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Fluorescence in the diagnosis of malignancy

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FLUORESCENCE IN THE DIAGNOSIS OF MALIGNANCY

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INTRODUCTION

For centuries the solution to the enigma of cancer has been diligently sought by the wisest minds, using the most ingenious methods of inquiry they could devise. But an understanding of the cause and nature of this disease still eludes us.

Treatment by complete excision or destruction of the malignant tissue, before it has grown so large that to do so would kill him, offers the only hope at present to the individual who is found to have cancer. Thus, until the basic processes and causes of cancer are understood, and prevention or specific therapy is possible, earlier detection of the malignancy is the only means of decreasing the toll of cancer.

All methods of diagnosis rest upon the observation that malignant cells look and act different from normal cells. The assumption is made that the basic metabolic processes of the cancer cell, and perhaps therefore of the cancerous individual, are different from those of the normal. Measurement of these differences, or the results of these differences, is the present goal. The search for measurable chemical, enzymatic, or physical differences has utilized almost every mode of human perceptions.

This paper traces the development of one method of observing physical differences between normal and malignant cells, outlines its present applications, presents some theoretical aspects of possible relationships between fluorescence and malignancy, and indicates directions in which further investigation is being made.

THE PHENOMENON OF FLUORESCENCE

Fluorescence is defined as the property possessed by certain compounds and substances of absorbing radiant energy over a characteristic range of wavelengths, and converting this energy into chemical or radiant energy of the same or longer wavelengths, such as heat or visible light.(13) Just how this is done is not known. It is presumed to involve electronic rearrangement.

Physical characteristics of fluorescence are not easy to account for. The fluorescence of a substance in solution is proportional to its concentration only in very dilute solutions. At higher concentration, its fluorescence may disappear completely. Changes in solvents, or in the pH of the solution may quench the fluorescence or alter its color or brightness. Each fluorescent substance has a specific and characteristic band or pattern of wavelengths, energy from which will excite its fluorescence.

As commonly used, ultraviolet light furnishes the energy for the excitation of visible fluorescence. Ultraviolet light is invisible, being in the range of wavelengths (below 3800 Å. units) not perceived by the human eye, which is only stimulated by radiations having wavelengths of from 3800 Å. in the violet region to 7600 Å. in the red region of the spectrum.

Some of the sources of ultraviolet rays commonly used in studying fluorescent substances are low voltage tungsten lamps, carbon arcs, and mercury arcs, which are preferred by most workers.

Special filters and monochromators are often used to provide a beam having only a very narrow range of wavelengths within the ultraviolet region. Special glass or quartz must be used in the optical systems so as not to absorb more than a small fraction of the ultraviolet rays.

A substance can be studied either by recording the pattern of the emitted visible fluorescence when it is struck by ultraviolet light, or by using a continuous spectrum of ultraviolet light for excitation, and measuring the location and amount of absorption which has occurred in the process of excitation of the substance being tested.

Fluorescence of substances can be observed or measured in several ways: 1. directly by the eye in a darkened room, 2. through a spectroscope visually, 3, through a spectroscope with a photographic recording apparatus, or 4. with a photoelectric spectrophotometer.

Technical discussion of the many variations and combinations of equipment used for study of fluorescence phenomenon is beyond the scope of this paper. It is obvious, however, that progress in the use of fluorescence phenomena in diagnosis of malignancy has been dependent in a large measure upon invention and refinements of the tools, since only sensitivity and accuracy of observation of these physical phenomena allows detection of differences between the normal and the cancerous cell, tissue, or individual.

EARLY OBSERVATIONS OF FLUORESCENCE

The term fluorescence was coined by Stokes in 1852 to describe the luminosity which he observed when he examined the mineral fluorite under ultraviolet light.(66) The general foundation of the physics and optics of fluorescence phenomena were laid down by Wood in 1903 and Lehman in 1910.(73, 42)

Helmholtz in 1896 and Hess in 1911 observed fluorescence in the eyes of insects and in the lens of the eyes of vertebrates. (27, 30) Nearly all animal cells and tissues when examined were found to possess the property of fluorescing in filtered ultraviolet light by Stübel in 1911.(68) He also observed that pigment, hemoglobin, and hemoglobin derivatives diminish or quench this fluorescence, and that bacterial growth or tissue necrosis changes the color of the fluorescence.

Bommer in 1927 reported observing spontaneous fluorescence of various human internal organs under ultraviolet light. (4) By hanging an ultraviolet light over his operating table, Havlicek attempted to differentiate tissues by their fluorescence, his findings being published later by Dankwortt.(26) In 1934 Dankwortt studied and described the appearance of normal tissues under ultraviolet light. He reviewed the literature, and mentioned the possibility of bringing "the luminescent substance stored in malignant tissue" to fluoresce in ultraviolet light.(8)

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I. Fluorescence of Malignant Tissue

The first association of fluorescence with malignancy was Policard's observation in 1924 of red fluorescence in the central necrotic portion of induced rat sarcomas. He believed this fluorescence to be due to hematoporphyrin, a product of bacterial decomposition of blood.(52) Körbler, in 1931, observed fluorescence in certain human carcinomas, and attempted inconclusively to prove that it was caused by primary metabolic differences between tumor cells and normal cells.(41)

The different colored fluorescences of particular gastro-intestinal tumors were described by Sutro and Burman in 1933. They advocated use of ultraviolet light in the examination of pathological tissues, stating that often small areas of tumor could be located by their fluorescence which would otherwise be missed. These workers were unable to find a specific correlation between the fluorescence reaction and the pathological diagnosis of tissues, however.(69)

Chloromas, named for their green color in ordinary light, were found to fluoresce brilliant red in near ultraviolet light by Thomas in 1938.(71) He extracted this red fluorescent material and proved it to be protoporphyrin. He also found the same substance in the fluorescent centers of lymph nodes in two cases of myelogenous leukemia.

Gougeret and Patté in 1939 reported success in the differentiation by its fluorescence of a basal cell carcinoma of the skin from a spinocellular carcinoma.(22) Roffo and his coworkers first reported in 1939 that precancerous skin lesions fluoresce a strong bluish-white in ultraviolet light. They have used this fluorescence to differentiate precancerous lesions from hyperkeratoses.(56)

Utilizing the observation of Goodman in 1928 that skin melanomas, including those appearing unpigmented in ordinary illumination, appear strikingly dark against the normal skin fluorescence when viewed under ultraviolet light,(21) Terry in 1943 used ultraviolet light for diagnosing unpigmented nevi and for locating unpigmented metastases from multiple melanomas.(70)

Herly in 1944, after examining under ultraviolet light 200 specimens of breast tissue removed at operation, described the colors of fluorescence characteristic of each tissue and of various disease processes. Malignancy, he found, characteristically shows a strong purple fluorescence, shading to tan, orange, and brown. By this method of examination, as checked by routine pathological sections, he missed only one diagnosis of malignancy, as against one error by frozen section. Because of its accuracy and simplicity, he urged adoption of ultraviolet light examinations for quick diagnosis during operation. (28)

In searching the literature, I have not found confirmation of this work of Herly. If this method is as reliable in other hands as it is for its originator, it would seem worthy of trial, especially in smaller hospitals with limited lab facilities.

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II. Fluorescence of Tissue Extracts

Since 1942, many conflicting and inconclusive reports have been published dealing with a blue fluorescent substance found in extracts of human cancerous livers. Penn in 1942 first described the substance as being present in lipoid extracts of human cancerous livers, and absent in similar extracts of normal livers.(50) Hieger, 1942, disputed these findings,(31) as did Jones and May in 1944, who found the blue fluorescent substance in normal as well as in cancerous extracts, both with spectra of peak absorption at 2550 to 2600 Å, but not characteristic of polynuclear hydrocarbon. When methylcholanthrene was added to normal liver before the same extraction process, however, it was found to be concentrated in the same fraction as the unknown blue fluorescent substance.(39)

Penn and Kaplan, in 1947, on repeating the original work, found that the same fluorescent substance was present in both cancerous and non-cancerous liver lipoids, but stronger in cancerous extracts. They found the absorption spectra to be closely similar to that of methylcholanthrene.(51)

Jones and Jamieson replied in 1947 that they had not been able to recognize in tissue extracts any polynuclear hydrocarbons by ultraviolet spectrophotometry. Their fluorescent extract closely resembled anhydrous vitamin A in physical

characteristics, and had no carcinogenic activity. However, their extract did not contain sterols so they concluded that their findings may not be contradictory after all. (38)

Hieger, in 1947, on the other hand, finds carcinogenic activity in the cholesterol fraction of such material. Cholesterol, he states, is a slow carcinogen: probably other much more potent carcinogenic substances isolated along with the cholesterol give such extracts their power of carcinogenesis. He believes these unknown carcinogenic substances are not found only in extracts of cancerous livers, but are also found in many human tissues, cow cream, etc. (32)

These experiments have not produced as yet any observations useful in diagnosis of malignancy. Studies potentially, perhaps, more fruitful for diagnosis are those reported in 1947 by Sano and Spiegel-Adolph. Ultraspectrophotometric observation of extracts of human tissues show characteristically the following results: peaks of absorption by normal liver, at 2650 Å, by carcinoma, at 2600 Å, and by inflammatory lymph nodes, at 2500 Å. Further studies of these phenomena are now in progress. (57)

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III. Fluorescence of Urine

As early as 1869, blue-green fluorescence of normal urine on exposure to ultraviolet light was noted by Jaffé.(34) Many workers have searched for the cause of this color.

Longecker in 1922 concluded that the familiar urinary constituents are not responsible for the fluorescence of normal urine.(44) Kinnersby, Peters, and Squires in 1925 found that the urochrome fraction of urine fluoresces blue when diluted to a pale yellow.(40) Systematic observations on eight normal individuals by Squires and by Squires and Jeffree in 1928 showed that the daily output of fluorescent material was independent of the volume of urine excreted, and that the fluorescent material bore no close relation to any of the tested urinary constituents. (63, 64)

Simon in 1939 studied the question with considerable care and came to the conclusion that there was no relationship between the fluorescence of urine and any disease process, and that it had no merit as a test for cancer. (58)

However, Stevens and Dick in 1946 again report the finding of a fluorescent substance in human urine, which on examination with a spectrophotometer yielded two sharp bands in the blue region of the spectrum, at 4100 and 4400 A. The physical characteristics of this substance indicated to these workers

that it was closely related to known carcinogenic substances.(65)

Studies on a red fluorescent and a blue fluorescent substance in urine were reported by Rabinowitz in 1949. Using a source of filtered ultraviolet lightand a fluorophotometer with a red and a blue filter, and reading the intensity of the fluorescence in arbitrary units on a galvanometer, he has found that the values for the blue substance (B) and the red substance (R) vary in a characteristic manner. The B:R ratios have different and distinctive values in each group: normal, benign growth and pregnancy, and malignancy.

A striking finding in a group of 22 cases of malignancy, studied preoperatively and followed postoperatively for a year or more, is that the B:R ratios reverted to normal four to eight weeks after the operation. In two cases of subtotal gastrectomy for adenocarcinoma of the stomach, normal B:R ratios were found while the patients were enjoying good health for 12 and 20 months respectively postoperatively. Reversion of the B:R ratios to the previous malignancy pattern occurred along with clinical and X-ray evidence of metastases and local extension. (53)

This work is of particular interest in the light of the work of Figge's group on the role of red fluorescent porphyrins in carcinogenesis (see pp. 20). Rabinowitz presents his findings not as diagnostic of cancer at present, but as a study of the possible role of the red and blue fluorescent substances with relation to the mechanism of growth and its alterations. (53)

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IV. Fluorescence of Serum

In 1931 the first published observations of blood serum under ultraviolet light were made by Reche, who found that sera from diseased persons fluoresced various colors but sera from healthy persons did not fluoresce.(54)

Further studies on sera were made by Boutaric and coworkers in 1934 and 1935, using the method of addition of sera to solutions of fluorescent uranin. At first it was reported that sera from persons having cancer decreased the fluorescence of the uranin solutions, and that sera from normal persons did not. Later investigations showed the reaction to lack specificity--that is, the action was not limited to cancer sera, and some cancer sera lack the described capacity. The test was considered inconclusive and was dropped. (1, 5, 6)

Another test using human sera has been described by Herly, in 1950. He observes human sera by transmitted filtered ultraviolet light having peak wavelength at 3600 Å, using subjective comparison of the fluorescence, and a light meter with an arbitrary scale for measurement of the transmission of light. He finds that sera from animals and humans with cancer transmit more light and fluorescence than normal and nonmalignant sera. The color of the fluorescence in cancer changes to turquoise blue or purple from the normal yellow or green. (29)

Herly warns that possible sources of confusion in using this method are hemolized or fatty sera, presence of bilirubin, sex hormones, transfusions, hyperpyrexia, acidosis, toxemias, and pregnancy. He advocates following and rechecking all 'false positives', because he has found, by means of rat experiments with transplanted tumors, that the serum changes occur long before the tumor becomes visible or palpable. Another interesting finding is that surgical removal of these rat tumors will cause reversal of the reaction to normal in 21 days if all tumor has been removed. (29)

This method seems to present interesting possibilities. Before its general adoption is possible, refinements in measuring the observed changes will be necessary. This work has not yet been confirmed by other investigators.

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V. Affinity of Malignant Tissue for Fluorescent Dyes

A. Gross Examination after Intra-Vital Staining

A great deal of experimentation was carried out by many workers in attempts to find satisfactory methods of selective intra-vital staining of body tissues, and especially of malignant tissues. Among these earlier experimenters are Weil, 1916, Simpson and Marsh, 1926 and 1927, Ludford, 1929, Duran-Reynals, 1939, Zahl and Waters, 1941. (11, 45, 60, 72, 74)

Sorsby, Wright, and Elkeles in 1942 summarized the findings and difficulties thus: acid dyes were concentrated to some extent in tumors, but in the histiocytes, fibroblasts, monocytes, and lympho-cytes of the stroma rather than in the tumor cells; acid dyes are not concentrated in brain tumors; basic dyes are very toxic; ampho-teric dyes investigated to date are concentrated in some tumors, not all, and frighten the patient and the anesthetist by staining the skin deeply. (62)

It was in 1942 that Auler and Banzer observed the tenden-cy for injected fluorescent hematoporphyrin to accumulate in neoplastic tissues of rats. (2) No further studies were made on this phenomenon, however, until it was rediscovered independently by Figge, Weiland, and Manganiello in 1948 during the course of experiments on cocarcinogenesis of hematoporphyrin in mice. (17)

The fluorescent hematoporphyrin has been found to concentrate in all tumors of mice tested, as well as in placenta, embryo, lymph nodes, and omentum, and in traumatized tissues. All other porphyrins tested concentrated in the various tissues similarly. Addition of a metal atom, Zinc, to the porphyrin molecule did not interfere with its concentration in the same tissues. This metal-containing substance has been used in therapy with some success to sensitize deep tumors to penetrating radiation.

In addition to the diagnosis and location of tumors by the fluorescence of these porphyrin compounds, a further possibility for diagnosis is being investigated--the possibility of developing radioactive metalloporphyrins for diagnosis of deep tumors, and possibly also for treatment of lymphatic leukemias. (17, 18)

Meanwhile, Moore in 1947 reported that after preoperative intravenous injection of sodium fluorescein, brilliant yellow fluorescence of tumors was seen at operation. Tumors placed deeply could not be located by this method, and it was least useful in tumors of the colon, stomach, and breast. However, for tumors just under the peritoneum, or for tumors of the brain and spinal cord, good results were obtained (out of twelve CNS tumors tried, eleven were well located.) Areas of edema and cysts are also observed to fluoresce, but necrotic tissue did not concentrate the fluorescent dye. (46)

Further success in using preoperative injection of fluorescein for location of CNS lesions is reported by Moore, Peyton, French, and Walker, 1948, and Hubbard and Moore, 1949. Tumor tissue appears brilliantly yellow-green against the less fluorescent normal brain tissue. Needle biopsy before opening the dura widely is used for exact localization of the tumor tissue. Direct examination under ultraviolet light of the site of excision of tumor is made after removal of all recognizable tumor tissue, often revealing the presence of remaining tumor tissue. In one series of 46 patients, 44 correct diagnoses were made by this method as checked by routine pathological sections, and one was questionable by both methods.

Meningeal, glial, and metastatic tumors can be well localized by this method. The authors advocate its use especially for subcortical lesions, and for differentiating infiltrating malignant tissue from surrounding normal tissue. The dye is also concentrated in areas of edema, in abscesses, and in cortical areas in some persons which seem to be associated with epilepsy.

The authors are working on the development of radioactive diiodofluorescein for localization and diagnosis of tumors preoperatively, and on a radioopaque fluorescein derivative to aid in X-ray diagnosis of malignancies. (33, 48)

In a preliminary report, Moore, 1948, states that results of location of CNS tumors using diiodofluorescein with a Geiger counter are good,(11 out of 12 correct diagnoses, negative as well as positive.47)

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V. Affinity of Malignant Tissue for Fluorescent Dyes

B. Microscopic Examination

The fluorescence microscope has been used for several years in the solution of many special problems: histological studies, Haitinger, 1933, '34, '35, Jenkins, 1937, Ellinger, 1940, Dempsey, 1944, Loewenstein, 1944 (9, 12, 24, 35, 43), diagnosis of tuberculosis, Richards, 1941 (55), and diagnosis of malarial and other protozoal parasites, Patton and Metcalf, 1943, and Bock and Oesterlin, 1939 (3, 49).

The remarkable photomicrographic studies of a fluorescent hydrocarbon absorbed in protoplasm of cancer cells made by Graffi were published in 1940. (23)

The application of this work to the diagnosis of malignancy was made by Friedman in 1950. He developed a staining technique using several fluorescent dyes, which, when applied to vaginal smears and examined under a microscope with filtered ultraviolet illumination, gives to malignant cells a characteristic orange or reddish brown fluorescence. Morphologic details are also shown well by this method. For differentiating malignant from normal cells, this method gives three criteria: morphologic differences, degree of fluorescence brilliance, and variations of nuclear and cytoplasmic colors. The method is equally useful for examining other fluids such as sputum, ascitic fluid, and pleural fluid. (19)

Preliminary observations by Sluter at the University of Nebraska confirm the findings of Friedman.

Further, comparison of results of parallel smears on numerous patients shows almost exact correlation in the degree of malignancy as diagnosed by 1. morphologic staging using the method of Papanicolaou, and 2.by the degree of fluorescence brilliance and shift of fluorescence color to the bright orange in the malignant cells, using the method of Friedman. (61) See Figure 1.

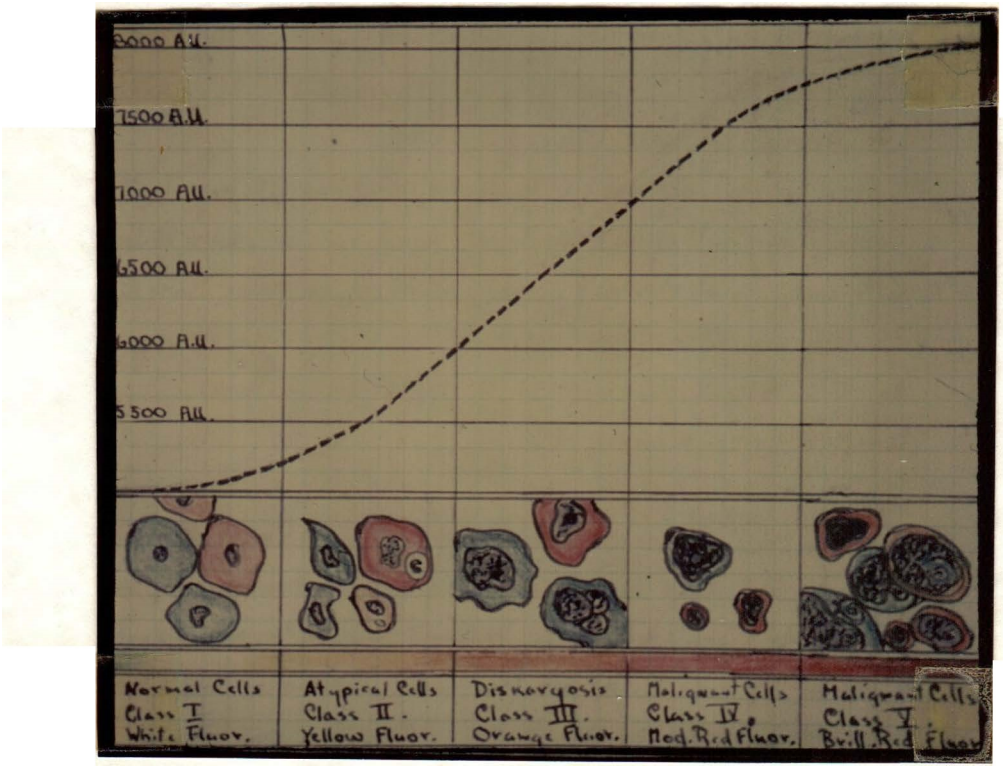

Figure 1. Above, cells staged as to malignancy according to the method of Papanicolaou. Below, line represents typical readings for brilliance of fluorescence and amount of orange coloring present according to the method of Friedman.

DEVELOPMENT OF THEORIES RELATING FLUORESCENCE AND MALIGNANCY

Fluorescence phenomena are curiously intertwined with the tangled knot of the cancer problem. As we have seen, fluorescence enters into diagnosis, location and treatment. Some hints that it may somehow be related to cause have been under investigation for some time.

As early as 1930, Kennaway and Hieger found that the fluorescence spectra of many of the known carcinogenic substances were similar, having peaks between 4000 and 4400 Å. At that time they suggested fluorescence studies as a preliminary screening in the search for other carcinogenic substances. (39)

Bruce in 1941 exploded by careful fluorescence and carcinogenicity analysis of a great many substances the attractive theory that the carcinogenicity of a compound is directly proportional to its fluorescence. (7)

A chance observation in 1941 has led a group at the University of Maryland School of Medicine, headed by Figge, along a chain of observations and experiments, story of which reads like a detective novel--with clues, red herrings, unlikely coincidences. But the story is not finished. The identity of the villain has not yet been discovered.

Quite by accident, Strong and Figge in 1941 saw brilliant red fluorescence in the orbital region of a mouse being dissected under ultraviolet light. Having readily accessible mice from many

strains, they observed other mice, and found that the Harderian glands of the cancer-susceptible strain of mice gave off brilliant red fluorescence but that glands of mice of a cancer-resistant strain did not demonstrate this fluorescence. (67)

Many animals were examined. The fluorescence of the Harderian glands was found not only to parallel closely the cancer susceptibility of the various strains of mice, but also to parallel the susceptibility to cancer induction among various laboratory animals. (16)

The red fluorescence of Harderian glands of mice had been discovered by Derrien and Turchini in 1925, and proven to be an indication of the presence of porphyrins. (10) Therefore, a study of porphyrins was taken up.

In 1944, Figge proposed the theory that "cells subjected to abnormally high concentrations of certain porphyrins become hypersensitive to the action of normal or abnormal growth-stimulating factors, or to relatively weak, natural or artificial carcinogenic stimuli." (14)

In attempting to apply this theory to human beings, a search for porphyrins in cancer susceptible organs was made. It had been observed that comedones of the face, especially the nose, fluoresced red in ultraviolet light in almost all persons tested, but in markedly varying degrees, Figge, 1942. (13) Red fluorescent deposits on female genitalia were then discovered, and proven to contain porphyrins. (15)

Red fluorescence of female genitalia had previously been been discovered, in 1929 during routine examinations under ultra-violet light by Hauser, who related these porphyrin deposits to decomposition of blood by bacterial action. (25)

Figge and coworkers undertook a systematic study in a large group of women to determine whether these red fluorescent deposits could lead to a diagnostic test for cancer. The results showed that they are present in most women in varying degrees at different times during the menstrual cycle. The red deposits were found in appreciable amounts in 40 % of the women tested. The deposition of porphyrins occurs intermittently over many years: it is greatly increased in foul lochia: and it may be caused by bacterial decomposition. These observed facts suggested to the group that porphyrins may be the link which connects multiparity, chronic infection, and increased incidence of carcinoma of the cervix. From this set of experiments, it was concluded that the red deposits are not useful as a diagnostic test. (36, 37)

The work on genital porphyrins yielded no immediate returns as to diagnostic tests, but the findings were considered strong indication of some relationship between porphyrins and carcinogenesis. Studies on on mice of cocarcinogenesis of porphyrin and methylcholanthrene were next undertaken. And here, in a study begun as pure research, it was found that injected hematoporphyrin localized well in the tumors—a propensity which has already been

used as a diagnostic test, and which is being investigated further for other possible diagnostic applications. (see pp. 13, 14)

The studies on cocarcinogenesis are still being carried on.

SUMMARY

1. A complete understanding of fluorescence phenomena and their relation to the whole problem of cancer has not yet been reached. Some technical aspects of fluorescence phenomena and their observation and measurement are presented.

2. The history of the observation of fluorescence is traced.

3. The development and present application to cancer diagnosis of fluorescence of tissues, tissue extracts, urine, and serum are outlined. Discovery and use of the affinity of malignant tissues for fluorescent dyes, both after intravital injection and in microscopic preparations are described.

4. The evolution of theories relating the fluorescent porphyrins to carcinogenesis, and the investigation of their role in cancer production are reviewed.

I wish to express my appreciation for the interest and assistance of S. J. Sluter, Jr., and for his permission to reproduce Figure 1.

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