MICROSPECTROPHOTOMETRY OF CELL NUCLEI STAINED WITH THE FEULGEN REACTION

IV. Formation of Tetraploid Nuclei in Rat Liver Cells During Postnatal Growth

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INTRODUCTION

It has previously been reported that liver cell nuclei of *adult* rats compared with other cells, have a higher average DNA content, while there is no difference in DNA content between the hepatocyte and other types of cells in *young* rats. It is, moreover, generally recognized that the nuclei having the higher and lower DNA content represent the tetraploid and diploid nuclei respectively (14, 17, 18, 25, 35, 36, 47, 50, 66). The proportion of such diploid and tetraploid nuclei in a liver changes in the course of postnatal growth but in rats weighing about 50 gm. the total number of diploid nuclei in the liver equals that of tetraploid (36).

A detailed study on the nuclear population of liver parenchyma was undertaken with special reference to the formation of tetraploid nuclei. The experiments reported here were designed to learn how the ploidy proportion changes in accordance with the growth of rats, and to follow the increase in total number of nuclei of a liver, using microspectrophotometry as a tool for identification of the ploidy class.

Ploidy classes of liver cell nuclei were identified by their relative DNA content as determined by microspectrophotometry, the nuclear class predominating in several other tissues besides liver being taken as diploid. Accordingly, the term "ploidy" as used in this paper does not refer to the number of chromosome sets, but to the modal Feulgen value of discrete nuclear classes which means DNA classes as proposed by Swift (62).

Methods

A. Estimation of Relative DNA Content of Individual Nuclei:

The method of estimating relative DNA content per individual cell nucleus was much the same as in the previous reports of this series, and made use of the microspectrophoto-

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metric apparatus of Koana-Naora type which is characterized by being free from the Schwarzschild-Villiger effect (S-V effect) (35, 36). In previous communications (32, 34), Naora has pointed out the possible existence of the Schwarzschild-Villiger effect in the equipment hitherto employed and he constructed the improved apparatus in which this effect is eliminated. It should be noted that the involvement of Schwarzschild-Villiger effect was challenged by Ornstein and Pollister (42) who were, in turn, critically answered by Lison (28), and Naora (35).

Livers and some other tissues were fixed with 20 per cent formaldehyde solution for 3 or 24 hours. Sections of appropriate thickness were prepared in the usual way and stained with the Feulgen reaction, F, MB, and B reaction mixtures being employed as in the previous communication (36, 51).

There are a number of sources of error in the determination of the relative DNA content per individual nucleus by means of microspectrophotometry. The main ones are associated with the apparatus, the measuring procedures, including preparation of a sample, and the quantitative nature of Feulgen reaction. These problems were repeatedly discussed by Sibatani and Naora, and the sources of error were minimized or eliminated (32, 33, 35–37, 40, 51–55, 57–59). The present experiment was attempted under the conditions recommended by their results. Therefore, the measurements made in the present experiments may be regarded as valid and the amount of the Feulgen dye per nucleus determined microspectrophotometrically may be expected to be proportional to DNA content per nucleus.

About 100 liver cell nuclei, circular in outline and assumed to be spherical, were selected quite at random out of representative sections of individual livers, and their relative DNA contents were estimated by the amount of Feulgen dye per single nucleus. The details of the measuring procedure have been published (37). The results were expressed in the form of frequency histograms. Cell nuclei of a single specimen usually fell into two or more discrete groups, and showed approximately normal distribution within a group. Each of such discrete groups was regarded as a separate ploidy class. The frequency within different ploidy classes could thus be computed and was expressed as percentage of the total nuclear population in a given specimen. If two groups slightly overlap in the histogram obtained, segregation into each polyploidy class was made on the statistical basis of normal distribution. In such cases, the value obtained for the frequency (percentage) is in error to a certain extent. The frequency (percentage) given in tables and figures represents the mean values of at least two such determinations. Although nuclei measured were selected quite at random, there is a possibility that the frequencies in higher ploidy classes were to some extent below the real value because only nuclei preserved completely in a given section were used for measurements, while the probability of nuclei within the higher ploidy classes being "chipped" by sectioning should, owing to their large size, be greater than that of nuclei of lower ploidy classes. However, the errors from this source and grouping into each polyploidy class were considered not to be too high to distort the over-all results to be communicated here.

B. Estimation of the Total Number of Parenchymal Cell Nuclei in a Liver:

This was obtained by a modification of the method of Brues and coworkers, based upon the nuclear count in histological sections (7, 10).

The total number (N) of nuclei of a certain cell type in a given tissue specimen can be obtained by the equation

$$N = \frac{n \times v}{f} \tag{1}$$

in which n is the nuclear density, or the number of nuclei per unit volume, and v the volume of a representative sample (R) taken appropriately from the tissue specimen to be studied,

and f indicates the fraction of the total tissue, by weight or volume, that this sample, R, represents. This method of calculation assumes uniform distribution of the cell types studied throughout the whole tissue. In the case of rat liver, the validity of this assumption can be demonstrated (Table I, see below).

1. Preliminary Treatment of the Liver.—The liver was carefully removed from the carcass and weighed, its total wet weight being W. Then a small piece, of about 1 gm., was cut therefrom, and its wet weight taken as w. This sample was then fixed in 20 per cent formaldehyde solution for 24 hours, dehydrated through ethanol, butanol, and xylol and then immersed in liquid paraffin. The volume and nuclear density of the liver was calculated from determinations on this sample. The fraction of this sample in total liver is $f = w/W$.

2. Estimation of the Apparent Volume of a Tissue Sample Fixed and Dehydrated.—Since the nuclear density was to be measured using tissue sections, the volume of the tissue to be estimated had to correspond to the one occupied by the representative sample preserved in a state comparable to that of the mounted histological sections. So, the tissue sample was first fixed in 20 per cent formaldehyde solution for 24 hours, dehydrated through etha-

TABLE I

The Volume of a Whole Liver Estimated from Individual Lobes

Body weight of the rat: 75 gm.

Lobes	Volume
	cm^3
I	2.19
II	2.09
III	2.14
IV	2.14
V	2.10
Average	2.13 ± 0.016*

* Standard error.

nol, butanol, and xylene, and then immersed in liquid paraffin. After the volume of this sample had been measured, it was embedded in paraffin and sectioned. Since in fixation and dehydration the sample lost most of its water, lipides, and soluble material, the residue was composed primarily of proteins and nucleic acids. Thus the tissue mass was very porous, and its real volume was much reduced from that of the original fresh sample. However, the value of the volume required here is not the real, but the apparent one occupied by this tissue mass, inclusive of organic residue and its interspaces, which are now filled with immersion medium. The estimation of the tissue volume was made with samples immersed in liquid paraffin. The small difference in apparent tissue volume to be expected between such samples and the ones after sectioning, proved to be negligible; the actual measurement of the size of a section (10 μ thick) showed that it did not deviate seriously from the one measured in liquid paraffin. Thus the volume (v) of the sample to be measured consists of two terms: v_1, the volume occupied by the tissue residue, and v_2, the volume occupied by the liquid paraffin infiltrating all the interspaces within the sample, R. v_1 is now readily estimated from Archimedes' formula:

$$v_1 = \frac{w_{air} - w_{lp}}{\rho_{lp}} \tag{2}$$

where w_{air} and w_{lp} indicate the weight, respectively, in air and in liquid paraffin of the sample (dehydrated tissue residue) and ρ_{lp} the specific gravity of liquid paraffin. v_2 is simply given by

$$v_2 = \frac{w_m}{\rho_{lp}} \tag{3}$$

where w_m is the weight in air of the liquid paraffin infiltrating the interspaces in the sample.

Actually, the tissue sample immersed in liquid paraffin was weighed in liquid paraffin to obtain w_{lp}, and then it was placed on a filter paper and well blotted to remove liquid paraffin from the surface. In this state the interspaces within the sample were filled

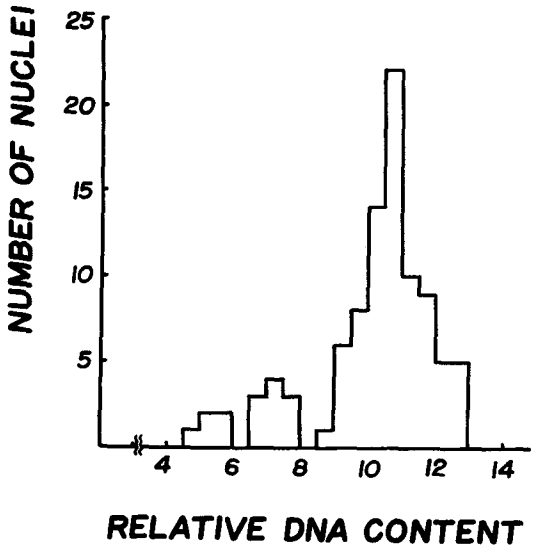

FIG. 1. Various ploidy classes in the liver of a rat, weighing about 100 gm. Measured nuclei were not sampled strictly at random in order to emphasize the occurrence of the intermediate class of DNA content.

with liquid paraffin. This was then reweighed in air, to get $w_t = w_{air} + w_m$. Now the volume of the sample is given by

$$v = v_1 + v_2 = \frac{w_{air} - w_{lp} + w_m}{\rho_{lp}} = \frac{w_t - w_{lp}}{\rho_{lp}} \tag{4}$$

This method of estimating tissue sample volume was checked by an experiment shown in Table I. In this, the apparent total volume of the liver preparation $V_a = v/f$ was calculated from estimations of v, using samples taken from five different lobes of a liver. The close agreement of five independent estimates suggests the reliability of the method described.

3. Estimation of Nuclear Density.—The nuclear density (n) is defined by the number of parenchymal nuclei per cm.^{3} of the mounted sample of the liver. If the number of parenchymal nuclei in a known area (s) of microscopic field (actually counted in an enlarged pro-

jection upon the screen) is c and the mean radius of nuclei r, n is obtained from the following equation,

$$n = \frac{c}{2(r + d\xi)s} \tag{5}$$

where $2d\xi$ is the depth of focus of the microscopic lens used. In the present experiments, the value of $d\xi$ was 0.5 μ.

Thus, the total number of parenchymal nuclei in the liver is obtained with the final formula:

$$N = n \cdot V = \frac{c \cdot W}{2(r + d\xi)s \cdot w} \cdot \frac{w_t - w_{lp}}{\rho_{lp}} \tag{6}$$

C. *Animals Employed:*

Albino rats of unspecified strain were grown on semisynthetic stock diet. Altogether, about 30 rats were sacrificed at the desired growth stage and the livers were sampled by

TABLE II

The Ploidy Frequency of the Individual Lobe Obtained from the Same Rat as Used in Table I

Lobes	Ploidy frequency			
	Diploid	Triploid	Tetraploid	Octoploid
	per cent	*per cent*	*per cent*	*per cent*
I	9.9	—	88.4	1.7
II	11.5	0.8	86.9	0.8
III	12.4	—	86.4	1.2
IV	10.7	—	88.3	0.9
V	11.8	0.8	87.4	—
Average	11.3		87.5	

the procedures described above. In experiments of growth inhibition, the minimum quantity of the stock diet required for maintaining life was given to young rats initially weighing 25 to 30 gm. for about 1 month, while control animals from the same litter were fed *ad libitum* on the same stock diet.

RESULTS

A. *Occurrence of Parenchymal Nuclei of Different Ploidy Class in a Single Liver Tissue:*

One liver specimen of a normal rat, weighing about 100 gm., was surveyed microspectrophotometrically for the occurrence of nuclei of different ploidy classes. The results are shown in Fig. 1 as a histogram. Parenchymal nuclei of this liver fall into three groups, group I with the lowest, group II with the medium, and group III with the highest DNA content. Most of the nuclei measured belong to group III. If we assume that the group I nuclei represent the diploid, it seems probable that groups II and III are of triploid and of

tetraploid type, respectively. However, nuclei of triploid type are poorly represented in the normal liver. It is doubtful whether the nuclei belonging to the intermediate class represent the ones which are just synthesizing their DNA in interphase (see (8, 9, 49)).

B. Polyploid Frequency in Different Lobes of the Liver:

Tissue sections prepared from five separate lobes of the liver of a rat weighing 75 gm. were examined for the occurrence of different nuclear groups. Frequencies (percentages) of nuclei in these nuclear groups, including diploid, triploid, tetraploid, and occasional polyploid (tentatively assumed to be

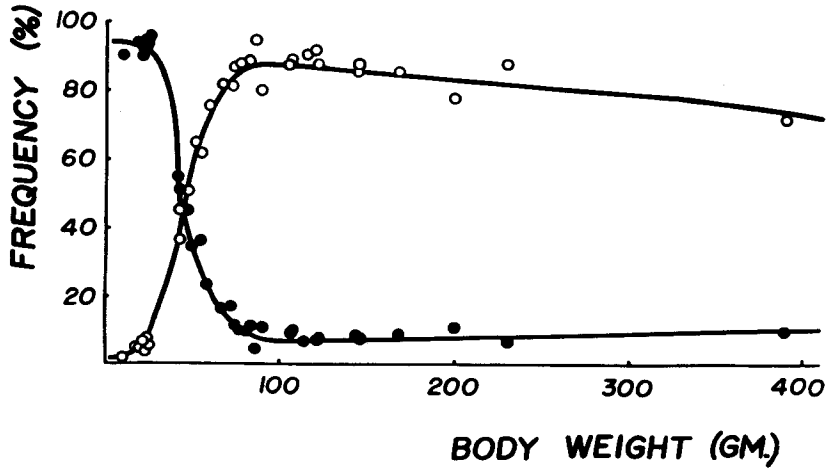

FIG. 2. Variation of the frequency of diploid and tetraploid nuclei in rat livers at different stages of postnatal growth as expressed by the body weight.
—●—●—: frequency of diploid nuclei.
—○—○—: frequency of tetraploid nuclei.

octoploid type) are shown in Table II. As seen in the table, the frequencies of the diploid or tetraploid type in different lobes agreed very well. This finding indicates the uniform distribution of nuclei of different ploidy in the whole liver. This fact warrants the validity of evaluating the ploidy frequency of hepatocyte nuclei with a single small specimen of the liver tissues.

C. Change in Frequency of Diploid and Tetraploid Nuclei of Liver Cells in Postnatal Growth:

Frequencies of diploid and tetraploid nuclei of liver cells were studied with representative specimens throughout the course of postnatal growth of the rat. The results are summarized in Fig. 2 and Table III. Most of liver cells of newborn suckling rats exhibited DNA values of diploid type, nuclei of the higher

ploidy classes being very few, if any at all. This type of ploidy proportion in the nuclear population was retained until the body weight of about 25 gm. was reached, when the young were at the weanling stage.

Just after the weanlings started taking the stock diet by themselves, *i.e.* the body weight of around 30 gm., a marked decrease in frequency (percentage)

TABLE III

*The Ploidy Frequency of the Liver of Rats in Postnatal Growth**

Body weight	No. of animals	Ploidy frequency	
		Diploid	Tetraploid
gm.		*per cent*	*per cent*
11	1	90.0	2.0
22–26	5	93.2 (91.3–95.5)	5.4 (3.4–7.1)
39–42	4	53.2 (51.0–55.0)	42.4 (36.0–45.0)
47–59	4	35.6 (24.2–45.0)	63.2 (51.0–75.4)
67–81	5	13.4 (10.2–18.4)	85.9 (81.0–89.1)
86–124	6	7.7 (4.0–11.0)	89.8 (80.0–95.0)
144–166	3	9.9 (8.8–10.7)	88.4 (87.3–89.2)
200–230	2	8.7 (6.3–11.0)	83.4 (78.0–88.7)
395	1	10.0	71.0

* The individual figures represent average values and their ranges.

of diploid nuclei began to occur. This was accompanied by the appearance of parenchymal nuclei with higher DNA contents, probably of tetraploid type, which then markedly increased in frequency. In livers of rats weighing about 50 gm., the frequency of the diploid nuclei equaled that of the tetraploid ones. The tetraploid frequency further increased significantly with the growth of the rat. In contrast to the increase in tetraploid nuclei, a very marked decrease in diploids took place.

At above 80 to 100 gm. of the body weight, there was no longer any further

increase of tetraploid or decrease of diploid nuclei in the liver tissue. A small number of nuclei with DNA contents higher than that of tetraploid nuclei (called "polyploid" hereafter) appeared in adult rats weighing more than 100 gm. and seemed to increase in frequency as the mature rat grew further. The frequency of the tetraploid nuclei, therefore, showed a decrease in spite of the

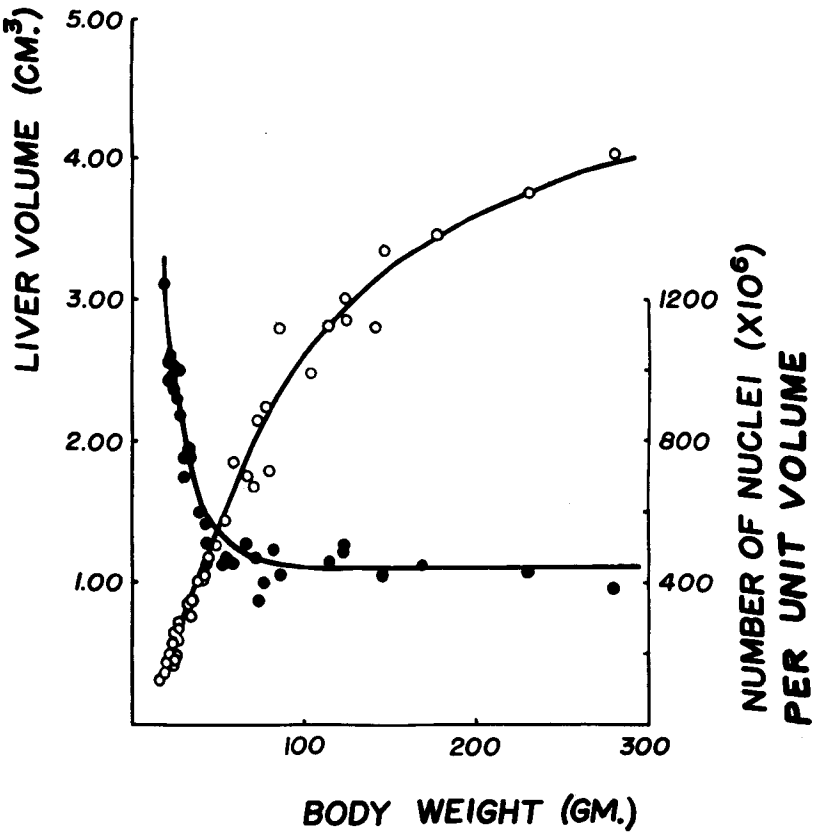

FIG. 3. Variation of the tissue volume after infiltration of liquid paraffin and nuclear density of livers of rats in postnatal growth as expressed by the body weight.
—○—○—: liver volume.
—●—●—: nuclear density.

gradual increase in total number of the tetraploid nuclei in livers of older rats. It seems that in adult rats weighing around 200 gm., the nuclear population of this liver is, so to say, in an equilibrium with respect to the ploidy proportion, although there is a slight change in frequency of polyploid nuclei.

In this connection, it should be noticed that the substitution of tetraploid for diploid nuclei in rat liver occurs just at the growth stage at which the Feulgen

dye content of diploid nuclei reaches the minimum value, as shown in the previous report (part III (36)).

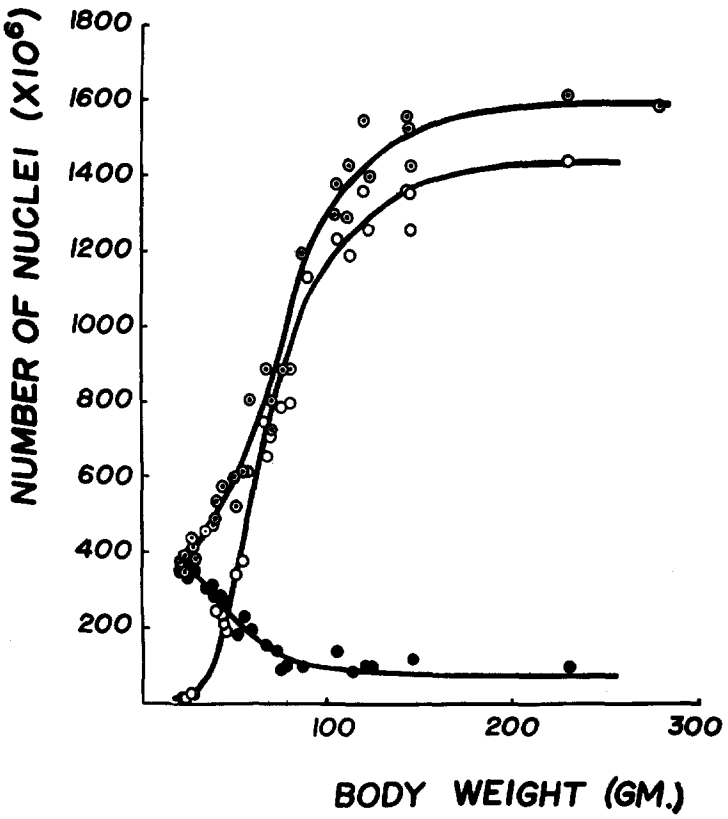

FIG. 4. Variation of total number of nuclei (—⊙—⊙—), of diploid nuclei (—●—●—), and of tetraploid nuclei (—○—○—) in the whole liver of rats at the different stages of postnatal growth as expressed by the body weight.

D. Changes in Nuclear Density and Total Number of Nuclei of Rat Livers in Postnatal Growth:

The results of measurements of nuclear density and apparent volume of fixed liver tissue, which were conducted with the same material as employed in the above experiments, are summarized in Figs. 3 and 4 and Table IV. The apparent total volume of the liver represents the volume occupied by the whole liver preparation, fixed, dehydrated through fat solvents, and infiltrated with liquid paraffin, but retaining its histological architecture. The deviation of this value from the real volume of the fresh liver may not be uniform with individual livers examined, but since the nuclear density also was estimated with

the same material, this deviation is cancelled. Thus, the final value of the total number of nuclei in the liver is not affected by our failure to estimate the real volume of the liver.

The microscopically estimated values of the total number of nuclei in the liver of rats agree well with those determined biochemically.

TABLE IV

*The Total Tissue Volume, Nuclear Density, and Total Number of Nuclei of the Liver of Rats in Postnatal Growth**

Body weight	Total tissue volume	Nuclear density ($\times 10^6$)	Total no. of nuclei ($\times 10^6$)
gm.	*cm.*^{3}		
20.6–22	0.37 (0.32–0.42)	971 (785–1238)	347 (319–391)
24–29	0.51 (0.39–0.73)	891 (601–1136)	438 (345–683)
39–42	1.05 (0.98–1.17)	519 (458–587)	541 (475–588)
51–59	1.49 (1.20–1.86)	434 (433–436)	648 (520–810)
67–86	2.07 (1.72–2.81)	439 (339–512)	898 (724–1199)
106–124	2.76 (2.44–3.00)	510 (466–568)	1405 (1279–1549)
144–146	3.05 (2.75–3.34)	496 (420–571)	1463 (1403–1523)
230–280	4.08 (3.73–4.44)	413 (391–435)	1607 (1591–1623)

* The individual figures represent average values and their ranges.

In young rats, a marked increase in number of liver nuclei occurs. The increment of nuclei per gram body weight is greater than that of the adult rat (Fig. 4) as is also demonstrated by the higher mitotic indices in younger rats as shown in Fig. 5. Above 150 gm. body weight, the rate of increase in number of liver cell nuclei falls down to a low level until the total number of nuclei of the whole liver reaches an almost constant value at the growth stage of 200 gm. body weight.

Nuclear density is highest in the youngest animals, and gradually lowered until it reaches a practically constant value at about 50 gm. body weight.

E. Number of Diploid and Tetraploid Nuclei in the Whole Liver:

The total number of nuclei of each ploidy class is easily estimated from the values of the frequency (percentage) of nuclei in this class and the total number of liver nuclei. The results are shown in Fig. 4. A marked increase of tetra-

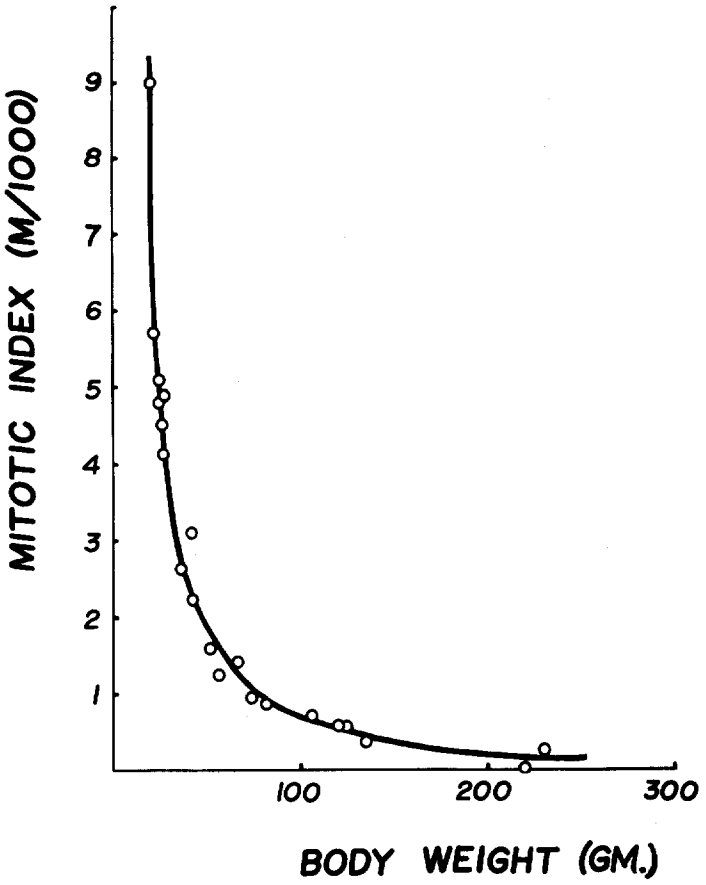

FIG. 5. Change in mitotic index of the rat liver in postnatal growth as expressed by the body weight.

ploid and decrease of diploid nuclei in total number per liver take place in younger rats, while no remarkable change occurs in adult rats weighing around 200 gm. As easily seen in Fig. 4, the curve of increase in number of tetraploid nuclei shows a logarithmic form. The intersection of the two curves corresponds to the body weight of 45 gm., at which the liver has diploid and tetraploid nuclei in equal number. It should be noticed that the rapid replacement of nearly all the diploid nuclei with tetraploid ones is completed during the growth from 30 to 70 gm. of the body weight. It suggests, therefore, that the synthesis

of DNA in a liver occurs at a remarkably high rate in this particular period, because here a marked increase in total number of nuclei of the liver is attended by the extensive tetraploidization, or doubling of DNA content per nucleus, which now comes to comprise almost the entire population of the hepatic nuclei.

TABLE V

*Influence of the Growth Rate on the Appearance of Tetraploid Nuclei in the Rat Liver**

Animal	No. of animals	Body weight at the beginning of experiment	Experimental period	Maximum body weight during experiment	Average growth rate
		gm.	*day*	*gm.*	*gm./day*
Control	3	33 (30–35)	33	129 (121–142)	3.1
Experiment	2	28 (25–30)	33	44 (42–46)	0.7

Liver weight	Liver volume	Nuclear density ($\times 10^6$)	Total No. of nuclei ($\times 10^6$)	Ploidy frequency	
				Diploid	Tetraploid
gm.	*cm³*			*per cent*	*per cent*
6.01 (5.75–6.50)	3.06 (2.84–3.34)	475 (420–512)	1451 (1402–1549)	7.6 (6.7–8.8)	90.6 (89.2–91.7)
2.19 (2.13–2.25)	1.06 (1.00–1.11)	438 (398–478)	460 (442–478)	72.5 (71.7–73.3)	26.7 (26.7–26.7)

No. of nuclei of individual ploidy classes ($\times 10^6$)		Mitotic index (No. of mitotic figures per 1000 nuclei)
Diploid	Tetraploid	
110 (104–123)	1305 (1251–1420)	0.57 (0.57–0.57)
334 (317–350)	123 (118–128)	0.43 (0.28–0.57)

* The figures represent average values and their ranges.

F. Influence of the Growth Rate on the Frequency of the Ploidy Class and the Number of Nuclei of the Liver Cells:

Five young rats of a litter, weighing 25 to 30 gm., were divided into two groups. The control group was fed with sufficient amount of the stock diet, while the experimental group received a small amount of the same diet which permitted the animal to survive, but not to grow much. Both groups were kept under these conditions for 33 days, whereupon the mean body weight of

the control and experimental groups turned out to be 129 and 40 gm., respectively. Most of the hepatic cells in experimental rats show the type II cell classified by Sibatani (56) with homogeneous distribution of cytoplasmic RNA.

The livers were studied as before, and the results are presented in Table V. The ploidy proportion of the control liver is quite typical of adult rats, the predominant nuclear class being tetraploid, whereas the population of the hepatic cell nuclei in the experimental group consists of 72.5 per cent diploid and 26.7 per cent tetraploid type. These figures are rather close to the ones of normal young rats weighing about 40 gm. (see Table III). Such facts may indicate that the change in the ploidy proportion of the liver cell nuclei is independent of the age of animals, but is rather conditioned by the growth state or maximal body weight the animals have ever attained. Such observations are reminiscent of the dependence of the average DNA content of nuclei of the rat liver on the body weight and not on the postnatal age (52).

The total number of liver cell nuclei of the experimental liver was estimated as 460×10^6. This value is comparable to that of the normal liver obtained from young rats weighing about 40 gm., although there is a slight difference. It may thus be suggested here that the number of parenchymal nuclei of the liver is, like its ploidy proportion, controlled by the growth state of the animal.

Now the total number of the tetraploid and diploid nuclei, respectively, of these livers can easily be estimated from figures just obtained. As shown in Table V, the total number of the tetraploid nuclei in the experimental group is smaller, and that of the diploid cell greater, than the corresponding ones of young rats which grew at the normal rate to attain the body weight of 43 gm. This observation suggests that the shift of the predominating ploidy class of the liver cell nuclei might depend on the physiological or nutritional conditions of cells or of the whole body, say, the growth stage.

DISCUSSION

It is well known that the average DNA content, estimated by gross biochemical techniques and pioneered by Boivin and Vendrely (6), of liver cells of adult rats is always much higher than that of other tissues such as kidney, pancreas, etc. (17, 18, 25, 50, 65, 68). At the same time, microspectrophotometric measurements on individual nuclei of the rat liver have been made by several workers, and they have always reported polyploid nuclei in adult rat liver (2, 13, 14, 35, 36, 44, 47, 66). Therefore, it has been considered that the higher values of DNA content obtained with the gross chemical techniques are due to the occurrence of such polyploid nuclei in the adult rat liver.

Recently, a slight decrease followed by a gradual increase of DNA content per cell, as estimated by the biochemical analysis during postnatal growth, has been observed by Sibatani and Fukuda (50). Such an observation involves

two factors to account for the variation of mean DNA content. First, the possibility of the true variation of DNA content of the individual cell nucleus should be considered. Secondly, polyploid cells might gradually appear in the population of the liver cells during growth, even if DNA content of the individual cell does not show any significant variation during this period. However, recent studies on the total amount of Feulgen dye in the individual cells by microspectrophotometry showed a rhythmic and slight variation during postnatal growth of animals, as previously reported (36). Considering this observation together with the formation of tetraploid cell nuclei in postnatal growth of animals, it may be suggested that the gradual increase of mean DNA content per nucleus determined by the biochemical techniques may be due to both the gradual appearance of polyploid cells and the gradual and slight alteration of DNA content of the individual cell.

Although tetraploid nuclei are observed in the liver of the rat, mouse (62), and man (27, 31, 61), the occurrence of such higher ploidy classes does not necessarily characterize the liver cells in general, for the liver cell of the salamander is the diploid type as reported by Truong and Dornfeld (67).

Recently, Swartz (61) studied the occurrence of polyploid cells in the liver of man ranging in age from 2 weeks to 90 years, and concluded that his results are consistent with the hypothesis that the development of polyploidy in mammalian organs is dependent on the anterior pituitary growth hormone. Moreover, the finding of Di Stefano and Diermeir (11), who found the restoration of polyploid proportion in the liver of the hypophysectomized rat by administration of a pituitary growth hormone, is quite consistent with such an hypothesis.

Several morphological mechanisms have been proposed for the formation of polyploid cells (16, 30, 71). Generally speaking, there are two possible mechanisms. First, polyploid cells may arise under rather pathological or abnormal conditions: the mitosis of binucleate cells, fusion of two nuclei, or failure of chromosomes to separate. In these cases, the polyploidization is certain to follow the mitotic process. Secondly, polyploid cells may arise without the formation of visible chromosomes. The investigation of such a mechanism in connection with the synthesis of DNA bears on the important problem of whether the process of DNA synthesis is linked with the initiation of the mitotic process or not. Can we, then, explain the predominant occurrence of tetraploid nuclei in the liver of adult rats on the basis of pathological changes or accidental division alone? Two possible answers to this question are considered. First, a small number of tetraploid nuclei, which appeared accidentally or pathologically by the abnormal mitosis or duplication of DNA, might proliferate enormously to yield a great number of tetraploid nuclei for about 1 month, and the population of diploid nuclei would concomitantly be destroyed and finally disappear. As the second possible explanation it might be con-

sidered that interphase diploid nuclei are directly transformed into the tetraploid ones under certain cell-physiological and/or cell-ecological conditions.

According to the first possibility, we should observe a lot of destroyed cells in the liver. It is not difficult to estimate the probability, P, with which microscopists can expect to observe the disintegrating nuclei in the liver if the decrease in number of diploid nuclei is due to the nuclear lysis. If μ denotes the mean disintegrating time of the nucleus,

$$P = \frac{\int_{-\mu}^{\mu} |dN_{di}|}{N} \approx \frac{2\mu \left|\frac{dN_{di}}{dt}\right|}{N}$$

where N is the number of total hepatocyte nuclei and dN_{di}/dt is the rate at which the diploid nuclei are changed during dt. Since the exact μ is not known in the present

TABLE VI

Calculated Probability, P, of Degenerating Nuclei of the Liver being Detected Microscopically

Body weight	P^* ‰
gm.	
25	28.2
40	37.6
50	23.1
70	8.9
90	2.9
110	1.2
140	0.38

* These values were approximately calculated with the assumption that the degenerating time is 1 day, which may be accepted as the minimum time.

experiment, the probability, P, can not directly be computed from the above formula. However, it has been known that it may take a day, at least, until a "dead" nucleus disappears in the liver tissue as the result of nuclear disintegration (43). Then the probability, P, should satisfy the following inequality,

$$P \geq \frac{2\left|\frac{dN_{di}}{dt}\right|}{N} \qquad (\because \mu \geq 1)$$

where the time scale is taken in days. Table VI shows the calculated lowest values of P at the several stages of growth, using the data of Fig. 4 and the data shown below about the age of experimental animals. If the diploid nuclei had disintegrated and then disappeared, these cells would have been observed with a probability not less than such values. Actually, however, with livers of young and adult rats, the damaged nuclei are scarcely ever observed microscopically. Also no evidence of DNA loss has been obtained by tracer experiments of these types of material (15,

21). Even if the multiplication of the tetraploid nuclei could be explained by the first assumption, the disappearance of diploid nuclei can certainly not be accounted for.

Can we now explain the decrease in diploid nuclei and the increase in tetraploid nuclei under the second possibility? To answer this we shall mathematically analyze the results of the present experiment.

According to the second possibility, it is feasible to assume (1) that the multiplication of nuclei involves the diploid nuclei only, and these nuclei never disintegrate and disappear from liver tissue, and (2) that tetraploid nuclei originate from the interphase diploid nuclei *directly*, *i.e.* without the intervening formation of a mitotic figure. Then the total number of the mitotic nuclei in a liver at a given instance, MN, is

$$MN = M_{di} \cdot N_{di} \tag{7}$$

where M and N indicate the observed fractional mitotic index and the total number, respectively, of parenchymal nuclei of a liver, and M_{di} and N_{di} indicate the fractional mitotic index of diploid nuclei alone and total number of diploid nuclei of the same liver.

Since the mitotic index of the diploid cell in 1000 diploid nuclei, M_{di}, can be calculated from the observed values using equation (7), we can obtain the following two equations:

$$-\frac{dN_{di}}{dt} = \frac{M_{di}(1 - 2\alpha)}{\tau} N_{di} \tag{8}$$

and

$$\frac{dN_{tet}}{dt} = 2\frac{M_{di}\alpha}{\tau} N_{di}, \tag{9}$$

but

$$\tfrac{1}{2} < \alpha < 1,$$

where α, N_{tet}, and τ indicate the probability of the newly arising tetraploid nuclei in the diploid nuclei divided through mitosis at a given instance, the total number of tetraploid nuclei of the liver and the mitotic time, respectively. In this case, α, τ, and M_{di} may be the function of time "t."

In general, the equation (8) is integrated to yield

$$N_{di} = A \cdot e^{-\int \frac{M_{di}(1-2\alpha)}{\tau} dt} \tag{10}$$

where A is a constant.

In the case of present studies, the body weights of normally growing rats are measured as the relative value of the growth time "t," since there is a proportional relationship between the growth time "t" in days and the body weight in gm. within the limit of about 25 gm. to 130 gm. of the body weight as shown in Fig. 6. According to this figure, the average increment of the body weight per day of rats, fed on the stock diet described above, is 3.1 gm. Then, the growth time "t," employed below, was calculated from the body weight and the daily increment, 3.1 gm. per day.

As shown in Fig. 7, we can fortunately obtain a straight line correlation between the growth time "t" and $\log N'_{tet}$ or $\log N'_{di}$ where $N'_{tet} = C - N_{tet}$, $N'_{di} = N_{di} - D$ and C and D are constants, respectively, by transformation of the coordinates. Furthermore, it should be noticed here that these two lines are parallel. But with young rats of 25 to 40 gm. body weight, no such parallel and straight lines are obtained. Therefore, even though M_{di}, α, and τ are the functions of "t," their product, $\frac{M_{di}(1 - 2\alpha)}{\tau}$, takes a constant value except during the early growth period.

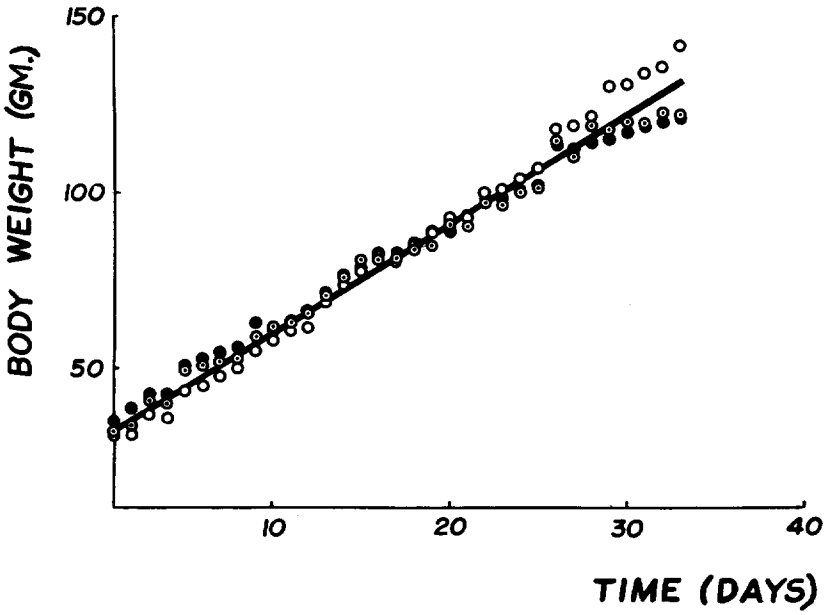

FIG. 6. Straight correlation between the body weight and the growth time in day (—●—●— and —⊙—⊙— ♀; —○—○— ♂).

This fact gives us the following formula instead of equation (10):

$$N'_{di} = A \cdot e^{-\frac{M_{di}(1-2\alpha)}{\tau}t} \tag{11}$$

Then, we can obtain the following equation from equations (9) and (11):

$$N_{tet} = C - B \cdot e^{-\frac{M_{di}(1-2\alpha)}{\tau}t} \tag{12}$$

where C and B are constants. Since $N'_{tet} = C - N_{tet}$,

$$N'_{tet} = B \cdot e^{-\frac{M_{di}(1-2\alpha)}{\tau}t}. \tag{13}$$

In equations (11) and (13), it should be noticed that if we plot $\log N'_{di}$ and $\log N'_{tet}$ in the ordinate, two equations (11) and (13) form two parallel straight lines.

Such a graphic parallelism is consistent with the observed result except during the early growth period. Accordingly, the appearance of the tetraploid nuclei can be explained by the hypothesis that tetraploid nuclei originate from the interphase diploid nuclei in normal polyploidization or duplication of DNA and the number of diploid nuclei consequently decreases. The ability of diploid nuclei to divide can be

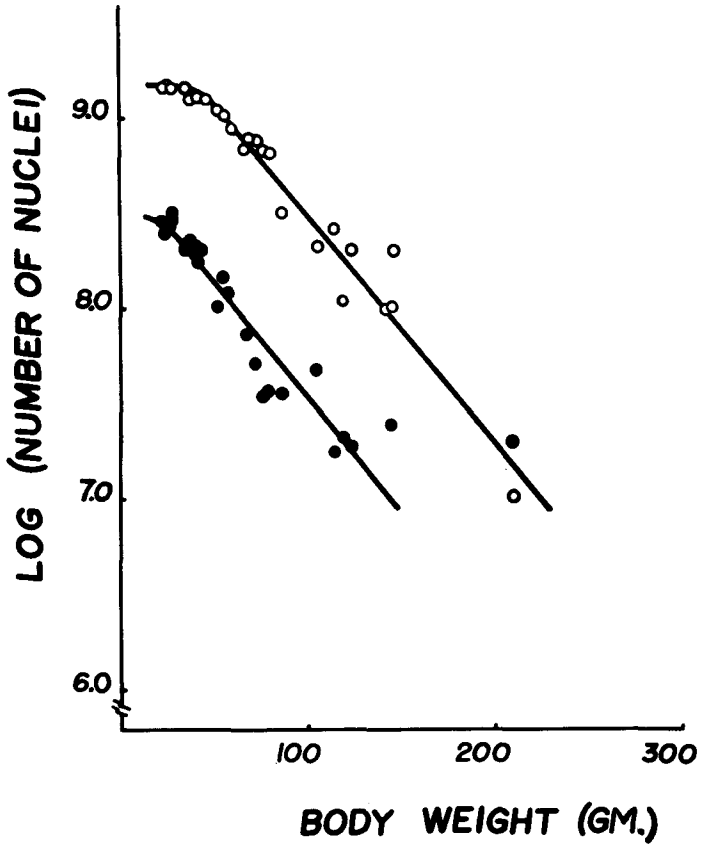

FIG. 7. Correlation between log N'_{tet} or log N'_{di} (see text) and growth as expressed by the body weight.

—●—●—: diploid nuclei.
—○—○—: tetraploid nuclei.

demonstrated with regenerating livers (37). Moreover, Bloch *et al.* have recently reported that the adult rat liver apparently contains a cell population consisting almost exclusively of group B cells which are thought to correspond to the heterosynthetic phase, while cells of group A are in the autosynthetic interphase among the diploid type having the ability to proliferate (5).

However, the process at the early growth stages cannot be explained by the above hypothesis. In cases of these stages, *i.e.* 25 gm. to 40 gm. of body weight, the graphi-

cal characterization shown in Fig. 7 indicates that $M_{di}(1 - 2\alpha)/\tau$ is the function of the growth time "t." As seen in Fig. 2, these stages correspond to the onset of polyploidization in the liver. It must be admitted, however, that the exact mechanism of the multiplication of hepatic cells at such young growth stages remains an open question.

M_{di}, α, and τ can be calculated from the observed data if the data to be used for this calculation are confined to the growth period of 40 gm. to 130 gm. of the body weight, because equations (11) and (13) satisfy the observed phenomenon within this period. Then, Figs. 8, 9, and 10 show the calculated ratio of mitotic diploid nuclei to total diploid nuclei, M_{di}, percentage of the polyploid nuclei arising from the diploid nuclei produced by mitosis, α, and the mitotic time, τ, from equations (7), (8), and (9).

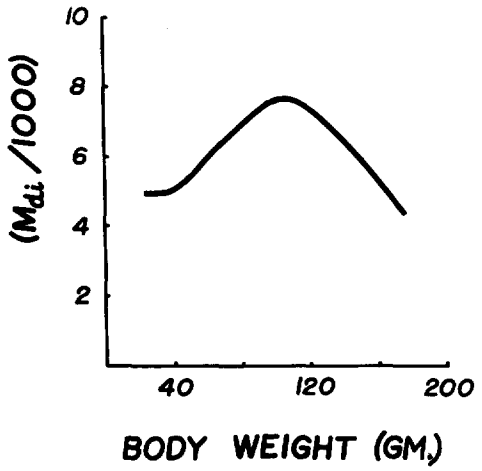

FIG. 8. Change in the calculated ratio of the mitotic diploid nuclei to the total diploid nuclei in postnatal growth as expressed by the body weight.

As shown in Fig. 8, M_{di} shows the maximum value at the stage of rats weighing 100 gm. to 110 gm. It does not follow, however, that the fraction of dividing diploid nuclei in total diploid nuclei is maximum at this stage, because M_{di} is the function of the mitotic time "τ" and furthermore, τ is not constant throughout the growth period. The mitotic time "τ" calculated with the present experimental results seems to agree with those from other sources (20, 29, 48, 52, 70). Furthermore, it is of biological interest that the mitotic time is dependent upon the physiological conditions of cells.

As described above, the value of M_{di} means the following:

$$M_{di} = \frac{\tau}{l} \tag{14}$$

where l denotes the mean generation time of the diploid nucleus. If the mean interphase time, l_i, is plotted against the body weight, the top line in Fig. 10 is obtained

which shows additional evidence pertinent to this discussion. As seen in this figure, it is suggested that the hepatic diploid nuclei of the rats weighing about 70 gm. are cell-physiologically and/or cell-ecologically destined to have a short generation time or be subjected to a rapid rearrangement for polyploidization.

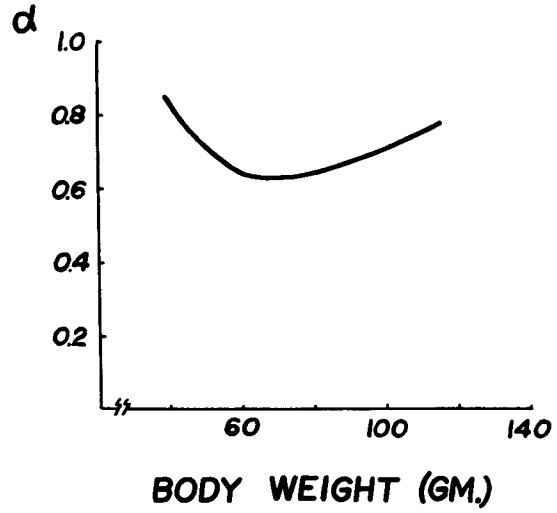

FIG. 9. Change in percentage of the polyploid nuclei arising from the diploid nuclei produced by mitosis in postnatal growth as expressed by the body weight.

Since $M_{di}(1 - 2\alpha)/\tau = k$ where k is a constant, we can obtain the following equation by substitution of equation (14) into it:

$$1 - 2\alpha = lk. \tag{15}$$

However, $l_i = l - \tau$, $l \gg \tau$. Therefore, the following equation is obtained:

$$1 - 2\alpha = l_i k. \tag{16}$$

This formula indicates that the potentiality for polyploidization of a nucleus is in direct proportion to the interphase time. Furthermore, such a finding may suggest that the polyploidization needs a sufficient time to arrange the mechanism for maintaining the higher amount of DNA in the nucleus. This suggestion may be very significant in considering the biological or genetical role of DNA in a cell.

As described above, it is, on the one hand, morphologically possible that polyploidization always follows abnormal mitosis. For example, polyploid cells in liver are said to arise through the mitosis of binucleated cells (30), fusion of two nuclei, or failure of meta- or anaphase chromosomes to separate (71). On the other hand, the hypothesis proposed here concerning tetraploidization in the liver assumes that tetraploid nuclei originate from diploid nuclei *directly*. This means that tetraploidization does not follow mitosis.

The rapid multiplication of parenchymal nuclei takes place in livers of rats

that are in the stage of remarkable polyploidization. Therefore, if polyploidization is assumed to occur in accordance with the first hypothesis mentioned above, a great number of the mitotic figures, some of which represent simple multiplication of nuclei and others polyploidizing nuclei, should be microscopically evident in such livers. In the case of such polyploidization, the prob-

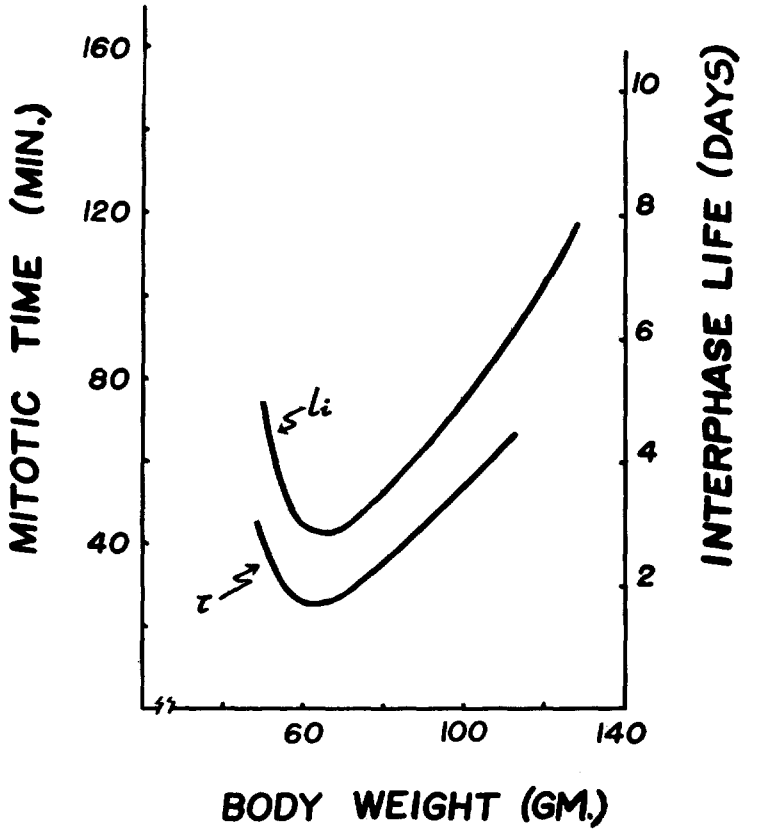

FIG. 10. Variation of the calculated mitotic time and the mean interphase time at the different stages of the postnatal growth as expressed by the body weight.

ability, M_p, of these nuclei, proceeding to polyploidization, being observed microscopically in the liver of the present experiment is

$$M_p = \frac{\int_{-\tau}^{\tau} dN_{tet}}{N} \approx \frac{2\tau \frac{dN_{tet}}{dt}}{N} \tag{17}$$

because the appearance of one polyploid cell follows one mitotic figure regardless of the form of the abnormal mitosis required to produce the polyploid cell described above.

If the values of τ estimated above are used in the formula (17), the mitotic figures leading to the polyploidization should be detected with the probability of M_p given in column 2 of Table VII, in order to account for the phenomenon of polyploidization in the normal liver by the first hypothesis. The mitotic indices to be expected here are listed in column 3 of Table VII. Contrary to this expectation, however, the actual mitotic indices observed experimentally are smaller than half of the expected values.

If it is again assumed, contrary to our hypothesis, that all of the mitotic figures observed microscopically in the present experiments are involved in the

TABLE VII

Calculated Probability, M_p, of the Microscopic Detection of Mitotic Figures for Tetraploidization of Liver Cell Nuclei

Body weight	M_p* (‰)	Total mitotic index to be expected by the hypothesis based on the abnormal mitosis (‰)	τ‡ to be expected by the hypothesis based on the abnormal mitosis
gm.			*min.*
40	11.2	14.0	26.7
50	6.4	8.5	12.9
70	2.6	3.8	11.7
90	1.7	2.5	20.3
110	1.2	1.8	29.2
140	0.79	1.2	50.0
170	0.51	0.76	75.0

* These values were approximately calculated with values of τ which were obtained with equations (8) and (9).

‡ These values were approximately calculated with the assumption that all the observed mitotic figures are to lead the tetraploidization. Therefore, these show maximum values of the mitotic time under the condition that a tetraploidization follows a mitosis, and in the actual case of such condition, values of the mitotic time may be remarkably less than such values.

polyploidization, the values of τ listed in column 4 of Table VII can be approximately obtained from the equation (17). However, if this mitotic mechanism of polyploidization were the true one, the frequency of mitosis leading to polyploidization should be smaller than that of the total mitotic figures observed, because a rapid multiplication of hepatic cell nuclei occurs in the livers of young rats. Thus it is quite natural that actual values of τ to be obtained under the assumption of such an hypothesis should be markedly *less* than that shown in column 4 of Table VII. As long as we adhere to the mitosis-polyploidization hypothesis, it follows that a remarkably short mitotic time would be required for the mitosis leading to the polyploidization. It seems more reasonable to suppose that values of τ calculated from equations (8) and (9) are of

approximately the correct magnitude while that derived from the mitotic hypothesis is inadequate.

These findings are taken to indicate that the assumption that a mitosis is required for tetraploidization of a diploid nucleus cannot explain the results of the present experiments. The word "tetraploidization" used in this paper means the duplication of DNA content of the nucleus. Therefore, it is concluded that *the synthesis of DNA is not necessarily followed by the mitosis.* It has recently been reported that colchicine is not directly concerned with DNA synthesis in spite of blocking mitosis at the metaphase, (see the work of Bloch on the occurrence of polyploidy in the colchicine-treated fibroblast (4)). Since it has also been reported in various experiments that the synthesis of DNA takes place in the interphase period (1, 12, 19, 62, 64, 69), it may be assumed that most of the liver cells in adult rats synthesize new molecules of DNA in the interphase period, and thereafter keep themselves continuously in the interphase condition without the formation of *visible chromosomes.* This consideration leads to the idea that *the mechanism of the DNA synthesis is not directly linked to the mitotic mechanism.* There are several reports that nuclear division other than mitosis is often encountered in animal tissues (38, 41, 60), but, notwithstanding such nuclear divisions, DNA is undoubtedly synthesized (38). It seems, then, possible that there is a mechanism of DNA synthesis categorically separated from the nuclear division in the cell and, that after synthesizing DNA, one of at least three different events may occur: the nucleus may enter a mitosis, it can commence division of other types, or remain as an interphase nucleus. Cell-physiological and/or cell-ecological conditions might decide which one of these mechanisms shall prevail.

The reproduction of the nuclear content that is not followed by chromosome movement and cytoplasmic division has been observed and called endomitosis (16, 48). Such a process of endomitosis may occur with a typical prophase. According to the results obtained in the present investigation, polyploidization of liver cells during the postnatal growth of rats cannot be explained by such a mechanism, because endomitosis, in the sense described above, is accompanied by the formation of visible chromosomes. There is, however, another case of endomitosis where the visible change in the microscopical structure of the interphase nucleus is not observed, although the nuclear content is reproduced. The second possible mechanism of polyploidization described above corresponds to this latter mechanism of endomitosis, where the reproduction of DNA, but not of the morphological unit, takes place in a nucleus.

With rats weighing 150 gm. and upwards, no remarkable alteration in the nuclear population in the liver is observed with respect to ploidy. Such a fact suggests that the cellular population reaches a cell-ecological equilibrium state among nuclei of different ploidy classes, and livers of rats weighing more than 150 gm. are in a stable state in respect to the ploidy composition of the cell

population. Though this composition of the cell population is rather rigid in normal rats, malignant tissues may show variability in this point as reported by Bader (3), Swift (22, 63), and others (26, 45). It should, moreover, be noted that in such a population, composition and multiplication of each polyploid class are controlled by the growth state of animals, but not by the age. This finding shows the close similarity between the control of these properties and that of DNA content of individual cell nuclei.

Lecomte and de Smul, Rasch *et al.* have reported that polyploid frequency is markedly altered by dietary conditions (24, 46, 23), *e.g.* by protein-low diets. The former workers especially, have tested the effect of dietary conditions with young rats (body weight of 60 to 65 gm.) and have reported that the tetraploid and octoploid frequencies are shifted by low-protein diet. Their results are, in general, inconsistent with the present results, although there are some differences in the experimental methods in the two laboratories. However, according to the results of Sibatani *et al.* (50), average DNA content per nucleus, as determined biochemically, of growth-depressed rat livers shows no difference in comparison with control livers taken from rats of the same body weight. This finding indicates that the cellular population in the liver scarcely changes during the depression of growth of young rats. Since it has been found that the DNA content in an individual cell nucleus of growth-depressed rat livers does not differ from that of the suitable control animals, the average value of DNA content per nucleus as determined biochemically indicates a relative measure of the development of polyploid cells. The reason for the discrepancy between Lecomte and de Smul's and our results still remains an open question.

On the contrary, the cell population of the liver in starved rats does not change in spite of the reduction in their body weight (unpublished data (39)). This finding suggests that the control of cell population is a rigid one in rat livers and that the population once established in the liver is no longer alterable unless some special condition, for example, regeneration, is induced.

SUMMARY

1. DNA contents of the individual parenchymal nuclei of rat livers during postnatal growth were estimated by microspectrophotometric apparatus, and different ploidy classes of nuclei were classified by their DNA contents. With the same material the total number of parenchymal nuclei in the liver was counted microscopically.

2. If the DNA content of nuclei encountered most frequently in several tissues represents the diploid class, the ploidy classes of the rat liver cell nuclei correspond to di-, tri-, tetra-, and octoploid, with the di- and tetraploid ones predominating considerably.

3. In suckling rats (below 25 gm. of body weight) the liver parenchyma is

composed almost exclusively of cells with diploid nuclei, whereas in young rats (above 80 gm.), of tetraploid nuclei. In the growth stage between 25 and 80 gm., there is a remarkable replacement of the diploid nuclei by the tetraploid ones. However, in the liver of adult rats weighing more than 150 gm., any increase or decrease in the frequency of diploid and tetraploid nuclei is hardly observable. In such rats, the nuclear population of the liver parenchyma seems to reach a cell-ecological equilibrium which is considered to be a stable one.

4. It is shown that such nuclear populations and the total number of nuclei in a liver are controlled by the growth state, and not by the age.

5. The decrease in the total number of diploid nuclei and the increase in tetraploid nuclei in the growing livers of rats weighing from 40 up to 130 gm. can both be explained by the hypothesis that the tetraploid nuclei originate from the interphase diploid nuclei without involving mitosis. This hypothesis implies that mitosis is confined to the reproduction of diploid cells alone.

6. It is suggested that, in general, the synthesis of DNA does not necessarily result in the formation of visible mitotic chromosomes.

7. Mitotic time and generation time of diploid nuclei and the percentage of the tetraploidization from diploid nuclei are calculated and discussed.

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