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THESIS TITLE: A Quantitative Cytophotometric Analysis of Tumor Cell Growth Patterns that Occur in Human Intestine Adenocarcinoma

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The author wishes to dedicate this thesis to his parents, Joseph and Rose Nudo; to his brothers and sister, David, Raymond, and Tracey; and to his girlfriend Marlene, whose support and encouragement made this study possible.

ABSTRACT

A QUANTITATIVE CYTOPHOTOMETRIC ANALYSIS OF
TUMOR CELL GROWTH PATTERNS THAT OCCUR IN
HUMAN INTESTINE ADENOCARCINOMA

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Master of Science

Youngstown State University, 1984

Eight adenocarcinomas of the colon were sectioned into divisions and examined microspectrophotometrically for DNA content employing the Feulgen reaction. The results indicate that 75% of these tumors contain an elevated concentration of DNA in the central division. The DNA elevation is mainly due to the environmental pressures in each individual tumor which results in the formation of aneuploid cells. Populations of cells that are active in the synthesis of DNA result in the elevation of the mean DNA value in the periphery of the tumor, which occurs in the remaining twenty-five percent of the tumors sampled. The mean DNA values and number of aneuploid cells were correlated with the possible age of the tumors and probability for metastasis. The older tumors were shown to metastasize to lymph nodes and other organs, while the other tumors exhibit the ability to metastasize but were excised from patients before this occurred.

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CHAPTER I

Introduction

Colon cancer afflicts every country in the world to some degree. Unfortunately, the United States has a relatively high incidence of colon cancer. Large bowel adenocarcinoma is this country's most common malignant tumor, excluding skin cancer (Carter, 1976). Nearly 50,000 deaths per year are attributed to colon carcinoma (Leibovitz, 1976). This is second only to lung cancer. About one half of these deaths are due to metastasis of the cancer to other vital organs. The most common sites of large bowel cancer metastasis are the liver and lungs. The kidneys, adrenal glands, brain and bone can also be invaded with less frequency. Metastasis occurs mainly through the lymphatic channels. Direct extension of the tumor into surrounding tissue may also account for the spread of the cancer (Carter, 1976).

There are certain characteristics which may make certain individuals candidates for developing colon cancer. Individuals having survived a previous colon cancer have a high probability of developing colon cancer again (Lipkin, 1975). Statistics indicate that relatives of patients with large bowel adenocarcinoma have an increased probability of developing colon cancer three times greater than that of the general population (Lipkin, 1975). He also states that within the past thirty years the death rate of male patients demonstrating colon cancer has changed very little. Nineteen of every 100,000 male patients die as a result of the cancer. The cure rate of colon cancer

has not changed remarkably in the past twenty years. The US News and World Report notes that 43% of people with colon cancer were cured in the years 1960-63 as compared to 50% cured in the years 1973-80, in the Dec. 12, 1983 issue. These statistics make a strong case for continued research in this area in order to hopefully rid the human race of this devastating disease.

In order to understand the characteristics which constitute the diseased state of the colon, the histology and biology of the normal colon must be understood. The large intestine is approximately 180 cm in length. It begins at the ileocecal valve which separates the small and large intestine. The first portion of the large intestine is the cecum. This proceeds into the ascending, transverse, and descending colon respectively. The colon terminates at the anus.

Microscopically the colon is lined with many glands which range in depth of 0.5 mm in the colon to 0.75 mm in the rectum. These glands are primarily responsible for water reabsorption. Mucous secretion is the responsibility of the goblet cells. Also present within the submucosa of the colon are scattered lymphatic nodules which are responsible for immune purposes (Leeson, 1981). See figure 1.

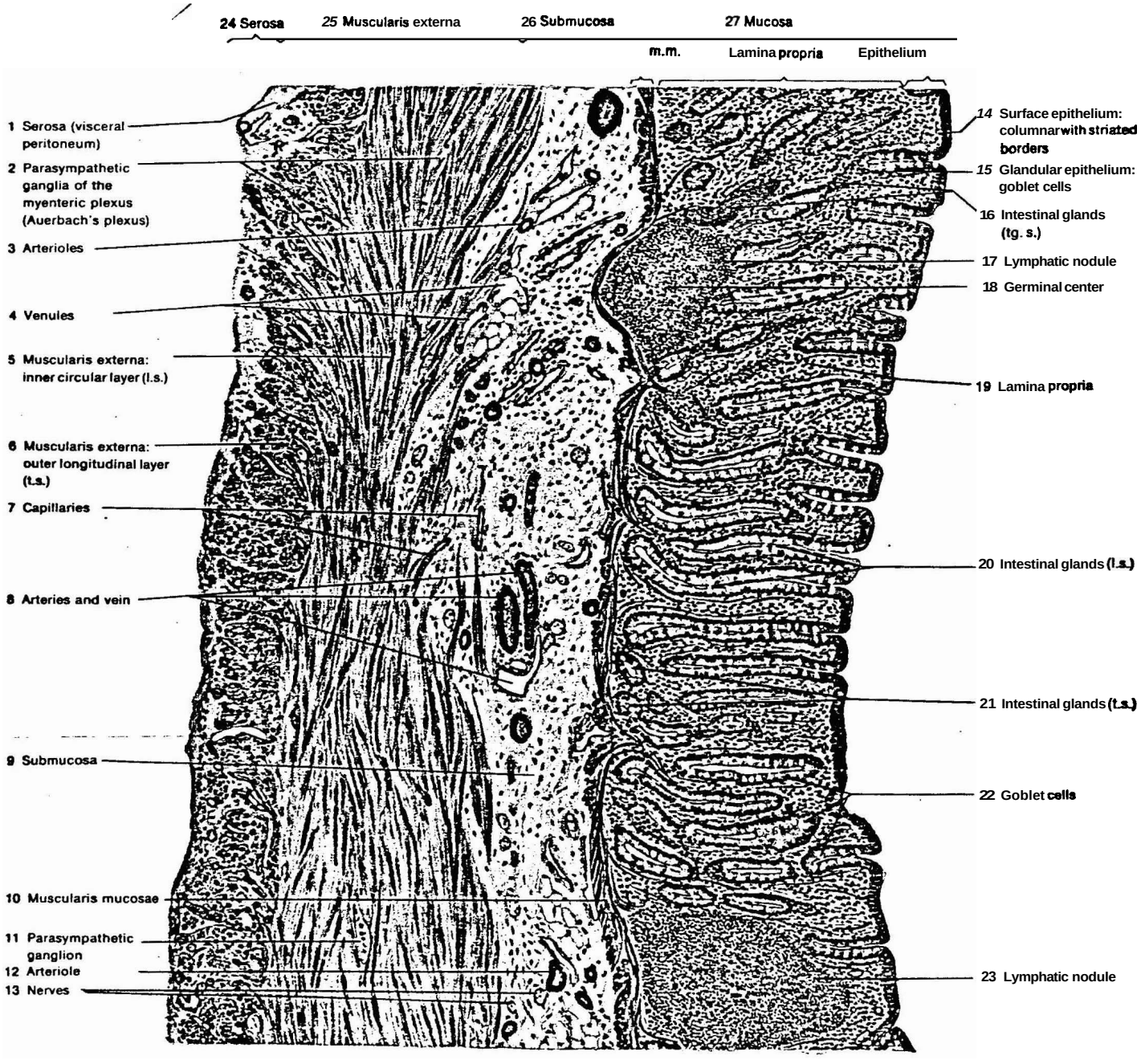
Early observations of the physiology of intestinal cell renewal by Bizzorero was contained in the medical literature of 1888. He recognized high mitotic activity in the crypts of the small intestine and of the gastric pits of the stomach. Bensley (1898) made accurate observations of the cell migration from the area of mitosis toward the lumen surface of the stomach. Friedman (1945) carried out radiation experiments to reinforce the concept of rapid cell proliferation in the

FIGURE 1.

Illustration of the Large Intestine

Figure 1

LARGE INTESTINE: COLON (WALL, TRANSVERSE SECTION)



Colon: a sector of *the wall.* Stain: hematoxylin-eosin. 53×.

Illustration found in Atlas of Human Histology. 5th ed. Mariano S. H. deFiore. 1982. Lea & Febiger, Philadelphia, Pennsylvania. p. 157.

gastrointestinal tract. Radioactive phosphorus was used by Lebland (1964) to measure mitotic indices to estimate cell renewal rates. These initial studies enable experimenters to understand the basic principles of cell proliferation kinetics in the gastrointestinal tract. Presently, the colon is studied extensively in regard to the underlying mechanism of rapid cellular proliferation. An important observation of maturing and fully mature cells is that they migrate in coherent sheets toward the lumen of the gastrointestinal tract (Lipkin, 1962). Cell division, as is shown experimentally with tri-tiated thymidine, occurs in the lower two-thirds of the colon crypts. This zone is referred to as the proliferative zone. Synthesis of de-oxyribonucleic acid (DNA) occurs in 15 to 20 percent of cells found in this zone (Lipkin, 1976). Chance seems to determine whether these proliferative cells continue as proliferating elements or differen-tiate into mature cells. As cells move through the transitional zone they are destined to differentiate. Cells showing cytodifferentiation may still divide while moving through the transitional zone (Winawer, 1969). As cells move out of the transitional zone to the luminal sur-face all proliferative activity ceases. At the luminal surface new stimuli present may be responsible for the cells' final differentiation and maturation. The average time of the proliferative cell cycle is 20 hours. The cycle of cell birth, migration, and extrusion leads to a steady state condition, with the rate of cell birth and cell loss being equal. Proliferation is found to be one cell per 100 cells per hour and the turnover rate is the same, thus producing an equilibrium of cell loss and cell birth. The replacement time for the cells lining the colonic surface is four to eight days (Lipkin, 1963).

The function of cell proliferation and differentiation are influenced by many intra and extracellular factors. Some of these external factors are available blood supply, hormones, stress, diurnal variation, serum enzymes, mitotic inhibitory substances, cholinergic neural activity, and surgical resection (Cooper, 1973). Internal regulators also control the cell cycle.

The cell cycle is composed of a series of specific phases including the inter and mitotic phases (Swift, 1950). The interphase is usually designated as G_0, G_1, S, and G_2 while mitosis occurs in M. The resting stage is designated as G_0. The cells in this stage may remain in the proliferative zone for an undetermined amount of time. Once a cell commits itself to division it must produce the appropriate ribonucleic acid (RNA) and resultant proteins to prepare the cell for DNA synthesis. This stage is known as G_1 or the presynthetic gap stage. The DNA replicates in the gastrointestinal epithelium every 14 to 18 hours (Winawer, 1969). After the DNA is replicated, the cell produces the appropriate RNA and proteins responsible for the M phase or mitosis. The postsynthetic gap (G_2) period may last six hours or longer. Mitosis (M) and following cell division may now take place. The overall cell cycle takes approximately two days (Bottomley, 1973). See figure 2.

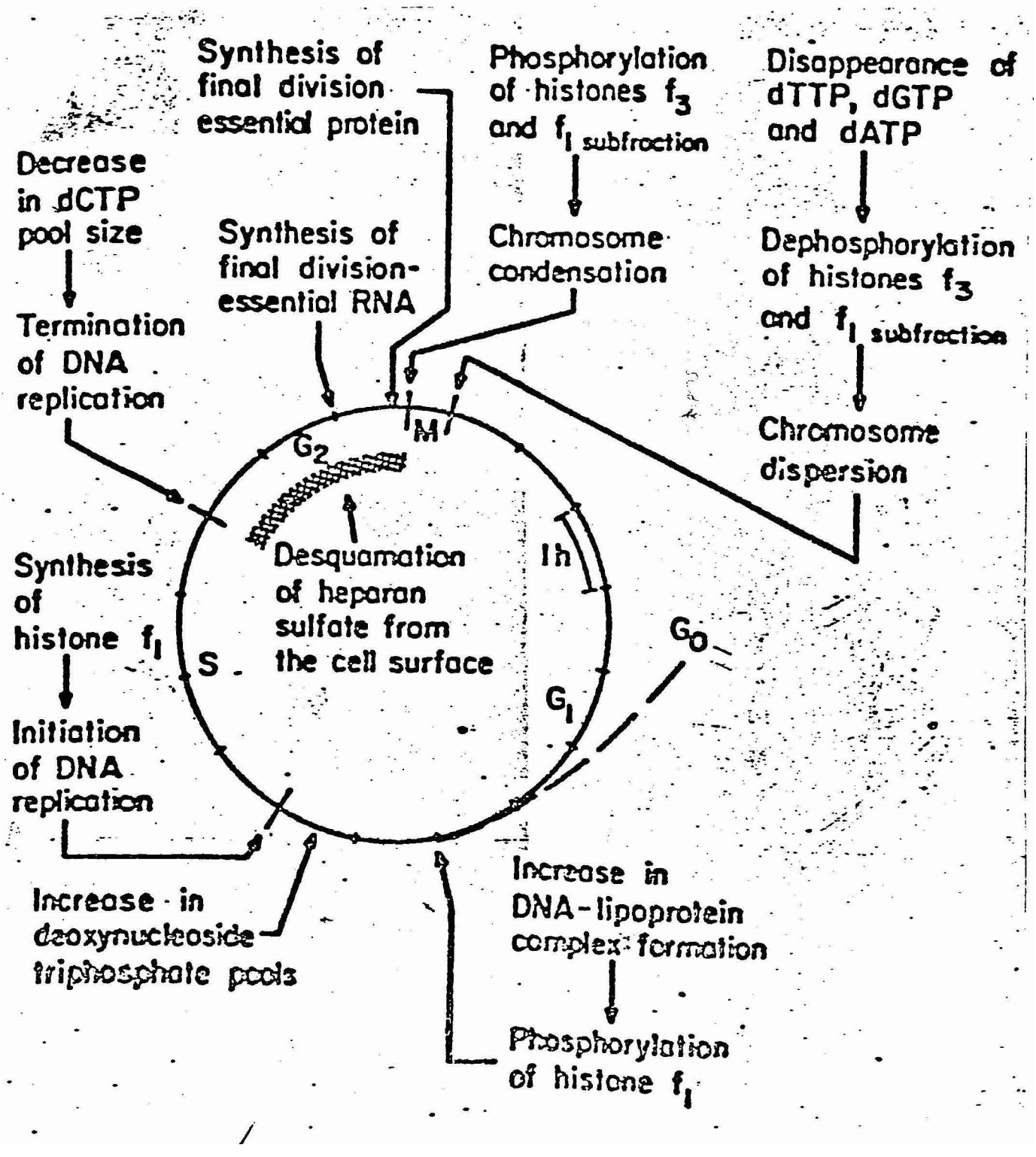
Other factors associated with the cell cycle have been shown to influence cell differentiation in the human colon as outlined by Lipkin (1973). Among these are multiplicity of controls which bring about appearance and disappearance of metabolic pathways during critical stages of cell differentiation. The activity of cyclic guanosine monophosphate (cGMP) is associated with the induction of

FIGURE 2.

Cell cycle

Figure 2

Cell Cycle



proliferation. This mononucleotide is primarily found in the crypts of the colon. As the cells move through the transitional zone and to the lumen, cyclic adenosine monophosphate (cAMP) becomes the predominant mononucleotide present. Cyclic AMP is responsible for a decrease in cellular proliferation and eventually cellular maturation. Cyclic GMP and AMP interact with histone proteins which function in recognition and regulation of the genes. Histone proteins must interact with nonhistone proteins to provide the proper gene for template activity and eventually the necessary protein. Histone protein functionally represses genes. Gene activation and cellular proliferation are found to be regulated by the nonhistone protein. The differential activation of the genes themselves likely proceed in a coordinated manner in proliferating cells to produce the changes observed in the cell cycle and during differentiation. For example, genes coding for the enzymes thymidine kinase and thymidylate synthetase are active during proliferative cell activity. These enzymes are responsible for producing thymidine monophosphate (TMP) which is incorporated into the replicating DNA. In differentiating cells DNA synthesis is terminated when these genes are repressed. Synthesis of DNA will terminate when a deficiency of adenine, guanine, thymine, or cytosine occurs because of gene repression. The control of thymidine kinase is one of the major mechanisms involved in the inhibition of cellular proliferation. Genes responsible for the production of alkaline and acid phosphatase are only active when colon cells are differentiated (Deschner, 1970).

The cell membrane plays an important role in cellular differentiation. As the cell migrates to the luminal surface of the crypt it comes in contact with certain molecules which bind to specific cell

receptors on the cell membrane. These molecules cause a release of macromolecules (cGMP, CAMP) which are capable of modifying gene activation and control (Lipkin, 1973).

As noted, events leading to and influencing gene activation or repression are very complicated and influenced by many factors. Any variation or deviation of these factors may result in mutant cell formation. The repression of these mutant cells may not occur resulting in possible formation of a tumor.

Many interactions between inherited and environmental factors are involved in the evolution of cellular changes that lead to neoplastic transformation of colonic epithelium cells (Cooper, 1973). The main function of the colon is water reabsorption (Haenszel, 1971). This function can result in the increase of the concentration of insoluble toxid substances. Simultaneously, bacterial putrefaction of undigested food material may create carcinogens. The bulk of the diet and speed of movement of fecal material influences this process (Haenszel, 1971). Excessive cell loss from the colon is the result of exposure to these transforming substances. In response to the cell loss, the proliferative zone increases in the crypt cells. Also the time between successive mitosises is decreased (Bleiberg, 1977). Iverson (1970) observed sensitivity of colonic epithelial cells involved in DNA synthesis when carcinogens were present. It was found that the cells of the colon increase their DNA synthetic rate in response to agitation. The carcinogen now can transform many S phase cells. The body's immune system is usually capable of eliminating these resulting transformed cells, except when inherited defects

potentiate the action of carcinogens and increase the possibility of neoplastic development. At this time progressive phases of abnormal cell development occur.

Transformed cells have a growth advantage over the adjacent normal cells (Nowell, 1976). Regulatory control of thymidine mono-phosphate synthesis is lost resulting in continued DNA production (Lipkin, 1974). An increase in RNA and protein synthesis is observed. There is also an observable cell accumulation on the luminal surface of the colon. These accumulations are known as a hyperplasia or neoplasm (Lipkin, 1974).

The loss of normal cellular regulation in hyperplasia results in definite shifts in DNA content of cells. This DNA shift will result in a ploidy level shift (Avtanilov, 1976). These ploidy shifts occur because of an unequal partitioning of chromosomes. Numerical alterations and/or structural reorganization of chromosomes may also occur (Makino, 1964). He notes that these abnormalities may be studied in metaphase arrested cells. These abnormalities are represented by chromatid breaks, translocation, stickiness, and fragmentation of chromosomes (Makino, 1964). Nondisjunction may occur which results in chromosomal elimination (Ohno, 1971). Many times these mutations are deleterious to the resulting cells. Surviving cells may contain three or four doses of a certain chromosome while other chromosomes may be represented only once. These cells do not correspond to any whole multiple of a haploid number and are considered aneuploid (Emson, 1967).

Chromosome rearrangement was first thought to be a marker of neoplastic formation (Makino, 1964). Studies carried on by Makino

(1964) and Emson (1967) demonstrated that no set chromosomal morphology pattern leads to the transformation of cancer cells. Since the amount of DNA in the nucleus corresponds to chromosomal number, this marker may be studied for clues by which neoplastic formation may be controlled. The measurement of cellular DNA content offers advantages in the study of neoplasms, for it does not depend on morphological differences in chromosomes and bears a more direct relationship to the cell genotype (Atkin, 1956). Quantitative cytophotometric methods which were used throughout this study accomplished this reasonably well. Cell cycle kinetics can be studied by this method, and the resultant histograms offer visual changes, that is, ploidy shifts in DNA levels.

Past studies indicate that a significant fraction of cells in a neoplasm are found to be in a resting state (G_0) allowing only 4.5 to 28 percent of this population remaining to actively proliferate (Clarkson, 1965). This study demonstrates that the presynthetic gap (G_1) time is increased (more cells in G_1) because much more DNA synthetic proteins are necessary in aneuploid cells. As expected the DNA synthesis stage is also prolonged. Clarkson (1965) states that the S phase can be completed in 17 to 34 hours in the diseased colon. The postsynthetic gap (G_2) which produces mitotic proteins, also takes longer than the normal colon cells. Mitosis in normal and neoplastic tissue cover the same time period (Barthold, 1979). The overall duration of cell kinetics is longer in neoplasms than in normal tissue.

The formation of a tumor from a neoplasm is the next stage of the disease. These tumor lesions are now described pathologically as adenomatous because they are derived from epithelium of glandular origin

(Thomas, 1982). Nowell (1976) states that a large number of cells may be affected by a carcinogen but a tumor that ultimately develops is usually the progeny of a single cell, or at most a few cells. This establishes the stem line theory of tumors. Histological research of tumors show a diversity in the cell populations within a tumor. These different subpopulations arise by mitotic abnormalities which produce genetically altered cells (Stich, 1960), although just where in a tumor these cellular populations may be located has not yet been determined. Many of these variants are eliminated because of a metabolic disadvantage or immunological destruction. Subpopulation precursors are those cells which have additional selective advantages over the original tumor cells. Natural and artificial selection processes such as nutritional changes are responsible for the continuous emergence of successive clones in a tumor (Kerbel, 1979). Cell Loss is also responsible for tumor growth kinetics. Frindel (1968) observed a large majority of cells die or migrate out of a tumor. Tumors are able to lose 95 percent of their daily cell production without any problems regarding elimination.

Malignancy is the next stage in the biology of the tumor. A tumor that has a potential for metastasis contains many subpopulations, each with a different ability to metastasize. These metastatic cells do not result from random survival of cells released from the primary tumor, but from a selective growth of specialized subpopulations that mutate readily (Talmadge, 1982). A large degree of aneuploidy and a great loss of morphological differentiation are characteristic of the

malignant potential of a tumor (Nowell, 1976). Collagenolytic activity is seen in malignant cells. This activity allows metastatic cells to migrate from the tissue into the plasma or lymph and back into the tissue in another site in the body (Barret, 1978).

Early treatment of colon carcinoma is important before metastisis is able to occur. Carter (1976) states that radical surgery is the only universally accepted curative treatment for large bowel cancer. Unfortunately, diagnosis of these tumors sometimes occurs too late to insure complete success of surgical excision (Carter, 1976). The main problem with chemotherapeutic agents is that many cells are in a state of rest in a tumor (Clarkson, 1965). Only cells active in DNA synthesis are affected by the drug. After the time of treatment has passed, these resting cells may begin to proliferate. Radiotherapy also has not been proven effective against these tumors. Immunotherapy is now being experimentally tested in colon cancer cases (Carter, 1976).

The development of cancer is a complicated process which contains many variables. Medical science must find a variable in early tumor development that can be treated effectively to insure a decrease in the incidence of the cancer or at least the seriousness of the disease.

The purpose of this study is to examine human colon tumors in various stages of development; by serially examining them, it is intended that highly proliferative areas be determined. Also by these means the overall growth pattern of the cells can thus be elucidated.

CHAPTER 2

Materials and Methods

The colon adenocarcinomas and control tissue were obtained surgically by Dr. Armin Banez, a surgeon employed by the Youngstown Hospital Association. Upon removal, the tumors were immediately placed in 10 percent buffered Formalin solution and transported to Youngstown State University. This noncoagulent fixative was chosen because of its known property of maintaining tissue integrity. The tissue was fixed in the Formalin solution for a time period of 14 hours, followed by dehydration in a graded ethanol series, cleared in Xylene, and subsequently paraffin embedded. This was accomplished by use of a programmable tissue processor. Each individual tumor was dissected into three sections designated left, middle, and right divisions, and these were examined serially. The fixation, dehydration, and clearing process as well as embedding process is outlined in Table 1.

A Tissue-Tec microtome was employed in order to section tissue at a depth of six microns. The resulting paraffin tissue ribbon was then placed in a warm water bath (59° C) to prevent folding and insure proper adhesion on an albuminized glass slide. Once the tissue is oriented on the slide it is allowed to dry on a slide warmer.

Cytochemical Methods

The nuclear Feulgen staining reaction was employed throughout this study. This reaction enables the quantitative measurement of DNA within a cell nucleus (Feulgen and Rossenback, 1924; as modified by

TABLE 1

Fixation Procedure

TABLE 1.

FIXATION PROCEDURE

A. Formalin 10% for 12 hours

B. Formalin 10% for 2 hours

C. Ethanol 70% for 2 hours

D. Ethanol 95% for 2 hours

E. Ethanol Abs., three washes for 2 hours each

F. Xylene for 1/2 hour

G. Xylene for 1 hour

H. Paraffin for 1 hour

I. Paraffin for 2 hours

J. Block tissue

TABLE 2

Deoxyribonuclease Protocol

TABLE 2.

DEOXYRIBONUCLEASE PROTOCOL

A. Bring slides through graded ethanol series to distilled water.

B. Immerse in 0.05% solution of DNAase for 4 hours at 38° C.

C. Place in 5% TCA at room temperature for 10 minutes.

D. Pass through distilled water and stain.

REAGENTS

25 mg DNAase

37 mg $MgSO_4$

6.8 ml of 0.1 M Citric Acid

18.2 ml of 0.2 M Na_2HPO_4

25 ml of distilled water

Therrien, 1966; and Bryant and Howard, 1969). A stain control slide subjected to deoxyribonuclease action before Feulgen staining is included in order to remove DNA and to abolish any staining. Table 2 describes the procedure used for the deoxyribonuclease treatment.

To Summarize Tissue Treatment:

The specimens are subjected to the following sequence of events:

1. The paraffin sections are cleared in xylene to remove the wax from the tissue.
2. The tissue slides are then hydrated by being immersed in a graded alcohol sequence which concludes in distilled water. This step is needed to remove the xylene and bring the tissue back to its natural state.
3. Hydrolysis of the tissue is achieved with a solution of 5N hydrochloric acid for 42 minutes at room temperature. This procedure hydrolyzes adjacent hydroxyl groups to aldehyde. The acid also removes purine groups.
4. Rinse in distilled water.
5. The slides are stained for one hour in freshly fortified Schiff's reagent with potassium-meta-bisulfite. The stain reduced the adjacent aldehyde groups on the deoxyribose to form the characteristic magenta color. One dye molecule combined with the two adjacent aldehyde groups, thus the stain is quantitative for the amount of

DNA in the cell. The stain is purchased from the Fisher Scientific Company (C.I. #42500).

6. Rinse the slides three times in freshly prepared 10 percent potassium-metabisulfite rinse (Yemma, 1972). This step is necessary to prevent reoxidation of the dye from the magenta color to the leukofushin color.
7. Rinse in distilled water.
8. The slides are subjected to a graded ethanol series in order to dehydrate the tissue.
9. The tissue is immersed in xylene to remove the ethanol and prepare the slides for the mounting media. The coverslips are affixed to the slides with permount.

A summary of this procedure is presented in Table 3.

Spectrophotometric Method

A Zeiss type 01 microspectrophotometer with a quartz iodine light source is used to quantitate the Feulgen stained tissue. The process of microscopy and spectrophotometry were employed to measure and compute the amount of DNA in the individual nuclei. Microspectrophotometry of DNA content in the nucleus may be used as an objective test for the evaluation of cell ploidy and cell population heterogenisity (Avtandilov, 1976). The objective employed is a Zeiss oil immersion objective X100 Numerical Aperature 130, at an optover setting of 1.25X, with a Zeiss immersion oil, 518CDM5884. For the selection of the desired wavelengths a continuous interference filter monochromater is utilized.

TABLE 3

Staining **Procedure**

TABLE 3.

STAINING PROCEDURE

A. Xylene for 7 min.

B. Ethanol Abs. for 7 min.

C. Ethanol 95% for 7 min.

D. Ethanol 70% for 7 min.

E. Distilled water rinse

F. HCl (5N) for 42 min. at room temperature

G. Feulgen Stain - 4 parts Schiff's reagent to 1 part potassium-metabisulfite (10%) for 1 hour

H. Potassium-metabisulfite for 10 min.

I. Potassium-metabisulfite for 5 min.

J. Potassium-metabisulfite for 5 min.

K. Distilled water for 5 min.

L. Distilled water rinse

M. Ethanol 70% for 10 min.

N. Ethanol 70% for 5 min.

O. Ethanol 95% for 5 min.

P. Ethanol Abs. for 5 min.

Q. Xylene

R. Mount coverslips with permount

The instrument alignment and phototube linearity response are checked before any readings are taken. The maximum and minimum wavelengths of the dye are determined by an absorption curve (Figure 3). The two wavelengths determined by the absorption curve are 560 and 505 nm.

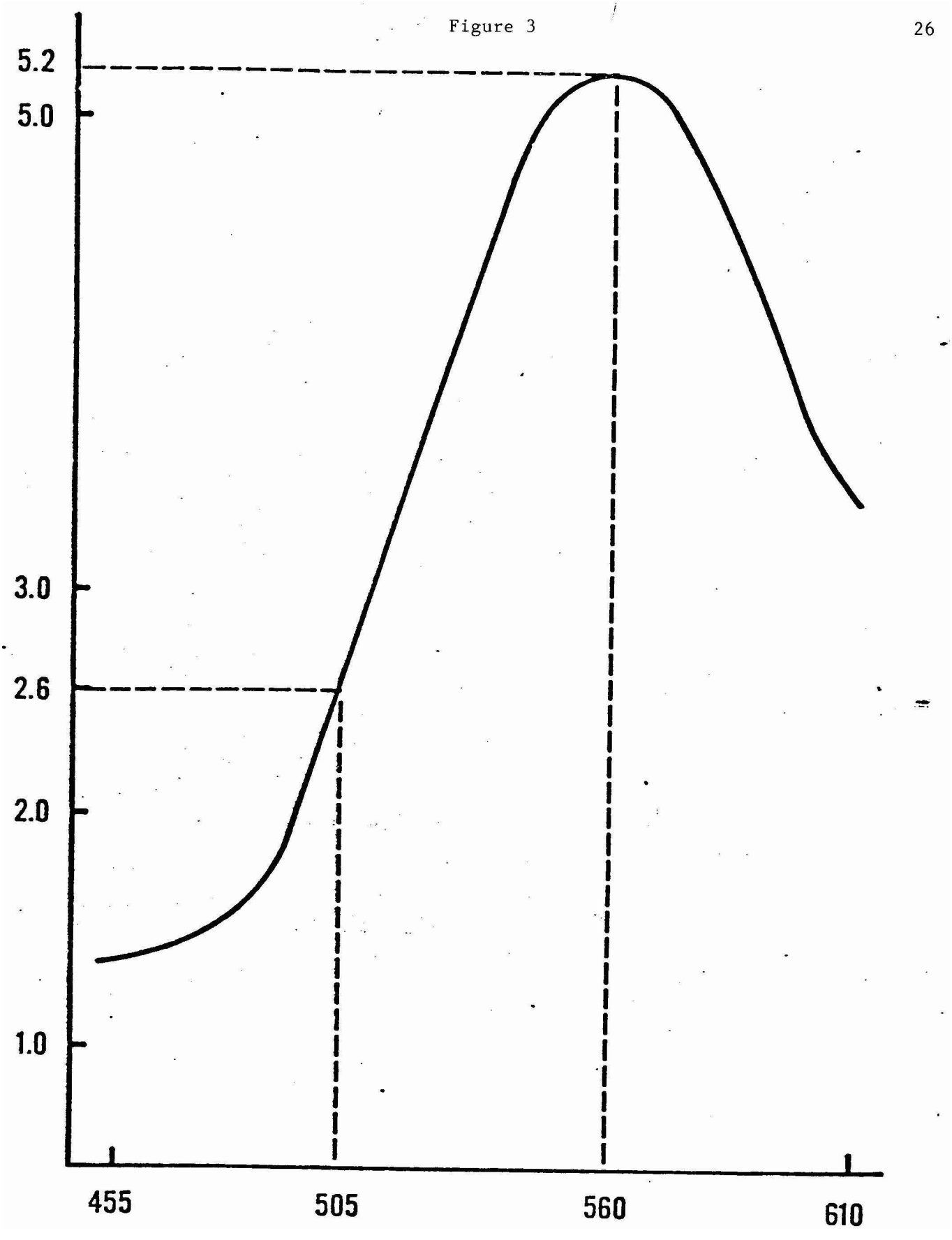
The relative amounts of DNA measured by the microspectrophotometer are determined by the two wavelength method (Patau, 1952; and Ornstein, 1952). Mayall and Mendelsohn (1970) show this method is chosen because it eliminates a direct measurement of nuclear materials. This method is less subject to errors due to parts of the material being out of focus (Atkin, 1969). Nuclear materials that are not heterogeneously stained are also compensated. All tissue slides are concurrently hydrolyzed and stained to insure uniform staining of the DNA content within the cells.

According to the absorption curve the maximum extinction (E_2) occurs at 560 nm. The second wavelength is chosen by calculating half of the maximum extinction which is found to occur at 505 nm (E_1). After this is determined, nuclei are picked randomly to be read. Each nuclei is centered in the aperature which is slightly larger than the nucleus. The aperature specific for the nuclei is 6.3 microns in diameter. Readings are made in this order: on the nucleus at 560 nm (Is_{560}), on the nucleus at 505 nm (I_{s505}), background at 505 nm (I_{o505}), and background at 560 nm (I_{o560}). The amount of chromophore (M) is determined using these readings and employing the formula, $M = KAL_1C$. The constant K is not included in the calculations. L is calculated by the equations $L_1 = (1-T_1)$, and $L_2 = (1-T_2)$ where T is the transmittance. Q is determined by $L = (1-T_1)$, and $L_2 = (1-T_2)$, where T is the transmittance. Transmittance is determined by $T_1 = I_{s560}/I_{o560}$

FIGURE 3.

Absorption Curve

Figure 3



and $T_2 = I_{s505}/I_{o505}$. The ratio between L_1/L_2 equals Q. This value (Q) is needed to eradicate the influence of unoccupied area measured around the nucleus. This is allowed because the extinction ratio at the two wavelengths is 2:1. The Q value is converted to the C value in a table determined by Patau in 1952. The correction factor C is used in the calculation for the distributional error.

A computer program written by Dr. John Yemma was used to calculate the relative DNA values utilizing the Amdahl main frame computer at Youngstown State University.

CHAPTER 3

Results

Frequency histograms of DNA measurements facilitate the observations made in this study. In this way, major and minor shifts in the nuclear DNA are easily detectable. Where tables are used they will aid in the characterization of data while graphs aid in the characterization of cells whose DNA content appears to uniquely characterize ploidy levels. The mean DNA values are used to assess the nuclear kinetics involving changes in DNA of the cells.

Since the two-wavelength method of microspectrophotometry was utilized, it was necessary to generate an absorption curve for the dye-molecule complex under investigation (Figure 3). A normal tissue control was used to establish this curve, and since all tissues used in this investigation were treated and stained under the same conditions simultaneously in order to eliminate any experimental error, the established wavelengths representing the maximum absorption and the half-maximum, 560 nm and 505 nm respectively were employed in obtaining the results through this study.

The control tissue used in this study were obtained by biopsy and after microscopic examination was regarded as normal diploid tissue by a pathologist. The mean DNA content of this tissue was 11.07 arbitrary units. This value is used to describe diploid or the 2C condition of the cells. Fifty-eight percent of the 300 sampled cells are contained in the diploid state. Cells containing replicated DNA

and proceeding through the anaphase stage of mitosis contain twice the diploid value (22.14). The cells at this value are considered 4C. The DNA values between the diploid and diploid replicated 4C areas are in the synthesis phase (S) of the cell cycle. The large amount of cells in the synthesis phase is attributed to an irritation in the area of the biopsy. Also, colon epithelia has a rapid proliferation rate which accounts for the large number of S phase cells. Cells with a DNA content slightly larger than the 2C level are just beginning to synthesize their DNA. The DNA content approaching the tetraploid level are in the late stages of DNA synthesis. Using these parameters the tumor histograms are examined and compared in order to interpret the data.

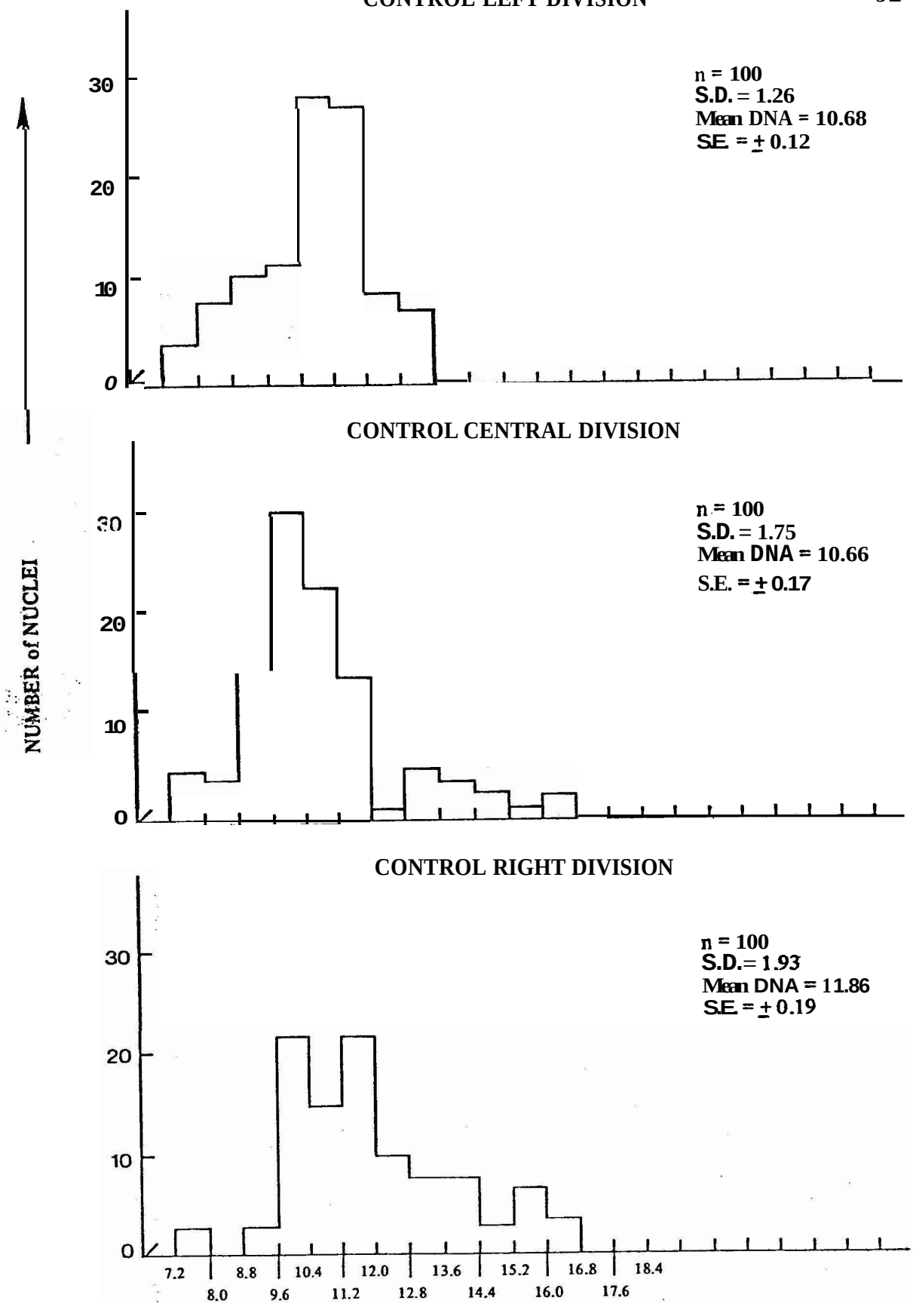
A DNA content above the tetraploid (4C) level that appears within a population is interpreted to be indicative of cancerous tissue pre-diagnosis supports this interpretation. For example, a cell that contains an octoploid (8C) amount of DNA will have a DNA value of 44.40. The tumors in this study contain many cells that are not found in a defined ploidy level. These cells are described as being aneuploid which is characteristic of cancerous tissue. As mentioned previously aneuploid cells are commonly found in tumors of all physiological types. Table 3 describes the number of aneuploid cells contained in each quadrant of each tumor examined.

FIGURE 4.

Control Left Division

Control Central Division

Control Right Division


CONTROL LEFT DIVISION
n = 100
S.D. = 1.26
Mean DNA = 10.68
S.E. = ± 0.12
CONTROL CENTRAL DIVISION
n = 100
S.D. = 1.75
Mean DNA = 10.66
S.E. = ± 0.17
CONTROL RIGHT DIVISION
n = 100
S.D. = 1.93
Mean DNA = 11.86
S.E. = ± 0.19
NUMBER of NUCLEI
30
20
10
0
7.2
8.0
8.8
9.6
10.4
11.2
12.0
12.8
13.6
14.4
15.2
16.0
16.8
17.6
18.4
RELATIVE AMOUNTS of DNA
.8 arbitrary units

Tumor 1

Tumor 1 was a whitish-tan tissue measuring approximately 4 x 3.5 x 1.5 cm. The pathology report continues to describe the tumor as an infiltrating moderately well differentiated adenocarcinoma. This tumor has invaded the pericolonic fat region of the descending colon. Of the five mesenteric lymph nodes examined, three show metastatic potential. A liver biopsy illustrates ischemic tumor necrosis which is highly consistent of metastatic disease. The number of aneuploid cells present are consistent with a well developed tumor of standing duration.

The histograms representing the cellular population of tumor 1 contain a mean DNA level approaching the tetraploid range. The reason for this is consistent with the presence of a large number of aneuploid cells (31) found in all the tumor quadrants. The majority of aneuploid cells (14) is illustrated in the central division histogram. A few cells in this section are approaching the octoploid (8C) level. The left division has the next highest number of aneuploid cells (12). The central and left sections have similar mean DNA values which is as expected reflected in the similar number of aneuploid cells demonstrated in the representative histograms. The right division demonstrates a lower mean DNA value and thus contains a lower number of aneuploid cells.

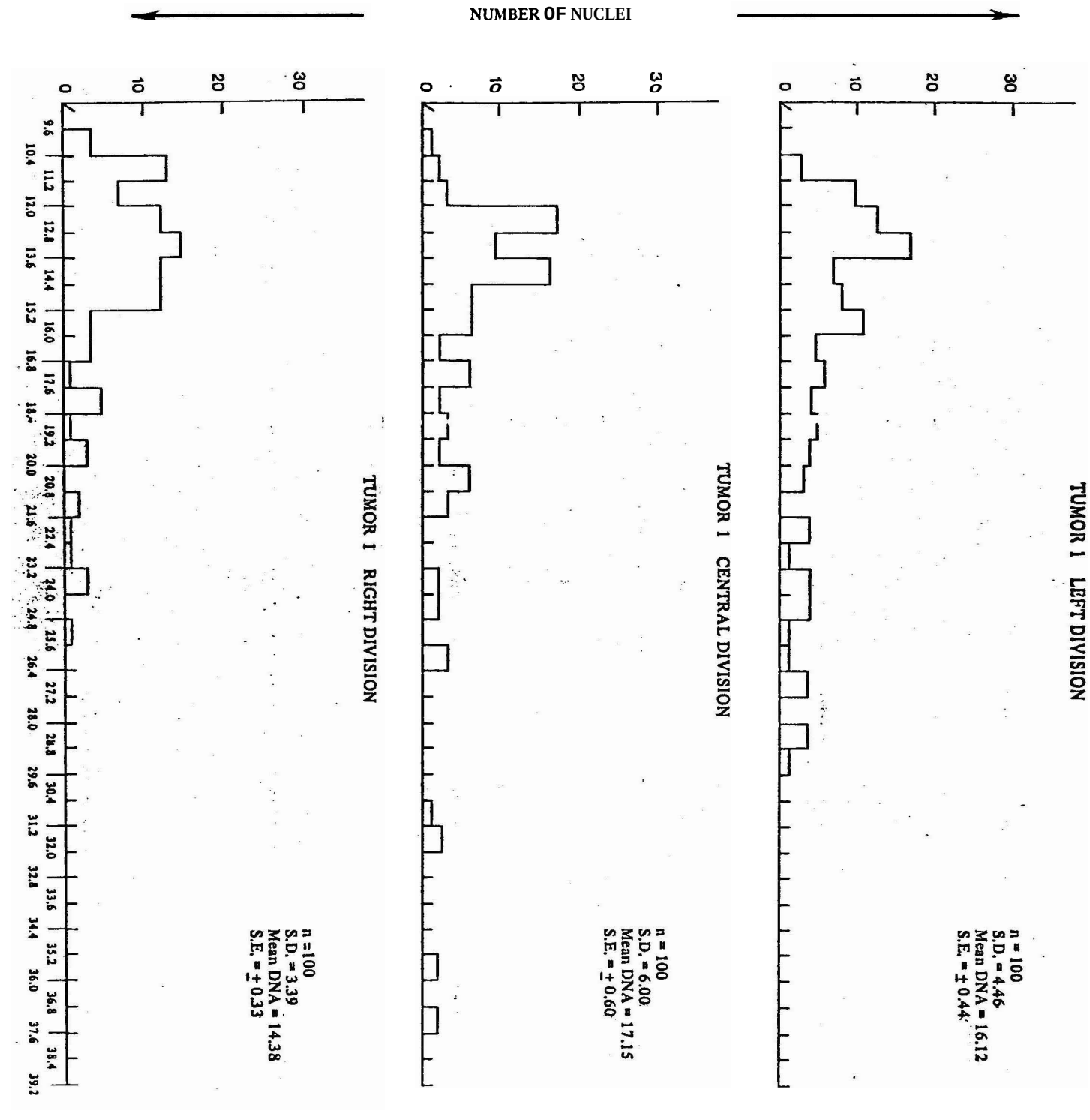
The cells contained in the diploid to tetraploid category are very active in DNA synthesis. The accelerated activity of proliferation within this tumor can be expected to result in a greater number of mutants among those cells.

FIGURE 5.

Tumor 1 Left Division

Tumor 1 Central Division

Tumor 1 Right Division


NUMBER OF NUCLEI
TUMOR 1 LEFT DIVISION
n = 100
S.D. = 4.46
Mean DNA = 16.12
S.E. = ± 0.44
TUMOR 1 CENTRAL DIVISION
n = 100
S.D. = 6.00
Mean DNA = 17.15
S.E. = ± 0.60
TUMOR 1 RIGHT DIVISION
n = 100
S.D. = 3.39
Mean DNA = 14.38
S.E. = ± 0.33
0
10
20
30
9.6
10.4
11.2
12.0
12.8
13.6
14.4
15.2
16.0
16.8
17.6
18.4
19.2
20.0
20.8
21.6
22.4
23.2
24.0
24.8
25.6
26.4
27.2
28.0
28.8
29.6
30.4
31.2
32.0
32.8
33.6
34.4
35.2
36.0
36.8
37.6
38.4
39.2

Tumor 2

Tumor 2 was described pathologically as an infiltrating, well differentiated adenocarcinoma with extension to the pericolonic soft tissue of the proximal transverse colon. This irregular, annular mass, obstructs the lumen and measures approximately 0.6 to 0.7 cm in diameter. Also excised were seven mesenteric lymph nodes which were negative for metastatic disease.

The tumor sample measured contained eight aneuploid cells. Five of these aneuploid cells are contained in the central division. As expected, this section also has the highest value for the mean DNA level in this tumor. The right and left divisions share the other three aneuploid cells. Their mean DNA values are similar.

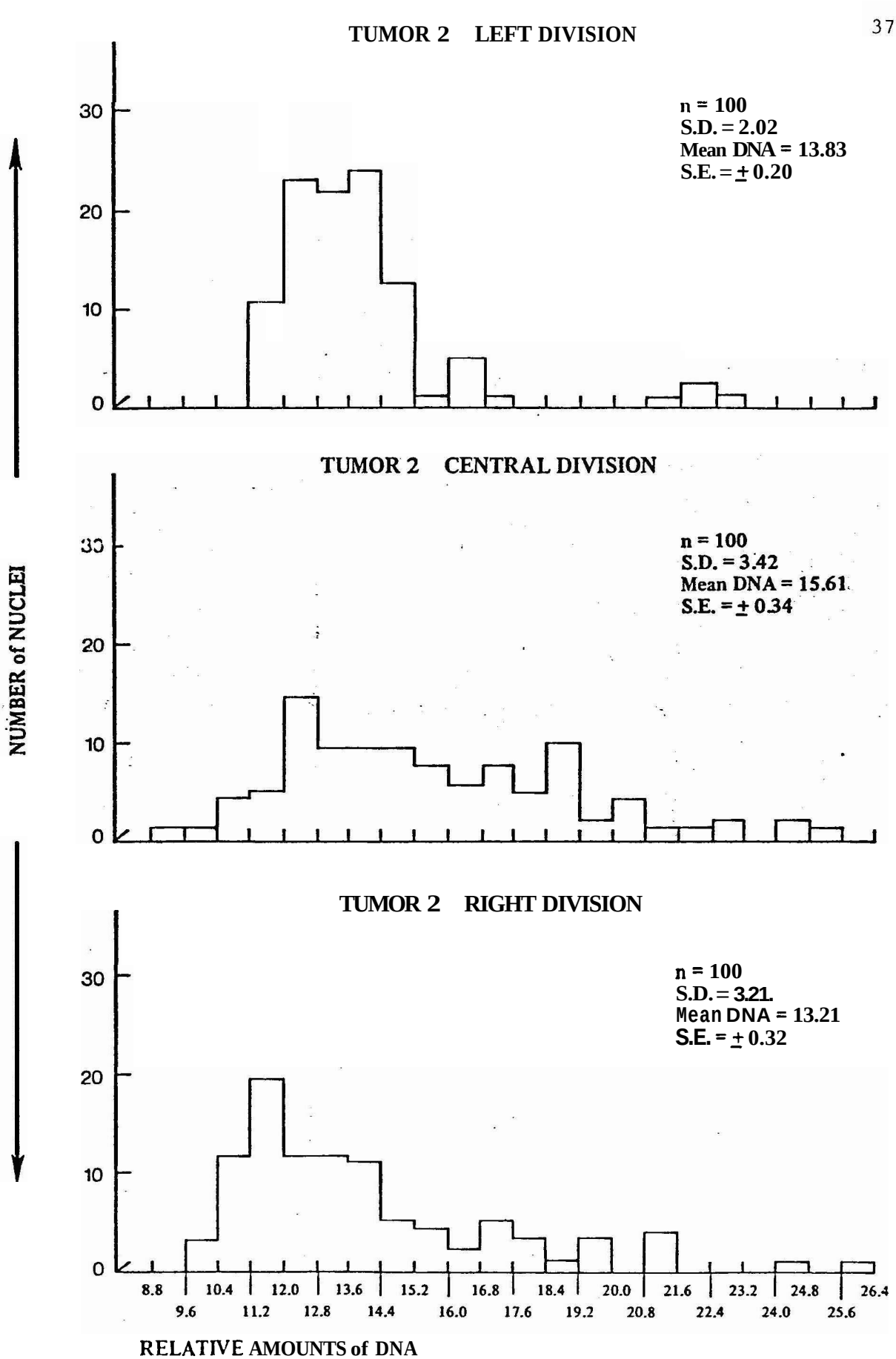
The central section contains many cells in the late DNA synthesis phase. This may be the reason for the number of aneuploid cells observed in this division. The other two divisions have the majority of their diploid to 4C cells in the early stages of DNA synthesis. There is less chance for mitotic mutations occuring and giving rise to aneuploid cells. These divisions are essentially diploid in DNA content.

FIGURE 6.

Tumor 2 Left Division

Tumor 2 Central Division

Tumor 2 Right Division


TUMOR 2 LEFT DIVISION
n = 100
S.D. = 2.02
Mean DNA = 13.83
S.E. = ± 0.20
TUMOR 2 CENTRAL DIVISION
n = 100
S.D. = 3.42
Mean DNA = 15.61
S.E. = ± 0.34
TUMOR 2 RIGHT DIVISION
n = 100
S.D. = 3.21
Mean DNA = 13.21
S.E. = ± 0.32
NUMBER of NUCLEI
30
20
10
0
8.8
9.6
10.4
11.2
12.0
12.8
13.6
14.4
15.2
16.0
16.8
17.6
18.4
19.2
20.0
20.8
21.6
22.4
23.2
24.0
24.8
25.6
26.4
RELATIVE AMOUNTS of DNA
.8 arbitrary units

Tumor *3*

Tumor *3* was a semi-annular, slightly raised ulcerated neoplasm measuring *4.5* x *3.5 x 0.5* cm. This tumor is described as a moderately differentiated, deeply infiltrative adenocarcinoma in early stages of development. The tumor penetrated through the muscularis into the serosal soft tissue. There were no metastatic precursors found in eight regional lymph nodes.

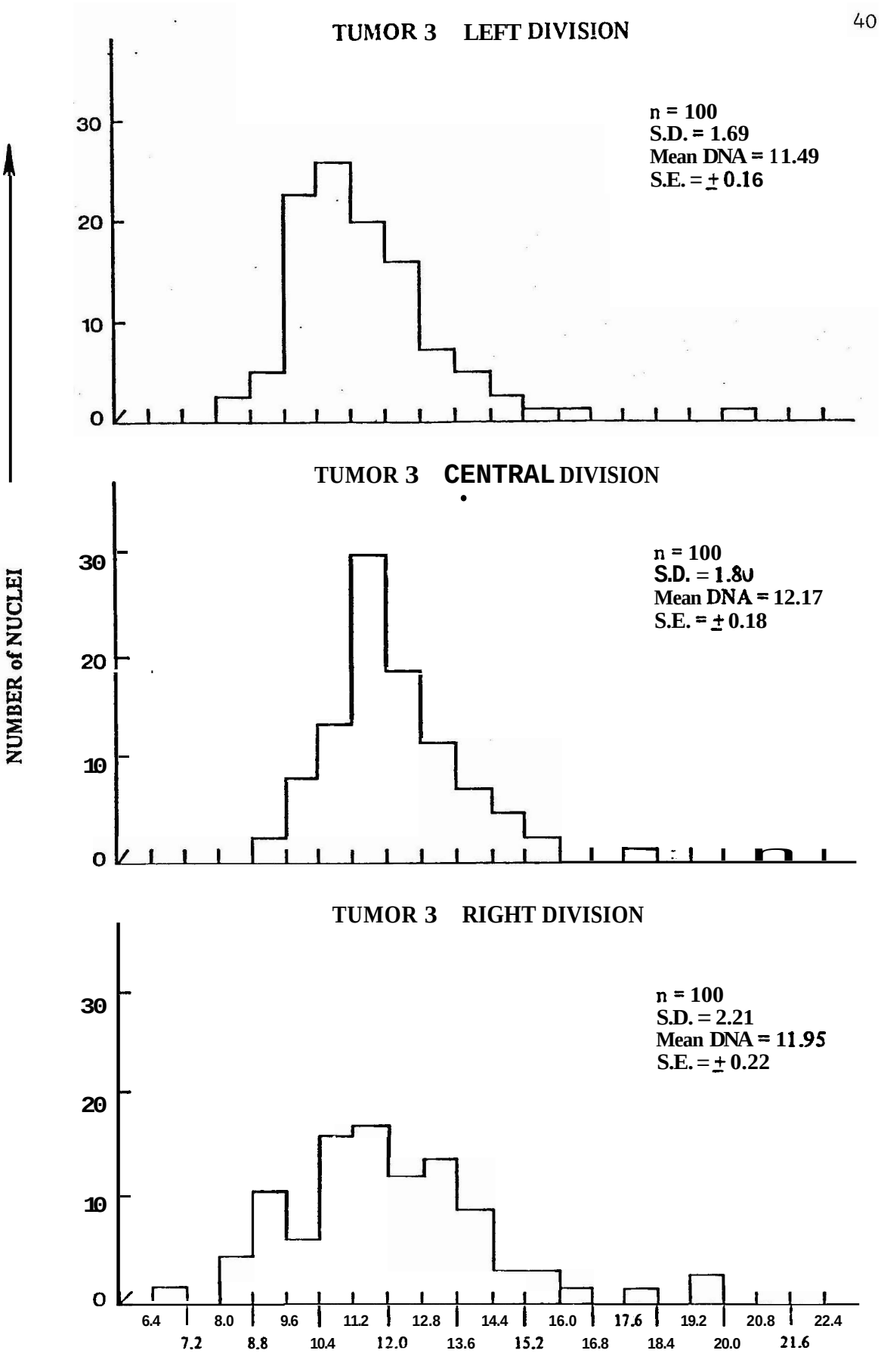
No aneuploid cells were found in the population of measured cells. The three divisions' mean DNA values are similar to that seen in normal tissue. Certain environmental factors may be present that limit mutant cells becoming aneuplerotic or in this case may be due to the age of the tumor. The majority of cells present in this tumor are actively synthesizing their DNA. Tumor growth may eventually change environmental conditions that will result in the appearance of aneuploid cells.

FIGURE 7.

Tumor 3 Left Division

Tumor 3 Central Division

Tumor 3 Right Division


TUMOR 3 LEFT DIVISION
n = 100
S.D. = 1.69
Mean DNA = 11.49
S.E. = ± 0.16
TUMOR 3 CENTRAL DIVISION
n = 100
S.D. = 1.80
Mean DNA = 12.17
S.E. = ± 0.18
TUMOR 3 RIGHT DIVISION
n = 100
S.D. = 2.21
Mean DNA = 11.95
S.E. = ± 0.22
NUMBER of NUCLEI
0
10
20
30
6.4
7.2
8.0
8.8
9.6
10.4
11.2
12.0
12.8
13.6
14.4
15.2
16.0
16.8
17.6
18.4
19.2
20.0
20.8
21.6
22.4
RELATIVE AMOUNTS of DNA
.8 arbitrary units

Tumor 4

The tissue of tumor 4 was grayish-white and irregular shaped. The mass measures 8 x 5 x 5 cm. This colon adenocarcinoma is moderately well differentiated with extension to the serosa and parametrium. There is no metastatic carcinoma seen in the ten pericolonic lymph nodes excised.

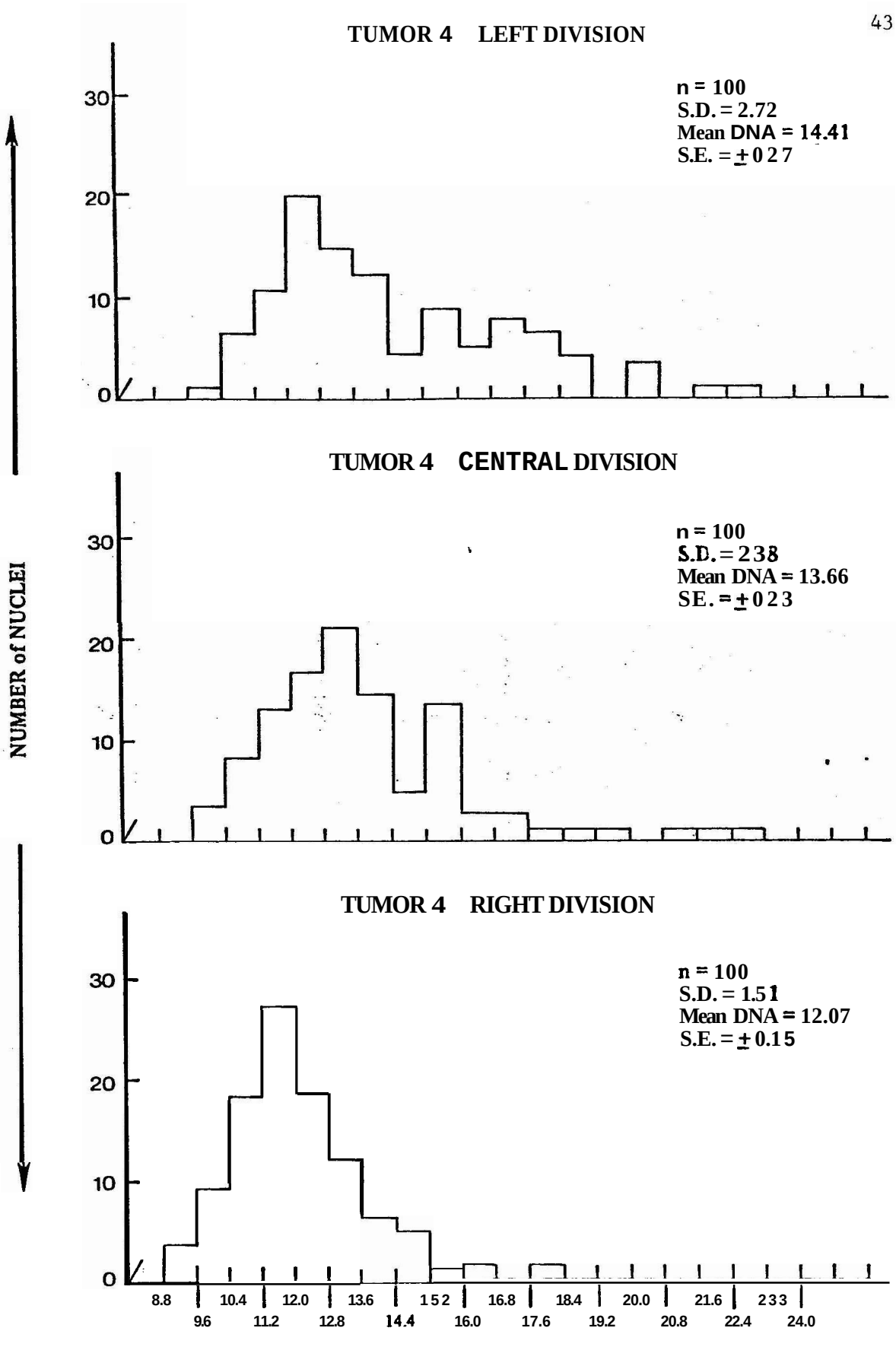
The cells measured in this tumor yield two aneuploid cells. One cell is seen in the central division and one in the left division. These sections also show many cells in advancing stages of DNA synthesis, many of these cells may be aneuploid. The left section contains the highest mean DNA value due to more cells approaching the tetraploid level. The right division's cells are quiescent in comparison to the rest of the tumor. This results in a lower mean DNA value as well as the fact that the tumor is not developing an appreciable number of aneuploid cells via mutation of dividing cells.

FIGURE 8.

Tumor 4 Left **Division**

Tumor 4 Central **Division**

Tumor 4 **Right Division**


TUMOR 4 LEFT DIVISION
n = 100
S.D. = 2.72
Mean DNA = 14.41
S.E. = ± 0 2 7
TUMOR 4 CENTRAL DIVISION
n = 100
S.D. = 2 3 8
Mean DNA = 13.66
SE. = ± 0 2 3
TUMOR 4 RIGHT DIVISION
n = 100
S.D. = 1.5 1
Mean DNA = 12.07
S.E. = ± 0.1 5
NUMBER of NUCLEI
30
20
10
0
8.8
9.6
10.4
11.2
12.0
12.8
13.6
14.4
15 2
16.0
16.8
17.6
18.4
19.2
20.0
20.8
21.6
22.4
23 3
24.0
RELATIVE AMOUNTS of DNA
.8 arbitrary units

Tumor 5

Tumor 5 was a moderately well differentiated adenocarcinoma of the colo-rectal region. This tumor has invaded the muscular wall and has spread to the adjacent fat. Adenomatous polyps were observed in the area of the tumor. Seventeen mesenteric lymph nodes were observed for metastatic neoplasm. The tumor did not metastisize to these glands.

The central division contains 75% of the aneuploid cells sampled in this tumor and also contains the highest mean DNA value. The remaining aneuploid cell is confined to the right division.

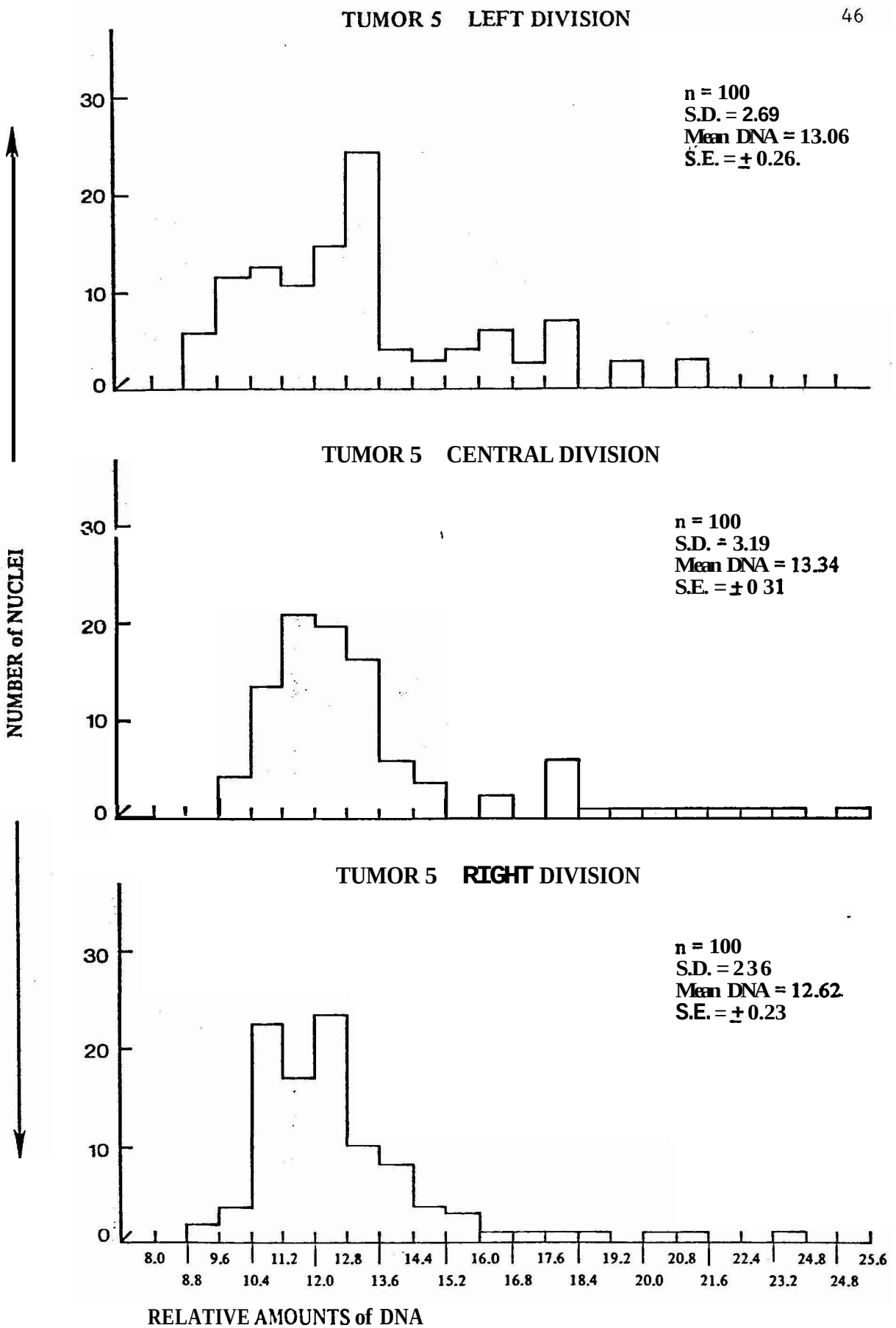
The cells within normal ploidy limits are seen to be actively synthesizing their DNA. The left and central divisions have the majority of cells in the S phase. The right section has the lowest mean DNA value and has 29% of its cells in a resting stage.

FIGURE 9.

Tumor 5 Left Division

Tumor 5 Central Division

Tumor 5 Right Division


TUMOR 5 LEFT DIVISION
n = 100
S.D. = 2.69
Mean DNA = 13.06
S.E. = ± 0.26.
TUMOR 5 CENTRAL DIVISION
n = 100
S.D. = 3.19
Mean DNA = 13.34
S.E. = ± 0 31
TUMOR 5 RIGHT DIVISION
n = 100
S.D. = 2 3 6
Mean DNA = 12.62.
S.E. = ± 0.23
NUMBER of NUCLEI
0
10
20
30
8.0 9.6 11.2 12.8 14.4 16.0 17.6 19.2 20.8 22.4 24.8 25.6
8.8 10.4 12.0 13.6 15.2 16.8 18.4 20.0 21.6 23.2 24.8
RELATIVE AMOUNTS of DNA
.8 arbitrary units

Tumor 6

The sixth tumor was an ulcerated lesion about 4.5 x 3.5 cm and approximately 0.5 cm deep. This tumor was a well differentiated adenocarcinoma of the colo-rectal area. The serosa of the sigmoid colon had been invaded by the lesion. Metastasis to one of the nine peri-rectal lymph nodes was observed.

Two of the three aneuploid cells were found in the middle section of this tumor among cells measured. This section also had the highest mean DNA value due more than likely to the presence of aneuploid cells in the S phase. The right division contained the remaining aneuploid cell.

The normal cells of this tumor were actively synthesizing their DNA. The central section had 26% of the cells in the late stage of synthesis. As a result, the mean DNA values are similar in these two areas

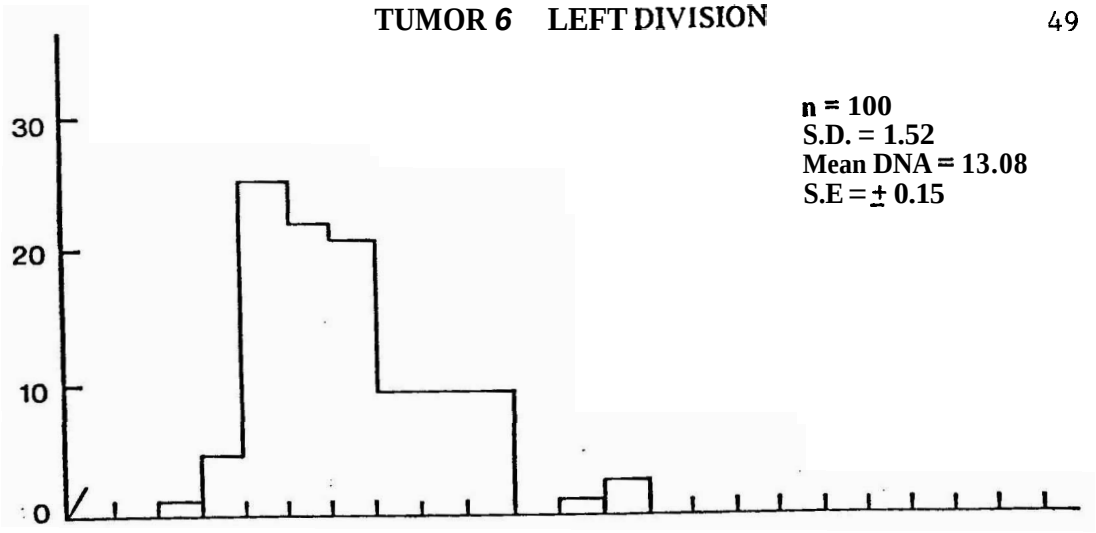
FIGURE 10.

Tumor 6 Left Division

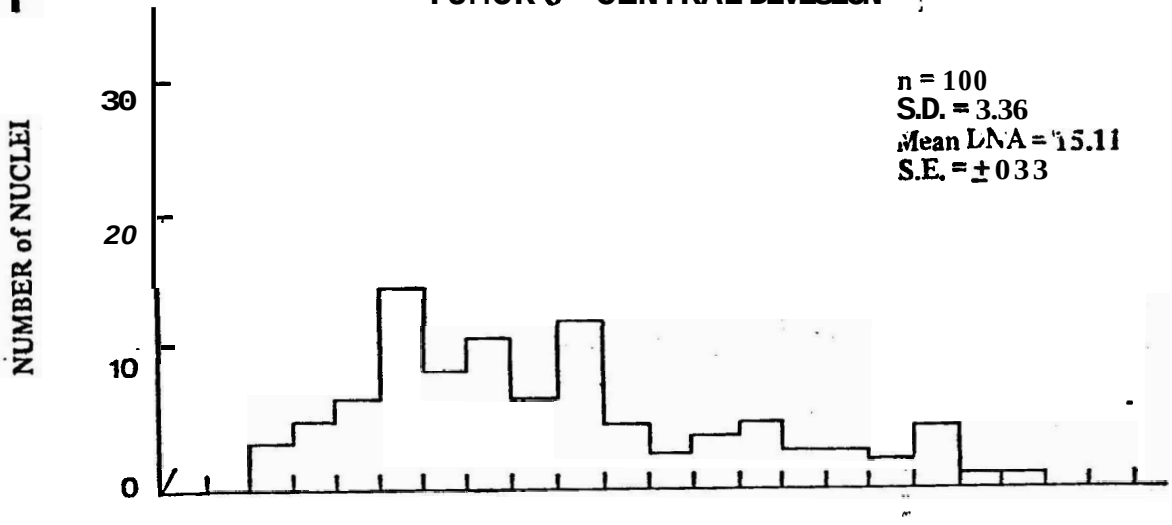
Tumor 6 Central Division

Tumor 6 Right Division

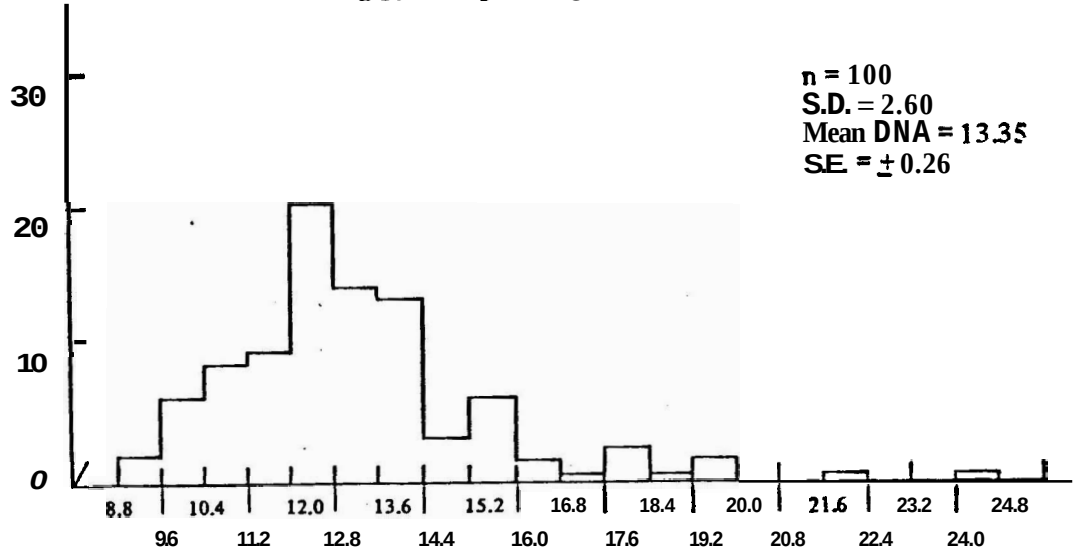
TUMOR 6 LEFT DIVISION



TUMOR 6 CENTRAL DIVISION



TUMOR 6 RIGHT DIVISION



RELATIVE AMOUNTS of DNA
.8 arbitrary units

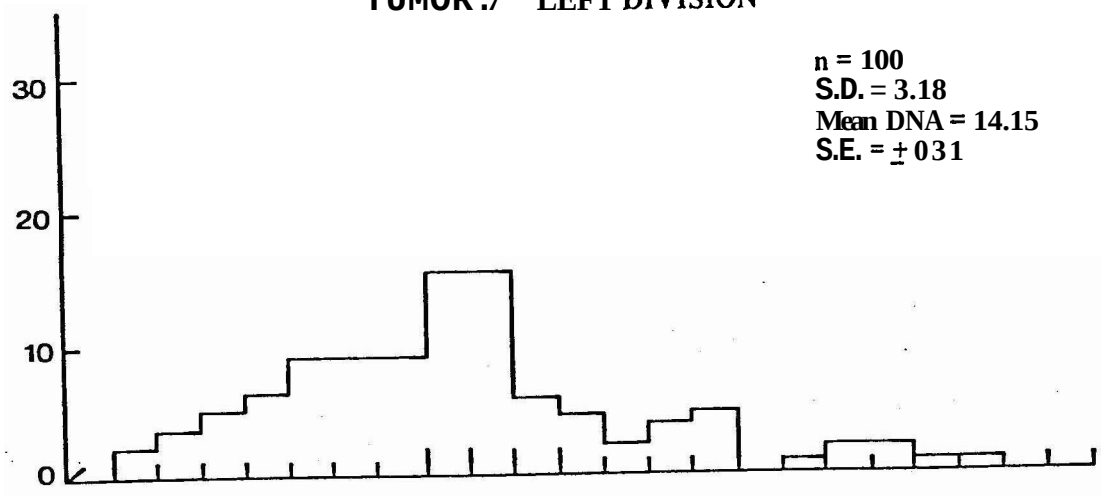
FIGURE 11.

Tumor 7 Left Division

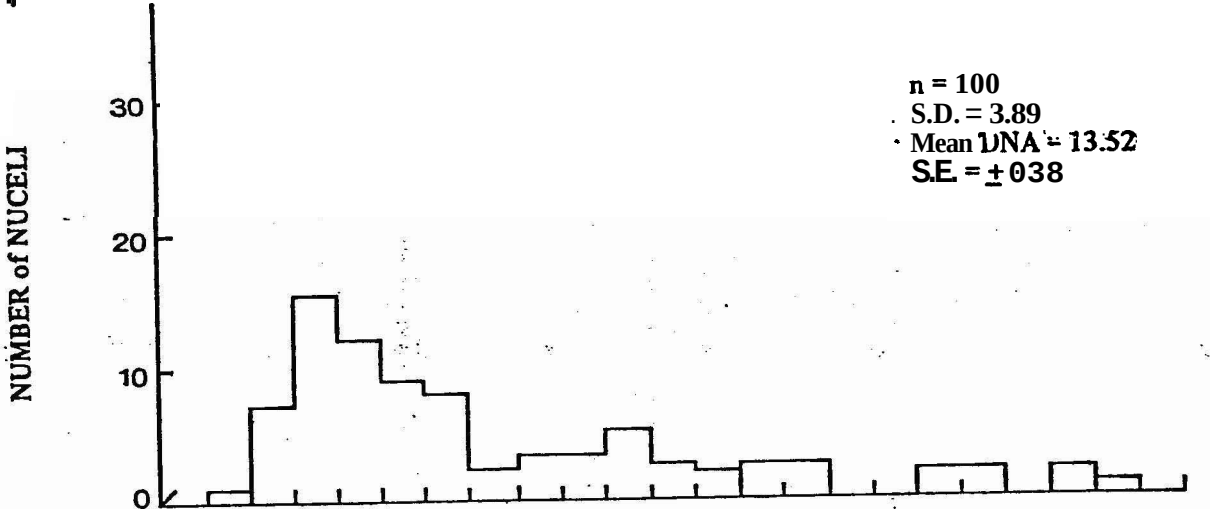
Tumor 7 Central Division

Tumor 7 Right Division

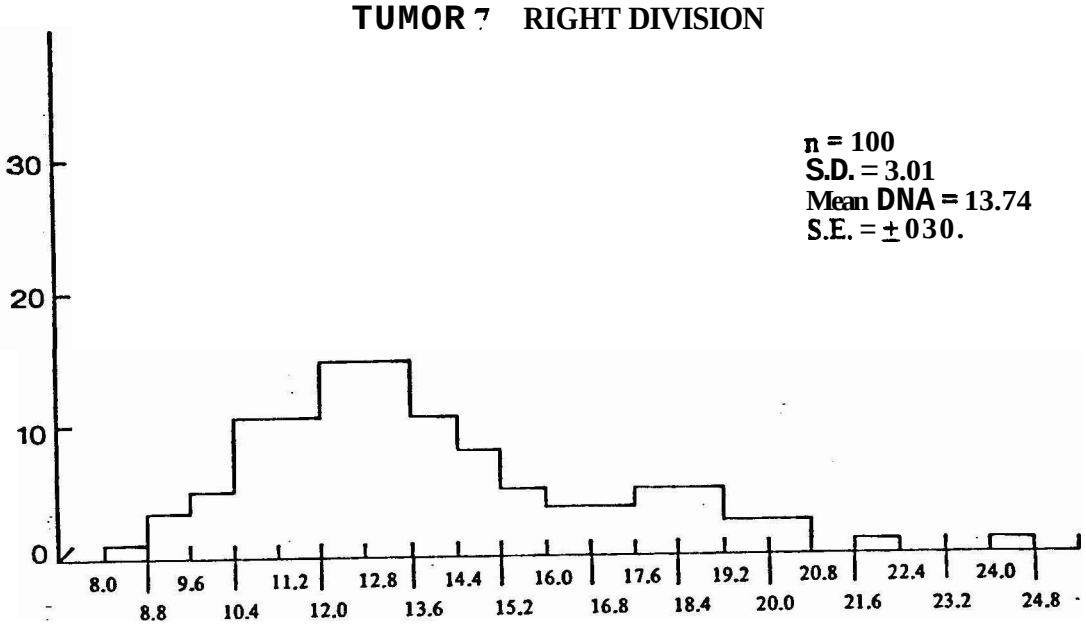
TUMOR .7 LEFT DIVISION



TUMOR 7 CENTRAL DIVISION



TUMOR 7 RIGHT DIVISION



RELATIVE AMOUNTS of DNA
.8 arbitrary units

Tumor 8

This tumor was described as a pinkish-tan, moderately elevated, fungating mucosal mass. The lesion was well circumscribed and measures 3 x 3.5 cm. This tumor occupied half the circumference of the colon lumen. The adenocarcinoma is moderately well differentiated with invasion into the inner muscular layer. Four lymph nodes were negative for tumor involvement.

Four of the five aneuploid cells detected among those measured were in the central area of the tumor. The remaining aneuploid cell was contained in the right division. These two areas of the tumor have a greater mean DNA concentration than the remaining left section. Again, the number of S cells contribute to this factor. The middle section contained the largest amount of these cells. The right division also has a number of cells involved in DNA synthesis. A total of 51% of the cells in the left section were quiescent. This fact accounts for the low DNA concentration average.

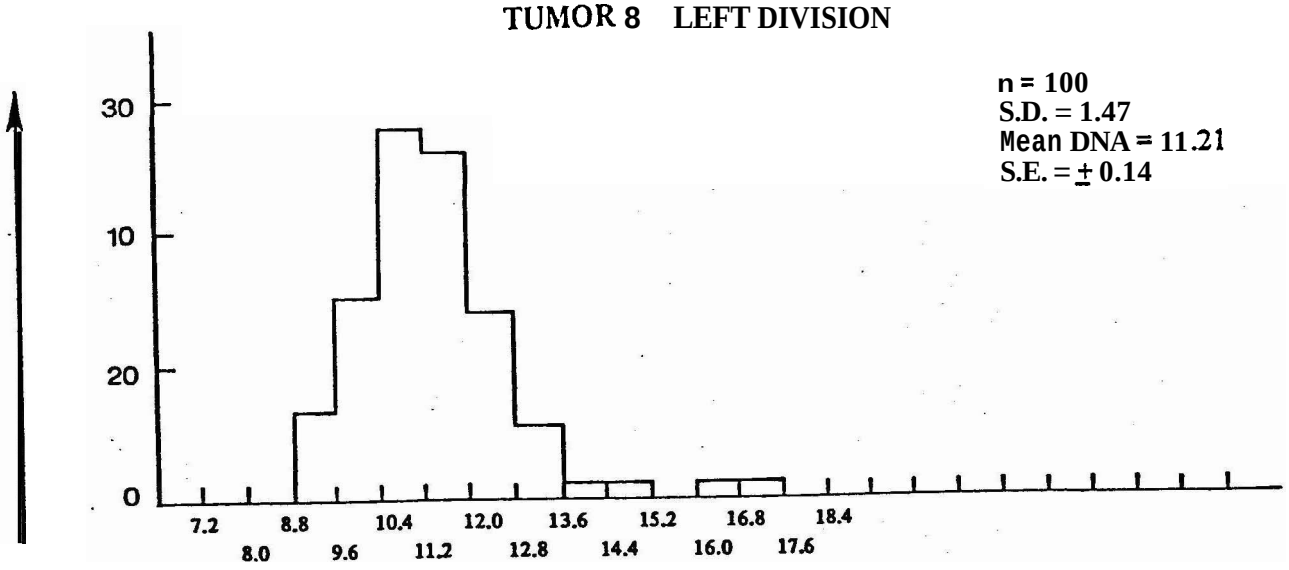
FIGURE 12.

Tumor 8 Left Division

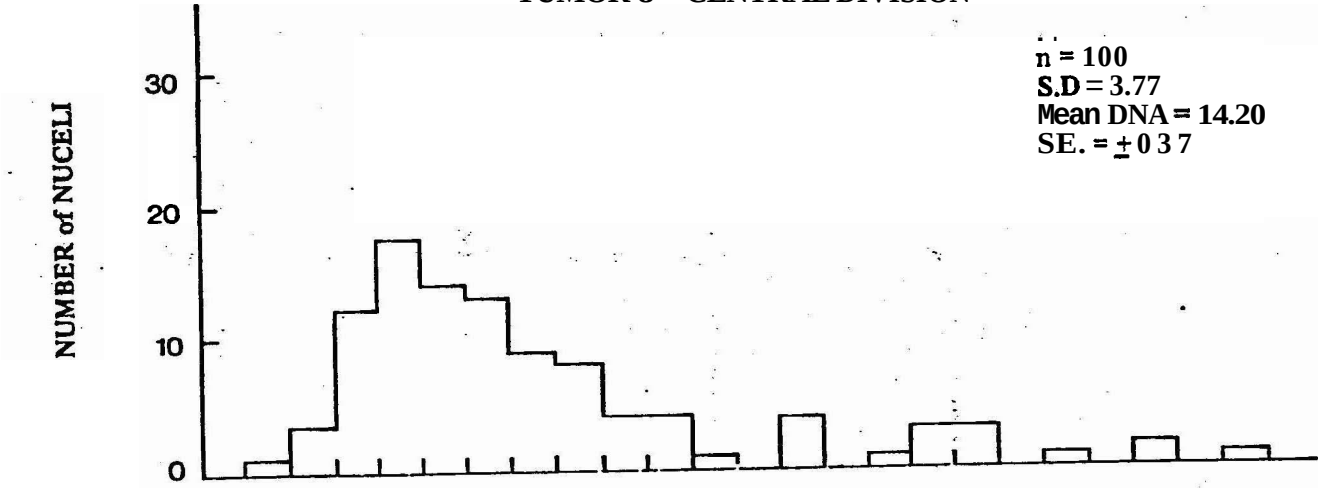
Tumor 8 Central Division

Tumor 8 Right Division

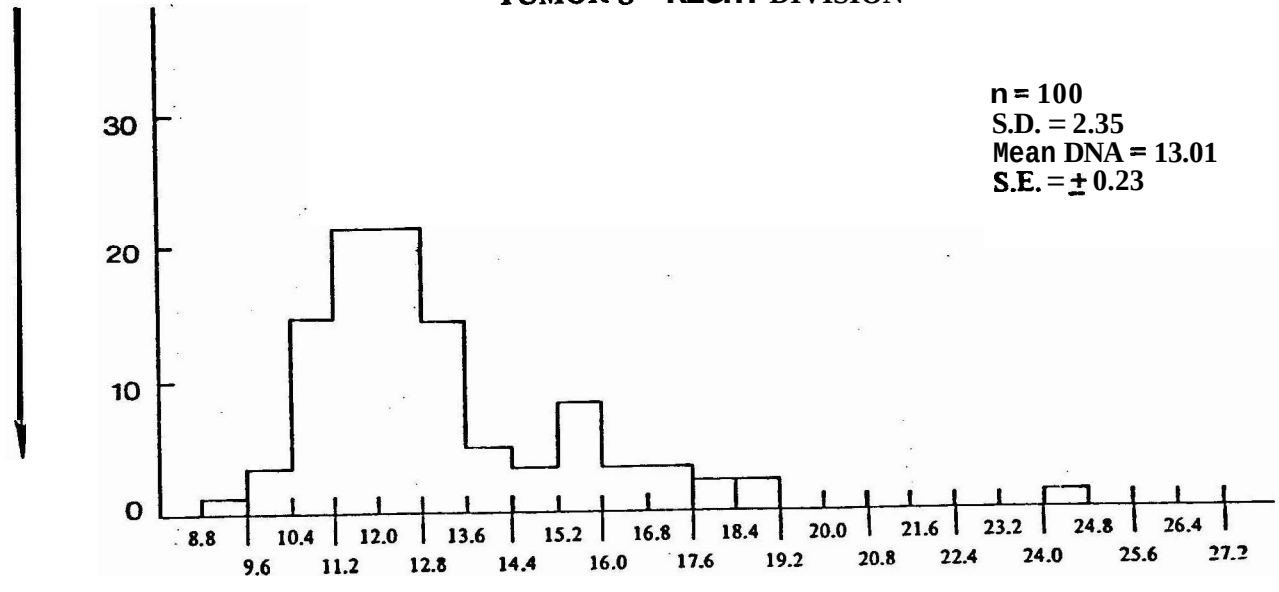
TUMOR 8 LEFT DIVISION



TUMOR 8 CENTRAL DIVISION



TUMOR 8 RIGHT DIVISION



RELATIVE AMOUNTS of DNA
.8 arbitrary units

TABLE 4

Number of Aneuploid Cells

Table 4

Number of Aneuploid Cells

	Left Division	Middle Division	Right Division	Total per Tumor
Tumor 1	12*	14*	5*	31
Tumor 2	1	5	2	8
Tumor 3	0	0	0	0
Tumor 4	1	1	0	2
Tumor 5	0	3	1	4
Tumor 6	0	2	1	3
Tumor 7	2	4	1	7
Tumor 8	0	4	1	5
Total	16	33	11	60
Percent	2%	4%	1.4%	2.5%

*Total cells counted in all cases – 100.

Percent of nuclei in the diploid to tetraploid range – 97.5%

The T test was employed to measure differences between the means of aneuploid cells in each division. The central quadrant was significantly different than the two peripheral divisions. The peripheral divisions were not determined to be significantly different from one another.

CHAPTER 4

Discussion

In the past quantitative cytophotometry has been used to investigate DNA changes that occur in cells during the cell cycle, both those which are normal as well as those that appear abnormal (Atkin, 1969; Emson, 1967; Avtandilov, 1973). Recently these methods have been used to a great advantage to investigate DNA changes in pre-cancerous and cancerous cells (Heenan, 1975; Leibovitz, 1976). These studies have mainly centered on DNA changes that occur during the administration of chemotherapeutic drugs to cancer patients (Carter, 1976). This method enables investigators to monitor the effectiveness of such chemical agents.

The purpose of this study is to characterize tumors obtained from the human colon, and to assess them on the basis of DNA changes within the population of cells comprising the tumor. An attempt is made to discover cellular growth patterns that may take place in emerging or young tumors as well as those occuring in one that has aged and is defined as a differentiated cancer. Since this type of tissue characteristically gives rise to metastasizing cells, the likely origin and characterization of cells involved in movement from the defined tumor area to lymphatic tissue, and then to other tissues, were studied and are discussed.

In an effort to make comparisons between normal and cancerous populations of colon cells, normal cells were first investigated and

characterized (Figure 4) and thus served as the control tissue for this study. Cytophotometric analysis of this tissue demonstrated that it is composed of diploid cells with most cells in the 2C, unreplicated DNA category and G_1 phase of the cell cycle. There are however, cells in the S phase as would be expected as well as some entering the G_2 phase. These cells are at or near the 4C replicated state. The 2C cells have a mean DNA value of approximately 11.07, an average of the three divisions representing the control tissue (Figure 4). However, since colon tissue is a highly proliferative tissue (Leblond, 1964), it may be assumed that many of the cells on or near this value are in early S phase. The 4C or G_2 cells would be expected to be at or near the 22.15 mean DNA value as they enter this phase. Care should be exercised when these values are used in this way, for they must be considered approximate values due to the dynamic nature of cells comprising colon tissue. Growth here is very rapid and DNA synthesis is almost a continual process, this coupled with those cells completing this event as well as the appearance of occasional aneuploids tend to obscure this value.

Koss (1977) using a microfluorometric analysis of normal colon tissue also found that the majority of cells were in the 2C category with many cells in the S and early 4C range. Microfluorometry enables the experimenter to sample a large number of nuclei in a short period of time. However, when compared to the data obtained by microspectrophotometry, the method of analysis used in this study, it compared favorably with that data and similar results were obtained.

Seven of the eight cancerous tumors (87.5%) examined in this study contain aneuploid cells (Table 4), which are those higher in DNA content than the 4C value obtained in the control. This consideration will tend to shift the histograms to the right and increase the mean DNA value established for the controls. The presence of aneuploid cells result in the prolonging of all stages of interphase in the cell cycle. The pre-synthetic gap (G_1) is increased because more synthetic protein is required before aneuploid cells can enter the synthetic phase (S). The S phase also takes longer in aneuploid cells because there is a greater amount of DNA to synthesize as compared to normal tissue. Proteins synthesized to prepare the cell for mitosis occurs in the post-synthetic gap (G_2) stage, which is also prolonged in aneuploid cells because of an increased number of chromosomes. Mitosis basically occurs in the same time period regarding aneuploid and normal cells. Barlogie (1978) found a 91% incidence of aneuploid cells in malignant tumors of different tissues. The percent of aneuploid cells in the sampled populations in the experimental tumors range from 10.3% (Tumor 1) to 0.7% (Tumor 4). The majority of nuclei (97.5%) in these experimental tumors are in the 2C to 4C region of the cell cycle. The 2C cell populations have been found in all tumors (Ludwig, 1973; Freidlander, 1973). Taylor (1983) reported that populations of 2C cells vary in concentration in breast tumors from 10% to greater than 70%. This published data correlates well regarding the large number of 2C to 4C cells found in the histograms presented in this paper.

Hyperdiploid cellular populations are defined as having a majority of cells in the 2C to 4C range in addition to a certain

percentage of aneuploid cells. Atkin (1957) described this hyperdiploid configuration when he observed that the average DNA content of tumors were slightly elevated in comparison to normal tissue. Cells examined by Stich (1960) showed that the average amount of DNA was slightly deviated from the 2C value. Many other studies in which tumors were examined gave a hyperdiploid histogram as a result of aneuploid cells in these tumors (Avtandilov, 1973). For example, in studies done by Inui (1967), mammary carcinomas had DNA levels within the 2C to 4C range with some hyperdiploid characteristics. The DNA distribution patterns of occult sclerosing and frank papillary carcinoma of the thyroid were near the 2C values with aneuploid characteristics (Izuo, 1971). Barlogie (1978) examined aneuploid cells and found characteristics to a variety of hyperdiploid histograms in 24 to 26 tumors. Adenocarcinomas consistently show aneuploid values usually in the hyperdiploid range but also have cell populations resembling normal tissue (Stich, 1960). It was determined in this study that seven of the eight sampled tumors had hyperdiploid cells within the population.

Aneuploid cells were not present in Tumor 3 and the analysis and resulting histogram does not demonstrate an appreciable number of hyperdiploid cells (Figure 7). The average DNA content of this population of cells closely resembles that of normal tissue. This is expected since it is in the early stages of tumorgenesis and had not demonstrated the presence of any metastisizing cells. The pathological examination of this tissue supports this data.

The pathological description of differentiated as well as undifferentiated tumors revealed throughout this study that morphological

characteristics of cells and thus tumor tissues can be correlated well with recorded DNA changes in the representative histograms. Aneuploid DNA apperns are expected in well differentiated carcinomas where the age of the tumor is significant. It is evident that well differentiated tumors are more likely to be derived from low ploidy stem cells or those closer to 2C characteristics which can give rise to cells of higher ploidy levels. All of the tumors examined in this study were described pathologically as moderately to well differentiated adenocarcinomas.

However, the degree of aneuploidy or percentage (Table 4) of aneuploid cells found in a tumor does not always demonstrate a direct relationship with the degree of tumor differentiation or the degree of invasiveness. This accounts for the variety of varying number of aneuploid cells contained in the experimental tumors. For example, Tumor 1 contains the greatest amount of aneuploid cells (10.3%). The remainder of the tumors decrease in aneuploid cell content until there are none present in Tumor 3. It is evident that although cells become aneuploid during tumorgenesis and is a prerequisite to metastasis, other factors are involved which determines whether and when they will become invasive.

That all of these tumors, however, eventually become invasive to the surrounding tissue of the lumen of the colon, points to the existence of an intrinsic factor or factors which determine the period at which these cells become invasive. Invasive adenocarcinomas however were found in most cases to contain aneuploid DNA content and is, however, a necessary requirement of tumorgenesis and metastasis. It was found that only one of the eight tumors examined had metastasized

to another organ. Tumor 1 had metastasized via the lymph nodes into the liver. The histograms of this tumor demonstrated an elevated DNA level almost reaching the 8C level (Figure 5). It is evident that a condition which increased the process of malignancy was characterized by the presence of near 8C cells or polyploid cells. That the other tumors examined have the potential to metastasize must be deduced from the data presented for the majority of malignant tumors contain hyperdiploid characteristics and the potential to become polyploid.

This paper correlates the age of the tumor or time of diagnosis to the mean DNA value and metastasis. The older the tumor the greater the chance of accumulating aneuploid cells of a higher mean DNA value. The higher the mean cellular DNA value the greater the potential for metastasis as these cells become polyploid. Since Tumor 1 metastasized to the liver and also contains the highest mean DNA value among the experimental tumors, it is safe to assume that this was a long standing tumor. Tumor 6 metastasized to the lymph nodes and demonstrated a hyperdiploid mean cellular DNA value. This tumor may have been excised earlier than Tumor 1. Five tumors showed hyperdiploid histograms and no evidence of metastasis to regional lymph nodes. These tumors with hyperdiploid characteristics contain metastatic potential, however were not present in the patients for as long a duration as Tumor 1. They were more than likely excised before they could reach this potential, thus these tumors were fairly young. Tumor 3 was diagnosed early in its development which accounts for the 2C histogram and the 2C mean DNA value.

The comparison of DNA content in different divisions of a tumor is unique to this study. Seventy-five percent of the experimental

tumors have a higher concentration of DNA contained in the central section where abnormal cells had existed prior to recognized tumor growth. Of the 800 cells sampled in the middle section, 4% of these cells were aneuploid. This percentage was found to be consistent with the hypothesis that cells progress from the diploid to aneuploid level of DNA, and then polyploid. The left divisions contain 16 or 2% aneu-pliod cells out of the 800 cells sampled. The lowest percentage of aneuploid cells (1.4%) was found in the right sections of the sampled tumors (Table 4). An elevated mean DNA value can also be seen in the central division without the presence of aneuploid cells, this data is consistent with the prior stated hypothesis since these cells appear to be in the process of increasing their DNA. Tumor 3 does not contain aneuploid cells within its population but has a slightly elevated mean DNA value in the central division due to more cells being involved in DNA synthesis.

Twenty-five percent of the tumors examined do not have their mean DNA concentration elevated in the central section. Tumors four and seven contain a higher mean DNA concentration in the left division. Tumor four has a total of two aneuploid cells, one in the central section and one in the left section. The increased mean DNA value in the left division is due to a large number of cells committed to DNA synthesis. The central division of Tumor 7 contains four aneuploid cells as compared to the two aneuploid cells found in the left division. As in Tumor 4 the left division of this tumor showed a greater number of S cells or cells that are committed to the progression to aneuploids. As stated earlier, the amount of S cells and aneuploids will shift histograms to the right thus resulting in an increased mean DNA value.

Aneuploid cells are the result of chromosomal change that occurs in a tumor. Particular environmental pressures can influence the development of tumors (Carter, 1976). Tumors benefit from the presence of a diversity of cells, because of the regulatory constraints imposed on individual members of the cell populations as may occur through the administration of chemotherapeutic agents (Fidler, 1982). These populations are characterized by a particular chromosomal constitution which in turn determines DNA content. Fidler observed these characteristics and revealed that cells isolated from one tumor have been shown to differ with respect to growth rate, karyotype, cell surface receptors, immunogenisity, and capacity for metastasis. This data explains the differences found in the cellular populations represented by histograms demonstrating mean DNA values, and amount of aneuploid cells found in the sampled tumors.

Tritiated thymidine studies have shown tumor growth occurs on the peripheries of tumors (Cole, 1963). In this study he noted an accumulation of cells on the advancing edge of the neoplasm. This accumulation of cells is likely due to the rate of rapid cell division taking place in these areas. It is probable that this growth pattern is centrifugal because the tumor grows from the middle to the periphery. The cells actively dividing on the periphery of the tumor have a lower DNA content and are in the 2C DNA category compared to the cells that are located in the central and slower growing area of the tumor.

Seventy-five percent of the central divisions examined have an increased DNA concentration along with an increased number of aneuploid cells. Mitotic abnormalities which produce aneuploid cells may come

about because of reduced circulation in certain portions of a rapidly growing tumor (Nowell, 1976). Hanna (1982) studied tumor cells in culture and discovered the presence of hypoxia in the tissue colony as it increases in size. The cells exposed to this stress either die or survive. The survival of these cells usually leads to the production of a new subpopulation which thrives in this environment (Ohno, 1971). A loss or reduction of nutrients as well as oxygen can be expected when certain parts of the tumor may become hypoxic. The surviving aneuploid cells may have a certain genetic composition which enables them to reproduce in this environment.

As previously stated, elevated levels of DNA in the central divisions are shown in 75% of the tumors studied in this experiment. This increase is due to aneuploid cells which are a result of environmental stress and stimuli causing mutated mitosises. Each tumor is in an environment which dictates the characteristics seen in the resulting histograms of their DNA.

In conclusion, time of diagnosis or age of the tumor seems to strongly influence DNA changes as shown in the resulting histograms. The older the tumor the higher the mean DNA value. The probability for the occurrence of metastasis also occurs in older tumors because of this elevated DNA value.

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