^{3}H-ACTINOMYCIN D AUTORADIOGRAPHIC AND FEULGEN-CYTOPHOTOMETRIC INVESTIGATION OF THE DNA CONTENT OF NORMAL HEPATIC CELLS AND HEPATOMA CELLS

by

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ABSTRACT

The following experiments were designed to study and compare the DNA content of tumor cells in transplanted mouse hepatoma vs. normal hepatic cells by means of autoradiography using ^{3}H-Actinomycin D, and by means of Feulgen cytophotometry. These techniques indicated that the hepatoma cells contain an elevated amount of both nuclear and cytoplasmic DNA.

The average DNA content of hepatoma cells estimated cytophotometrically following Feulgen staining was in the hyperoctaploid range. The ^{3}H-Actinomycin D binding was also much greater in the hepatoma cell nuclei than in normal liver diploid or tetraploid cell nuclei. The number of silver grains per square microns was more than double in the nuclei of hepatoma cells (200.78 $\pm$ 2.40 grains/100 μm^2 nuclear area) than in the nuclei of normal hepatic cells (94.33 $\pm$ 3.57 and 94.02 $\pm$ 1.83 grains/100 μm^2 nuclear area in diploid and tetraploid cells respectively).

The autoradiographic data showed a two-fold increase in the ^{3}H-Actinomycin D binding to the cytoplasmic DNA (probably mDNA) of hepatoma cells (27.03 $\pm$ 0.18 grains/100 μm^2 cytoplasmic area) as opposed to the amount of binding that was found in the cytoplasm of normal hepatic cells (12.41 $\pm$ 0.20 grains/100 μm^2 cytoplasmic area).

Both, the Feulgen-cytophotometric measurements and the ^{3}H-Actinomycin D binding, were shown to be specific for the detection of DNA, since both the Feulgen stain and the radioactive label disappeared after incubation of tissue sections with DNase, yet incubation with RNase did not affect the subsequent labeling pattern.

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INTRODUCTION

The present study was undertaken to compare the distribution of nuclear, nucleolar and cytoplasmic DNA content in hepatoma cells and in normal liver cells.

One of the most striking features of malignant tumors is the abundance of cells having an abnormal, usually elevated, nuclear DNA content (Atkin and Richards, 1956; Bader, 1959; Ogawa _et al._, 1959; Rabotti, 1959; Stich and Emson, 1959; Stich _et al._, 1959; Stich, 1960; 1963; Stich and Steele, 1962; Leuchtenberger, 1958; Kit, 1960), while only a very small percentage of all cancers studied so far consist of cells with an amount of DNA in the diploid range (Stich, 1963). Another common feature of tumor cell populations is that they have an abnormal, usually mixaploid karyotype (Maini and Stich, 1961; Hauschka, 1961; Hellström, 1959; Kraemer _et al._, 1971), with not only high modal chromosome numbers per cell, but with a high variability of this chromosome number too. Potter (1968) noted that, out of 41 transplantable hepatoma lines studied, there were only seven with normal karyotypes, and in the rest of the 35 hepatomas, no two were alike in karyotype. By contrast, normal animal cells exhibit very little karyotypic variability (Hsu, 1961).

The transformation of normal into malignant cells has been long associated with hereditary changes (Boveri,

1914; Winge, 1930). The chromosome analysis in some of these early investigations was based almost entirely on studies of ascites tumors (Hansen-Melander _et al._, 1956; Stich _et al._, 1960; Makino, 1957), because it was, and it still is, very difficult to study the karyotype of solid tumors. Since it is known that the amount of nuclear DNA corresponds in general to the number of chromosomes per nucleus (Leuchtenberger, 1954; Freed and Hungerford, 1957), one can make a direct and simple determination of the DNA content per nucleus using Feulgen cytophotometric measurements (Swift and Rasch, 1956).

Determining the nuclear DNA content in cancer cells with Feulgen-cytophotometry, rather than using karyotype analysis, offers certain additional advantages because it can be applied to sectioned material, so that it makes it possible to combine DNA estimation with a histological examination of the cancer cell morphology on the same section.

Although Feulgen-cytophotometry is still a useful and well established tool in the quantitative analysis of nuclear, i.e. chromosomal DNA content, it is not a sensitive enough method to detect "small amounts" of DNA in cells, i.e. nucleolar and mitochondrial DNA, both of which are part of the "total" cellular DNA content, and are therefore important in a thorough investigation of differences between normal and tumor cells with respect to their DNA distribution.

Studies with ^3H-Actinomycin D for the detection of DNA have demonstrated however, the specific binding of these molecules to the DNA present in cells, and that they, on the other hand, can be used to label "small amounts" of DNA too with a high degree of specificity in autoradiographic preparations (Ebstein, 1967; 1969; Camargo and Plaut, 1967; Simard, 1967).

The molecular mechanism for the binding of Actinomycin D to both the DNA of intact cells and to isolated DNA has been the subject of considerable study (Reich and Goldberg, 1964; Bolund, 1970; Wells and Larson, 1970; Muller and Crothers, 1968; Kersten, 1971; Hamilton et al.,1963). Two possible models have been proposed for Actinomycin D-DNA complex formation: (1) hydrogen binding of the Actinomycin D chromophore to guanine residues in DNA, in combination with packing of the Actinomycin D cyclic peptide chains into the small groove of the double helix (Hamilton et al., 1963); (2) intercalation of the Actinomycin D chromophore into the DNA adjacent to any Guanine-Cytosine (G-C) base pair, which then produces a distortion of the adjacent helical structure, greatly disfavoring binding of another Actinomycin D closer than six pairs away, and it is followed by a conformational adaptation of the Actinomycin D peptide rings to the DNA backbone (Muller and Crothers, 1968). The more recent studies by Wells and Larson (1970); Waring (1968);

Sobell *et al.* (1971) and Bolund (1970) are consistent with the intercalation model of Muller and Crothers (1968). The presence of a suitable DNA configuration has also been stressed by these authors as one of the most important factors for the formation of Actinomycin-DNA complex, and it has been shown that Actinomycin D binds well to double-stranded DNA, whereas single-stranded DNA and DNA-RNA hybrids show little or no binding (Bolund, 1970; Wells and Larson, 1970) because of the slightly different configuration of these latter molecules compared to that of double-stranded DNA.

In the present study the distribution of DNA (nuclear and cytoplasmic) was examined and compared in transplanted mouse hepatoma and in mouse liver hepatic cells using both the classical method of Feulgen cytophotometry and the more sensitive cytochemical technique of ^{3}H-Actinomycin D autoradiography. The specificity of these methods, i.e. Feulgen cytophotometry and ^{3}H-Actinomycin D autoradiography for DNA, was demonstrated by the absence of the Feulgen stain and the radioactive label respectively, after DNase treatments. Both of these techniques have shown that the normal hepatic cells differ greatly from the hepatoma cells with respect to the amounts of both nuclear and cytoplasmic (probably mitochondrial) DNA and with respect to certain morphological features as well.

This study is an effort to provide a better understanding of some of the more basic differences that distinguish hepatoma cells from comparable normal cells, and perhaps to stimulate interest in others to investigate further the differences observed here, in other tumor cells and their normal counterparts.

MATERIALS AND METHODS

Animals Used:

The experiments to be described below were all performed with transplanted mouse hepatoma tumors and normal mouse livers. The tumors were obtained from a hepatoma tumor-bearing line (Jax. Code: BW7756) of an inbred strain (C57L/J) of tumor-susceptable mice. This strain is maintained at the Jackson Laboratory (Bar Harbor, Maine) where the tumors are transplanted serially by subcutaneous passage using 5 to 6 week old animals. The origin of these inbred mice is discussed in some detail by Strong (1955). The normal livers were also obtained from the same strain of mice (C57L/J) as the above, but in this case from healthy specimens which served as controls.

After the transplant, the mice were kept for approximately two and a half weeks in our laboratory on Purina lab. chow with water available *ad libitum*. This proved to be an adequate length of time for the tumor to develop to a sufficient size (approximately 2 X 1 X 1 cm, when excised).

The experimental plan outlined in Text-Fig. 1 was performed in triplicate. In each experiment, containing four tumor-bearing and two normal mice, 7 to 8 week old animals weighing 22-24 gm. were used. For the first

two experiments only males and for the last experiment only females were selected. The mice were sacrificed by dislcoating their neck (U.F.A.W. Staff eds., 1967), thus avoiding anesthesia.

<u>Experimental Plan for the Preparation of ^{3}H-Actinomycin D Labeled Liver and Hepatoma Tissues, and for Enzyme Extractions Prior to Autoradiography</u>:

The tumors were removed from the hepatoma-bearing mice and rapidly diced into approximately one cubic millimeter pieces. Care was taken that no grossly necrotic hepatoma be included in the tissue samples (Kit, 1960) since cytologic details would be difficult to interpret in necrotic tissue.

The livers excised from the normal control mice were cut into similarly small pieces. Each dissection was carried out rapidly (less than 2 minutes per animal) and the tissues were kept wet constantly with freshly prepared Locke solution (Galigher and Kozloff, 1964) during dicing.

Tissues obtained in the above manner from each animal were processed in three parallel experiments, run under similar conditions, differing mainly in the time sequence of incubation with the isotope ^{3}H-Actinomycin D, and the mode in which this incubation was done. These three procedures are outlined in Text-Fig. 1.

Text-Figure 1: Outline of the experimental plan for three parallel procedures, showing sequence of fixation, and incubation with the ^{3}H-Actinomycin D used in each.

Experiment I

Tissues Fixed

↓

Sections cut and
Slides prepared

↓ DNase treated → ↓
↓ RNase treated → ↓
↓ Non treated → ↓

All slides
Incubated in ^{3}H-Actinomycin D

Experiment II

Tissues Incubated
in Locke solution
containing
^{3}H-Actinomycin D

↓

Tissues Fixed

↓

Sections cut and
Slides prepared

Experiment III

Tissues Fixed

↓

Incubated
in
^{3}H-Actinomycin D

↓

Sections cut and
Slides prepared

In the first experiment the small cubes of liver and hepatoma were immediately fixed for 2 hours in 3% glutaraldehyde at 4°C (Sabatini et al., 1963) buffered to pH 7.2 with 0.1M sodium phosphate buffer (Nunn, 1970). This was followed by three rinses in the phosphate buffer alone, prior to postfixing for 1 hour in 1% osmium tetroxide buffered as with the primary fixative. The tissues were then rinsed in several changes of distilled water, dehydrated in graded series of alcohol and in three changes of propylene oxide, transferred for 1 hour into a one part propylene oxide, one part complete Araldite mixture and then embedded in Araldite (Glauert, 1962). 0.5 μm sections were cut with glass knives on a Porter Blum MT-1 ultra-microtome.

To provide control slides in which the DNA had been selectively removed, and thus to ensure that only DNA was being labeled during the subsequent incubation with ^{3}H-Actinomycin D, control sections were treated for 4 hours at 37°C with a solution of 0.30 mg deoxyribonuclease (Worthington DNase 1X crystallized) per milliliter of 0.03M magnesium sulfate brought to pH 6.0 with 0.1N NaOH (Deitch, 1966).

It was also necessary to carry out RNA extraction to make certain that no labeled Actinomycin D was binding to the RNA. Thus, under the same conditions, other slides were incubated in a solution of ribonuclease (Worthington RNase 2X crystallized) using 0.30 mg/ml adjusted to a pH

6.5 (Deitch, 1966).

After both enzyme digestions, removal of hydrolyzed fragments of nucleic acids was facilitated by washing the slides in three changes of 5% trichloroacetic acid at 4°C for 15 minutes as suggested by Swift (1966), and the acid was later removed with several changes of distilled water.

These enzyme treated sections, together with untreated ones, were incubated for 4 hours at room temperature with a solution of 20 μCi/ml tritiated Actinomycin D, a product of Schwarz Bioresearch Inc. (Orangeburg, New York), specific activity 8.4 Ci/mMole and a concentration of 0.5 mCi/ml. The solution also contained two drops of 0.5% Tween(20).

The incubation was carried out in a moist chamber by placing a large drop of the ^{3}H-Actinomycin D solution over each section as in the method used by Ebstein (1967, 1969) and by Camargo and Plaut (1967). After incubation the excess isotope was washed off with several changes of distilled water and then the slides were taken through an ethanol series (2 minutes each in 50%, 70%, 85%, 95% and 100%), which has been reported to greatly reduce the background without affecting the specific binding of ^{3}H-Actinomycin D to the DNA (Ebstein, 1969). These slides were later rehydrated and air dried.

The second experiment differed from the first only in that here, even prior to fixation, the tissues were incubated as unfixed (Ebstein, 1969) one millimeter cubes for 4 hours at room temperature in 0.2 ml Locke solution containing 50 μCi ^{3}H-Actinomycin D, and that the incubation itself was followed by several changes of fresh Locke solution to remove excess radioactivity. They were subsequently fixed, embedded and sectioned in the same manner as in the first experiment.

In the third experiment, incubation was carried out after double fixation in glutaraldehyde and osmium tetroxide, using 0.2 ml distilled water, which contained the 50 μCi ^{3}H-Actinomycin D, and otherwise under conditions identical to the previous experiment as far as incubation time and temperature were concerned. Distilled water was substituted in the place of Locke solution to wash off the excess radioactivity at the end of the incubation period. The embedding and sectioning procedures that followed were identical to the previous two experiments.

The 0.5 μm sections prepared for the two latter experiments were not subjected to enzyme treatments.

Autoradiography and Staining Procedures:

Sections obtained by all three of the above experiments were dipped in total darkness in Ilford L-4 emulsion

(Ilford Nuclear Research, Ilford, Essex, England) at approximately 32°C, diluted 1:1 with double distilled water (Kopriwa, personal commun.). The slides were allowed to dry at 25°C in vertical position and then stored for exposure at 4°C, in light proof black plastic boxes for 10-12 weeks. The preparations were developed, after exposure, in Kodak D-19 developer at 20°C for 3 minutes, and after a short rinse in distilled water, fixed for 3 minutes in 24% sodium thiosulfate, then rinsed in three changes of distilled water for 2 minutes in each (Kopriwa, personal commun.).

Following development, the slides were stained in 1% Toluidine blue saturated with sodium borate for 35-40 minutes. They were then transferred to a Coplin jar of distilled water to which 2-3 drops of acetic acid has been added until the stain was seen running off from the emulsion; then the slides were rinsed in three changes of distilled water, air dried and mounted in Permount.

To determine differences between normal liver and hepatoma cells, with respect to the total amounts of DNA in each, the silver grains, indicating the ^{3}H-Actinomycin D deoxyribonucleic acid binding sites, were counted separately over the nuclei and over unit areas of cytoplasm.

Grain counts were made over ten randomly chosen parenchyma nuclei (easily distinguished from the smaller and oval nuclei of von Kupffer cells) per animal in each

of the three experiments and for all three groups of animals used. At the same time two measurements were taken at right angles to each other for each nucleus, along the short and the long axis respectively, using an ocular micrometer calibrated in microns (μm) by means of a reference stage micrometer. The total area occupied by each nucleus could then be calculated in square microns. The mean grain counts per nucleus were then determined for the normal liver diploid and tetraploid cell population and for the hepatoma cell population respectively. The mean grain concentration was subsequently also expressed per a standard (100 μm^2) nuclear area. Silver grains were also counted per unit area (123.43 μm^2) of cytoplasm using a calibrated grid placed in the eyepiece of the microscope, recording grain counts over twenty such randomly selected areas per animal. The mean grain concentrations thus obtained per unit area of cytoplasm were then converted, and expressed per a standard 100 μm^2 cytoplasmic area. Standard errors of the means were calculated for all experimental data above.

Background counts were made on "cold" control sections which were dipped, exposed, developed, fixed and stained in a manner identical to the experimental slides, but which were <u>not</u> subject, at any point, to incubation in the isotope. The experimental means obtained per nucleus were corrected by subtraction of the background fog using the method suggested by Baserga and Malamud (1969) and

according to the equation given by Stillström (1963). When the mean grain concentration was expressed per standard area (100 μm^2), the corresponding background fog per standard area (100 μm^2) was simply subtracted from this mean experimental value.

Photomicrographs were taken with Leica M4 camera mounted on a Leitz Orthoplan microscope using Kodak Panatomic-X 35mm film and a Leitz yellow-green filter.

Cytophotometry:

For the cytophotometric analysis of nuclear DNA content, approximately two cubic millimeter pieces of the tumors and the livers were removed from four of the hepatoma-bearing and from two control (C57L/J) mice respectively, and were fixed in Carnoy's ethanol: acetic acid (3:1) for 2 hours (Galigher and Kozloff, 1964). They were then dehydrated in alcohol, cleared in xylene and embedded in Tissuemat (m.p. 56.5°C, Fisher Scientific Co.) and sectioned at 15 and 20 µm. The sections were stained by the Feulgen technique (Pearse, 1968). Control sections were deoxyribonuclease (Worthington DNase) extracted at 37°C for 4 hours, using the method of Deitch (1966), prior to Feulgen staining. The sections were then mounted in oil of refractive index 1.560 (R.P. Cargille Inc., New York), corresponding to the refractive index of the cytoplasm. Matching of the refrac-

tive indices of the mounting oil and the cytoplasm prevents error due to extraneous light scattering (Swift and Rasch, 1956).

The cytophotometric measurements were made on a Zeiss cytophotometer of the type described by Pollister and Ornstein (1959). Since the peak absorption of the dye is known to be 540 mμ (Deitch, 1966), the measurements were taken at this absorption peak by the one wavelength core method described by Swift and Rasch (1956). In this method, accuracy is enhanced by measuring light absorption through a core or plug in the center of the nucleus, and then using the core diameter, nuclear diameter and Feulgen absorption value to calculate DNA content per nucleus, routinely expressed in arbitrary units (A.U.) which represent Feulgen DNA content per nucleus. Sixty such measurements were taken for normal and for hepatoma nuclei and the average calculated values used as a measure of the relative concentration of DNA per nucleus. Prior to each such reading the nuclear dimensions were recorded, using the ocular micrometer as described in the section on autoradiography which were necessary to select the proper core diameter and were also used in the classification of nuclei. The Feulgen reaction is well established as a reliable mode of measuring nuclear DNA (Swift, 1953; 1966; Deitch, 1966).

The DNA content of two classes of normal hepatic cell nuclei were measured, diploid and tetraploid, and com-

pared to that of the hepatoma tumor cell nuclei. For each nuclear class, diploid, tetroploid and hepatoma respectively, the standard error of the mean relative DNA concentration per nucleus was also calculated.

RESULTS

The Microscopic Morphology of Normal Liver in Mice:

The liver consists of four main lobes which are situated beneath the diaphragm, and are attached to it. Three of these lobes are partially divided by a deep bifurcation. The lobes are surrounded by a thin fibro-elastic capsule which is overlaid with reflected peritoneum. Strands of this connective tissue capsule project inward to form the inner supporting framework for the parenchyma. In this inner connective tissue within the portal canals are the components of the hepatic triad; a branch of a hepatic artery, portal vein and bile duct, usually accompanied by few delicate lymphatic vessels.

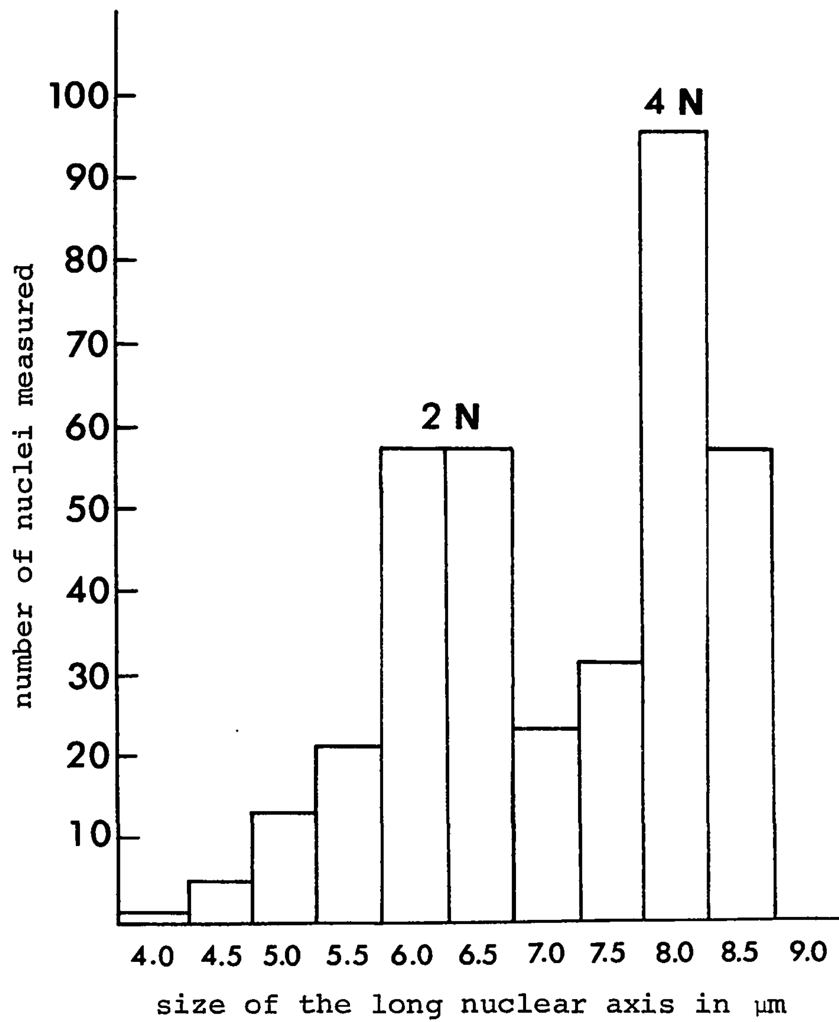
The parenchyma consists of cords or plates of liver cells radiating outward from a central vein. Between these cells pass microscopic bile canaliculi, but they can only be demonstrated by special staining techniques. The hepatic cords are separated by sinusoids lined by two kinds of cells: the typical endothelial cells with darkly staining, small, elongated nuclei and the phagocytic stellate cells of von Kupffer containing somewhat larger and more oval nuclei with small but prominent nucleoli. The hepatic cells themselves are large, polyhedral in shape and they have round, centrally located, vesicular nuclei with one

or more nucleoli (Figs. 1, 2) [Fekete, 1956; Hummel, Richardson and Fekete 1966].

In the present study hepatic cells with nuclei of various size were observed, probably corresponding to a diploid and tetraploid nuclear population which was not at all surprising in view of the fact that the liver is known to be a polyploid tissue (Bloom and Fawcett, 1968; Arey, 1964). In order to distinguish between these two classes of nuclei correctly on the basis of nuclear size, the measurements of the long axis of nuclei, from all nuclei measured (n = 360), were grouped onto a histogram (Text-Fig. 2). This histogram then revealed two distinct peaks, one for diploid and one for tetraploid nuclei, with a clear cut-off point separating them. One group of nuclei (diploid) had an average long axis of 6.09 μm, while the second group of nuclei (tetraploid) had an average long axis of 8.17 μm. The average nuclear area was calculated as 28.56 μm^2 for diploid nuclei and 49.94 μm^2 for tetraploid nuclei (Table 1). These latter (tetraploid) nuclei were in slight majority. All mean nuclear dimensions as well as sample sizes are given in Table 1.

Both nuclear classes contained prominent and mostly multiple nucleoli (Fig. 1). The average lengths of the long axis of nucleoli were found to be quite close, 1.34 μm and 1.44 μm respectively for diploid and for tetraploid cells

Text-Figure 2. Histogram illustrating the distribution of normal hepatic cell nuclei (diploid and tetraploid) according to size in six mice. The long axis of nuclei (n=360) measured with ocular micrometer.



100
90
80
70
60
50
40
30
20
10
number of nuclei measured
2 N
4 N
4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5 8.0 8.5 9.0
size of the long nuclear axis in μm

Table 1: Measurements of Nuclear Size in Liver and Hepatoma Cells.

Tissue	Mean Nuclear Dimensions			Number of nuclei
	Short axis	Long axis	Area in μm^2	measured
Liver				
Hepatic cell presumably diploid	5.94	6.09	28.56	177
Hepatic cell presumably tetraploid	7.85	8.17	49.94	183
Hepatoma				
Parenchymal cell	11.53	12.62	115.47	660

All linear measurements given in μm.

(For range of nuclear size see Text-Figs. 2,3).

(Table 2). In the diploid cells the long axis of nucleoli ranged in size from 0.5 - 2.5 μm, while in the tetraploid cells this range extended to 2.75 μm (Text-Fig. 3).

Nuclear and nucleolar size measurements reported previously by Shea and Leblond (1966) differ somewhat from those observed in this study, since they reported the following mean nuclear diameters: 6.41 μm for diploids and 7.89 μm for tetraploids (also taking the long axis into consideration), whereas the nucleolar diameters reported by these authors were 1.22 μm and 1.26 μm in diploid and in tetraploid cells respectively. These differences could easily be attributed to the different strains of mice used and the different types of fixatives used in the two studies.

Besides the above mentioned cell types, binucleate cells with two small nuclei were also observed (Fig. 3), but not too frequently. These cells have multiples of the ordinary diploid number of chromosomes. There was no mitotic activity seen, which is according to expectation since mitoses are usually rare in normal liver of adult animals (Fekete, 1956; Bloom and Fawcett, 1968).

The 0.5 μm toluidine blue stained sections used in this study were advantageous in showing not only a good nuclear detail, but also the variability of the cytoplasm and the mitochondria in the latter (Fig. 3). This distribution and staining of mitochondria was similar to the one noted by Ham (1965).

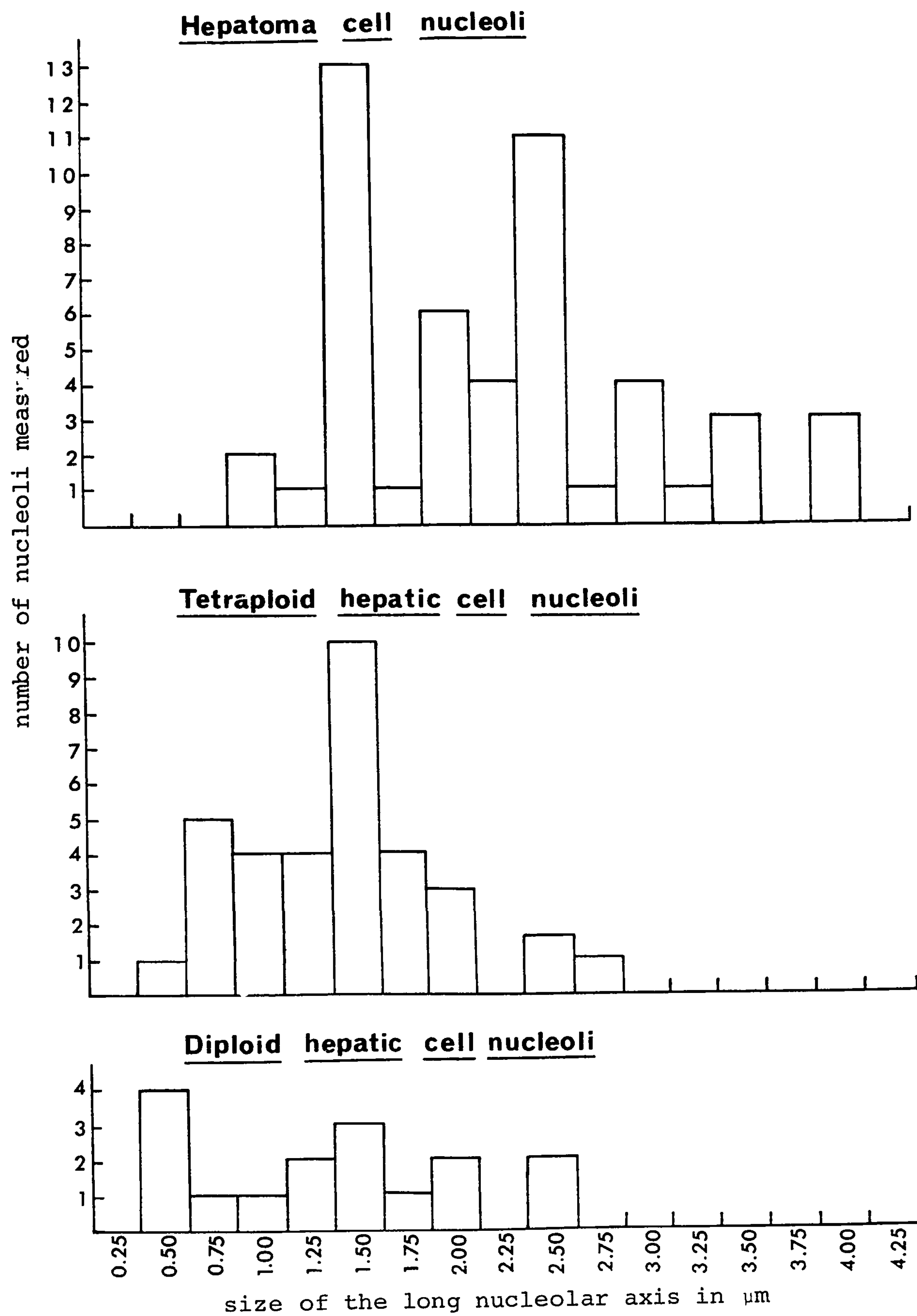
Table 2: Measurements of Nucleolar Size in Liver and Hepatoma Cells.

Tissue	Mean Nucleolar Dimensions			Number of Nucleoli
	Short axis	Long axis	Area in μm^2	measured
Liver				
Hepatic cell presumably diploid	1.04	1.34	1.30	16
Hepatic cell presumably tetraploid	1.20	1.44	1.48	34
Hepatoma				
Parenchymal cell	1.88	2.27	3.61	50

All linear measurements given in μm.

(For range of nucleolar size see Text-Fig. 4).

Text-Figure 3: Histogram illustrating the size distribution of nucleoli in diploid, tetraploid normal liver cells and in hepatoma parenchymal cells. The long axis of nucleoli measured with ocular micrometer.


Hepatoma cell nucleoli
Tetraploid hepatic cell nucleoli
Diploid hepatic cell nucleoli
number of nucleoli measured
size of the long nucleolar axis in µm
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00
2.25
2.50
2.75
3.00
3.25
3.50
3.75
4.00
4.25

<u>The Microscopic Morphology of the Transplanted Hepatoma in Mice (C57L/J)</u>:

The subcutaneously transplanted hepatoma cells grow and form a discrete mass under the skin. The tumor thus developed, is a fairly soft whitish tissue with a few hemorrhagic nodules. Hepatomas are usually divided into two major types, those involving principally the biliary ducts better known as cholangiocarcinomas or cholangiomas, and those involving mainly the liver parenchyma cells, which are the real hepatomas or hepatocellular carcinomas (Firminger, 1955). The majority of hepatomas are of the latter type. It is not uncommon, however, to find cholangiohepatomas (Firminger, 1955) which are composed of both cell types.

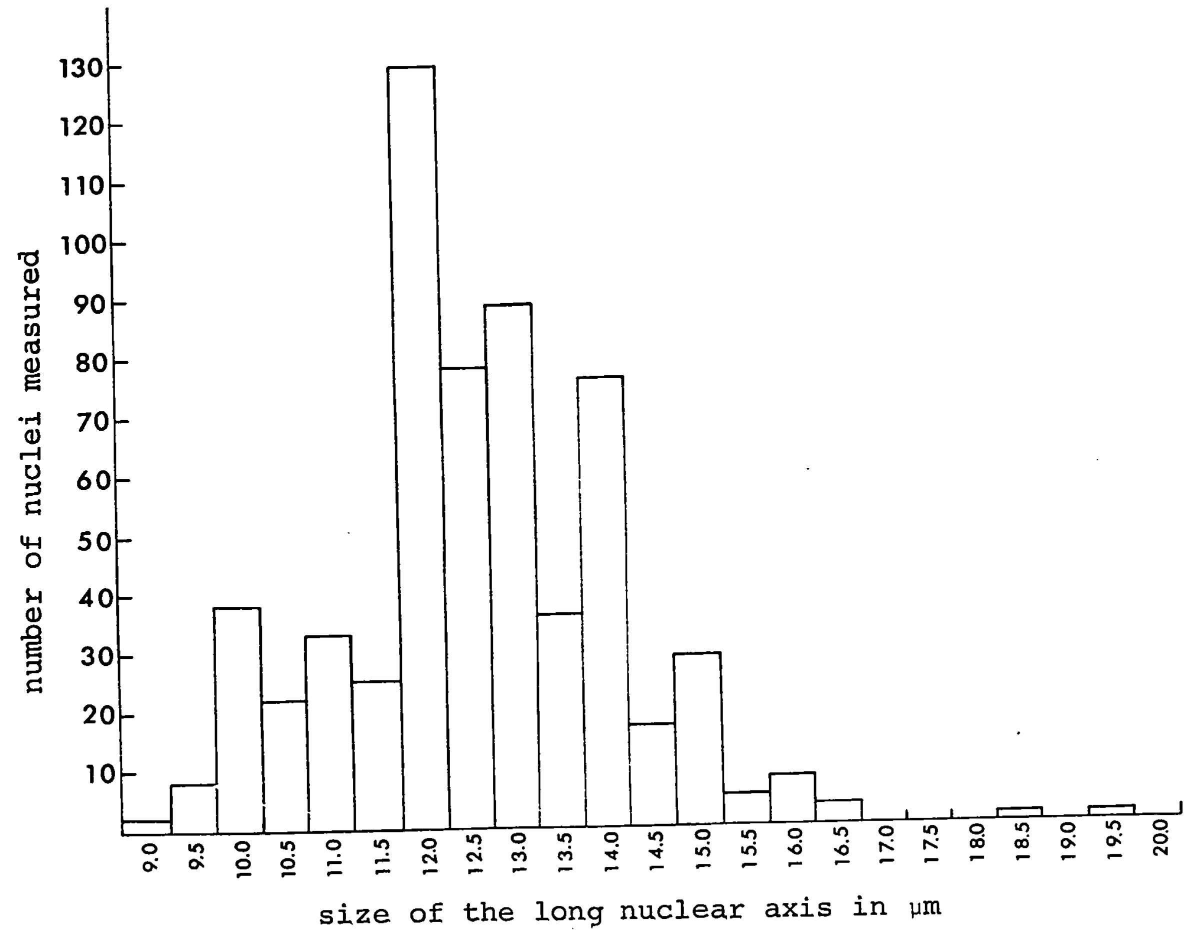
The transplanted hepatomas used in this research, were the hepatocellular type. The microscopic morphology of this hepatoma mimics the normal liver to some extent in that the parenchyma retains a vague cord like arrangement, but with cells which are atypical. Although the cords are still separated by sinusoids, the normal architecture characteristic of liver is lost (Murphy, 1966). The tumor cells are usually larger than their normal counterparts; giant and bizarre cell forms are also not uncommon among them (Cloudman, 1956). The cytoplasm of the tumor cells may often be vacuolated (Figs. 5, 6), and it has been shown with the elec-

tronmicroscope that it contains all of the organelles characteristic of normal cells (Koss, 1968, Williams, 1970).

In the present study too, it was found that the nuclei of hepatoma cells were commonly much larger than the normal liver diploid or even tetraploid nuclei and that they exhibited a greater variation in size and shape (Figs. 4,5,6). Most of these nuclei contained many darkly staining chromatin clumps which are interpreted usually as condensed portions of the chromatin, i.e. heterochromatin (Figs. 4,5,6). The chromatin also seemed to be frequently located near the periphery, referred to by Koss (1968) as "margination of chromatin" and apparently characteristic of neoplastic nuclei (Figs. 4,5). The mean nuclear dimensions for hepatoma are also shown in Table 1. The hepatoma nuclei ranged in size between 9.0 µm and 16.5 µm (Text-Fig. 4), when measured along their long axis, with an average nuclear area of 115.47 μm^2 (Table 1). In some of these nuclei, very large and frequently irregular nucleoli were seen (Figs. 4, 6). The mean nucleolar diameter was 2.27 µm (Table 2), with a range between 1.0 µm and 4.0 µm also taking the long axis into consideration (Text-Fig. 3).

Mitoses were observed very frequently among these tumor cells, as would be expected from a rapidly proliferating neoplastic tissue (Figs. 7,8).

Text-Figure 4: Histogram illustrating the distribution of hepatoma cell nuclei (n=660) of twelve mice according to size. The long axis of nuclei measured with ocular micrometer.



130
120
110
100
90
80
70
60
50
40
30
20
10
number of nuclei measured
9.0
9.5
10.0
10.5
11.0
11.5
12.0
12.5
13.0
13.5
14.0
14.5
15.0
15.5
16.0
16.5
17.0
17.5
18.0
18.5
19.0
19.5
20.0
size of the long nuclear axis in μm

^{3}H-Actinomycin D Autoradiography Results:

The information obtained from this study indicates a significant difference between normal hepatic cells and hepatoma cells with respect to the amount of ^{3}H-Actinomycin D bound by each. This difference was seen on the autoradiographed sections when the concentration of silver grains was measured in the nuclei and in the cytoplasm of the normal liver and hepatoma cells respectively. Furthermore, this observation suggests a difference in the DNA content of these cells and/or a difference in their ^{3}H-Actinomycin D binding ability.

In the normal liver of all animals a polyploid series was discernable on the basis of differences in nuclear dimensions, as it was mentioned earlier (Table 1), as well as on the basis of mean grain counts per nucleus (Table 3). As shown in Table 3, the average number of grains found per tetraploid nucleus (expressed in terms of nuclear area) was almost double that per diploid nucleus of the same tissue. Proof that the ^{3}H-Actinomycin D was indeed binding to DNA was provided, when the results obtained from the enzyme treated control sections were compared to the results of the untreated sections. There were no significant differences observed between the RNase treated sections of Experiment 1 (Figs. 12, 20, 21) and the non treated sections of Experiment 1 (Figs. 9, 14, 15); Experiment II

Table 3: Mean Grain Counts, Representing ^{3}H-Actinomycin D Binding to the Nuclear DNA of Normal Hepatic Cells and Hepatoma Cells in 18 Mice*

Tissue	Corrected Mean Grain Count/Nucleus ± Standard Error				
	Experiment I			Experiment II	Experiment III
	Untreated	RNase treated	DNase treated		
Normal Liver					
Hepatic cell presumably diploid	(n=31) 26.16 ± 1.08	(n=33) 27.54 ± 1.04	(n=21) 0.23 ± 0.15	(n=31) 27.45 ± 1.16	(n=38) 26.86 ± 0.71
Hepatic cell presumably tetraploid	(n=29) 47.44 ± 0.73	(n=27) 46.26 ± 0.97	(n=39) 0.15 ± 0.08	(n=29) 45.61 ± 1.00	(n=22) 47.09 ± 1.26
Hepatoma					
Tumor cell	(n=120) 219.87 ± 2.77	(n=120) 227.55 ± 2.99	(n=120) 0.16 ± 0.04	(n=120) 232.75 ± 2.32	(n=120) 234.15 ± 2.10

*Light microscope autoradiographs of 0.5 μm Araldite sections.

Nuclear sample size shown in brackets.

(Figs. 10, 16, 17) or Experiment III (Figs. 11, 18, 19) as far as the average number of grains per nucleus or per 100 μm^2 nuclear area were concerned (Tables 3,4). On the other hand, when the DNase treated controls of the same experiment were examined under the microscope, the mean grain counts were found to be drastically reduced in both the normal (Fig. 13) and hepatoma (Fig. 22) tissues (Tables 3, 4).

These results show that in this system, ^{3}H-Actinomycin D was binding specifically to DNA, as described in the introduction, since when the DNA was extracted with DNase, no ^{3}H-Actinomycin D binding occurred.

In the hepatoma, dense clumps of grains were observed in the nucleus proper and many times near the nuclear membrane (Figs. 14, 17, 18, 19, 21), corresponding in position to the heterochromatin clumps seen in our stained but not autoradiographed preparations, and a particularly heavy concentration of silver grains was observed around and over most nucleoli, in the area usually occupied by the nucleolus-associated heterochromatin (Figs. 15, 16, 17, 20, 21).

Table 3 also shows that ^{3}H-Actinomycin D was bound to a much greater extent to hepatoma nuclei than to normal liver diploid or tetraploid nuclei. This became quite clear when mean grain counts were expressed either as grains per nucleus or when expressed as mean grain concentrations per 100 μm^2 nuclear area. The ^{3}H-Actinomycin D binding was

Table 4: Mean Grain Concentration Per 100 μm^2 Nuclear Area, Representing ^3H-Actinomycin D Binding to Nuclear DNA in 18 Mice*

Tissue	Corrected Mean Grain Concentration/100 μm^2 Nuclear Area ± Standard Error				
	Experiment I			Experiment II	Experiment III
	Untreated	RNase treated	DNase treated		
Normal Liver					
Hepatic cell presumably diploid	(n=31) 94.33 ± 3.57	(n=33) 96.13 ± 3.09	(n=21) 0.75 ± 0.47	(n=31) 93.04 ± 2.74	(n=38) 95.21 ± 2.57
Hepatic cell presumably tetraploid	(n=29) 94.02 ± 1.83	(n=27) 94.18 ± 2.19	(n=39) 0.33 ± 0.16	(n=29) 91.07 ± 2.04	(n=22) 92.70 ± 1.77
Hepatoma					
Tumor cell	(n=120) 200.78 ± 2.40	(n-120) 204.03 ± 1.97	(n=120) 0.09 ± 0.04	(n=120) 202.52 ± 2.05	(n=120) 204.44 ± 2.07

*Light microscope autoradiographs of 0.5 μm Araldite sections.

Nuclear sample size shown in brackets.

found to average at 219.87 grains per hepatoma nucleus, as compared to the 47.44 grains average per tetraploid and the 26.16 grains average per diploid nucleus (Table 3). It was also found that the number of silver grains per square microns was more than double in the nuclei of hepatoma cells than in the nuclei of normal liver cells (Table 4).

Another important result obtained in this study is the observation of ^{3}H-Actinomycin D binding outside the nuclei. This binding can not be interpreted as being due to coincidental grain scattering, since it is obvious on inspection that in all three experiments the mean grain concentrations per 100 μm^2 of cytoplasm were similar on all non-enzyme treated sections, as well as on the RNase treated controls of Experiment 1 (Table 5, Figs. 9-12, 14-21). However, as shown in Table 5, a radical decrease was observed in the grain density per 100 μm^2 cytoplasmic area after treatment with DNase in both the normal and hepatoma tissues of Experiment 1 (Figs. 13, 22). Furthermore, when the average number of grains per 100 μm^2 cytoplasm was compared in the normal liver cells and in the tumor cells of hepatoma, a more than two-fold increase was observed in the cytoplasm of the latter (Table 5).

The results obtained with all three experimental methods were very similar, indicating that neither of the three procedures facilitated binding with ^{3}H-Actinomy-

Table 5: Mean Grain Concentrations Per Standard (100 μm^2) Cytoplasmic Area, Representing ^{3}H-Actinomycin D Binding to the Cytoplasmic DNA of Normal Hepatic Cells and Hepatoma Cells in 18 Mice*

Tissue	Corrected Mean Grain Count/100 μm^2 Cytoplasm $\pm$ Standard Error				
	Experiment I			Experiment II	Experiment III
	Untreated	RNase treated	DNase treated		
Normal Liver	(n=120)	(n-120)	(n=120)	(n=120)	(n=120)
Hepatic Cell	12.41 $\pm$ 0.20	12.41 $\pm$ 0.18	0.10 $\pm$ 0.04	12.31 $\pm$ 0.19	12.35 $\pm$ 0.19
Hepatoma	(n=120)	(n=120)	(n=120)	(n=120)	(n=120)
Tumor cell	27.03 $\pm$ 0.18	26.97 $\pm$ 0.18	0.11 $\pm$ 0.04	27.00 $\pm$ 0.16	27.00 $\pm$ 0.16

*Grain counts made over unit area (123.43 μm^2) of cytoplasm in light microscope autoradiographs of 0.5 μm Araldite sections and then converted to standard area (100 μm^2).

Sample size in brackets.

cin D to a greater extent than the other two (Tables 3, 4, 5; Figs. 9-11 and 14-19).

Results of Feulgen Cytophotometry:

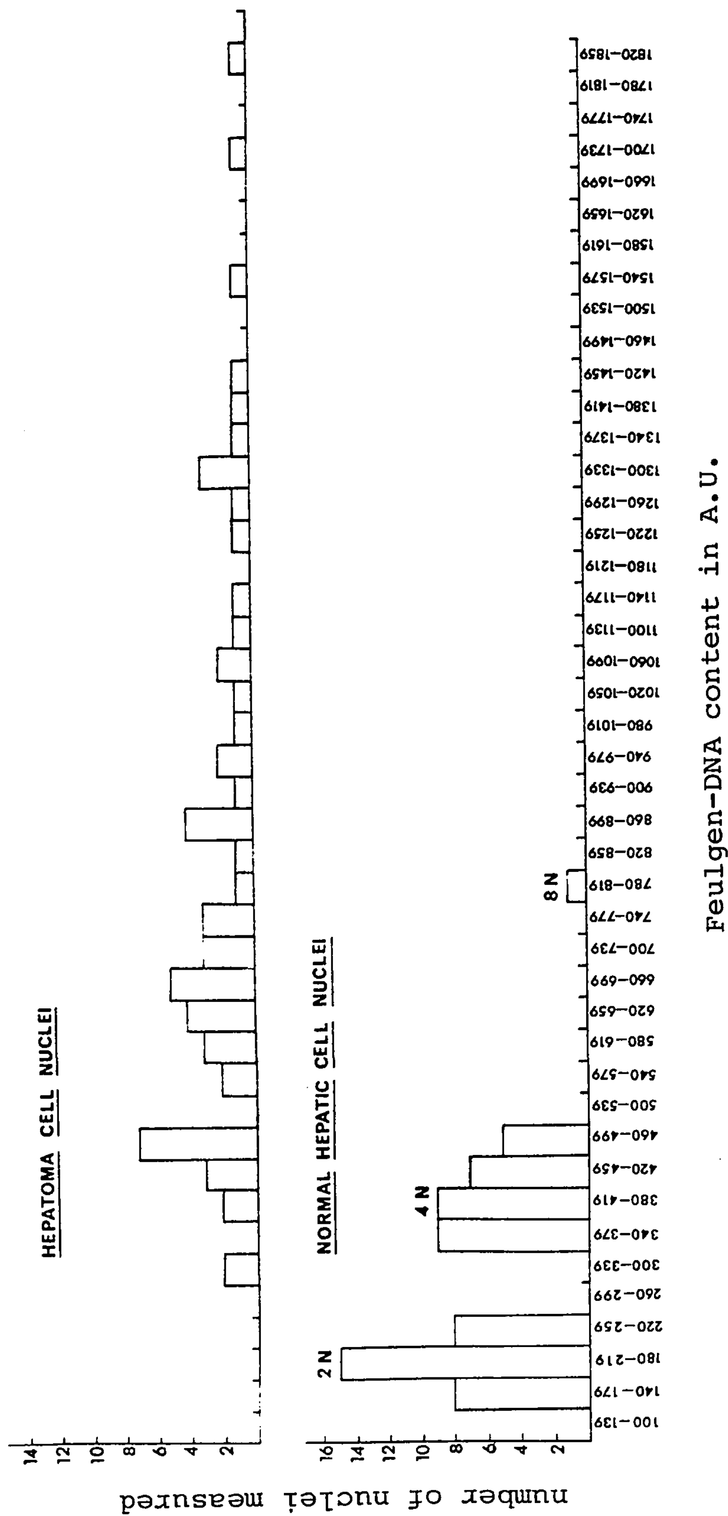
The characteristic polyploid series expected in liver nuclei on the basis of cytophotometric measurements of Feulgen dye-binding is presented in Table 6. There was a clear separation observed, into diploid, tetraploid and octaploid nuclei, on the basis of Feulgen-DNA content. The average amount of DNA found per nucleus was expressed in arbitrary units (A.U.) and it was 200.30 A.U., 407.70 A.U. and 810.00 A.U. (Table 6) for normal 2N, 4N and 8N hepatic cells respectively. The criterion of DNA content may be considered more exact than that of size in characterizing a polyploid series.

This regular series of DNA values found in liver nuclei served as a point of comparison for the wide range of DNA values found in hepatoma cell nuclei. The DNA values found per hepatoma cell nucleus in this study ranged between 302 A.U. and 1830 A.U. (Text-Fig. 5) with an average DNA value of 825.93 $\pm$ 117.20 A.U. (Table 6). With such a wide range of DNA values and such a large standard error we realize that there is a great diversity in the DNA content of these nuclei. The data on hepatoma nuclei presented as a histogram (Text-Fig. 5) suggests that about 30% of the

Table 6: Average Feulgen-DNA Content of Hepatic Cell and Hepatoma Cell Nuclei in 6 Mice ± S.E., Expressed in Arbitrary Units (A.U.).

Tissue	Feulgen-DNA Content in A.U.	Number of cells measured
Liver 2N	200.30 ± 4.59	30
Liver 4N	407.70 ± 7.48	30
Liver 8N	810.00	1
Hepatoma	825.93 ± 117.20	60

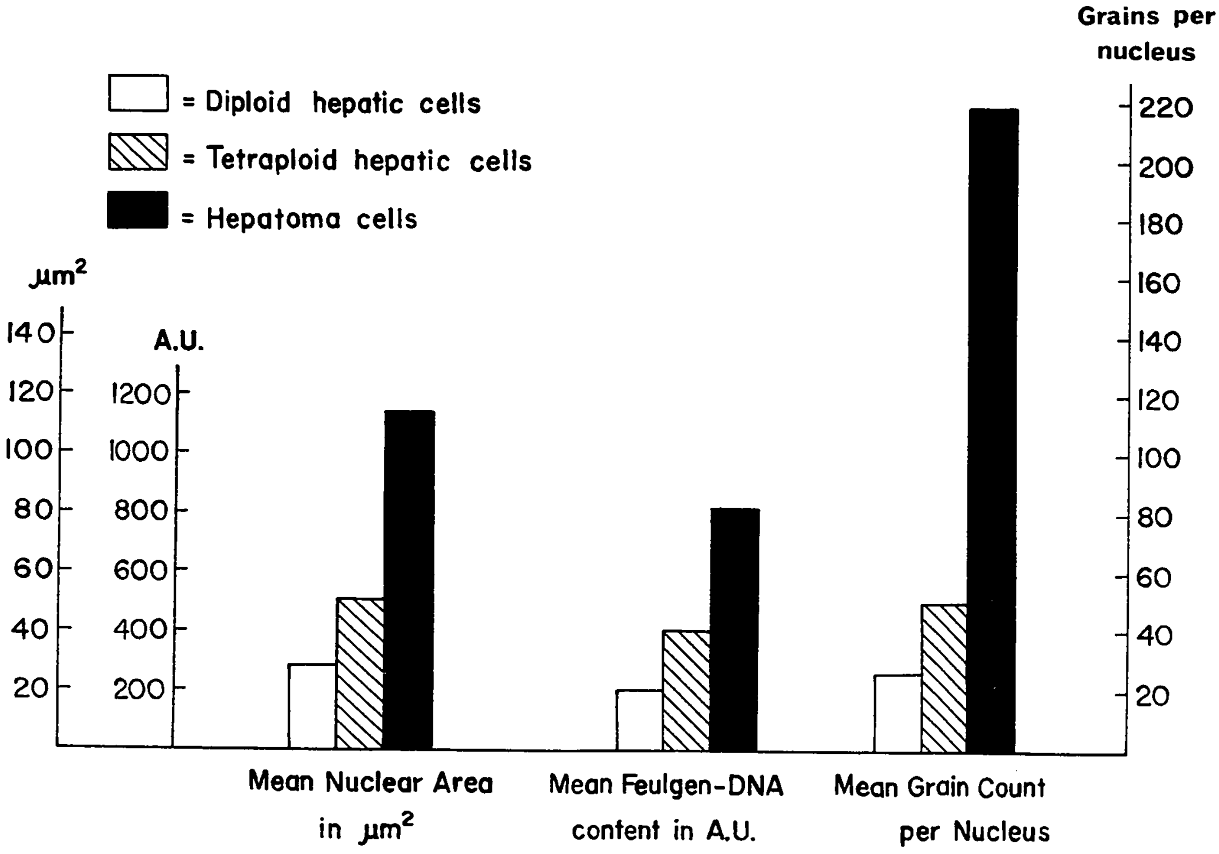
Text-Figure 5: Histogram showing the nuclear distribution according to Feulgen-DNA content in normal liver cells and in hepatoma cells. The Feulgen-DNA content per nucleus expressed in arbitrary units (A.U.).


HEPATOMA CELL NUCLEI
NORMAL HEPATIC CELL NUCLEI
2 N
4 N
8 N
Feulgen-DNA content in A.U.
number of nuclei measured
14 12 10 8 6 4 2
16 14 12 10 8 6 4 2
100-139 140-179 180-219 220-259 260-299 300-339 340-379 380-419 420-459 460-499 500-539 540-579 580-619 620-659 660-699 700-739 740-779 780-819 820-859 860-899 900-939 940-979 980-1019 1020-1059 1060-1099 1100-1139 1140-1179 1180-1219 1220-1259 1260-1299 1300-1339 1340-1379 1380-1419 1420-1459 1460-1499 1500-1539 1540-1579 1580-1619 1620-1659 1660-1699 1700-1739 1740-1779 1780-1819 1820-1859

hepatoma cells fell into the hypertetraploid category, with another 30% in the octaploid and hyperoctaploid range, while the rest (with the exception of only a few which could be considered straight tetraploid) exhibited higher polyploidy and aneuploidy, some in the 16N range and higher (Text-Fig. 5).

These Feulgen cytophotometric results helped to reinforce the data on nuclear size measurements and the autoradiographic findings. The results of all three methods are presented for comparison in Text-Fig. 6. This figure clearly shows that a direct proportionality exists with respect to nuclear size, Feulgen-DNA content and ^{3}H-Actinomycin D binding in the normal liver polyploid series. In the hepatoma cells the nuclear size and Feulgen-DNA content is approximately double that of the tetraploid cells, while the ^{3}H-Actinomycin D binding per nucleus is approximately four times that of the tetraploid cells. This increased ^{3}H-Actinomycin D binding may imply a difference in the state of the DNA of tumor cells, which will be discussed in the next section.

Text-Figure 6: Histogram summarizing the results, and allowing comparison between nuclear area, Feulgen cytophotometric measurements and ^{3}H-Actinomycin D binding to nuclear DNA.


= Diploid hepatic cells
= Tetraploid hepatic cells
= Hepatoma cells
µm²
140
120
100
80
60
40
20
A.U.
1200
1000
800
600
400
200
Grains per nucleus
220
200
180
160
140
120
100
80
60
40
20
Mean Nuclear Area in µm²
Mean Feulgen-DNA content in A.U.
Mean Grain Count per Nucleus

DISCUSSION

The principal points for discussion can be formulated into the following questions: (1) Does this study confirm our knowledge of cell characteristics and nuclear DNA distribution in a polyploid series? (2) In what way does the cell characteristics and nuclear DNA distribution of normal cells (liver) differ from those of a tumor cell (hepatoma)? (3) Is there a difference in the amount of cytoplasmic DNA, as detected by the concentration of radioactivity (^{3}H-Actinomycin D binding), between these normal and hepatoma cells?

Nuclear Characteristics and Cytophotometry:

It is well known that the liver is a polyploid tissue (Bloom and Fawcett, 1968; Arey, 1964; Ham, 1965). The data presented in this study offers confirmation once again of this fact in the mouse (C57L/J) liver as observed by a variety of measurement techniques employed (i.e. nuclear size data, cytophotometric and autoradiographic data).

Diploid and tetraploid nuclei were observed in the present study (based on nuclear measurements) with almost equal frequency (Table 1, Text-Fig. 2), whereas octaploid nuclei were extremely rare (only one observed - Text-Fig. 5).

In one recent paper by Shea and Leblond (1966) a diploid and tetraploid cell population was similarly characterized, in mouse liver, by measurements of nuclear diameter. Their measurement values differ slightly from the ones reported here which, as already mentioned in the results, can be attributed to the different fixatives and/or the different strains of mice used in the two studies. Another point which should be kept in mind is that the classification of cells into various ploidy groups on the basis of size data alone is always somewhat arbitrary, since in thin histological sections one can usually measure only a slice through the nucleus. Thus it is impossible to tell with certainty whether the slice is through the equator or through the tip of the cell, just to mention the two extremes.

It has been documented, however, for some time now with another and more reliable method, Feulgen cytophotometry, which measures the DNA content of the nuclei, that the liver is indeed a polyploid tissue (Swift, 1966; Sandritter, 1966; Deitch, 1966). For cytophotometry at least 15-20 μm tissue sections are used, which ensures that the section contains whole nuclei. The cytophotometric measurements of Feulgen stained liver nuclei presented in this study (Table 6; Text-Fig. 5) also confirm these previous cytophotometric observations, since the amount of Feulgen dye

bound by the tetraploid liver nuclei was double the amount bound by diploid nuclei of the same tissue, giving, the expected ratio of 2:4:8 for diploid, tetraploid and octaploid nuclei respectively (Table 6).

The hepatoma nuclei did not present such a regular nuclear size distribution (Text-Fig. 4) as the normal hepatic cell nuclei. Therefore, using only nuclear size measurement values (Table 1) and the histogram (Text-Fig. 4) prepared from this data, it was only possible to estimate approximately the degree of ploidy in the hepatoma parenchyma population. Although this size data was inadequate by itself, it was certainly useful in showing the extreme variability of the hepatoma nuclei (Text-Fig. 4), a phenomena characteristic of tumor cells in general. All these hepatoma nuclei measured were larger than the diploid or tetraploid nuclei of normal liver cells (Text-Fig. 2,4). On the basis of size distribution alone, the hepatoma nuclei appeared to be primarily octaploid and hyperoctaploid, but with a possible range of ploidy extending between hypertetraploid and 16-ploid (or higher) in a continuous distribution (Text-Fig. 4).

Our Feulgen cytophotometric measurements reinforced the estimated values above, since the average Feulgen-DNA content for hepatoma cell nuclei was found to be in the hyperoctaploid range, 825.93 A.U. (Table 6), while the DNA values for individual hepatoma cell nuclei ranged between

302 A.U. (tetraploid range) and 1830 A.U. (16N range, or higher) [Text-Fig. 5].

Nuclear enlargements and hyperchromasia are two of the key morphologic features associated with cancers for many years now (Koss, 1968). But only within recent years, with the accumulation of biochemical, Feulgen cytophotometric and karyotype data, did it become possible to demonstrate on a quantitative basis that the nuclei of cancer cells usually contain more DNA than comparable normal cells and that the amount of DNA in tumor nuclei is often variable, reflecting hyperploidy, polyploidy or aneuploidy.

Although the increase in DNA and the degree of hyperchromasia is assumed to be proportional to the number and size of chromosomes per nucleus, Miles and Koss (1966) demonstrated on transplanted human tumors with known chromosome complement that this theory has certain limitations. Richards and Atkin (1960) too, reported that after examining a variety of human cancers, they found that most of them contained more than the expected amount of DNA relative to their chromosome complement. These authors claimed, however, that this could be explained, assuming that the chromosome numbers of these cancer cells were in excess of the expected number. On the other hand, the results of an earlier investigation by Richards _et al._, (1956) on ascites tumor, showed a good correlation between the karyotype and

the amount of DNA in these tumor cell nuclei. Since many cancers show variation in the number of chromosomes, even from cell to cell, the stemline karyotype is difficult to determine, or there is no stemline karyotype at all, so that both of the above observations are quite possible simultaneously.

All the apparent discrepancy in the literature seems to point to the fact that cancer cell nuclei exhibit great variability which is manifested both by their chromosome complement and DNA content and by the physical state of their DNA. Most malignant tumors have amounts of DNA different from the normal value and are characterized by a polyploid or aneuploid DNA stemline (Sandritter, 1966). But as mentioned earlier, by no means do all cancers exhibit a distinct stemline karyotype. This accounts for the fact that the abnormal distribution of DNA values in these tumors could not always be correlated to the expected chromosome number, either because the karyotype itself differed in all of the nuclei, or perhaps only in some of the nuclei within the same cancer (Koss, 1968).

It is extremely difficult to make real generalizations about neoplastic tissues, because cancer cells differ from one another almost as much as they differ from normal cells (Meek, 1961).

Autoradiography

Nuclear DNA:

The specificity of ^{3}H-Actinomycin D autoradiographic technique for labeling DNA has been demonstrated once again in the present study. Several previous investigations have established that Actinomycin D binds specifically to DNA (Bolund, 1970; Hamilton *et al.*, 1963; Muller and Crothers, 1968; Sobell *et al.*, 1971; Wells and Larson, 1970; Simard, 1967; Dingman and Sporn, 1965; Fraccaro *et al.*, 1966; Harbers *et al.*, 1963; Goldstein *et al.*, 1966; Ebstein, 1967; 1969; Camargo and Plaut, 1967). Some of these were biochemical, others involved autoradiography at the light or electron microscope level.

The present study has shown that after tissue treatment with ^{3}H-Actinomycin D, radioactivity appeared in the nuclei of both normal and tumor cells. It has also shown that this label was still present in identical amounts when the tissues were previously extracted with RNase, while pretreatment with DNase resulted in the absence of ^{3}H-Actinomycin D binding. These results with enzyme extractions, using RNase and DNase, were similar to the observations of Kawamata *et al.* (1965) and Ebstein (1967; 1969), and according to expectation, if ^{3}H-Actinomycin D was indeed bound specifically to DNA. Furthermore the labeling pattern

in both hepatic cell nuclei and in the hepatoma cell nuclei clearly followed the distribution of DNA, i.e. the number of silver grains bound to nuclear DNA showed an accurate relationship to nuclear class, and thus paralleled the results obtained by Feulgen cytophotometry.

The nuclear size measurements discussed earlier served a dual purpose in this investigation, since they were also necessary for the calculation of mean grain counts per nucleus (i.e. per nuclear area), and so, for the interpretation of ^{3}H-Actinomycin D binding to nuclear DNA.

The average grain count per diploid liver cell nucleus (in Experiment I) was 26.16 $\pm$ 1.08, while that for tetraploid nuclei was 47.44 $\pm$ 0.73; thus the amount of ^{3}H-Actinomycin D binding in normal tetraploid cells was nearly double that of diploid cells, which closely reflects the cytophotometric data presented previously, and indicates that the ^{3}H-Actinomycin D binding was proportional to the degree of ploidy. It is also noteworthy that since the mean nuclear area of the diploid cells was 28.56 μm^2 and that of the tetraploid cells 49.94 μm^2, when the number of grains were calculated per unit area (100 μm^2) to reflect the DNA concentration, the binding of ^{3}H-Actinomycin D per unit area was nearly identical in diploid and tetraploid nuclei, 94.33 $\pm$ 3.57 and 94.02 $\pm$ 1.83 grains per 100 μm^2 respectively (Tables 1,3,4). This data shows that the DNA

in these diploid and tetraploid liver cell nuclei is distributed with an equal density. The evidence was consistent throughout all three experiments.

In examining the autoradiographic data from the hepatoma nuclei, we must keep in mind that these nuclei exhibited a wide range of size distribution (Text-Fig. 4) and also a wide range of DNA content as measured by Feulgen cytophotometry (Text-Fig. 5). While there was an average of 219.87 $\pm$ 2.77 grains over these hepatoma cell nuclei (in Experiment I), which is a value approximately four times higher than that found in normal tetraploid nuclei, the mean nuclear area of the hepatoma cells was only about double (115.47 μm^2) that of tetraploid hepatic cells (49.94 μm^2). In expressing this difference more meaningfully in terms of grain density (or DNA concentration) per unit area (100 μm^2), we calculated the value of 200.78 $\pm$ 2.40 grains per unit area over the hepatoma cell nuclei, as opposed to the values of 94.33 $\pm$ 3.57 and 94.02 $\pm$ 1.83 grains per 100 μm^2, for diploid and tetraploid hepatic cells respectively. These figures seem to indicate that ^{3}H-Actinomycin D binding and hence DNA concentration was twice as dense in hepatoma as in normal cell nuclei, and that there was no longer a regular increase in the number of grains corresponding to the nuclear area and to the degree of ploidy.

In view of these results it is interesting to postulate how this difference observed between the nuclei

of normal hepatic cells and hepatoma cells can be accounted for: (A) Is there actually a greater amount of DNA per square microns in the hepatoma cell nuclei? or (B) Is there something peculiar about the DNA of tumor cells that facilitates its ^{3}H-Actinomycin D binding ability? The occurrence of one naturally does not exclude the other, on the contrary, probably a combination of the two postulates is closest to reality.

Our Feulgen cytophotometric results have shown that the amount of DNA in the hepatoma cell nuclei examined, was indeed elevated, showing a wide range of variation from cell to cell relative to the DNA in normal liver cell nuclei, thus indicating that the hepatoma cell population was both polyploid and aneuploid. This pattern is characteristic of numerous types of cancers investigated, as reported by Mellors, 1955; Kit, 1959; 1960; Meek, 1961; Stich _et al._, 1960; Atkin and Richards, 1956; Stich and Emson, 1959; Kit and Gross, 1959; Klein, 1951; Leuchtenberger _et al._, 1954 and it is due to the gross remodeling of the chromosome complex which generally takes place during neoplastic progression(Petrakis, 1953; Freed and Hungerford, 1957; Kit and Griffin, 1958; Daoust, 1963; Maini and Stich, 1961; Stich, 1960; 1963). While polyploidy and aneuploidy is responsible for the major increase in DNA content as observed with Feulgen cytophotometric studies, it is interesting to speculate on two other possibilities which may

partly contribute to a DNA increase or change in cancer cells. These are: (1) the selective replication of certain DNA regions, referred to as gene amplification, and (2) the presence of exogenous source of DNA due to incorporation of viral genomes into the host cell genome, which are then capable of replicating themselves, either selectively or together, with the host cell genome.

A great number of viruses (some 120 oncogenic viruses) with either DNA or RNA as their genetic material have been associated with cancer induction in animals (Arehart, 1971; Bader, 1965; Becker, 1972; Dulbecco, 1966; 1967; Farber, 1968; Green, 1970; Habel, 1968; Pierce, 1970; Sambrook, 1968; Temin, 1967; Todaro and Green, 1966), but none so far has been shown definitely capable of initiating the carcinogenic process in humans (Arehart, 1971).

Only less than half of the tumor viruses are DNA viruses, and their introduction into host cells has been an artificial one, under laboratory conditions. It was shown, however, by various experiments that the DNA viruses can incorporate their DNA directly into host cells which then copies its own DNA plus the viral DNA at each cell division. Cell replication is needed for the initial transfer of information, as with the SV40 virus (Todaro & Green, 1966; Farber, 1968), or the virus turns on the synthesis of host DNA as in the case of polyoma virus (Dulbecco, 1966; 1967).

The picture is more complex with RNA tumor viruses, but it appears that DNA synthesis is also needed for transformation by at least one such virus, the Rous sarcoma virus (Bader, 1965; Temin, 1967). The majority of viruses known to cause cancer in animals have an RNA core (Culliton, 1972). These RNA tumor viruses were shown to have a special enzyme, the RNA-directed DNA polymerase also known as reverse transcriptase which catalyzes the flow of genetic information from RNA to DNA (Temin and Mizutani, 1970; Baltimore, 1970), in contrast to the established "central dogma" of Watson and Crick (Watson, 1970). It has been suggested by several researchers that the presence of this reverse transcriptase in RNA tumor viruses explains, for the first time, the mechanism by which genes in the RNA of a virus can be incorporated into the DNA of a cell, where they might function like any other genes (Culliton, 1972), or where their replication may go "uncontrolled", and with the aid of their genetic information they may transfer normal cells into cancer cells.

Extensive gene amplification is the other factor (mentioned earlier) which may partly account for the increased amount of DNA in the hepatoma cell nuclei. The selective replication of the ribosomal cistrons was first observed in the oocytes of amphibians, fish, and some insects (Brown and Dawid, 1968; Perkowska, *et al.*, 1968; Gall *et al.*, 1969). This unique form of genetic control, by selective

synthesis of certain genes, has its counterpart in the giant polytene chromosomes of dipteran flies (DuPraw, 1970; Armelin *et al.*, 1970). The partial genomic replication of the (already redundant) ribosomal RNA locus results, in amphibian oocytes, in a very significant multiplication of the sites at which ribosomal RNA (rRNA) can be synthesized. Markert and Ursprung (1971) concluded, that the genes for rRNA are enriched by at least as much as 1000 times in *Xenopus* oocytes compared to somatic cells in the same species.

To explain the necessity for extra copies of ribosomal DNA (rDNA) in excess of the amounts present in somatic cell nucleoli, one can make a comparison between the rates at which rRNA molecules are synthesized in oocytes as opposed to their rate of synthesis in somatic tissues. As an example, the *Xenopus* oocyte at maturity contains some 1.1×10^{12} rRNA molecules (Perkowska *et al.*, 1968). At the rate of ribosome generation occurring in rapidly dividing somatic tissues, the synthesis of the oocyte rRNA would require 1.7×10^{5} days, if the replication of the ribosomal cistrons would not have occurred (according to the calculations of Perkowska *et al.*, 1968). Reduction of this time by a factor of $1 - 1.5 \times 10^{3}$, the approximate number of nucleolus organizers (amplified portion of DNA) per nucleus, brings the required synthesis period down to 3 - 6 months, assuming that each nucleolus contains one set of ribosomal

cistrons (Brown and Dawid, 1968).

The selective replication of the ribosomal genes in the oocyte system is thus the specialized mechanism underlying the massive synthesis of rRNA which is probably accumulated as a stockpile, to become engaged in protein synthesis at a later stage during embryogenesis, and which is actually needed to support development far into embryonic life (Brown and Dawid, 1968).

While it seems then that under normal circumstances the activation or stimulation of the structural genes can meet the needs of the cells as far as rRNA synthesis and protein needs are concerned, when these activities are inadequate to meet increased cellular demands (as in the case of oocytes), the efficiency of the system is enhanced by the selective replication of certain gene sequences.

In the tumor cells too, according to our hypothesis, a similar system of gene amplification may account for the increased amounts of RNA generally reported in cancer cells (Fukuda *et al.*, 1970; Phillips and Phillips, 1971) and for the production of a variety of proteins either not found at all in comparable normal adult tissues, or only produced in significantly lesser amounts in the latter (Abelev, 1968; Farina *et al.*, 1968; Pitot, 1968). Both surface and intracellular tumor-specific antigens (Haughton and Amos, 1968; Hellström and Hellström, 1969) as well as antigens previously thought to be characteristic of

embryonic tissue, have been identified (Abelev, 1968; Gold *et al.*, 1968; Nishi, 1970) in tumors. These alterations in cell phenotypic expression, typical of cancer cells, may be based on alteration of the genotype.

The possibility that these new gene products (proteins) in tumors might reflect a selective replication of certain DNA sequences has been investigated in two separate systems; in mouse myelomas (Krueger and McCarthy, 1970) and in rat hepatomas (Shearer, 1971). In both studies the experimental approach for detecting gene amplification relied on a technique known as molecular hybridization, using so-called saturation annealing experiments. These investigators confronted a given amount of tumor DNA, as well as normal DNA of comparable and various other tissues, with increasing amounts of radioactive tumor RNA(from the same tumor), and determined the point at which no further DNA-RNA hybrid molecules were formed by each. This so-called saturation value indicated the proportion of DNA in tumor and in normal tissue respectively, that was complementary to tumor RNA. The tumor RNA must contain transcripts of the genes coding for tumor proteins and therefore should anneal to a greater extent with DNA enriched for these base sequences than with DNA having no copy or only a single copy of each.

While Krueger and McCarthy (1970) found that both the rate and extent of tumor RNA annealing to homologous

tumor DNA was significantly greater than to normal DNA, suggesting that, indeed, a somatic amplification of specific genes may have occurred in mouse myelomas, Shearer (1971), detected no such significant differences between tumor and normal tissues with respect to the rate or degree of annealing or in the thermal stability of the hybrid molecules formed. Obviously several more tumors need to be examined before final conclusions can be drawn as to whether the mechanism of gene amplification is basic to tumors in general or not.

Before considering the second question, namely - is there something peculiar about the DNA of tumor cells that facilitates its ability to bind ^{3}H-Actinomycin D? - one should take a brief look at how the Actinomycin-DNA complex is formed and what favors this complex formation. As already pointed out in the introduction, two possible models have been proposed to explain the interaction of Actinomycin to DNA: - (1) The hydrogen bonded "outside binding model" (Hamilton *et al.*, 1963) and (2) The "intercalation model" (Muller and Crothers, 1968). The most recent studies (Bolund, 1970; Sobell *et al.*, 1971; Wells and Larson, 1970; Kerstein, 1971) favor the model proposed by Muller and Crothers (1968), stressing that probably the most important factor for Actinomycin association with DNA is the presence of a suitable DNA configuration.

Another well established fact related to the Actinomycin-DNA complex formation is that while double stranded DNA binds Actinomycin well, hybrid polymers containing one strand DNA and the complementary strand RNA bind little or no Actinomycin, and similarly single stranded DNA (or denatured DNA) binds poorly or not at all (Wells and Larson, 1970). These latter molecules were shown to have a slightly different configuration than bihelical DNA.

It might seem worthwhile, therefore, to go back and examine the normal cell nuclei and the tumor cell nuclei with respect to the physical state of their DNA and postulate if this could account for the difference (if any) in their ^{3}H-Actinomycin D binding ability.

As already mentioned earlier, the hepatoma cell nuclei exhibited some degree of hyperchromasia, a phenomena characteristic of most cancer cell nuclei, which could be the result of a marked increase in heterochromatic regions Koss (1968). Several authors (Gellhorn et al., 1966; Harbers and Vogt, 1966; Sandritter et al., 1965; Grunicke et al., 1970) have reported that tumor cells from highly deviated tumors contain a higher portion of their total DNA in heterochromatin than cells of several nonmalignant tissues. Another indication that the heterochromatin is indeed increased in cancer cells is the prominence of nucleoli together with an increase in nucleolus-associated

heterochromatin (Koss, 1968). Cells of certain tumors have been shown to contain more nucleolar DNA, than comparable normal cells (Busch and Smetana 1970). This nucleolus-associated chromatin, on the other hand, is also part of the condensed heterochromatin (Roels, 1966).

Simard (1967) presented quantitative data from high resolution autoradiographs on the "preferential" binding of ^{3}H-Actinomycin D to the heterochromatin in hamster fibroblasts (strain BHK). He found that the radioactivity (the silver grains) appeared to be concentrated in the nucleolus-associated chromatin and in the heterochromatin region of chromosomes to about equal extent, and that the radioactivity was extremely low in the extended euchromatin and in the "nucleolar body". Ebstein (1967; 1969) also found that the grain density over the lateral loops of lampbrush chromosomes (which are in a highly extended state) was very low, and that detectable label was only found along the chromomeres (heterochromatin enlargements of an extended chromonema) of the lampbrush chromosomes. Camargo and Plaut (1967) came to a similar conclusion from examining autoradiographed salivary gland squash preparations, i.e., that the puffed regions of polytene chromosomes bind less ^{3}H-Actinomycin D than normal regions. Two possible explanations were offered for these findings by Simard (1967) which seem to be of interest here. The first dealt with

the amount of DNA found in the two forms of chromatin, heterochromatin and euchromatin respectively. The second explanation was in terms of preferential binding to one or the other form of chromatin.

There is much controversy and very little data available concerning his first explanation, i.e., the amount of DNA in the two forms of chromatin, since they are difficult to isolate. However, according to Frenster *et al.*, (1963), who succeeded in isolating and separating condensed and diffused chromatin from sonicated nuclei, the condensed chromatin contains up to 80% of the nuclear DNA (DuPraw, 1968). The presence of higher grain density over the heterochromatin in autoradiographs could then be interpreted in terms of simply more DNA being available for binding with ^{3}H-Actinomycin D. Relevant to his second explanation is that in euchromatin the DNA molecules are in an extended form with evidence of local strand separation which usually accompanies active RNA transcription. Therefore, these euchromatin regions (or loops) can be compared to single stranded DNA, or more likely, to DNA-RNA hybrid molecules formed during transcription, which were shown to bind very poorly to Actinomycin (Wells and Larson, 1970). They would more likely bind molecular species that stimulate RNA synthesis, thus stabilizing strand separation which in turn favors active transcription (Frenster, 1965). The tightly coiled DNA of the heterochromatin, on the other hand, is charac-

terized by an increased stability of the DNA helix, with no evidence of RNA transcription or strand separation, and as a result of its configuration it binds ideally with Actinomycin.

Perhaps at first it would appear that there is a contradiction in this interpretation, since the nucleolus is the most active site of RNA synthesis (Perry, 1964), and the nucleolus-associated chromatin is part of the heterochromatin (Roels, 1966). However, the nucleolar DNA consist of both rDNA (that DNA which contains the cistrons coding for rRNA) and other types of DNA. The amount of rDNA, that codes specifically for 18s and 28s RNA in mammalian cells is only a very small part, i.e. about 0.005 - 0.01% (McConkey and Hopkins, 1964; Steele, 1968) of the total nuclear DNA (Busch and Smetana, 1970). Thus, it follows that only a small fraction of the nucleolar DNA exists as extended strands and it is active in RNA synthesis (Busch and Smetana, 1970), the rest is probably condensed heterochromatin which therefore binds well with ^{3}H-Actinomycin D.

The findings in our study could also be interpreted according to this view, assuming that the hyperchromatic hepatoma cell nuclei probably contain more heterochromatin than comparable normal liver cell nuclei and therefore bind ^{3}H-Actinomycin D more readily.

Cytoplasmic DNA:

Our autoradiographic evidence suggests that differences between hepatoma cells and their normal counterparts with respect to DNA content are not restricted to nuclei, but exist in the cytoplasm as well. In addition to the amount of ^{3}H-Actinomycin D localized in the nuclei, a definite amount of radioactivity was observed in the cytoplasm of both normal hepatic cells and hepatoma cells, most probably corresponding to mitochondrial DNA (m-DNA).

The average number of silver grains found per standard unit area (100 μm^2) of hepatoma cell cytoplasm (in the first experiment) were 27.03 $\pm$ 0.18, more than double the amount found per normal hepatic cell cytoplasm (12.41 $\pm$ 0.20) [Table 5]. These values were very similar in all three experiments (Table 5) and were well above background, therefore they could by no means be considered negligible.

Treatment of both tissues with DNase before incubation in ^{3}H-Actinomycin D resulted in the disappearance of this cytoplasmic label (as was the case with nuclear label), while pretreatment with RNase had no effect on the distribution of radioactivity (Table 5).

In view of these findings it seems reasonable to propose that ^{3}H-Actinomycin D, indeed, binds to cytoplasmic DNA under normal circumstances, that is, in normal liver cells, and that the increased amount of label probably

indicates an increased amount of cytoplasmic DNA in hepatoma cells. This increase could result from an uncontrolled multiplication of the mitochondrial genome without being followed by the actual division of mitochondria as such, since the actual number of mitochondria per cell is said to be reduced often in the malignant state. The increase in m-DNA could also be in the form of mitochondrial gene amplification and/or due to the presence of significantly multiplied viral genomes in the mitochondrion proper (Kara et al., 1971; De-The et al., 1969; Gazzalo et al., 1969). Both possibilities were already discussed in some detail in relation to nuclear DNA content.

The m-DNA molecules isolated from different types of normal tissues occur mostly as circular monomers which have a remarkably uniform average contour length of about 5 μm (Piko et al., 1968; Sinclair et al., 1967; VanBruggen et al., 1966; Roodyn and Wilkie, 1968). In contrast, the m-DNA molecules of tumor cells show a tendency toward broader and more heterogenous distribution of contour lengths as compared with m-DNA isolated from normal tissues (Takuzo, 1968; Nass, 1969; Korb, 1971; Clayton and Vinograd, 1967; Clayton et al., 1970). All tumor cells, either with viral or non viral etiology, so far investigated, seem to display an increased content of dimeric forms of m-DNA, containing two monomeric genomes (Clayton et al., 1970) as

well as oligomeric forms of m-DNA (Clayton and Vinograd, 1967; Riou and Delain, 1971). The recent investigation of Riou and Delain (1971) on mitochondria obtained from tumors induced by Adenovirus 7 and Adenovirus 12 as well as on mitochondria obtained from embryo hamster fibroblasts transformed *in vitro* by induced SV40 virus, confirmed the previous findings. They found that the m-DNA of tumor cells transformed by Adenoviruses and of cells transformed by SV40 consisted of an abnormally high proportion of oligomers, including molecules 13 times the monomer size (Riou and Delain, 1971), as compared with m-DNA of control cells used in their experiments and with m-DNA of other normal tissues (Clayton *et al.*, 1968). They also found circular dimers with double the contour length of monomers in the m-DNA of both, but they were in relatively low proportion in the cell line transformed by SV40. An increased content of dimeric m-DNA molecules was reported recently among other tumor cells in the avian leukemic myeloblasts by Korb (1971).

These circular dimers and oligomers have been first thought to occur only in mitochondria of human leukemic leucocytes (Clayton and Vinograd, 1967), but Clayton *et al.* (1969) have shown that oligomers are also abundant in some human solid tumors.

Now that the presence of high proportions of these dimeric and oligomeric forms of m-DNA in cells has been

linked generally to a physiological state like that of malignancy (Korb, 1971; Riou and Delain, 1971; Clayton *et al.*, 1969; 1970), and in view of our results of increased amount of ^{3}H-Actinomycin D binding in the cytoplasm of hepatoma cells as compared to normal hepatic cells, it seems reasonable to postulate that they represent an "abnormal" multiplication of whole m-DNA molecules or amplifications of only segments of m-DNA in tumor cells and thus also account for our observations.

Nucleoli:

The nucleoli were also examined in the present study in order to compare their appearance and size in the normal hepatic cells and in hepatoma cells (Table 2).

The number of nucleoli were also investigated earlier in some detail by Shea and Leblond (1966), in mouse liver hepatic cells (among many others). They found that the number of nucleoli in diploid and tetraploid hepatic cells respectively, were proportional to the degree of ploidy. This correlation between ploidy and nucleolar number was not noted however, in the present study. Shea and Leblond (1966) found a maximum of six nucleoli per diploid and eleven nucleoli (almost exactly double) per tetraploid nucleus, most probably due to the presence of six and twelve nucleolar organizers respectively. Although it is

generally agreed upon by researchers in this field that the number of nucleoli per nucleus increase proportionally to increase in ploidy, it is not uncommon to find that in some cells there is a decrease from this maximal expected nucleolar number, as was the case in the present study. This may be explained by the fairly common occurrence of nucleolar fusion (Shea and Leblond, 1966; Swift, 1959; Bernhard and Granboulan, 1968).

We observed mostly two, but occasionally three nucleoli in the diploid hepatic cells. In tetraploid cell nuclei, there were usually two or three nucleoli, and infrequently up to five nucleoli could be seen. The hepatoma nuclei, although highly polyploid, mostly contained only one large nucleolus and rarely more than three. These hepatoma cell nucleoli were much larger than those found in normal hepatic cells (Table 2) and they were frequently irregularly shaped. Both of these nucleolar features have been discribed in connection to other cancer cells too (Kopac and Mateyko, 1958; Busch and Smetana, 1970).

According to certain investigators, the number of nucleoli in some cancer cells is usually increased, probably due to the frequent polyploidy of nucleolus organizer regions (Busch and Smetana, 1970). Since nucleoli often fuse after their formation, at least in normal cells (Swift, 1959), their number should never be taken as strictly indicative of the degree of ploidy.

Just as multinucleolar nuclei occur in many normal cells, the number of nucleoli may decrease beyond the normal value in cancer cells (Busch and Smetana, 1970). In actual fact, a single nucleolus has been found in the majority of cells from human hepatomas, whereas two-three nucleoli are normally present in human hepatic cells (Berman, 1951; Busch and Smetana, 1970). A similar situation exists in the different types of Morris hepatomas in rats, where the number of nucleoli per nucleus average 1.3, while in the normal liver cells of adult rats, the number of nucleoli average 2.7 per nucleus (Unuma *et al.*, 1967).

It seems then that the only generalization that can be drawn is that in tumor cells the nucleolar number is either increased or decreased beyond normal values of corresponding normal cells (McGrew, 1965).

SUMMARY

The present investigation on the "total" DNA content (nuclear and cytoplasmic DNA) of hepatoma cells, as compared to the DNA content of normal hepatic cells, resulted in a clearer understanding of some of the differences which exist between these cells. The study has revealed that these hepatoma cells differ from their normal counterparts by having certain distinct nuclear features, such as nuclear enlargement, variability in size and shape and by being hyperchromatic and often exhibiting "margination of chromatin". In addition they also differ in that they contain a greater amount of nuclear DNA, which was shown to be in the mean hyperoctaploid range, and in that this DNA seem to bind with ^{3}H-Actinomycin D probably more preferentially, than normal nuclear DNA. Additional information has been obtained about the cytoplasmic DNA, probably m-DNA of these cells, which clearly suggested that hepatoma cells also contain more cytoplasmic DNA than normal liver cells.

Several postulates have been offered in an attempt to explain the peculiar ^{3}H-Actinomycin D binding to hepatoma DNA which resulted in a much greater concentration of label per unit area of hepatoma cell nucleus.

It is understood, however, that all these differences are probably the result, rather than the cause, of malignancy and that much more comparative work, - tumor vs. comparable normal tissue, - needs to be done on this system, as well as on other tumor systems, before we can come to definite conclusions and to some kind of generalization concerning the DNA of cancer cells.

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APPENDIX

The sections used for the cytophotometric analysis of nuclear DNA content were hydrolyzed, prior to the use of Feulgen reagent, in 1N HCl at 60°C for six minutes and then transferred briefly into 1N HCl at room temperature, then rinsed in distilled water. Six minutes in 1N HCl is considered the optimum time of hydrolysis by Pearse (1968), for tissues fixed in Carnoy's ethanol:acetic acid (3:1).

FIGURES

Unless otherwise indicated all photomicrographs and autoradiographs are of material fixed in glutaraldehyde and post fixed in osmium tetroxide, embedded in Araldite resin, sectioned at 0.5 μm and stained with Toluidine blue.

On all photomicrographs and autoradiographs 1 cm = 10 μm.

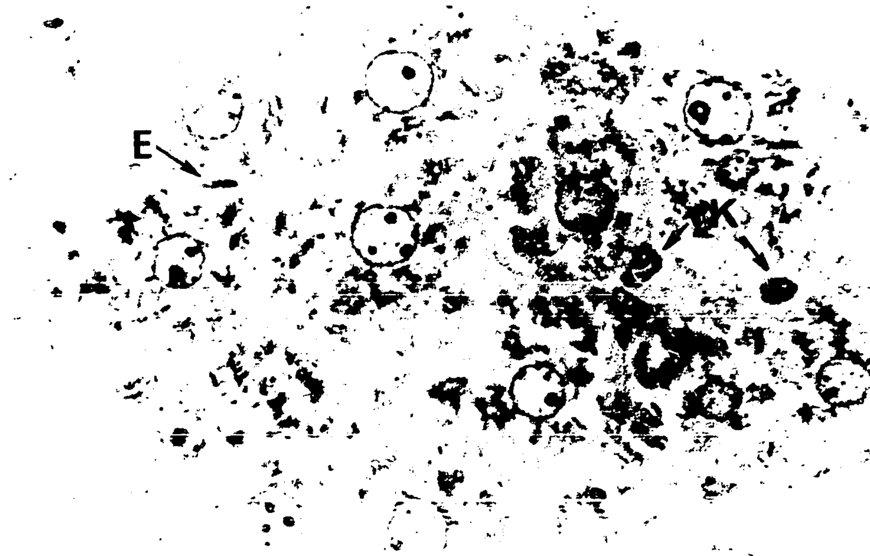
<u>Figure 1</u>: Photomicrograph of normal mouse liver showing hepatic cords separated by sinusoids. The sinusoids are lined by two kinds of cells, the endothelial cells (E) and the phagocytic von Kupffer cells (vK). The hepatic cells have round centrally located vesicular nuclei with one or more nucleoli. At least two different size of liver cell nuclei may be distinguished, probably diploids and tetraploids.

X1000

<u>Figure 2</u>: Photomicrograph of normal mouse liver, showing round, vesicular hepatic cell nuclei; a small elongated darkly staining endothelial cell nucleus (E) and somewhat larger, more oval von Kupffer cell nuclei (vK). Cell outlines are indistinct.

X1000

1



2

Figure 3: Photomicrograph of normal hepatic cells showing binucleate cell (BC). The darkly staining spots in the cytoplasm of the hepatic cells are mitochondria.

X1000

3

Figure 4: Photomicrograph of transplanted mouse hepatoma. The hepatoma cell nuclei are large and exhibit great variation in size and shape. These nuclei contain large irregular nucleoli (mostly one or two) and darkly staining chromatin clumps, i.e. heterochromatin, which is often located near the nuclear membrane (arrows).

X1000

Figure 5: Photomicrograph showing hepatoma cell nuclei of various size. The giant size nucleus in the right upper corner (arrow) contains many darkly staining heterochromatic regions. The cytoplasm of these cells appears vacuolated.

X1000

4

5

<u>Figure 6</u>: Photomicrograph showing hepatoma cell nuclei of various shape and size. In the nuclei, large and irregular nucleoli are seen, not exceeding three nucleoli per nucleus. Darkly staining chromatin clumps (heterochromatin) are also evident. The cytoplasm is delicately vacuolated.

X1000

6

Figure 7: Photomicrograph of transplanted mouse hepatoma, showing three cells just undergoing mitosis (arrows).

X1000

Figure 8: Photomicrograph, showing hepatoma cell nuclei of various size, containing mostly one large nucleolus per cell and occasionally two nucleoli. A mitotic figure is also seen in the field (arrow).

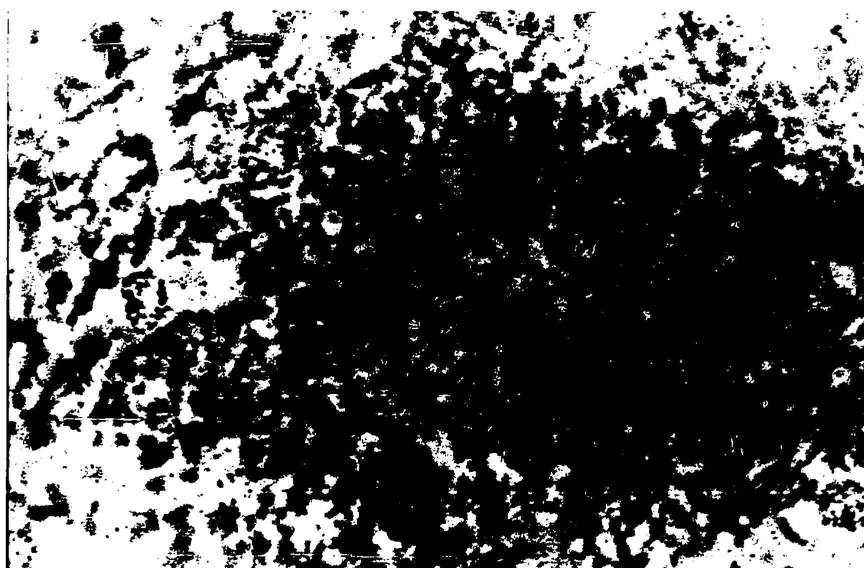
X1000

7

8

Figure 9: Autoradiograph of normal mouse liver following the incubation of 0.5 μm sections with ^{3}H-Actinomycin D, as described in Experiment 1. The silver grains are mainly distributed over the hepatic cell nuclei, but the cytoplasm is also labeled.

X1000



9

Figure 10: Autoradiograph of normal mouse liver incubated in ^{3}H-Actinomycin D as unfixed tissue as described in Experiment II. The label is seen mainly over the nuclei of hepatic cells, however, their cytoplasm is also distinctly labeled.

X1000

Figure 11: Autoradiograph showing labeled normal liver hepatic cells incubated in ^{3}H-Actinomycin D right after double fixation of tissue in glutaraldehyde and osmium tetroxide according to the plan described in Experiment III. The grains are distributed over the hepatic cell nuclei mainly, but the cytoplasm is also labeled.

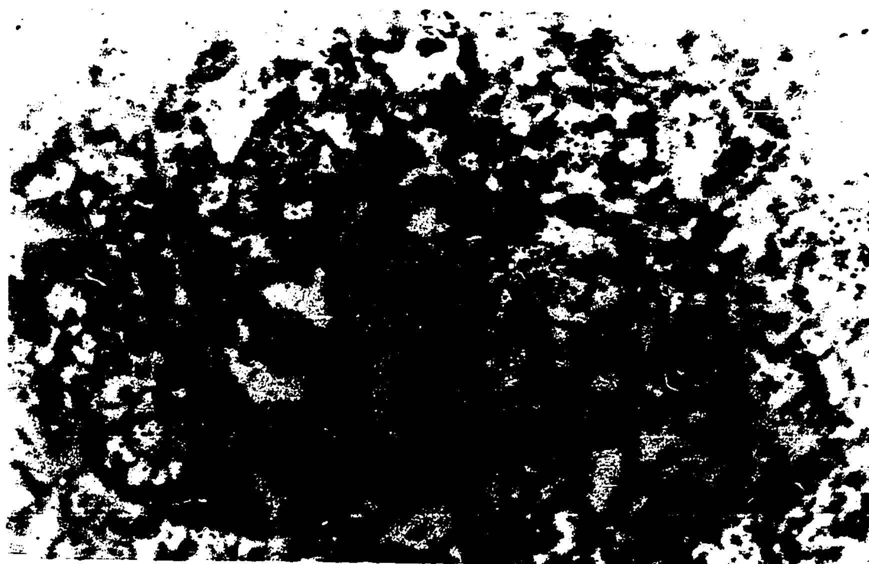
X1000

10

11

Figure 12: Autoradiograph of normal mouse liver treated with RNase prior to incubation in ^{3}H-Actinomycin D as described in Experiment I. The grains are mainly over the nuclei, but the cytoplasm is also distinctly labeled.

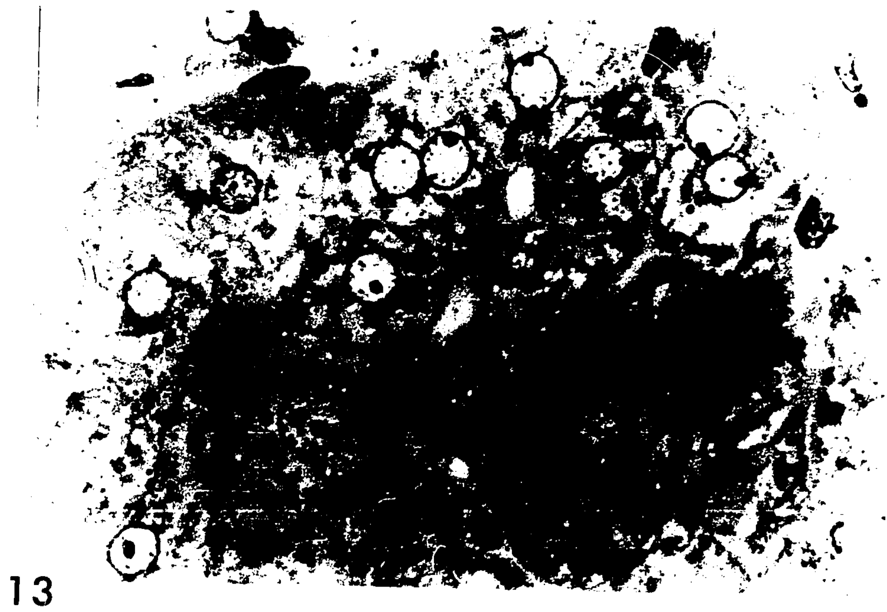
X1000



12

Figure 13: Control section of normal mouse liver treated with DNase prior to incubation in ^{3}H-Actinomycin D as described in Experiment I. Autoradiograph.

X1000



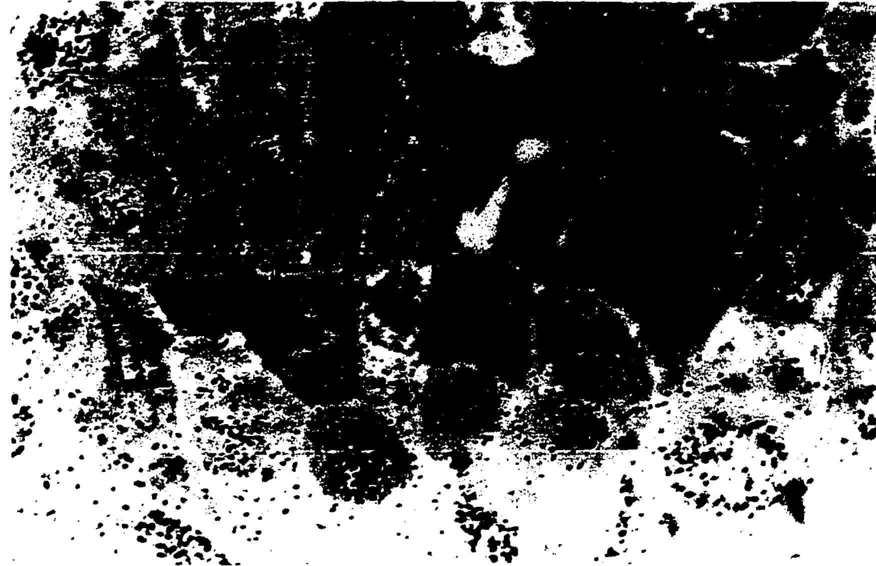
13

Figure 14: Autoradiograph of transplanted mouse hepatoma following the incubation of 0.5 μm sections in ^{3}H-Actinomycin D as described in Experiment I. The nuclei are heavily labeled, a dense concentration of grains is seen near the nuclear membranes in the hepatoma cell nuclei indicated by arrows. The amount of cytoplasmic label is also considerable.

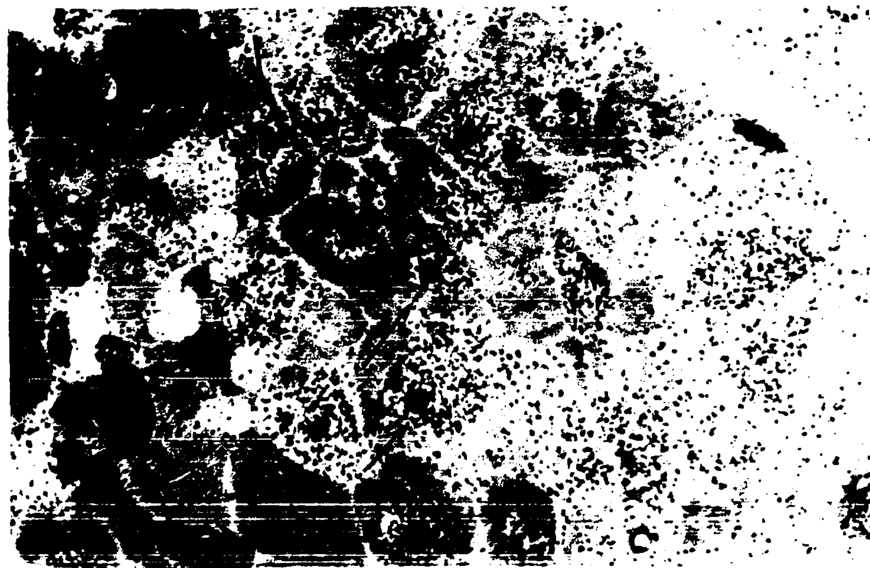
X1000

Figure 15: Autoradiograph prepared according to the plan described in Experiment I showing heavily labeled hepatoma cell nuclei and a considerable cytoplasmic label. A heavy concentration of grains is seen over and around the nucleoli (arrows) in the area occupied by nucleolar DNA.

X1000



14



15

Figure 16: Autoradiograph of transplanted mouse hepatoma incubated as unfixed tissue in ^{3}H-Actinomycin D as described in Experiment II. The giant hepatoma cell nuclei are heavily labeled and there is a considerable amount of label over the cytoplasm as well.

X1000

Figure 17: Autoradiograph prepared according to the plan described in Experiment II showing a heavy concentration of silver grains near the nuclear membranes of the hepatoma cell nuclei and over the nucleoli. The latter indicated by arrows. The cytoplasm is also heavily labeled.

X1000

16

17

Figure 18: Autoradiograph showing heavily labeled hepatoma cells which were incubated in ^{3}H-Actinomycin D right after double fixation with glutaraldehyde and osmium tetroxide according to the plan described in Experiment III. There is a heavy concentration of label near the nuclear membranes.

X1000

Figure 19: Autoradiograph of transplanted mouse hepatoma prepared according to the plan described in Experiment III. Both the nuclei and the cytoplasm of hepatoma cells are heavily labeled.

X1000

18

19

Figure 20: Autoradiograph of transplanted mouse hepatoma treated with RNase prior to incubation in ^{3}H-Actinomycin D as described in Experiment I. There is a heavy concentration of grains over the nuclei and a considerable amount of label over the cytoplasm of hepatoma cells.

X1000

Figure 21: Autoradiograph of RNase treated section prepared according to the plan described in Experiment I showing heavily labeled hepatoma cell nuclei with a particularly heavy concentration of grains near the nuclear membranes and over the nucleoli (arrows). The cytoplasm is also heavily labeled.

X1000

20

21

Figure 22: Control section of transplanted mouse hepatoma treated with DNase prior to incubation in ^{3}H-Actinomycin D as described in Experiment I.

Autoradiograph.

X1000

22