*) with fast atom bombardment, mass spectrometry, and nuclear magnetic resonance spectra led to the conclusion that the active ingredient was most likely a structural isomer of dihematoporphyrin ether (DHE). Concentration of the "active" ingredient led to the introduction of Photofrin II into experimental trials in late 1983.

In our study we used a well-defined murine tumor system, in a standard series of histopathologic and fluorescence microscopy experiments, to compare the activity of Photofrin and the concentrated, presumably active fraction of HpD (Photofrin II).

MATERIALS AND METHODS

Animal and tumor system.—All mice were 14-16 weeks old and weighed between 30 and 35 g at the time of treatment. The following tumor system was used: SMT-F, a spontaneous mammary tumor that arose in the flanks of DBA/2Ha mice, was provided by Roswell Park

ABBREVIATIONS USED: HPLC=high-pressure liquid chromatography; PDT=photodynamic therapy; PMT=photomultiplier tube.

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Memorial Institute, Buffalo, NY (*10*). Tumors were harvested fresh from the mice and were minced with the use of fine scissors. Transplanted tumors were propagated intradermally in the right flank of each mouse by injections of 0.1 ml fresh tumor inoculum prepared with a concentration of 5×10^5 viable tumor cells/ml suspended in RPMI-1640 medium (GIBCO, Grand Island, NY). Cell viability was assessed according to the cell's ability to resist lysis and exclude trypan blue dye (GIBCO). The mouse tumors generally were palpable at 5 days and reached a size of 5–7 mm at 10–14 days, at which time treatment was started. At that size, the small tumor was homogeneously white, and spontaneous tumor necrosis was minimal or absent.

Hematoporphyrin derivative.—Photofrin (HpD) and Photofrin II (the active fraction, DHE) were obtained from Photofrin, Inc., Cheektowaga, NY, and were stored in the dark and refrigerated until used. For treatment, the Photofrin and Photofrin II were diluted 1:4 with 0.9% NaCl solution and were injected ip.

Procedure.—When tumors were of the appropriate size (as indicated above), the animals were shaved in the tumor area and given ip injections of Photofrin and Photofrin II in doses equal to 10 mg/kg body weight. The remainder of the experiment was done in the dark, including housing of the animals. Control tumor-bearing animals received light without administration of either Photofrin or Photofrin II. Post injection (24 hr), the experimental animals were treated with the laser light delivery system (*see* below). The mice were anesthetized with ketamine HCl (Parke-Davis, Morris Plains, NJ) and each was covered with a metal shield with a circular hole that exposed the tumor. Animals were sacrificed by halothane (Halocarbon Laboratories, Inc., Hackensack, NJ) anesthesia 48 hours after PDT. Tumor tissue was excised immediately and fixed in 3% glutaraldehyde-5% Formalin in phosphate buffer (pH 7.4). Samples then were dehydrated in graded alcohols, cleared in xylene, and embedded in paraffin. Sections (6 μm) were cut, stained with hematoxylin and eosin, cleared of paraffin in xylene, and then dried. Sections were examined with an Axiomat microscope (Zeiss, Thornwood, NY) and photographed with Panatomic X film (Eastman Kodak Co., Rochester, NY).

Animals destined for fluorescence studies were sacrificed 24 hours post injection of 10 mg Photofrin or Photofrin II/kg. Tumors were excised, immediately embedded in Tissue-TEK II (Miles Laboratories, Inc., Naperville, IL), and frozen at −80°C. Sections (6 μm) were cut on a cryostat, placed on acid-cleaned slides, and stored at −80°C until fluorescence microscopy was performed.

Laser light delivery system.—Laser irradiation was performed with an Innova 20 argon ion laser (Coherent Radiation, Palo Alto, CA) stimulating a PRT-95 dye laser (Coherent Radiation). The dye laser was tuned to emit radiation at 630 nm. The radiation then was coupled into a 400-μm fused silica fiber by means of a fiber optic coupler (Model #316, Spectra-Physics Inc., Mountain View, CA). The output end of the fiber was terminated with a microlens that focused the laser radiation into a circular field of uniform light intensity. Laser irradiation emanating from the fiber was monitored with a power meter (Model #210; Coherent Radiation) before and after treatment.

Mice were placed underneath an aperture that controlled the area of light illumination on the tumor site. The area of illumination was approximately 1 cm^2. Total laser energy density ranged from 20 to 100 J/cm^2, with a power density of 150 mW/cm^2. The intensity of light on the tumor surface was calculated by measuring the intensity of the light emitted from the laser and dividing this number by the area (cm^2) treated. The total light dose was calculated as intensity in watts per square centimeter, multiplied by the treatment time in seconds, and was expressed in Joules per square centimeter. A total of 50 light-treated tumors (5 animals each at energy densities of 20, 40, 60, 80, and 100 J/cm^2 for both Photofrin and Photofrin II) were examined.

Laser microirradiation and fluorescence detection.—The system used in this study was described previously (*11*). Basically, the beam from a continuous wave helium-cadmium laser (Liconix, Sunnyvale, CA), operating at a wavelength of 442 nm, was directed into an epifluorescence (Zeiss) RA microscope and focused on the tissue. Even though the 442-nm wavelength of the HeCd laser is not at the absorption peak for hematoporphyrin, enough absorption occurs at this wavelength to generate fluorescence. The laser beam, with a power of approximately 200 nW, was focused to a 5-μm spot size, with the use of a 16X Neofluar objective. Fluorescence from the cell was directed upwards through the epifluorescence beam splitter and barrier filter (Zeiss; #FT510 and #LP590, respectively) and was focused onto the face of a PMT (Model #9862 B/070; EM Glencom Inc., Plainview, NY). The signal generated by the PMT was acquired and processed by a multichannel analyzer (#TN-1710; Tracor Northern, Middleton, WI). Fluorescence intensity was measured by counting photons as a function of time. Because there is a decay in fluorescence intensity as a function of time during laser stimulation, the highest photon count during a 100-msecond time period was recorded for each tissue section. Measurements were made in all areas of the tumor tissue. Generally, the tumor was arbitrarily divided into 4 equal areas, and within each area 10 measurements were made. Averages of the 40 measurements for each tumor are presented in table 1. Thirty animals were examined: 10 received Photofrin, 10 received Photofrin II, and 10 were used as controls.

RESULTS

Histopathology

Inspection of the tumors 48 hours post HpD-PDT revealed no visual evidence of necrosis in control animals that received 100 J/cm^2. Histologically, those tumors that received either 80 or 100 J/cm^2 in both treatment groups demonstrated total destruction of the

TABLE 1.—*Fluorescence detection after laser microirradiation*

Fluorescence, photon count/ 100 msec	Mouse group[a]		
	Controls	Photofrin treated	Photofrin II treated
Mean absolute value/mouse	2,053	2,267	2,357
	2,042	2,159	2,347
	1,779	2,080	2,255
	1,777	1,904	2,256
	1,904	2,250	2,501
	1,856	2,257	2,325
	1,973	2,273	2,768
	2,011	2,313	2,401
	1,853	2,192	2,347
	1,932	2,160	2,468
Average value ± SD	1,918±91.6	2,185±111	2,402±141
Increase (%) over average control value		267 (13.9)	484 (25.2)

[a] 10 mice in each group were examined.

tumor tissue (figs. 1c, 2c). Those tumors that received 40 or 60 J/cm^2 in both treatment groups demonstrated coagulation and liquefactive necrosis in more than 80% of the tumor; usual tumor cellular architecture was preserved only at the base and around the peripheral extremes of the tumor (figs. 1b, 2b). At total light doses of 20 J/cm^2, the animals that received Photofrin II had 60-80% necrosis of their tumor; usual tumor structure was seen at the base and periphery of the tumor (fig. 2a). In contrast to those animals that received Photofrin II, the animals that received Photofrin and total light doses of 20 J/cm^2 had only small areas of patchy necrosis scattered throughout the tumor. The usual tumor architecture was essentially preserved. These tumors, however, did appear to have evidence of vascular engorgement, with vascular thrombosis and extravasation of red blood cells into the surrounding perivascular stroma (fig. 1a).

Fluorescence Detection

Animals were sacrificed at 24 hours after injection of 10 mg/kg Photofrin or Photofrin II, and random fluorescence measurements were made throughout the tumor tissue by means of the microirradiation and fluorescence detection system described above. Relative to control animals that received no injection, animals that received Photofrin II had a 25.2% increase in total tumor tissue fluorescence as compared to a 13.9% increase in those animals that received Photofrin (table 1). The Student's *t*-test was performed to check the significance of the mean values, and the results were found to be statistically significant at $P = .01$.

DISCUSSION

Despite the long history of the clinical use of HpD and the current interest in it as a tumor localizer and a therapeutic agent, the mechanisms of its preferential accumulation and retention in malignant tissue, as well as the relationship between chemical structure and photosensitizing efficacy, remain obscure. The relationship between the properties of the excited states of porphyrins and their photochemical behavior is still a subject of intensive investigation.

Hematoporphyrin derivative used for clinical studies proves to be a complex mixture of 4 major porphyrins that are somewhat variable in composition. Therefore, it is critically important to establish the structures of the components of the hematoporphyrin derivative and to determine their individual biologic activities. Furthermore, the knowledge of which porphyrins are active and which are inactive in vivo is a prerequisite for understanding how tumor damage is caused and for searching for more effective porphyrin photosensitizers.

Several porphyrins in HpD previously were separated by HPLC and gel filtration methods and have been identified as hematoporphyrin, hydroxyethylvinyldeuteroporphyrin, and protoporphyrin (*12-15*). In addition, numerous investigators have tested these components both in vitro and in vivo and have shown that the component of HpD thought to be responsible for the in vivo photosensitization is a nonpolar, hydrophobic structure that migrates with fraction 7 on the HPLC analysis (*14-17*). Fraction 7 contains a large number of minor components, one of which has been identified as protoporphyrin. It has been impossible to isolate the other components of fraction 7 for use in in vitro and in vivo experiments due to the small amounts present of each of these components. Fraction 7 also was shown to be a significantly more specific tumor localizer than was HpD when tumor, skin, and muscle were compared (*18*). The cellular uptake of the HpD components increased with decreasing polarity consistent with the nonpolar nature of fraction 7. Thus the increasing photosensitizing efficiency with decreasing polarity of the components mainly is due to increased cellular uptake. The components of fraction 7 subsequently have been enriched in Photofrin II.

Berns et al. (*19*) in 1981 performed HPLC separation of both HpD (Photofrin) and the "active" fraction (Photofrin II) and found that the active fraction contains most of the same components as the HpD but that the relative proportions of the amount that is contributed by fraction 7 is increased in Photofrin II.

Our data are in agreement with previous studies, inasmuch as we found that Photofrin II increases (25.2% vs. 13.9% with Photofrin) the relative fluorescence of the tumor tissue, as compared to the fluorescence values for controls that received no HpD, and that smaller total doses of light are necessary to produce the desired effect of bringing about tumor necrosis.

These findings clearly are of considerable clinical significance because the only known drawback to the use of HpD-PDT is the potential for normal tissue damage due to the drug-induced effect of ultrasensitivity to sunlight. Patients receiving HpD treatment are warned to avoid exposure to sunlight for at least 4-6 weeks. Light exposure during that time may result in symptoms ranging from mild erythema to massive edema and subse-

quent skin sloughing. Presently, the doses required are high enough to have these deleterious effects, but if the photosensitizer can be modified so that it is more tumor specific, and therefore less amounts are required for optimal response as well as decreased amounts of light, PDT would become a much more sophisticated approach, with minimal side effects, to the treatment of tumors. Such may be the case with the "enriched" Photofrin II, although this possibility as yet has not been proved. Dougherty (*20*) recently proposed two approaches to the use of Photofrin II: that it be used 1) for patients with early stage tumors and generalized photosensitivity, for whom low doses can be used in conjunction with moderate-to-high light doses, and 2) for patients with large tumors, for whom larger doses of Photofrin II can be used to provide deeper biologic responses (due to higher tumor levels), without enhancing the photosensitivity now encountered with the use of HpD (*20*).

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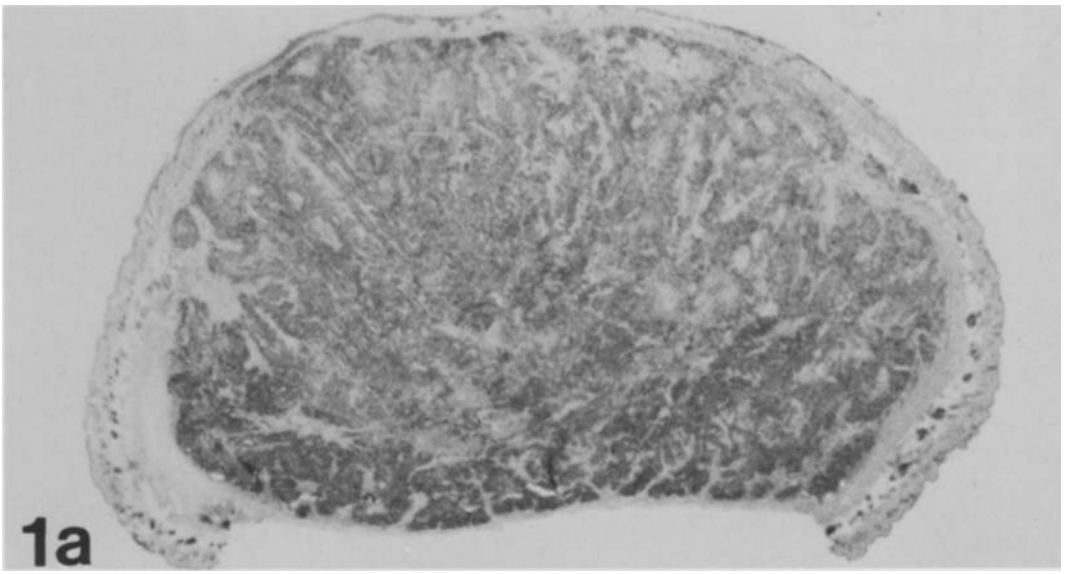
FIGURE 1.—Photomicrographs of SMT-F tumor removed 48 hr after illumination with light and Photofrin at a) 20 J/cm^2, b) 605 J/cm^2, and c) 100 J/cm^2. a) Note only minimal necrosis with vascular thrombosis. × 65

FIGURE 2.—Photomicrographs of SMT-F tumor removed 48 hr after illumination with light and Photofrin II at a) 20 J/cm^2, b) 60 J/cm^2, and c) 100 J/cm^2. a) Note 60-80% tumor necrosis, with usual tumor structure seen only at the base and around the periphery. × 65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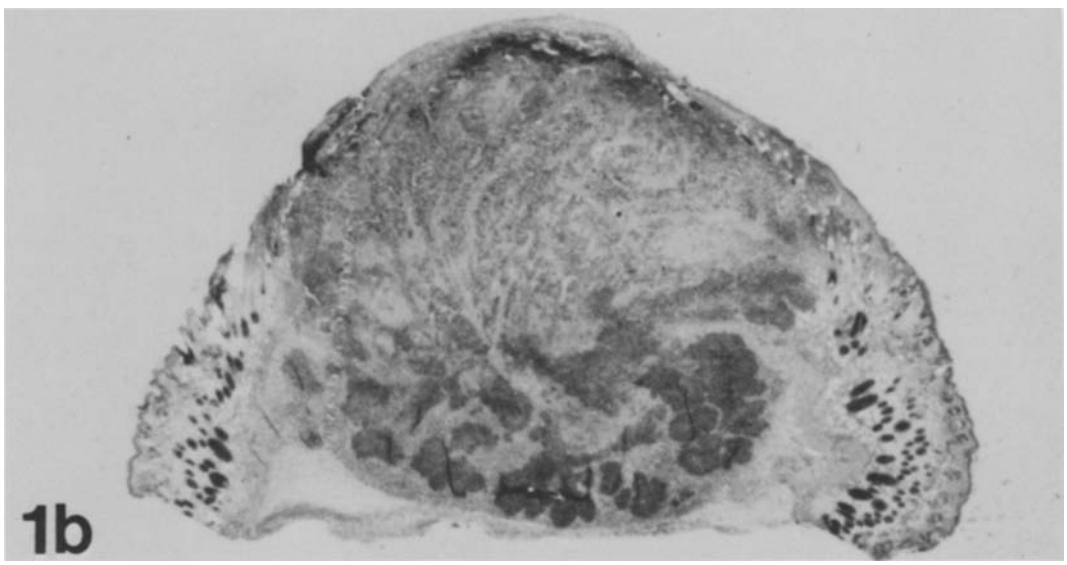
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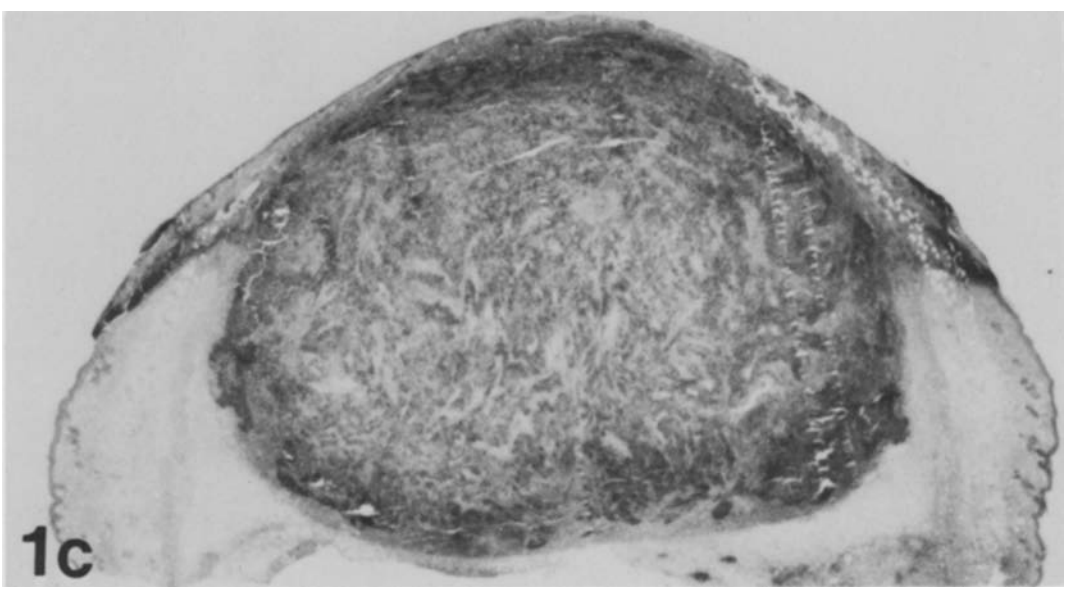
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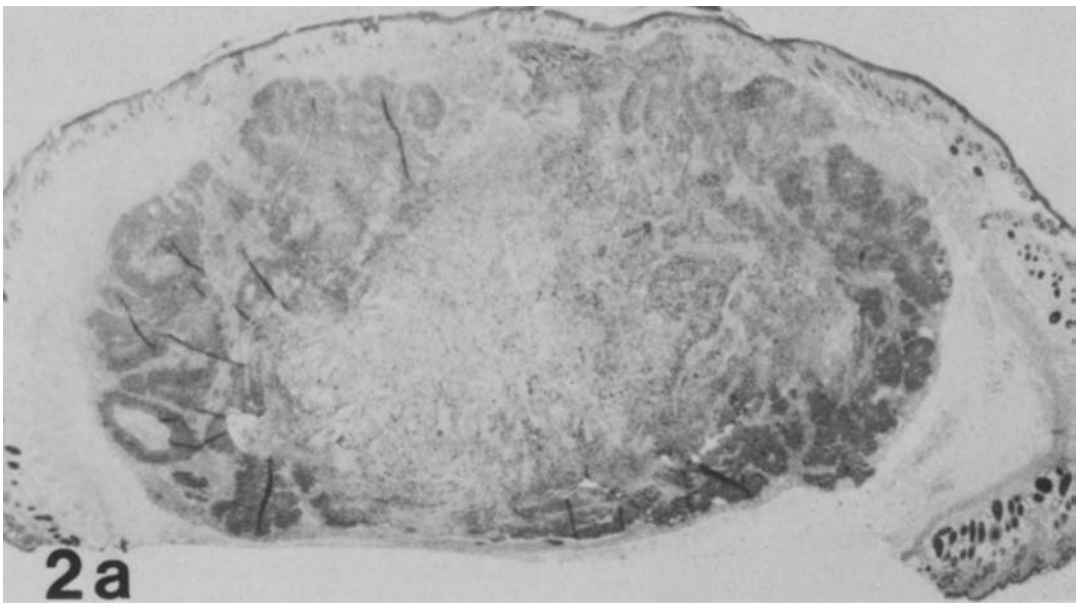
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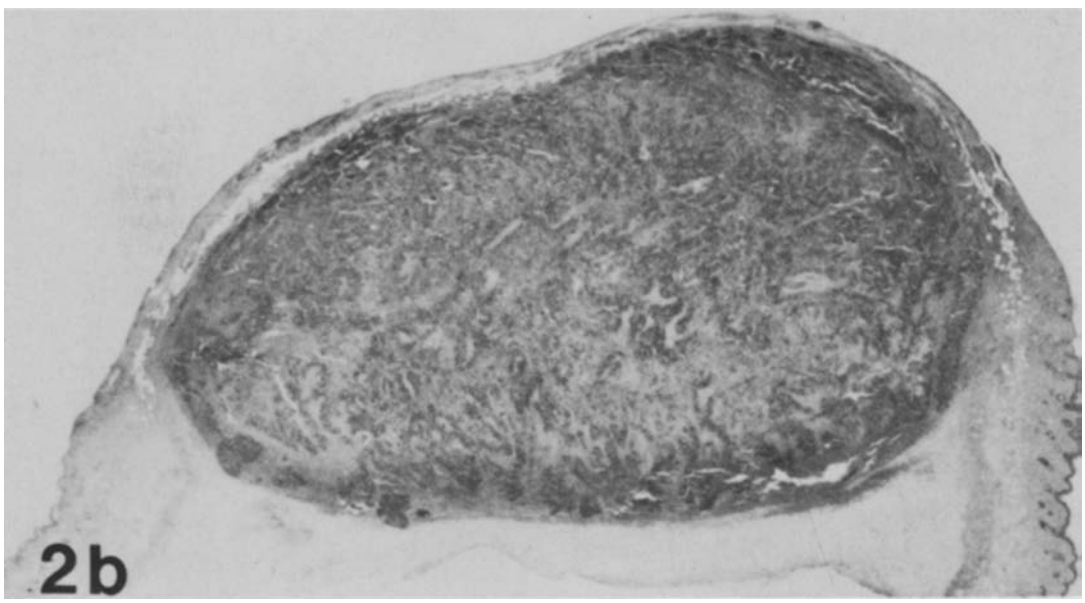
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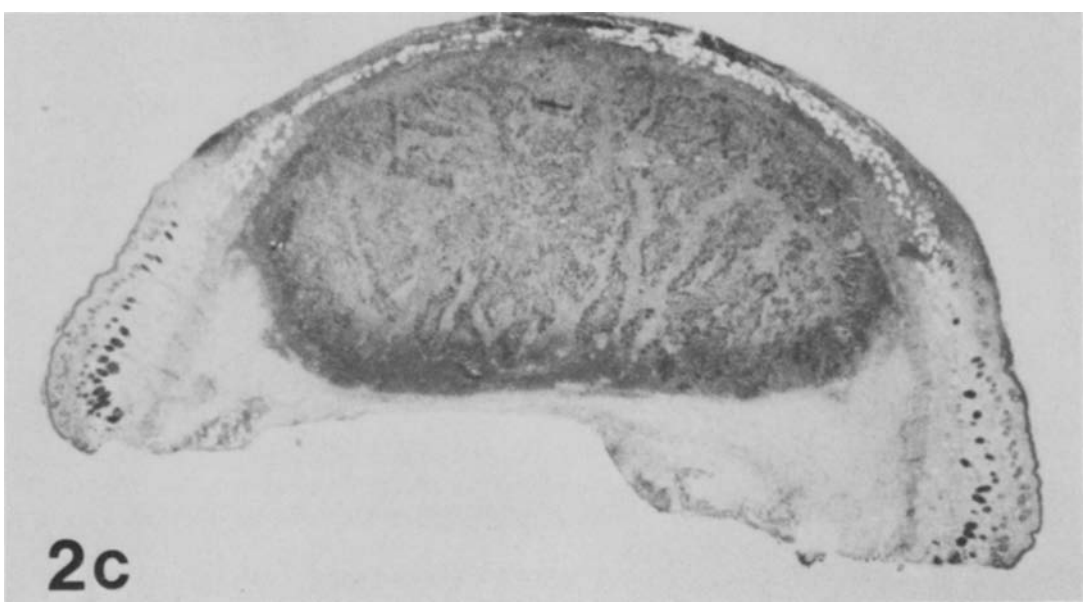
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