A Cytochemical study on the nucleus of Sarcoma 180 cancer cells from Crocker Albino Mice, grown in Vitro

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A CYTOCHEMICAL STUDY ON THE NUCLEUS OF SARCOMA 180
CANCER CELLS FROM CROCKER ALBINO MICE,
GROWN IN VITRO

A THESIS
SUBMITTED TO THE FACULTY OF ATLANTA UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE

BY
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ABSTRACT

A cytochemical study of the nucleus of Sarcoma 180 cancer cells from Crocker albino mice, grown in vitro, was made. The cancerous cells were treated with various reagents in an attempt to elucidate the nature of the nucleus. The Feulgen nuclear reaction revealed the presence of large quantities of DNA (deoxyribonucleic acid) within the nucleus of these malignant cells, which was not only evenly distributed throughout the structure, but also concentrated near one end of the nuclear membrane in some cells.

Cells were treated with 0.2% and 1.0% DNAase (adjusted to a pH of 6.0) and incubated at 37° C. for a one hour period. Treatment with the enzyme caused rapid breakdown of nuclear material leaving an apparently empty structure except for the presence of very conspicuous nucleoli. The enzyme revealed an apparent thickened nuclear membrane. The thickening was probably a result of the penetration of the membrane by the enzyme, or due to a breakdown of the chromatin material that has long been established as being in association with the nuclear membrane.

Some of the cultures were treated with varying concentrations of a soap-like detergent, digitonin. When cells were treated with strong concentrations of the substance (0.5%, 1.0%, and 5.0%) and incubated for a 12 hr. period, both the nuclei and cytoplasm became solubilized. Whereas, when weak concentrations (0.1% and 0.2%) were utilized, only the cytoplasm was solubilized leaving the nuclei intact. This property of digitonin, wherein nuclei were left intact after treatment, may be due to the
inability of weak concentrations of the soap-like detergent to breakdown the disulfide bonds of the proteins of which the nuclear membrane is composed, thus allowing the nuclei to maintain their integrity.
ACKNOWLEDGMENTS

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CHAPTER I

INTRODUCTION

Cowdry (1955) defined cancerous cells as being those which characterize malignant tumors irrespective of their origin, and malignant tumors as being those which can thrive at the expense of the organism and unless eliminated will cause death.

Homburger (1957) referred to Sarcoma 180 as being an extremely unspecific mouse tumor which grows in almost all strains. Snell (1941) described a sarcoma as being a malignant tumor composed of cells of the connective tissue type, since it is formed on the connective tissue plan; that is, developing its own stroma and blood vessels so that the stroma is in contact with the tumor cells.

Early attempts by biological investigators to establish fundamental differences between normal and malignant cells of the same tissue type have failed to bring out any striking variation in chemical make up, enzyme content, metabolism or structure. According to Cowdry (1955), in a discussion of the biochemistry of malignant tissue, no conclusive evidence existed which revealed any qualitative differences in chemical composition between normal and malignant tissues. He therefore concluded that whatever differences exist are of a quantitative nature, the biological significance of which is difficult to evaluate.
It has been reported by a number of investigators (Cowdry, 1955; Busch, 1962; Williams, 1963) that the chromatin material of malignant cells appeared to be conglomerated near the periphery of the nuclear membrane and that in all stages of mitotic division the nuclear membrane appeared to be intact. In view of these findings, this study was done in an attempt to elucidate the cytochemical nature of the nucleus of cancerous cells of mouse Sarcoma 180 grown in vitro.
CHAPTER II

REVIEW OF LITERATURE

According to Busch (1962), cancer cells possess a number of properties which are biologically different from most cells of the host. For example, the cells have darker stained nuclei, more chromatin, and a higher frequency of mitosis than cells of the tissue origin. He further stated that cancerous lesions exhibit the important pathological properties of invasion of normal tissues and entry into cellular masses, such as muscle cells, and the most unusual property of all, metastasis, which is the escape of cancer cells from the original locus of the tumor and passage via blood or lymphatic tissues to another tissue or organ where the lesion lodges and grows into a secondary tumor.

He observed that unlike many cells in man, the cancer cell is immortal in tissue culture. He noted that among the other characteristics of these cells is the occasional but characteristic multiple mitosis, and the resistance to conditions which would produce serious damage to other cells. For example, tumor cells have remained viable as long as three days in an environment containing cyanide and totally lacking in oxygen; wherein, cells of the brain or heart die within minutes under the same conditions.

Busch concluded that the biological characteristics of the cancer cell would seem to indicate that it is a cell which has specialized growth, particularly abnormal growth, and able to survive under adverse conditions. He
further postulated that perhaps the only pathognomonic histological characteristic, which already differentiates tumor cells from other cells, is the occurrence of multiple mitotic spindles which result in multiple divisions of the cancer cell.

In studying isolated nuclei of rat liver cells, Anderson (1953) observed that the effects of DNAase and the proteolytic enzymes, trypsin and chymotrypsin, were characteristic and distinct. When DNAase (1 mg./ml. solution) was introduced under the coverslip containing isolated nuclei, the gel-like structure of the nuclei was rapidly broken down with a consequent loss of physical and image density as observed under the dark contrast microscope. The clearing process required about 1-4 min. at room temperature and appeared to take place evenly throughout the nuclei. Brownian movement of the nucleoli and granules (which were generally condensed in the nuclei) gave further evidence of loss of gel structure. RNAase in contrast (0.001-1.0 mg./ml.) caused the nucleoli to condense into dark irregular masses, but caused no general clearing of the nuclei. The proteolytic enzymes trypsin and chymotrypsin (0.01-1.0 mg./ml. solutions) both caused rapid disintegration of nuclei leaving only shreds of the ruptured nuclear envelopes.

He concluded that these effects were sufficiently distinct to allow tentative identification of the types of enzymes responsible for the alterations observed in aged homogenates, and that the general clearing of the nucleus at pH 7.1 with a slight thinning of the nuclear envelope appeared to be due to both proteolytic activity and DNAase.

According to Barton (1951) the action of crystalline DNAase on nuclei may be conveniently studied and quantitatively characterized by using isolated
nuclei as a substrate. As a result of studies performed, he reported that the following conclusions may be drawn: (1) Only a part of the DNA can be removed by the enzyme. (2) No protein is released with the DNA. (3) Calcium is the only other component of the nucleus released along with the DNA so far discovered.

Barton indicated that the enzymatically resistant fraction cannot be removed by repeated digestion with fresh enzyme, or by washing with salt or buffer solutions of moderate ionic strength. He, however, observed that the fraction can be removed as a complex of nucleic acid and protein by a strong salt solution, 1 or 2 M NaCl. This investigator further reported that the amount of resistant fraction in various species was found to be as follows: beef thymus leucocyte nuclei, 77%; carp sperm, 82%; Astéris sperm, 74%; turtle (Caretta) erythrocyte nuclei, 80%; Pecten, 89%; Mytisus sperm, 84%; Arbacia sperm, 74% and Mactra (Spisula) sperm, 40%. In all except Mactra, the basic protein is of the histone type. In Mactra the basic protein more closely resembles a protamine. As a result of these investigations, Barton postulated that the resistant DNA is a part of a complex of nucleic acid and protein held together by bonds other than electrostatic, probably co-valent.

Harrington and Koza (1951) studied the effects of X-radiation on deoxyribonucleic acid and on the size of grasshopper embryonic nuclei using methyl green stain and the Feulgen reaction for DNA determinations. They observed that with these two stains it is possible to analyze two different properties of the nucleic acid. The Feulgen is specific for the DNA content and the methyl green probably indicates the presence of DNA in the normal state of polymerization, commonly called the "highly polymerized state".
They indicated that most workers agreed that when the nuclear reaction takes place under properly controlled conditions, the Feulgen method gives a relatively accurate estimate of the DNA in nuclei, and that ultraviolet studies by other investigators identified the Feulgen positive material in the nucleus as DNA. They further reported that splenic and pancreatic deoxyribonucleases have been used to remove the Feulgen positive bands (presumed to contain large amounts of DNA) from the salivary chromosomes of Drosophila.

They concluded that although there is evidence that the reaction does not indicate the total amount of DNA present, some workers (Ris and Mirsky, 1949) have shown that it is valid to use Feulgen reaction with the photometric system to determine the relative amount of DNA in nuclei. Pertaining to this conclusion, they indicated that Leuchtenburger (1950) found Feulgen methyl green ratios to be equal in normal nuclei.

While studying the effects of ribonuclease on the metabolism of nucleic acids, protein, and respiration in onion root-tips, Brachet (1954) observed that when intact onion root-tips were treated with 1 mg./ml. for a few hours prior to immersion in water containing a labeled amino acid (radioactive glycine or phenylalanine), there was considerable inhibition of the incorporation of amino acid into proteins. He also observed that ribonuclease immediately slowed down deoxyribonucleic acid synthesis, leading to cytological abnormalities, while it first stimulated ribonucleic acid metabolism. In contrast to these striking findings of changes in protein and nucleic acid metabolism, he observed that the oxygen consumption of the root-tip meristematic tissue remained unaffected by ribonuclease, even after treatment for 15 hours.
He further observed that when sections of roots previously treated in vivo for 3 hours with ribonuclease were stained in accordance with Mazia's (1953) method for proteins, an empty space became visible around most of the nucleoli. He concluded that this finding might mean that the nucleolus plays a very important part in protein synthesis. He stated that these findings were in agreement with the experimental data obtained in the same laboratory by A. Ficq (1953).

Williams (1963) studied the effects of pituitary digest on the growth rate of Sarcoma 180 cancer cells in mice. The cells were cultured in vitro employing the hanging drop slide culture method. He observed that in most cultures, cellular outgrowths extended from the peripheries of the explants into the surrounding medium, and that after 48 hours of incubation, the cellular outgrowths appeared circular and that mitotic figures were present in many cells. He further reported that the cells contained large nuclei with conspicuous nucleoli, and that most of the cells were mononucleated; however, occasionally, bi- and multinucleated cells were observed. The nuclear material was not always centrally located, but in many instances concentrated at one end of the cell.

Williams' conclusions were in agreement with those of Cowdry (1955). He concluded that polyploidy was fairly common in malignant cells and that it promoted an increase in the size of the cells. He further indicated that some of the multinucleated cells were perhaps produced by fusion of several mononucleated cells accompanied by the enclosure of all of them in a single cell membrane and that such occurrences are typical of malignant cells.

In studying cell division (mitosis) Mazia (1953) treated synchronously dividing sea-urchin eggs with a natural detergent, digitonin, obtained from
the common foxglove plant, in an attempt to isolate the mitotic apparatus. Dilute alcohol at a minus 10°C. was used to stop the mitotic process with least damage and chemical change to the mitotic structure. The detergent dispersed everything in the cell except the mitotic apparatus which was left intact. He stated that earlier attempts to isolate this structure using peroxide and Duponal were successful, but the method involved the creation of unnatural sulfur bridges (S-S) which did not exist in the original cell. Molecules were oxidized with hydrogen peroxide (H₂O₂) to remove hydrogen (H) from the natural sulfhydryl groups (S-H) of the mitotic apparatus. After this treatment, the reaction could then be reversed with thioglycolic acid which has a sulfhydryl group. The hydrogen of these groups joined with sulfurs of disulfide bonds and the bonds were broken by reduction back to sulfhydryl groups. He concluded that as a result of these findings, even though biologists are a long way from understanding how the mitotic apparatus is formed, the availability of the isolated mitotic apparatus and cell centers should permit investigators to feed chemical information into the theory of mitosis as it evolves.
CHAPTER III

MATERIALS AND METHODS

The experimental animals used in this investigation were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. A shipment of 10 Crocker albino mice were received at the Atlanta University Laboratory on April 21, 1964. The mice were of the BALB/CJ strain, three of which were innoculated with Sarcoma 180 cancer material on April 17, 1964, prior to being shipped. The mice were of mixed sex and approximately five weeks old when received. The 10 mice were designated experimental animals and the three that contained tumor material were designated as donor animals, from which cancerous tumor tissue was removed 10-15 days after the initial transplantation and transplanted to the mice in the experimental group. The mice were placed in individual containers, fed burger bits dog chow and received water ad-libitum.

All operations were performed under aseptic conditions to prevent contamination of the tumor material. The host animals containing the Sarcoma 180 tumor material were sacrificed by anesthetizing the animals with ether and by careful decapitation. The operations were performed after securing the animals in a stationary, inverted position in a dissecting pan. A longitudinal incision was made in the abdominal wall of the animal after carefully swabbing the cancerous area with 90% alcohol. The tumor was then
removed by the use of sterile scalpels, forceps and scissors. After removal, the tumor was then divided into two portions, one for explantation in vitro, and the other for in vivo transplantation into two or three of the remaining experimental animals, in order to maintain the tumor material supply. The cancerous tissue used in the explantation (tissue culture) process was placed in a Petri dish containing Earle's solution and covered until used.

The technique proposed by Homburger (1957), was employed in making tumor transplantations. The tumor was minced into fine pieces by means of curved scissors and aspirated into a trocar (special needle no. 13), or inserted into it by means of curved forceps. The trocar containing the tumor material was inserted subcutaneously into the axillary region of the experimental animal and its contents forced out into the desired region by the plunger of the needle. The tumor material was secured in the desired location by grasping the end of the plunger and needle with the forefinger and thumb while still being inserted under the skin and carefully withdrawing it from the region. In this manner, the tumor material supply was maintained throughout the period of experimentation.

Instruments used during the transplantation and explantation processes, and all glassware utilized were sterilized at 175°C. for one hr. after being thoroughly cleaned. All glassware was rinsed in hot tap and distilled water after washing. Cannisters were used for the storage of glassware and instruments until used.

After the removal of the tumor as explained earlier, the tissue for explantation was then macerated, while still in Earle's solution, with a scalpel and tissue teasers into small pieces of similar sizes. The pieces of Sarcoma 180 tissue were then removed from the original Petri dish
containing Earle's solution with forceps and placed into another containing Earle's solution. The dish was then covered and allowed to stand.

All procedures pertaining to the culturing process were performed in an isolated constant temperature room under a covered glass chamber designed for preparing tissue cultures. The room and chamber were rendered aseptic by a thorough washing of walls and floors with a saturated sodium hyperchloride solution one or two hours prior to commencing the culturing process. The walls and sides of the chamber were also swabbed and sprayed with a 90% alcohol solution.

The culture medium used in this investigation consisted of one drop each of fowl plasma and embryonic extract. The dessicated plasma, control no. 464397, and tissue culture reconstitution fluid, control no. 444924, with which the liquid plasma was prepared, were obtained from Difco Laboratories, Detroit, Michigan. The plasma was rendered soluble by dissolving the contents of the 5 ml. vial of dissicated plasma in 4.5 ml. of reconstitution fluid. It was then refrigerated until used in the culturing process.

The chicken embryonic extract was prepared from 9 day old chick embryos, obtained from fertilized eggs secured from the Georgia State Hatchery, Atlanta, Georgia. The eggs were incubated at 37°C. for 9 days prior to the preparation of the extract. A small hole was made in one end of the egg by cracking the shell with the handle end of a scalpel after the shell was swabbed with 90% alcohol. The cracked shell and membrane were removed with forceps and the embryos obtained by grasping the neck between the curved end of a pair of forceps and gently lifting them from the yolk material. The embryos were then placed in a petri dish containing Earle's solution (control no. 464662). After rinsing in Earle's solution, the
embryos were placed in the open end of a 10 cc syringe and the plunger inserted. They were then expressed into a graduated cylinder to which an equal volume of Earle's solution was added. The mixture was then stirred and allowed to stand for 30 min. After settling, it was then equally divided and placed into four centrifuge tubes and centrifuged at 2,000 RPM's for 30 min. After centrifugation, the supernatant was removed and placed in small test tubes and refrigerated until used.

In preparing the single depression slide cultures, 6 coverslips were placed in the bottom of a Petri dish using a pair of watchmaker forceps. One drop each of the prepared fowl plasma and embryonic extract was then pipetted onto the coverslip and allowed to stand in the covered Petri dish for approximately 5 min. or until coagulated. Upon coagulation of the culture medium, a small piece of the cancerous tissue was pushed down into the clot until it was in contact with each coverslip. This procedure was done in order that the cells growing out from the tissue might adhere to the coverslip. They were then inverted over the wells of depression slides and sealed in place with melted paraffin. After the paraffin was placed around the edges of the coverslip and depression slide by use of a camel's hair brush, the cultures were incubated at 37°C.

The explants were observed at various intervals for evidence of the outgrowths of individual cells from the tissue mass. Providing outgrowths were present, the cultures were incubated for 48 hrs. before treatment with the various chemicals used in the investigation.

The first cultures obtained in this manner were divided into two groups. The first group of 12 cultures, designated Group I, controls, was stained with Harris' hematoxylin and eosin after Bouin's solution fixation. The second group, designated Group II, was fixed with Carnoy's fixative and treated with the Feulgen nuclear reaction for the detection of
DNA (deoxyribonucleic acid).

All subsequent cultures were designated as groups in a similar manner, after various chemical treatment and staining. Group II and III cultures were treated with 0.2% and 1.0% DNAase (deoxyribonuclease), respectively. The DNAase enzyme solution was prepared according to the method of Kunitz (1950), which consisted of dissolving 5 mg. of the crystalline desoxyribonuclease in 4.95 ml. of distilled water and the solution adjusted to a final pH of 6.0. The 0.2% DNAase solution was prepared in a similar manner.

The cultures of Group II and III were treated with the enzyme solutions by carefully removing the coverslips from the well of the depression slides and pipetting two drops of the DNAase solution onto the original clot containing outgrowths of many individual cells. The coverslips were left in an upright position and the depression slide "ringed" with glycerin and inverted over the coverslip. The cultures were then incubated at 37°C. for one hour. After the period of incubation, they were fixed in acetic-alcohol formalin at 5°C. for 24 hrs. and stained, using a modification of Lillie's methyl green-pyronin staining technique. The technique consisted of using pyronin in a saturated acetone solution after dehydration and differentiation with methyl green, thus, avoiding irregular extraction of pyronin by the acetone. Untreated cultures were also stained in the same manner for making comparative studies of the DNA content of the nuclei of cancerous cells.

Smear slides of isolated nuclei treated with 1% DNAase were also made. Nuclei were isolated by homogenizing cultures containing outgrowths of individual cells in a small amount of Earle's solution. After homogenization, the solution containing suspended nuclei and cellular fragments was placed in centrifuge tubes and centrifuged at 10,000 RPM's for 10 min.
Centrifugation was done in a refrigerated centrifuge at -10°C. After centrifugation, the supernatant was discarded and the fractionate treated with an equal volume of 1.0% DNAase. This was allowed to stand for 12 hrs., after which the DNAase solution was pipetted off and discarded. One drop of the fractionate was pipetted onto a clean slide and stained with one drop of Lillie's methyl green-pyronin. Coverslips were placed over the smear, pressed firmly to the slide, and sealed in place with paraffin. Untreated smear slides of isolated nuclei were stained with acetocarmine for making comparative studies.

The crystalline desoxyribonuclease and digitonin utilized as the last chemical agents with which cultures were treated were obtained from the National Biochemical Corp., Cleveland, Ohio.

From a stock supply of 20 ml. of a 5.0% digitonin solution prepared by dissolving one gram of powered digitonin (a soap-like detergent) in 19 ml. of Earle's solution, the following percentage solutions were prepared: 0.1%, 0.2%, 0.5%, 1.0%, and 5.0%. A series of 12 cultures each was treated with these concentrations of digitonin solution and designated Groups IV, V, VI, VII, and VIII, respectively.

Cultures treated with the soap-like detergent were again incubated at 37°C. for 12 hrs. The treatment consisted of filling the well of depression slides with the desired concentration of the digitonin solution and sealing the coverslip in an inverted position over the well of the slide. The cultures were then incubated at 37°C. for 12 hrs. After this period of incubation, the cultures were fixed with Bouin's solution, stained with Harris' hematoxylin and eosin, and mounted onto clean slides using a synthetic resin.
CHAPTER IV

EXPERIMENTAL RESULTS

These findings were based upon microscopic observations of nuclear changes resulting from treating the cells of mouse Sarcoma 180, grown in vitro, with various chemical reagents in an attempt to elucidate the cytochemical nature of the nucleus. The results below are based on representative samples of a series of tissue culture experiments.

When a series of control cultures were fixed in Bouin's solution and stained with Harris' hematoxylin and eosin, the nuclei appeared to be somewhat bluish in color. Whereas, the cytoplasm had a rather red appearance. Nearly all of the tumor cells were similar in size, but varied somewhat in shape. Some cells appeared spherical, polygonal, and sometimes spindle in shape (Fig. 1). The cells possessed a large amount of basophilic cytoplasm which often contained tiny vacuoles.

The nuclei were highly hyperchromatic and fairly uniform in size, but varied in shape. An apparent heavily stained nuclear membrane enclosed the fine "dustlike" particles of chromatin and three to four large basophilic nucleoli. The nuclear material was not always centrally located, but rather, appeared in many instances to be concentrated at one end of the nucleus (Fig. 2). Many of the cells observed were evidently bi- and multinucleated, based upon the large quantity of chromatin material present in the nucleus. Some cells appeared to be undergoing nuclear division, but in all such instances the nuclear membrane appeared to be completely
Fig. 1. This photomicrograph shows varied shapes of tumor cells. X 1000.

Fig. 2. Photomicrograph showing position of nuclear material in the nucleus of Sarcoma 180 cells. X 450.
intact.

Those cultures stained with the Feulgen nuclear reaction revealed evidence that DNA (deoxyribonucleic acid) was present. The presence of large amounts of DNA was evident in that the nuclei of Sarcoma 180 cells gave a dark red to purplish color when treated with Schiff's reagent after acid hydrolysis (Fig. 3). Almost all of the cells treated with this reagent revealed similar quantities of DNA; but in those cells that were identified earlier as being bi- and multinucleated, DNA appeared to be present in greater amounts based on the large quantity of chromatin material depicting the characteristic purplish color that indicated the presence of DNA. In some cells in which the nuclear material was concentrated at one side of the nucleus the Feulgen reaction revealed that most of this material was chromatin, since it gave positive color reactions for DNA.

When the two groups of cultures designated Group III and IV were treated with 1.0% and 0.2% solutions of the enzyme, DNAase, respectively, and incubated for one hour, it was observed that the dense material occupying the nucleus of these cells began to disintegrate almost immediately. After 30 min. of incubation it was observed that the gel-like structure of the nuclei had rapidly broken down with a loss of image density as compared with nuclei of untreated cultures. After one hour of incubation, the nuclear membrane surrounding the chromatin material appeared to become somewhat thicker than the nuclear membrane of untreated cells.

Nuclear breakdown occurred at a much faster rate upon treatment with the 1.0% DNAase solution than that observed when cells were treated with the 0.2% enzyme solution. Although at the end of one hour of incubation
Fig. 3. Photomicrograph of chromatin material in cells treated with Schiff's reagent. X 1000.

Fig. 4. Photomicrograph of isolated nuclei after DNAase treatment showing nucleoli. X 1000.
the amount of nuclear material affected by the two concentrations paralleled each other, it could be observed that the nuclear membrane of the cells treated with the greater enzyme concentration became thicker at a much higher rate.

It was clearly evident when cultures were observed after acetic-alcohol formalin fixation and Lillie's methyl green-pyronin staining that almost all of the nuclear material was destroyed by the enzyme. This observation was based on the fact that the nuclei no longer revealed the characteristic bluish green color of the basic stain, methyl green as was seen in the untreated cultures fixed and stained in a similar manner.

The smear slides containing isolated nuclei that were treated with 1.0% DNAase and stained using methyl green-pyronin also revealed nuclei that appeared rather devoid of nuclear material, except for the presence of one or two very conspicuous nucleoli (Fig. 4). In some instances, as many as three to four nucleoli were present within the nucleus of the same cell. Occasionally, nuclei containing minute "dust-like" particles could be discerned. When those isolated nuclei treated as already described were compared with untreated nuclei stained with acetocarmine, it was revealed that the nuclei contained a gel-like structure that stained bright red in color with dense "dust-like" particles dispersed or enmeshed throughout the nucleus (Fig. 5).

When cultures were treated with 0.5% and 1.0% digitonin and incubated for 12 hrs., it was observed that the cytoplasm of the cells began to break down at a very rapid rate with a gradual disintegration of the nucleus, commencing first with a slow breakdown of the nuclear membrane.
Fig. 5. This photomicrograph shows the amount and dispersal of chromatin material in an isolated nucleus. X 1000.

Fig. 6. Photomicrograph of disintegrated nuclei and cytoplasm after treatment with 0.5% digitonin. X 450.
Once the nuclear membrane had been penetrated by the soap-like detergent, it appeared that the entire cell continued to disintegrate until only very fine threadlike projections of the cytoplasm remained intact with the small rod-shaped nuclei that remained (Fig. 6). Upon treating cultures with a 5.0% digitonin solution, breakdown of the cells occurred almost immediately leaving only slender threads of both the cytoplasm and nucleus.

It was observed that when cells were treated with weaker concentrations of digitonin, 0.1% and 0.2%, the cytoplasm disintegrated at a much slower rate and that the nuclear membrane was not rapidly penetrated by the soap-like detergent. As a result the nuclei were apparently left intact. Best results were obtained utilizing the 0.2% digitonin solution, on the basis of the presence of intact nuclei that were observed as isolated structures with hardly any obvious cytoplasm attached after treatment with the soap-like detergent for 12 hrs. at 37°C. (Fig. 7).
Fig. 7. Photomicrograph of intact nuclei after treatment with 0.2% digitonin. X 1000.
CHAPTER V

DISCUSSION

When the nuclei of Sarcoma 180 cancer cells grown \textit{in vitro} were stained with Harris' hematoxylin and eosin prior to any chemical treatment, it was observed that the chromatin material became distinct and in most instances evenly distributed along the nuclear membrane. Occasionally there were cells with clumps of chromatin in the center of the nucleus in close proximity with the nucleolar structure (probably nucleoli associated chromatin). In other cells the chromatin material was concentrated near one end of the nucleus. These findings are similar to those of other investigators (Busch, 1962; Cowdry, 1955; Williams, 1963; Koller, 1963). According to Koller, it had been observed as early as the 19th century that the shape and size of the nuclear structure of cancer cells were more variable than in normal cells. Busch and Cowdry indicated that such variability in tumor tissue increased with malignancy.

Koller observed that indentation, furrowing, elongation and even budding may occur in the nuclei of tumor cells and that most frequently there may be an increase in the amount and distribution of chromatin material. Therefore, on the basis of similarities in nuclear structure observed during this investigation, it may be concluded that such nuclear features are characteristic of malignant cells. Other characteristics observed during this investigation that may be used as criteria for
distinguishing malignant cells on the basis of nuclear organization, were those such as polyploidy, and bi- and multinucleated cells. Such criteria as these have been used by cancer researchers in their attempts to establish nuclear differences that might be used to distinguish normal from cancerous cells.

Recent works by many investigators (Anderson, 1953; Barton, 1951; Brachet, 1954) have shown that when DNAase and RNAase were prepared according to accepted methods, they were specific for DNA and RNA, respectively. Such were the findings during this study, for when cells of Sarcoma 180 were treated with DNAase, its effects upon the nuclear material and its organization were similar to those observed by these and other investigators. When DNAase was used to treat malignant cells grown in vitro and incubated for a 30-min. period, the nuclei of these cells gradually assumed an empty appearance and the nucleoli appeared to condense into irregular masses. After a one hr. incubation period in cells which were fixed with acetic-alcohol formalin and stained using methyl green-pyronin, the nuclear envelope (membrane) was more distinct and appeared to be much thicker than the membrane of the untreated cells. In some nuclei, especially those isolated by centrifugation (see Fig. 5), the main body of the nucleus was filled with very fine reticulations and a few granules were sometimes seen along the inner edges of the nuclei. However, in the nuclei of other cells very conspicuous granules were observable.

These observations appear to be similar to those observed by Anderson (1953), who while studying isolated rat liver nuclei indicated that after treatment with DNAase many such structural characteristics of nuclei could be distinguished that otherwise would not be discerned. He
also observed a sharper definition of the nuclear envelope and attributed this factor to alterations of the membrane due to the isolation technique, involved, or that such thickening might have been caused by the enzyme as it penetrated the nuclear envelope. He also observed that treatment with nucleases resulted in the formation of numerous intranuclear particles together with condensed nucleoli and concluded that the gradual clearing observed in incubated homogenates is believed to be due primarily to the action of DNAase.

It has long been known that the Feulgen reaction is specific for DNA. Results obtained during this investigation with the Feulgen reaction after acid hydrolysis revealed large quantities of DNA material in the nuclei of Sarcoma 180 cancer cells. These findings appear to be substantiated by the work of Harrington and Koza (1951) in their study of the effects of X-radiation on desoxyribonucleic acid and on the size of grasshopper nuclei using methyl green and the Feulgen reaction for DNA determinations. They observed that two different properties of DNA were revealed by the two stains. The Feulgen reaction was specific for the DNA content and methyl green probably indicated only the presence of DNA in its normal state of polymerization.

The effects of varying concentrations of digitonin (0.1%, 0.2%, 0.5%, 1.0%, and 5.0%) upon Sarcoma 180 cancer cells were found to be rather characteristic and distinct in that the soap-like detergent caused disintegration of both cytoplasmic and nuclear material depending upon the concentration used. Mazia (1953) used digitonin for isolation of the mitotic apparatus from synchronously dividing sea urchin eggs. Earlier
attempts to isolate the structure using Duponol (sodium lauryl sulfate) and hydrogen peroxide were successful, but involved the creation of unnatural disulfide bridges (S-S) which did not exist in the original cells. He further indicated that since the mitotic apparatus contained relatively few disulfide bridges, digitonin was concentrated enough to solubilize the other materials of the cell and yet not solubilize the mitotic apparatus.

Since digitonin possessed this unusual property it was thought by the present investigator that if weak concentrations of digitonin were used it would digest the cytoplasm and leave the nuclear membrane intact. Therefore, upon the treatment of Sarcoma 180 cancer cells with varying concentrations of digitonin it was observed that 0.1% and 0.2% concentrations would cause the cytoplasm to disintegrate, leaving the nucleus intact, while stronger concentrations (0.5%, 1.0%, and 5.0%) caused disintegration of both the cytoplasm and nucleus.

It has been established that the nuclear membrane is composed essentially of a lipo-protein complex in which the polar groups of the lipid are believed to be "masked" by the non-polar groups of the protein. Thus, since the essential active components of the nuclear membrane are protein in nature, it may be possible that it also contains disulfide bridges (S-S). Therefore, it is reasonable to assume that the reactions of digitonin on intact cells in which the nuclear membrane was not dissolved may be explained on the basis that weak concentrations of digitonin did not attack the disulfide bridges of this structure causing it to maintain its integrity, while strong concentrations solubilized the cytoplasm and penetrated the nuclear membrane, causing the nuclear material to become solubilized.
CHAPTER VI

SUMMARY AND CONCLUSION

1. A cytochemical study has been made on the effects of various reagents on the cells of Sarcoma 180 from Crocker albino mice, grown in vitro.

2. When untreated cells were studied it was revealed that they possessed many nuclear features which may be used as characteristics for distinguishing malignant from normal cells.

3. The Feulgen nuclear reaction was used to determine the organization and location of DNA within the nucleus of malignant cells. The reaction revealed the presence of large quantities of DNA concentrated near one end of the nuclear membrane in some cells and scattered throughout the nuclei of others, such as observed in mono-, bi- and multinucleated cells.

4. When cells were treated with 0.2% and 1.0% desoxyribonuclease and incubated for one hour at 37°C, the chromatin material of the nucleus was rapidly broken down, leaving an apparently empty structure except for the presence of very conspicuous nucleoli, while the nuclear membrane appeared thicker after treatment with the enzyme than that of untreated cells.

5. Treatment of cells with varying concentrations (0.1%, 0.2%, 0.5%, 1.0%, and 5.0%) of the soap-like detergent, digitonin, revealed that strong
concentrations of the substance will dissolve the cytoplasm and nucleus of cells, but if treated with weak concentrations, only the cytoplasm will be dissolved leaving the nuclei intact.

6. The nuclear membrane probably elicited a negative response to 0.1% and 0.2% digitonin and remained intact due to the structure being made up of a lipo-protein complex of which the protein part contained disulfide bridges (S-S) that were not dissolved by weak concentrations of digitonin.
LITERATURE CITED


