A STUDY OF THE SMEAR PREPARATIONS OF LIVERS OF RATS FED THE AZO DYE, m'METHYL-p-DIMETHYLAMINOAZOBENZENE.

by

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PREFACE

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INTRODUCTION

The morphology of the nucleus in fixed tissue smears will be studied from week to week as carcinogenesis progresses. Although the smear technique is extensively used in practical medical cytology, it has not yet been utilized in any investigation of carcinogenesis. However, a similar method, the squash reparation, has been employed in the study of carcinogenesis in the mouse skin. This did not include a detailed study of nuclear morphology, but was concerned mostly with nuclear size changes and chromosome changes. Methylcholanthrene, one of the phenanthrene series, was the carcinogenic agent. In this study, m'-methyl-p-dimethylaminoazobenzene, an azo dye, will be the carcinogen and the rat's liver will be the site of carcinogenesis. It is hoped that the results obtained will be comparable, indicating that the carcinogenic process may be the same regardless of the organ, carcinogenic agent, and kind of animal. Mainly, it is hoped that some of the confusion and controversy regarding the characteristics of cancer nuclei and their evolutionary changes will be clarified. In order that the problems involved in this investigation can be better understood, the following review of the pertinent literature is presented:

I. Cytology in General. (1)

Cytology, (the study of the cell) is a field which is being intensively investigated at present. This includes cytochemistry, cytomorphology, cytophysics and cytogenetics. Many tools and techniques have been utilized such as the ultramicroscope, the electron microscope, micro-

dissection apparati, polarisation optics, x-ray diffraction, new micro-techniques, and last, but not least, the smear technique. The smear technique consists essentially of crushing a piece of fresh tissue, secretions, sediment, or other material, along a slide and dropping it immediately in fixative. The smear consists of a thin layer of individual cells. As can be readily appreciated, this process has several advantages over the usual methods of tissue preservation, in addition to being very practical. Effects of autolysis are kept at a minimum, since the smear is fixed immediately. For the same reason, all manner of fixation artefacts are minimized. All cells are simultaneously fixed and are fixed in the same manner and to the same degree. There are no fixation current effects. Each cell can be individually studied and there is better preservation of the minute detail. The one big disadvantage is that the general architecture is lost, since the smear consists only of individual cells, and sectioned material is necessary to demonstrate this. The method depends on satisfactory preparation of the smears. Dry smears have been used but the cellular detail is not as definite. Cells and nuclei lose their sharp contours, become swollen and flattened, and do not take up stain as well. Although the Vincent Memorial Hospital Staff (2) says that dry smears are "adequate for interpretation," Papanicolau and Traut (3) state that they may be sufficient for endocrinologic and other evaluation, but are not satisfactory for cancer diagnosis. Papanicolau and Traut, who popularized this method, recommended equal parts of ethyl ether and absolute ethyl alcohol for fixation. This has been used almost uniformly. The stain technique varies somewhat with each laboratory, the important thing being that it be satisfactory and the microscopist familiar with it. Papanicolau and Traut complained that

the usual stains used for sectioned material did not differentiate nuclei sufficiently and stained cytoplasm and blood so heavily that in thick and bloody smears individual cells were not well differentiated. They devised a stain of their own and recommend that omission of the counterstain, which can be done subsequently to make a complete study of all cellular elements, may facilitate recognition of carcinoma cells in small numbers.

In the living cell the nucleus appears to be optically homogeneous, having no structures but the nuclear membrane and the nucleolus. Because of this, results of micromanipulation and the fact that the chromatin structure in fixed material is so variable, many authors have questioned the significance of the appearance of the fixed nucleus. Frey-Wyssling (4) suggests that since different fixing methods reveal similar nuclear structure, its pre-existence is likely. Benseley (5), using the freezing-drying method with its instantaneous fixation, found a nuclear structure similar to that in sectioned material and thinks this proves that the nucleus is not, in fact, homogeneous. DeRobertis and Nowinski, summing up, (1) state that "all the facts permit us to affirm that, although the living nucleus may be optically homogeneous, this does not signify that there is structural homogeneity. This structure, although it may be somewhat altered by fixatives, is characterized by the segregation of the chromatin in special regions of the nuclear space. Nevertheless, the image of fixed nuclei should not be accepted without a critical attitude because, besides true structure, there are others, such as the linin, which seem to be the result of a protein precipitation." Ayres, who used unfixed, stained, cell suspensions, says that in fixed preparations chromatin precipitates on the inner nuclear wall, network and outer nucleolus obscuring outline and detail. (6) On fixing, the

fibrils of the nucleus are dehydrated and become accessible to staining. They usually clot together as a result of the adhesive action of the coagulated proteins of the nuclear sap. (4) In stained and fixed meterial the structure of the nucleus is distinguished by its great complexity and varies with the fixative. The parts of the nucleus are: the nuclear membrane, the structure of which is disputed; the nuclear sap, which is unstained or lightly acidophilic; the nucleoli; filaments with linin, (oxychromatin, more acid-staining chromatin and basichromatin, more basic-staining chromatin;) and the coarser chromatin flakes, otherwise known as plasmosomes or false nucleoli. (4) Inclusion bodies may also be found in the nucleus associated with certain virus diseases. Green (7) has found crystals in the nuclei of mouse lung, mose liver and other tissues, which are protein and lipid in nature, and seem to be correlated with age, tumors, and chronic inflammation. Other workers have also observed crystals in nuclei. In cytomorphosis, the nucleus often stains more intensely and is shrunken. Simultaneously its structural details are progressively lost. (Pyknosis) However, each type of tissue must be studied separately in this regard. The most reliable criteria of cell death seems to be a diffuse stain of the nucleus and cytoplasm by vital dyes. (In living cells, the dye is found in granules and vacuoles.) Postmortem, the nucleus resists autolytic changes longer than the cytoplasm. It becomes pyknotic and shrunken with loss of structural detail. Later karolysis and loss of stainability, with or without karyorrhexis, occur. (1)

The definition of the nucleolus is not precise in the literature and often includes nucleolus-associated chromatin, and even larger heterochromatic parts of the chromatin. Caspersson offers the following definition to resolve these difficulties: "dense, rounded, as a rule optically homogeneous, endonuclear bodies consisting of proteins in high con-

centrations, rich in diamino acids and associated with the cytoplasmic protein formation." (8) The nucleolus vigorously collects most basic dyestuffs, but stores eosin. Its reaction to dyes is strongly proportional to the fixation and the method. It has a specific affinity for methyl green. Protein crytalloids, which sometimes replace nucleoli, grow in small nuclear vacuoles. (4) It has been demonstrated in plant cells that the nucleoli are in intimate relationship with specific chromosomes with secondary constrictions. The point of union is referred to as the organizer of the nucleolus. The nuclear material is derived from all the chromosomes present, but it is accumulated and organized only in the region of the organizer of the nucleolus. (8) Caspersson, in his investigation of cells with ultraviolet absorption techniques, (8) states that several endocellular organelles participate in the bulk of cytoplasmic protein formation, which he refers to as the "system for cytoplasmic protein formation." The most important parts of this system are the nucleolus associated chromatin, the nucleolus, and the nuclear membrane system. The chemical composition of the nucleolus is related to genetic control. Rearrangements in heterochromatic regions of chromosomes in Drosophila or sex differences lead to a change in the ratio of protein to nucleic acid and in the type of protein. (1) Caspersson notes that the increase of nucleolar mass is a conspicuous phenomenon during cytoplasmic protein synthesis. It increases, "sometimes enormously," during intensive growth. Nucleoli are very small or absent in cells which do not form any cytoplasmic protein. Exceptions were found only in cells prepared for very rapid growth--i.e. dormant plant embryo. During differentiation, large nucleolar masses were found and presumed to be due primarily to gene products. (8)

According to Frey-Wyssling, chromatin is a morphological concept for regions of the nucleus showing identical staining behavior. (4) Chromatin structure varies much: grenules varying in size from very small, (dust), to very large; occasionally a chromatic membrane along the edge of the nucleus; and often deposited on the inner karyotheca and around the nucleolus, making them stand out. Benseley, using the freezing-drying method, describes chromatin as distributed in the form of a large number of small, low pyramidal masses, distributed along the inner surface of the nuclear membrane; a small number of larger masses in the interior of the nucleus; and an investment of chromatin around the nucleolus. Sometimes a fine network can be seen in the background joining the various chromatin masses. This is known as the reticulum or linin network, which is a protein framework with embedded nuclein acids. (5) The heterochromatin is the regions of the chromatin which after cell division do not lose their high nuclein acid content,--i.e. chromosome parts with preserved spiral structure. The euchromatin is the regions of the chromatin in which the nuclein acids are decreased after cell division. (4) The heterochromatin absorbs less stain than that generally found in chromosomes, (euchromatin) and is often associated with the nucleolus. (9) There is some controversy about whether the chromatin elements, which are considered to be uncoiled chromosomes, form a definite structure or are in the form of freely dispersed particles. Frey-Wyssling is "convinced" the former is true.

Chromosomes can be studied by fixation with minimum fixation artefacts. (1) Chromosomes are composed of chromonemata, the only part in the interphasic nucleus and matrix. The chromonemata are fibrils and each chromosome contains one to four of them. Polytene chromosomes, first found in the salivary glands of the larval fly, "most probably"

arise from a series of successive longitudinal divisions of a common chromonema until a giant chromosome results. (1) These have also been found in other insect tissues, crown gall tissue, under non-cancerous experimental conditions, and in tumors. It has been postulated that the number of nucleoli can be used as an index of polyploidy, (which is occasionally found in normal cells) and polytene chromosomes, because more than the ordinary number of nucleolar organizers would be present and more nucleoli would be present. Also, it has been postulated that because the nucleolar organizers must be closer together in polytene chromosomes, the nucleoli are larger due to coalescing of adjacent organizers. (10) Chromosome sizes vary in general with nuclear volume, from organ to organ, and animal to animal. They vary least in embryos and most in older animals. The volume seems most likely to change when the cell is differentiating or modulating in accordance with quantitative changes in chromsome function. (11, 12) It has been proposed that differences in chromosome size from one normal cell type to another depend on euchromatin development. (11)

The cells of a given tissue do not vary in size irregularly, but depend on the laws of growth. Heidenhain's Law of Growth in Constant Proportions states that a given tissue contains cells whose volumes are within a geometrical line. (13) Jacobj observed a geometric progression: Mn/Mc, 2Mn/2Mc, 4Mn/4Mc, 8Mn/8Mc ... where Mn is the mass of the nucleus and Mc is the cytoplasmic mass. (14) The cytoplasmic volume and the nuclear volume are directly proportional. There exists an optimum equilibrium, the nucleocytoplasmic index, which cannot be exceeded, (within limits) without important changes in cell physiology. Np = Vnucleus/Vcell - Vnucleus. "The lack of maintenance of the nucleo-

plasmic ratio would seem to act as a timulus to cell division." (1) In general, the younger cells have more columinous nuclei. Nuclear sizes also vary with the individual animal, and from organ to organ in the same animal. Sheirer found in the lactating and non-lactating breast that secreting nuclei were larger than non-secreting nuclei. (15) Ehrich noted that as organs increased in functional acitivity the nuclei increased in size. (13) Ludford thought that the greater the metabolic activity, the greater the total volume of the nucleolar material. (16) The relative size of the nucleus is also less at high temperatures compared with low temperatures. (17) Most authors agree that there is considerable shrinkage of the nucleus with fixation, except for Sokoloff, who used a great variety of fixatives in his investigations and claimed that they played an "insignificant role" in nuclear size. (18)

All hepatic cells are essentially similar. There is no cytologic evidence that specialized groups exist. (19) Hepatic cells are polygonal in shape with six or more surfaces. Usually there is one large round nucleus, quite vesicular with a smooth membrane, one or more very prominent nucleoli and a few small chromatin dots. The cytoplasm varies tremendously with glycogen, fat and protein inclusions, a cytocentrum, very variable mitochrondria, a Golgi net, and occasional neutral red staining vacuoles. (20) In normal rat liver the chromatin in fixed preparations often seems precipitated on a network. The intermediate space is made up of a continuous mass constituting the organic residue of the karyolymph, which has a very minutely granular aspect and is faintly Feulgen positive. (5) Weatherford found in an average of 1.03% of hepatic nuclei of presumably normal dogs and other canidae highly refractive, hexagonal, prism-like crystals, 7-12 micra long, which were

strongly acidophilic or strongly basophilic. As a result of many chemical tests, he concluded that they were protein in nature and were derived from a purine base. They were unaltered by fasting, anaphylaxis and intravenous hemoglobin administration. (21) Green found in 2% of hepatic nuclei of mouse livers eosinophilic inclusion bodies. In dividing cells of mouse livers after fixatives not preserving mitochondria, Green also found basophilic granules which were not Feulgen positive around and within the spindle areas. (7) Discrete bodies occupy most of the cytoplasm of liver cells of rats. Part of these have the characteristics of mitochrondria, and part do not. The latter are found in cells around portal spaces, in foci of regeneration, and tumors. They are basophilic and contain ribonuclein acid. They increase toward the central vein, forming clumps and a palisade arrangement in the margin of liver columns. (22) Often binucleate cells are seen in the liver. Because of this there was much controversy about the mechanism of cell division in the liver. However, it seems generally agreed now that amitosis does not occur and that the binucleat cells originate as the result of daughter cells failing to divide in mitosis after the nucleus has. (19) Another controversy concerns whether or not the hepatic cells ever arise from bile duct cells. Maximow and Bloom in 1930 conceded that probably some of them do. Mitotic figures are rare, 1 in 10-20,000 nuclei normally. (23) Biesele states that the larger normal rat liver chromsomes are not due to an increased number of distrote strands and are not polytene because the rhythmic nuclear volume doublings are not underlaid by rhythmic chromosome volume doublings, (except in polyploidy and there the total chromsomeal material doubles and not the volume of individual chromosomes) and there is a maximum of only six nucleoli in all tissues with all sizes of diploid

nuclei.[11] Beam says that "efforts to use the nucleoli, as has been done in certain other material, to determine the chromosome number was found unreliable for the hepatic cell," but does not say why.[19] Chromosome volume varies with age in the rat liver, being larger soon after birth and maintained or augmented with maturity and old age. [12] In normal liver the frequency and magnitude of polyploidy is increased in the regions of the central vein over other areas, thought to indicate continuous slow replacement of normal hepatic cells, occurring mostly in that area. [24] Chromosome volume in normal rats does not vary in proportion to the cytoplasmic concentration of ribonucleic acid, or with the relative development of heterochromatin and nucleoli. It does parallel the total concentration of B vitamens--except inositol. In normal rat organs, the chromosome columes, in order of decreasing size, are as follows: liver, kidney, adrenal, lung, small intestine and spleen.[12]

After partial hepatectomy, there is a latent period of about 24 hours during which the liver increases 50-60% in size with no significant change in the number of cells. During this time the mean volumes of cytoplasm, nucleus and nucleolus increase 2.6, 2.2 and 4.1 times respectively. [25] The second day, nuclear and nucleolar areas are largest, with a mean increase in nuclear diameter of 5%. [24] After cell division starts, there is a decrease in the mean cell size which remains a little more than normal for 12 days. [26] No amitoses or abnormal mitoses were seen. Bruce and Marble state that the percentage of mitoses varies widely from hour to hour and is different in different livers so that a single count tells nothing of growth rate. The mitoses are evenly distributed and there is no preponderance near bile duct cells. Twenty-four hours after hepatectomy, the mitosis rate averages 2.13% and decreases

from then on. (25) Biesele found no new chromosomal complexity not present in controls in regenerating rat liver, no increase in chromosome size or increase in frequency of polyploidy. (27) Sulkin says there is an increase in polyploidy, but he uses the nuclear measurements as a measure of polyploidy, which Biesele deplores. Sulkin found a decrease in binucleate cells in restored liver 28 days after the operation. If only 25-45% of the liver is removed, there was no increase in binucleate cells or in "polyploidy." (28)

II. Cytology of the Cancer Nucleus.

The cancer cell has been much studied and many observations have been made of it such as: pleomorphism, aniscocytosis and poikilocytosis, abnormal cytoplasmic nuclear ratio, abnormal nuclear-nucleolar ratios, extreme hyperchromasia, condensation of chromatin, irregular nuclear pattern and chromatin network, giant cells with nucleoli, multinucleated cells, vacuolated and degenerate cytoplasm, sharp nuclear borders, abnormal distribution of cytoplasm, wrinkled nuclei, large nucleoli, cell size larger than the mother tissue, numerous mitoses, phagocytosis causing inclusions, inclusions due to chromatin extruded from a hyperchromatic nucleus, a sepcific Golgi apparatus, variation in chromosomes about the diploid number, asymmetrical and atypical mitoses, irregular fragmentation of nuclei, grouping of cells in smears, eccentric nuclear positions, nuclei stripped of cytoplasm, atypical staining properties, decreased mutual adhesiveness, irregular sheet formation, and "qualities words cannot describe." The appearance of large amounts of blood and leucocytes on a smear is considered suspicious, and invasion of large clumps of cells by polymorphonuclear leucocytes is "highly suggestive." (5) Undifferentiated carcinomas all look similar on a smear and are dis-

tinguished from differentiated carcinomas by their absence of cytoplasm and indistinct outline of cellular borders. (2)

Is there such a thing as a characteristic and diagnostic malignant cell cytologically? This question has been much disputed, although at present it seems as though the majority opinion is the affirmative. Hauptmann states that there are "many suggestive changes of malignancy, none constant enough or prominent enough to have diagnostic significance." He adds that though it is possible in some tumors to have one detail of the cell constant and characteristic enough to be diagnostic -- "general application of such a rule does not seem justified." MacCallum teaches "it is not possible with the means now at our command to distinguish with certainty a cell of the epithelium which has this (malignant) exaggerated power of growth from a cell of the epithelium of a benign tumor or even a normal cell." (29) Borst says definite morphologic characteristics of carcinoma cells do not exist and the autodestructive type of growth must be regarded as the most important, if not the only, histologic proof of carcinoma. (30) Shairer says that there is no evidence of such clearcut differences between malignant and benign. (15) Hansemann, Arnold, and Boveri uphold this viewpoint that there is nothing positively diagnostic. (31) On the other hand MacCarty affirms that the cancer cell can be absolutely diagnosed. (32-37) Quensel, Heiberg, Zadek, Kerp, Hertwig, Aichel, Sokoloff, Hartmann and other German workers uphold this view, as well as other English, (Dudgeon), and American workers, (McCormack, Strohl, Haumeder, and Fidler.) Papanicolau and Traut state that "the typical malignant cells are unmistakable," but these are less numerous. There are many more atypical, abnormal cells which cannot definitely be classified. He recommends that the vaginal smear should be considered as an

accessory or preliminary method of diagnosis only. (3) Ackerman states that although in a few instances, such as mouse hepatomas with characteristic changes in mitochondria and the Golgi net, single cells may be enough different from surrounding normals to be recognized as neoplastic, no strictly specific characteristic of the cancer cell has been established yet. (38) Fidler and MacCarty, as well as many other pathologists, emphasize that in order to diagnose malignancy experience is necessary because the malignant cells have "qualities words cannot describe." (32)

Much time and effort has been spent measuring malignant nuclei, nucleoli and the whole cell. MacCarty, using fresh, unfixed, unembedded human tissues, found that the mean areas of the nucleoli of malignant cells are more than those of corresponding non-malignant cells. He also found that the difference between nucleolar areas in malignant and non-malignant cells is more than the difference between nuclear areas. In this material, the ratio of the nucleolar area to the nuclear area varied from 1/5 to 1/17 in malignant cells and 1/13 to 1/45 in non-malignant cells, including reparative regenerative cells. Under some chronic inflammatory conditions, he found another type of regenerative cell mixed with the others -- spheroidal or slightly ovoidal, relatively large nucleus with larger granules and one or more larger spheroidal or ovoidal nucleoli with nucleolar-nuclear diameters of 1/4. He at first designated these as "secondary cytoplasia," but later recognized them as cancer cells. From this he concluded that it "may now be positively stated that all malignant cells arise from the regenerative cells of normal tissue." MacCarty for years carried on quite a crusade trying to get pathologists to use fresh tissues (claiming that other tissues could not be used to demonstrate these nucleolar changes) by means of which all cancer cells could

be positively diagnosed early. (32-37) But the method was tedious, time-consuming and did not give the same results in other hands. (39) However, Naumeder, an associate of MacCarty's, confirmed his work by measuring nucleolar and nuclear areas and their ratios in carcinomas, normals, and cytoplasias from many sources. (40) Quensel in Germany, using body fluids and supravital stains, found average nucleolar diameters of 1-1.5 micra, occasionally 2-3 micra, in controls and 3-10 micra, occasionally 1.6 to 2 micra, in malignant cells. The ratio of nucleolus to nuclear diameters was .20 to .60 in malignancies and .14 to .20 in controls. However, he found that the nucleolar size changes did not occur in the sarcomas studied. (41) Quensel's work was confirmed by Zadek and Karp as well as other German workers. Zadek found a n/N ratio of 1/4 to 1/20 in malignancies against 1/25 to 1/100 in controls. (42) Hauptmann observed that the majority of cancers do have larger cells than those they originated from but he was not able to confirm the work of Quensel and MacCarty for all the cancers he studied. Metastatic tumors in his series often had only small nucleoli or none. (43) Guttman, using sectioned material and assuming that the third diameter was equal to the shortest or transverse diameter, calculated volumes of nuclei and nucleoli in cancer cells. He noted that the ratio of nuclear to nucleolar volumes in normals, hyperplastic tissues and benign and malignant tumors was not essentially different. The volumes of nucleoli in normals, however, were significantly lower than those of hyperplastic tissues, benign and malignant tumors. But, because of the marked variation in nucleolar volumes, he maintained that this criteria could not be decisive in determining carcinoma. Von Haam and Alexander measured 10,000 cells in benign and malignant tissues in paraffin sections, frozen sections and cell suspensions. Ninety-six percent of the

carcinomas had nuclear to nucleolar area ratios distinctly less than benigns. Likewise, corpus luteum cells had very low ratios.(44) Saxen, Stenius, Castren, McCormack, Strohl and Naidu also measured and found increased nucleoli in cancers. Stenius found nucleolar diameters of 3-4 micra in malignant bladder tumors and diameters of 1-2 micra in benign ones. (31) Castren was unable to find enlarged nucleoli in all types of sarcomas. Stowell found that the individual nucleoli size and the total nucleolar mass per nuclear section increased three times during protein diet. Using ultraviolet light, he also discovered that cells with largest nucleoli did not have an increased absorption of ultraviolet light and were probably not producing large amounts of cellular protein substances. Cells in which more rapid growth would be expected, regenerating tissue and rat hepatomas due to butter yellow, had somewhat smaller nucleoli, more nucleoli per nucleus and usually increased nucleic acid of nucleolus and cytoplasm. He was unable to demonstrate an entirely consistent relationship between nucleolar size and rate of growth or protein formation. Hepatoma cells had the smallest mean nucleolar and nuclear volumes and volume ratios of all the cell types studied. (25) Spantschin, in tar tumors, cancroids and tarred skin nuclei, discovered that the nuclei were increased in size, especially in the carcinomatous lesions. (45) Biesele found in mouse carcinoma that the nuclei fell into volume classes as in normals, though far from as distinct, presumably due to aneuploidy.(10) In methylcholanthrene skin carcinogenesis, Biesele also found that the nucleoplasmic ratio changed very little though there was an increase in volume of both the nucleus and the cytoplasm. (46) Haumeder also found that the cellulenuclear ratio was fairly constant for each cell type, in cancer. (40) A high nucleoplasmic ratio was at first

thought diagnostic for malignancy but is also found in embryos and is thought merely to mean fast growth and division. (Heiberg, Hertwig, Aichel, Sokoloff, Hartmann) Sokoloff thinks, therefore, that carcinoma is closely related to embryonal cells.

In the cancer cell nucleolus, increased size out of proportion to the increase in nuclear size, irregularity in shape, increase in number, and variation in number have been described. Ayres, using fresh cell suspensions stained with Azure-C, added fine nucleolar strands between the nucleoli (thought to be evidences of nucleolar division) and loss of polarity to the list. (39) Quensel, in serous exudates, noted that the nucleolar shape in malignancies is usually oval or polygonal, rarely round as in normals. (41) Saxen, in malignancies of the nasal cavity and sinuses, found extensive variation in size and number of nucleoli. Stenius found nucleoli in bladder tumors oval and irregular in shape. Stowell found increased ultraviolet light absorption of nucleoli in regeneration and protein repletion, and an insignificant increase in early regeneration, protein depletion, cells near hepatomas and in hepatomas. (47) Caspersson and Santesson studied various malignancies with the microspectrograph. They found two extreme cell types: the first type showed extreme stimulation and activity of the system for protein formation, and represented areas where the nutrition was good and the cells actively invading; the second type showed extreme stimulation but little activity, the nucleolar apparatus showed signs of intense function but no increased protein in the cytoplasm. Then these cells apparently cease to grow, rarely divide and eventually undergo necrosis. Both differ from normals in that the endocellular inhibitory mechanism has more or less ceased to function. They postulate that the nucleolus-associated chromatin and genes, which regu-

late nucleolar function, must play a prominent role not only in the malignant cell growth but also in carcinogenesis. A series of sarcomas studied gave them approximately the same results. (8) Hauptmann compared the chromatin structure in control and malignant nuclei. The control chromatin was usually dense and clumped. If net-like it was delicate and regular. In the malignant nuclei the chromatin was irregular with coarse strands forming a net structure. The meshes were irregular and varied greatly in size. Other nuclear chromatin arrangements formed a grid, or consisted of irregular, coarse granules, with perhaps a dense amorphous appearance. Occasionally the chromatin was condensed in a ring at the periphery of the nucleus.(43) Biesele noted a slight increase in displaceability of the nucleoli and basophilic chromatin under ultracentrifugal force.(48) Lewis studied a large number of malignant cells from animal carcinomas, sarcomas and human tumors. All malignant cells had some hypertrophy of the chromatin and nucleolar material. Some tumors had such hypertrophied nucleoli they resembled herpetic inclusion bodies. Others had greatly increased numbers and sizes of karyosomes. The malignant cells of each tumor had nuclear structures characteristic for them which were maintained through many generations of transplants in vivo. No one abnormality was found common to all tumors.(49) Hauptmann, among ninety, proved carcinomas and in smears divided all the malignant cells into five types: squamous cell, columnar cell, round cell, undifferentiated cell and oat cell.(45) Dudgeon and Barrett noted phagocytic properties in malignant cells leading to inclusions; Horning and Richardson found inclusions due to chromatin extruded from a hyperchromatic nucleus; DeFano and Ludford found a specific Golgi apparatus; MacCarty noted intranucleolar bodies and even a minute, active, motile body; Opie, in butter yellow rat hepatomas, found

swollen cytochondria, which may form conspicuous cell inclusions. Opie also noted basophilic bodies in the cytoplasm, which increased in number in butter yellow rat hepatomas and did not have the characteristics of mitochondria. These bodies disappeared under the action of ribonuclease and were assumed to consist of ribonucleic acid, probably associated with increased protein synthesis in tumors. (22)

There has been great argument about the significance of heterotypical mitoses, asymmetrical mitoses and the casting out and degeneration of chromosomes in malignancies or even whether they occur. Hansemann noted atypical mitoses.(50) One school of thought for a time believed that these atypical and heterotypical mitoses were the most characteristic thing about malignant nuclei. MacCarty saw occasional multipolar mitoses but never an asymmetrical, irregular one. Lewis, as well as Biesele and others, saw occasional mitotic figures which were abnormal. Levine contended that the multipolar spindles in the giant cells of animal tumors arise by mitosis of their several nuclei and that a uninucleate giant cell may give rise to a bipolar spindle.(51) Biesle, among others, found an increased mitotic frequency in malignancies. Biesele also found nuclei bearing multiples of the normal number of heterochromatic segments in hyperplasia, predominant in mouse skin carcinomas which he thought were explicable on the basis of multiple stranded chromosomes and polyploidy. (52) In the smear preparation however, mitoses are rare and have little significance. Levine studied the stages in chromosomal disintegration and diffusion in many tumors. He contends that chromosome proliferation without regard to nuclear organization is an early stage in chromosome disintegration and diffusion into the cytoplasm, suggesting that the chromosome substance stimulates the other cells to grow and divide.(51) To

sum up, it is gretty well agreed that atypical, asymmetrical mitoses and multipolar mitoses do occur but there is wide disagreement about the occurrence of heterotypical mitoses. Biesele, in a study with the squash preparation on mouse carcinoma, found that the nuclei fall into volume classes as in normals, though far from as distinct, due apparently to considerable aneuploidy. Class I had a maximum of four nucleoli and normal appearing chromosomes, presumed to be non-malignant or hypodiploid malignant nuclei. Class II, the prevailing class, had about the diploid number of chromosomes but they were twice normal size and there were about twice the normal number of nucleoli. Biesele thinks this enlargement is due to twice the number of chromonemata, due to endomitosis or growth of the chromonema without division of the centromeres. In malignant cells, chromosomes have been seen which have separated a little for a time and appeared double without any mitosis taking place. The increased number of nucleoli would be due theoretically to the increased number of nucleolar organizers, due to the presence of more chromonemata. The enlarged nucleoli are accounted for by coalescence of adjacent nucleolar organizers, because they would in such case be closer together. Class III contained tetraploids the same size as Class II, diploids with four times the number of chromonemata as normals and even more numerous, larger nucleoli. Class IV contained octoploids with chromosomes twice normal size, tetraploids with chromosomes four times normal size, diploids with chromosomes eight times normal size, plus more and bigger nucleoli.(10) Levine found essentially the same thing in many types of tumors -- animal tumors, human epitheliomas, Rous chicken tumor and rat sarcoma. He devided the nuclei into three classes proportional to the size and the chromosome number (finding that the chromosome number was a good index of the size) normal size and apparently

diploid; tetraploid and semi-giant; and giant cells with many chromosomes.(51) Biesele extended his investigations to many tumor types -- rat hepatoma 31, Walker carcinosarcoma 256, animal leukemia, human adenocarcinoma of the uterus and human mammary carcinoma and others.(10,11,12,27,46,48,52,53,54,55) In all he found the same enlarged chromosomes, often appearing double, which he took to be polytene. The increased nuclear volume and increased numbers of nucleoli, he reasoned, were sequelae of polytene chromosomes. The frequency of endomitosis was roughly proportional to the degree of malignancy. In addition, his finding that the enlarged chromosomes shrank under pepsin digestion more than normals confirmed that there was a true increase in amount of actual chromosomal material, not swelling or loose coiling. However, the proportion of polyploid division figures in all instances was greatly exceeded by the proportion of resting nuclei with more nucleoli than the diploid normals. All the normal tissues studied by Biesele and one benign uterine tumor had no increase in nucleolar number or enlarged chromosomes. However, in methylcholanthrene hyperplasia and other conditions enlarged chromosomes were found.(46) Lewis noted that many normal, as well as malignant, cells of tumors were undergoing mitosis. The normal cells in all types of tumors studied had the normal number of chromosomes and the malignant cells in some of the tumor types had more than the normal number of chromosomes. The chromosome number characteristic of each tumor survived unchanged through many transplants in vivo.(56)

Another frequent source of controversy concerns which is the most reliable criterion of the malignant cell. MacCarty says that the enlargement of the nucleolus is "unquestionably diagnostic." Zadek affirms that the increased number of large and polygonal nucleoli in small

nuclei must be looked upon as the most characteristic sign of tumor cells. Haumeder states: "The increased nucleolar size in relation to the nuclei is characteristic for the cell of malignant neoplasms found in transudates and exudates," and should be most helpful in diagnosis. She claims that many if not all the cells in every field were changed.(40) Quensel, Hauptmann et al say flatly that the nucleolus is diagnostic. Adams asserts that MacCarty's work does not give the same results in other hands.(39) Von Haam and Alexander say that the study of the nucleoli must be emphasized as a valuable help in diagnosis, though none of the changes which have been reported are absolutely characteristic for each carcinoma cell. (This seems to be the majority opinion at the present time as far as nucleoli go). At one time the nucleoprotoplasmic ratio was thought to be diagnostic, but it has since been discarded. Buttman believes that the nucleolar volume may be used only as an adjunct to differentiate malignant and non-malignant cells. Hauptmann indicates that it seems impossible to make a diagnosis of malignancy on the basis of cell size of nucleocytoplasmic ratio. Papanicolau, in his vaginal smear studies, states: "should put more emphasis on the presence of structual abnormalities in the cell and more particularly the nucleus. Inequality in cell size of the same group of nuclei is a frequent and important diagnostic criteria. Nuclear enlargement is an important criteria, especially with suggestive structural changes."(8) Borst thinks that the autodestructive type of growth must be regarded as the most important if not only histologic proof of malignancy, though certain cytologic changes may be helpful. Hansemann thought atypical mitoses were most characteristic and Heiberg thought variation in size and shape of the nucleus was. Ackerman notes that although there are characteristic mitochondria and Golgi apparatus in some mice hepato-

mas, there is no strictly specific characteristic of a cancer cell.(56)

Similar cytologic changes to those found in malignant cells are found in inflammatory lesions, benign tumors and reparative regenerative cells. These are especially apparent to the inexperienced. MacCarty even designated as "secondary cytoplasia" some peculiar nuclei found under chronic inflammatory changes, which he later recognized as true malignant cells. He went further to state that all malignant cells arise from the regenerative cells of normal tissue. The benign regenerative cells are recognized by their more delicate construction, fine chromatin granules, lighter stain and the small nucleolar size compared to the nuclear size. (55) Papanicolau stresses much the same criteria and notes that enlarged nuclei are to be found in vaginal smears which, however, are of normal aspect and structure. (See the paragraph on the regenerating rat liver).

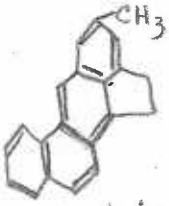
III. Precancer and Carcinogenesis.

Szodoray considers that most modern authors "regardless of the morphologic structure present, consider as precancerous changes from which sooner or later in a great percentage of instances cancer develops." He further claims that the transition is slow and morphologically unfixable and that there is no characteristic change of precancer.(57) It has been noted that inflammatory change is not an essential precursor to cancer due to carcinogenic hydrocarbons and that cirrhosis is not an essential precursor to the neoplastic reaction either. MacCarty, as has already been noted, believes that all malignant cells arise from reparative regenerative cells. Des Lignerous studied several types of so-called precancerous material. Rous tumor and spontaneous mammary carcinoma (with hormone administration) showed no precancer stage. No changes at all were noted before the cancer cells appeared. 3:4-benzpyrene and methyl-

cholanthrene always had a precancerous stage. When the cell reached a certain state of constitutional alteration, carcinoma occurred without the aid of further chemical. Non-specific irritants produced the same effects as in the chemical precancers but no cancer resulted. There was an increased rate and frequency of tumors when both chemical and non-specific irritants were employed.(58) Ackerman states that there is no evidence of sudden morphologic change but gradual transition of changes turning imperceptibly into carcinoma.(58) Rusch and Kline designated three phases in tumor formation: period of 1-3 months between two carcinogen periods. After a lag in the rest period the tumors quickly formed during the second carcinogen period. There was no difference in the rate or incidence of tumors with a one month rest but with an increase to 3 months, the incidence of tumors dropped 54 to 42%. Croton oil, a non-specific irritant, applied during the rest or critical period resulted in an increased incidence of tumors then.(59)

Several studies have been made of methylcholanthrene carcinogenesis in mouse skin. Page, using paraffin and frozen sections, (with about the same results) found an immediate increase in cell, nuclear and especially nucleolar sizes. These reached a maximum after about one week of treatment, then increased again with the advent of the carcinoma. The same results were obtained with cholanthrene as were obtained with methylcholanthrene but benzene applied in the same manner did not result in increased size of the nucleus or the nucleolus. Page concluded that one of the actions of these carcinogens seems to be a direct stimulating effect on nucleus and nucleolus.(60) Cowdry and Paletta found an increase in cell size with more of an increase in the volume of the cytoplasm than of the nucleus so that a lower nucleocytoplasmic ratio resulted. In 18 hours

there was an increase in cytoplasmic ribonucleic acid, maximum for 3-10 days, and then a decrease. The mitosis rate increased progressively, then fell, and then rose again just before the cancers appeared. The chromosomes were increased in size by the 2nd day. This included 13% of the metaphases by the 3rd day and from then on was somewhat lower. He also noted an increase in nucleolar displacability.(61) Biesele found diplochromosomes and other polytene chromosomes from the 2nd day on, but none were found in normal and benzene-treated mice. The frequency of diplochromosomes stayed at 8% through the first 2 months but later increased to more than 50% in the cancers. Mitotic frequency was increased at 2 days from a normal of 17 per 15,000 nuclei to 26.5 per 15,000 nuclei. At 9 days the mitotic count was 78 per 15,000 nuclei and at 20 days 149.7. There also were aberrations in the chromosome number by the 3rd day which "may have been more apparent than real." The nucleocytoplasmic ratio changed very little although there was an increase in volume of both the nucleus and the cytoplasm. There was a slight increase in displaceability of the nucleoli and basophilic chromatin under ultracentrifugal force. The cytoplasmic ribonucleic acid increased one-half the day after treatment, reaching a maximum at 3-10 days with an intermediate value at the 57th day. In one tumor it was found to be high again. Nuclei bearing multiples of the normal number of heterochromatic segments were seen from the 2nd day on and were predominant in cancers. Such nuclei -- which also contained increased numbers of nucleoli and were relatively large -- closely paralleled the metaphases containing enlarged chromosomes.(46)



Methylcholanthrene

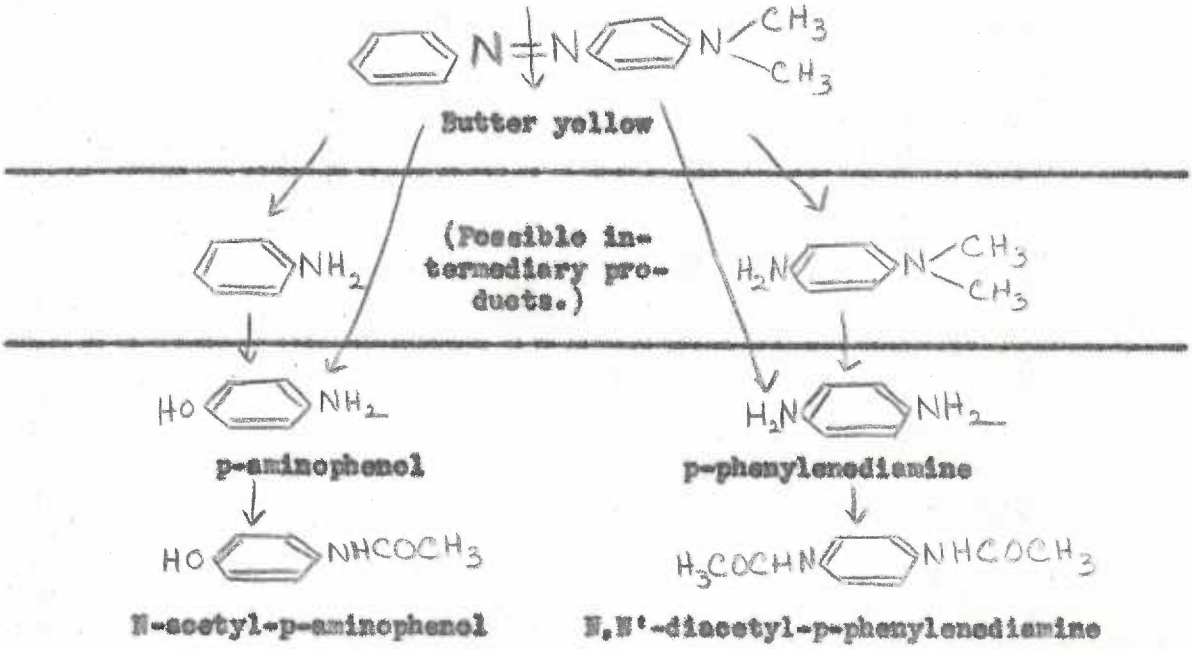
IV. m'MeDAB.

m'MeDAB
(m'-methyl-p-dimethylaminoazobenzene)

Butter Yellow
(p-dimethylaminoazobenzene)

m'MeDAB (see above structural formulas) is a derivative of the well-known carcinogen, butter yellow, which has been studied intensively for many years. m'MeDAB was first reported to be a carcinogen in 1945 when Miller and Baumann were studying the carcinogenic properties of a number of derivatives of butter yellow, which they had prepared. It was found to be more carcinogenic and more toxic than the parent drug.(62) Giese, Miller and Baumann, later in the same year, rechecked the carcinogenicity of m'MeDAB. The concluded that "m'MeDAB proved to be the most potent carcinogenic azo dye hiterto reported for the liver of the rat." On equivalent concentrations of dye, rats fed m'MeDAB invariably lost more weight, "developed a more severe cirrhosis," and formed large hepatic tumors more rapidly than butter yellow rats. When 0.048% (3/4 molar. Butter yellow is usually fed at 0.06% or 1 molar) was fed for 2½ months, the incidence of hepatic tumors 2 months later was 100%.(63) This has since been confirmed by many other workers. Cortell found that the minimum length of time the rat must be exposed was about 70 days, after which 80% to 93% developed tumors even though no more carcinogen was fed.(26) This has also been confirmed. The metabolism of butter yellow has been worked out to be somewhat as follows by Stevenson. The metabolites shown as follows were found in the urine and were unaffected by diet changes. (64) The blood level of butter yellow varies directly as the concentra-

tion of the drug in the diet. The metabolic rate of m'MeDAB is very probably similar.



There seems to be some controvery as to whether the intact dye, or its split products, or even its oxidation products are the true carcinogen. Miller and Baumann favor the intact molecule theory and point out that the evidence is more against than for the split product theory at the present time. Other factors which may affect carcinogenesis are factors which may alter the stability of the dye in the gastro-intestinal tract, affect its absorption rate, alter the oxidative or other means of dye destruction in the body or enter directly into the carcinogenic reaction itself, etc. (62) Although control diets containing butter yellow can be exposed at room temperature for at least 1-2 months without destruction of the drug or obvious rancidity, once diets containing corn oil become rancid, destruction of the dye is found. There was no destruction or rancidity with coconut oil or low fat diets but diets made rancid with linoleic acid will result in breakdown of the carcinogen, starting in a few days.(65)

The effects of diet and other metabolic problems relating to these carcinogens are being studied in many places at the present time -- such interesting phenomena as an increase in desoxyribonucleo-protein in the liver, decreased cathepsin-activating ability of butter yellow livers, inhibition of certain other liver enzymes and changes in liver homogenate coagulability have already been observed. The effect of diet on tumor formation has excited special interest. The rice-carrot diet has been used extensively in the study of these tumors because that was the diet employed by the original Japanese workers. However, it causes multiple deficiencies in the albino rat; they do not grow well and when the dye is added the food consumption is still lower with a resultant high percentage of complications and a high mortality. With semisynthetic diets however, the rats grow well and the tumor formation is similar.(66) The ordinary laboratory chow seems to retard carcinogenesis,(67) although Cortell found that the feeding of fox chow after the neoplastic focus, due to high carcinogenic semisynthetic diet, had arisen led to an earlier onset of tumor formation with significantly more metastases. Many factors have been reported which retard tumor formation due to butter yellow -- liver, yeast, casein plus riboflavin, protein plus B-vitamins, cystine plus choline, riboflavin, hydrogenated coconut oil, egg white, commercial synthetic detergents -- even though the protected animals ate more dye. The riboflavin level in the liver correlated well with the protective ability of the diet, according to Miller et al.(67) Also, rats receiving a low biotin diet were given subcutaneous biotin, which prevented deficiency symptoms but did not alter the protective ability of the diet. Some factors found to increase tumor incidence included pyridoxine, replacement of the crystalline B-complex by rice-bran extract, lowering riboflavin content to 1 mg. per kg., increasing corn oil to 20%, biotin and cystine. With m'MeDAB,

the inhibition of tumors due to dietary riboflavin and hydrogenated coconut oil was not nearly as marked as with butter yellow, even when the dye was fed at lower levels.(65) Giese et al suggest that "a very noticeable effect of diet should not be expected in experiments in which tumors are produced so rapidly."(65) Rice-bran concentrate, selenium, casein and casein plus methionine were found to retard m'MeDAB tumor formation. 29% nicotinamide, 20% corn oil and caloric restriction increased tumor formation. The effect of protein on weight and well being was much more marked than on tumor incidence. With m'MeDAB, dietary riboflavin led to a moderate increase in hepatic riboflavin compared to a marked increase with butter yellow. The m'MeDAB alone lowered hepatic riboflavine much more than butter yellow and other weaker azo dyes do.(68) (A similar riboflavin effect has been found with spontaneous mammary tumors and methylcholanthrene tumors.) A diet recommended by Miller et al as a control diet for medium tumor incidence is as follows:(65)

	Gm./kg.
Casein (vitamin low)	120
Glucose	790
Corn oil	50
Salts mixture	40
Butter yellow	0.6
Riboflavin	.001-.002
Thiamine hydrochloride	.003
Pyridoxine hydrochloride	.0025
Calcium pantothenate	.007
Choline chloride	.030
Halibut liver oil	1 drop/rat/month

According to P. N. Harris, who fed butter yellow to various strains of rats, there is no difference in tumor formation between the Evans, Sprague-Dawley and Harlan (from Wistar) strains. In Wistar strain rats, tumor formation was found to be somewhat retarded and in the Carworth Farms strain, tumor incidence was somewhat increased. None of the strain differences were marked however.(69)

The pathology of butter yellow varies little. Grossly Orr noted in rat liver at 2 months, occasional slight obscurring or exaggeration of the lobular pattern; at 11 weeks and more a variable coarseness not always evenly distrubed to all lobes, with yellow or pink nodules on a red-grey background; at 4 months, some greyish-white nodules -- not always carcinoma but accumulations of granulation tissue; later increasing size and incidence of tumors, usually multiple with variable color and consistency. Many cysts, usually multiple, were often seen. A few livers showed no granularity but a tough consistency and obscurred lobules on section.(70) Edwards, at 2 weeks, found the liver yellow, soft and glistening; at 100 days, the capsule was often pitted; at 150 days, a classical cirrhosis, usually with increased consistency, yellow or brown granules and nodules, wide flat-topped depressions and occasionally a shrunken white lobe with a corrugated capsule. On cut section the architecture was distorted with yellowish-brown nodules separated by strands of connective tissue. The shrunken areas were glistening, homogeneous and grey, often with multilocular cysts containing colorless or faintly yellow serous fluid. The various lobes were equally involved. The tumorous livers almost always were grossly cirrhotic. Early, small tumors were white, relatively firm nodules protruded from the liver surface. With extensive involvement, numerous localized nodules were scattered throughout the liver with increase in size and much irregularity in outline of the organ. The tumors varied from rubbery firm, white ones to softer, semi-fluctuant, purple to pearl-grey ones. Often one mass was definitely larger than the rest. Occasionally there was a large dependent mass with a relatively thin pedicle, rarely twisted with signs of hemorrhagic infarction. Microscopically, Sasaki and Yoshida thought that the tumors started

as hyperplasia of the portal parenchyma, continuously progressive until the carcinoma appears.(71) Others suggested that degenerative and regenerative changes played a role. Orr found that the usual sequence was proliferation of connective tissue with increased cellularity in the portal system; extension from the latter into the parenchyma with degeneration of contiguous liver cells; and atypical regenerative proliferation of bile duct and liver epithelium leading to non-architectural nodular hyperplasia (and microscopically hobnail liver) in which a certain percentage of tumors arose. In a few instances, those livers which grossly showed no granularity but a tough consistency, the predominant change was at the centers of the lobules, but no tumors occurred. Orr believed the primary effect is destructive and that the proliferative changes are regenerative in nature. He also noted that cancer could arise while the cirrhosis was still reversible.(70) Edwards, as early as 2 weeks, observed extensive fatty deposits demonstrable with osmic acid and Sudan IV. Usually cirrhosis was not microscopically observed before 100 days with bands of connective tissue in irregular fashion leading to considerable distortion of the architecture. Narrow bands were composed of fibroblasts and collagen plus occasional narrow capillaries. Wide bands contained pigmented macrophages, numerous proliferating bile ducts and many small blood vessels. Pigment was found early in the Kupffer cells, later in the macrophages mostly, and none in bile duct epithelium. Two types of pigment were observed: brown, granular, positive to Prussian blue -- probably on the basis of hemolysis due to butter yellow administration; and globular, pale canary yellow, acid-fast, positive to osmic acid. At 60 days bile duct proliferation was apparent, not infrequently associated with cysts lined by a single layer of flat or cuboidal epithelium and sur-

rounded by a small amount of connective tissue. Edwards says these cysts are not part of a tumor -- the cells are uniform and similar to lesions that occur with non-carcinogenic hepatotoxins -- though Orr refers to these lesions as "cystadenoma." At first this bile duct proliferation was confined to portal areas but later extended into the lobule. The overgrowth at times replaced extensive areas of parenchyma with resulting white, flat-topped depressions microscopically. The connective tissue supporting the ducts gradually increased until the ducts appeared as scattered islands in a dense collagen matrix and the epithelium of the ducts atrophied and degenerated, being replaced by shadow cells. With or without fully developed tumors, there were "numerous suggestive areas," increase in size and loss of vacuolization of the parenchymal cells of part or all of a lobule, with associated increase in size of nuclei, increased prominence of nucleoli and decreased glycogen. Edwards claims that the non-neoplastic nature of the less extensive areas of bile duct proliferation was obvious and that the evidence against neoplastic nature of the more extensive areas included: that the cells closely resembled each other; there was no stratification; the nuclear-cytoplasmic ratio was unchanged; mitoses were rare and not atypical; they were not transplantable -- in two instances; there was no local invasion of blood vessels and there were no metastases. Other authors have interpreted this bile duct proliferation as adenocarcinoma.(72) Orr classified the tumors as liver cell carcinoma, bile duct carcinoma or bile duct cystadenocarcinoma. Edwards classified them as hepatoma, types I or II, or adenocarcinoma. Hepatomas were characterized by epithelial cells in cords alternating with endothelial-lined sinuses. Type I was well-differentiated and occasionally encapsulated. The cells were large with abundant acidophilic cytoplasm and well-defined

cell margins. The nuclei were large and vesicular with single large, prominent nucleoli. These were arranged in cords alternating with sinuses, often separated by delicate reticulum. They resembled hepatic tissue closely and occasionally were difficult to differentiate from regenerating parenchyma in the cirrhotic process, but they were characterized by papillary structures, cysts lined by liver-like cells, wide cords or sheets of cells and invasion of blood vessels. No extrahepatic metastases were observed and not infrequently acinar structures closely resembling hepatic parenchymal cells with transitions to the cord arrangement were noted. These structures were also noted in hyperplastic atypical nocules, apparently early neoplasm. For these latter reasons, Edwards thought that some of his hepatomas, Type I, might be benign. Type II, which was more common, had cords of cells with poorly outlined cell margins, in many areas suggestive of a syncytium. The cytoplasm was faintly stained, usually basophilic and the nuclei were large and vesicular with large prominent nucleoli. The structure was strikingly like liver with cords, often covered with delicate reticulum, alternating with sinuses. Sinuses varied from wide, almost cavernous, to almost collapsed, giving a solid character to the tumors. This tumor also contained acinar structures, cysts and transitional forms with some cells resembling hepatoma, Type I. The adenocarcinomas were characterized by cuboidal or columnar epithelium in acini surrounded by connective tissue. Almost every one classified as adenocarcinoma was associated with hepatoma, but not with alternating epithelial cells and sinuses. Adenocarcinomas were differentiated from bile duct proliferation by their cellular stratification, numerous and atypical mitoses, irregularity in size and shape of cells, increased nuclear-cytoplasmic ratio, solid sheets of cells present and papillary structures, invasion

of blood vessels, metastases and transplantability. They are differentiated from the acinar structures in hepatoma by their characteristic relationship to connective tissue and the loss of blood vessels. The stroma in hepatomas is scanty and reticular; in adenocarcinoma much more abundant with numerous fibroblasts and variable collagen. Two cases contained membranous bone and one hylaine cartilage in addition. The tumors were usually devoid of demonstrable fat. Small amounts were noted in areas of necrosis, usually in macrophages and occasionally in nearby tumor cells. In addition, some peculiar, usually acidophilic inclusion bodies were noted in some of the tumor cell's cytoplasm. Although it is generally agreed that the hepatomas arise from the parenchymal cells, there is disagreement concerning the cell of origin of the adenocarcinomas. Edwards believes that the adenocarcinoma is so non-specific that it is difficult to indicate the cell of origin on cytologic bases alone, contrary to Orr who thought the adenocarcinoma arose from bile duct cells on this basis. The spleen was found to be at first grossly enlarged and firm, later even decreased in size with a pitted surface and light adhesions. Microscopically, the pulp was engorged and there were extensive deposits of iron-containing pigment. According to Edwards, there was no fibrosis in the spleen although other workers say that there was. Lymphosarcoma has been produced in the spleen with implantation of butter yellow pellets in that organ. (73) The kidneys grossly had a dark brown cortex at two weeks, contrasting sharply with the medulla. The lining cells of the convoluted tubules and occasional stromal macrophages of the cortex contained a granular brown pigment. The mediastinal and regional nodes have been found to contain metastatic tumor in some instances and iron-containing and canary yellow pigment. They were prominent and greyish-brown grossly. In the peritoneum hemorrhagic ascites was often found; the omentum was often adherent to the

tumor and occasionally invaded; and sometimes the peritoneum was studded with pink or purple implants, mostly on the mesentery and along the hilum of the spleen. Often there was direct extension of tumor into the spleen and occasionally the diaphragm was invaded as well as the portal vein. Occasionally, tumor was found in the lungs as numerous purplish-grey subpleural nodules. In some instances, multiple papillomas of the forestmmach have been found, which consisted of a papillary overgrowth of squamous epithelium over a plug of connective tissue.

The pathology of m'MeDAB, in 200 rats, was studied by Richardson and Wachtnebel.(74) Cunningham et al also studied m'MeDAB changes found in rat livers up to 12 weeks, to a lesser extent.(75) Gross changes were first apparent by 6 to 9 weeks, when the liver would be enlarged, brownish-yellow, with multiple cysts and nodules of proliferating ducts. By 12 weeks, the gross appearance of the liver was that of cirrhosis. Tumors, which appeared grossly from 19 to 29 weeks, were the same as those seen in the butter yellow rats. No sex difference was noted. Microscopically, proliferation of bile ducts and parenchymal cells in interlobular areas was the first change seen, at about 3 weeks. By 6 to 9 weeks, a nodular cirrhosis was evident with formation of multiple bile duct cysts, often chronically inflammed. Adenomatous hyperplasia, fatty infiltration and various stages of necrosis were present with the cirrhosis. Focal clusters of hyperchromatic giant cells were first seen at 9 weeks and were numerous at 12 weeks. Transition of these cells into tumors was demonstrated. Malignant neoplasms of liver cell origin were hepatoma, adenocarcinoma and anaplastic carcinoma. The malignant bile duct carcinomas were adenocarcinoma and papillary cystadenocarcinoma. Malignant neoplasms of stromal origin were fibrosarcoma and angiosarcoma. One animal developed a benign

biliary adenoma, which were common, and no malignancies. The biliary adenomas consisted of proliferated ducts lightly invested with connective tissue. In the simple biliary adenocarcinomas, there were commonly mitoses, alteration in cell size and shape and much collagenous tissue stroma. The papillary biliary cystadenocarcinomas, differed from the simple type by its papillary arrangement and often excessive mucous production. Large and small cell hepatomas were seen. The large cell type was well differentiated and closely resembled normal liver but the small cell type was less differentiated with a more hyperchromatic, less vesicular nucleus and scanty, basophilic cytoplasm. Large and small cell varieties of the adenocarcinoma were also found, sometimes mixed with the hepatomas. These were composed of cuboidal and columnar cells in acini or glandular form with an abundant stroma. The anaplastic carcinomas were composed of small cells with large, dense nuclei and many mitotic figures. These appeared to radiate from a central blood vessel. The fibrosarcomas were composed of compact groups of elongated spindle-like cells with large nuclei with many mitoses. The angiosarcomas were composed of anaplastic cells with common mitoses forming irregular channels.

In some cases, true bone, cartilage and calcification was present. All the types of tumors metastasized, commonly to lung, peritoneal cavity and lymph nodes. Other changes observed were hyperplasia of bone marrow in all and myeloid metaplasia in the liver, spleen and lymph nodes.

Primary carcinoma of the liver in the human is rare in the United States, but rather frequent in the Japanese, Chinese, Malayan races and South Africans. Cirrhosis, parasitism or long-standing intrahepatic ductal disease are often associated with it and have been indicated as the cause, or at least a predisposition. There are two main types, the

cholangioma and the hepatoma. The hepatoma is apparently derived from parenchymal cells with the cells arranged in cords and a capillary stroma. It frequently produces bile. The cholangioma is thought to be derived from bile duct cells with the cells arranged in ducts and a fibrous stroma.(76) In addition there is a cholangiohepatoma and benign tumors of both types with transitional forms, which may be difficult to differentiate.(38)

PART I.

MATERIALS AND METHODS IN GENERAL

I. MATERIALS AND METHODS IN GENERAL

A. The Rats.

All experiments in this series were conducted with the ordinary white albino laboratory rat, Sprague-Dawley strain, purchased from the Biochemistry Department's rat colony at the University of Oregon Medical School. In the colony, they had all been given a diet of laboratory chow and water ad libitum. The majority of the rats were adult male, about two and one-half months old, weighing 220-250 Gm. at the start of the experiments. All exceptions to this will be specifically noted. Each rat was kept in an individual cage tagged with the rat number; a different consecutive number being assigned to each rat. Each rat was weighed weekly, except for a small number of preliminary animals and the weight was recorded along with any unusual features such as hair falling out, lung disease, etc. Straight line graphs were made of the weekly weights for each rat. The experimental animals were subject to the same spontaneous and endemic diseases as the animals in the stock rat colony, although it would have been more desirable to have rats free from such diseases. Among these incidental findings were such conditions as tapeworm cysts in the liver, which were apparently primary in cats, fungus infections of the ears, labyrinthine disease, occasional spontaneous mammary tumors, acute pneumonia and a very common chronic pneumonia. Most of the spontaneous deaths of animals without large tumors were due to either acute pneumonia or much more commonly, chronic pneumonia.

B. Diet. (m'Methyl-p-dimethylaminoazobenzene)

All rats were given water and food ad libitum. Each rat consumed on the average, 15 Gm. of food a day -- although the animals receiving the carcinogen in their diet ate slightly less than the control animals. The control animals were placed on a basal semisynthetic diet as follows:

Casein	3600 Gm.
Glucose monohydrate (Cerelose)	14600 Gm.
Corn oil (Mazola)	1000 Gm.
Wesson salt mixture[1]	800 Gm.
Thiamine hydrochloride	60 mg.
Riboflavin	40 mg.
Calcium pantothenate	140 mg.
Pyridoxine hydrochloride	50 mg.
Choline	10 Gm.

This diet is similar to the one recommended by Miller et al(65) as a control diet for medium tumor incidence, so that it is hoped strong dietary influences on carcinogenesis can be eliminated as nearly as possible. It was made up in small quantities, which did not last more than two weeks. Therefore, although kept at room temperature, it did not have a chance to become rancid and thus possibly cause destruction of the carcinogen.(65) With this diet alone, the laboratory rat can be maintained in good health with good weight gain and without vitamin or other deficiencies almost indefinitely. For induction of tumors, rats were placed on the same basal semisynthetic diet containing 0.06%, or one molar, m'Methyl-p-dimethylaminoazobenzene. The m'Methyl-p-dimethylaminoazobenzene used, which will be hereinafter designated m'MeDAB, was obtained from the Biochemistry De-

partment of Stanford University, where it was synthesized. A one molar concentration is employed here because that is the concentration which has been used almost uniformly by other investigators and therefore, the rate and pathology of carcinogenesis should be very similar to that obtained by them. In some of the first experiments on technique, rats directly from the stock colony, which had been fed the ordinary laboratory chow, were used. This will be specifically noted in every case where it occurs.
1. Later, the Wesson salt mixture was exchanged for Salt Mixture No. 2, U.S.P.

C. Autopsies and Tissue Preparation. Perfusions.

Most of the animals were sacrificed by ether anesthesia, usually followed by decapitation with subsequent exsanguination. In a few cases, specifically noted in every instance, the animals died spontaneously a very short time before autopsy was performed. The thoroughness of autopsy varied a great deal proportional to the materials and technical help available at that particular stage of experimentation. In all cases, gross pathology was recorded with special reference to the liver, spleen and lungs. In all cases, sections of liver and usually spleen, were dropped in various fixatives at autopsy and then given to technicians for preparation of paraffin sections, after the necessary preliminary washing et al required for each particular fixative. In addition, stomach and bone marrow are often saved and in many cases, sections of nearly all the organs were obtained. The fixatives used were specifically recorded for each animal. In some of the animals, the liver was perfused at autopsy with various solutions, specifically noted in each case, as follows:

> After laparotomy, the portal vein and right leaf of the diaphragm were severed and a perfusion needle was introduced into the hepatic vein via the superior vena cava.

A hemostat was then placed on the superior vena cava, proximal to the needle, to prevent refluxing of perfusion fluids into the heart. The perfusion needle was an ordinary #20 bevelled syringe needle bent at about a 120° angle. The needle was connected, by way of a piece of rubber tubing fitted with a pinch clamp, to an ordinary 15cc. syringe, without plunger. The syringe containing the perfusing solution was mounted on an ordinary ring stand about one and one-half feet above the level of the liver preparation. Thus, the perfusing solutions would flow into the hepatic vein, through the liver and out of the portal vein. The rate of flow was found to be about 1/10cc. per second and the amount of flow could be measured in the syringe and controlled by the aforementioned pinch clamp on the rubber tubing. Once in awhile some difficulty was occasioned by clots getting into the needle. Also it was discovered that the rapidity with which the liver changed in color and emptied of blood and the areas in which it did so varied with the position of the needle in the hepatic vein (actually a large sinus in the rat). However, on the whole, this method worked very well.

All slides were prepared by the usual paraffin methods by experienced technicians. In all cases hemotoxylin and eosin was used as the stain; in some cases Giemsa, connective tissue or fat stains were experimentally tried in addition. (The Giemsa and H & E stains were made by experienced technicians; the connective tissue and fat stains by myself.)

All slides were studied in the usual manner with the binocular microscope.

D. Smear Preparations and Their Pathologic Interpretation.

Smear preparations of the liver and occasionally other organs were also made in every case, except for some of the early technique experiments. This was done as soon as possible after death by rubbing a slice of liver about 2 mm. thick between two slides until well smeared, or by smearing a liver slice 2 mm. thick on a slide with the aid of the flat of a scalpel. The thickness of the smears was controlled only by judgement and experience, an attempt being made to keep the smears as uniform as possible compared with each other and such as was desirable for microscopic study. The slides thus prepared were each immediately dropped into a fixative before they had a chance to dry in the air. It was discovered that only a very short fixation time was necessary for the smears, i.e. 10-20 minutes -- after which they were transferred to 70% alcohol and given to the technicians for staining with Ehrlich's hematoxylin, eosin and Orange-G. The resultant liver preparation, under the microscope, demonstrates mainly individual nuclei. There are very thick areas where the nuclei are indistinguishable from each other and many more thin areas where the hepatic and bile duct nuclei are diffusely scattered or slightly clumped and do not touch each other. In the background are found amorphous debris, blood cells, fibrin strands and occasional connective tissue cells and/or their nuclei. A very few of the scattered nuclei retain some cytoplasm, poorly preserved or not, but the thick areas exhibit much preserved cytoplasm although usually no detail can be seen because of the thickness. Occasional bile duct tubular structures can be seen. For all practical purposes, mitoses are not to be found in the smear preparation.

although chromatin and nuclear detail is particularly well preserved. The smear preparations had to be observed under the oil immersion lens of the binocular microscope for best detail, although the high dry lens could be utilized to discover pertinent areas for study.

E. Measurement Methods.

All measurements were made on nuclei of the smear preparations under oil immersion. The Bausch and Lomb micrometer attachment, individually calibrated for the microscope, was the measuring device employed. Two-hundred nuclei were measured in each rat in which measurements were taken, but the two-hundred nuclei measured were not usually all taken from the same slide, depending upon the number of slides available. No significant statistical difference, as determined by the Chi-square test, was found between different slides of the nuclei of the same rat with the method here utilized. At first, attempts were made to measure vertical diameters with the vertical focusing device of the microscope, as Biesele did, (10) but this was found to be extremely unreliable for us and was discarded early. The method finally evolved[1] utilized measurements of one diameter only of all nuclei under random fields spaced as far apart as feasible. The diameters measured were also random, depending upon which side of the nucleus the moving vertical crosshair of the micrometer happened to contact. The following calculations were made for each nucleus with the aid of the ordinary type of slide rule. (These include calculations based on measurements of nucleoli, which were also taken at first).

Assuming that D = one diameter of the nucleus and

that d = one diameter of a nucleolus:

---

1. In Part IV, under "Nuclear Measurements" is presented the evidence which led to the use of this particular method.

$.785\ D^2$ = Area of the nucleus.

$.5236\ D^3$ = Volume of the nucleus.

$.785\ d^2$ = Area of the nucleolus.

$.5236\ d^3$ = Volume of the nucleolus.

$.785\ d^2$ = Sum of nucleolar areas of the nucleus.

$.5236\ d^3$ = Sum of nucleolar volumes of the nucleus.

$\frac{.785\ D^2}{.785\ d^2}$ = $\frac{\text{Nuclear area}}{\text{Sum of its nucleolar areas}}$

$\frac{.5236\ D^3}{.5236\ d^3}$ = $\frac{\text{Nuclear volumes}}{\text{Sum of its nucleolar volumes}}$

From this data various straight line graphs were made and compared statistically by means of the Chi-square test at 0.05 level of significance:

$$\text{Chi-square} = \sum \frac{(A - T)}{T} = < 11$$

As can readily be appreciated, this method is very time-consuming so that not as many measurements were made as originally were planned.

PART II.

LIVER PERFUSION EXPERIMENTS

TABLE I.

THE LIVER PERFUSIONS

Diet.	Rat No. and Sex.	Physiological Soln. Perfusion.	Fixative Perfusion.
m'MeDAB.			
3 wks.	♂ 10 M.	R. saline.[1]	R. Regaud.
3 wks.	♂ 12 M.	R. saline.	None.[2]
3 wks.	♂ 13 M.	R. saline.	None.[2]
3 wks.	♂ 17 M.	R. saline.	R. Bouin.
3 wks.	♂ 18 M.	R. saline.	R. Regaud.
6 wks.	♂ 20 M.	R. saline.	R. Regaud.
2 wks.	♂ 27 M.	W. Ringer.[3]	W. 10% formalin.
2 wks.	♂ 28 M.	W. Ringer.	W. Bouin.
11 wks.	♂ 37 M.	W. Ringer.	W. Bouin.
11⅓ wks.	♂ 39 M.	W. Ringer.	W. 95% alcohol.
9 wks.	♂ 40 M.	W. Ringer.	W. 10% formalin.
7.3 wks.	♂ 62 M.	R. saline.	R. Bouin.
8.7 wks.	♂ 64 M.	R. saline.	R. VandeGrift.
8 wks.	♂ 65 M.	R. saline.	R. VandeGrift.
9 wks.	♂ 66 M.	R. saline.	R. VandeGrift.
9 wks.	♂ 67 M.	R. saline.	R. VandeGrift.
9 wks.	♂ 69 M.	R. saline.	R. VandeGrift.
1 wk.	♂ 70 M.	R. saline.	R. VandeGrift.
6 wks.	♂ 71 M.	R. saline.	R. VandeGrift.
Basic Ration.			
3 wks.	♂ 11 M.	R. saline.	R. Regaud.
3 wks.	♂ 15 M.	R. saline.	None.[2]
6 wks.	♂ 16 M.	R. saline.	R. Bouin.
6 wks.	♂ 19 M.	R. saline.	R. Regaud.
2 wks.	♂ 26 M.	W. Ringer.	W. 80% alcohol.
9 wks.	♂ 36 M.	W. Ringer.	W. Bouin.
11 wks.	♂ 38 M.	W. Ringer.	W. 95% alcohol.
7.3 wks.	♂ 61 M.	R. saline.	R. Regaud.
8.7 wks.	♂ 63 M.	R. saline.	R. VandeGrift.
8 wks.	♂ 68 M.	R. saline.	R. VandeGrift.
Laboratory Chow.			
	♂ 21 M.	W. Ringer.	W. 80% alcohol.
	♂ 22 M.	W. Ringer.	W. 95% alcohol.
	♂ 23 M.	W. Ringer.	W. 10% formalin.
	♂ 24 M.	W. Ringer.	W. Bguin
	♂ 25 M.	W. Ringer.	W. Regaud.

TABLE I.
(continued)

Diet.	Rat No. and Sex.	Physiological Soln. Perfusion.	Fixative Perfusion.
Laboratory Chow. (continued)			
	# 30 M.[4]	R. Ringer.	R. 80% alcohol.
	# 31 M.[4]	R. Ringer.	R. Bouin.
	# 32 M.[4]	R. Ringer.	R. 10% formalin.
	# 33 M.[4]	R. Ringer.	R. 95% alcohol.
	# 34 M.[4]	W. Ringer.	W. Regaud.
	# 41 F.	W. Ringer.	W. K_2CrO_4.
	# 42 F.	W. Ringer.	W. $KMnO_4$.
	# 43 F.	W. Ringer.	W. $ZnCl_2$.
	# 44 F.	W. Ringer.	W. $FeCl_3$.
	# 45 F.	R. Ringer.	R. $HgCl_2$.
	# 46 F.	R. Ringer.	R. Phosphotungstic acid.
	# 47 F.	R. Ringer.	R. Helly.
	# 48 F.	R. Ringer.	R. Oxalic acid.
	# 49 M.	R. Ringer.	R. Copper acetate.
	# 50 M.	W. Ringer.	W. Uranium nitrate.
	# 51 M.	W. Ringer.	W. Barium nitrate.
	# 52 M.	W. Ringer.	W. Cobaltous acetate.
	# 53 F.	W. Ringer.	W. Lead acetate.
	# 54 M.	W. Ringer.	W. $AgNO_3$.
	# 55 M.	W. Ringer.	W. Ammonium vanadate.
	# 56 M.	W. Ringer.	W. Molybdic acid.
	# 57 F.	W. Ringer.	W. Cadmium nitrate
	# 58 M.	W. Ringer.	W. $FeSO_4$.
	# 59 M.	W. Ringer.	W. Lanthanum acetate.

1. R means room temperature.

2. After perfusion with the physiological solutions, pieces of these livers were immediately fixed in 80% alcohol, 95% alcohol and 10% formalin. No fixative perfusion was done.

3. W means warm --- 37°-C.

4. These animals had been injected with various carcinogens months before with negative results. They were old, feeble and had extensive chronic lung disease.

II. LIVER PERFUSION EXPERIMENTS

These experiments were performed early in the study of m'MeDAB, while casting about for techniques which would best demonstrate its pathology. They are not intended to be a complete study of all the aspects of the perfusion technique. Ultimately, the smear preparation with its find delineation of nuclear detail was selected for this study, but earlier the paraffin sections of perfused livers were found to have advantages over paraffin sections of unperfused livers. Therefore, a study was made of various aspects of the perfusion technique.[1]

A. Comparison of Perfused and Nonperfused Livers:

1. Materials and Methods:

The majority of the rats in this comparison were male, 220-250 Gm. rats of the Sprague-Dawley strain. However, a few female rats and a few smaller, although full-grown rats were utilized. Three of the nperfused rats, (#7, 8 and 9) had been injected with Colchicine 9-10 hours before sacrifice. Colchicine stops mitosis in the metaphase but should not affect this comparison. Altogether, the livers of 58 different rats were perfused under many various conditions, (as mentioned below). (See Table I.) Twenty-nine of these had been maintained on laboratory chow; ten on basic ration and nineteen on basic ration containing 0.06% m'MeDAB. To compare with them there were more than an equal number of similar unperfused rat livers. These perfused and nonperfused livers can be compared not only in presumably normal rats, but at the different stages of

1. The liver perfusion technique used is described in Part I. under "Autopsies and Tissue Preparation. Perfusions."

liver change due to m'MeDAB. The observations noted exclude all those found due to variations in technique.

All animals were sacrificed immediately before the perfusions were carried out. Perfusion with the physiological solutions was continued until the liver was apparently bloodless (about 15cc.), and then perfusion was continued with the fixing solutions until the liver was seen to harden and take on the color of the fixative (about 15cc.). Various physiological and fixing solutions at varying temperatures were utilized -- the special effects of which are reported later in this series of perfusion experiments. The conditions of each perfusion are tabulated in Table I. After perfusion, small pieces of liver about 4 mm. thick and 2 cm. long were removed from the tips of the lobes and dropped directly into a fixative -- usually the same one the liver was perfused with.

2. Observations.

First it must be noted that there is wide variation in different rats with the same procedure, different pieces of liver from the same rat or different parts of the same slide. The following is the general overall picture which is seen.

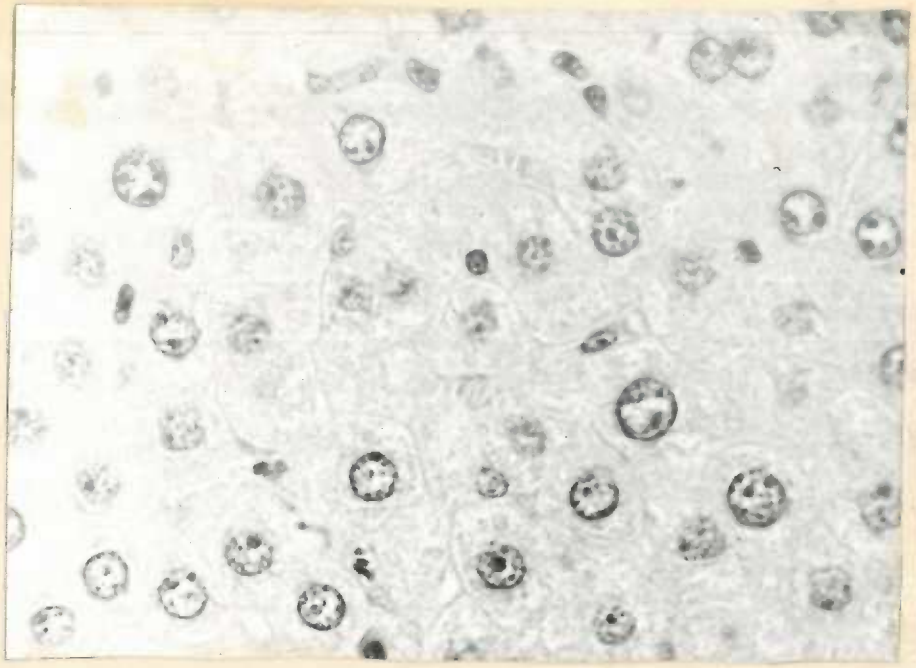
The principle outstanding difference between the perfused and nonperfused material is that in the perfused material the sinusoids are preserved and washed clear of blood cells and debris so that the overall architecture is more clearly delineated. In the unperfused material the cells seem swollen, the sinusoids and capillaries are obliterated or full of blood cells and debris and chromatin tends to be either washed out or precipitated in larger granules. In the perfused material the sinusoids are large and clearly demarcated with epithelial nuclei projecting into the lumens here and there. These sinusoids are often joined by capillar-

ies extending between the cells. Hepatic cords between the sinusoids are never more than two cells thick in normal livers and there tends to be a more delicate chromatin structure. Figure 1. demonstrates most of these differences beautifully. Questionably, the nuclei are more distinct in portal areas, compared to central areas of the lobule, in perfused livers.

Among the animals on m'MeDAB, the difference between perfused and nonperfused livers varies inversely with the number of weeks the drug has been fed and thus, the extent to which the architecture has been destroyed. Also, individual cells are more difficult to differentiate in m'MeDAB livers due to cellular proliferation alone.

3. Discussion and Summary:

The perfused livers decidedly have advantages over the unperfused preparations as far as demonstration of general architectural arrangement and uniformity of fixation goes. Moreover, there seems to be a tendency to preserve the delicate chromatin structure of the nuclei in the perfused material. The technique is simple and requires no complicated apparati. However, its advantages decrease with distortion of the architectural pattern and since m'MeDAB causes distortion of the architectural pattern of the liver, the advantages of this method become somewhat limited with increasing duration of time over which the drug is fed.



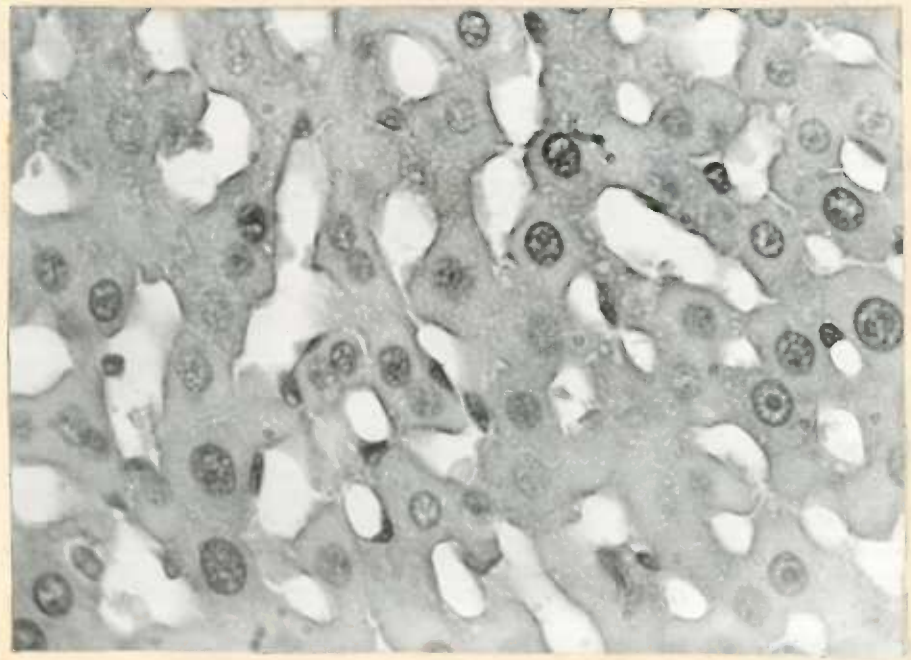


Figure 1. Comparison of a Perfused and an Unperfused Liver:

Rat # 9. Unperfused Liver, Bouin Fixation. Two weeks Basic Ration. H and E, 440x. Paraffin section.

Sinusoids and capillaries are almost obliterated, being very narrow and hard to see. The cells are swollen. The nuclear chromatin is clumped, centrally and peripherally. (With this high power it is difficult to see the blood and debris in the sinusoids). Nucleoli are of a solid color. Cell boundaries are fairly well distinguished.

Rat # 11. Regaud perfusion. Two weeks Basic Ration. H and E, 440x. Paraffin section.

The sinusoids are large and clearly demarcated with epithelial nuclei projecting into the lumens here and there. These sinusoids are often joined by capillaries extending between the cells. Dark particles of chromatin are seen at the periphery of the nucleus and there is a chromatin network centrally. Nucleoli consist of a central light area with a dark rim. Cell boundaries are not well distinguished except where capillaries demarcate them. (This difference in cell boundary delineation is largely due to the difference in fixatives).

B. Saline versus Ringer's Solution:

1. Materials and Methods:

The rats and methods utilized are the same as previously described. The purpose of this series of observations is to determine whether Ringer's solution or normal saline is preferable to perfuse the rat livers with before the perfusion of the fixing solution. Altogether, 21 rat livers were perfused with saline and 37 were perfused with Ringer's solution. (See Table I.) After perfusion with these physiological solutions, the livers were perfused with various fixing solutions, etc.

2. Observations:

After differences between sections in quality of staining, extent of dehydration, etc. were eliminated, no appreciable difference was found between Ringer's and saline perfusion.

3. Discussion and Summary:

It was thought that perhaps Ringer's solution would be more physiological than saline and lessen any artefacts that might arise during the perfusion. Such is apparently not the case or the difference is too minimal to be noticeable under these conditions.

C. Influence of Temperature:

1. Materials and Methods:

In an attempt to discover whether the temperature of the perfusing fluids might not have an effect on the quality of the liver sections obtained, a comparison was made between the results obtained with perfusion fluids kept at room temperature and perfusion fluids kept at 37° C., or approximately body temperature. It was felt that the latter temperature might be more physiologic than room temperature. Altogether, 26 rats were perfused with room temperature fluids and 32 were perfused with fluids heated to 37° C. More particularly in this regard, rats # 21-25 were all perfused with Ringer's solution heated to 37° C. Rats # 30-34 were all perfused with Ringer's solution at room temperature and then each with the same fixative as in rats # 21-25. The rats and methods used were the same as previously described.

2. Observations:

In comparing the series # 21-25 with series # 30-34, it seemed at first that the warm perfusions were slightly superior. The cytoplasm in the portal areas of the room temperature perfusion slides seemed denser, pinker and more granular. The sinusoids seemed somewhat more obliterated in the room temperature perfusion material. However, after restaining and comparing with other perfusions, very little if any difference could be seen between the room temperature and 37° C. material.

3. Discussion and Summary:

Again it seems that what would seem to be more physiologic and

beneficial to the end result is not, or the differences are too slight to observe. Perhaps the whole procedure has already altered the normal physiology, even immediately after death, so much that a temperature change no longer makes any difference; or, the reverse may be true -- the perfusion and immediate fixation, etc. take place before a factor like temperature change has a chance to take much effect. Probably this is more likely.

D. Comparison of Fixatives:

1. Materials and Methods:

Exclusive of the heavy metal perfusions, which will be described later, six fixatives were used for all the liver perfusions. These were 80% ethyl alcohol, 95% ethyl alcohol, 10% formalin, Bouin's, Regaud's and VandeGrift's.(77) Three rat's livers were perfused with 80% alcohol; four with 95% alcohol; four with 10% formalin; eight with Bouin's fixative, eight with Regaud's fixative and nine with VandeGrift's fixative. (See Table I.)

The H and E stained paraffin sections obtained from these rat livers were studied microscopically and are compared here for the effects of the different fixatives. Particularly studied in this respect were the rats which had not been on the carcinogen, especially # 21-25 and #30-34. (See Table I.)

2. Observations: (See Figure 2.)

80% ethyl alcohol. The general architecture is well preserved. Hepatic nuclei are very well defined with sharp nuclear membranes, one or more prominent, eccentric nucleoli and a finely distributed granular chromatin. The hepatic cell cytoplasm is coarsely granular. The cell boundaries are fairly distinct. Sinusoid endothelium is fairly well preserved as are the von Kupffer cells. Bile ducts and vessels are well delineated.

95% ethyl alcohol. The general architecture is clearly discernible. Hepatic nuclei are very well defined with sharp nuclear membranes, prominent central nucleoli and a granular, even chromatin network. The

hepatic cell cytoplasm is coarsely granular with clumps of granules. Near vessels, the cytoplasm is more densely acidophilic with fewer granules. Cell boundaries are fairly well preserved. Sinusoid endothelium is well preserved and von Kupffer cells are prominent. Bile ducts and vessels are well delineated.

10% formalin. In many places the sinusoids are obliterated and the general architecture is none too clear. Hepatic nuclei are very distinct with distinct nuclear membranes, but appear small with a halo formation around them. The chromatin is granular and diffuse but somewhat dark. Central nucleoli are marked. Hepatic cytoplasm is coarsely granular and clumped. Cell boundaries are fairly well defined. Sinusoid endothelium is very well preserved and von Kupffer cells are prominent. Bile ducts and vessels are well delineated.

Bouin's fixative. General architecture is easily discernible and sinusoids are well preserved. Hepatic nuclei are fairly distinct with prominent nuclear membranes, distinct nucleoli and a granular, evenly disbursed chromatin. The hepatic cytoplasm is coarsely granular with clumping. Cell boundaries are fairly well defined. Sinusoid endothelium is fairly well preserved with distinct von Kupffer cells. Bile ducts and vessels are well delineated.

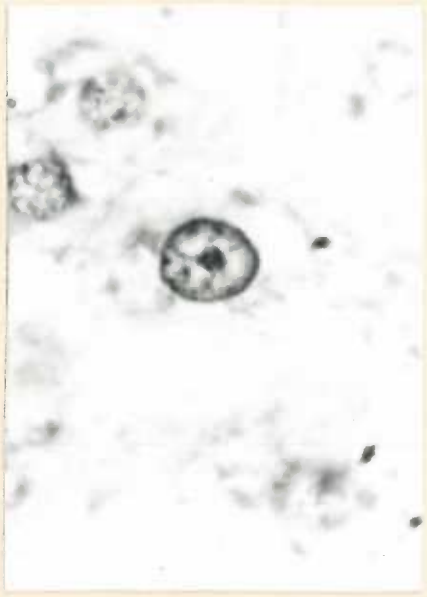
Regaud's fixative. Architecture is very well preserved and sinusoids are very wide open. Nuclei are fairly distinct with granular, diffuse chromatin, prominent nuclear membranes and distinct nucleoli. Hepatic cell cytoplasm is coarsely granular with a diffuse, pink background containing some vacuoles. Cell boundaries are not well defined except where capillaries cross between cells. Sinusoid endothelium is fairly well preserved and von Kupffer cells are distinct. Bile ducts and vessels are well delineated.

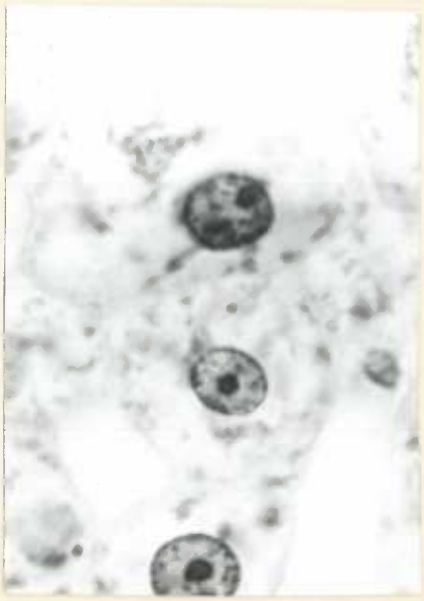
Vande&rift's fixative. General architecture is well preserved. Hepatic nuclei are distinct with prominent nuclear membranes, prominent nucleoli or central chromatin condensations and a granular, diffuse chromatin. Cytoplasm is granular and clumped. Cell boundaries are well defined. Sinusoid endothelium is well preserved, as are the von Kupffer cells. Bile ducts and vessels are well delineated.

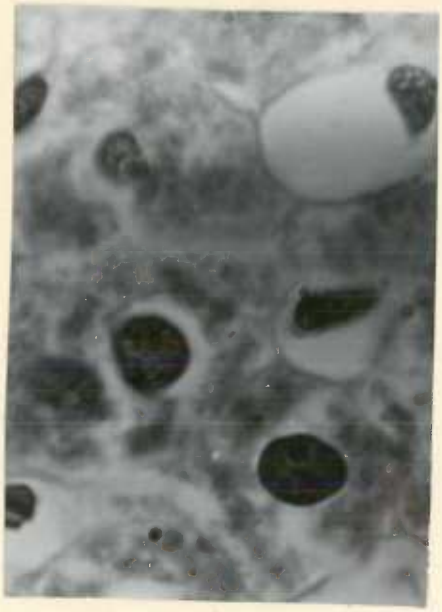
3. Discussion and Summary:

There is little apparent difference between the 80% and 95% alcohol fixation, except for the greater incidence of peculiar staining cytoplasm of cells near vessels with the 95% alcohol. The alcohols give excellent fixation with well controlled histocytologic configuration for this particular material. 10% formalin causes the shrinkage of nuclei with halo formation and the nuclear structure is darker and not as distinct as with the alcohols. Its only advantage over them seems to be the very good delineation of sinusoid endothelium. However, Vande&rift's fixative has this property without the poorer preservation of nuclei. Bouin's fixative is good but does not preserve nuclear structure quite as well as the alcohols or 10% formalin. Regaud's fixative lacks the fixation of cytoplasmic granules and boundaries that the other fixatives demonstrate. Although nuclear structure is more distinct than with Bouin's, it is less so than with the alcohols or 10% formalin. Rating the fixatives, with special reference to nuclear detail, in order:

1. 80% alcohol.
2. 95% alcohol.
3. Vande&rift's fixative.
4. Bouin's fixative.
5. 10% formalin.
6. Regaud's fixative.







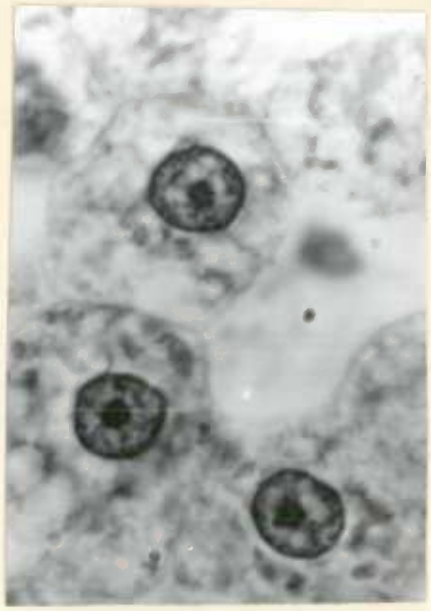


Figure 2. Comparison of Various Fixatives Used in Liver Perfusions.

Rat # 21
80% ethyl alcohol
(left)

Rat # 22
95% ethyl alcohol
(right)

Rat # 23
10% formalin
(left)

Rat # 24
Bouin's fixative
(right)

E. Giemsa Stains and Heavy Metal Perfusions:

1. Materials and Methods:

Opie, using the Giemsa and other stains, has reported finding an increased number of small basophilic bodies in the cytoplasm of butter yellow rat hepatomas.(78) Because of this, Giemsa stains were made of all the liver sections then available, i.e. rats # 1-25. These included normal control rats, rats which had been used in various perfusion experiments and rats which had been on m'MeDAB variable lengths of time. Most had had liver perfusions but a few had not. Various fixatives had been used in preparing the liver sections. Since the particular fixative used affects greatly the manner in which the Giemsa stain "takes" or whether it does at all, a great variety of results was to be expected on this basis alone, excluding the effects of carcinogenesis. Therefore, only rats in which the same fixative had been used were compared as to the effect of carcinogenesis on cytoplasmic basophilic bodies. Normal and/or control rats were also compared as to the effect of the various fixatives on the quality of stain obtained.

Opie believed that these basophilic bodies in the cytoplasm were not all mitochondria and that they contained high concentrations of nucleic acids, which would be expected to be increased in neoplasms. To support this theory he found that ribonuclease caused these bodies to lose their stain and become acidophilic. However, because of the markedly different effect which different fixatives have on the Giemsa stain, and thereby the presence, absence and number of cytoplasmic basophilic bodies, it was felt that they might be artefacts such as would be obtained from protein precipitation due to the heavy metals in the fixatives. Therefore, nineteen

normal rats were taken from the stock colony and each of their livers was perfused with a saturated solution of a different heavy metal. (See Table I. Rats # 41-59) Each heavy metal perfusion was preceded by a perfusion with Ringer's solution. All perfusing fluids were kept at 37° C. Most of these solutions fixed the liver, turning it hard, but the potassium dichromate, oxalic acid, barium nitrate, cobaltous acetate, lead acetate, ammonium vanadate, molybdic acid and lanthanum acetate did not and the liver remained of normal consistency. The silver nitrate solution apparently reacted with the Ringer's solution so that silver chloride was precipitated, plugging up the needle. This slowed this perfusion and the following molybdic acid one considerably. After perfusion, pieces of liver were dropped in 80% alcohol. Giemsa stains were made from paraffin sections of these livers and compared.

2. Observations of Giemsa Stains:

10% formalin.

Normal livers. (Rats # 1, 2, 5 and 23)

The nuclei have prominent central nuclear condensations, some definitely acidophilic, on a colorless, clear background or with a few clumps and strands of chromatin. Nuclear membranes are prominent and there is no chromatin network. The bile duct nuclei have heavier chromatin as does an isolated hepatic cell nucleus here and there. In the hepatic cytoplasm are clumps and aggregations of basophilic material, scattered evenly or applied close to the nucleus. In a few areas, they are more numerous at the periphery of the cytoplasm. Occasionally, they are aligned in a ring, a short distance from the nucleus. In some cases, they radiate out from the nucleus like the spokes of a wheel. In many cases, these clumps appear

like small, oval bodies. In a few of these slides the stain is too light.

80% alcohol.

Normal livers. (Rats # 9 and 21)

Hepatic nuclei are similar to that seen with 10% formalin. Almost all cells have moderate cytoplasmic basophilic stippling with a few indistinct particles. However, some slides show no cytoplasmic basophilia at all. Adjacent vessels, in some areas, are well defined, oval, basophilic bodies applied closely to the nucleus, radiating from it or scattered in the cytoplasm.

11 days m'MeDAB. (Rat # 4)

The stain is very uneven. Nuclei are unchanged. Only sporadically are basophilic particles seen but there seems to be an increased basophilic stippling toward the portal vein.

3 weeks m'MeDAB. (Rats # 7, 8, 12 and 15)

A few hepatic nuclei have darker and more numerous clumps and strands of chromatin, especially in the portal areas. The areas of bile duct proliferation contrast sharply, due to increased cytoplasmic basophilia there. Cytoplasmic basophilia is also increased at the edges of the sections and around veins. The cytoplasmic basophilia, in this case, consists of stippling with a few indistinct particles. In the portal hepatic cells, however, are a few clumps and aggregations of basophilic bodies around the nuclei and also some stippling.

6 weeks m'MeDAB. (Rat # 14)

There is little cytoplasmic basophilia anywhere except for a suggestion around vessels and at section edges. Many of the hepatic nuclei have a heavier chromatin, more basophilia and some clumps.

95% alcohol.

Normal liver. (Rats # 9, 15 and 22)

Nuclei are clear-cut with prominent central nuclear condensations, some definitely acidophilic on a light basophilic background with a few clumps and strands of chromatin and a dark nuclear membrane. Most cells have a moderate cytoplasmic basophilic stippling with a few indistinct particles or a few basophilic aggregations and clumps scattered here and there. There is some increased cytoplasmic basophilia around vessels, where some well defined, oval bodies are seen applied next to the nuclei, radiating from them or scattered in the cytoplasm.

11 days m'MeDAB. (Rat # 4)

Nuclei are unchanged. Cytoplasmic basophilia is scarce and particles are only occasionally seen.

3 weeks m'MeDAB. (Rats # 7 and 8)

It seems that chromatin clumps are more numerous in nuclei of the proliferating areas. The cytoplasm of the proliferating bile ducts has a light basophilic stippling but basophilia is absent elsewhere.

Bouin's fixative.

Normal livers. (Rats # 9, 16 and 24)

The stain is pale. Many nuclei are not stained at all. When they are, they usually have dark nucleoli on an acidophilic background with no chromatin seen. Only a few of these have dark nuclear membranes. Some nuclei however, have a deep basophilia with scattered dark particles of chromatin. There are all gradations of nuclei between these two extremes. There are large cytoplasmic basophilic clumps and aggregations, some definite oval bodies, applied close to the nuclei. These are especially prominent around vessels and at section edges.

11 days m'MeDAB. (Rat # 4)

Nuclei are the same. Isolated cytoplasmic basophilic bodies are seen in portal areas but they are scarce elsewhere.

3 weeks m'MeDAB. (Rats # 7, 8 and 17)

The stain is pale. In some slides the stain is a peculiar aqua color and nuclei are seen only occasionally. No cytoplasmic basophilia is seen except for a light stippling and a few indistinct particles in the cytoplasm of the proliferating bile ducts.

Regaud's fixative.

Normal livers. (Rats # 5, 11, 19 and 25)

Nuclei are not too well defined in some slides. They have prominent central nuclear condensations on a colorless or granular blue background and a dark nuclear rim. Many cytoplasmic basophilic particles are seen. They are for the most part scattered through the cytoplasm but some are found applied next to the nucleus, radiating out from the nucleus or along the edges of liver cords. Some are even concentrated at the cell periphery -- in fact, they are found in ever conceivable position. They are especially prominent around veins. In some slides, there is a background of basophilic stippling in addition to the particles, especially around portal areas. Around hepatic areas, the particles seem to be finer. Some of the particles resemble oval bodies and some appear like oval spaces with basophilic rims. Mostly however, they appear as large clumps and aggregations.

11 days m'MeDAB. (Rats # 4 and 6)

Nuclei are unchanged. In most areas there is more of a basophilic stippling in the cytoplasm than actual particles. This is increased in portal areas and around central veins. Many cells demonstrate little or no

basophilia. Many basophilic oval bodies are also seen however, around veins. Some of these are scattered aimlessly through the cytoplasm, some are applied next to the nucleus, some are radiating from the nucleus and some are arranged in parallel rows at the edges of liver cords.

3 weeks m'MeDAB. (Rats # 10 and 18)

Many nuclei do not show up well. Those seen often contain scattered dark chromatin granules. The areas of proliferation in the portal regions contrast sharply with the rest of the liver due to a deep cytoplasmic basophilia, which is also somewhat increased around central veins. In these proliferating areas are deep blue cytoplasmic granules or stippling and some indistinct and some distinct oval bodies. Elsewhere, distinct basophilic particles and bodies only are seen in the cytoplasm. These are found scattered throughout the cytoplasm, applied next to the nucleus, radiating from the nucleus like the spokes of a wheel and often oriented at the extreme cell periphery.

6 weeks m'MeDAB. (Rat # 20)

Hepatic nuclei have a granular chromatin but usually no nucleoli. Many cells have little cytoplasmic basophilia, ranging from none to a vague clump or two. Scattered among these cells, especially near areas of connective tissue proliferation, are other cells with deeply basophilic cytoplasms, stippled or finely vacuolated. Occasionally, entire nodules of these deeply basophilic cells contrast sharply with the surrounding parenchyma. In areas of cholangiofibrosis the proliferating bile ducts have a deeply stained, basophilic, stippled cytoplasm.

Potassium dichromate. (Rat # 41)

Hepatic nuclei have prominent central nuclear condensations with a diffuse blue, granular chromatin and a prominent nuclear membrane. Very slight basophilic stippling of the cytoplasm was seen at the edges of the

sections. No other basophilia was present.

Potassium permanganate. (Rat # 42)

For a considerable distance into each lobule all around the portal veins, parenchyma is destroyed and the necrotic remnants are stained a very deep blue. Elsewhere, cells are considerably shrunken and distorted. Hepatic nuclei have prominent central nuclear condensations on a colorless background with a few clumps and strands of chromatin and a prominent nuclear membrane. Occasional slight basophilic stippling of the cytoplasm is seen around portal veins and at section edges. No other basophilia is seen.

Zinc chloride. (Rat # 43)

Sinusoids are distended. Cytoplasm is very shrunken and distorted. Nuclei are stained a very dark blue so that little structure can be seen. Apparently there is a granular chromatin and occasionally, central nuclear condensations can be discerned. There is a moderate cytoplasmic basophilic stippling throughout, especially near nuclei. No other basophilia is seen.

Ferric chloride. (Rat # 44)

Hepatic nuclei have prominent central nuclear condensations, sometimes definitely acidophilic, and prominent nuclear membranes. Chromatin is in some places a diffuse granular blue; in other places lighter and structureless; and in still other places there is nothing but a clear, unstained background for the nucleolus. In one slide of four there was some cytoplasmic basophilic stippling, more marked in central areas, and cytoplasmic basophilic clumps resembling oval bodies scattered through the cytoplasm or radiating from nuclei like the spokes of a wheel. In the other slides, no basophilia was seen.

Mercuric chloride. (Rat # 45)

These slides vary tremendously. Central nuclear condensations are either prominent or only occasionally seen. Nuclear membranes are prominent. Chromatin structure ranges from a diffuse, granular, light brown to irregular dark clumps, which appear to be precipitated on the surface of the nucleus rather than part of its structure. Cytoplasmic stippling is entirely absent in many places and a brownish-purple color in others. Brownish-purple clumps and aggregations of material, some times resembling oval bodies, are seen in places in the cytoplasm of hepatic cells. These may be scattered throughout the cytoplasm, radiating from the nucleus or applied close to the nucleus.

Phosphotungstic acid. (Rat # 46)

There is much shrinkage and distortion of cells. Often the cytoplasm seems partially dissolved out. Many nuclei are not seen at all. Otherwise, they are dark, pyknotic, blue or blue, granular, indistinct bodies. Basophilic cytoplasmic stippling is massive, especially in portal areas. Occasionally, indistinct basophilic clumps are seen in the cytoplasm.

Helly's fixative. (Rat # 47)

Nuclei are not too well defined in some slides. They have prominent, central nuclear condensations on a colorless or granular, blue background and a dark nuclear rim. Many cytoplasmic basophilic particles are seen. They are for the most part, scattered through the cytoplasm, but some are found applied next to the nucleus, radiating out from the nucleus or along the edges of liver cords. Some are even concentrated at the cell periphery -- in fact, they are found in every conceivable position. They are especially prominent around veins. In some slides there is a background of basophilic stippling in addition to the particles, especially

around portal areas. Around hepatic areas, the particles seem to be finer. Some of the particles resemble oval bodies and some appear like oval spaces with basophilic rims. Mostly however, they appear as large clumps and aggregations.

Oxalic acid. (Rat # 48)

Lobules are collapsed. Cytoplasm is dissolved out leaving just a thin rim of medium blue stippling around the cell boundary. Hepatic nuclei have prominent central nuclear condensations, a light granular blue chromatin and a prominent nuclear membrane.

Copper acetate. (Rat # 49)

Very few hepatic nuclei are seen. Those seen have prominent central nuclear condensations, a light granular blue chromatin and a dark nuclear membrane. Cell boundaries are poorly defined. Slight cytoplasmic basophilic stippling is present around portal areas. Many basophilic clumps and aggregations are seen in the cytoplasm, some resembling oval bodies. These are found scattered through the cytoplasm, applied next to the nucleus or radiating out from the nucleus.

Uranium nitrate. (Rat # 50)

Cytoplasm is distorted, shrunken or dissolved out, especially in portal areas. Many large vacuoles are present in the cytoplasm. Some hepatic nuclei appear just as diffuse blue granular ovals. Others have prominent central nuclear condensations, a diffuse blue granular chromatin and prominent nuclear membranes. Moderate cytoplasmic basophilic stippling is seen in portal areas. No other basophilia is seen.

Barium nitrate. (Rat # 51)

The stain is very pale. In the central areas cytoplasm is dissolved out, leaving only a thin rim at the cell boundaries. No cytoplasmic

basophilia is seen. Only a few nuclei are stained at all. These have prominent central nuclear condensations on a diffuse blue background containing clumps and strands of chromatin and a dark nuclear membrane.

Cobalteus acetate. (Rat # 52)

The lobules are collapsed. Cytoplasm is largely dissolved out, especially in hepatic areas. This makes the extent of cytoplasmic basophilia difficult to evaluate. Nuclei tend to be quite darkly stained with prominent nuclear membranes and a diffuse granular chromatin. Central nuclear condensations are often seen. There is moderate cytoplasmic basophilic stippling in portal areas. Basophilic particles, often resembling oval bodies, are also present scattered through the cytoplasm, aligned next to the nucleus and in all the usual positions.

Lead acetate. (Rat # 53)

Not all the nuclei took the stain. However, central nuclear condensations are prominent, chromatin is blue and granular and nuclear membranes are prominent. There is a moderate cytoplasmic basophilic stippling with occasional indistinct clumps around vessels and at section edges.

Silver nitrate. (Rat # 54)

Hepatic nuclei have prominent central nuclear condensations on a colorless background with a few clumps and strands of brown chromatin or a diffuse brown background. Nuclear membranes are prominent. There is a light brown cytoplasmic stippling throughout the sections. There are many clumps and aggregations of brown material in the cytoplasm of the cells in the usual arrangements, already described. There are darker, black clumps which questionably resemble oval bodies.

Ammonium vanadate. (Rat # 55)

The stain is very pale. Few nuclei are seen. These have promi-

nent central nuclear condensations, prominent nuclear membranes and a colorless to blue background with occasional clumps and strands of chromatin. Occasional cytoplasmic basophilic stippling is seen near portal areas and at section edges. Occasional indistinct cytoplasmic basophilic clumps are seen.

Molybdic acid. (Rat # 56)

Lobules are collapsed. Cytoplasm is dissolved out in places, leaving only a rim around cell boundaries. Nuclei are very poorly seen. Usually if seen, they are just diffuse light blue ovals with no structure. Moderate cytoplasmic basophilic stippling is present throughout. Many cytoplasmic basophilic clumps and aggregations, often resembling oval bodies, are seen in the usual arrangements already described.

Cadmium nitrate. (Rat # 57)

Many nuclei are not seen, but many stand out. Nucleoli are usually obscurred, nuclear membranes are not prominent and the chromatin structure consists of dark clumps and strands on a blue background. No cytoplasmic basophilia is present.

Ferrous sulfate. (Rat # 58)

The stain is very pale. Most nuclei are not seen at all. If seen, they are medium blue ovals without structure. Occasional cytoplasmic basophilic stippling, aggregations and clumps are seen, in all the usual arrangements already described at section edges and around vessels.

Lanthanum acetate. (Rat # 59)

Cytoplasm is again dissolved out, making evaluation of the extent of cytoplasmic basophilia difficult. Central nuclear condensations are prominent, nuclear membranes are prominent and the background is usually colorless with a few clumps and strands of chromatin. Occasionally, there

is a diffuse blue granular chromatin structure. There are many cytoplasmic basophilic clumps and aggregations in the arrangements usually described, which are often not too well defined.

5. Discussion and Summary:

This experiment certainly has demonstrated the tremendous influence of the fixative on the quality of the subsequent Giemsa stain, which we already knew. The fixatives that proved to be absolutely worthless, as far as demonstrating basophilic cytoplasmic bodies go, include: potassium dichromate, potassium permanganate, zinc chloride, oxalic acid, barium nitrate, cadmium nitrate, 80% alcohol and Bouin's fixative. Other fixatives which are slightly better but still poor, include: ferric chloride, phosphotungstic acid, uranium nitrate, lead acetate, ammonium vanadate, ferrous sulfate and 95% alcohol. These are fixatives which demonstrated no basophilic bodies, but only basophilic stippling or basophilic stippling plus occasional ill-defined basophilic particles; fixatives which demonstrated only few basophilic bodies in certain areas of the slides, such as around veins and at section edges; and fixatives which were too uneven and erratic to be trusted. Perhaps under the proper conditions, some of these fixatives could be made to work. Still other fixatives were found to demonstrate the basophilic bodies well, but they caused serious artefacts, such as dissolving out of cytoplasm with shrinkage and distortion of the cell. These include: copper acetate, cobaltous acetate, lanthanum acetate and molybdic acid. It is interesting that three of them are acetates. Perhaps if the artefacts could be controlled (by diluting the fixative or changing the dehydration technique), these too could be made to work. Mercuric chloride and silver nitrate demonstrated the cytochondria well, but it is difficult to say whether they are acidophilic or basophilic since

they were stained a brown hue. Probably, the cytochondria were stained this way directly by salts of these heavy metals. 10% formalin gives a fair demonstration of the cytochondria, but Regaud's and Helly's fixatives are even better.

The basophilic bodies appeared, when demonstrated, much as Opie had described them. Moreover, as Opie described, they were more prominent in portal areas and in regenerating areas, such as demonstrated by the proliferating bile ducts in three weeks m'MeDAB livers and nodules of regenerating liver in six weeks m'MeDAB livers. As demonstrated by Opie, this basophilia decreases at some stages of carcinogenesis.(78) The six weeks m'MeDAB livers showed a great decrease in cytoplasmic basophilia compared with the three weeks m'MeDAB livers. However, it seems strange that the basophilia was often increased around central veins and at section edges, where there would be more contact with the fixative. This was especially prominent when the perfusion technique was used. Furthermore, why did some of these heavy metals demonstrate basophilic stippling without demonstrating basophilic bodies, while other heavy metals demonstrated basophilic bodies without basophilic stippling? Still other heavy metals demonstrated both. There seems to be no rhyme or reason to explain which heavy metals worked and which did not. Are these different phases of protein precipitation? Of course, protein precipitation <u>is</u> the basis of most staining and fixation, which leads us to the disadvantages of studying dead tissues. It is very difficult to say in this case what is artefact and what is not, as well as what is true and what is not.

F. Summary:

This is a series of experiments, dealing with the liver perfusion technique, performed while casting about for methods which would best demonstrate the pathology of m'MeDAB in the rat liver.

1. Perfused livers decidedly have advantages over unperfused livers histologically. However, this advantage decreases with the distortion of liver architecture, such as occurs with m'MeDAB.

2. There is no significant difference, histologically, between livers perfused with normal saline and livers perfused with Ringer's solution.

3. There is no significant difference, histologically, between livers perfused with fluids at room temperature and livers perfused with fluids at 37° C.

4. Livers perfused with different fixatives are compared histologically and rated, with special reference to nuclear detail, in order:

1. 80% ethyl alcohol.	4. Bouin's fixative.
2. 95% ethyl alcohol.	5. 10% formalin.
3. VandeGrift's fixative.	6. Regaud's fixative.

5. Livers perfused with various fixatives and heavy metals are compared histologically with the Giemsa stain as to demonstration of cytoplasmic basophilia. The significance of this is discussed.

PART III.

OBSERVATIONS OF THE SMEAR TECHNIQUE

III. OBSERVATIONS OF THE SMEAR TECHNIQUE

A. Versus Sectioned Material.

As can readily be appreciated by reviewing the histological observations made in the previous section on perfusion experiments, the nuclear structure of livers prepared by the paraffin method varies tremendously and is subject to much artefact. Because of this, attempts made to follow accurately nuclear structural changes in sectioned material during early m'MeDAB carcinogenesis were largely frustrated. This was very unfortunate since cancer is considered by many to be a disease of the nucleus or originating there, so that nuclear changes as carcinogenesis progresses are of great interest. For this reason, the smear technique was investigated and found to be highly superior. Artefacts are minimized by immediate fixation and minute nuclear structure can be easily seen and studied. In the sectioned material, nuclei vary widely in size and shape, even in normal livers, due to the plane in which they were cut, dehydration and distortion due to fixation. For the same reasons, nuclei are smaller than in the smear preparations. The whole nucleus is seen in the smear preparation. Chromatin is clumped and distorted very greatly in the sectioned material. In a normal liver section, chromatin varies widely from nucleus to nucleus but in a smear preparation of normal liver, chromatin is essentially similar from nucleus to nucleus. Definite changes in chromatin structure with m'MeDAB could be easily detected with the smear preparation that were not even suggested by a study of sectioned material of the same liver. Therefore, sectioned material was discarded as a means of demonstrating the evolution of the nuclear changes characteristic of cancer.

B. Fixatives:

1. Materials and Methods:

Three male rats were utilized in this experiment: Rat # 63 had been fed basic ration for 8.7 weeks; Rat # 64 had been fed the basic ration containing 0.06% m'MeDAB for 8.7 weeks; and Rat # 65 had been fed the m'MeDAB ration for 8 weeks. All three underwent the same procedure, as follows: All perfusion fluids and fixatives were at room temperature. Under ether anesthesia, the rat was decapitated and a liver perfusion preparation was immediately set up in the usual manner. After starting a liver perfusion with saline at the rate of about 1/10cc. per second, six small pieces of liver about 2 mm. thick were removed for the purpose of making tissue smears. The smears were made by rubbing each one of the liver pieces between two slides. Thus 12 slides were made per rat. Two were immediately dropped into a solution of one part ether to one part 95% ethyl alcohol; two into Schaudinn's fixative; two into Bouin's fixative; two into VandeGrift's fixative;(77) two into fuming formaldehyde; and two into methyl alcohol. The slides were then turned over to the technicians and stained with Ehrlich's Hemotoxylin, eosin and Orange-G. The slides were then studied histologically to compare the fixatives and to determine which fixative gave the best results as to demonstration of nuclear detail. Previous to this experiment, all liver smears made had been fixed in the alcohol-ether solution, the fixative recommended by Papanicolau for vaginal smears.(5) Although alcohol-ether fixation was satisfactory, it was thought that perhaps, for the liver smear, an even better one might be found.

2. Observations:

Bouin's fixative:

In one slide, although it is too pale in many places, large nuclei with diffuse and granular chromatin are seen. In all the other slides, nuclei show in many places simply as rings with the chromatin completely washed out, or seem shrunken and very pale. In isolated areas, chromatin network stands out very well, however. In some areas stain was precipitated on the slides.

VandeGrift's fixative:

Nuclei and chromatin are clear-cut and well defined. Chromatin is excellently preserved, diffuse and granular. Reticulum is well demonstrated. Prominent central nuclear condensations are observed, some definitely acidophilic.

Methyl alcohol:

Nuclei are well defined with a diffuse, granular, homogeneous chromatin. Acidophilic and basophilic vacuoles are occasionally found in the nucleus. Prominent central nuclear condensations, definitely basophilic, are seen in some areas. Portions of the slides are poorly stained, however.

Fuming formaldehyde:

Most of the nuclei appear as rings or acidophilic oval bodies, with no definite or only very faint chromatin discernible. Only in very small areas is chromatin demonstrated at all. In some places, nuclei appear distorted.

Schaudinn's fixative:

Many nuclei appear as blue rings. Some have a blue background containing indistinct or faint chromatin, especially in thin areas. In

other thicker areas, nuclei are distinct with clumped, black chromatin and prominent, acidophilic central nuclear condensations. Some nuclei have prominent nuclear membranes. Occasionally vacuoles are seen in the nucleus.

Ether-alcohol:

Nuclei are clear-cut and well defined. Chromatin is homogeneous, diffuse and granular. Vacuoles are seen in the nucleus, occasionally. Some nucleoli are discerned.

3. Summary:

Bouin's fixative, Schaudinn's fixative and fuming formaldehyde seem absolutely worthless for this material. Methyl alcohol is good but inconsistent. Ether-alcohol is good, but there is not as sharp and clear-cut delineation of chromatin detail as with VandeGrift's fixative. VandeGrift's fixative is the best of the six fixatives tested here, giving very excellent nuclear detail. There seems to be no difference in fixation between the control rat and the two rats under the influence of the carcinogen. The order of preference is, therefore:

1. VandeGrift's fixative.
2. Ether-alcohol.
3. Methyl alcohol.

C. Time of Fixation:

1. Materials and Methods:

Rat # 29, a 250 Gm. female rat with a mammary tumor, was taken from the stock colony. Under ether anesthesia, the rat was decapitated and allowed to exsanguinate as much as possible through the carotid artery. Laparotomy was performed and the liver was exposed. Tissue smears were made by cutting small slices of liver about 2 mm. thick from the tips of the large anterior lobes, flattening and rubbing them between two slides, and dropping the slides into ether-alcohol solution. One smear was made immediately, within about one minute after death, and subsequent smears were made every five minutes for one hour, i.e. one each at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 minutes. The stained finished slides were studied microscopically to determine the effect of the time of fixation on the nuclear structure. (The mammary tumor appeared well encapsulated with no evidences of degeneration).

2. Observations:

On each individual slide, there are some variations in the granularity of the nuclear chromatin. Nuclei which have crinkled edges and are fragmented or misshapen, have larger clumps and granules of chromatin than nuclei with more normal morphology. Moreover, in nuclei occurring in large clumps with much cytoplasm preserved, the chromatin detail is often obscurred by the overlying cytoplasm, etc. Therefore, only nuclei in similar positions are compared.

The smear made at one minute demonstrates, for the most part, nuc-

lei with a diffuse, homogeneous chromatin structure with very fine chromatin granules. The five minute smear may have some slight increase in the size of the chromatin granules but this is very questionable. All the later slides seem to definitely demonstrate an increase in size of the chromatin clumps and less homogeneity of the chromatin structure, which is not marked. However, this occurs in various degrees among these later smears and is not exactly proportional to the time of fixation. For example, the chromatin in the fifteen minute smear looks less clumped than that in the ten minute smear and the chromatin in the sixty minute smear is about like that in the thirty minute smear.

3. Summary:

There is a slight increase in the size of the chromatin granules and less homogeneity of chromatin structure, as the time after death at which the smear is made increases. The observation that fragmented and distorted nuclei also have increased chromatin granularity tends to confirm this. My conclusions are that the liver smears should be made within the first five minutes after death in order to obtain the very best nuclear delineation and detail. After the first five minutes, a chromatin clumping tends to occur, but fairly satisfactory smears can be made up to one hour. However, in a study such as this where a progression of fine nuclear changes is being followed, all smears should definitely be made as soon as possible after death -- not only to procure the best nuclear detail, but so that the smears from different animals will be comparable.

D. Giemsa Stains:

1. Materials and Methods:

Three male rats were utilized in this experiment: Rats # 66 and # 67 had been fed m'MeDAB for 9 weeks and Rat # 68 had been fed the basic control diet for 8 weeks. All three underwent the same procedure, as follows: All perfusion fluids and fixatives were at room temperature. Under ether anesthesia, the rat was decapitated and a liver perfusion was immediately set up in the usual manner. After starting a liver perfusion with saline at the rate of about 1/10cc. per second, eight small pieces of liver about 2mm. thick were removed for the purpose of making tissue smears, by rubbing each of the liver pieces between two slides. Thus 16 smears were made per rat. Two were dropped into alcohol-ether; two into Schaudinn's fixative; two into Bouin's fixative; two into VandeGrift's fixative; two into formaldehyde; two into methyl alcohol; two into Regaud's fixative; and two into Helly's fixative. The slides were then given to technicians for further preparation and Giemsa-staining. These were then studied microscopically to determine which fixative or fixatives gave the best results with the Giemsa stain, or if any of them did. It was thought that with the smear preparation, basophilic cytoplasmic material might be more easily demonstrated. Rats # 66 and # 67 should have less cytoplasmic basophilic substance in the liver, since they are developing hematomas, than the control Rat # 68.[78]

2. Observations:

Helly's fixative:

In most of these slides the stain is too faint and details are too blurred to observe anything clearly. Two of the slides are somewhat better. In these, however, the chromatin is not well defined and the little cytoplasmic basophilic substance seen is in the form of a diffuse stippling. No significant difference is seen between the rats.

Formaldehyde:

In many places the stain is faint and details are blurred. Chromatin is washed out or pale with large granules and clumps. In some places nucleoli show up well. In some places large, reddish granules are diffusely scattered through the cytoplasm. Much basophilic stain is precipitated on the slides. No significant difference is seen between the rats.

Regaud's fixative:

No chromatin detail can be seen. Nuclei are stained very dark greenish-black to dark blue. Cytoplasm demonstrates a very diffuse, light blue basophilic stippling. No significant difference is seen between the rats.

Bouin's fixative:

In many places nuclei are faint and blurred with no chromatin detail. In other places nuclei stain well with a homogeneous granular chromatin. Cytoplasm is stained a diffuse green color but no particles or stippling are present. No significant difference is seen between the rats.

VandeGrift's fixative:

Chromatin detail is excellent, but washed out or overstained in a few places. Nucleoli are often distinct. Cytoplasm is full of an amor-

phous green, granular material, but no definite oval bodies or particles can be differentiated. No significant difference is seen between the rats.

Methyl alcohol:

Stain is faint in many places. In other places nuclei stain well with a homogeneous granular chromatin. Most cytoplasm has no basophilia, but a few isolated clumps of cells have a diffuse light basophilic stippling. Possibly, a few more of these clumps are seen in the control rat than in the others.

Schaudinn's fixative:

Stain is faint. In some places chromatin is in large clumps or washed out and vacuolated. In other places nuclei demonstrate a homogeneous, granular, well-stained chromatin. Occasionally, prominent basophilic central nuclear condensations are seen. In some clumps of cells a fine diffuse cytoplasmic stippling is found and occasionally a few indistinct basophilic particles. Possibly, more of these cell clumps are seen in the control rat.

Ether-alcohol:

Rat # 67 (9 weeks m'MeDAB), and Rat # 68 (control), are similar. Chromatin appears well-stained, homogeneous and granular in some places. In other places, it is clumped, vacuolated or washed out. Occasional central nuclear condensations are seen. There is often a marked basophilic stippling of the cytoplasm. In Rat # 66 (9 weeks m'MeDAB), everything is stained blue. Nuclei generally appear as blurred blue objects. Cytoplasm demonstrates a diffuse blue stippling and occasionally, indistinct basophilic particles.

5. Discussion and Summary:

None of the smears demonstrated at all well basophilic bodies in

the cytoplasm. A few demonstrated erratically, basophilic stippling. It is quite possible that further experimentation with staining and technique methods would result in discovery of a good means of delineating cytoplasmic basophilic bodies in the smear preparation. Unless, of course, the cytoplasmic basophilic bodies are artefacts produced by the embedding method and may, therefore, only be seen in sectioned material. Ether-alcohol, Regaud's fixative, VandeGrift's fixative and possibly Schaudinn's fixative offer the most promise. No significant difference in cytoplasmic basophilia is seen between the control rat and the rats on m'MeDAB, except for a very questionable greater incidence of cell clumps containing cytoplasmic basophilia in the control animals.

E. Smears of Various Organs:

1. Materials and Methods:

Seven rats were utilized in this experiment: Rat # 69 had been fed the carcinogenic diet for 9 weeks; Rat # 70 for 1 week; Rat # 71 for 6 weeks; Rat # 73 for 7 weeks; Rat # 74 for 10 weeks; Rat # 75 for 2 weeks; and Rat # 72 had been fed the basic control diet for 7 weeks. All were decapitated under ether anesthesia and partially exsanguinated through the carotid artery. A saline liver perfusion was immediately set up in the usual manner, liver smears were made and then smears of various other organs were made. These were prepared the same way the liver smears were -- by rubbing thin tissue slices between two slides. Four kidney smears,(two from each kidney), four spleen smears and four testes smears, (two from each testis), were made from each of the rats # 69, 70 and 71. Four smears of the adrenals,(two from each adrenal), were made from each of the rats # 72, 73, 74 and 75. Half of the smears from each organ were fixed in ether-alcohol solution and half were fixed in VandeGrift's fixative. The slides were then given to technicians for further preparation and staining with Ehrlich's hematoxylin, eosin and Orange-G. These were then studied microscopically to determine whether the other organs lend themselves to the smear technique, as the liver does, and whether any nuclear changes occur in these other organs during m'MeDAB carcinogenesis. The two fixatives, VandeGrift's and ether-alcohol, cannot be properly compared for each organ as was planned because the VandeGrift's fixative reacted with the paper clips used to hold the slides apart. This resulted in precipitation of a brownish material on the slides, and usually a poor stain.

2. Observations:

Kidney smears:

These excellent smears look much like the liver smears, in general. There are many blood cells, much debris and numerous, larger, scattered single kidney nuclei, mostly without cytoplasm. Some clumps of cells are also seen occasionally. The kidney nuclei have much less variation in size and shape than the normal liver nuclei. They are smaller, more rounded and the chromatin structure is different. The nuclear membrane is very prominent, usually. No nuclear changes are seen, the chromatin appearing uniformly granular and homogeneous in all the three rats studied.

Spleen smears:

These slides demonstrate many different kinds of cells -- all kinds of blood cells, mostly mononuclears and connective tissue cells. The smears are very satisfactory histologically although occasionally there are areas where detail is blurred. Nuclear chromatin is homogeneous and granular, usually. This, of course, varies with the type of cell. No obvious difference is seen between the three rats, except for possibly the occurrence of a few larger nuclei without chromatin change, in the 6 and 9 weeks m'MeDAB rats. However, the great variety of cell types and their nuclei makes it difficult to rule out whether or not there actually is nuclear change.

Testes smears:

These slides also demonstrate a variety of cell types -- sperm, Sertoli cells, the stages of spermatogenesis, connective tissue cells, etc. Cells seem very distorted and fragmented in many areas. Again, with the variety of cell types which are present, it is difficult to rule out nuclear change. However, no obvious difference is seen between the three rats studied. Nuclear chromatin is homogeneous and granular, usually, varying

with cell type but not with weeks on m'MeDAB. What appear to be mitotic figures are often seen in all these slides.

Adrenal smears:

These smears are very poor. Only debris is seen on many slides. On other slides, fragmented, distorted, vacuolated nuclei are seen. In some areas where the nuclei are not too distorted, chromatin is not distinct. Very occasional, undistorted nuclei with granular, homogeneous chromatin are seen, however. Occasional cell clumps, also distorted, are seen. There is more variation in cell type than occurs in the liver smears, but not nearly comparable to that in the testes and spleen smears.

3. Discussion and Summary:

The kidney and spleen smears are excellent histological material; testes smears are fair but there is often fragmentation; and adrenal smears are no good at all with this particular technique. In the kidney smears, no nuclear changes are seen so they will not be studied further. No obvious differences due to m'MeDAB are seen in the spleen smears but the cell types are too numerous to be adequately studied in this present work. The same is true of the testes smears, in addition to the fragmentation often seen in them. This leaves the adrenal smears, which are completely unsatisfactory with this technique. If a satisfactory technique could be discovered, nuclear changes may be revealed in the adrenal. (Later, a satisfactory technique was developed by Doctor Howard L. Richardson, utilizing impressions of the adrenal. This would probably be more satisfactory for the testes also, since the testis is a more friable organ).

F. Summary:

1. Paraffin-sectioned preparations of liver are entirely unsatisfactory for demonstrating fine nuclear changes occurring during m'MeDAB carcinogenesis. The liver smear preparation is much superior for this purpose.

2. For the liver smear, VandeGrift's fixative is by far the best, for demonstration of nuclear detail, of six fixatives studied. Ether-alcohol is second and methyl alcohol is third.

3. Liver smears should be made within five minutes after death in order to obtain the very best nuclear delineation. Smears made up to one hour after death however, are fairly satisfactory.

4. Cytoplasmic basophilia is not at all well demonstrated in the Giemsa-stained smear preparations studied, though various fixatives were tried in an attempt to bring it out.

5. Kidney and spleen smears, prepared as are the liver smears, proved excellent histological specimens and testes smears are fair. No positive nuclear changes are observed in these during the early stages of m'MeDAB carcinogenesis studied. Adrenal smears prepared in this manner are completely unsatisfactory.

PART IV.

OBSERVATIONS OF LIVER SMEARS DURING CARCINOGENESIS

TABLE II.

RATS IN THE CARCINOGENIC SMEAR SERIES

Time on diet	Rats fed Basic Ration alone.	Rats fed Basic Ration with 0.06% m'Med4B
1 week.		# 70, 123, 151P[1].
2 weeks.	# 127P.	# 75, 146, 147, 161.
3 weeks.	# 9, 15.	# 7, 8, 12, 13, 17, 106, 134P.
4 weeks.		# 104, 122A, 180, 181.
5 weeks.		# 134P.
6 weeks.		# 14, 71, 98, 171.
7 weeks.	# 61, 72.	# 62, 73, 134P.
8 weeks.	# 68	# 65, 139P.
9 weeks.	# 36, 63.	# 40, 64, 66, 67, 69, 134P, 198.
10 weeks.		# 74.
11 weeks.	# 30, 135P.	# 37, 134P, 165, 168.
12 weeks.		# 39.
13 weeks.		# 183.
14 weeks.		# 139P.
15 weeks.		# 79P, 80P, 81P, 85P, 130P.
16 weeks.	# 127P.	# 88P, 90P, 122P.
17 weeks.		# 87, 137P, 138P, 194.
18 weeks.		# 174.
19 weeks.		# 78P, 79P, 85P, 96P, 137P, 138P, 175.
20 weeks.		# 90P, 129, 138, 139, 156, 159, 162, 173.

P means that the smears were prepared from material obtained by liver puncture biopsies.

TABLE II.

(continued)

Time on diet.	Rats fed Basic Ration alone.	Rats fed Basic Ration with 0.06 m'MeDAB.
21 weeks.		# 137, 169.
22 weeks.		# 128, 176, 178.

IV. OBSERVATIONS OF LIVER SMEARS DURING CARCINOGENESIS

A. Pathology:

1. Materials and Methods:

Table II. shows the rats which were studied in this series. Some of the animals were given gastric washings, blood intraperitoneally, etc., as parts of other experiments -- all procedures which did not affect the liver pathology. As noted in the table, 11 were control animals and 76 were fed the carcinogen, m'MeDAB. Control animals were maintained and studied under the same conditions as the rats fed the carcinogen. In the following pages detailed descriptions of the liver smears of each rat will be presented in chronological order according to the number of weeks on the diet -- first the control series and then the carcinogen series. Since the object of the experiment is to follow the nuclear changes in the liver smear as carcinogenesis progresses, animals which survived the carcinogenic diet more than 22 weeks are not included, although they are available. These animals, necessarily, all have carcinomas and the series already contains a representative number of these. This whole series of animals is part of the group studied by H. L. Richardson and Horace Wachtnebel,(74) so that the pathology of m'MeDAB and its tumors reported by them is the same as that found here. Their results are fully described in the introduction to this paper. In this series, all the m'MeDAB rats from 16 weeks on had obvious liver carcinomas, usually with metastases, demonstrable in the paraffin sections. Rats # 80, 15 weeks, had hepatomas. (This does not, of course, include those rats in which smears were obtained by puncture bi-

opsy, since tissue from them was not available for paraffin sections). The great majority of these rats, control and m'MeDAB, had pseudo-tuberculosis. This, microscopically, is a chronic focal pneumonia and chronic peribronchitis with occasional formation of large caseous abscesses. Several animals had acute pneumonia, parenchymatous degeneration of the kidneys or other sequelae of cachexia.

Most of the smears were made within five minutes after death by the methods already described. Some, however, were obtained by liver puncture biopsies. These are noted in Table II. Ether-alcohol or VandeGrift's fixative or both were used for fixation. Rats with numbers less than # 63 were fixed with ether-alcohol; the others with VandeGrift's fixative or both. They were all stained with Ehrlich's hemotoxylin, eosin and Orange-G.

Following this series of animals is a short summary of changes seen in the weight curves of the animals and a group of photographs[1] of some of the smears studied. The prime disadvantage of all cytologic methods is that it requires very close familiarity with the material in order to detect the minute changes present. The eye easily forgets these changes from slide to slide. The photographs were taken of the various liver smears at 970x, (oil immersion) and then enlarged to about 3070x. Thus nuclei can be directly compared and many of the changes seen can be easily demonstrated to observers unfamiliar with the material.

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1. I am indebted to Doctor H. L. Richardson, who took all these photographs.

II. Observations:

CONTROL SERIES

2 weeks. # 127. Male.

(Obtained by puncture biopsy).

Tremendous blood masses prevent really accurate observation in these slides. Chromatin is diffuse, finely granular and homogeneous. There are prominent central nuclear condensations which are round and regular. Occasional small chromatin condensations, probably chromatin net knots, are seen, usually peripherally in the nucleus.

3 weeks. # 9. Male.

Nuclei are round to slightly oval and uniform in size and shape. In this normal smear there are roughly three sizes of nuclei -- small, often ovoid, uniform nuclei, which are bile duct nuclei and have a slightly heavier chromatin than the hepatic nuclei; medium sized hepatic nuclei of uniform size and shape; and occasional larger hepatic nuclei, also uniform in size and shape. Many blood cells of all kinds, occasional connective tissue cells, fibrin strands, etc., are all present. Chromatin structure is homogeneous with a fine granularity and fine reticulum. Occasional chromatin net knots are seen.

3 weeks. # 15. Male.

Liver nuclei are normal with uniform, homogeneous chromatin. Occasional bile duct structures are present and occasionally, vacuoles within the nucleus are seen, as in most normal smears.

7 weeks. # 61. Male.

Nuclei are normal and unchanged. Occasionally clumps of cells

are seen which include the cytoplasm. In these clumps nuclear detail is often obscurred due to the thickness of the smear and the attached cytoplasm. (These clumps are seen in all the smears).

7 weeks. # 72. Male.[1]

These are normal nuclei with no chromatin change. Occasional vacuoles are seen and nucleoli are prominent.

8 weeks. # 68. Male.

These are normal nuclei, uniform in size and shape with a homogeneous, fine chromatin structure.

9 weeks. # 36. Male.

These are normal liver smears with normal, fine, granular homogeneous chromatin in the nuclei.

9 weeks. # 63. Male.

No change from the usual normal liver smear is present.

11 weeks. # 38. Male.

These are normal liver nuclei.

11 weeks. # 135. Male.[2]

(Obtained by puncture biopsy).

Much blood is present, preventing accurate observation of liver nuclei, in three-fourths of these slides. The nuclei seen are normal with prominent nucleoli and a homogeneous, finely granular chromatin.

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1. Evipal sleeping time -- 22 minutes. This is a liver function test which measures the time that the rats sleep while under the influence of the barbiturate, Evipal.(79)

2. This animal died after 25 weeks on basic ration. At autopsy, diffuse pseudo-tuberculosis and acute pneumonia were found.

16 weeks. # 127. Male.[1]

(Obtained by puncture biopsy).

The usual, normal, uniform type of liver cell nuclei are present. No giant forms are seen in these normal smears. Occasional vacuoles are seen in the nuclei and nucleoli are prominent, round and acidophilic.

---

1. This animal was discontinued after 7 months on the basic ration because of no change. No pathology was found.

3'MeDAB SERIES

1 week. # 70. Male.

No change in chromatin from the normal is seen. However, there are definitely more larger sized nuclei and occasionally a giant hepatic nucleus is seen. There is slight anisocytosis and poikilocytosis. Questionably, there are more binucleate cells in the cell clumps and possibly there are more numerous, smaller nucleoli per nucleus.

1 week. # 123. Male.[1]

(Obtained by puncture biopsy).

No chromatin change is present. There are a tremendous number of disintegrated cells. Questionable, more of the nuclei are oval, rather than round in shape.

1 week. # 151. Female.

No change from the normal is present. Nuclei are of uniform size and shape. Chromatin distribution is uniform.

2 weeks. # 75. Male.[2]

There are definitely more larger sized nuclei and there is increased variability in size and shape. In some of the nuclei reticulum is more prominent, dark strands connect the net knots. Chromatin is still diffuse, but in a few of the nuclei it is increased in density and has somewhat larger granules. Many of the nuclei are unchanged.

---

1. This animal died after 18 weeks, of carcinoma of the liver with diffuse abdominal metastases.
2. Evipal sleeping time - 36 minutes.

2 weeks. # 146. Female.

Chromatin is diffuse and homogeneous. No nuclear effect is noted. There are a large number of bile duct nuclei and bile duct structures on these slides, possibly more than normal.

2 weeks. # 147. Female.

Chromatin is unchanged from the normal configuration, homogeneous and finely granular. There are quite a large number of bile duct nuclei and bile duct structures on these slides, possibly more than normal.

2 weeks. # 161. Male.

Nuclei are mostly uniform in size and shape. Chromatin is of normal, diffuse homogeneity and fine granularity. Occasional unusually large nuclei are noted. Nucleoli are prominent, round, uniform and acidophilic.

3 weeks. # 7. Male.

Many nuclei have definitely, diffusely, increased density of chromatin, which is somewhat clumped in a few nuclei. There is increased prominence of the reticulum in a few nuclei, with strands extending between the net knots. Numerous, irregularly shaped hepatic nuclei are present and nuclei are of a larger average size.

3 weeks. # 8. Male.

Many larger sized nuclei are present and there is rather marked irregularity of nuclear shapes and sizes. Nuclear chromatin is usually increased in density but is still diffuse, homogeneous and finely granular.

3 weeks. # 12. Male.

There is tremendous variation in nuclear size. Many larger nuclei

are present. Chromatin is increased in density in many of the nuclei but it is still of a uniform, finely granular distribution. Occasional nuclei have a prominent reticulum with strands of chromatin connecting the net knots.

3 weeks. # 13. Male.

Chromatin is increased in density in many of the hepatic nuclei but it is diffuse, finely granular and homogeneous. Occasional very large nuclei are seen and the average size of the nuclei is larger. In some nuclei there is some increased clumping of chromatin. In the very large nuclei there seem to be more nucleoli, which are slightly irregular in shape and tend to be fusiform.

3 weeks. # 17. Male.

Nuclear change is slight -- diffuse increase in chromatin density in some of the nuclei, generally increased nuclear size, some irregularity of nuclear size and shapes, but no change in chromatin architecture, usually.

3 weeks. # 106. Female.

More of the large sized nuclei are present, occasional giant nuclei are seen and there is some variability in nuclear shape. Many of the nuclei have an increased chromatin density and many have a slight increase in clumping of chromatin. Slightly more nucleoli per nucleus, 5-6, are present and they are often fusiform.

3 weeks. # 134. Male.

(Obtained by puncture biopsy).

Nuclei are uniform in size and shape but more of the larger sizes are seen, as well as occasional giant nuclei. Chromatin is moderately in-

creased in density in many of the nuclei, but homogeneous and finely granular.

4 weeks. # 104. Female.

Many of the nuclei have normal chromatin. There is no apparent change in nuclear size or shape. However, many other nuclei have definitely increased chromatin clumping, a diffuse increase in chromatin density and many nucleoli.

4 weeks. # 122-A. Female.

Nuclei are, in general, of larger size, and more variable in size and shape than the normal. Occasional giant cells are seen. Chromatin is diffusely increased in density in most of the nuclei but homogeneous and finely granular. In a few of the nuclei there is an irregular clumping of the chromatin. In some areas there is an increased chromatin condensation about central nucleoli. Some nuclei show a more prominent reticulum.

4 weeks. # 130. Female.

Many nuclei of larger size are present, but most of the nuclei have normal chromatin. Occasional giant nuclei and occasional nuclei with diffusely increased chromatin density are seen, however. Prominent acidophilic nucleoli, in which no changes are seen, are present.

4 weeks. # 131. Female.

More nuclei of the larger size are present. There is variability in size and shape of nuclei. Occasional giant nuclei are present. Some nuclei have a normal chromatin but many have a diffusely increased chromatin density.

5 weeks. # 134. Male.

(Obtained by puncture biopsy).

There is irregularity in size and shape of nuclei. Nuclei are generally increased in size. Occasional giant nuclei are seen. Some nuclei have an increased reticulum with strands of chromatin between the net knots. In many of the nuclei there is a diffuse increase in chromatin density. Occasional nuclei have an irregular chromatin clumping.

6 weeks. # 14. Male.

Many nuclei have a finely granular, homogeneous chromatin which is increased in density. A few nuclei have an irregular clumping of chromatin. There is anisocytosis, poikilocytosis and a generally increased nuclear size. Occasional giant nuclei are seen. The great majority of these nuclei have a diffusely increased chromatin density.

6 weeks. # 71. Male.

All the nuclei show a change, usually a uniform increase in chromatin, which is homogeneous and finely granular. Many giant nuclei are seen. There is anisocytosis, poikilocytosis and a general increase in nuclear size. Some nuclei have irregularly shaped giant nucleoli. Some nuclei have a slight clumping of chromatin or an increase in size of the chromatin granules. Some nuclei show chromatin condensation about central nucleoli, and decreased chromatin density elsewhere in the nucleus.

6 weeks. # 98. Female.

All nuclei show an increased chromatin density, usually homogeneous and diffusely granular. Some nuclei have a slightly clumped or more granular chromatin. Occasional large nucleoli are seen, usually

round and uniform, but occasionally irregular. There is an increased general nuclear size. Many giant nuclei are present and there is aniso-cytosis and poikilocytosis. Many nuclei contain large clear vacuoles.

6 weeks. # 171. Male.

Nuclei are nearly all increased in size, variable in size and shape with diffusely increased chromatin density. Many giant nuclei are seen. Many nuclei contain vacuoles. Some nuclei, questionably, have an increased nuclear vesiculation. Chromatin is finely granular.

7 weeks. # 62. Male.

There is a tremendous number of the larger sized nuclei and many giant forms are present. There is aniscocytosis, poikilocytosis and a diffuse increase in chromatin density with a fine, granular, homogeneous chromatin. Occasional nuclei have some chromatin clumping and an increased granularity.

7 weeks. # 75. Male.[1]

All the nuclei are of the larger size and many giant nuclei are present. Most of the nuclei have a diffuse increase in chromatin density and a finely granular, diffuse chromatin architecture. Some nuclei have a moderate increase in chromatin granule size and a slight chromatin clumping. Some nuclei have much vesiculation.

7 weeks. # 134. Male.

(Obtained by puncture biopsy).

Most of the nuclei are of the larger size. Many giant forms are present. There is aniscocytosis and poikilocytosis. Nucleoli are round, uniform and not enlarged. All the nuclei are involved in a diffusely increased chromatin density. The chromatin structure is homogeneous, fine

1. Evipal sleeping time - 55 minutes.

and dust-like. One nucleus was observed which contained many black, bar-like structures -- undoubtedly chromosomes in a stage of mitosis.

8 weeks. # 66. Male.

Most nuclei are increased in size and many giant forms are present. There is aniscocytosis and poikilocytosis. There is a diffuse increase in density of chromatin, which is usually homogeneous and finely granular. Often, nuclei have a somewhat clumped chromatin. Occasionally there are nuclei with increased vesiculation and vacuolation. Occasional large nucleoli are seen, which are occasionally irregular.

8 weeks. # 139. Male.

(Obtained by puncture biopsy).

Tremendous amounts of blood prevent fine visualization on all the slides. The nuclei which are demonstrated are usually giant forms with large nucleoli and a diffuse, granular, slightly clumped chromatin density.

9 weeks. # 40. Male.

There is a general increase in nuclear size, aniscocytosis and poikilocytosis. Giant nuclei are very numerous and some are extremely large -- larger than the giant forms previously seen. There is a diffuse increase in chromatin density with a finely granular, homogeneous chromatin in most of the nuclei. Some of the nuclei exhibit an increased granularity. When nucleoli can be demonstrated, all are enlarged, acidophilic and often irregular.

9 weeks. # 64. Male.

All the nuclei are generally increased in size. Many giant forms

some huge, are present. There is anisocytosis and poikilocytosis. Usually, the nuclei have a fine, granular chromatin structure with a diffuse increase in chromatin density. Many nuclei have somewhat clumped chromatin with a slight increase in chromatin granule size. Occasional nuclei have increased vesiculation. Occasional medium sized nuclei are seen which have a condensation of chromatin around a large, irregular nucleolus and little chromatin elsewhere in the nucleus.

9 weeks. # 66. Male.

There is a general increase in nuclear size and many giant forms are seen. There is anisocytosis and poikilocytosis. In most of the nuclei, chromatin is homogeneous and finely granular with a diffuse increase in density.

9 weeks. # 67. Male.

There is a general increase in nuclear size and many giant forms are seen. There is anisocytosis and poikilocytosis. In most of the nuclei chromatin is homogeneous and finely granular with a diffuse increase in density.

9 weeks. # 69. Male.

Nuclear size is generally increased. There is poikilocytosis and anisocytosis. Not so many giant forms are seen. All the nuclei show some increase in chromatin density. Many have a diffuse chromatin with somewhat increased clumping and granularity. Nuclear vesiculation is often present. Many nuclei have large nucleoli, often fusiform.

9 weeks. # 134. Male.

(Obtained by puncture biopsy).

The general nuclear size is larger. There is poikilocytosis and aniscocytosis. Occasional giant nuclei with homogeneous, finely granular chromatin, which is diffusely increased in density, are present. Some of the other nuclei show this same chromatin picture. Most of the nuclei have a normal or decreased chromatin density. There are prominent acidophilic nucleoli which are round, uniform and occasionally enlarged.

9 weeks. # 198. Male.

There is a general increase in nuclear size, aniscocytosis and poikilocytosis. Not many giant forms are seen. Occasional hyperchromatic nuclei with enlarged nucleoli are present. Many bile duct structures are present. Some nuclei have a dust-like, homogeneous chromatin, diffusely increased in density. Some nuclei have a somewhat more granular and somewhat clumped chromatin, diffusely increased in density. Many nuclei have a normal chromatin structure and density. Many nuclei have large to huge, irregular nucleoli. Many medium to small nuclei with decreased chromatin density, or no chromatin at all, vesiculation, a dark nuclear membrane, and prominent nucleoli, which are not enlarged, are seen.

10 weeks. # 74. Male.[1]

Most nuclei are of the larger size. Many giant nuclei are seen. There is much aniscocytosis and poikilocytosis. Many of the nuclei have a finely granular, homogeneous chromatin which is diffusely increased in density. Many nuclei have a somewhat granular, somewhat clumped chromatin, which is diffusely increased in density. Many bile duct structures are seen.

11 weeks. # 37. Male.

Many of the nuclei appear unchanged, but many have a finely granular

1. Evipal sleeping time - 32 minutes.

homogeneous chromatin with diffusely increased chromatin density. Some of these have a somewhat clumped, more granular chromatin with diffusely increased chromatin density. Occasional giant nuclei are seen. There is a general increase in nuclear size.

11 weeks. # 154. Male.

(Obtained by puncture biopsy).

Many normal hepatic cell nuclei are seen. There is a general increase in nuclear size, aniscocytosis, poikilocytosis and occasional giant forms are seen. Chromatin density is diffusely increased in some of these nuclei with a finely granular to somewhat clumped chromatin arrangement. Occasional enlarged, round nucleoli are present.

11 weeks. # 165. Male.

All the nuclei are larger in size. There is aniscocytosis and poikilocytosis. Occasional giant nuclei are seen. Many nuclei have an increase in chromatin density. The chromatin structure in these varies from a diffuse, homogeneous, dust-like distribution to a somewhat clumped chromatin with an increased granularity. Many apparently normal nuclei are also present.

11 weeks. # 166. Male.

Many of the nuclei are normal. However, many are seen which have a general increase in size, demonstrate aniscocytosis and poikilocytosis and have an increase in chromatin density. The chromatin structure of these nuclei is homogeneous and finely granular, usually, or somewhat clumped with a slightly increased granularity. Occasional giant cells are seen and occasional uniform, round, enlarged nucleoli are present.

12 weeks. # 39. Male.

All the nuclei are enlarged, many giant nuclei are seen, and there is aniscocytosis and poikilocytosis. Chromatin density is often diffusely increased, ranging from a homogeneous, finely granular distribution to a somewhat clumped one with increased granularity.

13 weeks. # 183. Female.

Most of the nuclei are of the larger size, there is poikilocytosis and aniscocytosis and occasional giant nuclei are present. Many nuclei exhibit a diffuse increase in chromatin density with a chromatin structure ranging from homogeneous and finely granular to somewhat clumped with an increased granularity.

14 weeks. # 139. Male.

(Obtained by puncture biopsy).

Many of the nuclei are of normal size, shape and chromatin distribution. However, many of the nuclei are larger in size, there is poikilocytosis and aniscocytosis and occasional giant nuclei are present. Occasional nuclei have a diffuse increase in chromatin density, with chromatin structure of both the finely granular and the somewhat clumped varieties.

16 weeks. # 79. Male.

Nuclei are of a larger size. Many have a normal chromatin. Others have an increased chromatin density, with a finely granular distribution or the somewhat clumped variety. Many of the nuclei contain large, occasionally fusiform or irregular nucleoli -- even the nuclei with apparently normal chromatin. Occasional giant nuclei are present. Some of these are very hyperchromatin. There is marked aniscocytosis and poikilocytosis.

15 weeks. # 80. Female.

(Obtained by puncture biopsy).

All the nuclei are larger in size, there is much aniscocytosis and poikilocytosis and many giant nuclei are seen. Chrometin is usually diffusely increased in density, usually with a finely granular, homogeneous structure. Definitely larger nucleoli are present in the nuclei.

15 weeks. # 81. Male.

(Obtained by puncture biopsy).

Nuclei are generally larger with a finely granular to somewhat clumped chromatin arrangement, which is diffusely increased in density. There is poikilocytosis, aniscocytosis and occasional giant nuclei are seen. Occasional nucleoli of peculiar shapes are seen, but no giant ones.

15 weeks. # 85. Female.

The nuclei are of generally larger size, there is aniscocytosis and poikilocytosis and many giant cells are present. Some of the giant nuclei have a hypochromatic chrometin, but most of the nuclei have an increased density, of the usual finely granular or somewhat clumped structure. A few of the nuclei had such dense chromatin, containing large vacuoles, that they may possibly be neoplastic. Many of the giant cell nuclei have fusiform, irregularly shaped nucleoli.

15 weeks. # 138. Male.

(Obtained by puncture biopsy).

Nuclei are generally increased in size, there is aniscocytosis and poikilocytosis and occasional giant nuclei are found. Chrometin is usually diffusely increased in density with structures of either the finely granular or the somewhat clumped variety represented. Nucleoli or central

nuclear condensations are much larger. One slide demonstrates much more pleomorphism than the others and chromatin similar to malignancy.

16 weeks. # 88. Male.[1]

(Obtained by puncture biopsy).

Many of the liver nuclei have normal chromatin. Many have chromatin with diffusely increased density and finely granular or somewhat clumped chromatin arrangements. Most of the nuclei are enlarged and occasional giant nuclei are present. There is marked anisocytosis and poikilocytosis. Many of the nuclei, even those with normal chromatin, had giant, occasionally irregular nucleoli. Some of the nuclei demonstrated increased vesiculation and vacuolization.

16 weeks. # 90. Female.

Many of the liver nuclei have apparently normal chromatin. Most of the nuclei are larger in size, there is poikilocytosis and anisocytosis and occasional giant cell nuclei are seen. Many of the nuclei have a diffuse increase in chromatin density, of the finely granular and somewhat clumped types. Some of the nuclei are quite hyperchromatic. Most of the nuclei, even those with apparently normal chromatin structure, have definitely enlarged nucleoli.

16 weeks. # 122-B. Male.

There is extreme variability in the size and shape of the nuclei and the nucleoli. Folding of nuclear edges is often seen, especially in a smaller type of cell which tends to be elongated and often connected with bile duct structures. These are often bizarre enough to be neoplastic. All varieties of chromatin are seen -- often irregular clumping, often vesiculation and often the diffusely increased chromatin density described hereto-

1. This animal died in its cage after 25 weeks on m'MeDAB. Many liver carcinomas were present, with dèsseminated métastases.

fore. Many of the medium sized nuclei with normal or hypochromatic vesiculated, vacuolated chromatin and enlarged nucleoli, described since about 9 weeks, are seen. Some of these are so bizarre that they must be neoplastic. Some of the nuclei contain 20 or more nucleoli and many of the nuclei are unquestionably neoplastic, so bizarre are they. Giant, bizarre nuclei are also seen.

17 weeks. # 137. Male.

(Obtained by puncture biopsy).

Most of the nuclei are increased in size, there is poikilocytosis and aniscocytosis and occasional giant nuclei are seen. Chromatin is usually diffusely increased in density, of finely granular, homogeneous and somewhat clumped varieties. Some of the nuclei have apparently normal chromatin.

17 weeks. # 87. Female.

(Obtained by puncture biopsy).

There is extreme variation in nuclear size and shape, nucleolar size and shape and in the chromatin. Giant hyperchromatin nuclei with multiple, irregular nucleoli are seen. Many medium sized nuclei with single enlarged central nucleoli and normal or hypochromatic, vesicular, vacuolated chromatin are seen. Many nuclei with diffusely increased chromatin density of the types previously seen are also present.

17 weeks. # 139. Male.

(Obtained by puncture biopsy).

Most of the nuclei are increased in size, there is poikilocytosis and aniscocytosis and occasional giant nuclei are present. Chromatin is generally diffusely increased in density, with both the finely granular and somewhat clumped type of arrangement represented.

17 weeks. # 194. Male.

There is extreme pleomorphism, aniscocytosis and poikilocytosis of nuclei and nucleoli. All varieties of chromatin arrangement are present. Many of the nuclei are unquestionably neoplastic. Some of the carcinoma nuclei have a fine, dust-like chromatin like that of the normal liver nucleus, or diffusely increased chromatin density like that seen earlier. There are huge nuclei with multiple, bizarre nucleoli and many hypochromatic, medium-sized nuclei with large, central nucleoli and chromatin vesiculation.

18 weeks. # 174. Male.

Many disintegrated cells, bile duct structures and much connective tissue is present. Large and small, irregular nuclei with irregular nucleoli and a finely granular chromatin with a diffusely increased density are seen. Some medium-sized nuclei are seen that have very irregular chromatin clumping. Occasional bizarre, hyperchromatic nuclei of giant size are present. Many medium-sized nuclei with large, central or eccentric nucleoli and normal or decreased chromatin, which is often vesicular, are seen. There is great variation and every possible one seems to be present.

19 weeks. # 78. Female.[1]

(Obtained by puncture biopsy).

Many of the nuclei had apparently normal chromatin structure. Many had definitely enlarged nucleoli. Many had an increased chromatin density, finely granular or somewhat clumped. Occasional giant nuclei were seen. No obviously neoplastic nuclei were present.

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1. This animal was killed at 26 weeks. Adenocarcinoma of the liver with extensive omental metastases were found.

19 weeks. # 79. Female.[1]

(Obtained by puncture biopsy).

One solid mass of unquestionably neoplastic nuclei with every possible variation in size, shape and chromatin structure is present. Nucleoli are similarly extremely variable, often with connecting strands between the nucleoli. Often, however, the chromatin is homogeneous, finely granular and diffusely increased in density, although the nucleus may show all the other criteria of malignancy. Among these nuclei are found other nuclei of similar design, but very hyperchromatic.

19 weeks. # 86. Female.

In some areas, nuclei of uniform size and shape with finely granular, homogeneous chromatin or somewhat clumped, homogeneous chromatin, which is diffusely increased in density are present. Occasional huge nuclei with irregular, large nucleoli and hyperchromatic, irregularly clumped structure are present. Many nuclei with chromatin condensed around a large central nucleolus, a dark nuclear membrane and hypochromatic or absent chromatin structure are seen.

19 weeks. # 96. Male.[2]

(Obtained by puncture biopsy).

Many apparently normal nuclei are present. Many nuclei with an increased density of chromatin, of finely granular or somewhat clumped arrangement, are present. Most of the nuclei have enlarged, acidophilic nucleoli, which occasionally are irregular in shape. Occasional giant

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1. This animal died after 20 weeks of adenocarcinoma of the liver with diffuse lung metastases.

2. This animal was killed at 27 weeks. Advanced liver carcinoma with diffuse lung metastases was present.

nuclei with hyperchromatic chromatin and bizarre forms are present, probably neoplastic.

19 weeks. # 137. Male.

(Obtained by puncture biopsy).

Several clusters of very hyperchromatic, huge nuclei are present. They are very bizarre, irregular in shape and are probably neoplastic. The other nuclei also demonstrate extreme variability in size, shape and chromatin distribution, such as has already been described.

19 weeks. # 136. Male.

(Obtained by puncture biopsy).

Many bizarre, hyperchromatic giant nuclei with large, irregular acidophilic nucleoli are present. Some of these nuclei, while in every other way the same, have a finely granular, homogeneous chromatin of normal or increased density. All varieties of nuclear sizes, shapes and chromatin arrangements are present. Many smaller nuclei with eccentric single nucleoli and increased density of chromatin, with a fine, granular arrangement are present.

19 weeks. # 175. Male.

Most of these nuclei are of the type reported as far back as 12 weeks. They have enlarged central nucleoli, often vesicular, usually hypochromatic chromatin, dark nuclear membranes and are usually of medium sizes. In this slide, however, all sizes are present and many demonstrate great pleomorphism of the nucleoli and the nucleus. Occasional nuclei have an irregularly clumped chromatin. These must all be neoplastic nuclei. In addition, nuclei with a diffusely increased density of chromatin, with finely granular or somewhat clumped arrangements, are noted.

20 weeks. # 90. Female.[1]

(Obtained by puncture biopsy).

Most of the nuclei show the drug effect -- generally increased size and diffusely increased density of chromatin with finely granular or somewhat clumped arrangement. One clump of nuclei resembles large cell adenocarcinoma but the chromatin is like that of the drug effect. These nuclei have definitely enlarged, often irregular, central nuclear condensations.

20 weeks. # 129. Male.

Many pleomorphic nuclei, giant and small, are present. Nucleoli are rather prominent. These are unquestionably neoplastic.

20 weeks. # 138. Male.

Many nuclei demonstrate the drug effect of diffusely increased chromatin density, but in these nuclei it is more chromatic. Bizarre giant nuclei with irregular, clumped chromatin and large, irregular nucleoli are present. Adenocarcinoma nuclei, the medium-sized ones with hypochromatic, granular, vesicular, chromatin, dark nuclear membrane and large central nucleoli, are present in abundance.

20 weeks. # 139. Male.

(Obtained by puncture biopsy and at autopsy).

Many hyperchromatic nuclei with large central nucleoli and a granular chromatin are present. There is a large bizarre type with clumping and irregularity of the chromatin and irregular nucleoli. Smaller nuclei are seen with hypochromatic, vesicular chromatin, large central nucleoli and dark nuclear rims. There is some increased vacuolation. Some nuclei

1. This animal died after 27½ weeks, of liver carcinoma with lung metastases.

tend to be fusiform with folded edges, which are irregular. Many disintegrated nuclei and many granulocytes are present.

20 weeks. # 156. Male.

Much debris and many bile duct structures are present. Adenocarcinoma nuclei, the folded type of nuclei, the large bizarre type of nucleo and giant nuclei with very pleomorphic size, shape and nucleoli, but a diffusely increased, finely granular, chromatin structure are seen. Many nuclei, which apparently are like the drug effect nuclei, are seen.

20 weeks. # 159. Male.

Some of these slides demonstrate much necrotic debris and many white blood cells. Adenocarcinoma nuclei, the nuclei with folded edges and giant, bizarre, irregular nuclei with irregular nucleoli, are present. These giant nuclei often have irregular, clumped chromatin and, occasionally, hyperchromatic chromatin, but usually chromatin is of the finely granular variety, which is diffusely increased in density.

20 weeks. # 162. Male.

There are numerous cancer cells, large and small type adenocarcinoma. Nucleoli are quite prominent. Some of the small nuclei with folded edges, bizarre nucleoli and occasional pleomorphic forms, are present. Probably these are bile duct adenocarcinoma. Many nuclei with diffusely increased chromatin density are seen, some with and some without the other criteria of malignancy.

20 weeks. # 173. Male.

Hepatic adenocarcinoma nuclei, the folded form of nucleus with small and occasionally bizarre nucleoli, and the bizarre giant type of nuc-

lei are all present. The small elongated folded form is seen to lead into bile duct structures. Much necrotic debris and many white blood cells are seen. All possible variations in nuclear and nucleolar size and shape and all kinds of chromatin are present. Many of the neoplastic nuclei have a homogeneous, finely granular or somewhat clumped chromatin, which is diffusely increased in density.

21 weeks. # 137. Male.

(Obtained by puncture biopsy).

Numerous nuclei with the drug effect type of chromatin are seen, many of them of giant size. Some of these giant nuclei are quite pleomorphic and hyperchromatin. (Carcinoma ?) Some nuclear disintegration is observed. Many bile duct structures are present.

21 weeks. # 169. Male.

Most of the nuclei are small cell type adenocarcinoma, but drug effect nuclei, giant bizarre nuclei and the folded type of nuclei are also represented.

22 weeks. # 128. Male.

Small cell carcinoma (adenocarcinoma) nuclei, drug effect nuclei, and occasional large bizarre nuclei are observed. Most of the carcinoma nuclei have, as usual, the finely granular, homogeneous chromatin of diffusely increased density that is seen in the drug effect nuclei.

22 weeks. # 176. Male.

A smear of one of the large tumors reveals many of the small, elongated, folded type of nuclei, with occasional bizarre variations and many bile duct structures. A smear of one of the small tumors reveals

bile duct debris, giant nuclei with bizarre, irregular chrometin and nucleoli and some of the folded type of nuclei. A slide of a "hepatoma" reveals large and small cell adenocarcinoma nuclei with occasional pleomorphic variations.

22 weeks. # 178. Male.

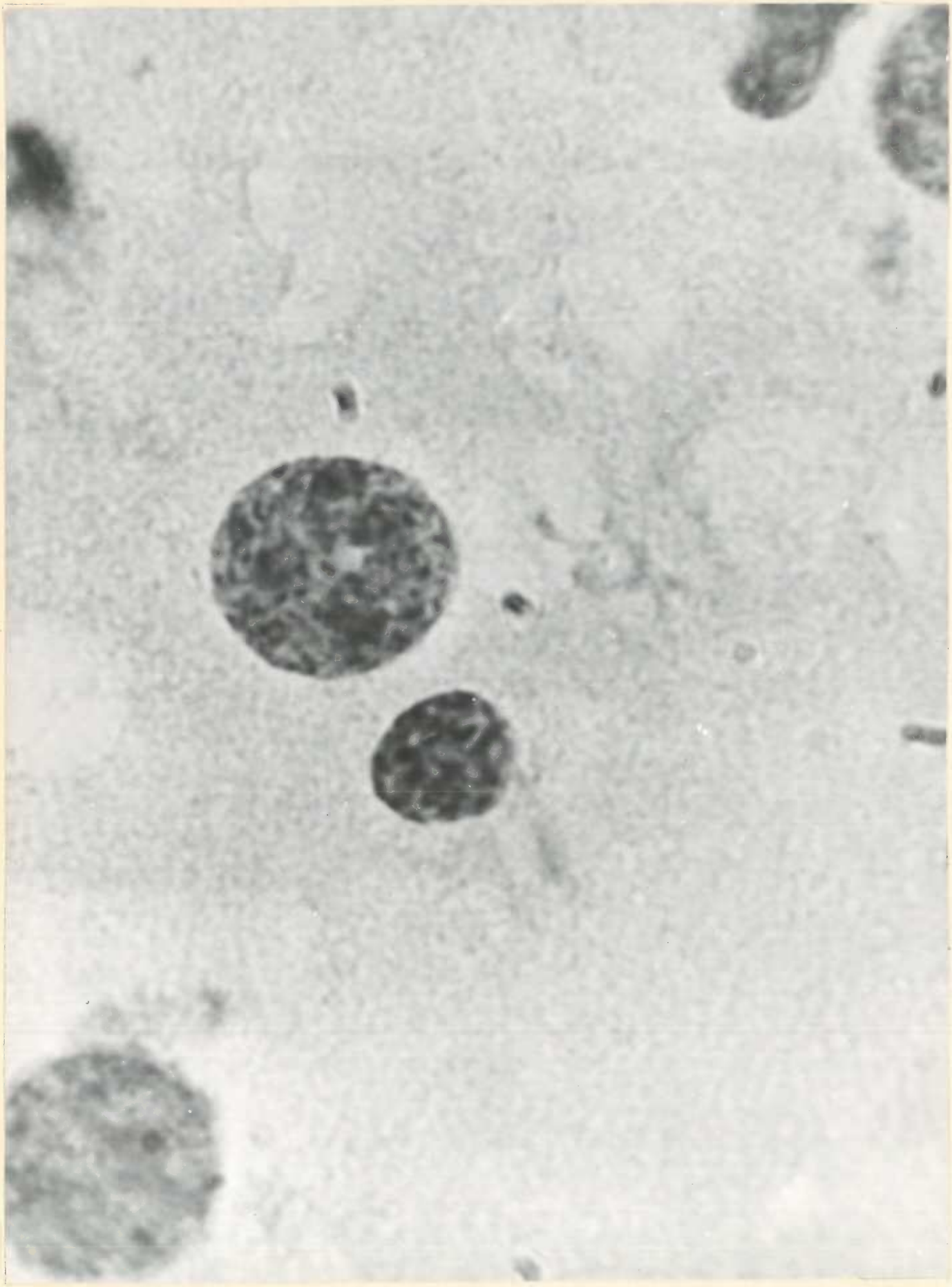
A smear of a small tumor reveals many bile duct structures, many of the folded type of nuclei with small nucleoli and not much pleomorphism, and many small adenocarcinoma-like nuclei with a granular, homogeneous chrometin. A smear of a large tumor reveals many small cell adenocarcinoma nuclei with numerous giant and bizarre forms.

THE WEIGHT CURVES

Most of these animals were weighed weekly and line graphs were made of these weights for each animal. All of the control animals steadily gained weight, becoming fat and often very large. Of course, they consumed slightly more food than the carcinogenic rats did, but this was kept to a minimum by the simple expedient of giving them as much food per day as the carcinogenic rats were able to consume and no more. The only spontaneous death in this control group was Rat # 135, due to advanced pseudo-tuberculous lung disease. This animal lost 80 grams in its last two weeks of life, but prior to that its weight gain was similar to that of the other control animals.

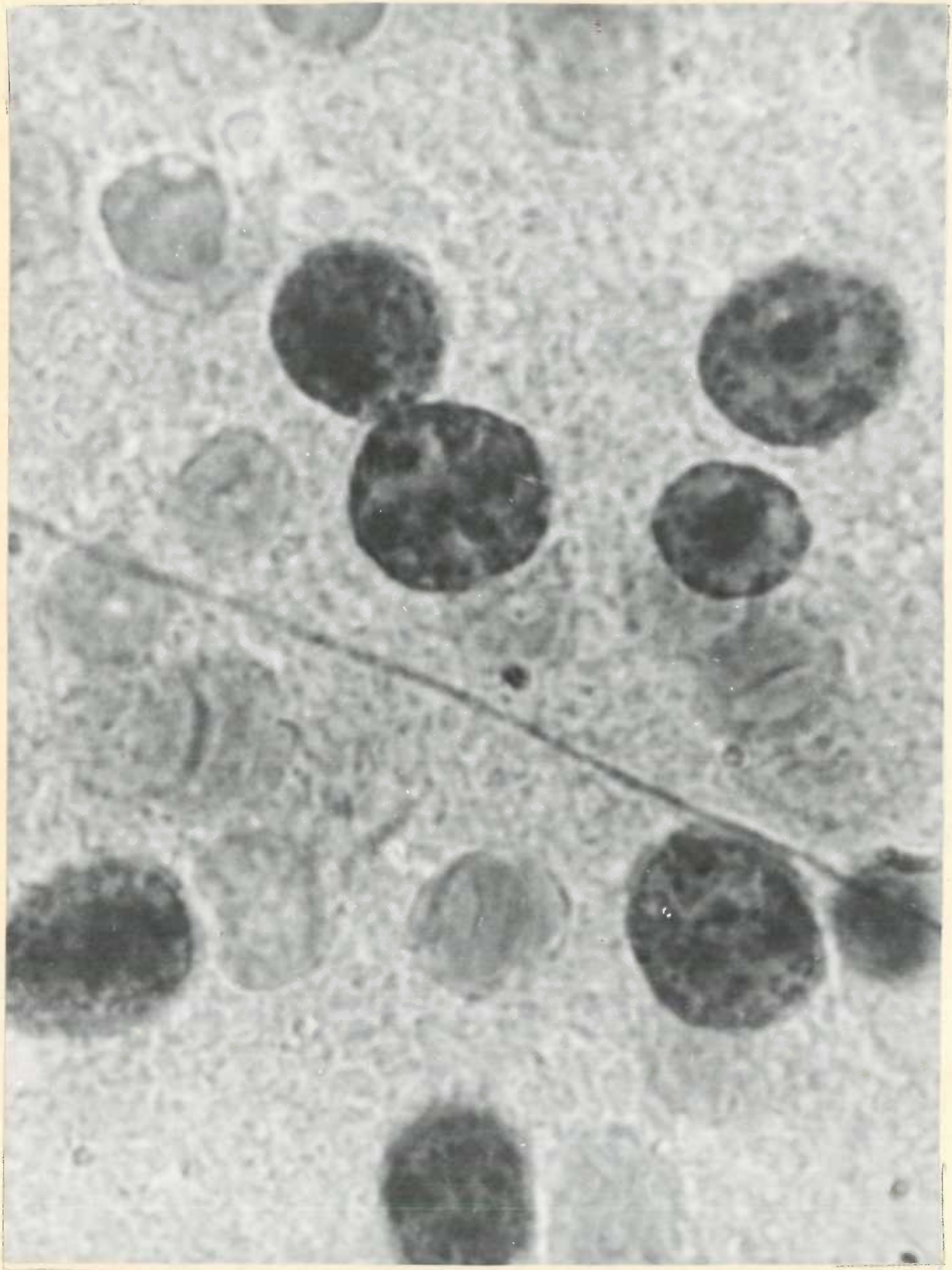
Many of the carcinogenic rat group also steadily gained in weight, but the gain was not nearly comparable to that seen in the control group and the animals did not have the prosperous, well-fed appearance of the control rats. Other rats in the carcinogenic group only maintained their initial weight. Still others gradually decreased in weight and eventually had to be sacrificed, often, because of extreme debilitation. These rats usually had an advanced pseudo-tuberculous lung disease. Often these rats would suddenly start to lose weight 1-2 weeks before death, as seen in the control Rat # 135, because of advanced lung disease. Animals which were given gastric washings with 95% ethyl-alcohol would lose a lot of weight by the next weighing, then gradually start uphill again. The rats born of a mother which had been on m'MeDAB, and then fed m'MeDAB themselves, # 122-A and # 122-B, reacted differently. Rat # 122-A, the female, never weighed

more than 15 grams, never gained any weight, and was killed at 7 weeks of age because of extreme debilitation. Rat # 122-B, the male, lived 19weeks and gained up to 100 grams, which is not comparable at all to similar rats of that age. The rats which developed large, full-blown tumors, would suddenly gain weight 2-3 weeks before death, presumably due to rapid growth of the tumor.



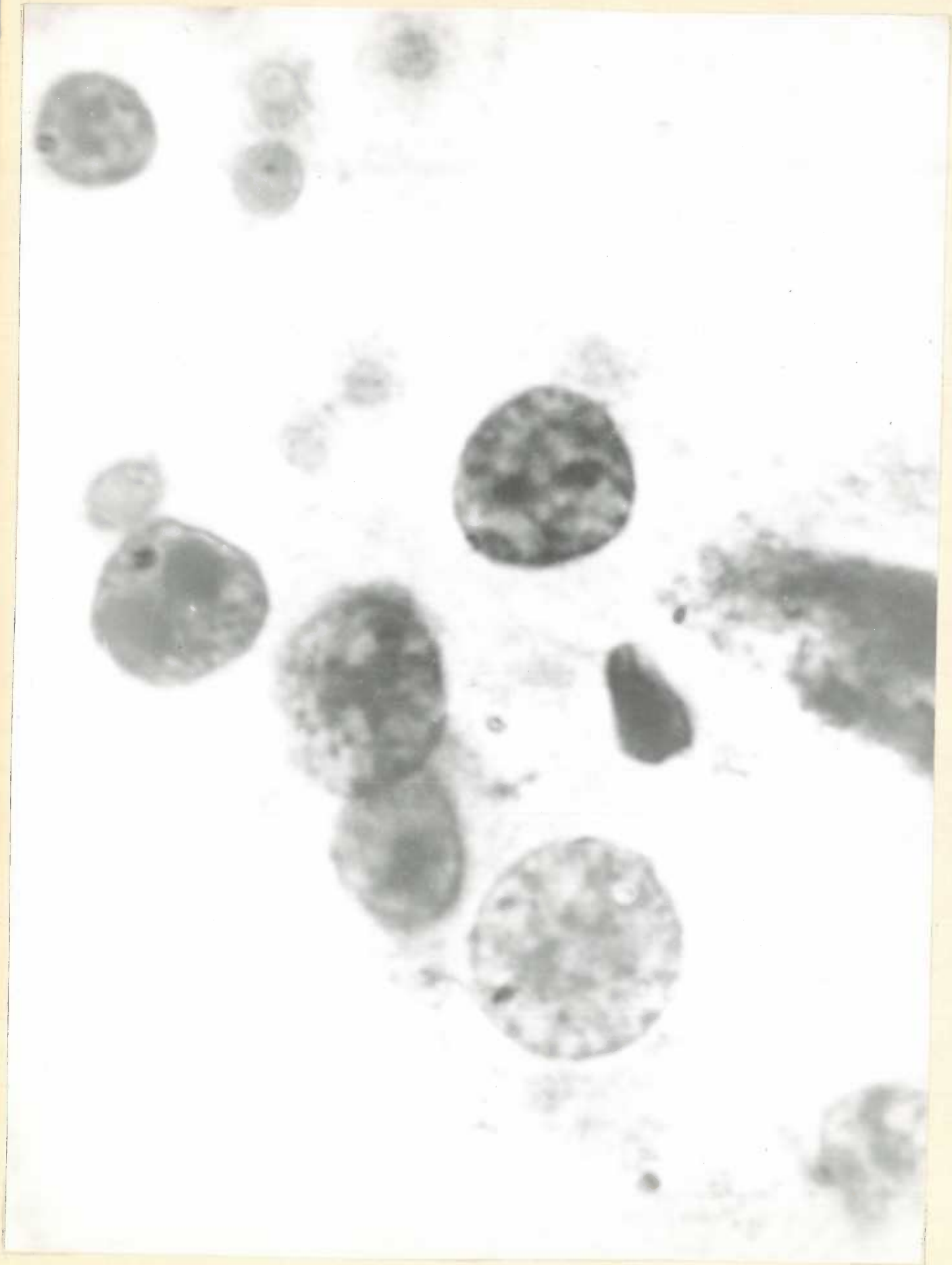
Rat # 63. 9 weeks basic ration.
VandeGrift's fixation.

In the center of the photograph are two normal liver nuclei. The larger one is a hepatic nucleus and the smaller one is a bile duct nucleus. The hepatic nuclear chromatin is homogeneous and finely granular with occasional net knots. There are probably four indistinct, round, uniform nucleoli. The bile duct nuclear chromatin resembles the hepatic nuclear chromatin except for its greater density. In the left lower corner is seen part of another rather blurred hepatic nucleus. Chromatin is similar. Both these hepatic nuclei are at the upper limit of normal hepatic nuclear size.



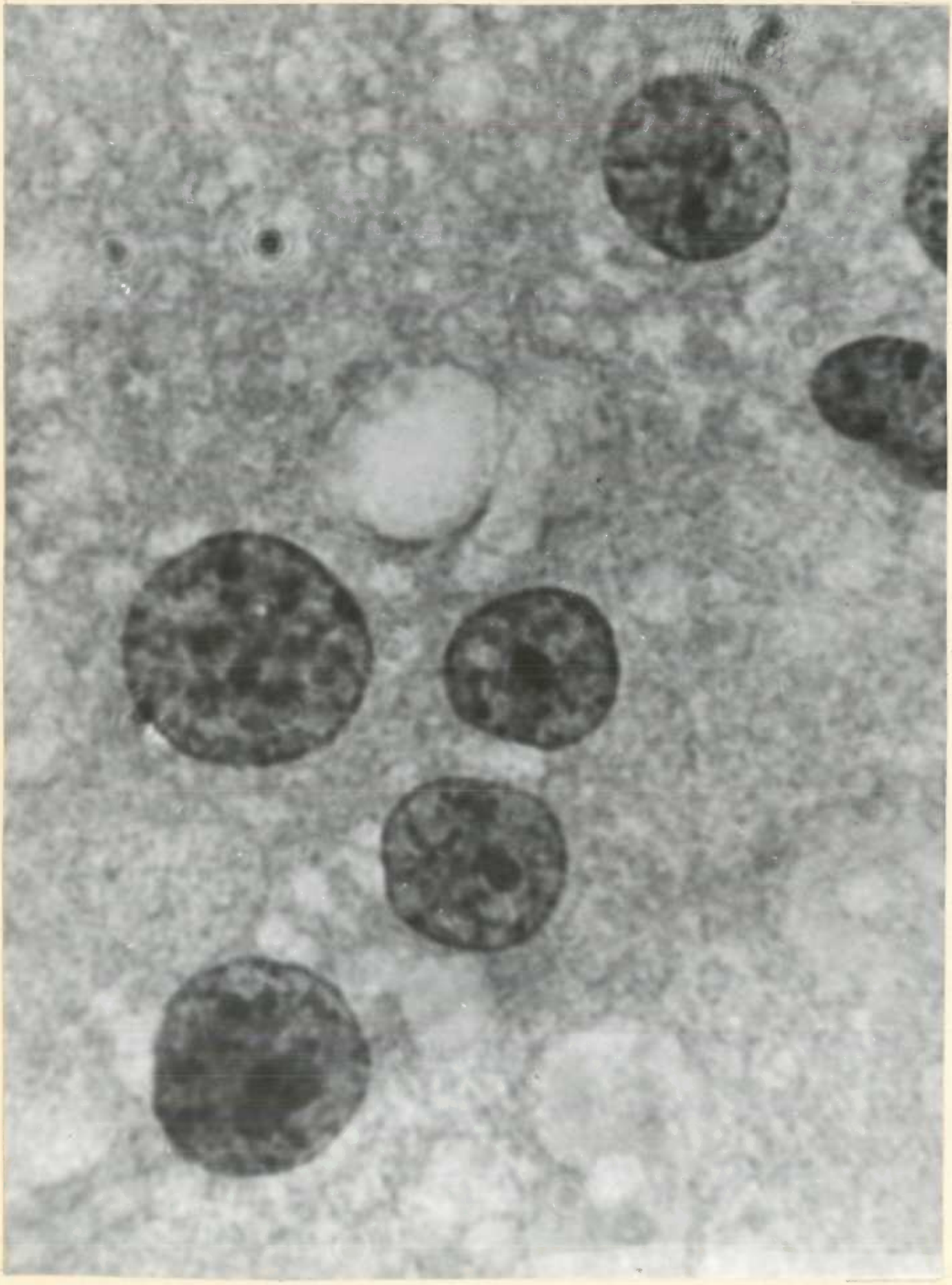
Rat # 83. 9 weeks basic ration.
Methyl alcohol fixation.

Six hepatic nuclei and two smaller bile duct nuclei are present. The scattered, round, shadowy forms in the background are red blood cells. Notice that with the methyl alcohol fixation, chromatin granules and detail are not as clear-cut as with the VandeGrift's fixation. Chromatin is homogeneous, finely granular with occasional net knots and uniform, round nucleili. The hepatic nuclei are round and uniform in size and shape.



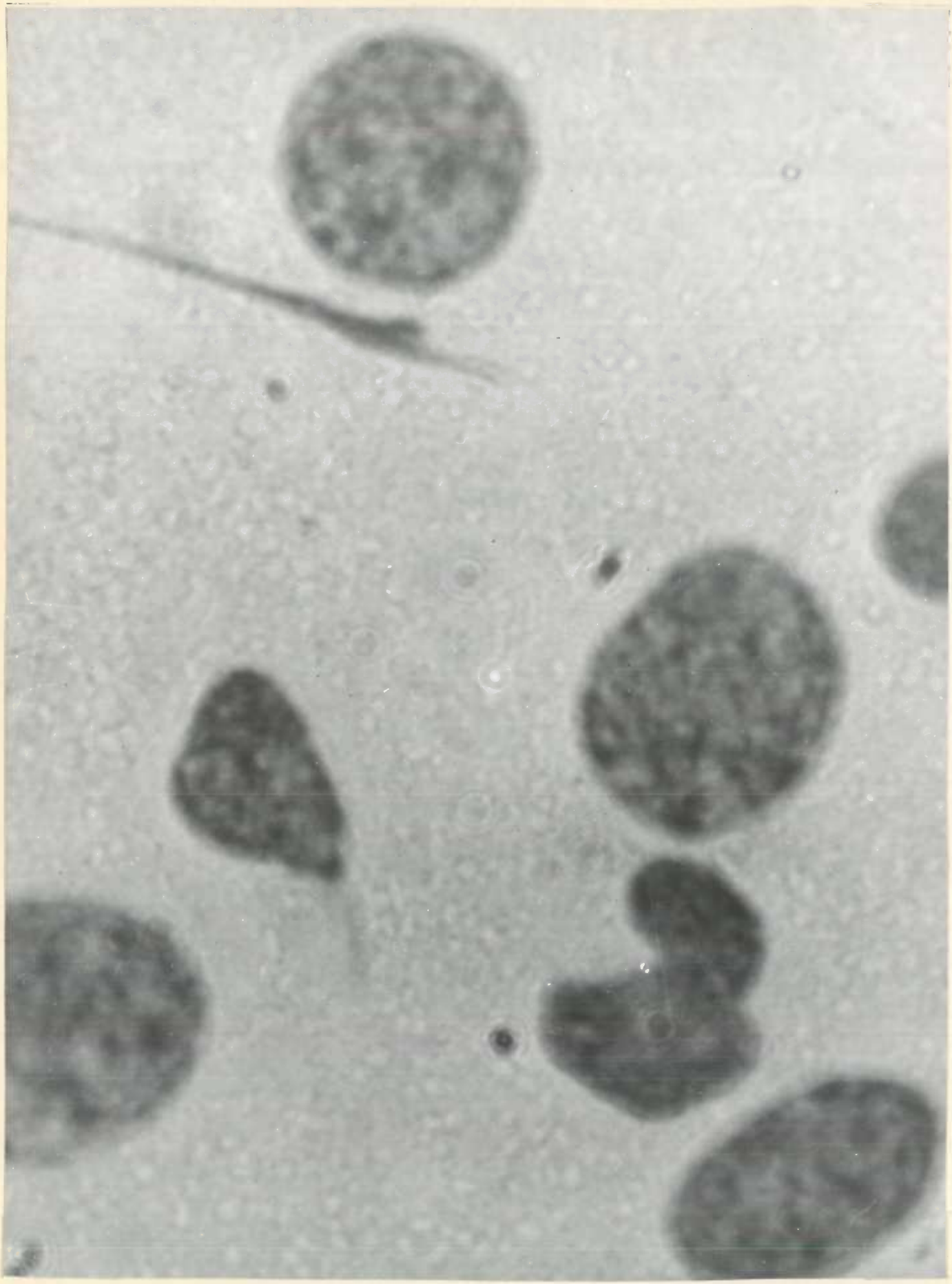
Rat # 15. 3 weeks m'MeDAB.
Ether-alcohol fixation.

No change is seen in these nuclei except for a slight aniscocytosis and poikilocytosis. Chromatin detail is less clear-cut with this fixation than either the methyl alcohol or VandeGrift's fixation.



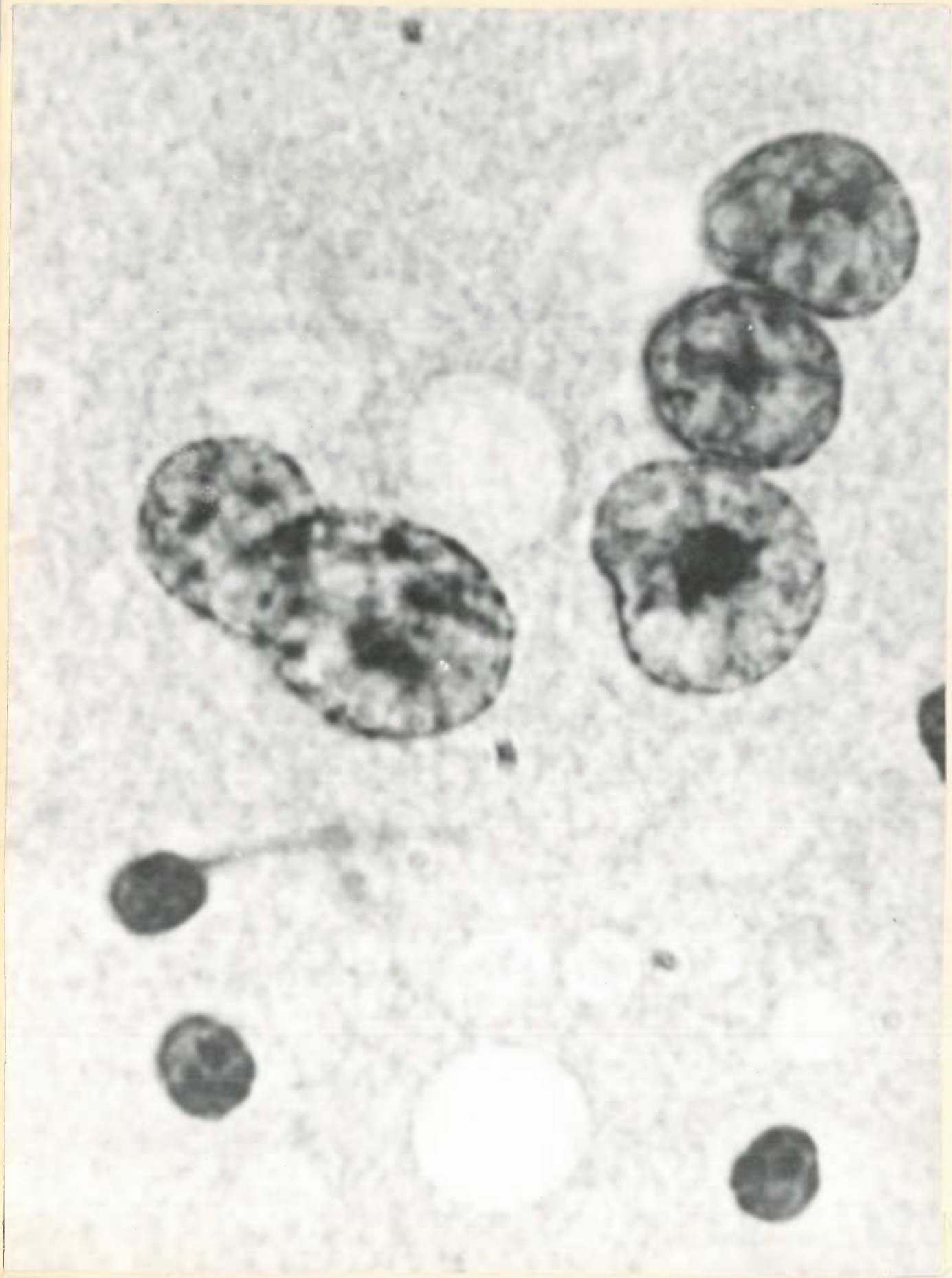
Rat # 71. 6 weeks m'MeDAB.
VandeGrift's fixation.

These nuclei are apparently taken from an area without much change. The nuclei are of larger, over-all size, and chromatin density is diffusely and slightly increased.



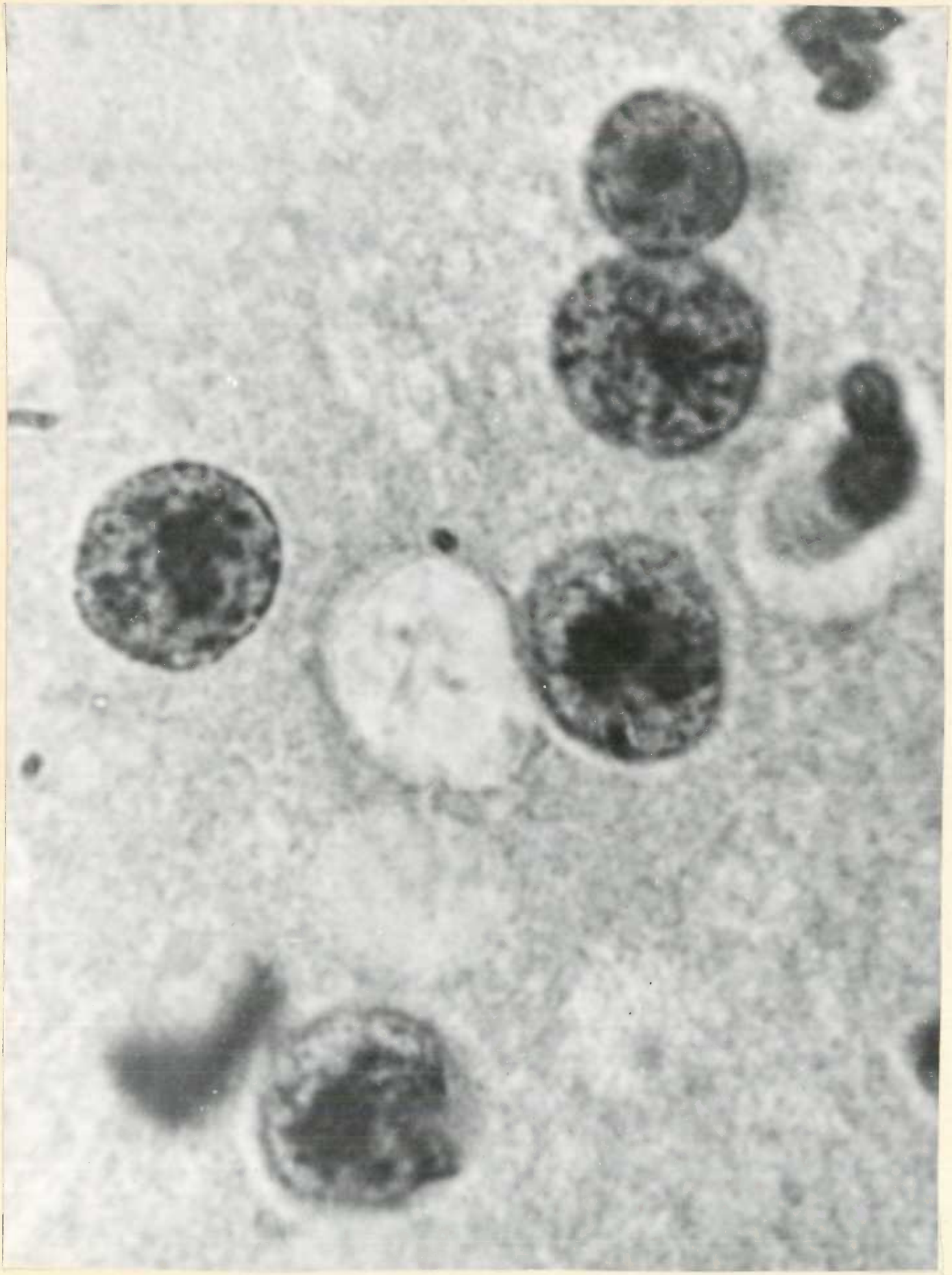
Rat # 73. 7 weeks m'MeDAB.
VandeGrift's fixation.

These are drug effect nuclei. Notice the diffuse increase in chromatin density. Nuclei are definitely larger, and there is some aniscocytosis and poikilocytosis. Unfortunately, this photograph is not quite in focus.



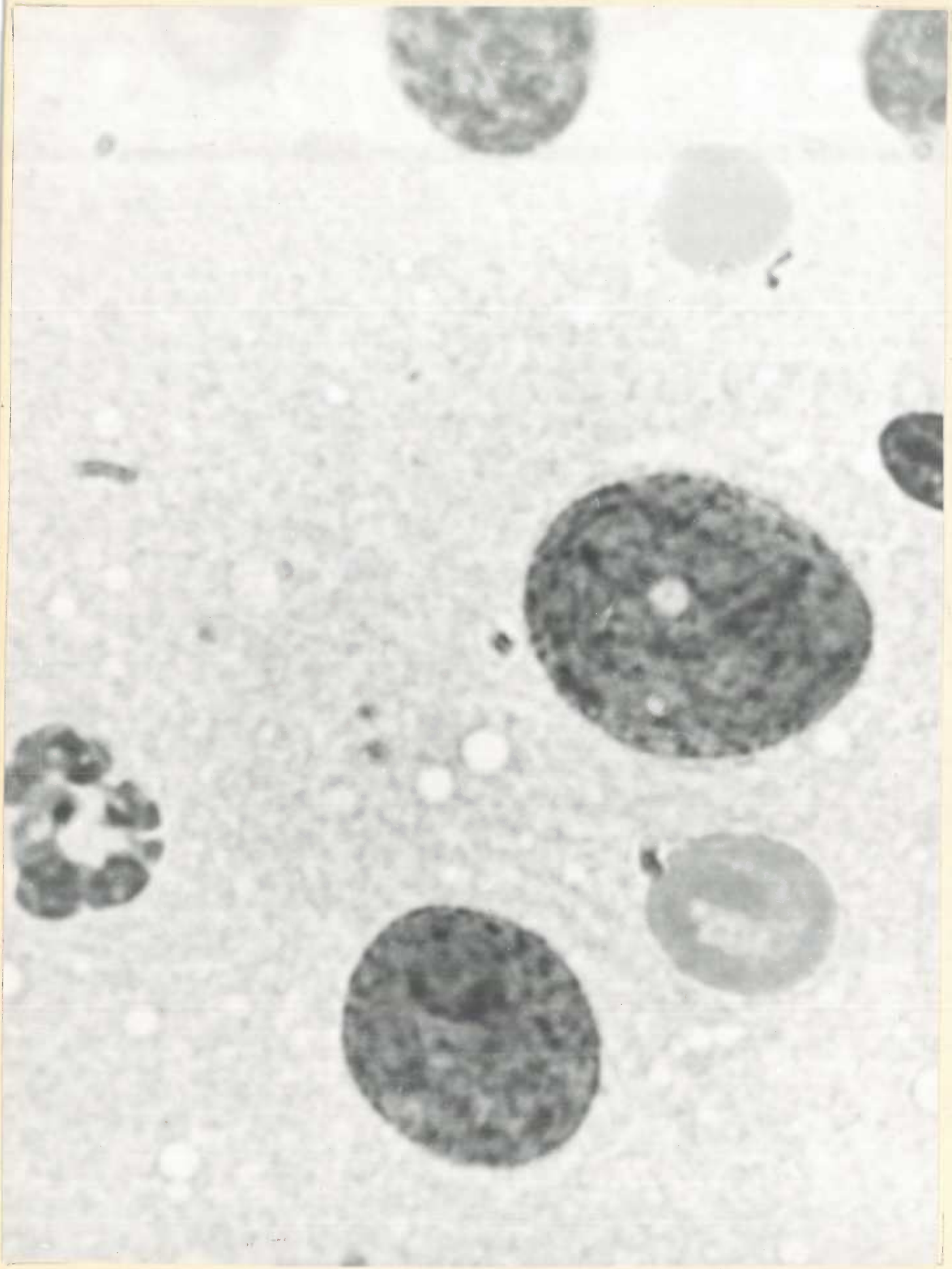
Rat # 65. 8 weeks m'MeDAB.
VandeGrift's fixation.

These nuclei are examples of the type seen when the drug effect nuclei decrease in number, as they sometimes do at about 8 weeks. One of the nuclei has an enlarged central nucleolus. All have dark nuclear membranes and much vesiculation and vacuolation in a somewhat hypochromatic chromatin. These nuclei are generally smaller than the drug effect nuclei and resemble somewhat small cell adenocarcinoma. There is slight aniscocytosis and poikilocytosis.



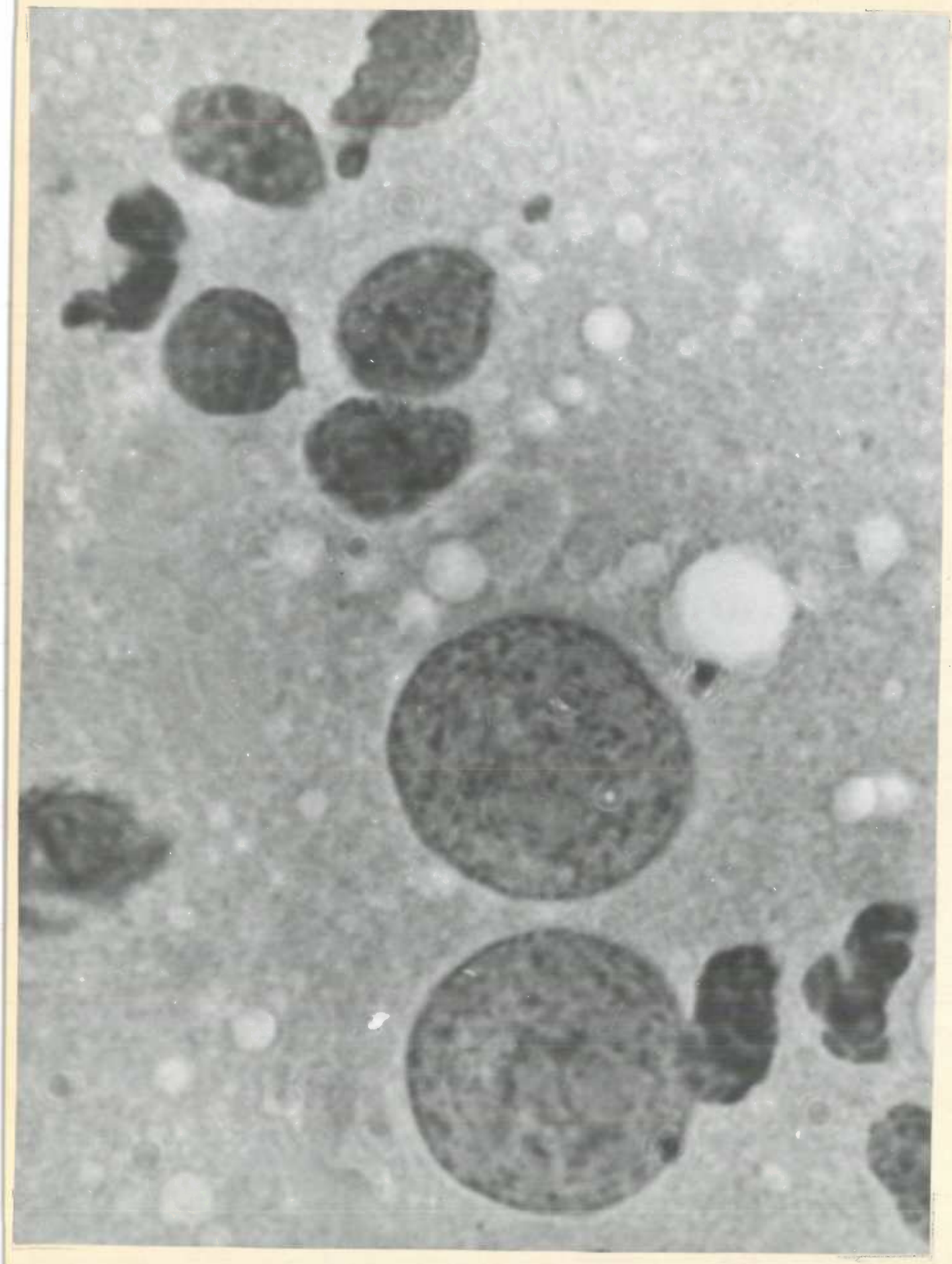
Rat # 64. 9 weeks m'MeDAB.
VandeGrift's fixation

These are the type of nucleus sometimes seen as early as six weeks. The nuclei are not increased in size. Chromatin is irregularly clumped and there is chromatin condensation about central nucleoli. Nuclear membranes are prominent. There is some anisocytosis and poikilocytosis.



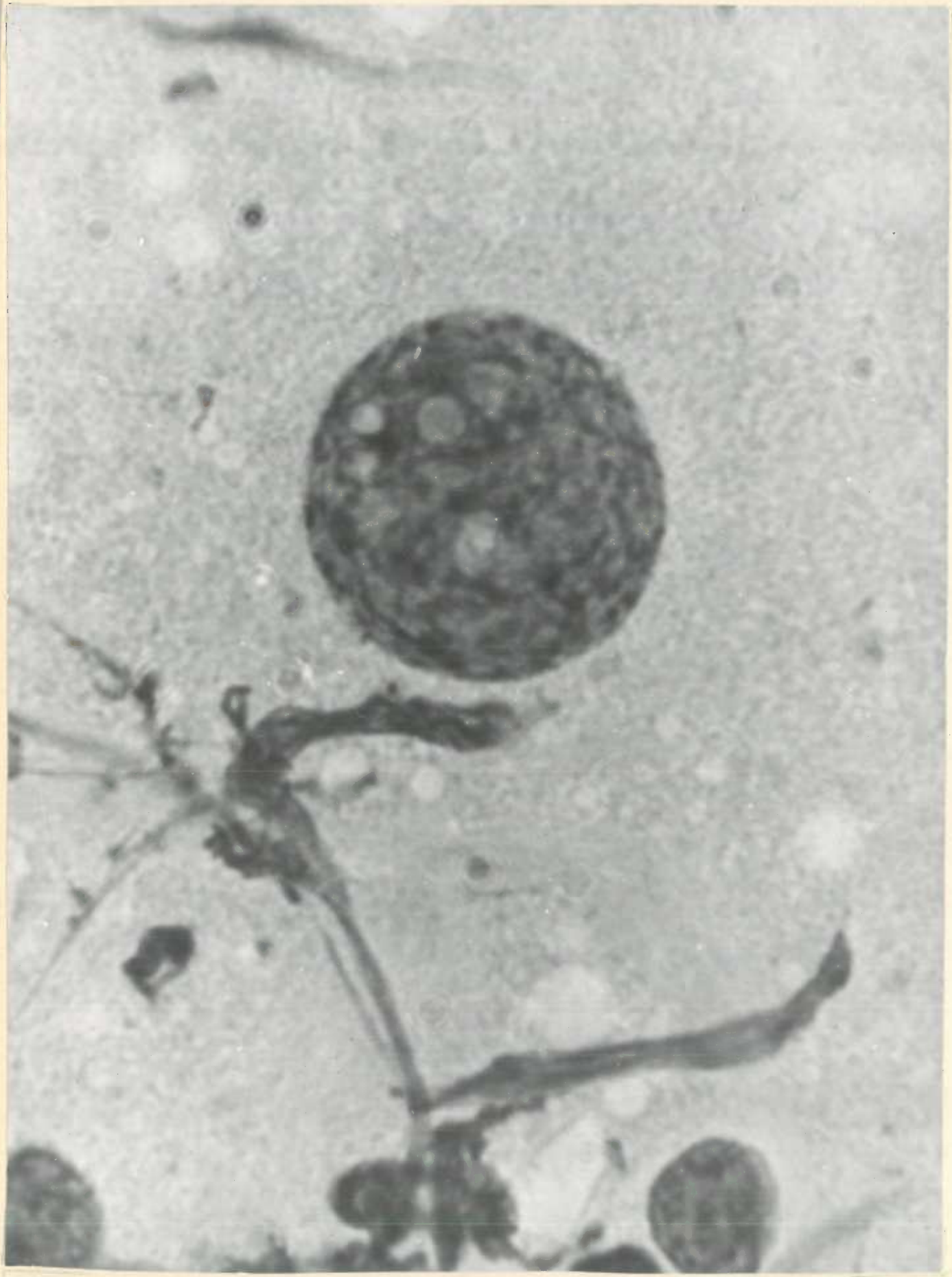
Rat # 40. 9 weeks n'MeDAB.
Ether-alcohol fixation.

These are typical drug effect nuclei. They are both enlarged and the larger one is a giant nucleus. Chromatin is diffusely increased in density, but the arrangement is still the homogeneous, finely granular type of the normal liver nucleus. Note the two clear vacuoles in the giant nucleus. There is a granulocyte at the left.



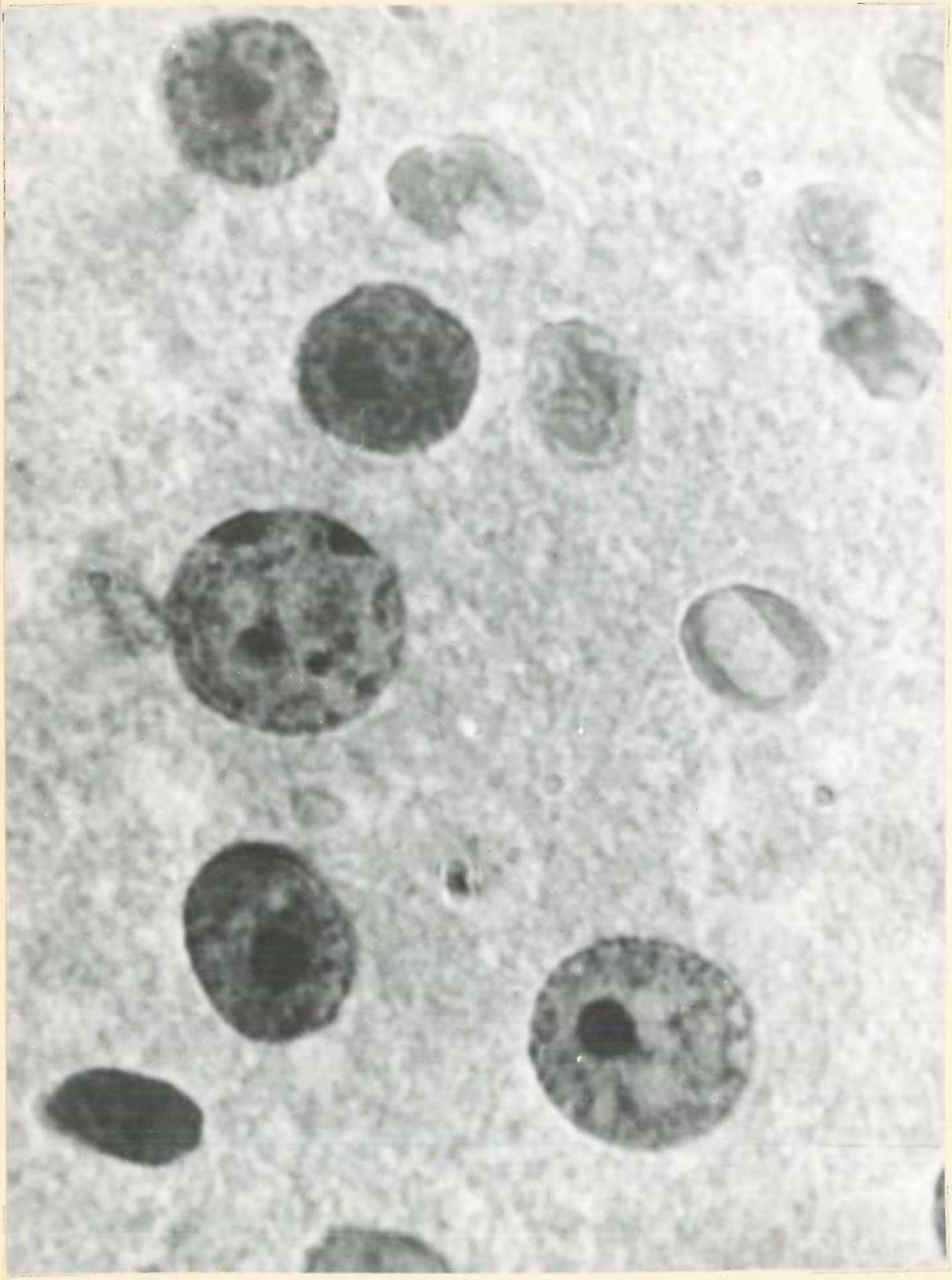
Rat # 74. 10 weeks m'MeDAB.
VandeGrift's fixation.

These are, also, typical drug effect nuclei. There is an increase in nuclear size and a diffuse increase in chromatin density. Chromatin is of normal, finely granular distribution. The bile duct nuclei show the drug effect too. There is aniscocytosis and poikilocytosis.



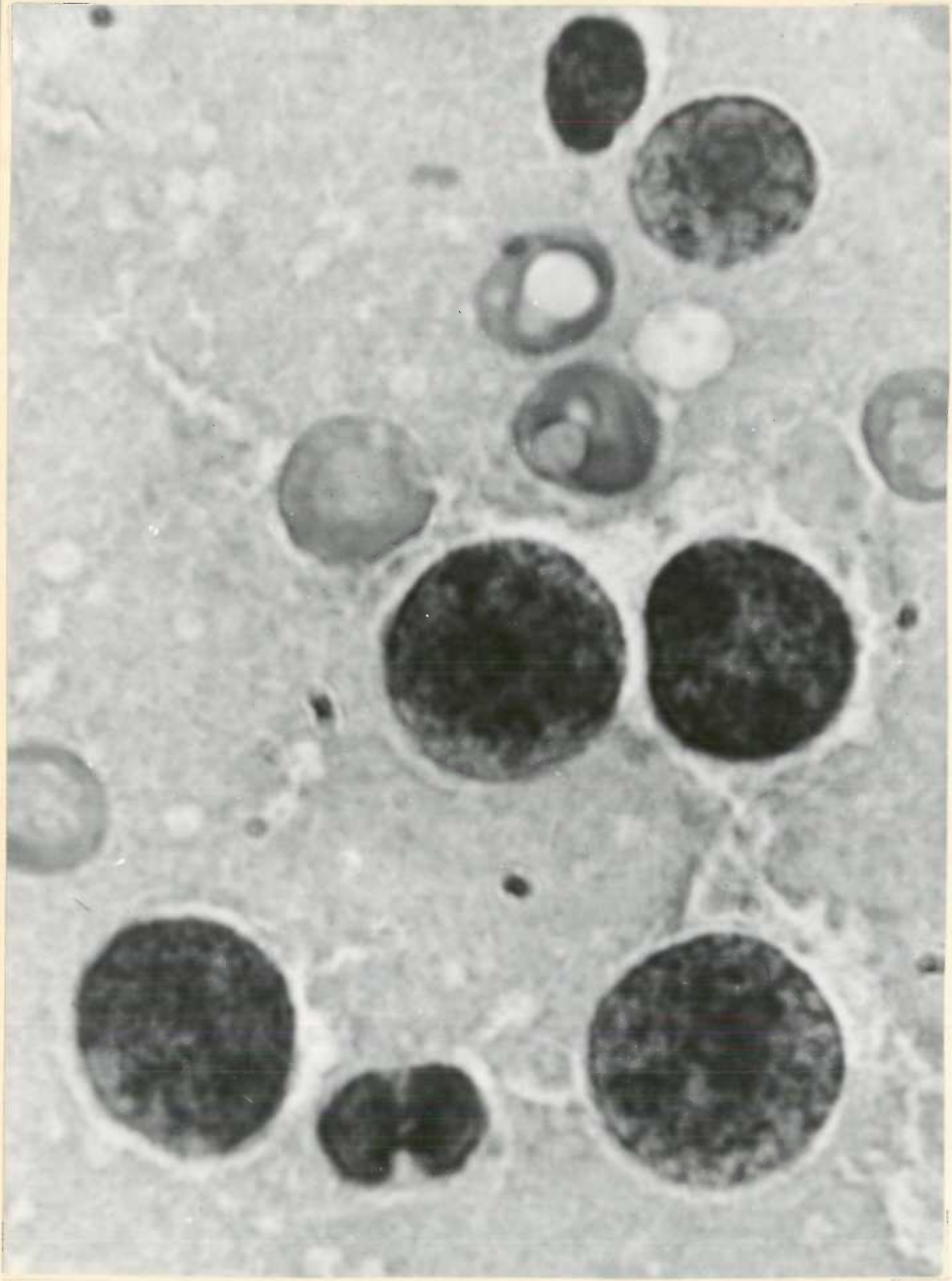
Rat # 74. 10 weeks m'MeDAB.
VendeGrift's fixation.

This is a giant drug effect nucleus. Chromatin is homogeneous, finely granular and diffusely increased in density. Two or three clear vacuoles are present in the nucleus. Below this large hepatic nucleus, are some fibrin strands and some bile duct nuclei.



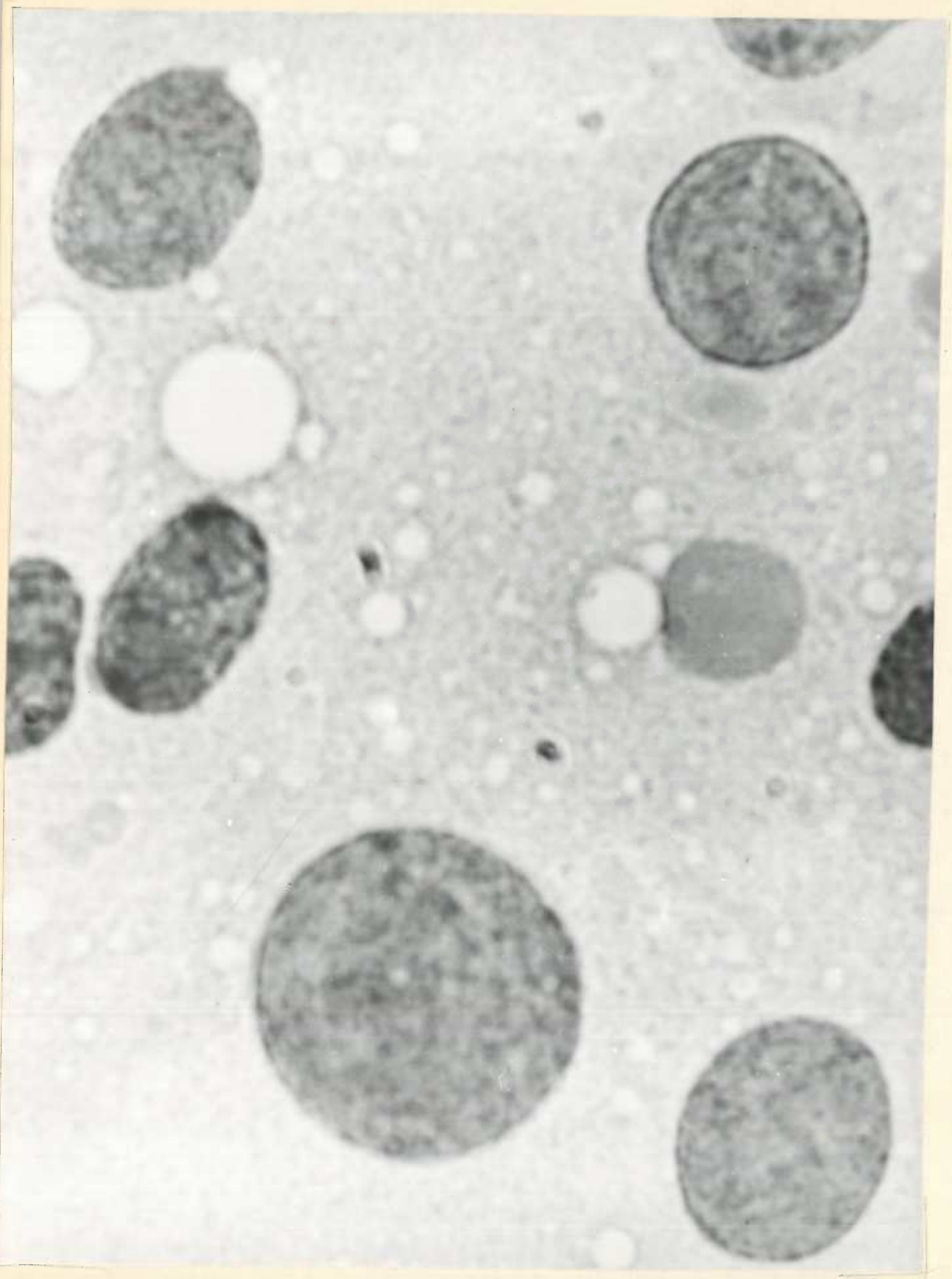
Rat # 37. 11 weeks m'MeDAB.
Ether-alcohol fixation.

These nuclei are about normal size. There is anisocytosis and poikilocytosis. Notice the oval nucleus at the lower left. Chromatin is of the drug effect type -- homogeneous and finely granular with a diffuse increase in density. Three of the nuclei have uniform, round, enlarged nucleoli.



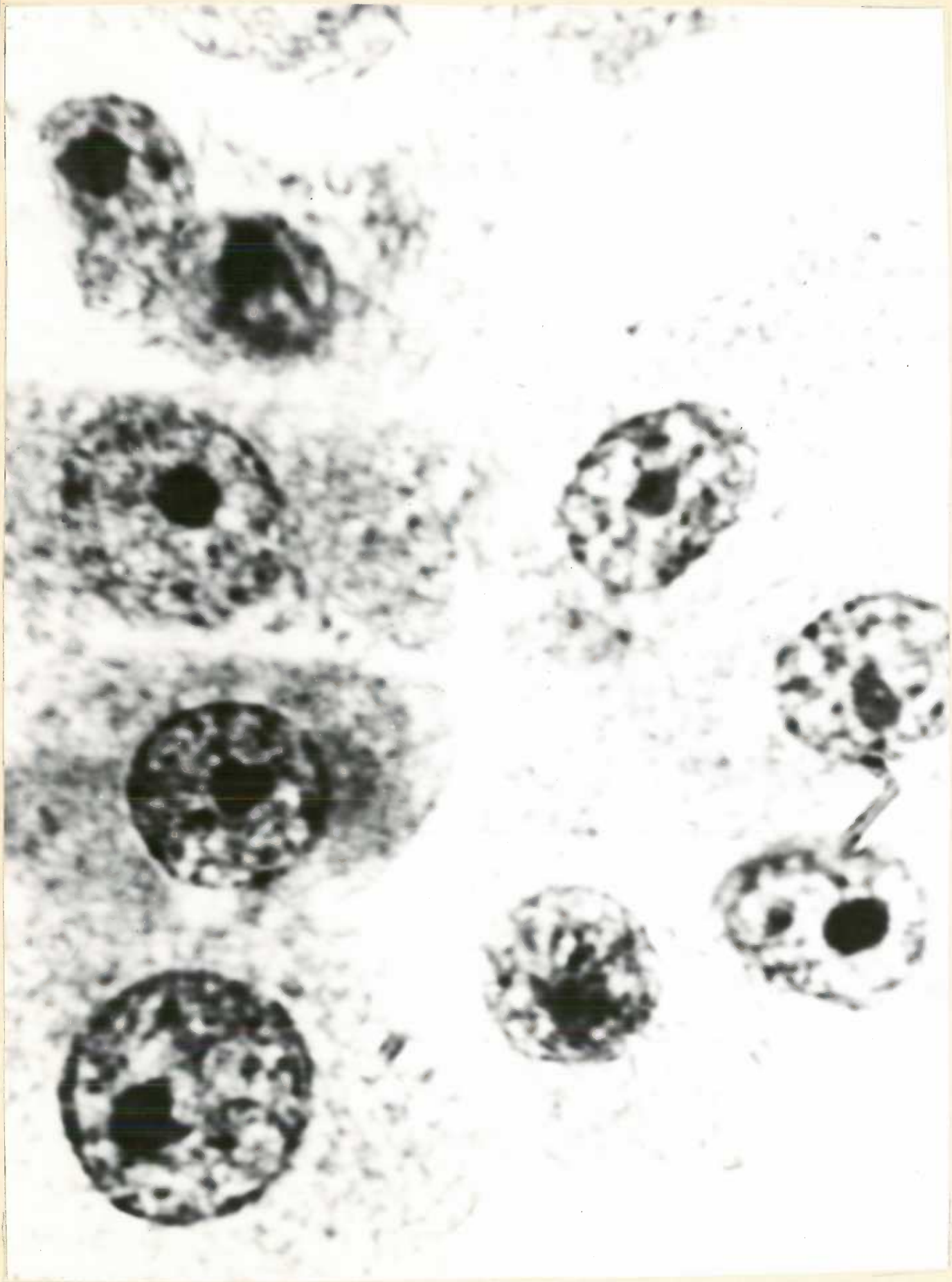
Rat # 38. 11 weeks m'MeDAB.
Ether-alcohol fixation.

These nuclei are of larger size than normal. There is some poikilocytosis and aniscocytosis. The chromatin is of the drug effect type -- homogeneous, with a diffuse increase in density. There may be a slight clumping of the chromatin here.



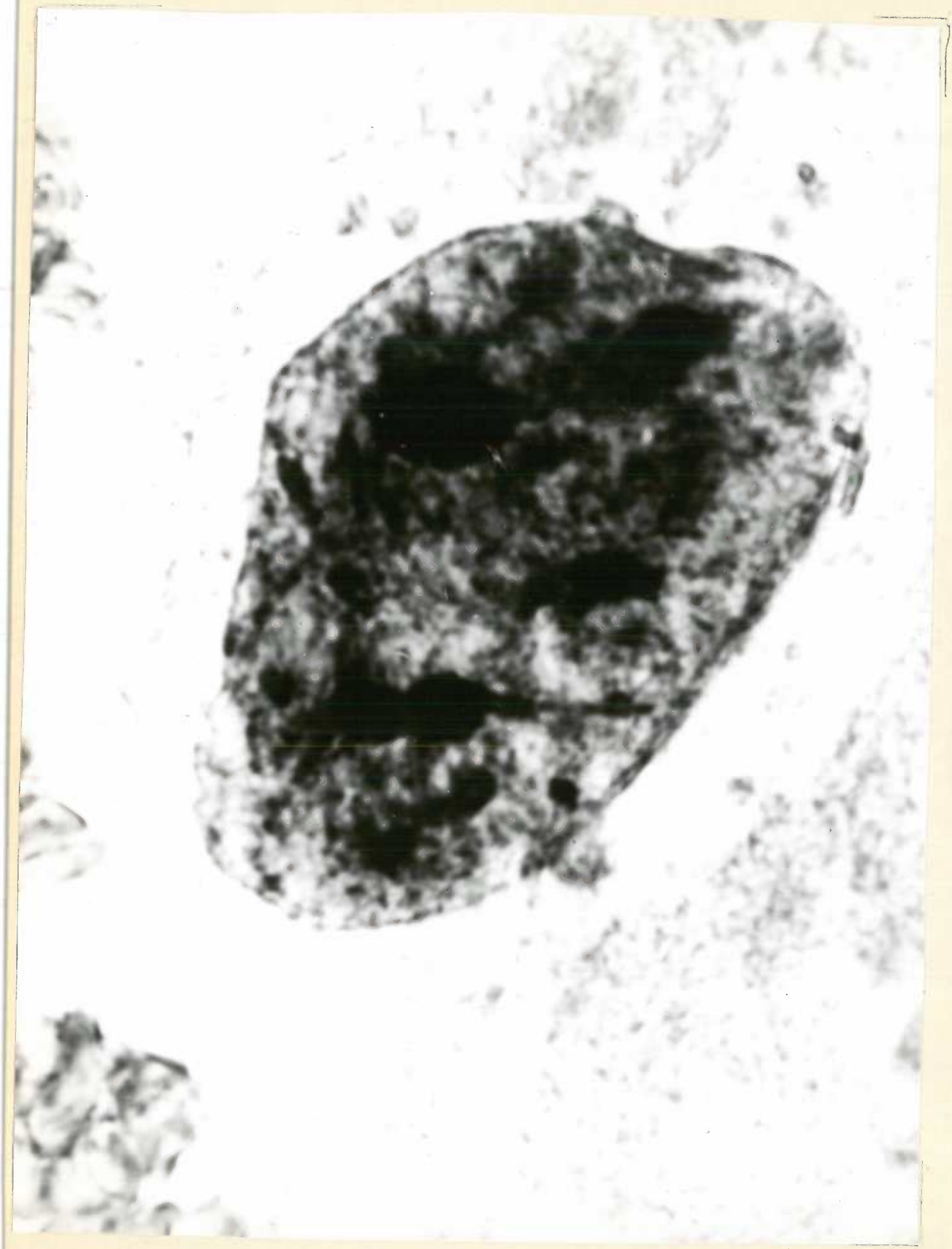
Rat # 39. 12 weeks m'MeDAB.
Ether-alcohol fixation.

These again, are drug effect nuclei. The nucleus at the center of the lower half of the picture is a giant nucleus. There is aniscocytosis, poikilocytosis and the usual chromatin distribution of drug effect nuclei.



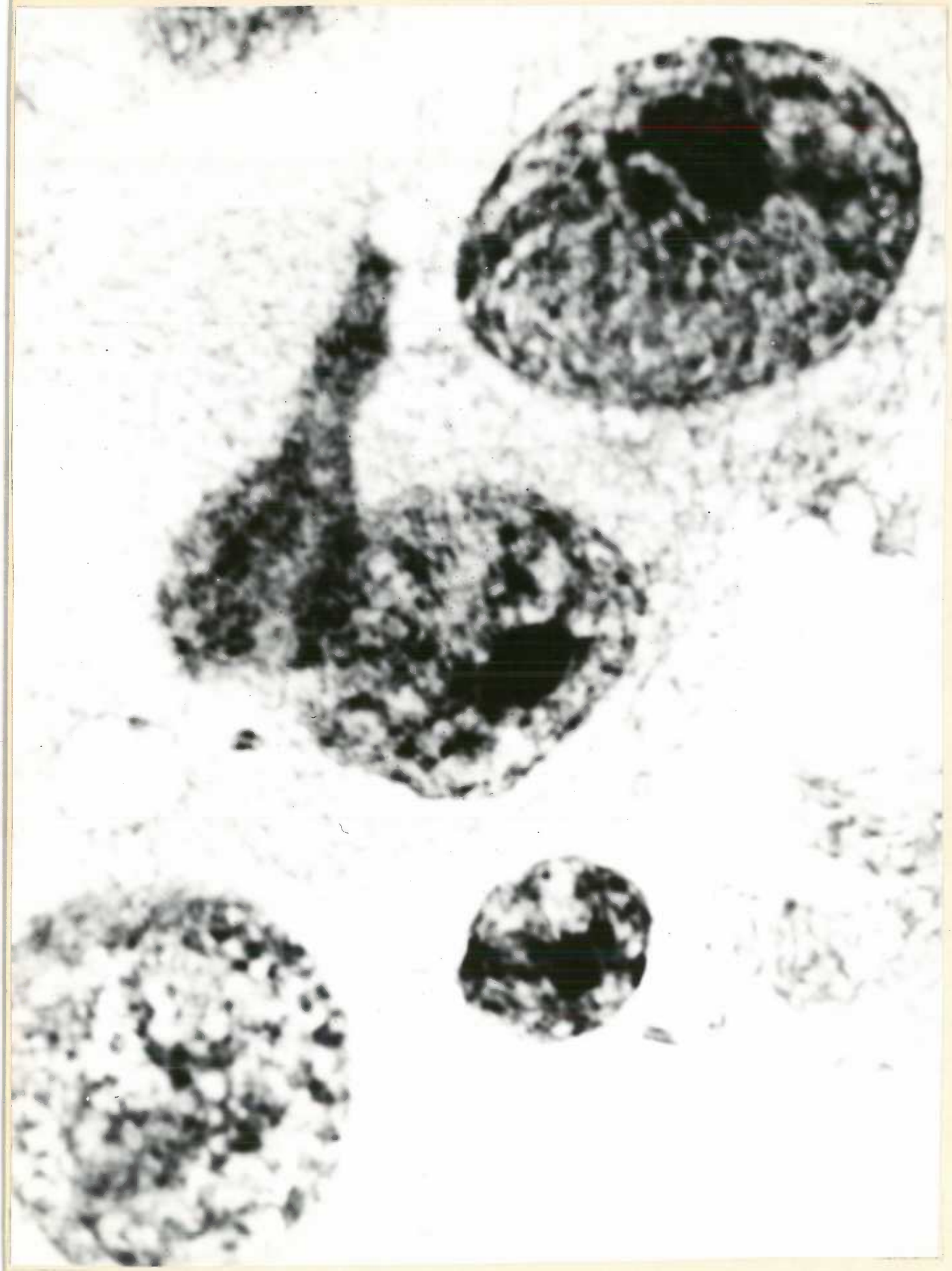
Rat # 139. 20 weeks m'MeDAB.
VandeGrift's fixation.

These are small cell adenocarcinoma nuclei. Notice how similar they are to the photograph of Rat # 65, 8 weeks m'MeDAB. There is aniscocytosis and poikilocytosis. Chromatin is vesiculated, vacuolated and irregularly clumped. Reticulum is prominent. Nucleoli are enlarged and somewhat irregular. Most of these nuclei are within the normal size range. Some of the nuclei at the left retain some cytoplasm.



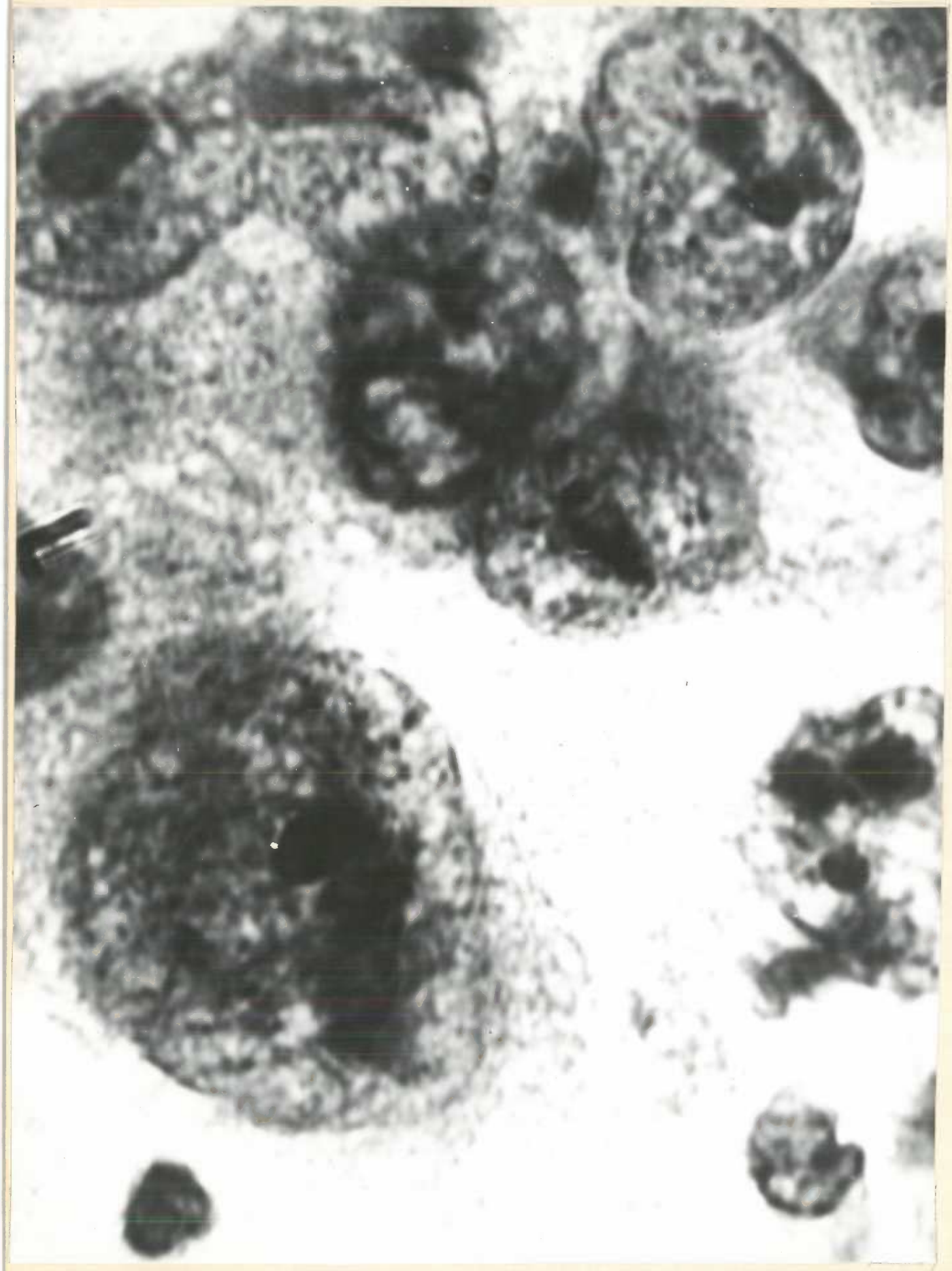
Rat # 139. 20 weeks m'MeDAB.
VandeGrift's fixation.

This is a bizarre neoplastic nucleus. It is of tremendous size -- this photograph is <u>not</u> enlarged more than the others. The shape is atypical and irregular. Chromatin is very pleomorphic, irregularly clumped and vesicular. Many bizarre and enlarged nucleoli are present. There are connecting strands between some of the nucleoli.



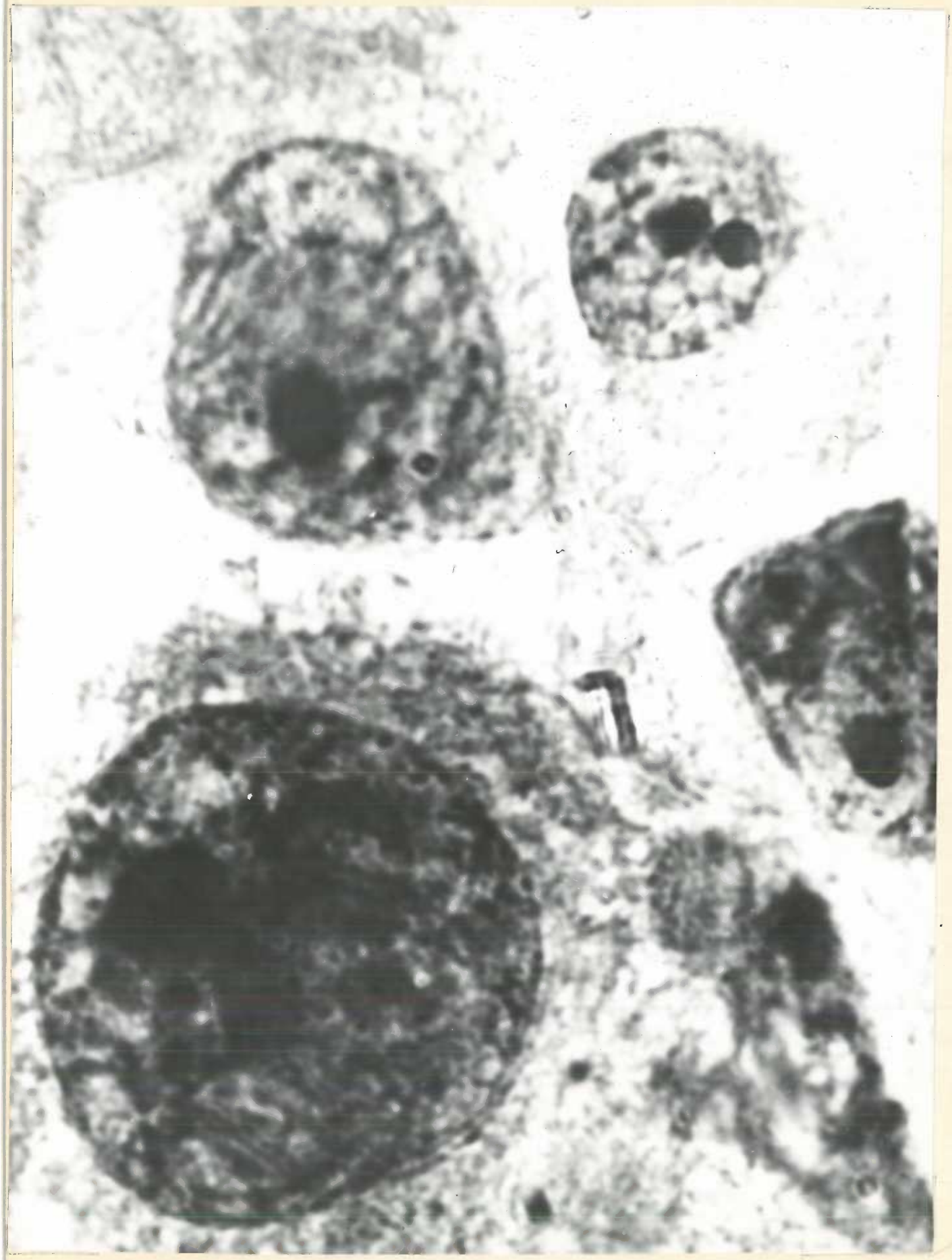
Rat # 159. 20 weeks m'MeDAB.
VandeGrift's fixation.

These are more neoplastic nuclei. Three are of tremendous size with giant, irregular nucleoli and bizarre, vesiculated, irregularly clumped chromatin. There is marked aniscocytosis and poikilocytosis. A smaller, similar nucleus is at the lower right and a disintegrated nucleus is overlying one of the large bizarre ones.



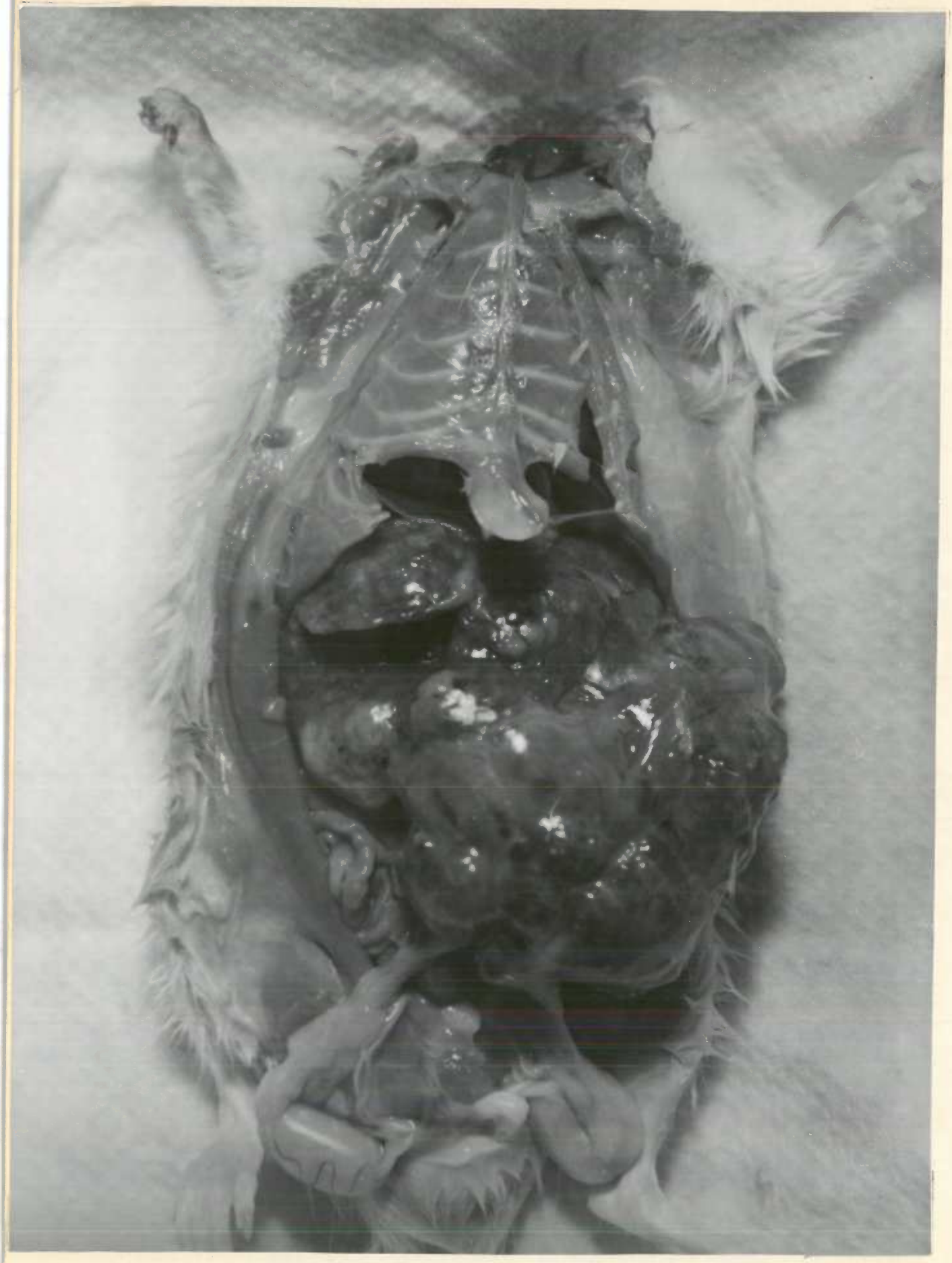
Rat # 139. 20 weeks m'MeDAB.
VandeGrift's fixation.

These are probably neoplastic nuclei. They demonstrate marked anisocytosis and poikilocytosis. They are of giant size with giant pleomorphic nucleoli. The chromatin, although much more vesiculated, resembles closely drug effect chromatin. They may be hepatoma nuclei.



Rat # 188. 20 weeks m'MeDAB.
VendeGrift's fixation.

These are still more giant, pleomorphic, neoplastic nuclei with giant pleomorphic nucleoli, aniscocytosis, poikilocytosis and vesiculated, vacuolated, irregularly clumped chromatin.



Rat # 138. 20 weeks m'MeDAB.
Gross specimen.

This is a photograph of a carcinomatous liver in situ. It completely fills the abdominal cavity. Adhesions to the fascia of the testicles can be seen. There are many different tumors, one much larger than the others. The uninvolved liver surface is coarsely nodular. There are many areas of hemorrhage and necrosis in the largest tumor.

III. Discussion and Summary:

It is obvious from a review of the pathology that although the liver was always diffusely involved with the precancerous change, not every lobule was affected similarly. Thus, often seen are different pieces of liver from the same rat with somewhat different degrees of precancerous change. The same thing applies to the cancerous changes found as well as the precancerous ones. For this reason, it must be realized that the liver smears are taken blindly and could, and apparently did in some cases, miss the areas of greatest precancerous or cancerous change. By "blindly" it is meant that the degree of change and architectural picture of the tiny piece of liver selected arbitrarily for smearing, (unless obvious tumors are present and the smear was made at autopsy) cannot be known. In addition to this factor, there is the variation in response to the carcinogen between the individual animals. Not every animal died of its carcinoma at exactly the same time, nor did every animal have the same kind or kinds of tumor as every other animal. Moreover, precancerous changes had not progressed to exactly the same stage at exactly the same time in exactly the same manner in every animal. Therefore, the progress of events in such a process as this carcinogenesis cannot be followed and reported with the mathematical precision one would like.

The very first change taking place in the smeared hepatic nuclei, apparently, is an increase in the average nuclear size. This was observed as early as one week and seems to occur before any obvious chromatin change does because it can be observed in the majority of the nuclei in the first 4-5 weeks smears, when only a few or none of these nuclei are also involved in chromatin alteration. The first change in chromatin was seen as early as 2 weeks. Two types of change were seen: (1) An occasional hepatic nuc-

leus, particularly the larger-sized ones, would demonstrate a diffuse increase in chromatin density, but the chromatin would usually retain its homogeneous, finely granular arrangement. In a smaller number of these nuclei, the chromatin arrangement would be slightly clumped with a slight increase in the size of the chromatin granules, but still diffuse and homogeneous. These nuclei are referred to as drug effect nuclei. (2) In a few of the early smears a much rarer type of change was observed. Strands of chromatin would connect some of the chromatin net knots irregularly throughout the nucleus, resulting in an unusual prominence of the reticulum. I believe that in some of the nuclei, this appeared to be an irregular clumping of the chromatin. By 3 weeks, the change in average size and the diffusely increased chromatin density in occasional nuclei was present to varying degrees in all the rats, as well as some anisocytosis and poikilocytosis. Before 3 weeks, although nuclear changes were seen in the smears, sectioned material was apparently normal. From then on, until about 8 or 9 weeks, these nuclei with chromatin change were found in increasing numbers until the majority of all the nuclei in the smear would be involved. Occasional giant nuclei, seen as early as 3 weeks, were observed. Anisocytosis and poikilocytosis gradually increased. There seemed to be many nuclei with increased vacuolation. In some of the smears, nucleoli seemed to be increased in number per nucleus and, occasionally, they seemed slightly enlarged, or even somewhat irregular and fusiform in shape. The most striking change, however, was the chromatin change. Changes in the bile duct nuclei paralleled those of the hepatic nuclei. In every slide, varying numbers of nuclei with apparently normal chromatin were seen. These were usually the smaller or medium-sized nuclei. In the sectioned material from 3-9 weeks, there is gradual development of a nodular cirrhosis and an

adenomatous hyperplasia of hepatic and bile duct cells.

As early as 9 weeks, another type of cell began to be observed. These were small or medium-sized nuclei with prominent central nucleoli and dark nuclear rims. The chromatin of these nuclei was most often hypochromatic, vesiculated and vacuolated. However, often it would be normal, and then these nuclei resembled very closely normal nuclei and often there would be the usual diffuse increase in chromatin density. Then they closely resembled the drug effect nuclei. Occasionally, chromatin would be condensed about the nucleolus. (This was seen as early as 7 weeks). Occasionally, the central nucleolus would be enlarged. When this was so, these nuclei could be differentiated from the small cell and large cell adenocarcinoma nuclei seen later only by the company which they kept. Among the true adenocarcinoma nuclei, are often seen nuclei of the same type which are so pleomorphic and bizarre that they are undoubtedly neoplastic. These bizarre forms may be giant with very irregular shapes, may have a bizarre hyperchromatic chromatin or may have many nucleoli or very large nucleoli of extremely unusual shapes with connecting links between the nucleoli. Often large vacuoles are seen, basophilic and acidophilic, in the nucleus. The large and small cell adenocarcinoma are differentiated by the size of the nuclei. One could assume that these bizarre forms are the only true carcinoma nuclei, but this is unlikely since the other nuclei seem to be participating in growth in the same area and there are all gradations between the two. They are too closely mixed up with each other and there are not that many of the bizarre forms. At any rate, they are first seen at about the time when the first carcinoma foci are known to be present. Where and when the change takes place it is difficult to say, and whether they arise from the nuclei with apparently normal chroma-

tin seen on every slide or from the drug effect nuclei is open to question. It seems very probable that all the carcinomas must arise from the drug effect nuclei and/or the nuclei with the other varieties of chromatin change.

From 9-14 weeks, or earlier in some cases, there are fewer giant nuclei and drug effect nuclei seen, though the general nuclear enlargement is still present. After 10 weeks, there is a gradual increase in pleomorphism, aniscocytosis and poikilocytosis, especially among the drug effect nuclei. From this time until carcinoma is unquestionably present, in both sectioned and smear material, nuclei and foci are often seen which are identical with the known types of carcinoma nuclei seen in the fully developed tumors. The only difference is that these earlier nuclei are isolated forms and that some of the extremely bizarre variations of them are not seen. The impression is that from about 10 weeks until the full-blown tumor is present, there is a "gathering of the storm" among the cancer nuclei which must be present in small numbers at about 10 weeks.(62, 63) During this period, occasional extremely large nuclei are seen, the drug effect is intensified and nuclei with irregular chromatin clumping are sometimes observed; occasionally hyperchromatic nuclei are seen; occasional giant, bizarrely shaped, elongated nucleoli exist; and most nucleoli seem definitely enlarged.

There are several types of carcinoma nuclei found in the fully developed tumors: The large and small cell adenocarcinoma nuclei have already been described; Some smears show almost exclusively extremely pleomorphic, very hyperchromatic, very bizarre, very large nuclei with just as bizarre nucleoli. These are usually seen in such large numbers only in tumors of the later weeks, (10-20) and are probably from the anaplastic

carcinomas; The hepatoma nuclei appear to be those that have the chromatin structure seen in drug effect nuclei. The differences are that the hepatoma nuclei are generally much larger with extreme variation in size and shape and have giant, irregularly shaped nucleoli which are often increased in number. It seems to be a logical assumption that these are hepatoma nuclei since the hepatomas arise from areas of adenomatous hyperplasia and the drug effect nuclei seem to arise about the same time and increase in frequency the same way the adenomatous hyperplasia seen in the sectioned material does; In some of the slides are smaller nuclei with an elongated shape, sharp corners and often a folding over of one edge or the other in places. The chromatin is usually similar to the drug effect nuclei and nucleoli are usually single, central and small. In some places these nuclei are seen to originate from tubular biliary ductal structures. Apparently, these are cholangioma nuclei. However, in some of the slides of these nuclei are found very bizarre, unquestionably neoplastic forms of this type of nucleus. These are probably bile duct adenocarcinoma. No fibrosarcomas or angiosarcomas, as described by H. L. Richardson and Borsos-Machtnebel[74] were seen. Among the carcinoma nuclei were the apparently unchanged, ever-present drug effect nuclei and some apparently normal nuclei.

The control animals demonstrated none of the liver changes described -- in smears, sections or grossly. The nuclear chromatin was always homogeneous and diffusely finely granular. Nucleoli were not enlarged, uniform and few in number.

B. Nuclear Measurements:

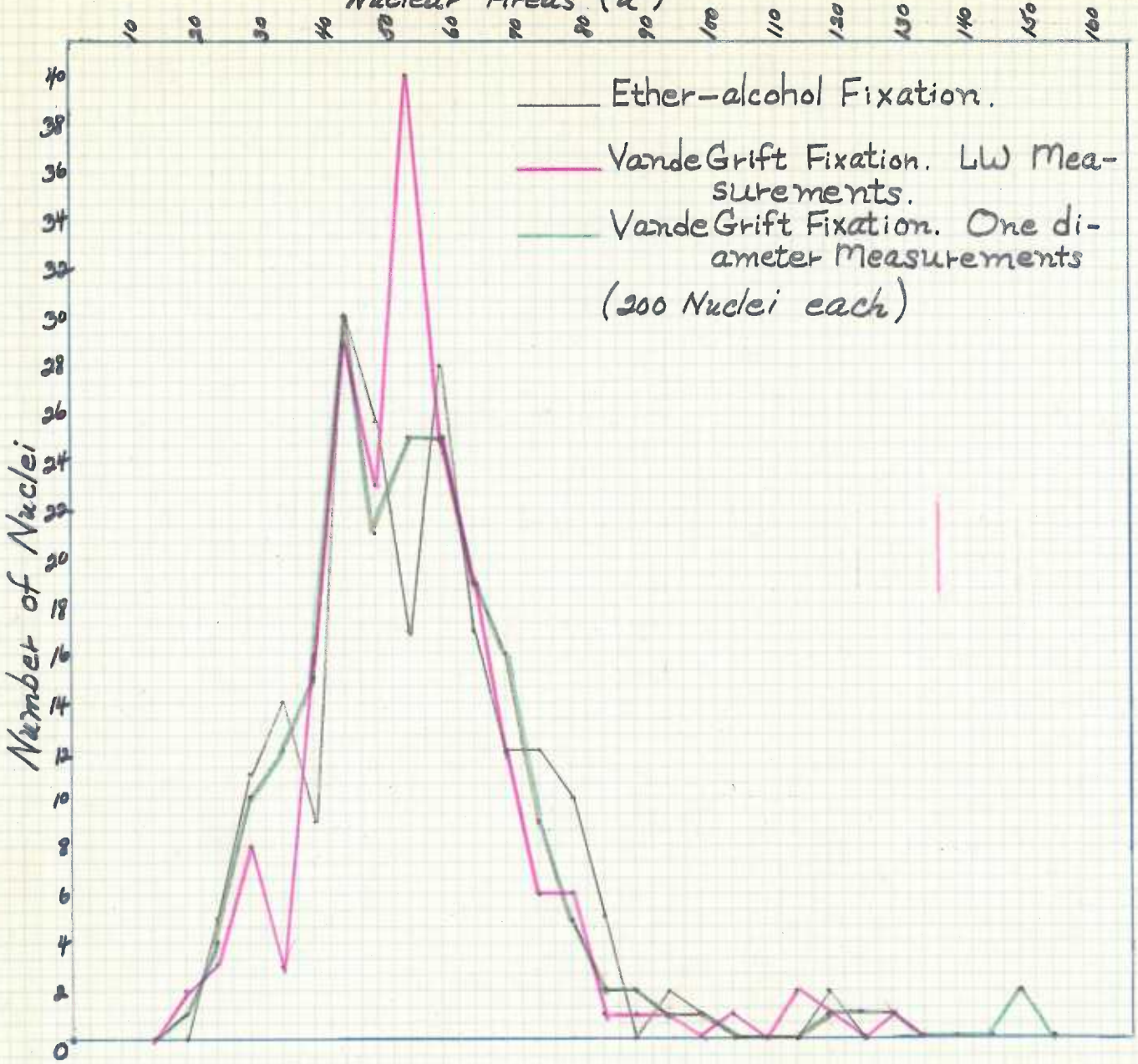
I. Materials and Methods:

Measurements were made of 400 nuclei from Rat # 63, a rat which had been fed the control diet for 9 weeks. 200 nuclei were from ether-alcohol fixed smears and 200 were from VandeGrift fixed smears. The object was to determine whether, since about half of all the liver smears ultimately made were fixed in ether-alcohol and half were fixed in VandeGrift's fixative, there is any significant difference between measurements made of ether-alcohol fixed nuclei and VandeGrift fixed nuclei. If not, measurements could be made of either at various stages of carcinogenesis and compared directly. Since two slides were made with each fixative, 100 nuclei were measured from each slide -- 20 nuclei from five different random fields. Measurements, calculations and graphs were made as already described in the separate section entitled "Materials and Methods in General" except that the length and width of the nucleus was measured instead of one diameter only. Moreover, in calculating nuclear volumes, the average of the length and width was taken as the third dimension of an ellipsoid. This method proved so very time-consuming, that recalculations of nuclear areas and volumes were made of the 200 VandeGrift fixed nuclei using only one diameter -- calculating the area as a circle and the volume as a sphere. The diameters used were the alternate lengths and widths already measured. Graphs were made of these areas and volumes to compare statistically, (Chi-square test) with the areas and volumes measured and calculated as ovals and ellipsoids. The object was

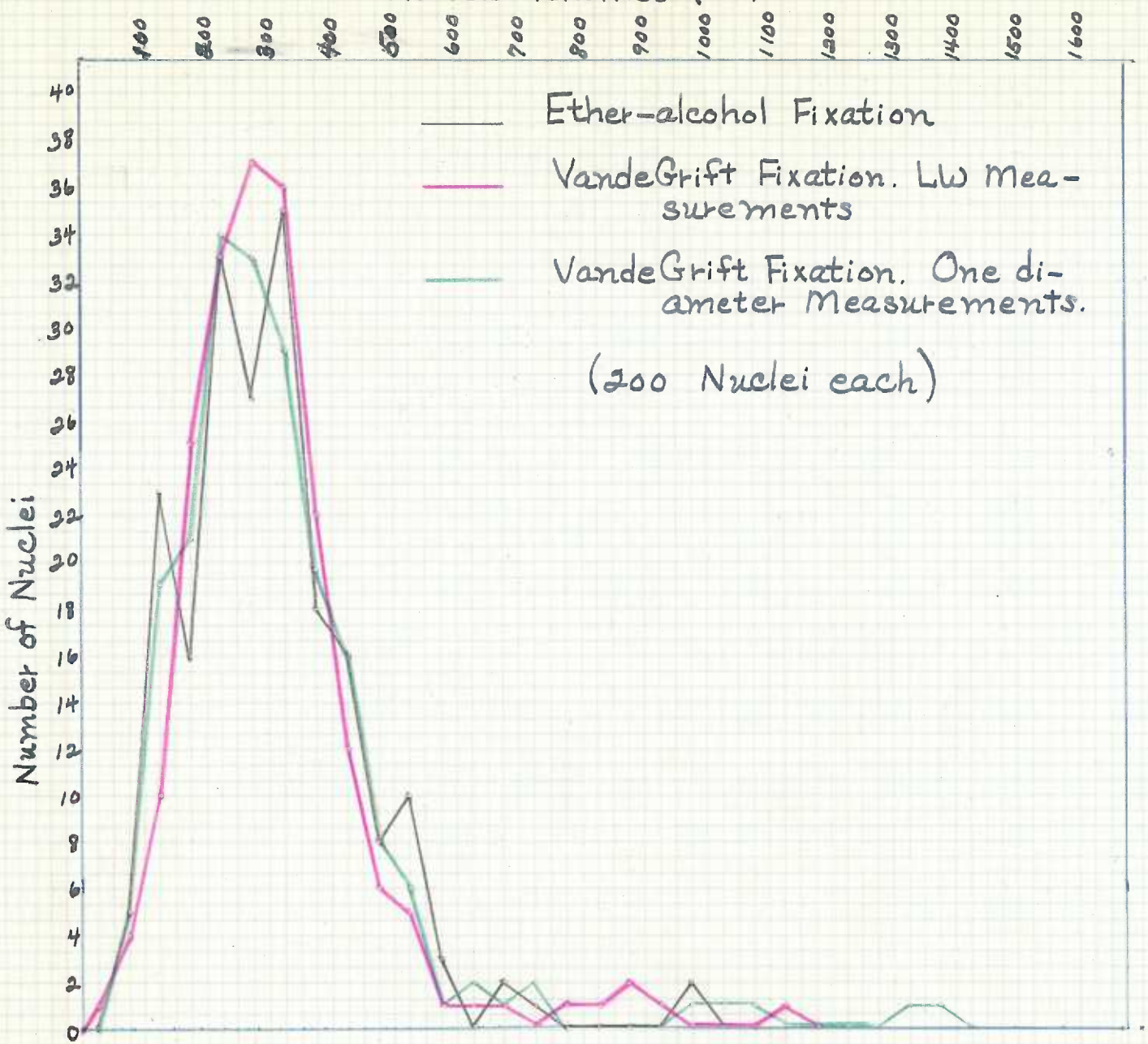
to determine if measuring two diameters gives significantly different results from measuring any one diameter. If no difference is found, much time and toil can be saved. In the VandeGrift fixed nuclei were clear-cut acidophilic nucleoli, while in the ether-alcohol fixed nuclei, nucleoli were not clear at all and difficult to measure. There was also marked difference in nucleolar delineation from slide to slide and even from area to area on the same slide. This will necessarily limit the value of nucleolar measurements.

After these procedures, 200 nuclei from each of several rats at different stages of carcinogenesis were measured in the same manner. Profiting from the control measurements, only one diameter and no nucleoli were measured. It was hoped that the increase in nuclear size seen in the smears could be objectively measured and more carefully analyzed. Areas and volumes for each nucleus for each rat were calculated as ovals and spheres, respectively. These data were then made into the graphs on the following pages, which are more or less self-explanatory. The rats measured were: # 70 -- 1 week m'MeDAB; # 8 -- 3 weeks m'MeDAB; # 73 -- 7 weeks m'MeDAB; # 74 -- 10 weeks m'MeDAB; # 39 -- 12 weeks m'MeDAB, # 177 -- 16 weeks m'MeDAB (small cell adenocarcinoma), and # 162 -- 20 weeks m'MeDAB (small and large cell adenocarcinoma).

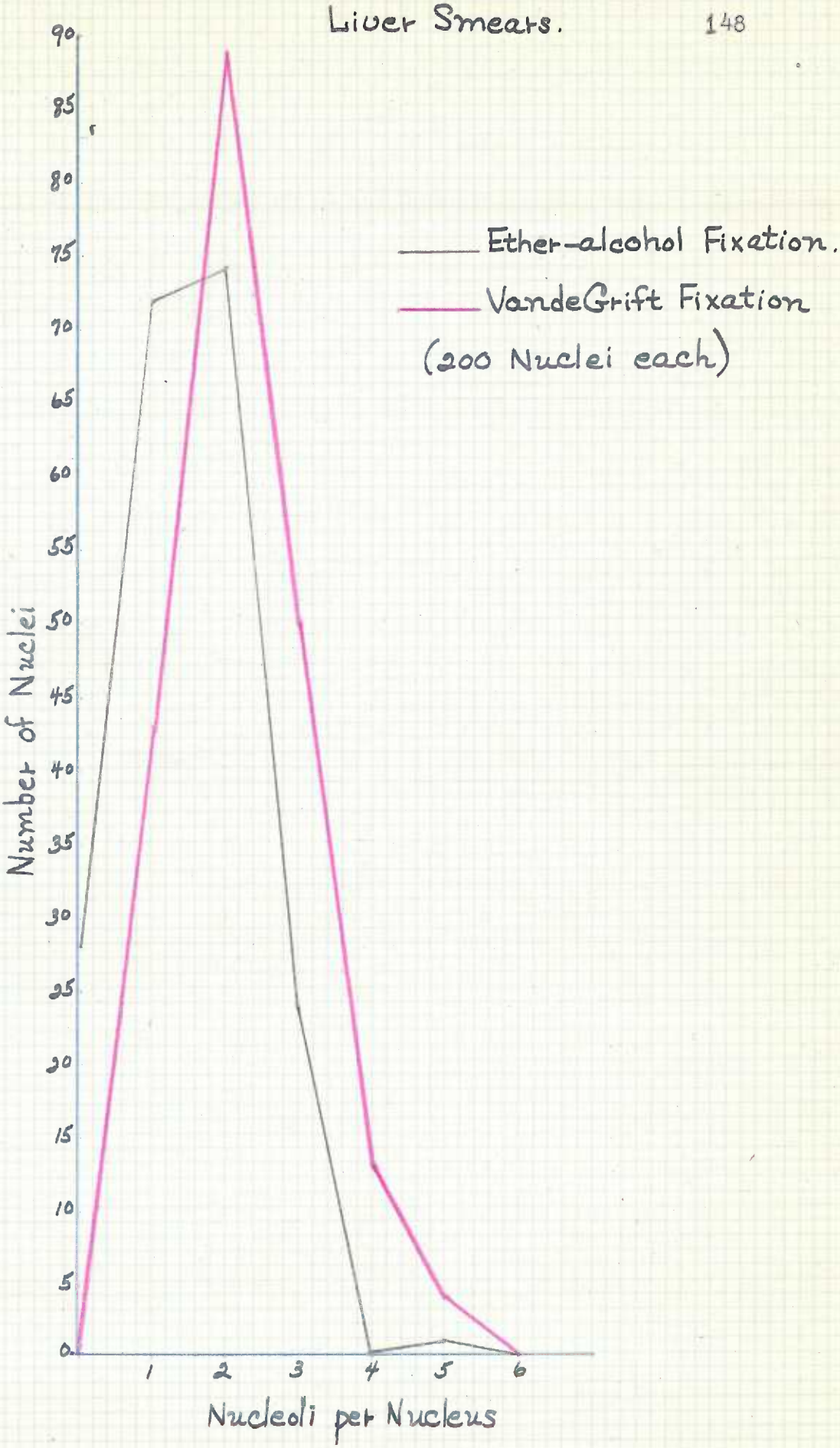
Rat # 63. 9 weeks basic ration. Liver Smears.



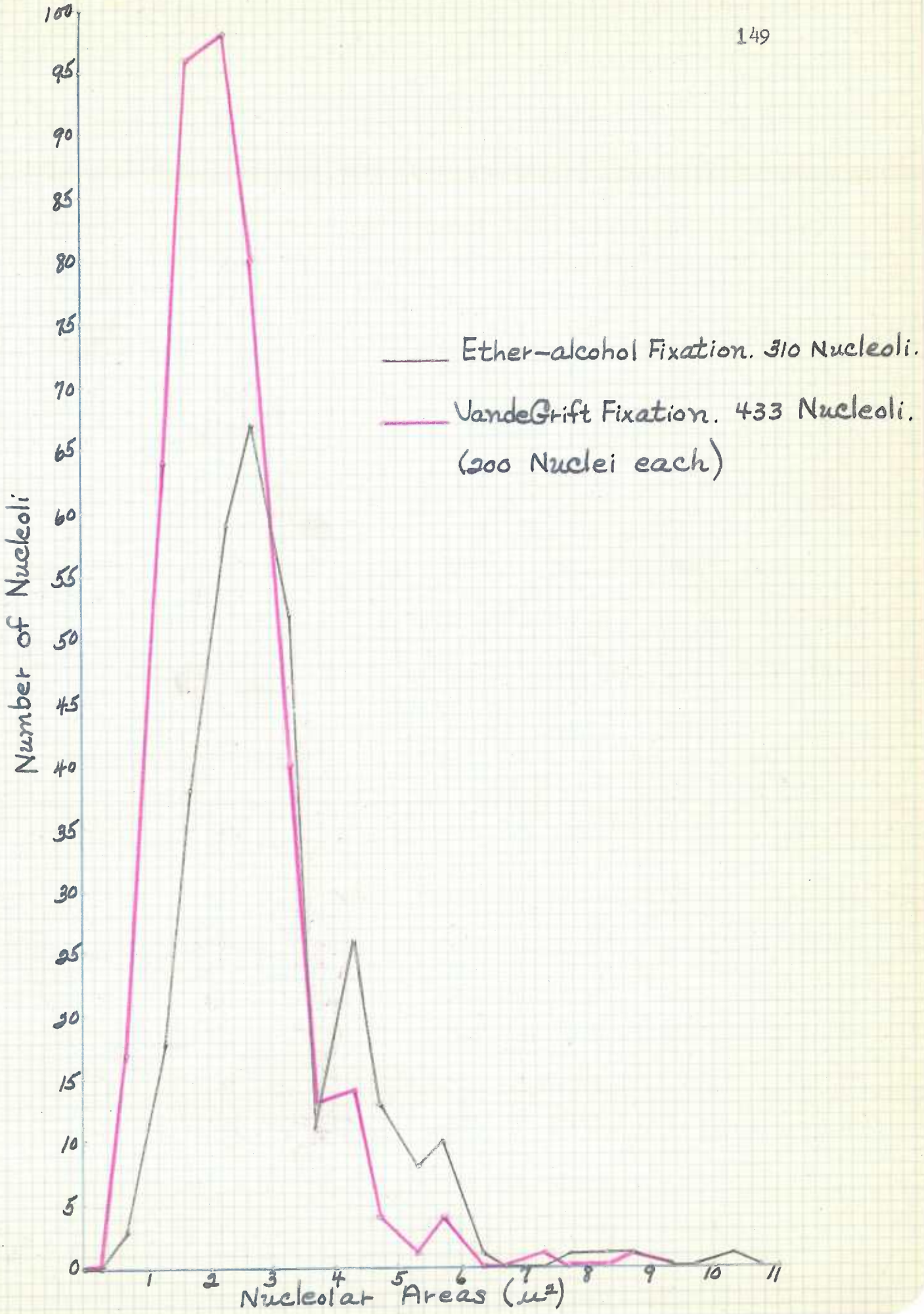
Rat # 63. 9 weeks basic ration. Liver Smears.



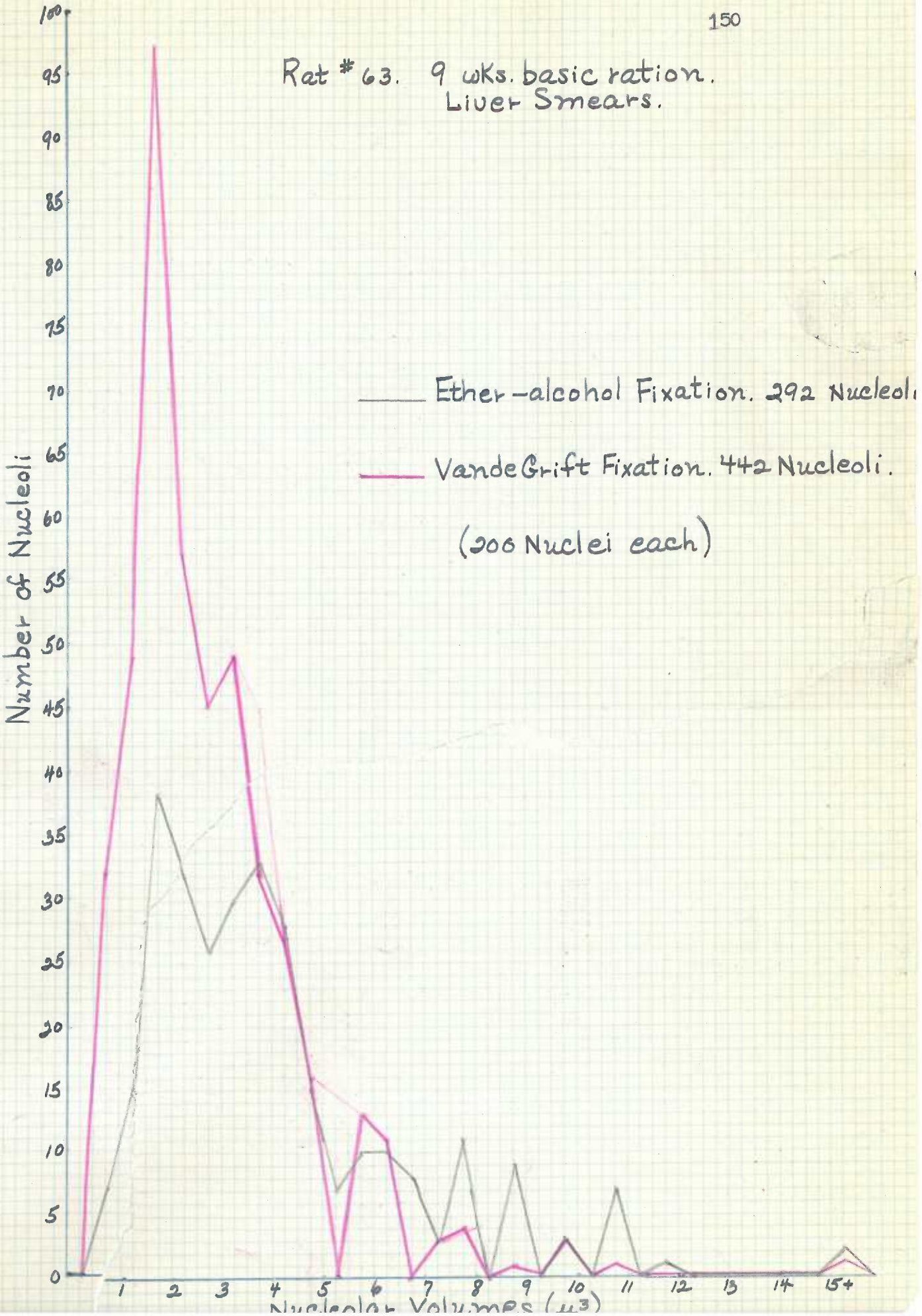
Rat # 63. 9 wks. basic ration.
Liver Smears.



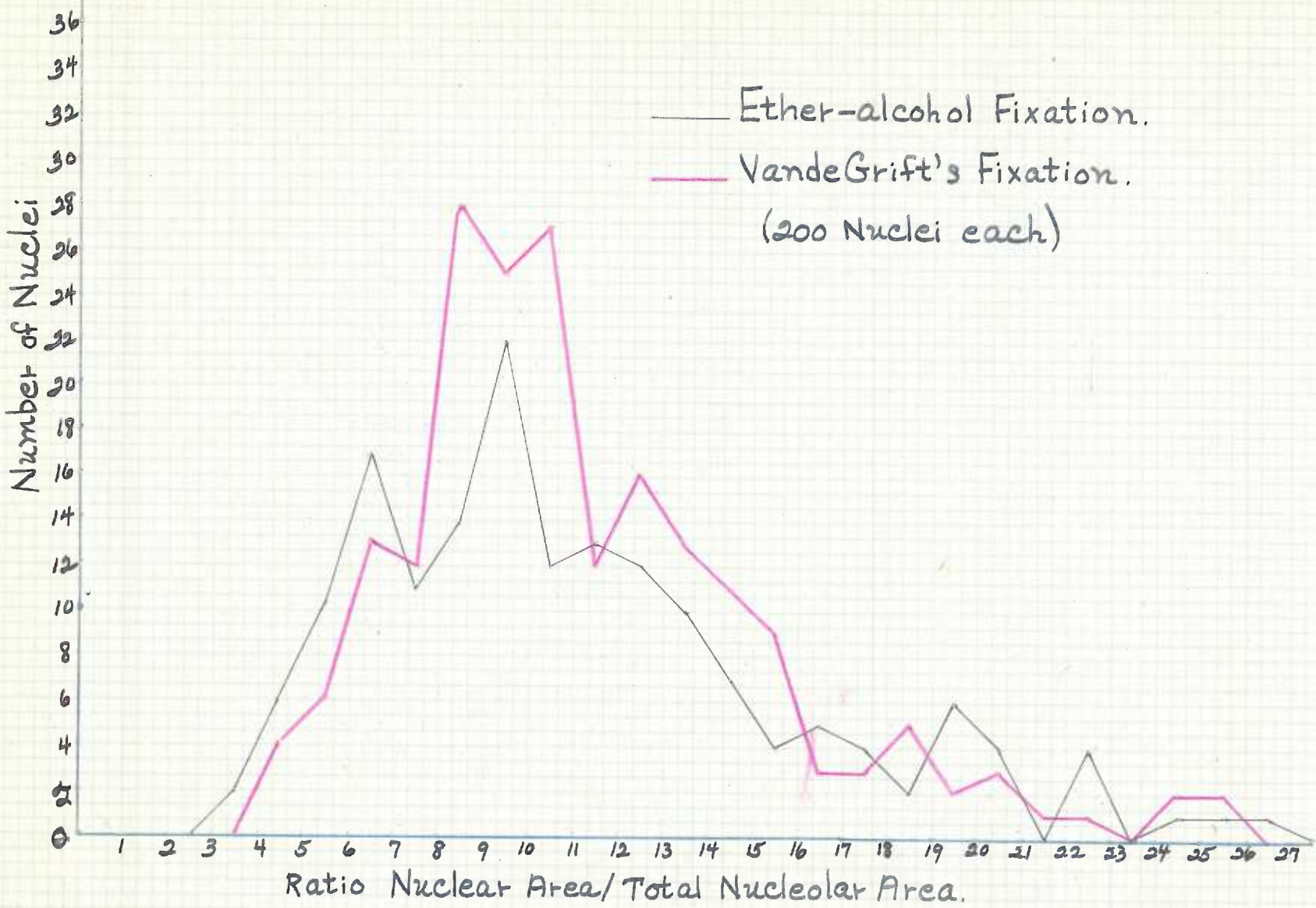
Rat # 63. 9 weeks basic ration. Liver Smears.



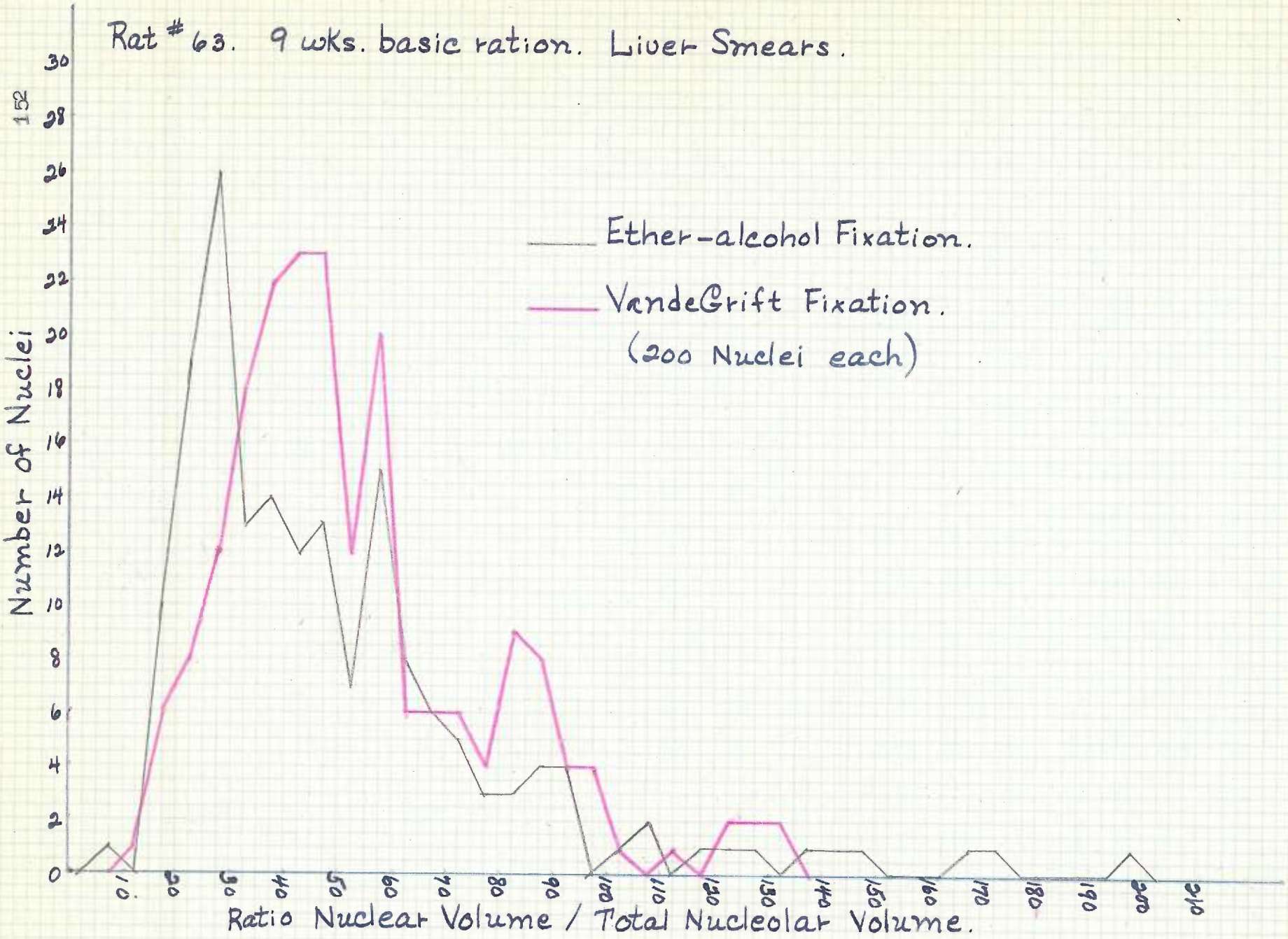
Rat # 63. 9 wks. basic ration.
Liver Smears.



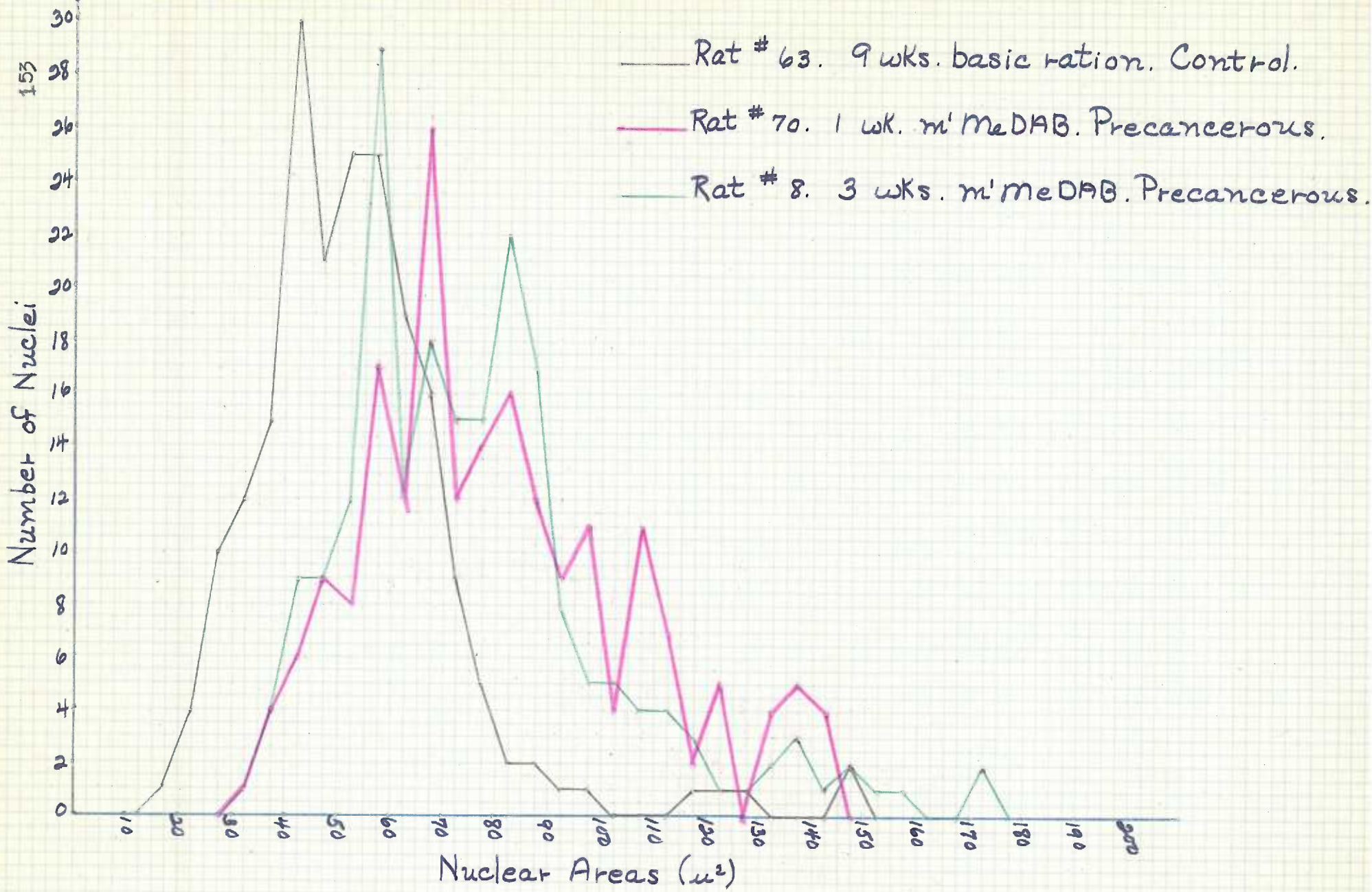
Rat # 63. 9 wks. basic ration. Liver Smears.



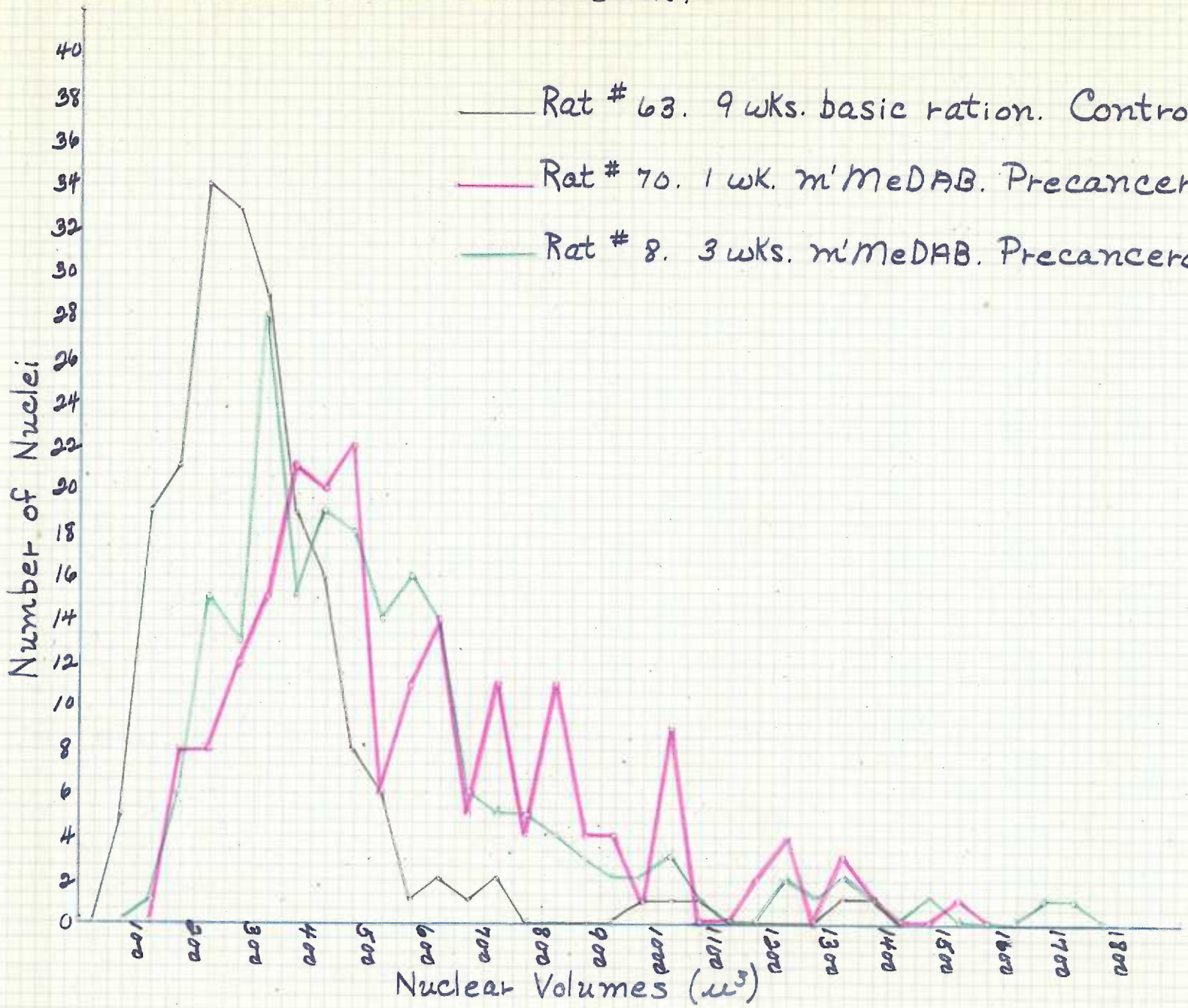
Rat # 63. 9 wks. basic ration. Liver Smears.



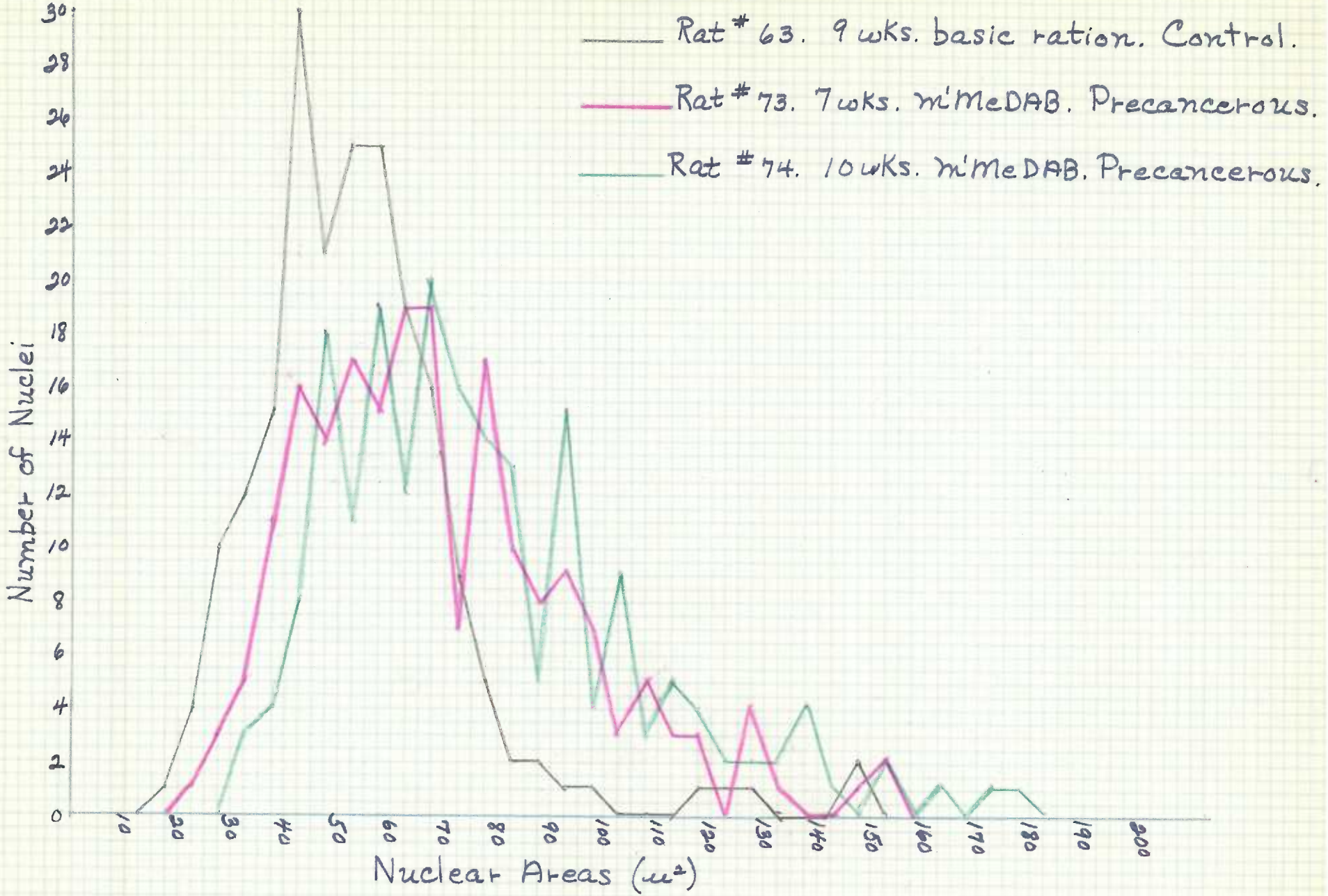
Liver Smears. 200 Nuclei each.



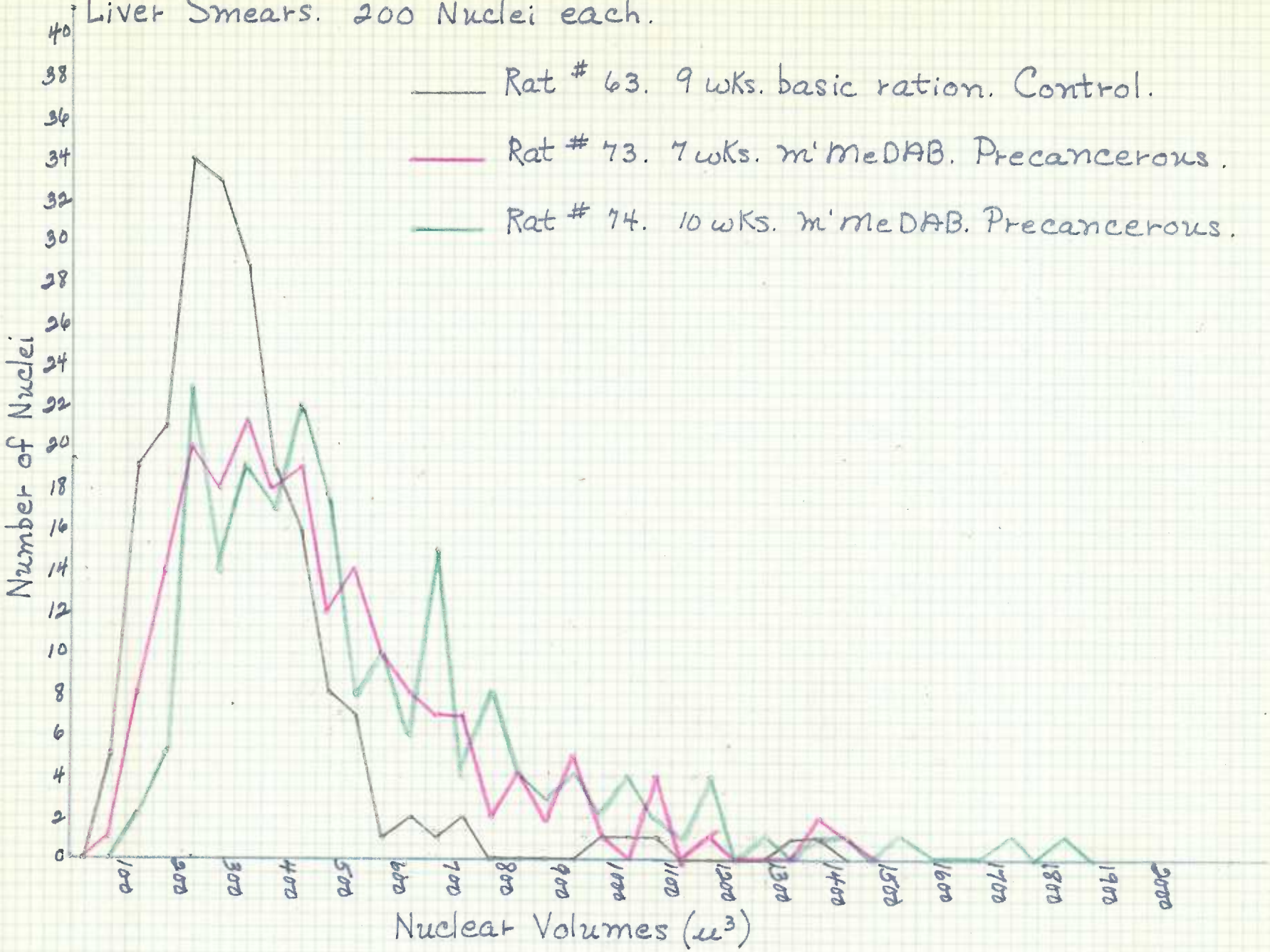
Liver Smears. 200 Nuclei each.

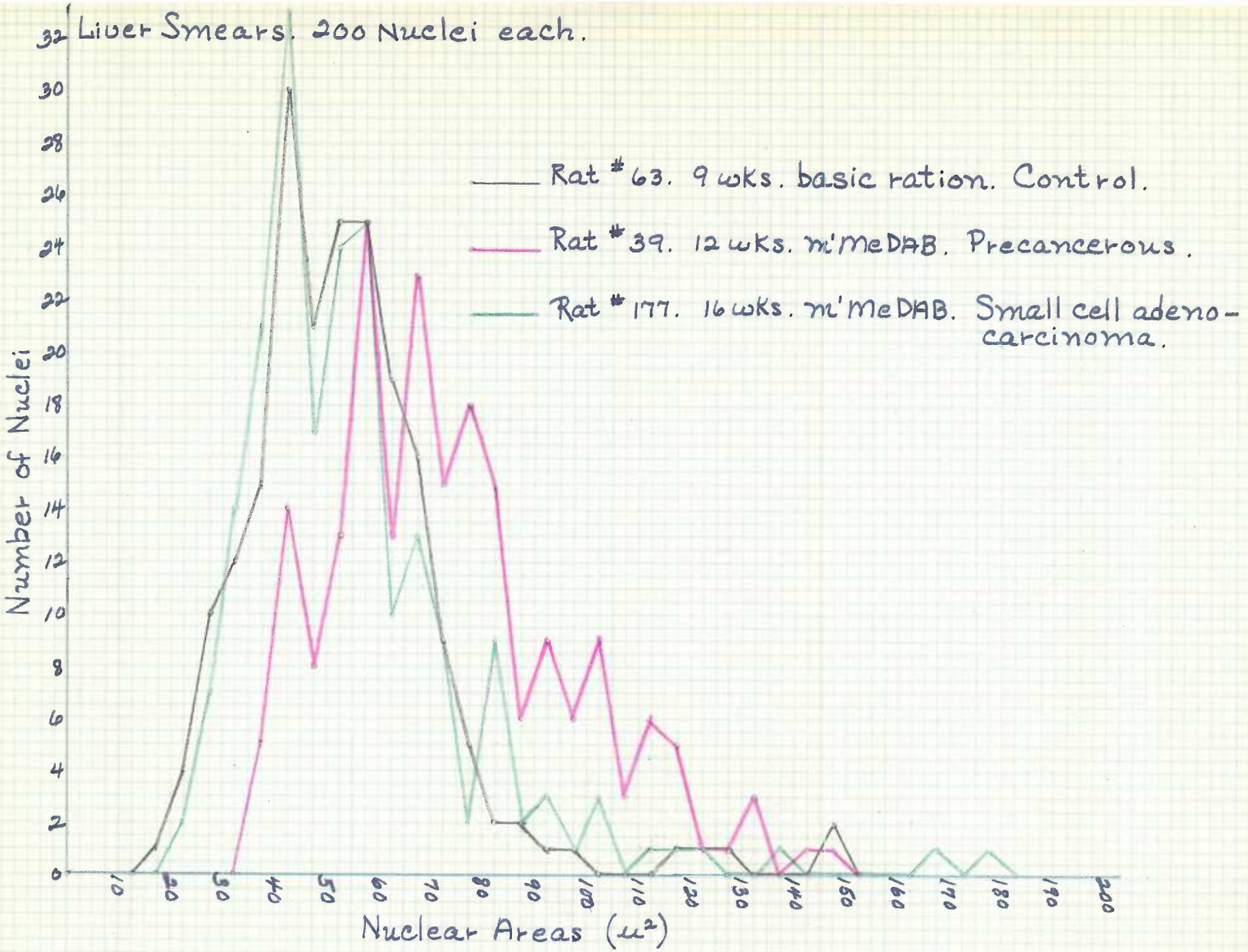


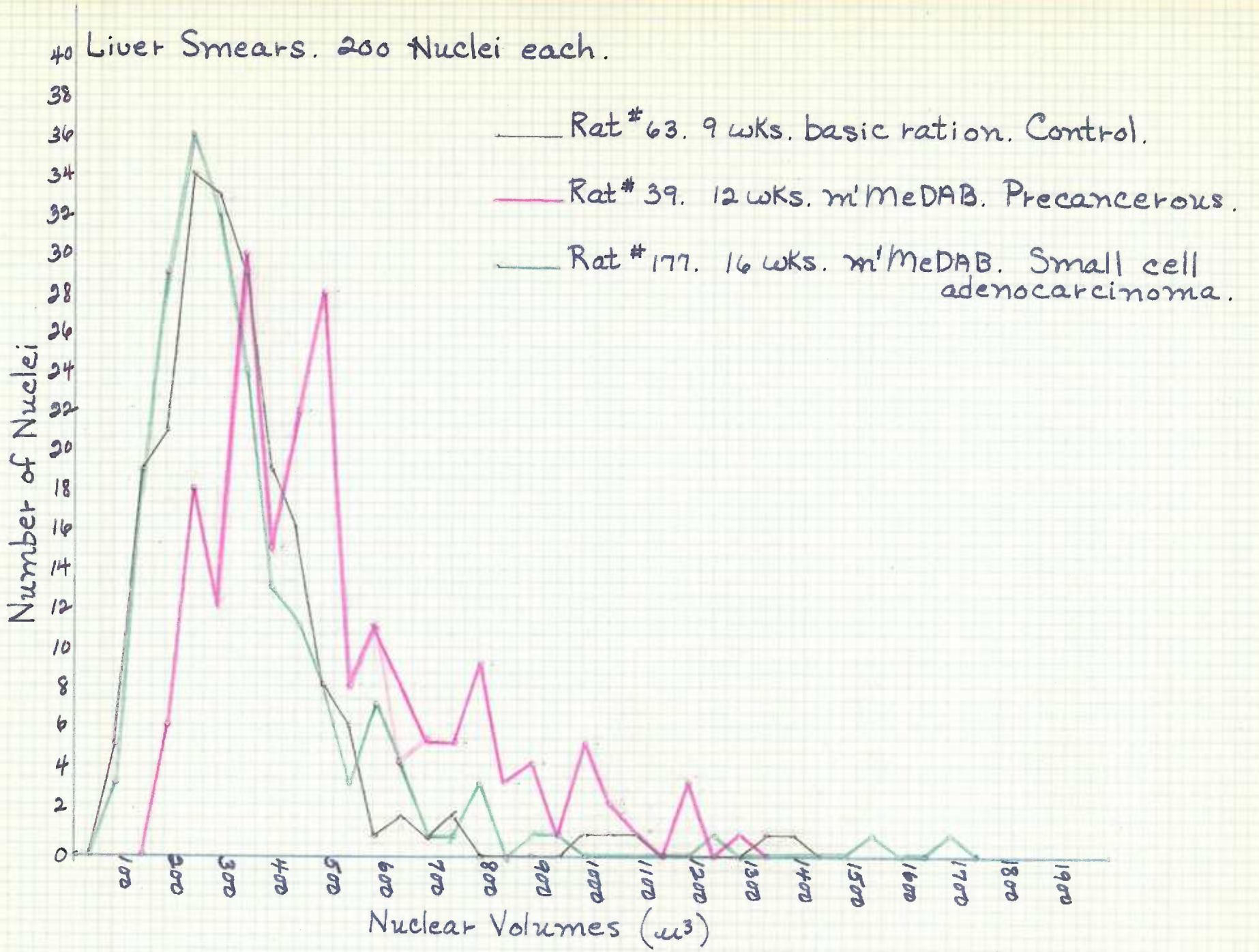
Liver Smears. 200 Nuclei each.

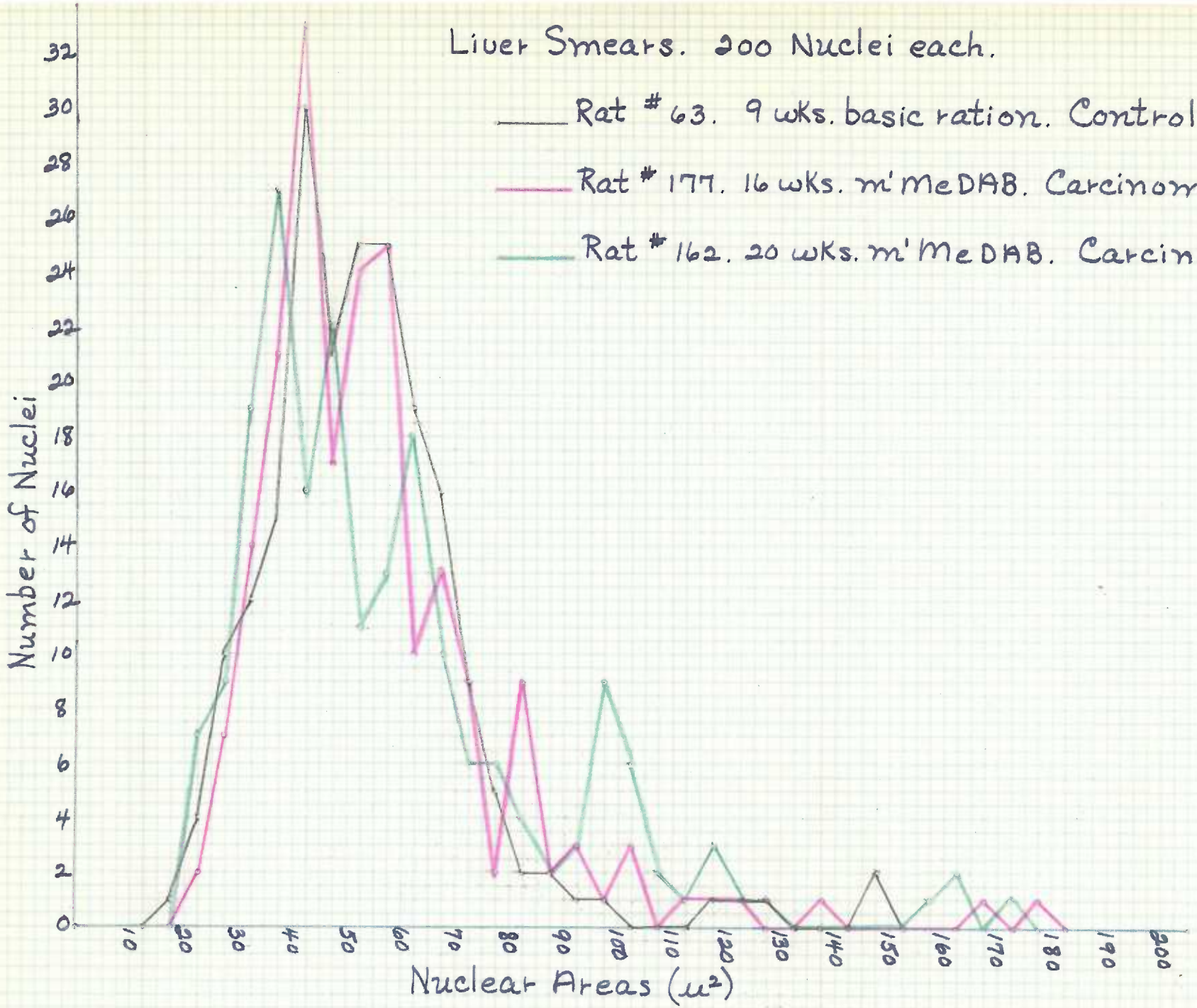


Liver Smears. 200 Nuclei each.




Liver Smears. 200 Nuclei each.
Rat # 63. 9 wks. basic ration. Control.
Rat # 39. 12 wks. m'MeDAB. Precancerous.
Rat # 177. 16 wks. m'MeDAB. Small cell adeno-carcinoma.
Number of Nuclei
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
Nuclear Areas (μ²)


Liver Smears. 200 Nuclei each.
Rat # 63. 9 wks. basic ration. Control.
Rat # 39. 12 wks. m'MeDAB. Precancerous.
Rat # 177. 16 wks. m'MeDAB. Small cell adenocarcinoma.
Number of Nuclei
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
Nuclear Volumes (μ³)
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400


Liver Smears. 200 Nuclei each.
Rat # 63. 9 wks. basic ration. Control.
Rat # 177. 16 wks. m' MeDAB. Carcinoma.
Rat # 162. 20 wks. m' MeDAB. Carcinoma.
Number of Nuclei
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
Nuclear Areas (μ²)

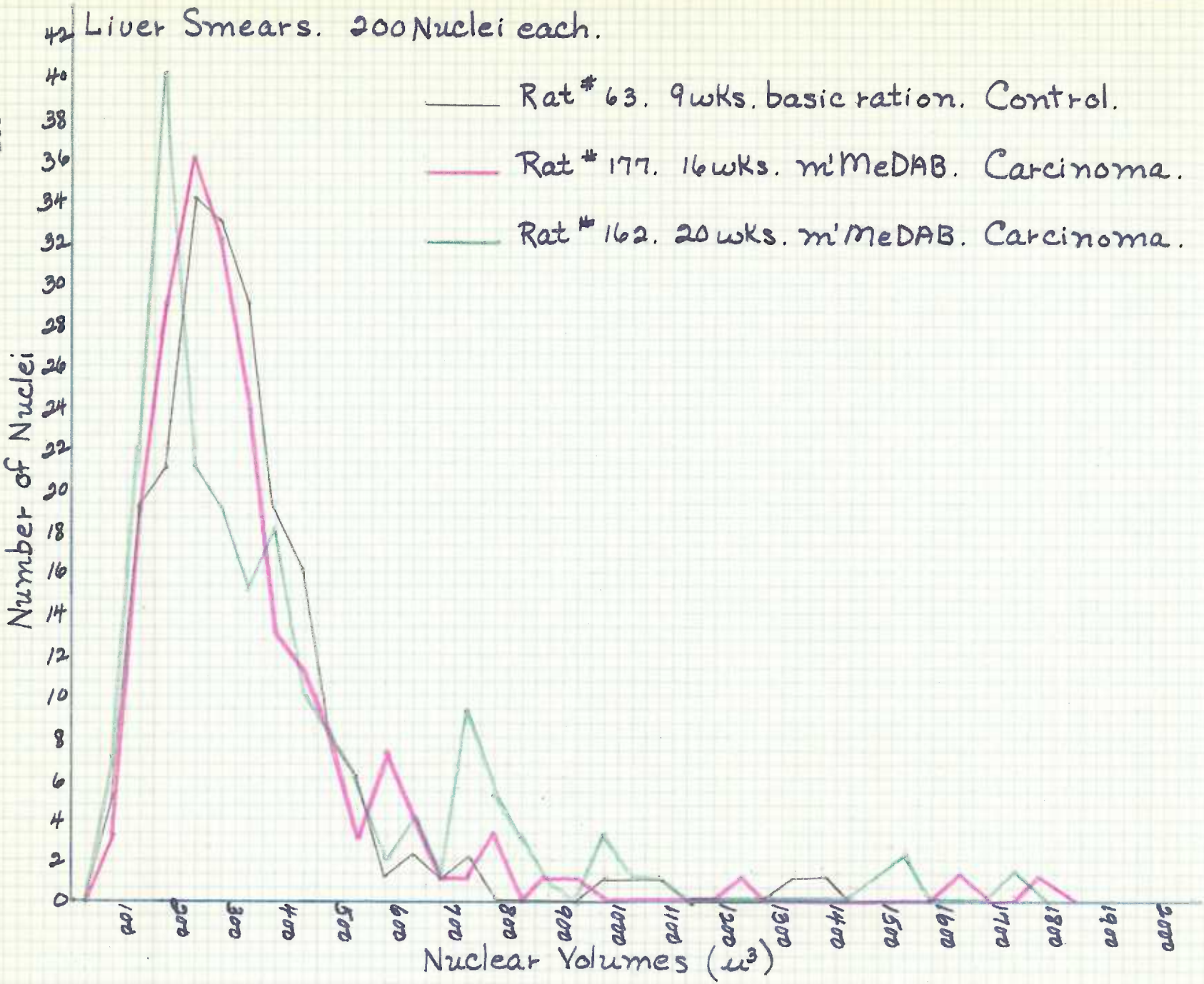

Liver Smears. 200 Nuclei each.
Rat # 63. 9 wks. basic ration. Control.
Rat # 177. 16 wks. m'MeDAB. Carcinoma.
Rat # 162. 20 wks. m'MeDAB. Carcinoma.
Number of Nuclei
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
Nuclear Volumes (μ^3)

TABLE III.

CHI-SQUARE VALUES. CARCINOGENIC SMEAR SERIES.

Rats	Chi-square	Area of Greatest Difference
# 68. 9 weeks. Control.		
Cf. ether-alcohol and VandeGrift's fixative:		
Areas.	7.74	40, 60.
Volumes.	5.55	400, 600.
Nucleoli/nucleus.	30.8	1, 4.
Nucleolar areas.	62.49	2, 6.
Nucleolar volumes.	58.47	2, 8.
Nuclear areas/total nucleolar area.	10.77	4, 8.
Nucleolar volume/total nucleolar volume.	23.23	50, 60.
Cf. length-width and one diameter measurements:		
Areas.	4.72	40, 60.
Volumes	5.99	1000, 1200.

m'MeDAB Rats.	Areas	Volumes	Areas	Volumes
0 wk. cf. 1 wk.	138.2	259.7	100, 120	200, 800
0 wk. cf. 3 wks.	74.8	82.4	20, 100	200, 800
0 wk. cf. 7 wks.	40.2	44.3	100, 120	800, 1000
0 wk. cf. 10 wks.	76.4	87.9	40, 120	200, 800
0 wk. cf. 12 wks.	82.4	77.4	40, 100	200, 800
0 wk. cf. 16 wks.	8.83	6.96	80, 100	200, 800
0 wk. cf. 20 wks.	30.24	28.0	60, 120	400, 800
1 wk. cf. 3 wks.	6.06	6.70	60, 120	1000, 1200
3 wks. cf. 7 wks.	12.8	11.80	40, 100	200, 600
3 wks. cf. 10 wks.	2.56	1.66	80, 120	600, 1200
7 wks. cf. 10 wks.	12.33	13.34	40, 120	200, 1200
10 wks. cf. 12 wks.	1.8	4.64	80, 120	200, 1200
12 wks. cf. 16 wks.	74.2	74.2	40, 120	200, 600
16 wks. cf. 20 wks.	15.42	16.30	40, 120	400, 600

Chi-square must be less than 11.0 for the 0.05 level of significance and the six classes which were used.

III. Discussion and Summary:

The Control Rat # 63. 9 weeks:

According to the Chi-square test, there is no significant statistical difference between the nuclear areas and volumes of the 200 nuclei of Rat # 63, fixed with VandeGrift's fixative and the 200 nuclei of Rat # 63, fixed with ether-alcohol. (Of course, these are not assumed to be true areas and volumes, but they are indexes of them). Moreover, in comparing areas and volumes calculated from measurements of one diameter and those calculated from measurements of two diameters, (already described) the Chi-square test says that there is no statistically significant difference. That is, there is a 95% chance that they are not significantly different. However, nucleoli are a different story. In every case in which calculations utilising nucleolar measurements were performed, except one, there is a large statistical difference between the graphs comparing the two fixatives. No conclusions can be drawn, therefore, from any calculations based upon nucleolar measurements. Because of the great difficulty in finding and measuring nucleoli according to the capriciousness of the stain from slide to slide, it is definitely felt that this is the real reason for most of the large statistical deviation and not the fixative difference. Moreover, no such difference is observed by looking at the nucleoli. Although Chi-square for the ratio of nuclear area to total nucleolar area is 10.77, this has to be fortuitous because the nucleolar areas are way off and so is the similar volume ratio. From then on, therefore, nucleolar measurements were discontinued, nuclei were measured as one diameter only and ether-alcohol fixed material and VandeGrift's fixed material were directly compared.

m'MeDAB Rats:

As noted in Table III, there is a large statistical difference between the control liver nuclei and all of the m'MeDAB liver nuclei, except for the 16 week smears of small cell adenocarcinoma. The next thing to be determined was what this difference consisted of. Looking at the graphs, observing the smears and noting the areas of greatest Chi-square difference, one would think that the mean had simply shifted -- that the average nuclear size was larger. By means of a statistical test,[1] it was determined that the mean of the control nuclear areas and the one week nuclear areas was definitely different. This is almost certainly true of the other m'MeDAB rats up to and including 12 weeks, since all but one were not statistically different from the one week nuclei and that one very slightly. Since the curve is shifted to the right, this means that the average nuclear size is increased. This coincides exactly with the observations of the smears -- that there is an increase in average nuclear size, seen as early as one week. It is not surprising that the smaller nuclei of the small cell adenocarcinoma are not statistically different from normal nuclei in size. It is unfortunate that the one week rat measured, happened to be the one week rat in which the most change was observed. Probably, all the one week rats would not show this much change from the normal. Animal variation and variables other than the carcinogen must be considered as a possible cause of the shift in the mean and the statistical difference between the normal and the carcinogenic curves; however, this is unlikely on the basis of observation of the smears and because there is no statistical difference between most of the precancer-

1. I am indebted to Doctor Hopkins of the University of Oregon Medical School Statistical Consultation Service for performing this.

ous nuclei from different rats at different precancerous times. Rat # 73 is the only precancerous, (7 weeks) one with a statistically significant difference from the other precancerous nuclei and it is slight. This may be due to the slight decrease in larger nuclei, which is seen sometimes at this time, or there may be other factors involved. Observations of the 7 and 10 week graphs would lead one to think that there was flattening of the curve compared to the other weeks, but a Chi-square test with 12 classes comparing 8 weeks and 10 weeks areas revealed no statistically significant difference. This then, is due to calculation artefact. It is quite understandable that the two malignant rats, (16 and 20 weeks) are statistically different from each other since they are different types of carcinoma.

In conclusion, it seems that there is an initial increase in average nuclear size within 1-3 weeks which is maintained without further change until a true malignant change occurs. Once malignant change occurs, anything can happen to the nuclear size. Nothing which resembles Biesele's (10) volume classes are discernible in these graphs, but the material is not adequate for this purpose. It seems to me that Biesele's is too.

PART V.

OBSERVATIONS OF LIVER SMEARS DURING REGENERATION

V. OBSERVATIONS OF LIVER SMEARS DURING REGENERATION

A. Materials and Methods:

Because such difficulty is often encountered in differentiating regenerating, rapidly growing cells from cancer cells and because there is some debate concerning the precancerous role of regenerating cells in some situations, it was desirable to compare smears of regenerating liver with precancerous ones.

The liver regeneration was induced by removing surgically, part of the liver, (65-70%) according to the technique of Brues and Marble.(26) The first successful operation was done under Nembutal anesthesia, (# 405) from which the animal never recovered consciousness, presumably because the damaged liver was unable to detoxify the drug. Thereafter, the operations were performed under ether anesthesia. It was not possible to maintain sterile conditions during the operations, but all possible practical precautions were taken. There was considerable mortality rate during surgery before our technique was perfected. The rats used had been taken from the stock colony and fed the basic ration for a few days before, and after surgery. These rats were similar in every way to the m'MeDAB rats. Six rats were studied; # 405 after 48 hours; # 410 after 48 hours, # 409 after 5 days; # 407 after 9 days; # 406 after 12 days; and # 408 after 17 days. These smears were studied and nuclear measurements were made and reported in the same manner as in the carcinogenic series.

B. Pathology:

405. Female. 48 hours.[1]

Liver Pathology:

Smear: The most striking change is in the nucleoli. They are very large, acidophilic and prominent. Some are truly giant. Smaller sizes are present too, however. There is definitely quite an increase in the number of nucleoli per nucleus. Usually, they are round and uniform, occasionally they are fusiform. All are well defined. The nuclei are definitely larger in average size and occasional giant forms are present. Nuclei are often irregular in shape. Except for an occasional nucleus with a more prominent reticulum, most nuclei have chromatin that is normal in density and distribution. However, there is tremendous vesiculation and clear vacuolation.

Gross: The incision shows no evidence of healing, though no signs of inflammation are present. Adhesions are present between the right lateral lobe of liver and stomach. The left lateral lobe and median lobes have been removed. The liver is exceedingly pale, greyish-yellow, with a smooth surface. The right lateral lobe appears to be enlarged. The liver is soft and friable. The spleen is much enlarged and dark purple.

Section: A fat stain demonstrates massive fatty change. The fatty metamorphosis is diffuse with much vaculation of hepatic cytoplasm. Architecture is normal.

---

1. This animal is the one operated under Nembutal anesthesia. The animal remained comatose, never recovering consciousness and was sacrificed after 48 hours.

Incidental Pathology:

Kidneys: Shock kidneys.

Spleen: Acute passive congestion.

Adrenals: Shock adrenals.

Lung: Severe pulmonary edema.

410. Female. 48 hours.

Liver Pathology:

Smear: This is much the same as that seen in Rat # 405 except that there is not the tremendous amount of vacuolation, though some is present. Acidophilic, well-defined nucleoli often assume bizarre shapes -- rod-like, fusiform, double nucleoli and connections between two distinct nucleoli. The chromatin is usually normal, though questionably there may be a slight diffuse increase in density in some of the nuclei. Occasional nuclei demonstrate a slight prominence of the reticulum and occasional nuclei demonstrate irregular chromatin clumping of all degrees. There may questionably be an increased number of the larger nuclei, above that seen in Rat # 405. There is some increase in the number of small bile duct nuclei.

Gross: Weight is 5.5 Gm. (Resected portion weighed 5.2 Gm.) The caudate and right lateral lobes which remain are much enlarged with a smooth, pale yellowish-red surface. There are adhesions of liver to omentum, diaphragm, stomach and small intestine. The portal vein and inferior vena cava are engorged. Distal to the tie used to cut off the missing lobes is a white infarcted piece of tissue. The spleen is much enlarged.

Section: There is much pigment in the Kupffer cells. An unusual number of small nuclei and binucleate cells seem to be present. Occasional very large nuclei are seen, and occasional giant cell groups. There is much fatty infiltration in some areas and hepatic cytoplasm is much vacuolated.

Incidental Pathology:

Kidney: Much pigment in the tubules, hyperemia.

Spleen: Acute, passive congestion.

409. Female. 6 days.

Liver Pathology:

Smear: There is a decrease in nucleolar pleomorphism and the number of nucleoli per nucleus, though these changes are still quite evident. Chromatin is often slightly and diffusely increased in density, but not comparably to the drug effect nuclei. Some chromatin demonstrates a granular, clumped distribution. There is quite an increase in the number of bile duct structures and nuclei.

Gross: The incision is healing well with no sign of infection. The liver weight is 10.2 Gm. The right lateral and caudate lobes which remain are much enlarged and engorged. There is dilatation of the remaining blood supply to the liver. The surface is smooth and reddish-brown. There is a small, firm, yellowish-white infarcted area distal to the tie used to cut off the missing lobes. There are adhesions of liver to omentum, diaphragm, stomach and intestine. Spleen is enlarged, smooth and reddish-purple.

Section: The liver architectural pattern appears somewhat disorganized and more cellular. Groups of small nuclei and many larger ones are seen. There is a slight proliferation of the bile ducts around the area of the hepatic trinity. Cytoplasm is often much vacuolated. There is focal fatty infiltration in some sections. Some groups of giant cells and some groups of cells resembling adenomatous hyperplasia are seen.

Incidental Pathology:

Spleen: Acute, passive congestion.

Kidney: Hyperemia.

Lung: Focal chronic pneumonia, chronic peribronchitis, hyperemia.

407. Female. 9 days.

Liver Pathology:

Smear: There is definitely a diffuse increase in chromatin density similar to that seen in the drug effect nuclei. Many nuclei have clumping of the chromatin, occasionally somewhat irregularly. There are still many large sized nucleoli but there are no so many of them per nucleus. They still often demonstrate irregularity in shape. Often single, large vacuoles are seen in the nuclei. The average nuclear size is larger;there are giant forms seen occasionally. There are many bile duct cells.

Gross: The incision appears to be healing well. The sutures are still in place. There is a small 5 mm. abscess at the base of the right lateral lobe of the liver. Liver weight is 11.8 Gm. The right lateral lobe and caudate lobe are tremendously enlarged, beefy-red and very firm. The surface is smooth. The portal vein and its

branches are tremendously dilated. The liver is adherent to the stomach, intestines, and omentum. The spleen is tremendously enlarged.

Section: The entire liver architecture is more cellular, but it is not distorted. Occasional large nuclei and occasional giant nuclei are found. There is focal lymphocytic infiltration. Sinusoid epithelium is prominent. Occasional new bile ducts are apparently growing out from the main portal ones. There is slight disorganization of biliary ductal cells and in portal areas. This is not at all comparable to the biliary ductal proliferation of m'MeDAB, which it resembles in some areas.

Incidental Pathology:

Spleen: Acute, passive congestion.

406. Female. 12 days.

Smear: There is an apparent decrease in the average nuclear size and fewer irregular forms are seen. (Are these new hepatic nuclei regenerated from bile duct cells?) There is not the large number of bile duct nuclei seen earlier. Chromatin is definitely, diffusely increased in density in some of the nuclei but the distribution is coarser and more clumped than in the drug effect nuclei. Many nuclei with normal chromatin are present. Nucleoli are often giant, often very numerous, but there is not the amount of pleomorphism that was seen formerly.

Gross: There is a small subcutaneous stitch abscess at the anterior end of the suture line, but otherwise the incision is well healed and the sutures are missing. The liver weight is 7.8 Gm. The remain-

ing caudate and right lateral lobes have tremendously hypertrophied, giving the appearance of a normal liver in size and weight. The liver is engorged with blood and the portal vein and its branches are tremendously dilated. The surface is smooth and beefy-red and the consistency is very firm. There are adhesions of the liver to the stomach, intestines, omentum and to each other. The spleen is very much enlarged.

Section: Architecture is well preserved and no giant cells are seen, although the average nuclear size appears to be larger.

Incidental Pathology:

Spleen: Acute, passive congestion.

Lung: Focal chronic pneumonia, focal chronic bronchitis.

408. Female. 17 days.

Liver Pathology:

Smear: There is definitely a diffuse increase in chromatin density in many of the nuclei. The distribution is finely granular and homogeneous, very like the drug effect nuclei. The nuclei are larger in average size and occasional giant forms are seen. Nucleoli are not well stained in these slides but are still enlarged, though not so pleomorphic. Occasional vacuoles are present.

Gross: The anterior end of the wound has opened a distance of 1 cm. and there is a 1 cm. subcutaneous stitch abscess underlying this opening. Liver weight is 9.5 Gm. The right lateral and caudate lobes are greatly enlarged and firm, being engorged with blood. The surface is smooth and dark reddish-brown, with a dry, white

infarcted area on the stump of the excised lobes. The portal vein is greatly dilated. The spleen is greatly enlarged.

Section: The liver architecture appears normal. In most places there are fewer larger cells than were seen formerly. There seems to be more binucleat cells, however.

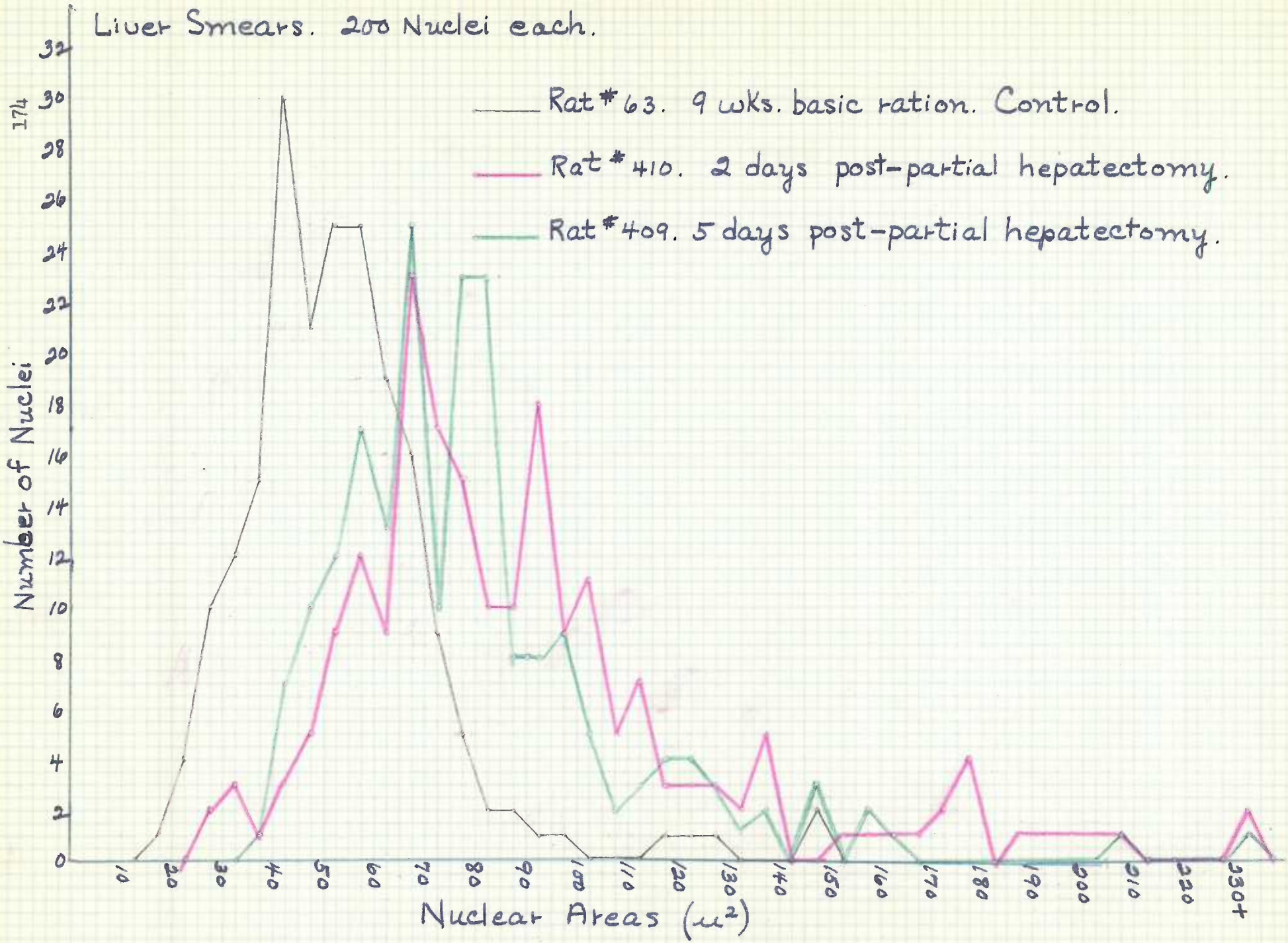
Incidental Pathology:

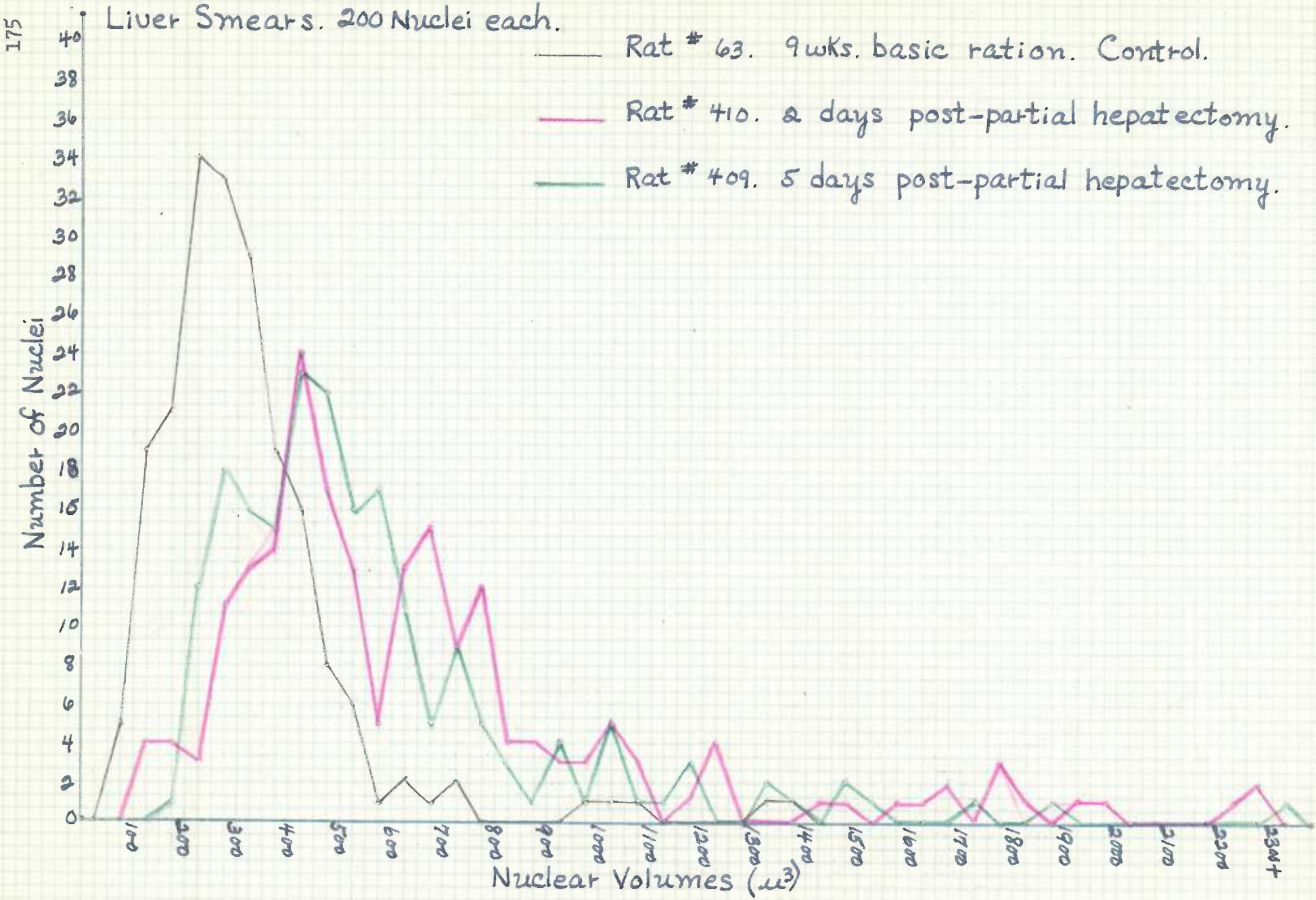
Spleen: Lymphoid hyperplasia, increased pigment.

Kidney: Hyperemia.

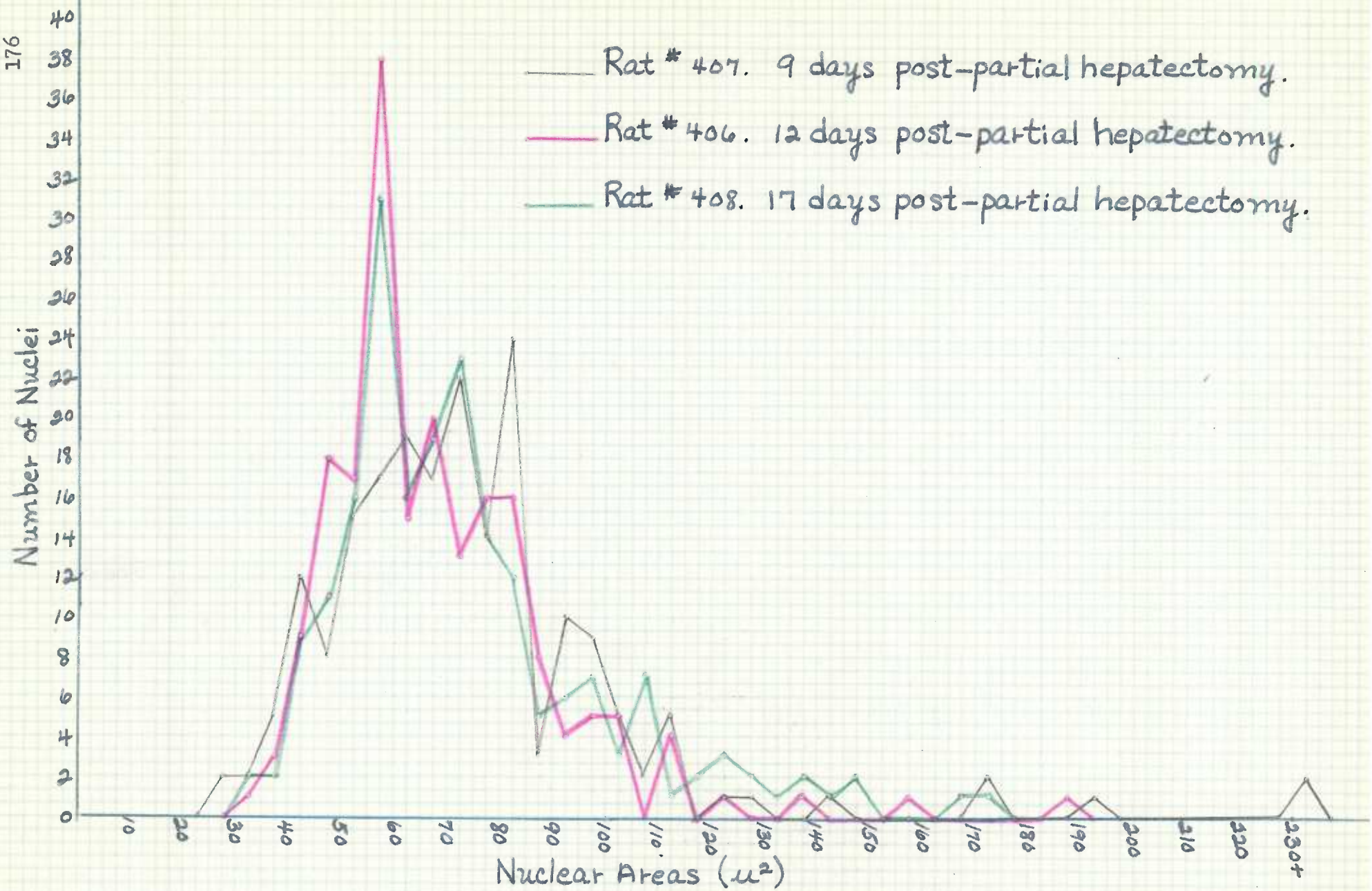
Adrenal: Hyperemia of medulla and zona reticularis, hyperplasia of zona reticularis.

Lung: Hyperemia, focal chronic pneumonia.


Liver Smears. 200 Nuclei each.
Rat #63. 9 wks. basic ration. Control.
Rat #410. 2 days post-partial hepatectomy.
Rat #409. 5 days post-partial hepatectomy.
Number of Nuclei
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
230+
Nuclear Areas (μ²)


Liver Smears. 200 Nuclei each.
Rat # 63. 9 wks. basic ration. Control.
Rat # 410. 2 days post-partial hepatectomy.
Rat # 409. 5 days post-partial hepatectomy.
Number of Nuclei
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
1900
2000
2100
2200
2300+
Nuclear Volumes (μ3)

Liver Smears. 200 Nuclei each.



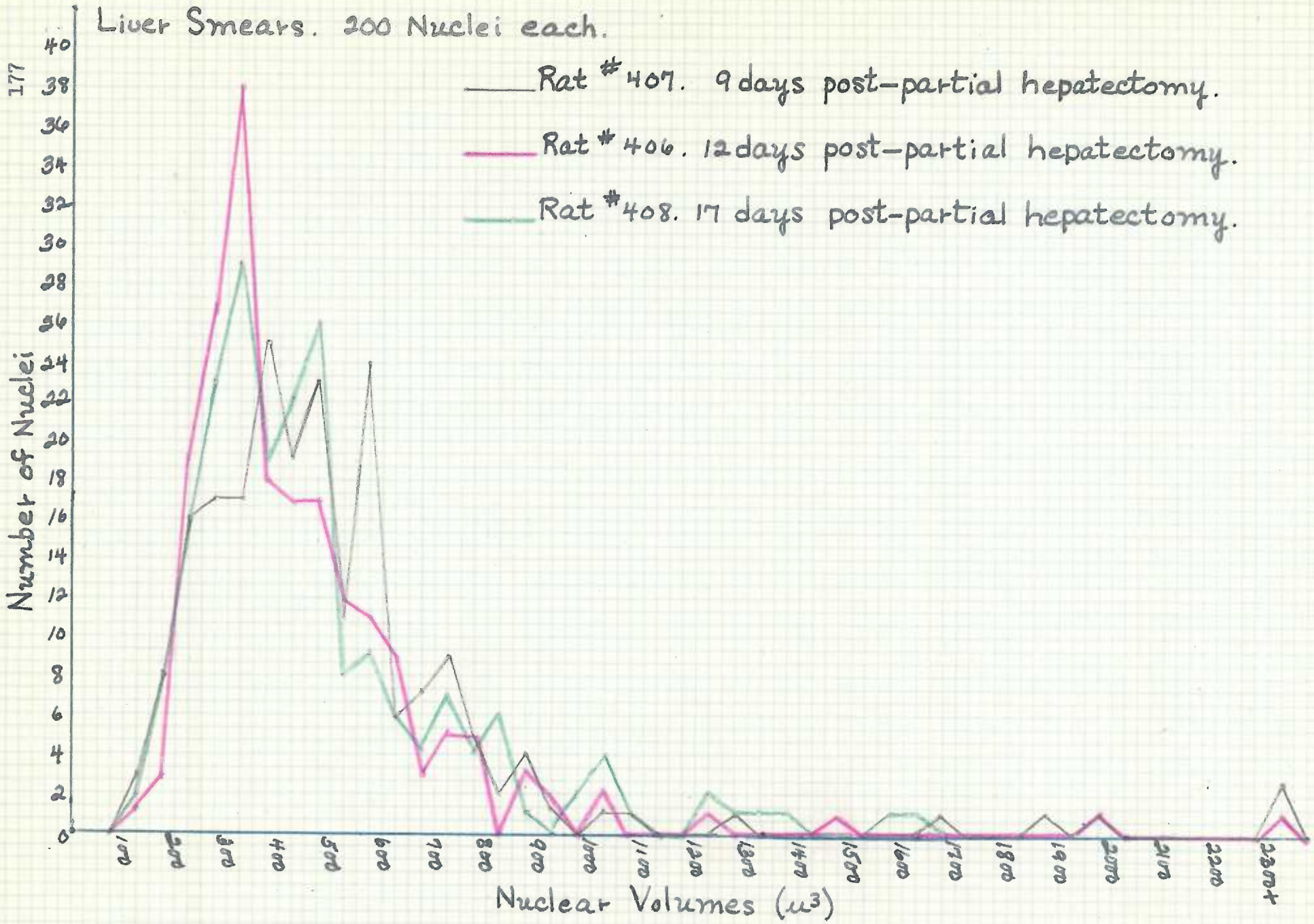

Liver Smears. 200 Nuclei each.
Rat #407. 9 days post-partial hepatectomy.
Rat #406. 12 days post-partial hepatectomy.
Rat #408. 17 days post-partial hepatectomy.
Number of Nuclei
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
1900
2000
2100
2200
2300+
Nuclear Volumes (μ³)

TABLE IV.

CHI-SQUARE VALUES. REGENERATING LIVERS SMEARS.

Rats	Chi-square		Area of Greatest Difference	
	Area	Volume	Area	Volume
Post-hepatectomy rats				
0 days cf. 2 days	141.24	101.7	60, 100	200, 800
0 days cf. 5 days	118.5	112.7	40, 100	200, 600
0 days cf. 9 days	81.4	66.9	40, 100	200, 600
0 days cf. 12 days	41.08	59.26	40, 120	200, 800
0 days cf. 17 days	75.0	88.1	20, 100	200, 600
Cf. m'MeDAB rats				
1 wk. cf. 2 days	3.12	6.98	60, 120	400, 800
1 wk. cf. 5 days	4.83	12.23	40, 120	200, 600
3 wks. cf. 9 days	4.68	3.50	40, 120	200, 1000
7 wks. cf. 12 days	16.34	20.68	40, 60	200, 400
10 wks. cf. 17 days	5.45	5.96	80, 120	400, 800

Chi-square must be less than 11.0 for the 0.05 level of significance for the six classes used.

C. Discussion and Summary:

One of the first changes, as in carcinogenesis, seems to be an increase in average nuclear size with occasional giant forms. However, unlike the carcinogenesis, this is accompanied by much more anisocytosis, poikilocytosis and marked nucleolar change. The nucleolar changes resemble closely those seen in many malignant nuclei. Chromatin change does not resemble the drug effect until the 17 days, at which time these smears are in every way almost indistinguishable from some of the m'MeDAB smears. The type of nucleus with prominent reticulum is occasionally seen, just as in m'MeDAB smears. The chromatin clumping, which occurs at 9 and 12 days most markedly, is more coarse and granular than in the drug effect nuclei. Some of these giant irregular nuclei with clumped chromatin and multiple, large, bizarre nucleoli closely resemble some malignant nuclei. The change in the sections does not parallel carcinogenesis markedly, except that there is fatty infiltration, growing bile ducts (regenerating, not proliferating), and occasional giant cells. There is no architectural distortion comparable to the m'MeDAB livers. This picture of the first 12 days of regeneration suggests what might occur if m'MeDAB carcinogenesis were speeded up to cover only that length of time. Or, conversely, when regeneration has slowed down, as in the 17 day rat, the smears closely resemble the drug effect nuclei.

The nuclear measurements also resemble m'MeDAB changes, except that they are faster and the end result in this case is a slow return to the normal. All the regenerating smears differ significantly from the normal. This appears to be, though no statistical test was done to prove it, due to a shift of the mean so that the average nuclear size is larger,

Again, no volume classes were seen, the curves resembling parabolas or probability curves. The significant difference from normal decreases in degree with number of days of regeneration. There is a slight borderline significant statistical difference between 2 day and 5 day smears, 5 day and 9 day smears and 9 day and 12 day smears, but none between 12 day and 17 day smears. Apparently, by 12 days the process is slowing down and returning to normal. The fascinating thing is that some of the graphs of regenerating nuclei do not differ significantly from some of the graphs of precancerous nuclei. This may be sheer coincidence, but taken in conjunction with the observations of the smears, one must conclude that regeneration of liver and this type of liver carcinogenesis are related to each other. However, in many ways, obviously, they are worlds apart.

DISCUSSION

Although the evolution of neoplastic nuclei in livers of rats fed the azo dye carcinogen, m'MeDAB, was closely observed with all the advantages offered by the smear technique, it still was not possible to tell definitely just when this or that particular nucleus had just become, or was about to become, neoplastic. The change was too gradual. It seems doubtful that it will ever be possible to identify most single neoplastic nuclei as such just by looking at them. No new characteristics of cancer nuclei were discovered. In fact, no one change was found that could be called characteristic. Many single nuclei were observed that were obviously and characteristically cancer, but these embodied combinations of several well-known cancer characteristics. The striking and well-known, conclusion seemed to be that any arrangement of chromatin, any kind of nucleolus and any type of nucleus can be found in neoplastic nuclei. The increase in degree of variability seemed the most constant characteristic.

Another intriguing finding is that although the precancerous nuclei often closely resembled nuclei of damaged, regenerating liver, they were also very different. There is a not too subtle difference between regenerating and proliferating cells. In the human, primary carcinoma of the liver is increased in frequency in cirrhotic or damaged livers. The carcinogenic process here is accompanied by a nodular cirrhosis. Perhaps, in humans, the cause of the cirrhosis causes the liver carcinoma, rather than that the presence of cirrhosis tends to predispose to liver cancer. Of course, one could turn the initial statement around and state that while

precancerous liver nuclei are different from regenerating liver nuclei, they are also very similar.

The increase in nuclear size and the increase in chromatin density early in carcinogenesis agrees with studies of methylcholanthrene carcinogenesis in the mouse skin. That is, increased numbers of chromosomes were found in the mouse skin, which should result in an increased chromatin density. The mouse skin workers did not study the chromatin structure when it was not undergoing mitosis or endomitosis, because their preparations were apparently unsuitable for this. On the other hand, mitotic figures are rare in the liver smears and could not be studied in this work. It is interesting to conjecture whether this diffuse increase in chromatin density seen early is found in all precancers, whether it is due to the drug directly, or whether it is due to the benign rapid cell growth that occurs, since regenerating liver nuclei closely resemble it in some stages. Probably, some of each element contribute. Methylcholanthrene is quite different structurally from m'MeDAB, the mouse skin is quite different structurally and physiologically from the rat liver and the tumors produced at these sites are quite different. Yet, the precancerous nuclear change is at least somewhat similar, although it is impossible to tell whether or not the chromatin morphological change in the mouse skin is really the same as that found in these rat livers. This study of nuclei undergoing carcinogenesis appears to be more or less unique. Carcinogenesis has been studied many times before, but not with all the advantages and demonstration of fine nuclear detail that the smear technique offers. Early chromatin changes had not been observed. The next step should be to study another organ, with another carcinogen in a similar manner. It would be valuable to study carcinogenesis in liver smears with a totally unrelated carcinogen, if one

could be found which is suitable. It is very probable that the process would be very similar, if not identical. Do all cancer nuclei arise by way of this gradual change in chromatin structure? If so, why? These are undoubtedly true carcinomas, identical with spontaneous carcinomas histologically and cytologically. Is the drug effect type of nucleus the precursor to all types of cancer, or all carcinomas?

GENERAL SUMMARY AND CONCLUSIONS

I. Paraffin sections of livers which have been perfused with a fixative are superior for histological study to livers which have not been perfused. 80 or 95% ethyl-alcohol is the best of several fixatives investigated for this purpose. Demonstration of cytoplasmic basophilia with various fixative or heavy metal perfusions and the Giemsa stain is investigated and its significance is discussed.

II. Liver smear preparations are much superior to sectioned material for demonstration of fine nuclear detail. Smears are best if made within five minutes after death and if fixed in VandeGrift's fixative, which was found to be superior to the five other fixatives studied. Cytoplasmic basophilia is not well demonstrated in the smear preparation with the Giemsa stain. Similar excellent smear preparations can be made from kidney and spleen.

III. Rat liver nuclei in smear preparations are studied closely morphologically from week to week as carcinogenesis, due to feeding the azo dye, m'MeDAB, progresses. Measurements are also made of these nuclei and statistically analyzed. The evolutionary changes in the nucleus leading to carcinoma are reported and discussed.

IV. Nuclei in smears from regenerating rat livers are observed and measured at different stages of regeneration. The changes observed during regeneration are discussed and compared with the changes seen during carcinogenesis.

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Rat # 63. 9 wks. basic ration.
Liver Smears.