CHANGES IN GLYCOGEN, RNA AND DNA CONTENTS IN LIVERS OF TUMOR-BEARING RATS
(Plate XXVI)

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The pathological and biochemical changes of tumor-bearing animals in comparison with those of normal ones have been a subject of repeated studies in the field of oncology. The inhibition of catalase in the liver (Appleman et al. 1950, Dounce & Shanewise 1950, Greenfield & Meister 1951, and others) and the increment of P32 incorporation into tissue deoxyribonucleic acid (DNA) (Payne et al. 1952, Kelly et al. 1951 and others) have been claimed as a sequence of tumor transplantation. In the course of cytochemical studies on the mitochondrial morphology in relation to chemical constituents in rat liver cells, one of the authors (Hori 1958) has found that the almost complete depletion of glycogen occurs accompanying a considerable increase of cellular basophilia in the liver of a tumor-bearing animal without remarkable alteration in morphology of the mitochondria. In the present study, the authors have undertaken to inquire in detail into what changes do occur in contents of glycogen, RNA and DNA in the liver of rats bearing tumor transplants. Microspectrophotometry of the DNA content was performed at the Kwansei Gakuin University under the direction of Professor Yoshio Ojima to whom the authors' thanks are due.

MATERIAL AND METHOD

The MTK-sarcoma III was inoculated intraperitoneally into Wistar rats weighing 70 to 120 gm. Rats bearing tumors were sacrificed 3, 6 and 8 or 9 days after the tumor inoculation; they are conveniently referred to as Group-A, -B and -C rats, respectively, in the following descriptions. The mean survival time of the hosts was 8.7 days. The livers from normal and tumor-bearing rats were fixed with formal-calcium and Gendre's fluid, and subjected to a series of cytochemical tests as follows:

Periodic acid Schiff reaction (PAS) according to the method of McManus (1948) was applied to the material which was fixed with subzero Gendre's fluid for the

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demonstration of glycogen. The Feulgen reaction according to Stowell's method for DNA, the toluidine blue staining method for RNA followed with or without the digestion test by ribonuclease (Worthington Biochem. Sales Co., Freefold, New Jersey, U.S.A.*), the bromphenol-blue method for basic protein according to Mazia (1953), and the light green method for protein, with or without hydrolyzation by HCl (Kaufmann et al. 1951), were applied to sectioned materials fixed with formal-calcium. Microspectrophotometric measurement of DNA in liver nuclei was performed according to Swift's method (1950). Regaud's staining was also applied to formal-calcium fixed tissues which were chromated with 3% potassium bichromate for 6 days at room temperature. Fixation with formal-calcium yielded good results as well as Regaud's original fixative consisting of formal and bichromate.

**Observations**

**Periodic acid Schiff reaction and histological remarks:** The cytoplasm of liver cells of well-nourished healthy rats was generally well-supplied with glycogen, the distribution of which was almost uniform throughout hepatic lobules (Fig. 5).

The livers from Group-A rats (sacrificed 3 days after the tumor transfer) contained PAS positive substances in much less amount than the controls. Through the digestion of sectioned material with saliva extract, the PAS positive substance was shown to be glycogen. Depletion of glycogen was observed in all of the hepatic lobules (Fig. 6), being especially remarkable in their periphery. Mitoses of hepatic sinusoids were also remarkable. The leucocytes were abundant among the anastomosing hepatic strands than in the controls.

In the material (Group-B) six days after the tumor transplantation, the liver was found mostly covered with a layer consisting of necrosed and viable tumor cells having the cytoplasm strongly positive to the PAS reaction (Fig. 12). Division of hepatic cells took place more frequently than in the former group. PAS reaction revealed further advanced depletion of glycogen in the livers (Fig. 7). Glycogen granules were mostly invisible in the cells at periphery of the lobules, while the centrolobular cells contained the granules in the form of globules or minute particles. The mitotic apparatus of hepatic cells was completely negative to the PAS reaction. The leucocytes in the hepatic sinusoid were more numerous in Group-B rats. The cytoplasm of the leucocytes showed slightly diffused red coloration when the PAS reaction was applied.

All of the livers from Group-C rats are characterized by cells which contained a small amount of glycogen. Certain liver cells contained only a single minute PAS positive granule in each (Fig. 8). Leucocytes in the sinusoid further increased in number and their cytoplasm was strongly positive to the PAS reaction. A con-

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* Kindly supplied by Dr. C. Leuchtenberger for the use of this study. The authors’ cordial thanks are due to her cooperation.
siderably strong tissue reaction was observed throughout the whole liver with a remarkable dilatation of sinusoids. Consequently the original architecture of the hepatic lobules was distorted. Mitoses of hepatic cells were frequent, though they seemed to be a little less in number than those in the livers of Group-B rats. Another remarkable item of change occurring in the liver of Group-C rats was a striking increase in stainability of hepatic cell nuclei with toluidine blue.

**The toluidine blue staining:** Examinations of the material stained with 0.1% toluidine blue solution have revealed that both the cytoplasm and nucleolus of the hepatic cell show blue coloration while their nuclear chromatin is metachromatically blue-violet (Fig. 1). After the treatment with 0.02% ribonuclease solution the stainability of the cytoplasm and nucleolus was completely lost, whereas the coloration of the chromatin changed from blue-violet to violet. This seems to imply that blue coloration obtained after toluidine blue staining is owing to the existence of RNA in the cellular constituents.

In comparison with the control rats the tumor-bearing rats show an increase in amount of RNA in the cytoplasm of hepatic cells. Such an increase was evident in the livers of Group-A rats and much more remarkable in those of Group-B and -C rats (Figs. 2-4). The cytoplasm contained a number of mitochondria-like bodies which were spherical and rod-like in shape. The peripheral zone of the bodies was stained blue and rather translucent in the inner part. Some of them occurred in close contact with the nuclear membrane while others aggregated into several masses scattered in the cytoplasm. The stainability of the nuclear membrane and chromatin, especially nucleolus-associated chromatin, was also strong in the livers of the tumor-bearing rats. The nucleoli increased both in number and volume. Generally they were irregularly shaped and often in contact with the nuclear membrane. The nucleolar or chromatic substance adhered to the inner surface of the nuclear membrane, so that the membrane displayed an abnormal thickening. The cytoplasm adjacent to the nuclear membrane stained blue in most cases more remarkably than the other cytoplasmic areas. Sometimes, the thick nuclear membrane extruded somewhat into the cytoplasm, thus bringing about a wavy and irregular surface of nuclei. Such a feature of hepatic cells was rather common in the tumor-bearing rats (Figs. 9 & 10). Based on the above findings the following speculation may be allowable that the presence of a tumor in the rats induces the metabolic activity of their hepatic cells to produce a large amount of RNA, and further that the increase of RNA amount in those cells may be closely related to the accelerated elaboration of the nucleus.

The hepatic nuclei of Group-C rats are generally characterized by a very strong affinity to the basic dye (Fig. 4). The majority of nuclei of this Group were rather small in size and densely stained with toluidine blue. It is therefore rather difficult
to study in detail the morphology of the nucleoli and chromatin in those cells. There was no evidence of the extrusion of nucleoli in those nuclei.

**Regaud's iron-hematoxylin staining:** It was aimed by the employment of this method to demonstrate the extrusion of the nucleolus in hepatic cells of tumor-bearing rats, since the evidence was expected to be found by the examination of the toluidine blue preparations. The examination failed however to demonstrate the extrusion of nucleoli. Instances of the thickening of the nuclear membrane, probably caused by the association with chromatic or nucleolar substance, and the existence of nucleoli of polymorphic nature were found to occur in the preparations here studied, in a similar way as in the toluidine blue preparations.

**Staining with light green and bromphenol blue:** These two methods for examination of test of basic protein did not yield any appreciable results: little difference was observed in stainability between the control and experimental materials.

**Feulgen nucleal reaction:** Upon examination of Feulgen stained preparations particular attention was concentrated on the possibility of extrusion of the nuclear DNA into the cytoplasm, on the assumption that if the extrusion of nucleolar material would occur concomitantly with the liberation of chromatin, especially nucleolus-associated chromatin, from the nucleus, the Feulgen positive substance should be found in the cytoplasm. Contrary to the expectation, no Feulgen positive material was detected in the cytoplasm either in control or in rats bearing tumor transplants.

**Microspectrophotometric measurements of the DNA contents in liver cell nuclei:** The DNA contents in liver cell nuclei of tumor-bearing rats were measured in formal-fixed and Feulgen-stained preparations by means of Swift's type apparatus. The results obtained are collected in Text-figure 1: four histograms at the left are presented by way of comparison of the data between the liver of a newborn rat and the livers of three tumor-bearing rats at the 9th day of tumor inoculation. The histograms at the right show the data from the liver of a normal rat in comparison with those from the livers of three rats which were killed 3, 6 and 8 days after the tumor transplantation. Measurements of the DNA contents in these two groups were carried out at different times and by different present authors. Accordingly, arbitrary units of the DNA contents used are slightly different by measurements.

**Comparison of DNA amount between the newborn and tumor livers A, B and C:** It has been widely accepted that the DNA amount of most liver cells of adult rats is twice the amount of those of the newborn rat. If the average amount of DNA

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1) They are conveniently referred to as newborn liver and tumor livers A, B and C in the histograms.

2) They are indicated as tumor-livers D, E and F in the histograms.
(1.6) in the newborn liver here estimated is assumed to be a diploid value (2x), its duplication (4x) is 3.2. Tumor liver A and B show a slightly higher average value than the expected 4x value, while tumor liver C shows the expected 4x value.

Comparison of DNA amount between the normal adult and tumor livers D, E and F: A normal liver and tumor liver F, which was obtained from a rat killed 8 days after the tumor transfer, showed a similar pattern of frequency distribution of the DNA content; the average value of DNA content is 3.77±0.10 and 3.78±0.12, for the normal and tumor liver F respectively. On the contrary, the DNA contents of tumor livers D and E which were obtained from rats killed 3 and 6 days after tumor inoculation, were slightly higher than those of the normal and tumor liver F, and the scatter ranges of DNA contents in the former two samples were also wider than in the latter ones. The standard deviation was 0.91 and 0.97 in the

Text-Figure I. Four histogram at left hand indicate relative amount of DNA in the liver cells of a newborn rat, and of three tumor-bearing rats at the 9th day of tumor inoculation, respectively. The histograms at right hand show relative amount of DNA in the liver cells of a normal adult rat, and of three tumor-bearing rats which were killed 3, 6 and 8 days after the tumor transplantation, respectively. For detail see text.

Text-Figure II. The histograms show nuclear diameters of the liver cells of a normal adult rat, and of three tumor-bearing rats which were killed 3, 6 and 8 days after the tumor transplantation. For detail see text.

8 days after the tumor transfer, showed a similar pattern of frequency distribution of the DNA content; the average value of DNA content is 3.77±0.10 and 3.78±0.12, for the normal and tumor liver F respectively. On the contrary, the DNA contents of tumor livers D and E which were obtained from rats killed 3 and 6 days after tumor inoculation, were slightly higher than those of the normal and tumor liver F, and the scatter ranges of DNA contents in the former two samples were also wider than in the latter ones. The standard deviation was 0.91 and 0.97 in the
former, and 0.62 and 0.73 in the latter, respectively.

On the basis of the above findings it is highly probable that the tumor exerts influence upon the host liver to raise the DNA content in its liver cells during the period of the most active growth of the tumor which ranges from about 3 to 6 days after tumor transfer, with or without a return of the DNA content to the normal value which occurs during the time from the 8th to the 9th day after the transfer, near the end of the tumor-animal’s life.

**Changes of diameter in liver cell nuclei**: The diameter of nuclei was measured in the normal liver and tumor livers D, E and F and the results are given in Text-figure II; the mean in length of long and short axes of the nucleus is expressed as a diameter in an arbitrary unit. Referring to these data it is clear that changes in the DNA content occurring during the tumor growth run closely parallel to changes in the size of nuclei of liver cells: namely, the average value of the nuclear diameter is 68 for the normal liver and 76, 77 and 70 for tumor livers D, E and F, respectively. It is therefore probable that an increase in DNA content of liver cell nuclei occurs concomittantly with an increase in the volume of cell nuclei in rats bearing tumor.

**DISCUSSION**

Gradual decrease of liver glycogen in tumor-bearing rats was observed in a series of experiments in the present study. Glycogen depletion in rat livers can also be induced by starving the rat for several days (Hori 1958). The livers of rats starved for 6 days showed, however, much more glycogen contents than those of rats bearing a tumor for 6 days. This seems to imply that the observed depletion of liver glycogen in the tumor-bearing rats may be attributed to, or be caused by the transplantation of that tumor.

There are a number of studies which indicate that the carbohydrate-containing proteins in serum increase beyond their normal levels both in patients with cancer and in animals bearing transplanted tumors (cf. Baldwin and Harries 1958). Probably, the liver glycogen may play a role in relation to the increase of serum carbohydrate-containing proteins, and further the marked depletion of liver glycogen as observed in the present study may be responsible for the increase of serum carbohydrate-containing proteins as above dealt with.

Blood sugar level and liver glycogen are under the control of various kinds of hormones, such as adrenalin, insulin, anterior pituitary and thyroid hormones, so that the disturbance of hormonal balance following the injury of endocrine glands which can be induced by various factors must be taken into consideration, so far as the fate of liver glycogen is concerned. Dalton and Peter (1944) studied the effects of tumor on the endocrine glands, and reported that marked depletion of
lipids occurred in the adrenal cortex of tumor-bearing mice. Umeda et al. (1957) observed involution of the thymus which was accompanied by a decrease in DNA and RNA contents. An increase of glycolysis has also been reported in the liver of leukemic mice by Hall (1944) and Burk et al. (1942).

Bennett (1956) proposed an interesting hypothesis pertaining to the transmission of nucleoprotein from nucleus to cytoplasm, indicating that some of the RNA newly formed in the nucleus may become attached to the inner nuclear membrane and be carried out onto the ergastolasmic portions of the endoplasmic reticulum by the "membrane flow." In the electron micrography study of salivary-gland cells of third instar larvae of Drosophila melanogaster, Gay (1956) has observed nuclear membrane out-pocketings and regarded them as a mechanism for transport of materials of chromosomal origin into the cytoplasm. Moses (1956) also observed out-pocketings of the nuclear envelope in a crayfish spermatid. Using a hemipteran insect as material, Anderson and Beams (1956) obtained electron micrographs which show that actual transport of nuclear substance takes place through pores of the nuclear membrane to the cytoplasm. As referred to above, evidence favorable to the idea of transport of nuclear material into cytoplasm is now available specially in the field of electron microscopy. In the present study, observations of toluidine blue stained preparations furnished evidence that basophilia of the hepatic cells increases to a great extent in tumor-bearing rats. In addition to the increase of cytoplasmic basophilia, the extrusion of nucleolar material and its hypertrophy observed in the hepatic cells of tumor-bearing rats seem to be evidence supporting the view that the transport of nuclear material, especially RNA, into the cytoplasm occurs in the livers, though no conclusive statement can be made without further supplementary experiments.

It has been shown that the tumor transplanted into animals exerts influence upon the turnover of tissue deoxyribonucleic acid. Payne et al. (1952) reported an increase of P³² incorporation into the liver DNA in rats bearing tumor transplants by way of comparison with the control animal. Kelly et al. (1950) and Kelly and Jones (1950) also obtained similar results. Effects of tumor transplants on nucleic acid content of host mouse tissues were investigated by Reddy and Ceredo (1951). They found that both DNA and PNA increased in the livers of mice bearing Crocker sarcoma 180 transplants, and regarded such a change as the above to be a result of increased cell proliferation.

The present study has yielded results indicating a slight, but significant increase of net DNA contents in liver cells of animals with tumor transplants. Though it seems difficult to expect accurate results in measurement of DNA contents by means of microspectrophotometry, the above evidence receives support from the finding that an increase in nuclear volume of liver cells of tumor-animals occurs
concomittantly with an increase in DNA contents of those cells.

Yeakel (1948) and Yeakel and Tobias (1951) reported that liver weight increased in rats bearing induced or transplanted tumors. Annau et al. (1951) also found an increased weight and mitotic activity in the liver of tumor-bearing rats and mice, and concluded that the increase in weight might be partly a consequence of mitotic activity induced by the presence of the tumor.

The increased mitotic activity in livers of tumor animals may be associated with the increased DNA content in liver cells observed in the present study, though there is no direct evidence to support that interpretation.

Since Vendrely and Vendrely (1948) found that the DNA content of isolated liver nuclei is approximately twice that of spermatozoa, a number of reports have been published pertaining to the DNA contents of various kinds of cells. Many authors agree that the DNA content of a nucleus is strictly correlated with its chromosome number (Alfert & Swift 1953, Davidson & Leslie 1950, Mirsky & Ris 1951, Thomson and Frazer 1954, Cole & Leuchtenberger 1956 and many others, see also the review by C. & R. Vendrely 1956), while some authors hold a view contrary to the above (Pasteels & Lison 1950, Lison & Pasteels 1951, Fautrez & Fautrez-Firlefyn 1953). According to them, the DNA content of the nucleus can vary with the physiological activity of the cell. Recently, several reports have become available regarding the views of Belgian workers. Lowe and Rand (1956) investigated the effect of cortisone on the DNA content of rat liver cells; they reported that the DNA content of liver nuclei fell during cortisone administration, the fall was progressive with continuity of hormone administration (by 20%), and the return to normal occurred 3 days after discontinuance of cortisone administration. LaCour et al. (1956) observed a considerable drop in the DNA content of the nuclei after exposure of plant root meristematic cells to low and high temperature. Leuchtenberger et al. (1956) observed in dwarf bulls a considerable variation of the DNA content per nucleus during spermatogenesis which is not followed by the shift of the chromosome number.

Bendich (1952) and Bendich et al. (1953) discovered the existence of different DNA fractions, DNA₁ and DNA₂, which show significant metabolic differences. According to them, the ratios of DNA₁ to DNA₂ may be related to the mitotic activity of the tissue from which the DNAs were derived. Morin et al. (1956) also reported the metabolic heterogeneity of DNA. They examined the incorporation of P³² into the DNA of the various fractions which were isolated by the method of differential centrifugation from mouse liver nuclei, and found evidence that the fractions rich in nucleolus-associated chromatin showed the highest specific activity among the fractions isolated. There are still additional reports which oppose the review as to the strict constancy and metabolic stability of DNA in the nucleus, or as to the
close correlation between the chromosome number and the DNA content per nucleus.

In reference to the reported evidence as above, it seems that the deviation of DNA content from the values which are expected from the chromosomal ploidy basis can not be discussed on the basis of mere change in the DNA content of the nucleus occurring during the mitotic cycle.

Fautrez (1956) and Fautrez et al. (1956) reexamined the correlation between the volume and DNA content of individual nuclei in different tissues, of the rat, and presented evidence that the average nuclear volumes for their three given classes (2C, 4C and 8C) follow exactly the same progression as the average DNA content. They showed further that within one of those classes the relation between the DNA content and volume is not very evident for the different individual nuclei, though there exists a relation at least to a certain extent.

If an increase of DNA contents with an increase of nuclear volume as observed in the present study should be related to change in DNA content of the nucleus during the mitotic cycle, an increase in nuclear volume should in turn occur during the mitotic cycle. In other words, if a close correlation should exist between the nuclear volume and DNA content, the volume of the nucleus just undergoing division should be twice that of the nucleus at the end of division. There is no direct evidence for the above supposition, and therefore, the present findings showing that the DNA contents and nuclear volume increased in liver cells of the tumor-bearing animals remain without clear explanation at present.

Wolstenholme and Gardner (1950) investigated histologically the livers of mice with transplanted testicular tumors and described the sinusoidal dilatation as usual while the destruction of the normal lobular architecture of the liver was induced by the dilatation. This is parallel to the present findings.

In conclusion, the marked depletion of glycogen, the gradual increase of RNA contents, the significant increase of DNA contents with a return to normal contents in the latter part of the tumor-animal's life, the increase of mitotic activity and the sinusoidal dilatation are a series of findings which have been derived from the observations of the liver cells of rats bearing tumor transplants. However, the authors hesitate to conclude simply that the existence of the tumor directly induces these changes, since some changes of similar character not uncommonly occur in rats without tumor when they are exposed to certain physiological and pathological conditions.

**Summary**

In the rats which received the transplantation of the ascites tumor, MTK-sarcoma III, the changes in contents of glycogen, RNA and DNA in the liver cells were investigated by means of a series of cytochemical and microspectrophotometric methods.
Gradual glycogen depletion was consistently observed from experiment to experiment in the liver cells of rats bearing tumor transplants. Every rat which died of tumor contained a very little amount of glycogen in their liver cells. The rate of the glycogen depletion of tumor-bearing animals exceedingly surpassed that of starved rats. It has remained unsolved in the present study whether the observed depletion of liver glycogen occurred in rats as a direct response to the enormous requirement of energy by tumor cells, or whether tumor transplants attacked hormonal glands of the host which control the glycogen storage in the liver. Whatever the causes may be, it is very likely that the glycogen depletion observed in the tumor-bearing rats may be attributable to the tumor transplants in hosts.

Contrary to the glycogen depletion, RNA (basophilia digestable by RNase) increased gradually with the prolonged existence of the tumor transplants. The increase in amount of RNA was observed both in the cytoplasm and in the nucleus. The thickening of the nuclear membrane and the hyperactivity of the nucleolus observed in the tumor animals seem to indicate the active participation of the nucleus in association with the accelerated production of RNA in the liver cells.

An increase of net DNA content (Feulgen-Schiff complex) took place in connection with an increase of the nuclear volume in liver cells of tumor-bearing animals during the period of the most active proliferation of the tumor. Its significance was discussed in relation to the DNA content of the nucleus.

Increased mitotic activity and dilatation of the liver sinusoids were also observed in livers of the rats bearing tumor transplants.

References

44. ——— and G.L. Tobias 1951. Ibid. 11: 830–833.

EXPLANATION OF PLATE XX

Fig. 1. Control rat liver.
Figs. 2–4 livers of tumor-bearing rats sacrificed on 3, 6 and 8 days after transplantation, respectively.

Figs. 5–8. Fixative: subzero Gendre’s fluid. Stain: periodic acid Schiff. Magnif. 100x, except Fig. 8 which is 400x.
Fig. 5. Control rat liver. Glycogen abundant, showing a uniform distribution throughout the hepatic lobules.
Figs. 6–8. Livers of tumor-bearing rats sacrificed on 3, 6 and 8 days after transplantation, respectively.
Note a gradual decrease of PAS positive substance in liver cells.

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Figs. 9 and 10. Showing the extrusion of nucleoli into the cytoplasm.

Fig. 11. Nuclei stained densely with toluidine blue.

Fig. 12. *Fixative*: Gendre's fluid. *Stain*: PAS. *Magnif.*, 400×. Showing tumor cells in contact with the surface of the liver, 6 days after the tumor transplantation. Note the positive reaction of the cytoplasm.

Fig. 13. *Fixative*: formal-calcium followed by the chromation with potassium bichromate for 6 days. *Stain*: Regaud's iron-hematoxylin. *Magnif.*, 1000×. Liver cells of tumor-bearing rat 6 days after transplantation. Mitochondria show no marked morphological change by the presence of the tumor.

Addendum. After this article was completed and sent to press, Leuchtenberger, Leuchtenberger and Uyeki (1958) published an article: Cytological and cytochemical changes in livers of white mice following intraperitoneal injections of DNA preparations from breast cancers of agouti C3H mice (Proc. Nat. Acad. Sci. 44: 700-705), in which they present approximately similar results to those obtained in this study, so far as the DNA contents are concerned.

要 旨

MTK-肉腫 III を移植せるシロネズミ肝のグリコーダー, RNA, および DNA の消長について

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Wistar 系シロネズミ (70〜120g) の腹腔内に MTK 腹水肉腫 III を移植し, 移植後 3, 6, 8 または 9 日後に宿主を殺して, 肝におけるグリコーダー (McManus PAS 反応), RNA (Toluidine blue 染色) および DNA (Feulgen-Schiff 複合体の顕微側光法による測定) の消長を調査した。

グリコーダーは正常なネズミの肝においては常に豊富に含有されているが, 肉腫を移植されたものにおいては, 次第に肝小葉周辺部より減少し, 宿主が死亡する 8〜9 日後にはほとんど肝全体に亘って消失してしまう。6 日間絶食させた, 肉腫を植えないネズミにおいてもこれほど極端なグリコーダーの消失は見られなかった。グリコーダーの消失とは逆に, RNA は肉腫移植後次第に増加する。RNA の増加は腫瘍の増大に関係があると思われるが, 仁の肥大・多形化・数の増加および核膜の肥厚が肝細胞において一貫的に観察された。一方, DNA も腫瘍動物において,やや増加する傾向が見られた。また肝細胞核の体積の増加も DNA 量の増加と平行して見られた。この外, 腫瘍動物肝においては, 有絲分裂の増加および sinusoid の膨張が観察された。