cin. Thereafter the dose was increased to 0.1 mg. One or more eggs in which death of the embryo took place were used as source of material for the next passage, and a control group of eggs (*i.e.*, without aureomycin) was also injected. At the end of the experiment residual virus activity was tested for by mouse as well as egg inoculation.

Altogether 12 experiments similar to that outlined in Table II have been performed, using the various dosages of aureomycin indicated above, and the results in every one have been almost identical. The virus seemed to survive in attenuated form for several passages, and then to disappear. For example, in the experiment outlined, there appeared to be little virus activity remaining after the fifth passage, and at the time of the ninth passage 2 successive attempts to demonstrate virus activity were completely negative.

Discussion and conclusions. The purpose of these experiments was to find out whether the phenomenon of acquired resistance could be demonstrated in the case of a filterable virus to the newer antibiotics. The virus of feline pneumonitis was employed, and the two antibiotics were terramycin and aureomycin. These two were used in subcurative dosages in mice following intranasal inoculation in one series of experiments, and aureomycin was studied in 12 experiments in varying dosages in embryonated eggs. In order to enhance the likelihood of the emergence of a resistant mutant, large inocula of virus were employed. Under the conditions of our experiments no evidence whatsoever of resistance could be demonstrated, but an interesting phenomenon was observed. In both the mouse series and the egg series virus seemed to persist through several passages in the presence of antibiotic in diminished amounts. Then, with no increase in the dosage of antibiotic, it died out. The significance of this finding is obscure. That it might be due to an actual increase in sensitivity of the virus to antibiotic is conceivable, although such an hypothesis is not proven by the data presented here.

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Cell Proliferation and Changes in the Large Granule Fraction During Hepatic Carcinogenesis.* (18805)

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Little quantitative study has been made of cell division during carcinogenesis, and in the few cases reported, mainly of skin carcinogenesis(1), estimates of cell proliferation were based on observed changes in the mitotic index. Such estimates are subject to error due to changes in the duration of the various phases of mitosis. An extreme example of such inaccuracy is given by the early impression that the main effect of colchicine is to stimulate mitosis, while later investigations showed its main effect to be the inhibition of cell division in the metaphase(2). The liver offers advantages for the study of cell proliferation in that it is possible to calculate the total number of cells present from the number of cells per g of tissue, a procedure introduced by Brues, Drury, and Brues(3) for the estimation of replacement of liver tissue during regeneration. Price, Miller, and Miller(4,5) have demonstrated a striking depression of the protein and pentosenucleic

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2. Krythe, J. M., and Wellensiek, S. J., *Bibl. Genet.,* 1942, v14, 1.

3. Brues, A. M., Drury, D. R., and Brues, M. C., *Arch. Path.,* 1936, v22, 658.

4. Price, J. M., Miller, E. C., Miller, J. A., and Weber, G. M., *Cancer Research,* 1949, v9, 398.

acid content of the large granule fraction in rat livers undergoing carcinogenesis by a variety of azo dyes. In almost all cases they analyzed such livers after 4 weeks of dye-feeding. Comparison of such dye-fed livers with each other and with normal livers was based on wet weight of tissue. More recently, the importance of the cell as a unit in the biochemical comparison of tissues has been emphasized by Price and Laird(6) and independently by Davidson and Leslie(7).

The present study was undertaken to follow in detail the proliferative response of the hepatic tissue and the sequence of changes undergone by the large granule fraction of the average liver cell of rats fed 3'-methyl-4-dimethylaminoazobenzene, during the early period of dye-feeding not previously reported. The significance of this period was emphasized by the finding that the depletion of the large granule fraction of the average liver cell was more extensive after 4 weeks of dye-feeding (data of Price, Miller, and Miller†) than it was after 4 months of dye-feeding (unpublished data of the present author). Nuclear counts on the liver homogenates were used not only to express the analytical results in terms of the average liver cell, but also to determine the total number of cells in the liver, thus permitting for the first time a quantitative description of the over-all proliferative response of a tissue undergoing malignant change.

Material and methods. The animals used were male albino rats of the Sprague-Dawley strain‡§ weighing about 200 g at the start of dye-feeding. They were fed *ad libitum* a semisynthetic diet‖ to which 3'-methyl-4-dimethylaminoazobenzene was added in the concentration of 0.058%. They were killed with ether at intervals of 7, 11, 14, 16, 18, 20, 22, 24, 26, 28, and 32 days after the initiation of dye-feeding. Two groups of controls were also studied: an initial control on a grain diet, and one after 30 days on the basal synthetic diet. For each analysis, the livers of 3 rats were pooled after perfusion with ice-cold 0.14 M NaCl, and squeezed through a plastic tissue mincer to remove as much of the connective tissue as possible(8). The liver mince was then homogenized by the technic previously described(8). The cellular particles were separated by centrifugation according to the method reported from this laboratory(9), except that the nuclei were washed three times. Only the large granule fraction was saved for analysis. Two samples of the whole homogenate were suspended in 3% acetic acid, and the nuclei were counted in a hemocytometer. By counting a minimum of 800 nuclei, duplicate counts were found to agree within 5%. From these counts the numbers of nuclei per g and per whole liver were calculated. To determine the nuclei per whole liver, the number per g was multiplied by the fresh weight of the liver in g, on the assumption that the cells collected after passage through the mincer (essentially the parenchymal cells) constitute the entire weight of the liver. When the weight of the mince was compared with that of the liver before mincing, it was found that the mince constitutes more than 90% of the fresh liver, and therefore little error is introduced by this assumption. The validity of the concept of the "average cell" has been discussed previously with respect to the pres-

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† Recalculated on a per cell basis, assuming a desoxypentosenucleic acid content per nucleus of 10.0 x 10^{-12} g, the value customarily found in this laboratory.

‡ Obtained from the Holtzman Rat Co., Madison, Wis.

§ The author is indebted to J. M. Price, J. W. Harman, E. C. Miller, and J. A. Miller for samples of the livers from the rats used by them in their histological study of carcinogenesis. *Cancer Research,* July, 1951.

‖ Casein 24%; vitab 2%; salts 4%; glucose monohydrate 65%; corn oil (containing 1 part in 600 of halibut liver oil) 5%; riboflavin to bring the total content in the diet to 1.5 mg/kg.

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TABLE I. Numbers of Nuclei and Avg Cell Wt in Livers of Rats at Various Times After Feeding of 3'methyl-4-dimethylaminoazobenzene Was Initiated.

Exper. group, days	Avg liver wt (g)	Nuclei/g of liver $\times 10^6$	Avg cell wt ($g \times 10^{-9}$)	Total nuclei/ liver $\times 10^6$
Initial control	12	181	5.5	2167
7	9.5	194	5.2	1833
11	9.5	188	5.3	1780
14	12	138	7.2	1653
16	9.9	229	4.4	2262
18	9.9	229	4.4	2262
20	9.9	223	4.5	2190
22	10.2	220	4.5	2230
24	9.7	229	4.4	2230
26	8.2	226	4.4	1847
28	9.7	329	3	3200
32	8.9	315	3.2	2800
Basal diet control (30 days)	11.4	171	5.9	1950

In each case 3 livers were pooled for homogenization and analysis.

ence of binucleate and polyploid cells in the rat liver(6). Protein-nitrogen by a nesslerization procedure(10), and pentosenucleic and desoxypentosenucleic acid by Schneider's method(11) were determined on the large granule fraction, and the analytical values expressed in terms of the average cell.

Results. The number of nuclei in the whole liver remained about 2000 x 10^6 throughout the first 26 days of dye-feeding, as shown in Table I, but between the 26th and 28th days there was a sudden increase of 50% in the total number of liver nuclei.

The number of nuclei per g of liver, however, rose early in dye-feeding, leveled off during the third and fourth weeks at about 226 x 10^6 per g, and then increased abruptly by 50% between the 26th and 28th days. Thus, the weight of the average cell decreased soon after the initiation of dye-feeding and leveled off during the third and fourth weeks at about 4.4 x 10^{-9} g. Between the 26th and 28th days the average cell weight again suddenly decreased to about ⅔ of its previous value, or to about ½ of the cell weight of the normal controls. This abrupt reduction in cell size corresponded precisely in time to the increase in the total number of cells noted above.

A proportionate decrease in the pentosenucleic acid and the protein-nitrogen of the large granule fraction of the average cell occurred throughout the experimental period (Table II). This decrease was at first gradual, but coincident with the increase in cell number and decrease in cell weight, a further abrupt halving occurred, with the result that the 28- and 32-day values were about the same as those recalculated from Price, Miller, and Miller, as given in Table II, and lower than those found for tumor-bearing dye-fed livers.

No appreciable desoxypentosenucleic acid was found in this fraction in any of the analyses carried out.

Discussion. During and for 4 days after the cell division which occurred between the 26th and 28th days, there must have been complete failure to achieve net synthesis of

TABLE II. Pentosenucleic Acid and Protein-nitrogen Contents of the Large Granule Fraction of Livers of Rats at Various Times After Initiation of Dye-feeding.

Exper. group	Pentose-nucleic acid $g \times 10^{-12}$ per cell	Protein-nitrogen $g \times 10^{-12}$ per cell	PNA/N ratio
Initial control	13	34	.38
7 days	6.7	26	.26
11	6.7	23	.29
14	6.7	29	.23
16	3.1	15	.21
18	5.8	21	.28
20	5.2	19	.27
22	4.9	21	.23
24	4.6	16	.29
26	6.2	20	.31
28	2.4	9.1	.26
32	2.6	11	.24
4 wk, Price*(4)	1.7	6.2	.27
4 wk, Price*(5)	1.9	8.9	.21
4 mo.†	7.2	12	.60
4 mo.†	6.8	17	.40
Hepatoma†‡	1.4	3	.47
Basal diet control (30 days)	8.5	32	.27

In each case 3 livers were pooled for homogenization and analysis.

* Avg of 2 analyses. Assuming DNA/nucleus = 10.0×10^{-12} g.

† Data from another experimental series studied by present author.

‡ Induced by 3'-methyl-4-dimethylaminoazobenzene.

10. Umbreit, W. W., Burris, R. H., and Stauffer, J. F. Minneapolis, Burgess Publishing Co., 1949, 191.

11. Schneider, W. C., *J. Biol. Chem.*, 1945, v161, 293.

the large granule protein and pentosenucleic acid, since the decrease in the average protein-nitrogen and pentosenucleic acid to 1/2 their previous value is more than the fall to 2/3 which would result from complete failure of synthesis of these components in the 50% of the cell population undergoing division. This effect may be due to a decreased anabolism, or to an increased catabolism, or to both, since these components presumably are undergoing active synthesis and degradation. These cell divisions induced in liver cells by the feeding of a carcinogenic dye differ from those initiated in liver cells by regeneration(6). In the sudden burst of cell division which occurred 24 hours after partial hepatectomy, the increase in the protein of the nuclear and small granule fractions and the pentosenucleic acid of the small granule and supernatant fluid fractions paralleled the increase in the desoxypentosenucleic acid per nucleus. Thus, during the prophase of this cell division net synthesis of certain cell constituents occurred, and evidence was presented to show that later, during the actual division, the large granule pentosenucleic acid also doubled, with the result that the daughter cells contained the full complement of cytoplasmic pentosenucleic acid and of nuclear and small granule protein.

In large measure the results of the present study corroborate the findings of a series of cell fractionations performed at longer intervals on the livers of rats fed 2-acetylaminofluorene(12). In that study an increase of almost 100% in the total number of cells in the liver occurred between the 4th and 7th weeks of dye-feeding. However, depression of the pentosenucleic acid, protein-nitrogen, and riboflavin of the large granules to values even below those found in tumor-bearing livers was completed before the cell division occurred.

These altered liver cells show some resemblance to the average cell of a hepatoma induced by 3'-methyl-4-dimethylaminoazobenzene, with respect to the large granule pentosenucleic acid and protein content, as shown in Table II. Whereas in the large granule fraction of normal liver cells, the protein-nitrogen content is about 20 times and the pentosenucleic acid content is about 10 times that found in a variety of tumors(13), in the 28- and 32-day dye-fed livers of the present study the amounts of these components were respectively only 3 and 2 times those characteristic of the tumors.

Cell divisions occurring subsequently to that studied here were accompanied by a statistically complete reproduction of large granule substance, since the pentosenucleic acid and protein-nitrogen contents at 4 months (Table II), in livers containing tumors, were actually somewhat higher than in the livers at 4 weeks. It however seems possible that occasionally another defective cell division might occur to produce daughter cells in which the quantity of these components was once more substantially reduced. Little more than one such defective division, occurring occasionally, would be required to produce a scattering of cells in which the large granule fraction resembled that of tumors with respect to protein and nucleic acid content.

Summary. The total number of hepatic cells was determined at frequent intervals during the first month of carcinogenesis in the livers of rats fed 3'-methyl-4-dimethylaminoazobenzene; the large granule fraction was also isolated from these livers and the quantity of pentosenucleic acid and protein-nitrogen in this fraction of the average cell was determined. The total number of liver cells remained in the normal range for 26 days, but between the 26th and 28th days a sudden increase of 50% occurred. No increase in total liver mass accompanied this increase in cell number, and evidence is presented to show that no net synthesis of large granule protein or pentosenucleic acid occurred during this cell division. This cell division without increase in cell substance is compared to the cell division induced in normal liver cells by regeneration.

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