A STUDY OF GLIOMAS AND OTHER CENTRAL NERVOUS SYSTEM LESIONS BASED ON FLUORESCENCE MICROSCOPY AFTER STAINING WITH ACRIDINE ORANGE

by

Bartolo M. Barone, B.S., M.D.

with the technical assistance of
John R. Gilbert, and Charles P. Hodge, F.B.P.A.

A thesis submitted to the Faculty of Graduate Studies and Research, in partial fulfilment of the requirements for the degree of Master of Science.

Department of Neurology & Neurosurgery,
McGill University,
Montreal.

April 1962.

TABLE OF CONTENTS

.....

I. INTRODUCTION

Quick frozen section of brain tumors, as well as the smear technique developed here at the Montreal Neurological Institute by the late Dr. W.V. Cone are extremely valuable means for arriving at a preliminary pathological diagnosis which can be relayed to the surgeon prior to the completion of an operation. We felt, however, that the fine histological distinction between benign and malignant intracranial lesions which arises in some cases could not be readily resolved by using the above techniques alone and that, perhaps, a technique could be devised to help clarify these problem cases. This reasoning would apply to extrinsic brain tumors as well, for example, meningeal fibroblastoma, perineurial fibroblastoma and carcinoma.

It is important to realize that in many instances the specimens of brain tumor, to be examined, are very small. The routine smear specimen consists of a fine thread of tissue aspirated through a biopsy needle.

In the recent past, many laboratories have employed fluorescence microscopy after staining with acridine orange, as a screening method to pick up malignant exfoliative cells in vaginal, bronchoscopic and ascitic smears; instances where the specimen to be examined was limited. The

technique itself is fairly simple and the time required to examine the stained specimen is relatively short, 8 to 10 minutes, and as a result, can be readily applied while the operation is still in progress.

Consequent to the various observations, namely, on the changes in RNA and DNA in tumor cells (Caspersson, 1947) and the ability of acridine orange to differentially stain RNA and DNA, (Schümmelfeder, 1957a), Von Bertalanffy and co-workers (1956a, 1957, 1958, 1959, 1959a, 1960) have written a long series of papers on the use of acridine orange as an analytical, histochemical method in the cyto-diagnosis of malignant cells. Preliminarily, the latter investigators employed this fluorchrome mainly in the study of cervical and uterine cancer, but it has since been extended to include pulmonary and ascitic malignancies as well. To-date, no one has applied it to a study of brain tumor smears.

Although the ultraviolet microscope has been used for histological and histochemical purposes for many years, there has recently been a considerable revival in interest in fluorescence microscopy and in its employment for a number of new techniques. This is partly due to the impetus provided by the fluorescent antibody methods whose general adoption has meant that ultraviolet microscopes are now to be found in many institutes which formerly possessed none. Partly, however, it is due to the increasing realization that,

despite its many disadvantages, fluorescence microscopy is capable of raising the sensitivity of any histochemical method whose final product in the tissues is fluorescent, or can be modified so as to be fluorescent. The phenomenon of fluorescence is exhibited by materials which, having absorbed light energy of a specific range of frequencies, can re-emit part of this energy at a higher wavelength level.

This report, then, describes both the techniques and the results of application of these techniques to a wide variety of intracranial lesions as regards whether or not a quick differentiation between benign and malignant tumor can be made using the acridine orange smear technique.

An analysis of cells obtained from cerebrospinal fluid and ventricular fluid is also included plus additional comments as to ancillary fields of investigation which appear profitable.

II. REVIEW OF LITERATURE

Many previous investigators have shown that the vital activities of cells are controlled to a great extent by complex conjugated proteins, nucleoproteins, which consist of one or more protein molecules combined with nucleic acids. These nucleic acids are esters of phosphoric acids and glucosides that consist of a pentose sugar (ribose or desoxyribose) and a cyclic derivative (adenine, guanine, cytosine, thymine or uracil). The desoxyribonucleic acid is found in the nucleus and the ribonucleic acid in the nucleolus and cytoplasm of animal cells. These two acids differ in many chemical and physical respects. However, both are precipitable from alkaline solutions by hydrochloric acid and ribonucleic acid is also precipitated by acetic acid. Both form salts with alkaline earths or heavy metals. Histochemical methods for their demonstration in tissues, have been based on reactions for all these constituents.

Before we enter into the controversy of whether acridine orange is a specific stain for ribonucleic and desoxyribonucleic acid and also the problem of localization of RNA and DNA in the cell, let us first review some of the earlier methods of studying these two chemical substances. Feulgen and Rossenbeck (1924) first developed the leucobasic fuchsin

method as a microchemical test for the DNA found in chromosomes. It depends on Schiff's aldehyde reaction and the liberation of the aldehyde groups of nucleic acid by mild hydrolysis in normal hydrochloric acid at 60 degrees centigrade, resulting in a violet colouration of the chromosomes. Since Pappenheim's (1899) and Unna's (1887) original work, it has been known that DNA, largely confined to the nucleus in mammalian cells, can also be preferentially stained by methylgreen. These two methods then, have received wide application in histochemical studies and have undergone varying forms of modifications by different investigators. (Brachet, 1933, 1940, 1947; Kurnick, 1952; Barka, 1959.) Whereas the localization of DNA has been a relatively simple problem histochemically, identification of RNA has given some difficulty. Taft (1951) reported that pyronin-Y was a specific stain for RNA but the other pyronins, such as pyronin-B were of no value in this regard. Kurnick (1952) substantiated this work so that now a combination of methyl-green and pyronin is again being employed as a differential nucleic acid stain. Presently, in this laboratory, we are beginning a study of paraffin fixed brain tissue sections comparing the methyl-green pyronin stain with acridine orange. This study, however, is beyond the scope of this paper, and will be treated in later publications.

With the advent of the enzyme treatment of tissue preparations, further advances in histochemical techniques ensued.

Chemically pure ribonuclease can be used either to pretreat an unstained specimen and then stain it; or depending on the technique, to stain the tissue and after visualization de-stain and treat with ribonuclease. Acridine orange staining can be compared with methyl-green pyronin or some other stain which according to some investigators is more specific for RNA and DNA; for example, Einarson's gallocyanin-chromalum method.

The story of the use of acridine orange as a tissue stain goes back to 1933, when according to Bukatsch and Haitinger, (1940) the latter author and Linsbauer first used a fluorescent microscope to study tissues before and after application of different fluorochromes. They stated that fluorescent microscopy was useful to study changes in the components of tissue not visible in ordinary light or to simplify many complicated histochemical methods down to one stain.. Much of the original work with fluorescent microscopic techniques was done on botanical problems in an attempt to delineate how plants took up certain materials and thence how they were distributed.

Bukatsch and Haitinger (1940) used coriphosphin-O (an acridine derivative) in a solution of 1:10,000. They observed greenish-yellow fluorescing nuclei and orange fluorescing cytoplasm. With acridine orange they observed green fluorescing cytoplasm. These studies were on living plant cells. Strugger, (1941) was the first to report that

acridine orange could be used to distinguish between living and dead protoplast, the former giving a green and the latter a red fluorescence. He postulated that by death one destroyed the ordered protein structure. Strugger used the fluorochrome, acridine orange, to study mainly plant cells and bacteria and felt that dead cells stored the basic dye more strongly than living cells, thus resulting in the red fluorescence. Schümmelfeder (1950a) confirmed Strugger's findings in animal cells. Schümmelfeder postulated that with death, the nuclear protein in the cell was split into protein and acid fractions and the acid fraction was further split into protein and nucleic acid. This breakdown resulted in both basic and acid groups which were now free to bind the acridine orange.

The acridine orange method in exfoliative cytology, is the result of studies on the role of nucleic acids in partial agenesis carried out by von Bertalanffy and co-workers in 1952. Pirozynski and von Bertalanffy (1952) observed that morphological nuclear changes were appreciated by changes in RNA content. Moreover, it was found by von Bertalanffy and Bickis (1956) using either fresh smears or fresh frozen sections, that staining with acridine orange permitted one to distinguish the red fluorescent basophilic inclusions in the cytoplasm, removable by ribonuclease, as well as green fluorescing nuclei. They felt that fluorescence microscopy, using the fluorochrome acridine orange, is an excellent means

of demonstrating the two types of nucleic acid of the cell. DNA appears as a yellowish-green and RNA of the cytoplasm appears in order of increasing concentration as brown, reddish-brown, orange to bright red fluorescence.

Localization of DNA and RNA

In 1933 Einarson called attention to the many functional stages which may be observed in the Purkinje cell. He clearly formulated the theory as to the formation of Nissl substance, which was later confirmed by Caspersson (1941) and Sandritter and Siegert (1954). The main points in the theory were as follows: the Nissl substance is formed around the nucleolus and diffuses through the nuclear membrane, where it forms a distinct nuclear cap and then passes into the cytoplasm. Since Einarson's work, there has been a tremendous amount of basic research on the localization of DNA and RNA, using various techniques, all based on morphological criteria. (Behrens, 1938; Brachet, 1947; Gulick, 1941; Mirsky and Pollister, 1942, 1943 and 1944).

Today, it is a well-established fact that DNA is limited chiefly to the nucleus and RNA to the nucleolus and cytoplasm of cells. Following the original description of the localization of DNA and RNA in cells, investigators began to quantitate the varying amounts of DNA and RNA in different pathological conditions. Caspersson et al. (1941a) in the study of human carcinoma, found that the cytoplasm of tumor cells contains larger amounts of RNA than corresponding normal cells.

Tha amounts varied in different parts of the same tumor, and were larger in areas of more rapid growth. Stowell (1942) using the Feulgen reaction with photometric quantitation has shown that some epidermoid carcinomas of mice and man (methylcholanthrene induced carcinomas in mice) and leukemic blood cells from the human subject, contain increased amounts of DNA. Claude and Potter (1943) estimated that in leukemic blood cells, the DNA comprised 40% of the chromatin complex. Pollister and Mirsky (1944) showed that rapidly dividing cells have high concentration of DNA and especially RNA, whereas the absorption band of mature cells show that they consist chiefly of protein. It has been stated, Stowell (1945), that nucleoproteins control the hereditary and vital functions of living cells and that DNA has important roles in mitosis, in polymerization of nucleoprotein and in the formation of proteins and of RNA of the nucleolus and cytoplasm. As evidenced from the above references, cytochemical studies have demonstrated that the cytoplasm of malignant cells contains increased amounts of RNA and in the minds of some investigators, suggests that the heterochromatic region of the chromatin plays a specific role in carcinogenesis.

Specificity of Acridine Orange

To date, there are numerous reports in the literature on the capacity of acridine orange to stain components of cells, removable specifically by either ribonuclease or

desoxyribonuclease, as the case may be. Gossner (1950) treated mouse and cow pancreas mounted in paraffin, fixed in Carnoy's solution, and employed the Veronal-acetate buffer system as described by Michaelis at pH 6.8. Dye concentrations used varied from 1:1,000, 1:5,000, and 1:10,000. Following treatment of the specimens with ribonuclease (0.1 milligram /cc. at 56°C. for 15 minutes to 1 hour), the specimens lost their red fluorescence. Liver and nerve cells were also studied and all showed high amounts of red fluorescing material removable by ribonuclease. Gossner pointed out that even after ribonuclease treatment, mast cells and cartilage retained their red fluorescence because of the acid mucopolysaccarides present (we were able to substantiate this in our case of chordoma). He studied live mouse ascites tumor cells and observed green fluorescing nuclei and cytoplasm with an acridine orange concentration of 1:10,000 at pH 6.8. Following treatment with formalin, both fluoresced red. Gossner also suggested that, because of this change in fluorescent colour, the acridine orange stain might be useful in a study of the changes in cellular elements occurring at the time of death of the cell.

Since Gossner's work, Schümmelfeder (1950a & b, 1957a) has carried out many extensive investigations on the histochemical significance of the fluorescence induced by acridine orange and has further enlarged and clarified the concepts as put forth by Gossner. Schümmelfeder in 1958 showed

that the cation of the basic fluorochrome, acridine orange, shows a gradual change of fluorescent colour from green through yellow and orange to red, when the concentration of the dye increases in aqueous solutions. The metachromatic fluorescence, he showed, is due to the formation of red fluorescent polymer cations of acridine orange, and the intermediate colours, yellow and orange,are due to different mixtures of green fluorescent monomer and red fluorescent polymer cations. He also showed that acridine orange in alcoholic solution becomes bound to nucleic acid in two ways: a) by electrostatic salt linkages and b) by cohesive forces. The affinity of acid substances in cells to the basic fluorochrome acridine orange was very strong, in his opinion, and this was best shown he felt, in his studies on ascites tumor cells.

The different fluorescence of RNA and DNA is due to their degree of depolymerization. The highly polymerized DNA of the untreated nuclei prevents the red fluorescence. After depolymerization, by boiling in water or by treatment with warm trichloracetic acid, cold perchloric acid or by formalin fixation, the DNA in the nucleus shows the same staining quality as cytoplasmic RNA (Bucher, 1946 and Schümmelfeder, 1957a). This can be well brought out by fixing fresh tissue in formalin and then staining with acridine orange.

The one big class of substances present in cells which tends to confuse the specific picture of acridine orange to stain RNA and DNA is the acid mucopolysaccarides; however, these substances can be differentiated quite easily from RNA by treating the specimen with ribonuclease, which will not interfere with the red fluorescence due to acid mucopolysaccarides, but which will eliminate the red fluorescence due to RNA.

Acridine orange continues to be used extensively in fluorescent microscopy, especially for the demonstration of nucleic acids, both in normal cells such as neurones and in malignant cells. Considerable controversy still reigns, however, over the interpretation of the specificity of acridine orange to stain RNA and DNA. Einarson, (1960) strictly speaking feels that this specificity is somewhat doubtful, yet application of ribonuclease to the prepared specimens after staining with acridine orange results in a loss of all the red fluorescing material. As a more specific stain, Einarson uses the gallocyanin-chromalum technique whereby the staining process consists in a selective binding of the dyelake cations to the phosphoric acid groups of the polynucleotides. Supposedly, the staining is of an exquisite progressivity and completely withstands alcoholic dehydration and clearing in xylene. The nucleoproteins become progressively occupied by the lake cations until

maximum occupation has taken place, resulting in a quantitative reaction for nucleic acids. Schümmelfeder (1957) used acridine orange to demonstrate early histochemical changes in Purkinje cells, following exposure of the animal subjects to high doses of radiation. He also undertook an exhaustive study correlating his results, using acridine orange, with those of Einarson using the gallocyanin-chromalum method, and also found that his results corresponded with the results of the more classical method for differentially staining RNA and DNA, namely, the methyl-green pyronin stain.

The Role of Acridine Orange as a Cytochemical Analytic Method

Santesson and Caspersson (1942) and Caspersson (1947) showed that in the tumor cell, the normal inhibitory mechanism for the protein forming system in the cytoplasm is checked or entirely absent and, as a result, the cells are recklessly trying to grow as rapidly as the supply of raw material allows. This never occurred, in their judgment, in the normal cell where, in all cases studied, the cell breaks down the system for protein production when the supply falls below a certain level. They concluded that this made it possible to identify an individual cell as being malignant by cytochemical analysis. The cause of this change, of course, is still unknown and when solved, will bring us closer to the etiologic basis of anaplasia.

Their observations indicated that the inhibitory mechanism for the protein forming system is to be found in the nucleolus associated chromatin or possibly, in those centres which regulate their apparatus.

Consequent to these various observations, namely, on the changes in RNA and DNA in tumour cells and the ability of acridine orange to differentially stain RNA and DNA, von Bertalanffy, and co-workers (1956, 1956a, 1958, 1959, 1960) have written a long series of papers on the use of acridine orange (3,6 tetramethyldiamino acridine) as an analytical histochemical method in the cytodiagnosis of malignant cells. Preliminarily, the use of this fluorochrome was limited mainly to the study of cervical and uterine cancer, but has since been extended to include pulmonary and ascitic malignancies (Bertalanffy, von, 1960).

Today, the acridine orange smear technique is widely used as a rapid screening method for malignancy. It is a routine procedure at many gynecological centres (Dart,1959; Hunter, 1959; Kaplan,1960). Holland (1961) has published an analysis of the application of the acridine orange smear technique in the office practice of gynecology, that is, as regards the detection of cervical carcinoma.

The various people working in cytopathology,as described above plus others (Sussman, 1959; Umiker, 1959) agree on the merits and reliability of the technique.

As alluded to previously, many investigators have used this method in the cytodiagnosis of malignant cells and in the study of histochemical changes in neurones (Schümmelfeder 1957) but none have applied it to smears of tumors affecting the nervous system.

III. MATERIAL AND METHODS

A. Plan of Study

Using the facilities of the Department of Neuropathology, we made smears of all tumour specimens submitted from the operating rooms, as well as smears from selected cases of surgical intervention for uncontrolled, focal cerebral seizures. The latter were used as non-tumour controls. The remainder of the tissue was processed in the routine manner in accordance with the technique of the department and the permanent sections so obtained were used as controls against which we could compare our results.

In our preliminary studies, we found that the staining method as described by Bertalanffy et al (1956) is the best method of achieving consistently reproducible results. Consequently, except for some minor variations, we employed their technique rigorously.

We were not primarily interested in measuring the fine quantitative differences in the degree of red fluorescence in the cytoplasm of cells, but in the all or none phonomenon which we felt would best serve the object of the experiment; that is, a rapid means of differentiating a benign from a malignant glioma, bright red fluorescence in the cytoplasm

of glial cells indicating malignancy and little or no red fluorescing cytoplasm indicating normalcy or neoplasia, benign in nature. This is, indeed, an over-simplification of the application of this type of technique. However, to be useful as a complementary method to the already employed smear and quick frozen section techniques, it would have to be clearcut with little room for speculative interpretation.

The examination of smears was set up as follows:

a) For the study of non tumor brain, the resection material from operations for focal cerebral seizures was employed. These were thirteen (13) in number and cases in which, on permanent histological section, did not show any evidence of tumor but did show, for the most part, varying degrees of gliosis. In the few specimens of epilepsy patients which, on permanent histological examination, showed some abnormal but not diagnostic features suggestive of something more than just gliosis, the case was discarded from this study and not used.

b) In the category of benign extrinsic brain tumors, there were seven (7) meningeal fibroblastomas (meningiomas), and four (4) perineurial fibroblastomas (acoustic neurinomas).

c) In the category of malignant extrinsic nervous system tumors there were two (2) fibrosarcomas, one affecting the median nerve, the other involving the region of the

temporalis fascia over a previous craniotomy site.

d) In the category of benign intrinsic brain tumors, there were six (6) piloid astrocytomas and one (1) oligodendroglioma.

e) In the group of malignant intrinsic brain tumors, there were seven (7) malignant astrocytomas and nine (9) full-blown glioblastoma multiforme.

f) In the metastatic carcinoma group, there was one (1) from prostate, two (2) from breast, one (1) from bowel, one (1) from kidney and one (1) from lung.

g) Cerebrospinal fluid specimens on two (2) patients with medulloblastoma and one (1) patient with meningeal sarcomatosis were examined and ventricular fluid in one (1) patient with known recurrence of a malignant astrocytoma involving the wall of the right lateral ventricle. There were also seven (7) cases examined of mixed etiology as well as one (1) case of a radiologically diagnosed brain stem tumor.

B. Microscopic and Photographic Setup

As a source of light, we employed the new Leitz Illuminator with the Osram HBO 200 high pressure mercury burner. This new housing has an improved light collecting system so that the exciting light output is very strong. Preliminarily, we used, as the exciting radiation, the 365 millimicron mercury spectral line. This was accomplished by filtering the remainder of the light with a UG-1 (4mm.) filter. This

filter also passes the red end of the spectrum in the neighbourhood of 700 millimicrons. To eliminate this, an absorption cell containing 3% copper sulphate solution 1 cm. thick was introduced into the system. To protect the eyes and the film from ultraviolet radiation, an ultraviolet absorbing filter, Euphos, was used between the objective and the eye pieces.

For most of the investigative work, we employed the 435 millimicron mercury spectral line by filtering the light with a BG-12 (8 mm.) filter. In order to obtain a black background, a barrier filter OG-1 (orange) was used between the objective and the eye pieces. This latter filter absorbs the bluish 435 millimicron line. As a result, more light reaches the specimen, giving a stronger fluorescence. Most investigators working with acridine orange use this latter series of filters. The microscope was a Leitz Ortholux containing a Berek condenser with a numerical aperture of 0.95. Three objectives were used: two apochromatic (x 12 - NA. 30, and x 40 - NA. 95) and one achromatic (x 45 - NA. 65). For the photographic setup a mirror reflex housing with a 35 mm. Leica camera back containing an intermediate lens to reduce the magnification in the microscope to one third was employed, resulting in a greater photographic field. (Figure 1.). By this method we were able to decrease the exposure time and this factor, together with the perfection of negative developing technique devised by Mr.

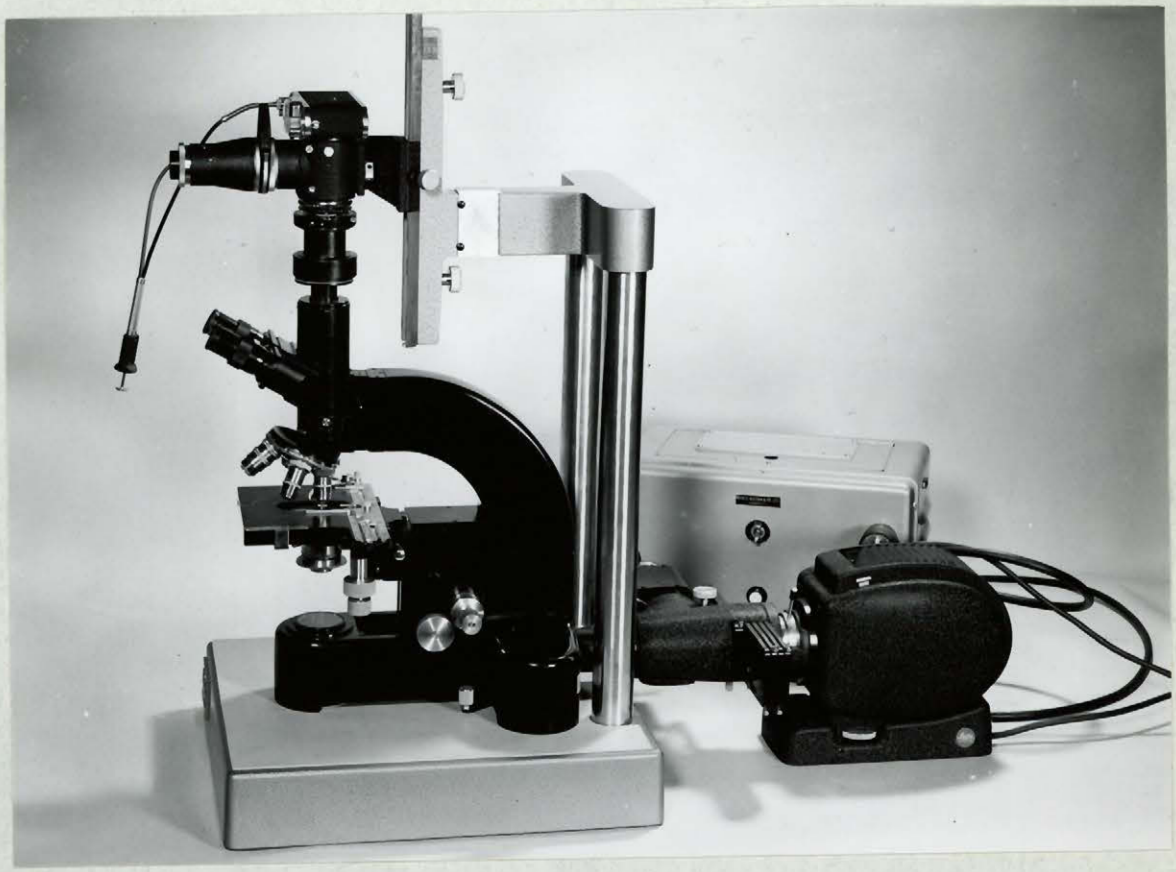

Figure 1. Microscopic and Photographic Setup.

Charles P. Hodge, F.B.P.A., the ASA of high speed Ektachrome Daylight film (Kodak) was effectively raised from 160 to over 650, thus decreasing our exposure times even more. A further more complete report, to be published, will give the details of the developing and printing methods. This developing technique enabled us to use the commercially supplied high speed Ektachrome-Daylight film with exposure times of 0.5 - 1.0 seconds for many cells in the field, and 2.5 - 5.0 seconds for single cells. Developing the film as a negative also afforded a better method of obtaining colour prints.

C. Experimental Method

Smears of fresh tissue were made using the two slide technique developed at this Institute by the late Dr. W.V. Cone. These were allowed to air dry and then were immediately fixed in a solution containing equal parts ether and 90% ethyl alcohol. The length of fixation varied and we found that a smear could remain in the fixative for a period of up to one week without interfering with the consequent fluorescence. The details of making the stock solutions of the stain and of the buffer are well described in the literature by Bertalanffy et al. (1960). Briefly then, the smeared slide was passed rapidly through graded alcohol solutions, namely 80-70-50%, to distilled water. It was then briefly rinsed in 1% acetic acid and rewashed in distilled water. The actual staining in the 0.01%

acridine orange* dissolved in phosphate buffer at pH 6.0 was for 3 minutes. The specimen was then transferred for one minute into 1/15 Molar phosphate buffer at pH 6.0 to remove the excess dye. Following differentiation for 2 minutes in 10 Molar calcium chloride, the specimen was rinsed with phosphate buffer and mounted under a cover glass in a drop of buffer. This done, the specimen was ready to be examined with the fluorescent microscope. The mercury arc lamp should be turned on approximately 10 minutes prior to the actual examination of the specimen, in order to ensure a constant activating light source. The photographic setup was accomplished in a darkened room. The amount of light involved in fluorescent studies is relatively low by comparison with that falling on the object, so that especially in visual work the aim must be to increase contrast by eliminating all other light from the field of view, while gaining the maximum amount of fluorescent light through the objective.

The ribonuclease**used was dissolved in glass distilled water in a concentration of 1 mg. per millilitre and the specimens were incubated at 37°C for 2 hours. Not all

* G.T. Gurr, supplied by ESBE Laboratories, Toronto, Canada

**supplied by Nutritional Biochemical Corp., Cleveland, Ohio.

smears were studied using the ribonuclease technique. In those cases where it was used, two parallel smears were made from the same case, one stained with acridine orange and the other treated with ribonuclease and then stained with acridine orange.

Following the visualization of the specimen and the taking of the photograph, a preliminary diagnosis of non-tumor, benign or malignant tumor was made. These findings were later correlated with the findings on permanent histological sections. The processing of the permanent sections was carried out by Messrs. John Gilbert and Gerard Papillon. The stains employed in the group of non-tumor brain and in the intrinsic brain tumors were: hematoxylin - phloxin-safranin, Mallory's phosphotungstic acid hematoxylin,Laidlaw's connective tissue stain, Luxol blue, Cajal's gold sublimate and Penfield's modification of the del Rio Hortega silver carbonate method. The extrinsic tumors were studied using the first four above-mentioned stains. In the case of carcinoma, routine use of the same four plus periodic acid-Schiff and a mucin stain was undertaken. All final diagnoses were reviewed by members of the Department of Neuropathology.

IV. RESULTS

In all cases studied, the final pathological diagnosis was clearcut and in no instance was there any question as to the validity of the diagnosis.

A. Non-Tumor Brain

Thirteen (13) specimens from either temporal lobectomies or partial frontal, parietal or occipital removals for focal cerebral seizures, when examined, all showed consistent findings. There was little or no red fluorescence of the glial cell cytoplasm and the nucleolus in all instances fluoresced yellow. Neurones showed varying degrees of red cytoplasmic fluorescence with a prominent red fluorescing nucleolus, Figure 2. The nucleus appeared as a large greenish-yellow fluorescing body with a vesicular arrangement of the contained chromatin. This group of cases, although not normal brain in nature, did comply with the original criteria of the experiment and in no instance was there very much red fluorescence in the cytoplasm of glial cells observed, Figure 3.

B. Benign Extrinsic Brain Tumors

There were seven (7) meningeal fibroblastomas in this group, none of which, on permanent section, showed any evidence of sarcomatous change. The acridine orange method

demonstrated large amounts of red fluorescing cytoplasm in all the cells comprising the tumor. This is well illustrated in Figure 4 where the cell whorls characteristic of meningioma can be identified. In this instance, the tumor cells possessed a yellow fluorescing nucleus with a red fluorescing cytoplasm.

The four (4) perineurial fibroblastomas studied did not show any red fluorescence in the cytoplasm of the tumor cells, Figure 5. The absence of red fluorescing cytoplasm in these eighth nerve tumors, again all of which were benign in nature, was very striking.

C. Malignant Extrinsic Tumors

Here we group the two (2) fibrosarcomas studied, one involving the median nerve in a 13-year-old boy, the other involving the temporalis fascia over a previous craniotomy site. Both showed large pools of red fluorescing cytoplasm with very bizarre-shaped yellowish fluorescing nuclei, some with a prominent red fluorescing nucleolus. As a check on the accuracy of the staining method, Figures 6 and 7 show these findings plus the occasional polymorphonuclear leukocyte with its greenish fluorescing bi- and tri-lobed nucleus, and no fluorescence in the cytoplasm. This manner of checking the stain advocated by Bertalanffy et al (1960) was also used by us as the prime method of standardizing the staining procedure to the point of achieving consistently reproducible results.

D. Benign Intrinsic Brain Tumors

There were six (6) piloid astrocytomas studied. Again, the degree of red fluorescing cytoplasm in the tumor glial cell varied from little to none. There was no doubt about this latter finding. The nucleus again fluoresced as yellow or greenish yellow, and in this instance the nucleolus consistently fluoresced as red, Figure 8. The size of the nucleus, in general, was somewhat larger than in the non-tumor astrocyte. In this study we dealt only with smears, and this latter finding may or may not be significant. We were able to examine only one oligodendroglioma and again, very little to no red fluorescence of the oligodendroglial tumor cell was seen Figure 9.

E. Malignant Intrinsic Brain Tumor

The seven (7) malignant astrocytomas, Figure 10, and the nine (9) glioblastoma multiforme, Figure 11, studied, all showed the same characteristics. The quasi semantic division of these two groups does not arise from the acridine orange interpretation, but from findings on permanent sections. Here in this Institute, a glioma is not classified as glioblastoma multiforme unless all of the following four criteria are met: pleomorphism, mitotic figures, necrosis and endothelial proliferation.

The acridine orange stain showed many cells with an enlarged yellowish fluorescing nucleus with varying amounts of red fluorescing cytoplasm. The degree of red fluorescence in the cytoplasm was always significantly more than

that seen in any of the preparations of the benign intrinsic brain tumors studied. Here again many, but not all of the tumor cells showed red fluorescing nucleoli.

F. Metastatic Carcinoma

A variety of metastatic lesions was studied. In all cases, the location of the primary had been confirmed either by prior or subsequent investigation. All were intracerebral metastases except for the one from the prostate, one from the breast and one from the colon, which were spinal and extradural in location. The fluorescent properties of all were very similar in that the cells were large, epithelial in nature, with an abundant amount of red fluorescing cytoplasm and a large, irregular yellowish-green fluorescing nucleus. The location of the nucleus within the cell varied from case to case, being mainly eccentric in location in the hypernephroma, Figure 12, and central in location in many of the remaining carcinomas studied, Figures 13 and 14. Again, a red fluorescing nucleolus was evident in many of the cells, Figure 12. As is evident from Figure 15 when these and other preparations were treated with ribonuclease, either the red fluorescence was lost or the ribonuclease destroyed the red fluorescence.

G. Cerebrospinal Fluid and Ventricular Fluid

1) Positive Results:

There were two patients in whom a diagnosis of medulloblastoma was made, one with known cauda equina lesions, the

other with a lesion in the 4th ventricle. Both revealed cells in the lumbar spinal fluid which were larger than monocytes, had an irregularly shaped nucleus that fluoresced yellowish and relatively large quantities of red fluorescing cytoplasm, Figures 16 and 17. The lumbar spinal fluid from the patient with autopsy proven meningeal sarcomatosis also showed cells with large pools of red fluorescing cytoplasm, Figure 18.

Another positive result was obtained from examination of cerebro - spinal fluid from a young child with multiple cranial nerve palsies and a radiologically diagnosed mass lesion of the brain stem. This specimen showed clumps of cells, 5-6 cells/clump, all of which possessed red cytoplasmic fluorescence.

The specimen of ventricular fluid was obtained from a patient with known recurrence of a malignant astrocytoma of the right frontal region. Again, cells arranged in clumps were identified. These cells were larger than white blood cells and besides possessing the red fluorescing cytoplasm also exhibited large yellowish-green fluorescing nuclei, Figures 19, 20 and 21.

The one specimen of ascitic fluid examined was from a patient with known carcinoma of the stomach and widespread peritoneal as well as visceral metastases. Here again cells were demonstrated typically tumoral in nature.

2) Negative Results:

The remaining seven cases of spinal fluid examination (mixed etiology) which showed no cells containing red cytoplasmic fluorescence can be subdivided as follows: three (3) metastatic extradural spinal tumors in adults, one (1) child with papilledema and headaches which was later diagnosed as optic neuritis, one (1) case of aqueduct stenosis, one (1) child with a glioma deep in the hemisphere and one (1) child with a radiologic diagnosis of medullablastoma and no tumor cells seen. This latter case did receive deep radiation therapy, and following sixteen courses of therapy re-examination of a sample of cerebro- spinal fluid showed many cells non-clumped with red cytoplasmic fluorescence and may have been macrophages.

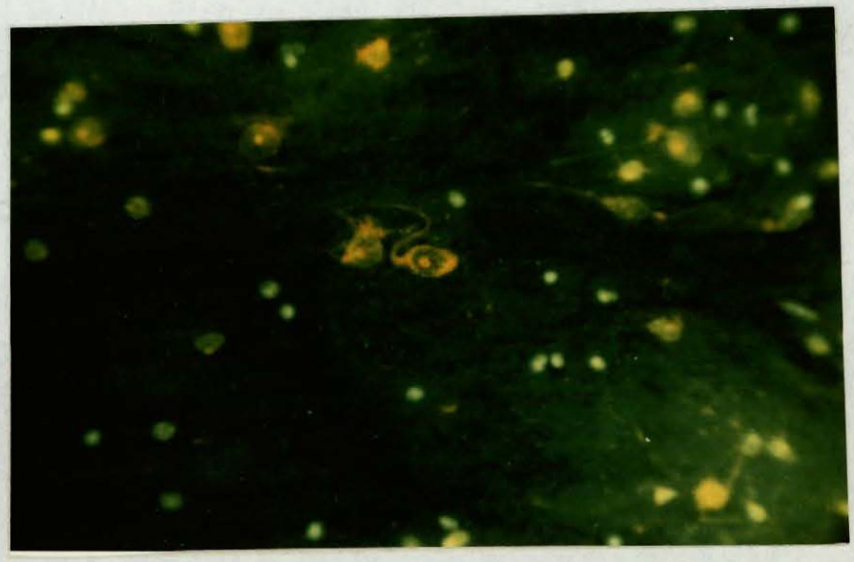

Figure 2. Temporal lobe gliosis (cortex) apart from the red cytoplasmic fluorescence in the neurones, the glial elements contain little to no red fluorescing material X 175.

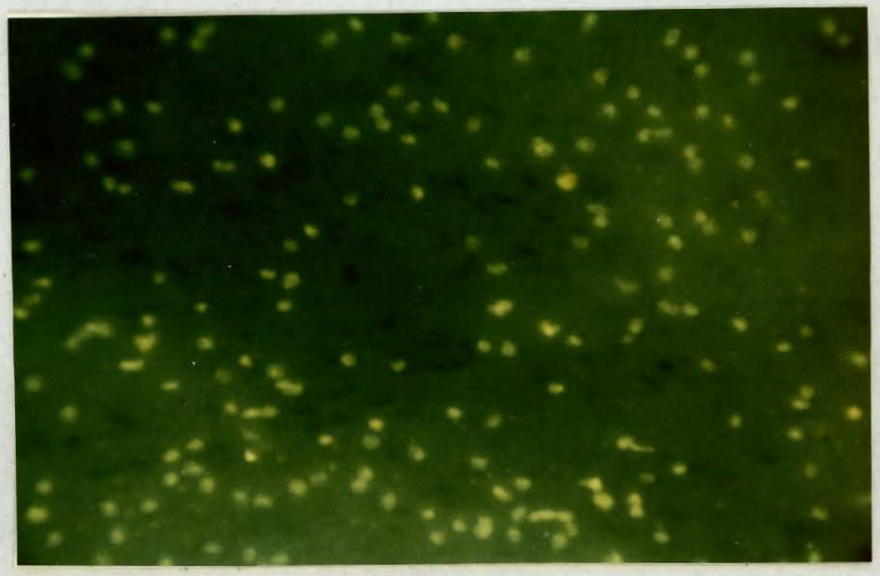

Figure 3. Temporal lobe gliosis (white matter) demonstrating the fluorescence of glial cells, nucleoli fluorescence yellow X 175.

Figure 4. Meningeal fibroblastoma - cell whorls with large amounts of red fluorescing cytoplasm X 175.

Figure 5. Perineurial fibroblastoma - striking absence of red fluorescing material in the cytoplasm. X 175.

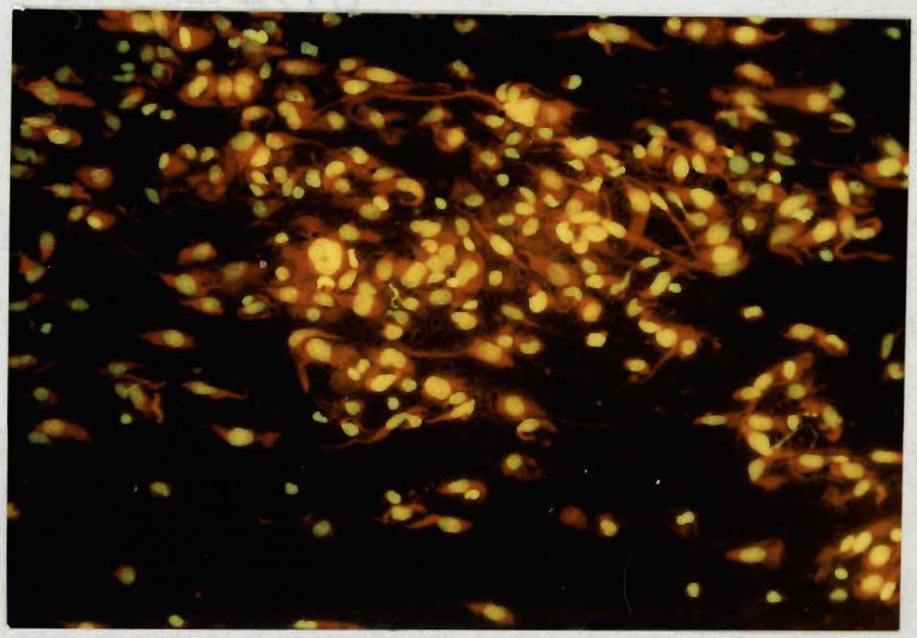

Figure 6. Fibrosarcoma - many large cells with an abundant amount of red cytoplasmic fluorescence X 175.

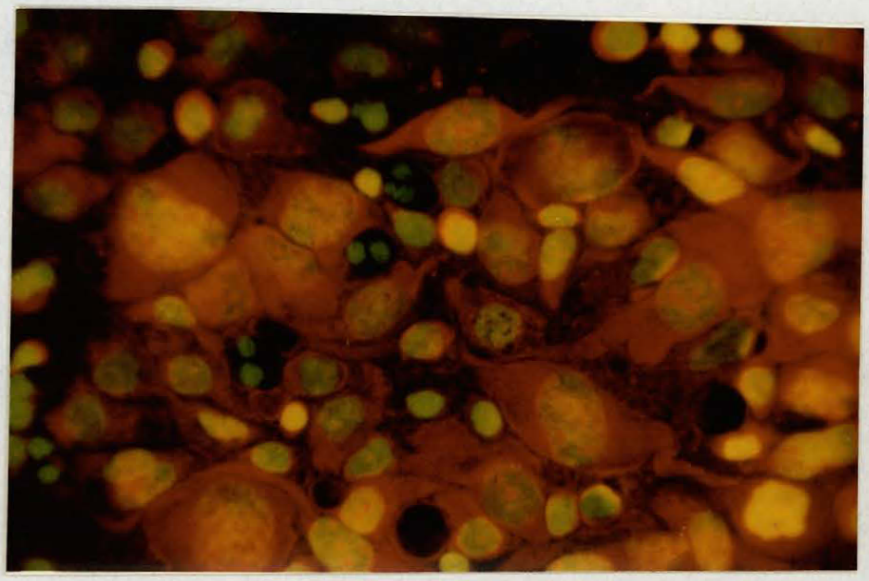

Figure 7. Fibrosarcoma - paired green dots are the nuclei of the polys, surrounded by very large cells containing abundant quantities of red cytoplasmic fluorescence. Note the red fluorescing nucleoli X 650.

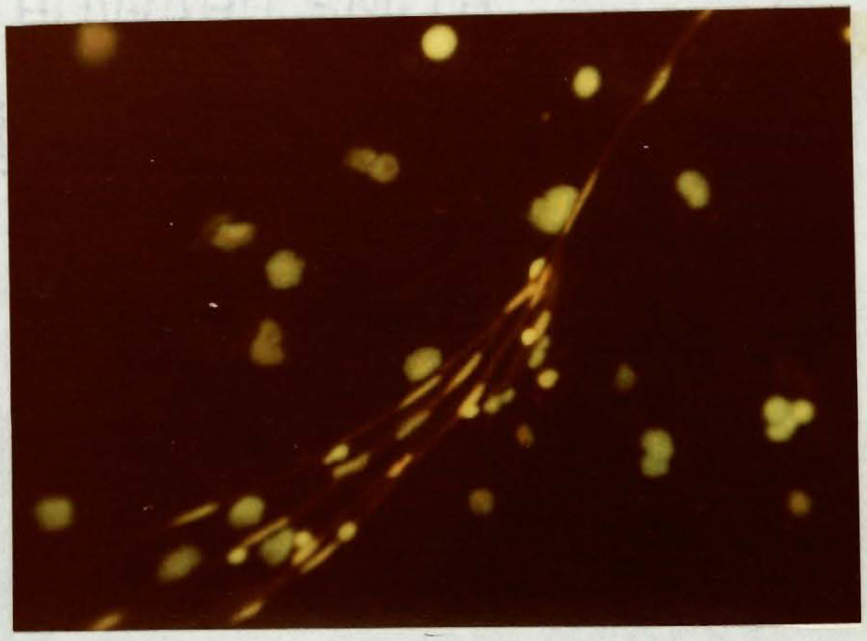

Figure 8. Piloid astrocytoma - again negligible red fluorescing cytoplasm of tumor glial cells, nucleoli fluoresce red as does the cytoplasm of the endothelial cells X 175.

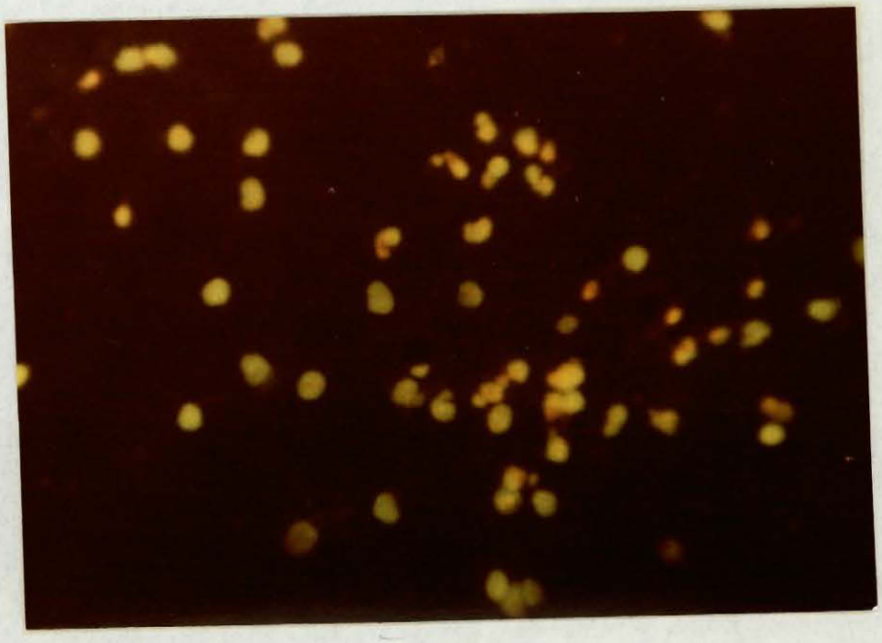

Figure 9. Oligodendroglioma - round nuclei, some possessing a red nucleolus, little red cytoplasmic fluorescence X 175.

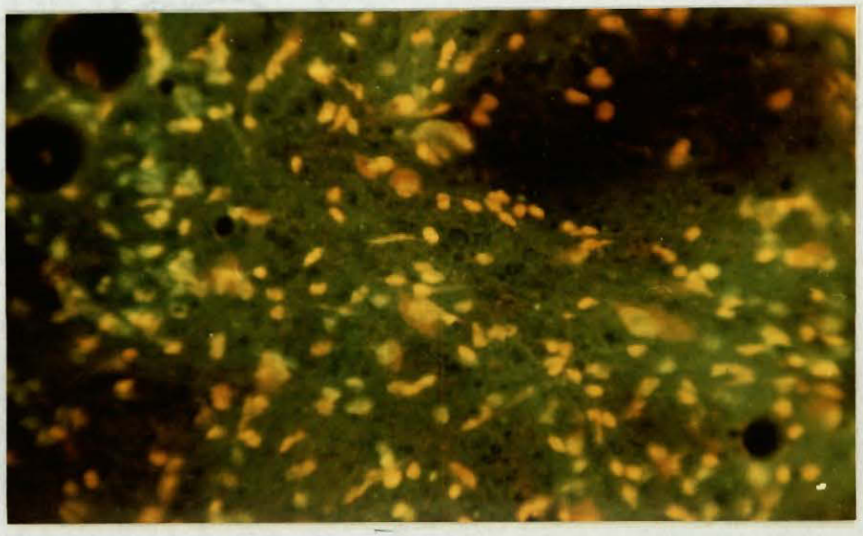

Figure 10. Malignant astrocytoma - tumor cells showing a large amount of red cytoplasmic fluorescence X 175.

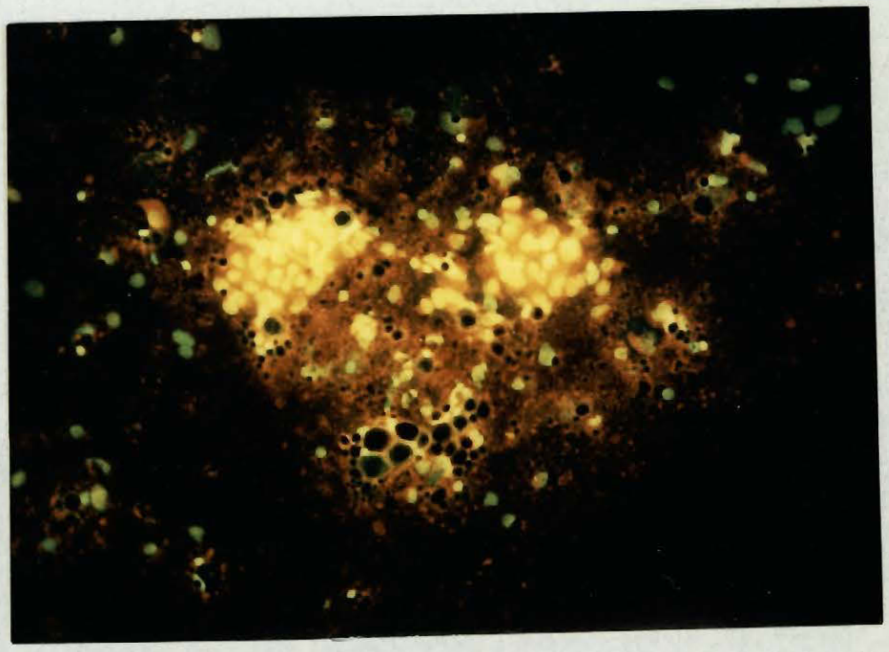

Figure 11. Glioblastoma multiforme - pleomorphism and again many cells with abundant red cytoplasmic fluorescence X 175.

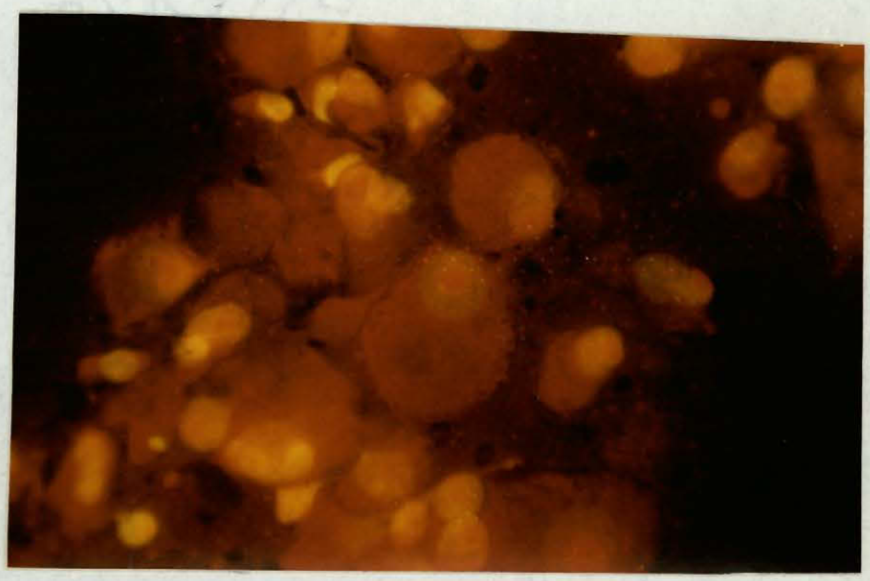

Figure 12. Hypernephroma, metastatic - eccentric nucleus with red fluorescing nucleoli and large pools of red cytoplasmic fluorescence X 650.

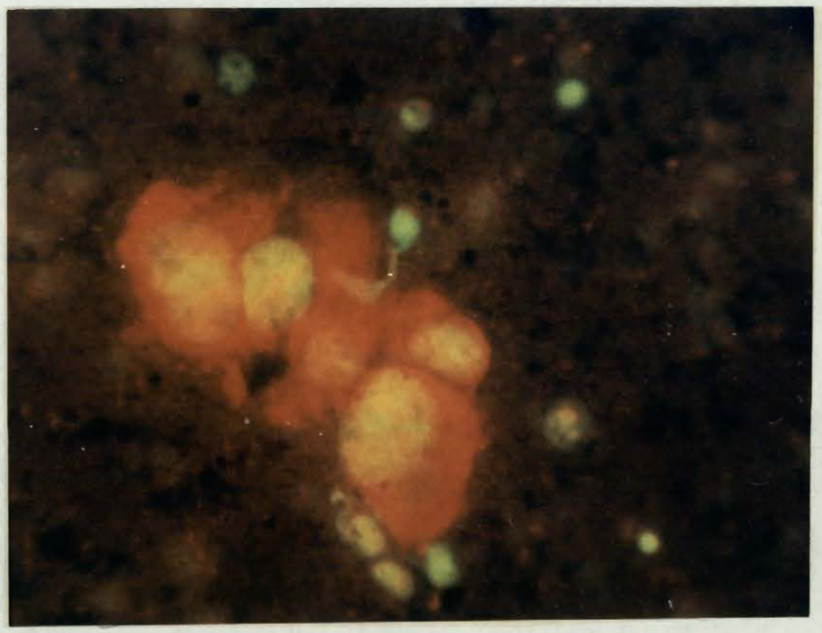

Figure 13. Bronchogenic carcinoma, metastatic - very large bizarre cells with multiple red fluorescing nucleoli. Nuclei of surrounding glial cells are also visible. X 650.

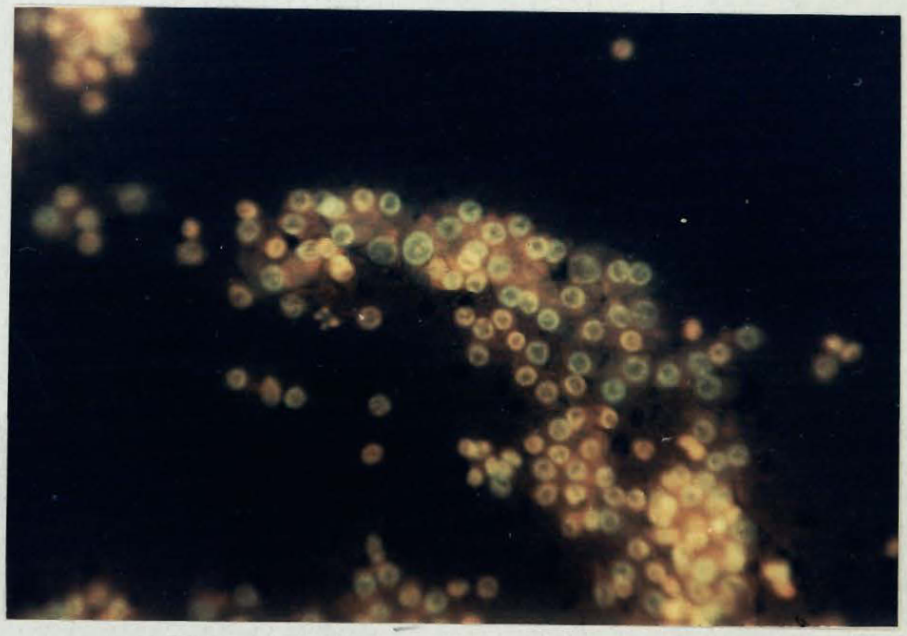

Figure 14. Metastatic adenocarcinoma (breast) - large nuclei with red cytoplasmic fluorescence X 175.

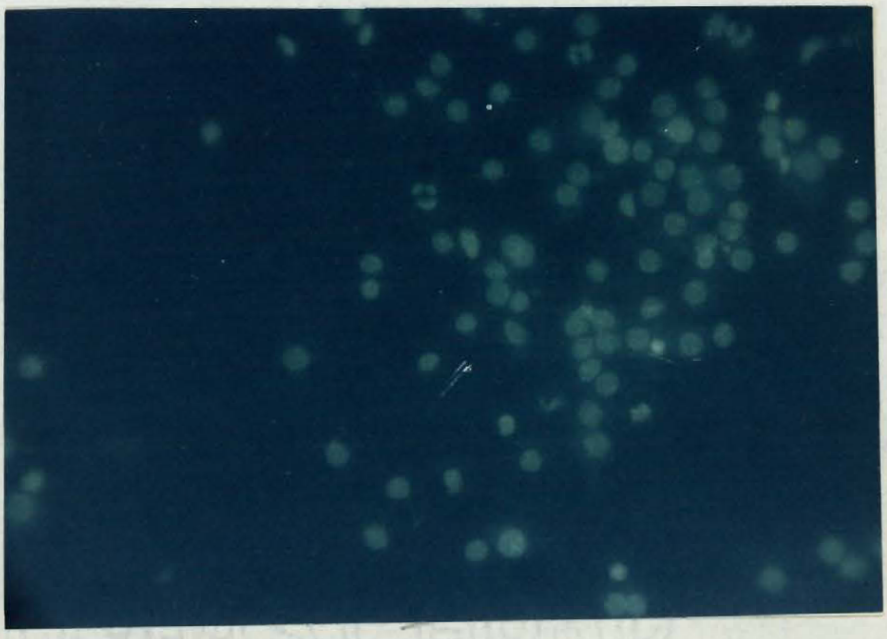

Figure 15. Metastatic adenocarcinoma (breast) - after treatment with ribonuclease resulting in a loss of red fluorescing material X 175.

Figure 16. Medulloblastoma - appearance of cells seen on examination of cerebro-spinal fluid X 650.

Figure 17. Medulloblastoma - small clump of abnormal cells with the nucleus of a lympocyte also visible in the field X 650.

Figure 18. Meningeal sarcomatosis - an example of the type of cell seen on examination of cerebrospinal fluid X 650.

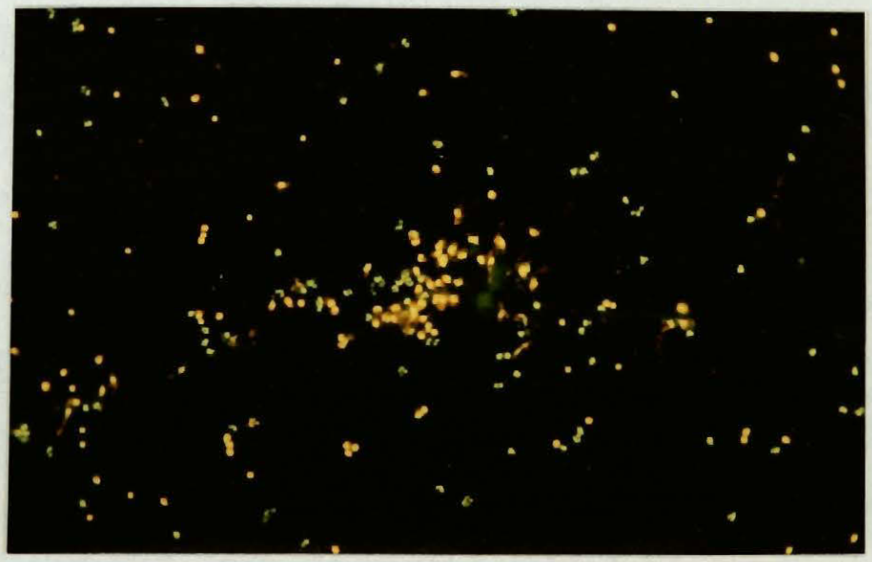

Figure 19. Malignant astrocytoma, recurrent - appearance of cells seen on examination of ventricular fluid X 175.

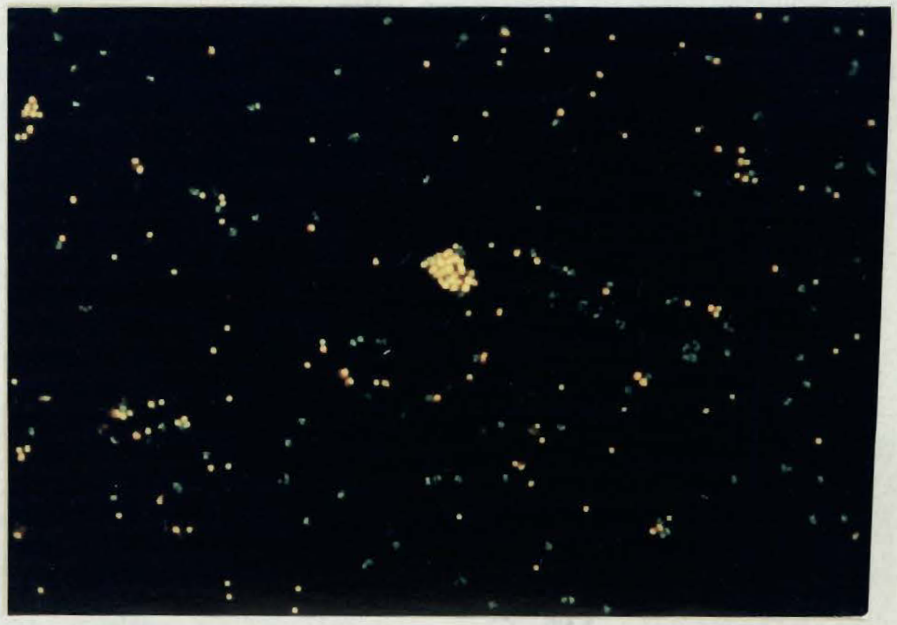

Figure 20. Same case as Figure 19 X 175.

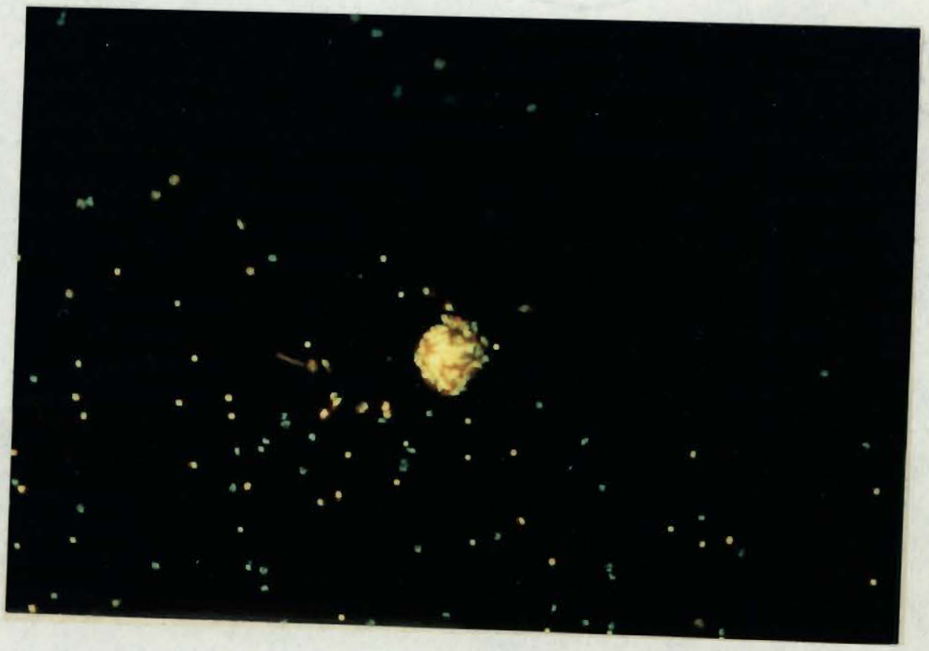

Figure 21. Same case as Figures 19 and 20 X 175.

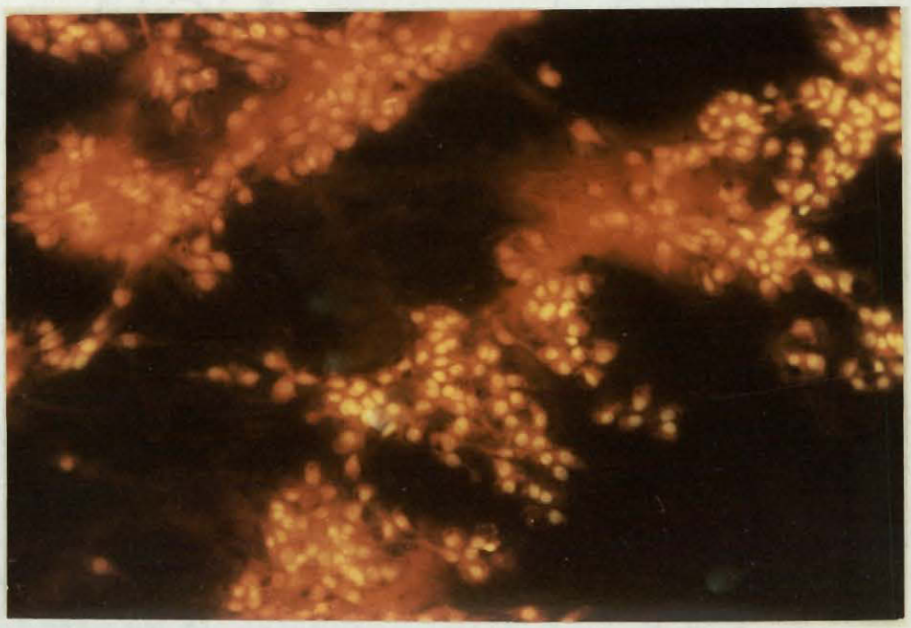

Figure 22. Chordoma - cells arranged in cords with abundant red cytoplasmic fluorescence. Note the amorphous, large area of diffuse red fluorescence about the cells in the lower left centre X 175.

Figure 23. Chordoma after treatment with ribonuclease, same case as Figure 22 - the red cytoplasmic fluorescence has disappeared, that due to RNA; the diffuse amorphous fluorescence remains, that due to the acid mucopolysaccharides present X 175.

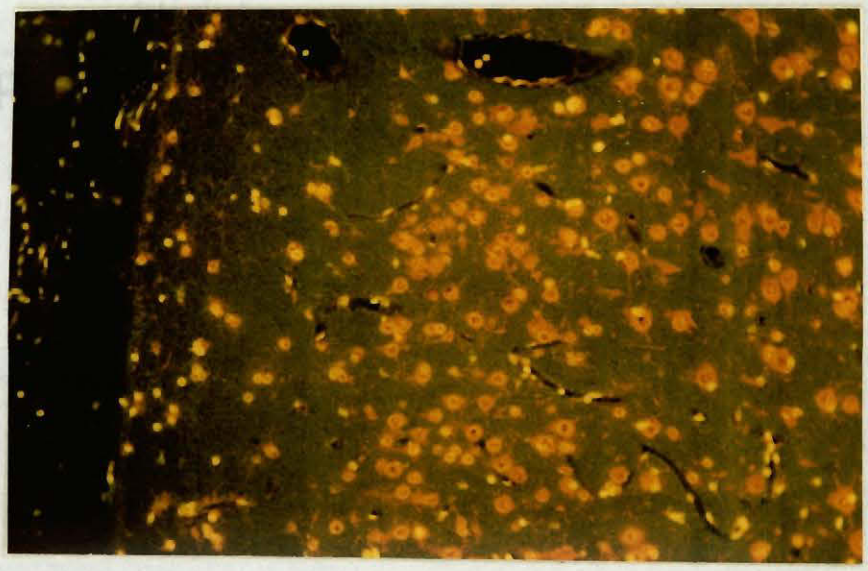

Figure 24. Cerebral cortex, Carnoy's fixative - demonstrating the typical morphologic and fluorescent properties of neurones and glial cells. Pial surface with the overlying meninges is visible at the left X 175.

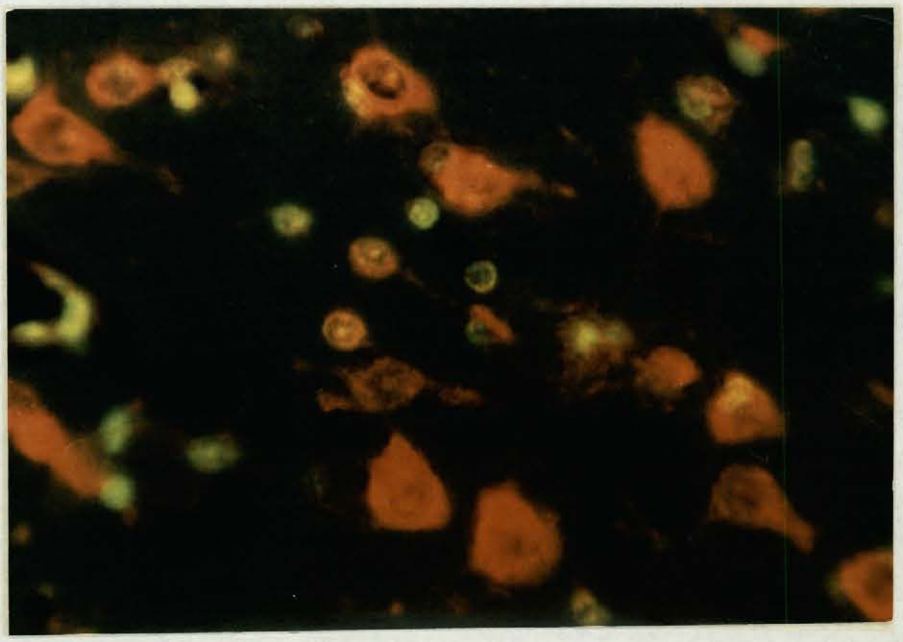

Figure 25. Same case as Figure 24 - demonstrating the same appearance under higher magnification X 650.

V. ANALYSIS OF RESULTS

An analysis of the results of all cases studied is shown in Table I.

It is interesting to note that in all but one group, the correlation between benign and malignant, as indicated by the red fluorescing cytoplasm, is very straightforward. The one tumor that showed no correlation was the meningeal fibroblastoma which in all instances possessed red cytoplasmic fluorescence to a degree that, according to the criteria employed,would have to be regarded as malignant. This, of course, is not a reflection as to the inaccuracy of the method nor to its specificity, but does represent a miscalculation in our thinking regarding the format of the experiment.

The specimens from patients suffering with focal cerebral seizures could theoretically be interchanged with one another. The morphology and the character and colour of fluorescence observed were strikingly similar. Neurones in these preparations could easily be distinguished from the non-tumor glial cells, both by their size and the degree of red fluorescence in the cell processes. The problem of differentiation of neurones from malignant glial cells is another question. Here, the degree of red cytoplasmic

SUMMARY

CLASS OF TUMOR	NUMBER OF CASES	FINAL DIAGNOSIS PERMANENT SECTIONS	ACRIDINE ORANGE INTERPRETATION	CORRELATION
NON TUMOR BRAIN	3	GLIOSIS, ETIOLOGY UNDETERMINED	NO RED FLUORESCENCE	YES
	2	HIPPOCAMPAL SCLEROSIS, ETIOLOGY UNDETERMINED	"	"
	2	HIPPOCAMPAL SCLEROSIS, SECONDARY TO BIRTH INJURY	"	"
	2	MENINGOCEREBRAL CICATRIX, POST TRAUMATIC	"	"
	1	GLIOSIS AND MULTIPLE CYST FORMATION, POST TRAUMATIC	"	"
	2	GLIOSIS, SECONDARY TO BIRTH INJURY	"	"
	1	GLIOSIS, POST INFECTIOUS	"	"
BENIGN EXTRINSIC BRAIN TUMORS	7	MENINGEAL FIBROBLASTOMA (MENINGIOMA)	RED FLUORESCENCE	NO
	4	PERINEURIAL FIBROBLASTOMA (ACOUSTIC NEURINOMAS)	NO RED FLUORESCENCE	YES
MALIGNANT EXTRINSIC TUMORS	2	FIBROSARCOMA	RED FLUORESCENCE	YES
BENIGN INTRINSIC BRAIN TUMORS	6	PILOID ASTROCYTOMA	NO RED FLUORESCENCE	YES
	1	OLIGODENDROGLIOMA	"	"
MALIGNANT INTRINSIC BRAIN TUMORS	7	MALIGNANT ASTROCYTOMA	RED FLUORESCENCE	YES
	9	GLIOBLASTOMA MULTIFORME	"	"
METASTATIC CARCINOMA	2	BREAST, ADENOCARCINOMA	RED FLUORESCENCE	YES
	1	PROSTATE, ADENOCARCINOMA	"	"
	1	COLON, ADENOCARCINOMA	"	"
	1	BRONCHOGENIC CARCINOMA	"	"
	1	HYPERNEPHROMA	"	"
CEREBROSPINAL FLUID	2	MEDULLOBLASTOMA	RED FLUORESCENCE	YES
	1	MENINGEAL SARCOMATOSIS	"	"
	1	BRAIN STEM TUMOR, TYPE UNKNOWN	"	"
	7	MIXED ETIOLOGY	NO RED FLUORESCENCE	"
VENTRICULAR FLUID	1	RECURRENT MALIGNANT ASTROCYTOMA	RED FLUORESCENCE	YES
ASCITIC FLUID	1	STOMACH, ADENOCARCINOMA WITH VISCERAL METASTACES	RED FLUORESCENCE	YES

fluorescence in tumor cells of glioblastoma multiforme or malignant astrocytoma is almost comparable both in amount and colour of fluorescence to that of the neurones. The other disturbing limitation, of course, is that our study was undertaken on smeared rather than on cut sections and much of the morphology of cells is, a priori, sacrificed. Therefore, in the cases of borderline malignancy, the very group in which this method was originally thought to have its greatest clinical application, the interpretation is not straightforward; the smeared neurones interfered.

This does not mean that the method is not applicable. What it does represent, perhaps, is the problem of refinement of the technique whereby a differentiation can be made between neurones and malignant cells. Daoust (1961) demonstrated a significant loss of tissue ribonuclease in liver carcinoma cells of the rat with a preservation of that in the normal surrounding hepatic cells. Incubating a specimen of brain tumor with adherent reactive brain may quench the cytoplasmic fluorescence of the neurone without damaging that of the tumor cells, thus enabling an accurate straightforward interpretation. To date, preliminary studies in this direction at our laboratory have not been too rewarding.

The group of benign intrinsic brain tumors and the malignant extrinsic brain tumors offered no problem; the results were obvious. It is interesting to speculate that the

oligodendroglioma may be a biologically more active tumor as evidence by the slightly greater degree of red cytoplasmic fluorescence than that seen in the piloid astrocytoma.

The metastatic carcinomas were striking. In no instance did confusion arise. It was impossible to interpret these specimens mistakenly, for the cell size, shape and staining characteristics stood out glaringly.

In dealing with lumbar spinal fluid and ventricular fluid, the problem of smeared neurones is no longer present. The fluorescent properties of the white blood cell elements after staining with acridine orange are well studied and described (Bertalanffy,1960a). It is a relatively simple matter for a cytologist, trained in the fluorescence microscopy technique, to differentiate a polymorphonuclear leukocyte from a lymphocyte, a monocyte or a plasma cell. With this accomplished, it is feasible to assume that any cell seen in either cerebrospinal fluid or ventricular fluid that is larger in size than the corresponding white blood cell is abnormal. The only other cells which could interfere are the ependymal cells and macrophages but they should be differentiated by their morphological characteristics.

In a patient, then, with an exfoliating medulloblastoma, malignant astrocytoma or metastatic carcinoma, should the exfoliative cells gain entrance into the subarachnoid spaces, one should be able to demonstrate these cells much more

readily than by the classical Papanicolaou technique where morphology alone is applicable.

To date, we have autopsy studies on two test cases. The first, a man of 36 years of age with a space occupying lesion deep in the left cerebral hemisphere: needle biopsy showed an inconclusive histological picture on routine smear. The diagnostic impressions ranged from meningeal fibroblastoma, ependymoma to glioma. The acridine orange smear was interpreted as malignant astrocytoma and, at postmortem, a glioblastoma multiforme was found. The second case was a woman, age 51, with a radiologic myelographic diagnosis of subarachnoid adhesions or diffuse tumor. The patient was in extremis and operative biopsy was not carried out. Acridine orange smear of lumbar cerebrospinal fluid demonstrated large cells with abundant amounts of red cytoplasmic fluorescense and, at postmortem, a diagnosis of meningeal sarcomatosis was made.

In the total series all cases studied with the diagnosis of tumor, except two, had final tissue diagnoses. One case where tissue was not available for routine pathologic study was the child with the midline fourth ventricle tumor who after receiving deep radiation therapy showed marked improvement both radiologically and symptomatically. The other case of medulloblastoma was confirmed by tissue section.

The second case where no final tissue diagnosis was available was of the child with the radiologically diagnosed brain stem lesion who improved following deep radiation

therapy. However, seven months later difficulty in walking ensued followed by a demonstrable subarachnoid block in the mid-thoracic region. Examination of lumbar cerebrospinal fluid at this stage revealed cells with large, slightly irregular yellowish fluorescing nuclei and large amounts of red fluorescing cytoplasm. Many of these cells were arranged in clumps. This finding was followed by a course of deep radiation therapy to the spinal axis with resultant regression of symptomatology and partial return of motor function.

The patient with the recurrent malignant astrocytoma has since died and autopsy was refused. In the patients with extradural spinal metastases we did not expect to see any tumor cells in the cerebro - spinal fluid, nor did we in the case of finally diagnosed optic neuritis and aqueduct stenosis. The same applies to the child with the deep seated cerebral glioma which did not communicate, grossly, with the subarachnoid space.

The one case which is somewhat difficult to explain in the group of cerebro - spinal fluid examinations is that of the youngster with the clinical diagnosis of medulloblastoma in whose specimen no tumor cells were seen. This probably represents a technical problem in sampling. Following sixteen deep radiation treatments, re-examination of lumbar spinal fluid revealed many cells with red

cytoplasmic fluorescence. These cells were larger in size than the previously seen cells associated with medulloblastoma, were not arranged in clumps and were interpreted as macrophages or gitter cells.

Recently, we have been fortunate to receive a specimen of chordoma removed from the lumbar region. Treatment of a parallel smear with ribonuclease resulted in a loss of the red cytoplasmic fluorescence but did not interfere with the red fluorescence due to the acid mucopolysaccharides present. This corroborates the original descriptions of the staining properties of acridine orange and substantiates the observation that acridine orange is a reliable means of studying the fluorescent properties of DNA and RNA, Figures 22 and 23.

VI. DISCUSSION

The application of this method in the study of diseases affecting the nervous system has great potental value. The results are consistently reproducible and two parameters of study are utilized: a) a differentiation of red cytoplasmic fluorescence, RNA (removable by ribonuclease) and of greenish-yellow nuclear fluorescence, DNA and b) changes in morphology.

The principal application, at present, is in the analysis of cerebrospinal fluid and ventricular fluid for malignant cells. The interpretation of malignancy on the basis of the degree of red cytoplasmic fluorescence is not clouded by the presence of neurones; and the morphologic characteristics of cells is preserved. In one case of medulloblastoma the acridine orange findings were confirmed by permanent histopathological sections. The other case responded well to deep radiation therapy. The acridine orange findings on the patient with autopsy proven meningeal sarcomatosis were also correctly interpreted as were the findings in the case of brain stem tumor with the development of a thoracic subarachnoid block, the case of ascitic fluid and the seven cases of mixed etiology.

The series of lumbar cerebrospinal fluid analyses is not large enough to permit any statistically valid conclusions, yet the findings warrant further investigation.

According to Schümmelfeder (1957), he as well as Haymaker et al, are already employing this method in the study of early reversible changes in the Purkinje and granule cells of mice cerebellum following high doses of radiation. Changes in both RNA and DNA can be studied simultaneously both before and after treatment with ribonuclease, desoxyribonuclease, warm trichloracetic acid or cold perchloric acid. Perhaps, the technique may also be of use in the study of brain tissue which at operation demonstrates epileptogenic activity. To date, the correlation between electrical abnormalities in epileptic brain and the demonstrable histopathology has not been adequately consistent.

As alluded to previously, the acridine orange smear method as applied to the study of intracranial brain tumors has limited immediate value. The primary reason is the fluorescent similarities between malignant glial tumor cells and neurones. The other disturbing finding, which also limits the method, is the observation that purely benign lesions, meningiomata, have abundant amounts of red cytoplasmic fluorescence in the tumor cells. As a rapid method of differentiating benign from malignant in smears of brain tumor tissue, the straightforward potentialities of the method have not as yet, been realized.

In an attempt to circumvent the difficulties inherent

in the smear technique we, in this laboratory, are currently undertaking a study of brain tumors utilizing Carnoy's fixed tissue embedded in paraffin. Following sectioning, the specimens will be examined using the same staining and microscopic methods previously described, Figures 24 and 25.

One other application of this technique is in the demonstration of nuclei in ultracentrifuged specimens of white matter. Wolfe (1961) is presently undertaking a biochemical study of myelin products submitted to ultracentrifugation. As a step in the experiment, it is necessary to rule out the presence of contaminating nuclei. This can readily be done by the aforementioned method with greater accuracy than with the more classical histopathologic techniques, and more conveniently than by having to rely on the availability and use of an electron microscope. This observation stems from the fact that the main advantage of fluorescent microscopy, which transcends all others, is its great sensitivity plus the high degree of contrast. As Price and Schwartz (1956) have pointed out, a 1% difference in transmitted light between two different areas of a cell can be demonstrated by fluorescence as a hundredfold difference. However, recognizing that fluorescent light is emitted in all directions from its point of origin in the tissues, the degree of resolution obtainable is less than that given by visible light methods.

Unfortunately, fluorescence is easily modified by

relatively minor changes in hydrogen ion concentration. This is a well recognized fact throughout the literature in this field. At times it is possible to intensify the degree of fluorescence to the point where it may be of some benefit. Varying the pH of our staining solutions from 5.3 to 6.5 in an attempt to intensify the resultant metachromatic fluorescence did show some predictable changes. At pH 6.0, the metachromatic effect was optimum as previously pointed out by Bertalanffy (1960a).

One of the major disadvantages of the acridine orange method is the impossibility of preserving the stained specimens indefinitely. This becomes an increasing problem with the number of investigators working with this method. As described by von Bertalanffy et al (1957, 1958) the stained specimen is mounted under a cover glass with a few drops of buffer. Air drying hastens the decay of fluorescent metachromasia as does the exposure of the specimen to the blue light. Use of a xylene solution of isobutylmethacrylate, in our laboratory, as a somewhat permanent mounting medium showed an appreciable destruction of the differentiation between red and yellow fluorescence and, hence, was of little value in this technique. The photographic method described by Turner (1960) also resulted in an appreciable loss of contrast and fluorescent colour differentiation, due to the relatively long exposure times required,

i.e. up to 55 seconds for degenerated, sparsely cellular fields. In an attempt to rectify these problems and also to be able to employ colour prints as demonstrable examples of the acridine orange method, the Department of Neurophotography was able to institute a new negative-developing technique which effectively lowers the exposure times necessary into a range when both the changes in fluorescent colour differentiation and the effects of drying were no longer a serious problem. Simultaneously, the colour negatives were available from which any number of colour prints could be made.

As a check on the staining capabilities of acridine orange as concerns cellular elements in nervous tissue, Raimondi (1961) has recently shown that when viewed with the electronmicroscope, astrocytes contain very little ribosome material (RNA) in their cytoplasm, whereas neurones contain vast quantities. The only disturbing feature, which applies also to the light microscopists' concept, is that oligodendroglia also contain large amounts of ribosome material in their cytoplasm. The latter, of course, is a finding which is quite revolutionary in scope to all the classical concepts of the morphological appearance of oligodendroglia. We too, were not ready for this observation, for in our material, the oligodendroglia showed very little in the way of red cytoplasmic fluorescence. The disparity may reside in the time interval between removal

of tissue and fixation.

Mention is made of a case of chordoma where after treatment with ribonuclease red cytoplasmic fluorescence disappeared whereas that of the acid mucopolysaccharides remained, again pointing to the specificity of acridine orange to obtain RNA.

Another stimulating finding is the disparity in staining characteristics of meningeal fibroblastoma and perineurial fibroblastoma. The former showing large quantities of red cytoplasmic fluorescence and the latter showing little to none. This may be an indication that the perineurial fibroblastoma is not a tumor from a mesenchymal stem cell and perhaps, phylogenectically, our thinking on these tumors should be re-evaluated.

The question of whether RNA differs significantly in the glial cells of non-tumor tissue and in glial brain tumors, as manifested by the changes in fluorescent colour of the nucleoli, is the object of our next series of experiments.

In conclusion, the present study suggests that the acridine orange staining method: a) can be readily and fruitfully employed in the analysis of cerebro-spinal fluid and ventricular fluid for malignant cells, b) may be used in the histochemical analysis of epileptogenic brain tissue or in brain tumors, and c) is of little immediate value in the rapid differentiation between a purely benign and a malignant brain tumor as applied to the smear technique.

VII. SUMMARY

A study of primarily intracranial tumors, based on fluorescence microscopy after staining with the fluorochrome, acridine orange, is discussed; along with a description of the techniques and the results of application of these techniques to a wide variety of intracranial tumors. A clinically rewarding application of these techniques to cerebro-spinal and ventricular fluids is mentioned, together with some comments as to the significance of these findings towards future avenues of study.

BIBLIOGRAPHY

Armstrong, J.A., and Niven, J.S.: Histochemical observations on cellular and virus nucleic acids, Nature, London. 180:1335-1336, 1957.

Barber, H.N. and Callan, H.G.: Distribution of nucleic acid in the cell, Nature, London 153: 109, 1944.

Barka, T., and Dallner, G.: The correlation of UV to feulgen absorption measured in ascites tumor cells, J. Histochem. 7: 409-415, 1959.

Beers, R.F., Jr.: Inhibition and activation of polynucleotide phosphorylase by acridine orange, J. Biol.Chem. 235: 726-736, 1960.

Behrens, M.: Über die Lokalisation der Hefenucleinsaüre in Pflanzlichen Zellen, Ztschr. f. physiol. Chem. 253: 185-192, 1938.

Bertalaniffy, F.D.: Fluorescence microscope method for detection of pulmonary malignancies, Canad. Med. Assoc. J. 83: 211-212, 1960.
: Fluorescence microscopy for cytodiagnosis of cancer, Postgrad. Med. 28:627-633, 1960a.

Bertalanffy, L. von, and Pirozynski, W.J.: Ribonucleic acid in cytoplasm of liver cells; its localization in hyperplasia and hepatoma produced by 2 acetylamino fluorene, AMA. Arch. Path. 54: 450-457, 1952.

__, and Pirozynaski, W.J.: Changes of cytoplasmic basophilia during carcinogenesis induced by 2 acetylamino fluorene, Exper. Med. & Surg. 13: 261-269, 1955.

__, and Bickis, I.: Identification of cytoplasmic basophilia (RNA) by fluorescence microscopy, J. Histochem. 4: 481-493, 1956.

__, Masin, F., and Masin, M.: Use of acridine orange fluorescence technique and exfoliative cytology, Science 124: 1024- 1025, 1956a.

__,__,__: A new and rapid method for diagnosis of vaginal and cervical cancer by fluorescent microscopy, Cancer II: 873-887, 1958.

__,__,__, and Caplan, L.: Detection of gynecological cancer. Use of fluorescnt microscopy to show nucleic acids in malignant growth, California Med. 87: 248-251, 1957.

__: Cancer diagnosis by fluorescence microscopy, Modern Medicine 27:112-113, Oct.1959.

__, and Bertalanffy, F.D.: Cytological cancer diagnosis: a new approach based upon acridine orange fluorescence microscopy, What's New #214: 7-9, Early Winter 1959a.

__,__: A new method for cytological diagnosis of pulmonary cancer, Annals of N.Y. Acad. Sci. 84:225-238, 1960.

Brachet, J.: Recherches sur la synthèse de l'acide thymonucléique pendent le developpement de l'oeuf d'oursim, Arch. de Biol. 44: 519-576, 1933.

__: La detection histochimique des acides pentosenucléiques, Compt. rend.Soc.de biol. 133:88-90,1940.

__: Nucleic acids in cell and embryo, Symp. Soc. Exper.Biol. No.1: 207-244, 1947.

Bradley, D.F., and Felsenfeld, G.: Aggregation of an acridine dye on native and denatured desoxyribonucleates, Nature, London, 184: 1920-1922, 1959.

Bucher, O.: Zur untersuchung Lebender Menschilicher Leukocyten in vitro, Experientia 2: 461-463, 1946.

Bukatsch, F., and Haitinger, N.: Beiträge zur Fluoreszenzmikroskopischen Darstellung des Zellinhaltes, Insbesondere des Cytoplasmas und des Zellkerns, Protoplasma 34: 515-523,1940.

Caspersson, T.: Chromosomin and Nucleic Acids, Nature, London 153: 499-500, 1944.

__: Studien über den Eiweissumsate der Zelle, Naturw. 29: 33-43, 1941.

__: The relation between nucleic acid and protein synthesis, Symp. Soc. Exper. Biol. No.1: 127-149, 1947.

__: Cell Growth and Cell Function: A Cytochemical Study. W.W. Norton, New York. 1950 p. 185.

__, and Santesson, L.: Studies on protein metabolism in the cells of epithelial tumors, Acta. radiol. Sup. 46: 1-105, 1942.

__, Nyström, C., and Santesson, L.: Zytoplasmatische Nukleotide in Tumorzellen, Naturw 29: 29-30, 1941a.

Claude, A., and Potter, J.S.P. Isolation of chromatin threads from the resting nucleus of leukemic cells, J. Exper. Med. 77: 345-354, 1943.

Darlington, C.D., and LaCour, L.F.: Handling of Chromosomes. George Allen & Unwin Ltd., London, 1950, p. 117.

Dart, L.H., Jr., and Turner, T.R.: Fluorescence microscopy in exfoliative cytology. Report of acridine orange examination of 5,491 cases with comparison by the Papanicolaou technic, J. Lab. Invest. 8: 1513-1522,1959.

De Bruyn, P.P.H., Robertson, R.C. and Farr, R.S.: In vivo affinity of diaminoacridines for nuclei, Anat. Rec. 108: 279-308, 1950.

Daoust, R.: Analyses histochimiques des nucleases dans les tumeurs experimentales et humaines. Paper presented at the meeting of the Clinical Research Club of Quebec, Montebello, Quebec, October 20, 1961.

__, Farr, R.S., Banks, H., and Morthland, F.W.: In vivo and in vitro affinity of diaminoacridines for nucleoproteins, Exper. Cell Res. 4: 174-180, 1953.

Einarson, L. Notes on morphology of chromophil material of nerve cells and its relation to nuclear substances, Am. J. Anat. 53: 141-175, 1933.

__: Cummings, J.N.: Modern Scientific Aspects of Neurology. Edward Arnold Ltd., London, 1960, Chp.1.

Elevitch, F.R., and Brunson, J.G.: Rapid identification of malignant cells in vaginal smears by cytoplasmic fluorescence, Surg. Gynec. Obstet. 112: 3-10, 1961.

Feulgen, R., and Rossenbeck, H.: Mikroscopisch-chemischer nachweis einer Nucleinsäure vom Typus der Thymonucleinsäure, Hoppe-Seyl. Z. 135: 203-248, 1924.

Fiers, W., and Stockx, J.: Ein neues Fällungsmittel für Ribonucleinsäure und seine Anwendung bei der Bestimmung von Ribonuclease, Naturw. 44: 115, 1957.

Ghadially, F.N.: Red fluorescence of experimentally induced and human tumors, J. Path . Bact. 80: 345-351, 1960.

Gossner, W.: Zur Histochemie des Strugger-Effektes, Verh. Dtsch. Ges. Path. 33: 102-108, 1950.

Gulick, A.: The Chemistry of Chromosomes, Bot.Rev. 7: 433-457, 1941.

Gundlack, Gerd. H., Stein, W.H., and Moore, S.: The nature of the amino acid residues involved in the inactivation of ribonuclease by iodoacetate, J. Biol. Chem. 234: 1754-1760, 1959.

Hamperl, H.: Fluorescence Microscopy, Arch.Path. 19: 838-839, 1935.

Henry, H. and Stacey, M.: Histochemistry of gram staining reaction for micro-organisms, Nature, London 151:671, 1943.

Hicks, J.S., and Matthaei, E.: Fluorescence in Histology, J. Path. Bact. 70: 1-12, 1955.

Hirsch, G.C.: Die Hebendbenbachtung der Restitution des Sekretes im Pankreas; die Restitution der Granulo nach Pilokarpinreizung ihr ort und ihre Zeit, Ztschr. f. Zellforsch. u. mikr. Anat. 15: 36-68, 1932.

Holland, J.C., and Ackermann, M.R.: Fluorescent microscopy in the diagnosis of cervical carcinoma: its application in office practice, Obstet. Gynec., N.Y. 17: 38-40,1961.

Hunter, D.T., Brown, D.N., and Redman, R.F.: Extracervical cancer detection by fluorescent cytology, J. Okla. Med. Ass. 52: 772-777, 1959.

Kaplan, L., Masin, F., Masin, M., Carleton, R., and Bertalanffy, L. von: Acridine orange fluorochrome in the study of normal and malignant epithelium of the uterine cervix, Amer. J. Obstet. Gynec. 80: 1062-1073, 1960.

Kit, S.: Base composition of the ribonucleic acids of normal tissue, tumors and tissue subfractions, Arch. Biochem. 88: 1-9, 1960.

Kleinschmidt, W.J.: The composition of RNA and DNA of normal and neoplastic tissue, Cancer Res. 19: 966-969, 1959.

Krogh, E.V., and Bergeder, H.D.: Experimental irradiation damage of the cerebellum demonstrated by Einarson's Gallocyaninchromalum staining method, Proc. IIIrd. Int. Congress of Neuropath., Brussels, 287-294, 1957.

Kurnick, N.B.: Histological staining with methyl-green pyronine, Stain Technol. 27: 233-242, 1952.

Lillie, R.D.: Histopathologic Technic and Practical Histochemistry. 2nd. Edition. The Blakiston Company, N.Y., 1954, pps. 11-15.

Love, R.: Distribution of RNA in tumor cells during mitosis, Nature, London 180: 1138-1139,1957.

McClung's Handbook of Microscopical Technique. 3rd edition. Paul B. Hoeber Inc., N.Y., 1950, p. 678.

Mirsky, A.E., and Pollister, A.W.: Nucleoproteins of Cell Nuclei, Proc. Nat. Acad. Sc. 28: 344-352, 1942.

__,__:Fibrous Nucleoproteins of Chromatin, Biol. Symposia 10: 247-260, 1943.

Naylor, B.: The history of exfoliative cancer cytology, Univ. Mich. Med. Bull. 26: 289-296, 1960.

Pappenheim, A.: Vergleichende Untersuchungen über die elementare Zusammensetzung des Rothen Knochenmarkes einiger Säugethiere, Virchows Arch. f. path. Anat. 157: 19-76, 1899.

Pearce, A.G.E.: Histochemistry, 2nd edition, J.A. Churchill Ltd., London, 1960, Chp. 8 & 26.

Perry, S., and Reynolds, J.: Methyl-gree-pyronin as a differential nucleic acid stain for peripheral blood smears, Blood II: 1132-1139, 1956.

Pollister, A.W., and Mirsky, A.E.: Distribution of Nucleic Acids, Nature, London, 153: 711, 1944.

Popper, H.: Distribution of vitamin A in tussue as visualized by fluorescence microscopy, Physiol. Rev. 24: 205-221, 1944.

Price, G.R., and Schwartz, S.: Oster, G., and Pollister, A.W.: Physical Technique and Biological Research. Cells and Tissues. Vol. 3, Academic Press, N.Y., 1956, p.91, 728.

Raimondi, A.: Personal communication, 1961.

Ranadine, N.S., and Korgaonkar, K.S.: Spectrophotometric studies on the binding of acridine orange to RNA and DNA, Biochim. Biophys. Acta. 39: 547-550, 1960.

Rozdilsky, B.: Personal communication, 1961.

Russell, D.S., and Rubinstein, L.J.: Pathology of Tumors of the Nervous System. Edw. Arnold Ltd., London, 1959 pp.241.

Sandritter, W., and Seigert, R,: Ultraviolettmikrospektrophotometrische Untersuchungen an Virusbeimpften Chorio-Allantoismembranen, Verh. Dtsch. Ges. Path. 38: 102-111, 1954.

Schümmelfeder, N.: Strukturänderungen des Protoplasmas bie dem Absterben der Zelle, Verh. Dtsch. Ges.Path. 33: 65-69, 1950.

__: Fluorochrome staining of living, surviving and dead protoplasm with basic dye, acridine orange; relation to cellular metabolism, Arch. path. Anat.318: 119-154, 1950a.

__: Fluorochromatization of medullated nerve fibers with acridine orange, Arch. path. Anat.319: 294-320,1950b.

__: Fluoreszenzmikroskopische und Cytochemische Untersuchunger Über Frühschäden am Kleinhirn der maus nach Rontgenbestrahlung, Proc. IIIrd Int.Cong. of Neuropath., Brussels, p. 295-307, 1957.

__, Ebschner, K.J., and Krogh, E.: Die Grundlage der Differenten Fluorochromierung von Ribo-und Desoxyribonucleinsäure mit Acridinorange, Naturw. 44: 467-468, 1957a.

__, Histochemical significance of the polychromatic fluorescence induced in tussues stained with acridine-orange. J. Histochem. 6: 392, 1958.

Stowell, R.E.: Photometric histochemical determination of thymonucleic acid in experimental epidermal carcinogenesis, J. Nat.Cancer Inst. 3: 111-121, 1942.

__: Thymonucleic acid in tumors, Cancer Res. 5:283-294,1945.

__: Fluorochromatization of medullated nerve fibers with acridine orange, Arch. path. Anat. 319: 294-320,1950b.

__: Fluoreszenzmikroskopische und Cytochemische Untersuchunger über Frühschäden am Kleinhirn der maus nach Rontgenbestrahlung, Proc. IIIrd. Int. Cong. of Neuropath., Brussels, p. 295-307, 1957.

__, Ebschner, K.J., and Krogh, E.: Die Grundlage der Differenten Fluorochromierung von Ribo-und Desoxyribonucleinsaure mit Acridinorange, Naturw. 44: 467-468, 1957a.

__: Histochemical significance of the polychromatic fluorescence induced in tissues stained with acridine-orange. J. Histochem. 6: 392, 1958.

Stowell, R.E.: Photometric histochemical determination of thymonucleic acid in experimental epidermal carcinogenesis, J. Nat. Cancer Inst. 3: 111-121, 1942.

__: Thymonucleic acid in tumors, Cancer Res. 5: 283-294,1945.

__, and Cooper, Z.K.: The relative thymonucleic acid content of human normal epidermis hyperplastic epidermis and epidermoid carcinoma, Cancer Res. 5: 295-301, 1945a.

Strugger, S.: Fluoreszenzmikroskopische Untersuchungen über die Aufnahme und Speicherung des Akridineorange durch Lebende und Tote Pflanzenzellen, Jena, Z. Naturw. 73: 97-134, 1941.

Sussman, W.: Detection of gynecological cancer by fluorescence microscopy Obst. Gyn., N.Y. 13: 273-277, 1959.

Taft, E.G.: The specificity of methyl-green pyronine stain for nucleic acids, Exper. Cell Res. 2: 312-326, 1951.

Turner, T.R.: Fluorescence Photomicrography of acridine orange stained exfoliative cytologic preparations, J.Biol. Photogr. Ass. 28: 9-10, 1960.

Umiker, W., Pickle, L., and Waite, B.: Fluorescence microscopy in exfoliative cytology: an evaluation of its application to cancer screening, Brit. J. Cancer 13: 398-402, 1959.

__, and Gadebusch, H.H.: Fluorescence microscopy: a review, Univ. Mich.Med. Bull. 26: 199-211, 1960.

Unna, P.G.: Ueber Weitere Versuchc, Farben auf dem Gewebe zu Erzeugen und die Chemische Theorie der Färbung, Arch.f. mikr. Anat. 30: 38-48, 1887.

Watzka, C.P. Personal communication 1961.

Wolfe, L.: Personal communication, 1961.

ACKNOWLEDGMENTS

I wish to express my appreciation to Professors Theodore Rasmussen, Arthur Elvidge and William Feindel for the privilege of carrying out this experimental study at the Montreal Neurological Institute. The entire surgical staff graciously granted me the opportunity to study their operative material. I am especially grateful to Dr. Gilles Bertrand for instilling the idea of this study and for his suggestions and guidance throughout the course of this work. Mr. John R. Gilbert, and Mr. Charles P. Hodge were most instrumental in perfecting and standardizing the all important technical aspects of this project. I am indebted to Mr. Gerard Papillon for his high quality work on the permanent histological sections and to Dr. Gordon Mathieson for free use of the departmental facilities.

I wish to thank Drs. Raymond Grossman and Stirling Carpenter, Mrs. "Pete" von Nida and Mrs. Garth Embree for help in translating the German manuscripts.

Mrs. Rona Fudge and Mrs. Elizabeth Campbell did much of the preliminary typing and the secretarial staff of the Institute, namely Miss Phyllis Angus, typed the final draft. A special thank you to my wife, Marianne for the tedious task of proofreading and for the many helpful corrections.

I should like to dedicate this thesis to the late Dr. William V. Cone under whose tutelage I was never privileged to study, but whose teachings have been most instrumental in my education.

This investigation was carried out during the tenure of a Post-Doctoral Fellowship, No. 13,522, National Institute of Neurological Diseases and Blindness, United States Public Health Service.