CELL SIZE, NUCLEAR CONTENT, AND THE DEVELOPMENT OF POLYPLOIDY IN THE MAMMALIAN LIVER

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The occurrence of polyploidy in the parenchymal cells of the livers of adult mammals has long been recognized, but its physiological significance has remained a mystery. At present there is no explanation, on a biochemical basis, of how polyploidy develops or of why.

A large body of evidence relating to the levels of enzymes and other proteins in tissues of individuals heterozygous for various protein deficiency states suggests that the amounts of proteins made are proportional to the "dosage" or number of structural genes which govern their synthesis.[1] If this evidence is translated to the individual cells of the mammalian liver, it would be expected, for example, that a tetraploid cell would make twice as much of gene-dosage-dependent materials as would a diploid cell. Since the liver of a single adult mammal may be composed of parenchymal cells of several orders of ploidy, the only way in which these different cells would be metabolically comparable, unless gene dosage did not apply, would be for their volumes to be directly proportional to their ploidies. In this way, the concentrations of cytoplasmic constituents would remain constant. The present investigation was undertaken to determine whether cell volume is directly proportional to ploidy.

Materials and Methods.—Animals and tissues: The major part of the work was carried out with albino Swiss mice bred at the National Institutes of Health. Livers were also obtained from normal and obese (ob/ob) C57 BL/6J mice, Sprague-Dawley rats, and Fisher rats bearing transplantable hormone-secreting sarcomas. Human liver was obtained at autopsy within 6 hr of death. In no case was the liver tissue affected by the pathologic process that caused death.

Preparation of parenchymal liver cells: Livers from all sources were separated into isolated single cells by the method of Rappaport and Howze.[2] This method has the advantage that prior perfusion of the organ, as with many of the techniques employing citrate, is not required. The livers were minced with razor blades and then stirred for 1 hr at 25° in a "dissociating" solution of 3×10^{-3} *M* sodium tetraphenylboron (TPB) in a 0.005 *M* sodium phosphate buffer, pH 7.8, containing 0.05 *M* sucrose and 0.14 *M* NaCl. The cell suspensions were then decanted and washed twice with cold dissociating solution without TPB.

For spectrophotometric work, the washed cells were suspended in the dissociating solution without TPB, and 1 ml of cell suspension (15,000/ml) was collected with gentle suction on a 19 × 42-mm Millipore filter with a pore size of 5.0 μ (SMWPO19OR). The filters were fixed in absolute ethanol, clipped onto 1 × 3-in. glass slides air-dried, and stained for DNA by the Feulgen method with a counterstain of Fast Green FCF.[3] After staining, the filters were dehydrated in ethanol, cleared in xylene, and mounted in Permount (Fisher Scientific Co.). Quantitative microspectrophotometry of the Feulgen-stained nuclei was carried out at 540 mμ with a Barr and Stroud GN2 integrating microspectrophotometer. The readings for individual nuclei clustered tightly about modal values that were in the ratio of 2:3.7–3.9:7.0–7.4. The departure of these ratios from the expected 2:4:8 appears to be the results of systematic errors introduced by the measurement of objects of different sizes in an aperture of fixed size.

Determination of cell size: Because of the difficulty in accurately measuring the volumes of single cells, cell size was determined from cell areas. The cells were suspended in the dissociating solution without TPB, and trypan blue at a final concentration of 1:1000 was added to stain the nuclei. To prevent distortion of their shapes, the cells were placed in a hemocytometer chamber

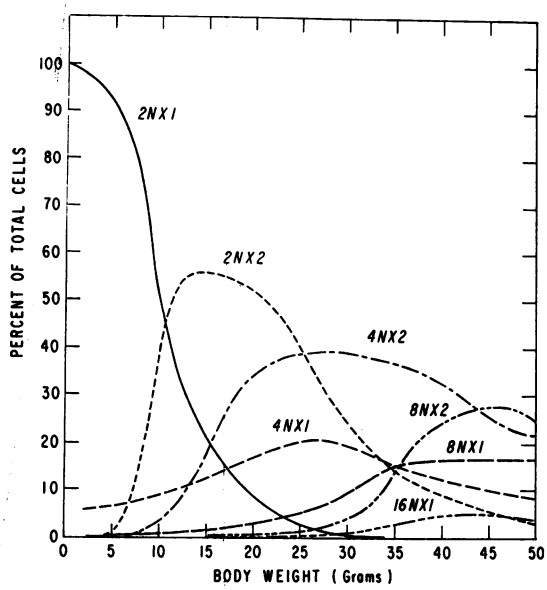

FIG. 1.—Relationship of the frequencies of various classes of parenchymal liver cells to body weight in the Swiss mouse.

covered with a thin cover glass. The images of individual cells (cells in clumps were not used) were projected onto paper by a prism projector at final magnification of 880 diameters, and the outline of each cell was traced. The nuclear diameter(s) was measured with an ocular micrometer to determine nuclear ploidy. The diameters clustered about means that were approximately in the ratios (1.26:1) expected for twofold differences in volume, and there was no overlap between groups.

The tracings of the cells were cut out and grouped according to cell ploidy and number of nuclei. Each group of tracings was weighed and the mean areas per cell were calculated using standards representing known areas. In most cases, the mean areas were determined for groups of 25 or more cells, and duplicate determinations agreed within 5%. The cell and nuclear sizes of the cells in suspension remained constant for several hours at 0°.

Results.—Development of the liver in the Swiss mouse: Over the range of body weight of 2.5–50 gm, the weight of the liver of the Swiss mouse is proportional to body weight and comprises about 5.5 per cent of body weight. The distribution of parenchymal cells in the various ploidy classes is illustrated in Figure 1. At birth, the cell population is almost completely 2N, but coincident with the time of weaning there is the appearance first of binucleate cells with 2N nuclei (cell ploidy 4N) and, somewhat later, of binucleate cells with 4N nuclei and mononucleate cells with 8N nuclei (cell ploidy 8N). In animals weighing over 25 gm, there is an increase in the proportions of cells of even higher ploidies, and nuclei as large as 32N are seen in the largest animals.

Contrary to earlier reports,[4,5] no evidence was found for cells or nuclei of intermediate ploidies (3N, 6N, etc.) or for a continuum of DNA contents and nuclear sizes in any cells from mouse, rat, or man.

The distributions of cells into the various ploidy classes may be represented by the calculated value of mean ploidy per cell. When this value is plotted as a function of liver weight (Fig. 2), a linear relationship is apparent, with the relative increase in mean ploidy per cell being almost the same as the relative increase in liver weight. The highest mean cell ploidies (up to 14.6N) were seen in the livers of female exbreeders.

Concomitant with the increase in mean cell ploidy, there is an increase in mean nuclear ploidy (Fig. 3). The relationship between the two is biphasic. The first limb corresponds to the increase in cell ploidy by the formation of binucleate cells ($2N \times 2 = 4N$) and a few mononucleate 4N cells. This does not produce a large increase in mean nuclear ploidy. The second limb, which extrapolates back to the

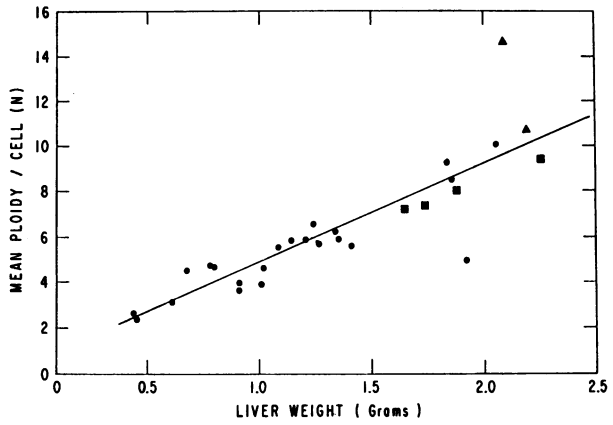

FIG. 2.—Relationship of mean ploidy per cell to liver weight. Circles represent female mice; squares, male mice; triangles, female exbreeders. Line calculated by method of least squares.

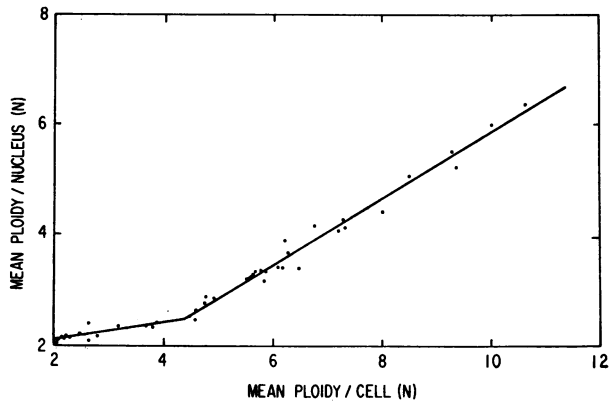

FIG. 3.—Relationship of mean ploidy per nucleus to mean ploidy per cell. The first part of the curve is largely attributable to the formation of 2N × 2 cells. The second part indicates an increase in nuclear ploidy with the fraction of binucleate cells remaining constant.

true origin of the graph, corresponds to an increase in cell ploidy by an increase in nuclear ploidy with little change in the over-all proportions of mononucleate and binucleate cells.

The size of parenchymal liver cells: Mean cell areas were determined for cells of various ploidies in Swiss mice ranging in weight from 2.5 to 50 gm, and are shown in Figure 4. For animals weighing more than 19 gm, there is no apparent dependence of cell size on animal size, but for smaller animals, the cells with ploidies of 4N or greater were somewhat smaller than those of the larger animals. In these determinations, mononucleate and binucleate cells of the same total ploidy are grouped together, since the mean areas of the two types of cells were found to be the same.

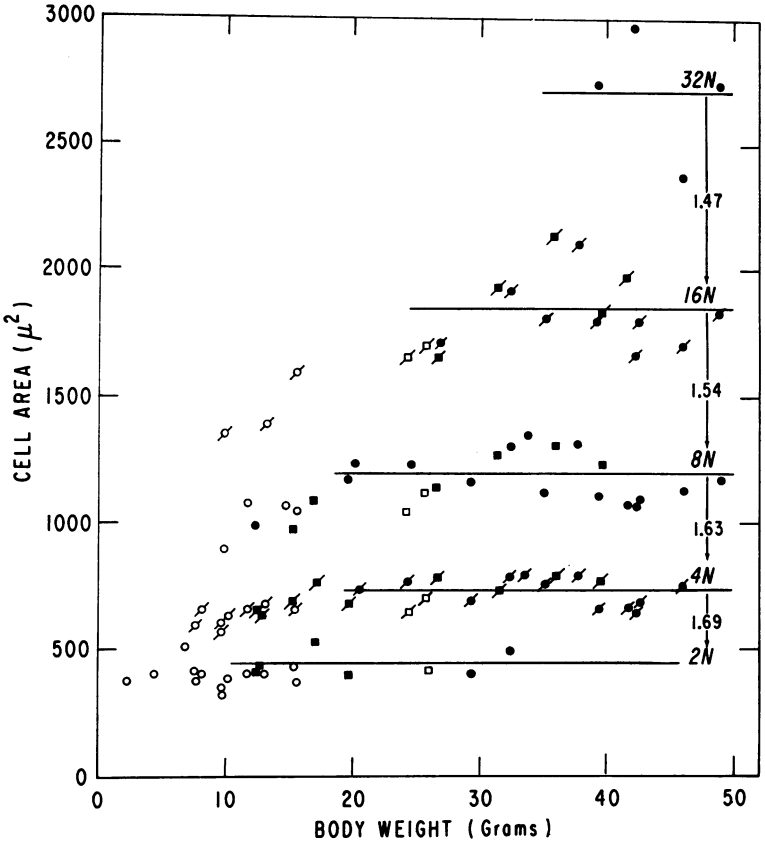

FIG. 4.—Relationship of cell areas to body weight in the Swiss mouse. The open figures indicate fasted animals; closed figures, fed animals; circles, females; squares, males. The points of the 4N and 16N cells have diagonal lines through them to distinguish them from the others. Except for the 2N cells, the lines representing mean cell areas are based on values for fed animals weighing more than 19 gm. The figures on the right represent the ratios of the mean cell areas.

To illustrate the relative differences in the areas of cells and nuclei, photomicrographs of cells of various ploidies are shown in Figure 5.

Relationship of cell size to nuclear ploidy: Two assumptions underlie the comparison of cell sizes on the basis of mean areas. One is that the mean area will accurately represent the size of a group of cells, even though the size of any single cell would not be so represented. The other is that there is a constant geometry for the cells of different ploidies, thereby allowing the comparision of mean areas. If these assumptions are valid, then for a twofold difference in volume, any common *linear* measurements should be in the ratio of $2^{1/3}$:1 (or 1.26:1), and any common *area* measurements in the ratio of $2^{2/3}$:1 (or 1.59:1).

Since the ratios of cell areas shown in Figure 4 were derived from the means of areas of cells from many different animals, it was felt that more accurate results could be obtained by making the comparisons for groups of cells within single animals. Cell suspensions from a total of 53 individuals (mice, rats, and deceased

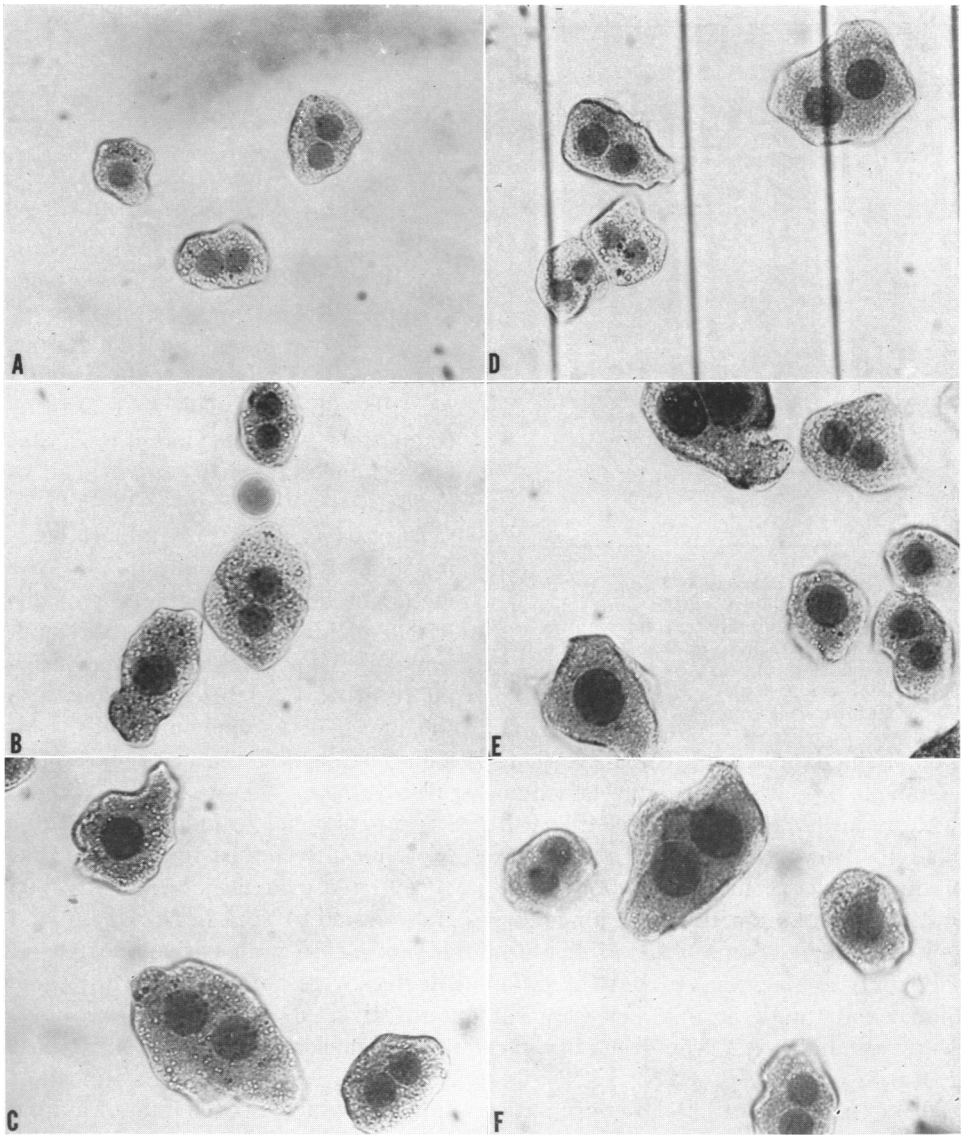

FIG. 5.—Photomicrographs of isolated mouse liver cells. All cells were photographed in a hemocytometer chamber through a 40× objective. Original magnification 700×. (*A*) 2N × 1 and 2N × 2 cells from female weanling; (*B*) 2N × 2, 4N × 2, and 8N × 1 cells from female exbreeder; (*C*) 4N × 2, 8N × 1, and 8N × 2 cells from same animal as in (*B*); (*D*) 2N × 2, 4N × 2, and 8N × 2 cells from mature male; (*E*) 4N × 1, 4N × 2, 8N × 1, 16N × 1, and 16N × 2 (in part) cells from the same animal as in (*D*); (*F*) 4N × 2, 8N × 1, 16N × 2 cells from same animal as in (*D*). Because of differences in the state of nutrition, the cell sizes in (*B*) and (*C*) are slightly different from those in (*D*), (*E*), and (*F*). The hemocytometer rulings in (*D*) are 50 μ apart.

human beings) were examined, and the results are summarized in Figure 6. The mean ratios are 1.68 ± 0.12, 1.61 ± 0.06, 1.53 ± 0.04, and 1.58 ± 0.20 for the areas of 4N:2N, 8N:4N, 16N:8N, and 32N:16N cells, respectively. The over-all

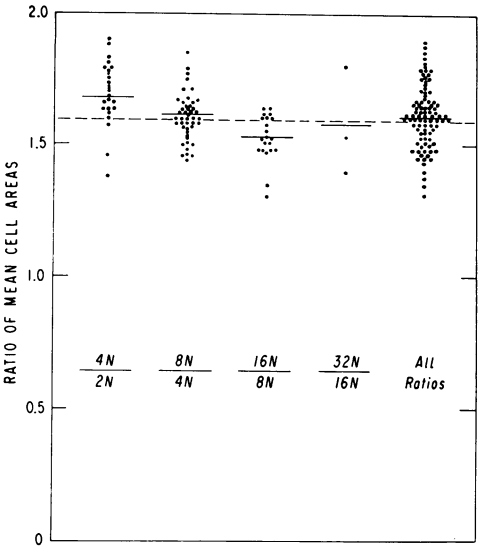

FIG. 6.—Ratios of mean cell areas for twofold differences in cell ploidy. Each point represents a separate determination carried out on cells from a mouse, rat, or human liver. The horizontal dashed line indicates a ratio of 1.59, the value theoretically expected for a twofold difference in mean cell volumes.

mean for 97 separate ratios is 1.61 ± 0.11. This corresponds to a ratio of volumes of 2.05 for cells differing twofold in ploidy. These ratios are not affected by variations in diet, the age, sex, or size of the animal, or by the presence of tumors.

The slight fall in ratios from 1.68 for 4N:2N to 1.53 for 16N:8N cells suggests that cell morphology may not remain strictly constant over the various size classes. Nevertheless, these values are still sufficiently close to 1.59 to be compatible with the hypothesis that cell volume is directly proportional to ploidy.

Discussion.—The only report bearing directly on the question of the metabolic behavior of the cells of different ploidies is that of Mortreuil-Langlois[6] who investigated the incorporation of C^{14}-labeled adenine into the nuclei of parenchymal cells. Although the data are quite scanty, there is a suggestion that the incorporation of adenine is proportional to nuclear volume or ploidy.

Many authors have *assumed* that cell size is proportional to nuclear volume or ploidy.[7] However, Yčas and collaborators[8] were recently forced to conclude that in animal cells accurate data were "rare." They cited evidence obtained in 1905 by Boveri working with sea urchin embryos and referred to work in *Drosophila* and ascites tumors. Some observations on amphibian larvae with various degrees of polyploidy are suggestive,[9] but the different degrees were not present in the same animal and the determinations were not quantitative. Data derived from plant tissues also indicate a proportionality of cell size and nuclear ploidy,[9, 10] and in yeast the ratios of volumes of 2N to 1N cells ranged from 1.91 to 3.15, with a mean of 2.60.[11] On the other hand, determinations in the cells of the epithelium of human amnia do not support the notion of a fixed relationship of cell size to nuclear ploidy. For a doubling in DNA content, the increase in both nuclear and cell volumes was considerably less than twofold.[12]

There is no *a priori* reason, except for the general assumptions about gene dosage discussed in the introduction, to assume that cell volume and nuclear ploidy need be directly proportional. In a sense, then, the direct test of this relationship becomes a test of the validity of the gene dosage concept in polyploid cells, and the results presented above are fully in accord with it. These data must, however, be considered only as being compatible with the operation of gene dosage; they do not prove it. Only the direct determination of such factors as the rates of RNA and protein synthesis and of the levels of specific enzymes in the cells of various ploidy classes within the same animal can do so.

If, for the present, the gene dosage concept is accepted in so far as polyploid liver cells are concerned, it is possible to analyze some of the consequences of the process of polyploidization. The concentrations of primary gene-dosage-dependent cell constituents will not differ among cells of different ploidies. Therefore, the liver parenchyma will be metabolically uniform, and the regional variations related to lobular architecture cannot be attributed to the variation in cell size. Furthermore, in view of the strict proportionality of cell size and of the constancy (or slight increase) in cell volume with animal growth, polyploidization by itself cannot improve in any obvious way the "efficiency"[13] of the cells.

There is, however, one consequence that will result from the formation of polyploid cells and that is a relative *decrease* in the total surface area of the parenchymal cells as the liver grows. Since surface area is proportional to $(\text{volume})^{2/3}$, the replacement of diploid cells by an equal volume of tetraploid cells will result in a decrease of total surface area by $(2 \times 1) - (1 \times 1.59)/2$, or 20.5 per cent. For a liver composed of 8N cells, the relative decrease in area, again in comparison to an equal volume of 2N cells, would be 36.7 per cent. This result appears paradoxical in that it is generally assumed that a maximization of surface area is desirable from the point of view of the influx and efflux of metabolites, but it is possible that there may be some physiological advantage to the liver as a whole to reduce the total surface area of the cells.

It has been reported that, after weaning, the number of parenchymal cells in the liver of mice remains constant[7] or even decreases,[14] and that there is little mitotic activity or "reparative" growth in the livers of mature animals.[15] If the increase in liver weight represents a commensurate increase in parenchymal cell mass, then it would be predicted that in the absence of cell division the mean ploidy per cell would increase proportionately with body or liver weight. The increase in cell mass could then be attributed solely to polyploidization of the cells, by the creation of binucleate from mononucleate cells and/or the increase in nuclear ploidy, as has been postulated by Helweg-Larsen[7] and Swartz.[16]

To test this hypothesis, the mean ploidy per cell was plotted as a function of liver weight (Fig. 2), and for a twofold increase in liver weight there was a 1.7- to 1.9-fold increase in mean cell ploidy. The fact that the increase in cell ploidy was not exactly proportional to the increase in liver weight indicates that the hypothesis of growth purely by polyploidization must be modified. One explanation is that a certain number of cells divide after nuclear replication so that daughter cells of the same ploidy as the original cells are produced. For this group of cells, the increase in cell mass would not be accompanied by a proportional increase in mean ploidy per cell. Another possibility is that polyploidization of the *nuclei* takes place by a mechanism such as that proposed for rat liver by Nadal and Zajdela.[17] They suggest that a binucleate cell divides to produce two mononucleate cells, each with a nucleus having twice the ploidy of the original nuclei. By this process, cell mass and, usually, nuclear ploidy increase more than does the ploidy per cell. Although this could occur in the Swiss mouse, it cannot represent the major mode of nuclear polyploidization. For the proportion of binucleate cells to remain constant (Fig. 3), most of the newly formed mononucleate cells would have to become binucleate. However, this would result in a large disproportion between

the increase in liver mass and the increase in cell ploidy, thereby violating the results shown in Figure 2.

The considerations just discussed also mitigate against the extension to the Swiss mouse of the proposal based on work in rats[18, 19] that liver growth occurs primarily from the repeated division of diploid cells. In the rat, nuclear ploidies above 4N are quite rare[17, 20] except in the largest animals, and the frequency of binucleate cells in the mature animal is relatively low (30–40%).[21] In the Swiss mouse, on the other hand, 8N nuclei are not uncommon, and binucleate cells predominate at all times after weaning. The development of a large population of 8N and 8N × 2 cells cannot be attributed to the replication of diploid cells.

Summary.—Isolated parenchymal cells were prepared from livers of mice, rats, and human beings. The sizes of cells of various ploidy classes were determined, and cell size was found to be directly proportional to cell ploidy. These results are compatible with the operation of gene dosage in the control of the rates of synthesis of cell constituents in polyploid liver cells.

The growth of the liver of the Swiss mouse appears to have three major phases: the multiplication of diploid cells; the formation of a large population of binucleate cells with diploid nuclei; and, finally, an increase in cell size, principally by polyploidization of the nuclei, with the proportions of mononucleate and binucleate cells remaining constant. No obvious advantage, except possibly a reduction in total cell surface area, accrues to the animal as a result of the process of cell polyploidization.

Note added in proof: Shea and Leblond[22] have recently reported that the number of nucleoli per mouse liver cell nucleus has a mode of 3 and a maximum of 6 in 2N nuclei and a mode of 6 and a maximum of 11 in 4N nuclei. These data indicate that the number of nucleoli and presumably their functional capacity are proportional to the ploidy of the nucleus.

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