Studies on the Intracellular Composition of Livers from Rats Fed 2-Acetylaminofluorene*  
A. K. Laird† and E. C. Miller  
(McArthur Memorial Laboratory, Medical School, University of Wisconsin, Madison 6, Wis.)

Price and his associates (17, 18, 19) found alterations in the amounts of protein, nucleic acids, and riboflavin in various fractions of the liver during the induction of liver tumors by a series of aminoaZO dyes of widely different carcinogenic activities. The changes induced by the more active carcinogens made the composition of the liver approach that of the tumors induced by the parent compound, 4-dimethylaminoazobenzene (20). Griffin et al. (6) determined the effect of 2-acetylaminofluorene (AAF) on the composition of rat liver during a prolonged period of feeding the compound. They found that the concentrations of total nitrogen, riboflavin, and pentosenucleic acid (PNA) decreased toward those found in the tumors induced by this carcinogen, while the concentration of deoxypentosenucleic acid (DNA) per gram of liver remained nearly normal.

In the present study, the intracellular changes in the content of nucleic acids, riboflavin, and protein-nitrogen were followed during hepatic carcinogenesis by AAF by analysis of the fractions obtained by differential centrifugation of liver homogenates.

METHODS

Male albino rats,1 with an initial weight of 200–220 gm., were fed ad libitum a grain diet (18) containing 0.05 per cent of AAF for the first 4 weeks. To prevent an excessive mortality, the concentration of the carcinogen was reduced to 0.04 per cent at 4 weeks, and all the rats alive at 6 times was carefully dissected away from the tumors, but the possible presence of a few microscopic tumors could not be excluded. Firm white tumors, less than 1 cm. in diameter and grossly free of necrosis, were used for fractionation. The tumors were obtained from other experiments in which the carcinogen was fed at a level of 0.05 per cent; tumors from six to twelve rats were pooled for each fractionation. Control analyses were made on the livers of four rats at the beginning of the experiment, and on two groups of four rats kept for 34 weeks on the same diet without the carcinogen.

All operations were carried out at 0–5° C. The livers were perfused via the vena cava with cold 0.14 M sodium chloride (28). Since liver tumors receive blood only via the hepatic arterial system (1), the tumors were not perfused under these conditions. Grossly, they did not appear to contain much blood, however. Tissue samples were fixed for microscopic examination, and then the livers or tumors were pooled, minced, and homogenised; an aliquot of the homogenate was separated into nuclear, large granule, small granule, and supernatant fluid fractions, as previously described (17, 18, 20).

Each of the liver fractions and the original homogenate was analyzed for protein-nitrogen (25), nucleic acids*, riboflavin (2), and PNA (8).

The nuclei in two aliquots of the homogenate were counted (19), and from these counts the numbers of nuclei per gram and per whole liver were calculated. The analytical results were expressed as the amount in the average cell and in the average whole liver. The liver cells consisted mainly of the parenchymal cells and the newly-formed cells within the lobules, since the connective tissue and the blood vessels and bile ducts of the perportal spaces are largely removed by passage through a mincer (17).

Tissues were fixed for microscopic examination in Moshman’s fixative (7) and in Regaud’s fixative (3); the sections were 10 μ thick. The DNA content of individual nuclei was measured by a microspectrophotometric method (14) after Feulgen staining of the material fixed in Regaud’s solution. Nuclear diameters were measured on an arbitrary scale with an eye-piece micrometer and an oil immersion lens. Only those nuclei were measured which were entirely within the section or from which only a very thin slice had been removed; this was determined by careful focusing.

RESULTS

Although the rats gained an average of 13 gm. during the first 4 weeks, six of the 36 animals died between the 3d and 6th weeks. These deaths were apparently caused by the severe hepatic damage. The level of carcinogen was lowered from 0.05 to 0.04 per cent at 4 weeks, and all the rats alive at 6

* The PNA analyses at 7 weeks were unreliable owing to technical difficulties and are not included.

1 Male albino rats, with an initial weight of 200–220 gm., were fed ad libitum a grain diet (18) containing 0.05 per cent of AAF for the first 4 weeks. To prevent an excessive mortality, the concentration of the carcinogen was reduced to 0.04 per cent at 4 weeks, and all the rats alive at 6.


1 Holtzman-Rolfsmeyer Rat Co., Madison, Wis.

Received for publication February 24, 1953.
weeks survived until they were killed for analysis or developed tumors at 5 or 6 months.

During the first 4 weeks, the livers lost approximately one-third of their weight (Table 1). However, the number of cells per liver (as determined by the nuclear counts) was unchanged, so that the average cell decreased in weight from approximately 7 to $4.5 \times 10^{-9}$ gm. The average cell weight remained at this low level for the remainder of the experiment; the cells of the tumors induced by AAF had an average weight of $2.5 \times 10^{-9}$ gm.

Between the 4th and 7th weeks the liver cell population nearly doubled, and further rapid increases occurred throughout the remainder of the carcinogenic period. An accurate estimation of the total number of cells per liver was not possible at 25 and 27 weeks, when large necrotic tumors were present, but the total population (liver and liver tumor cells) was at least $8 \times 10^6$ cells.

The amounts of DNA, PNA, protein-nitrogen, and riboflavin in each fraction of the average liver cell for each of the times studied are given in Chart 1. The amount of DNA per cell remained relatively constant, and in all cases, including the fractions of tumor, less than 10 per cent of the DNA were obtained at this time. The amounts of these constituents remained at their minimum levels in the nuclear fraction through the 14th week, but returned nearly to the initial level by the 25th

### TABLE 1

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Stock</th>
<th>Grain plus AAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver wt. (gm.)</td>
<td>13.7</td>
<td>9.7</td>
</tr>
<tr>
<td>Liver wt. (gm.)</td>
<td>148</td>
<td>280</td>
</tr>
<tr>
<td>Liver wt. (gm.)</td>
<td>6.8</td>
<td>4.5</td>
</tr>
<tr>
<td>Liver wt. (gm.)</td>
<td>2.06</td>
<td>2.06</td>
</tr>
<tr>
<td>Liver wt. (gm.)</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>Liver wt. (gm.)</td>
<td>22.8</td>
<td>55</td>
</tr>
<tr>
<td>Liver wt. (gm.)</td>
<td>5.9</td>
<td>8.00</td>
</tr>
<tr>
<td>Liver wt. (gm.)</td>
<td>37.1</td>
<td>198</td>
</tr>
<tr>
<td>Liver wt. (gm.)</td>
<td>5.5</td>
<td>6.0</td>
</tr>
<tr>
<td>Liver wt. (gm.)</td>
<td>92</td>
<td>125</td>
</tr>
<tr>
<td>Liver wt. (gm.)</td>
<td>2.92</td>
<td>5.0</td>
</tr>
<tr>
<td>Liver wt. (gm.)</td>
<td>3.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Liver wt. (gm.)</td>
<td>2.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Liver wt. (gm.)</td>
<td>2.54</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Including liver tumors.
granule and supernatant fluid fractions, the values generally began to approach the initial levels by the 7th or 14th week. The PNA content of the supernatant fluid of the average cell changed little throughout the experimental period. The analytical values for some of the liver cell fractions of rats maintained for 34 weeks on the same diet without carcinogen differed from those of the initial control animals, which had been maintained, prior to the experiment, on the stock colony diet of a commercial dog chow (Friskies, Carnation Co.) and the whole grain diet (12). All the protein values were higher in the diet controls than in the initial controls (Table 2); the PNA content of the large granules was lower, and that of the small granules higher, in the diet controls than in the initial control.

Since the decreases in the amounts of the large granules components were greater during the first 4 weeks than those of the other fractions and returned toward normal slowly (Chart 1), the intracellular distribution of the substances studied was quite different from normal throughout the period of administration of AAF. This altered intracellular distribution is similar to that of regenerating livers (16).

The tumor cells contained much less protein and PNA in each fraction than normal liver cells (Chart 1). The tumors also contained much less riboflavin. In two cases the riboflavin content of the tumors was 1.0 and 1.1 × 10⁻¹⁴ gm/cell; the surrounding liver tissue contained 6.6 and 7.5 × 10⁻¹⁴ gm/cell. In most cases the quantities of protein, PNA, and riboflavin in the tumor cells were less than one-half the minimum values observed for the livers of rats fed the carcinogen, but the amount of DNA was essentially the same in the tumor cells as in the average liver cells from normal or AAF-fed rats.

The total quantities of protein-nitrogen, PNA, and riboflavin in each fraction from the average whole liver were also calculated. During the first 4 weeks of administration of AAF, the amounts of these constituents decreased in each of the fractions. After 4 weeks, however, marked increases in the amounts of each of these constituents were found; the increases were due largely to the increase in cell number. By 7 weeks the amounts of protein, PNA, and riboflavin in each fraction except the large granules exceeded the amounts in the corresponding fraction of normal whole livers. The amounts of these constituents in the large granules of the whole liver exceeded the normal levels by 14 weeks.

**Microscopic observations.**—At 4 weeks the parenchymal cells were even more shrunken than at 4 weeks, so that there were approximately 50 per cent more cells per microscopic field. This increase in cellular density corresponds to the 50 per cent increase in the number of nuclei found per gram of fresh liver. The cytoplasm of the cells in the central lobular region was coarsely clumped, forming large basophilic masses. The normal lobular architecture was preserved, however, and there was no indication of new cell types.

At 7 weeks the hepatic cells were even more shrunken than at 4 weeks, so that almost twice the normal number were present in each microscopic field. The peripheries of the lobules were partially overrun by cells of a new type, which gave the appearance of clusters of nuclei rather than cells. Although these cells resembled bile duct cells in some respects, they rarely formed ducts with lumens. At this time the cells with the coarse basophilic cytoplasm, which were seen only at the center of the lobule at 4 weeks, extended almost to the periphery, leaving only a rim of apparently normal cells. In a few areas the lobular architecture was replaced by well-demarcated nodules of hepatic cells. Necrosis was rare and confined to small areas.

At 14 weeks the parenchymal cells lay in dense sheets, and there was complete disruption of the normal lobular architecture. Many areas composed almost exclusively of bile ducts were seen, and these were usually associated with masses of dense, small, round or spindled-shaped cells and, occasionally, polymorphonuclear leukocytes. Areas of cells with coarse basophilic cytoplasm alternated with areas of cells containing a finely granular and diffusely basophilic cytoplasm, with no apparent reference to a lobular pattern. Of the cells with the coarse cytoplasm, a few contained a single large sharply defined vacuole. A marked variation in nuclear size was apparent at this time, and cells with giant nuclei seemed to be concen-
trated in the neighborhood of bile duct clusters. Numerous minute blood capillaries were found throughout the epithelial sheets, but few blood vessels with muscular walls were seen. The capillary lumina were occasionally distended to such a large size that they suggested sinuses.

At 25 and 27 weeks, little if any of the original architectural pattern remained. In some areas distension of the bile ducts had resulted in the production of small cysts. There were scattered foci of polymorphonuclear and small round-cell infiltration in the periportal spaces and throughout the parenchyma. A few small foci of necrosis were seen. The parenchymal cells were extremely variable in size, and a mosaic was formed of masses of large cells alternating with masses of small cells. The large cells had a homogeneous, relatively acidophilic cytoplasm and are apparently identical with those described by Cox et al. (4) in their pathological study of the lesions induced by AAF in the rat. The small cells had a coarse, granular basophilic cytoplasm and resembled those present at earlier times during the administration of the carcinogen. No tumor tissue was included in the sections.

The diameters of the parenchymal nuclei in the control livers varied considerably, and there was a tendency toward three peaks (Chart 2). As noted by previous workers (24), there was also a tendency for nuclei of different sizes to predominate in the periportal and centrilobular areas. Four weeks of exposure to the carcinogen produced no apparent change in the size distribution of the nuclei of the parenchymal cells. At 25 weeks discrete nodules were present. In each nodule there was little variation in the nuclear diameters of the cells, but the characteristic nuclear diameter varied from nodule to nodule (Chart 2).

Microspectrophotometric estimation of the DNA content of individual nuclei by the Feulgen reaction indicated that most of the large cells were polyploid. For this purpose, the Feulgen-reacting material per nucleus was expressed as $E \times D^2$, where $E$ is the extinction coefficient ($\log I_0/I$) and $D$ is the nuclear diameter in arbitrary units. Of 27 nuclei studied, twelve had diameters of 7.0 to 8.5 and relative absorptions of 134–195; these were apparently diploid resting nuclei. Seven nuclei with diameters of 10.0–11.3 had relative absorptions of 283–460 and were apparently polyploid.
resting nuclei, while six nuclei with intermediate diameters of 8.3–9.5 and intermediate absorptions of 188–362 may have been diploid nuclei under observation. The low incidence of giant diploid nuclei, about 8 per cent in a sample which favors large nuclei, suggests that the very numerous giant hepatic cells of Cox et al. (4) are polyploid cells.

No mitoses were seen on examination of about 2,000 cells in the liver sections from the control animals or on examination of an equal number from the rats fed AAF for 4 and 7 weeks. At 14 weeks the mitotic index was about 1 per cent, and at 25–27 weeks it was almost 2 per cent.

The tumors analyzed were of the hepatoma type.

DISCUSSION

A considerable depletion of the PNA, riboflavin, and protein content of each fraction of the liver cells resulted from the administration of AAF to rats. In most cases the minimum levels were found at 4 weeks, but the minima may actually have occurred somewhat earlier or later, since the analyses were made at rather widely spaced intervals. Similar decreases, expressed per gram of liver, have been observed by Griffin et al. (6) and Rutman et al. (21). It appears likely that these early alterations were produced in interphase cells, since no mitoses were seen in the sections taken at 4 weeks and the number of cells per liver (as determined by nuclear counts) was essentially the same as in the control livers analyzed at the beginning. Within the next few weeks there was an extensive proliferation of these altered cells, so that by 7 weeks there were nearly twice and by 14 weeks 3 times as many cells per liver as at the start of the experiment. These cells were, on the average, only one-half to two-thirds as large as normal liver cells. As a result, although the amounts of PNA and protein on a cellular basis were lower than in normal liver cells, the amounts per whole liver were actually equal to or greater than normal by 8–14 weeks. This observation is in essential agreement with that of Griffin et al. (6). The increase in cell number between 4 and 7 weeks apparently resulted from a relatively sudden burst of mitosis, since no mitotic figures were seen in the sections taken at either 4 or 7 weeks and since the distribution of nuclear size classes of the portal and centrilobular regions were so similar in the control and 4-week livers. At 14 and 27 weeks, a mitotic rate of 1–2 per cent was observed.

The apparent recovery of the cells at 25–27 weeks illustrates the difficulties inherent in interpreting data on the basis of the “average cell” after the cell population becomes diverse. At this time there were a considerable number of large cells which probably contained greater quantities of the various constituents than the small cells also present. However, when averaged together these widely different cell types gave values approximating those obtained with normal liver cells.

The tumors induced by AAF were composed of cells with an average weight approximately one-third that of normal liver cells. The tumor cells also contained much less protein, PNA, and riboflavin than normal liver cells and, in most cases, less than half as much as the liver cells from rats fed the carcinogen for 4 weeks. On the other hand, the DNA content of these cells was essentially the same as that of normal liver cells or of the damaged liver cells analyzed at any stage of AAF ingestion. When calculated on a fresh weight basis, the DNA content of tumors collected in four experiments ranged from 8.9 to 4.9 mg/gm, or approximately twice that of normal liver. This observation is at variance with the finding of Griffin and his associates (6) that the DNA content on a fresh weight basis was approximately the same for normal liver and for the tumors induced by AAF. The AAF-induced tumors vary from rather diffuse neoplastic areas to firm white nodules, and the latter type, diagnosed microscopically as hepatomas, were used in this study.

On the cellular level, at least, the results of the present study are in contrast to the conclusion of Griffin et al. (6) that the cellular alterations induced during carcinogenesis with AAF are essentially different from those induced by 3′-methyl-4-dimethylaminoazobenzene. Although the alterations produced in the liver by the ingestion of the two carcinogens appear quite different when expressed on a fresh weight basis, both compounds caused a considerable depletion of the protein, PNA, and riboflavin contents of most of the fractions of the average cell. The AAF data of Griffin et al. (6) have been recalculated with reference to DNA by Davidson and Leslie (5), who thus demonstrated early depletion of cellular constituents and later increases to or above the normal level.
With both carcinogens, the large granule fraction was the most severely altered (9, 18). As in the case of AAF, the ingestion of 3'-methyl-4-dimethylaminoazobenzene caused extensive depletion of the large granule fraction of the average cell prior to a sudden burst of mitosis at about 4 weeks (9). In both cases the tumor cells are smaller than normal liver cells and contain less of each component, except DNA, than the normal liver cells (5, 8, 9, 16).

As in the case of carcinogenesis by the azo dyes (18, 19), the average composition of the liver cells after 4 weeks' exposure to AAF approaches that of the normal liver cells which arise at a later date. Only a few aberrant cell divisions might be required to convert some of these cells into cells with the composition of neoplastic cells (8). That cell divisions can occur in which the rate of accumulation of one cell constituent does not keep pace with the rate of cell division was shown with regenerating liver (16). Studies from this laboratory (10, 11, 13, 15) have suggested that the deletion of certain key proteins may be involved in the genesis of neoplastic growths. While data such as these show that a great decrease in the gross amount of protein per cell occurs during carcinogenesis (5, 9, 18, 19), the determination of the identities of such key proteins will depend on finer methods of analysis.

**SUMMARY**

1. Livers from rats fed 2-acetylaminofluorene for various times up to and including gross tumor development (4, 7, 14, 25, and 27 weeks) were homogenized and separated into nuclear, large granule, small granule, and supernatant fluid fractions, and each fraction was analyzed for protein nitrogen, nucleic acids, and riboflavin. The results were expressed in terms of the amount per average cell and per average liver. Livers from rats taken at the beginning of the experiment or maintained on the same diet without the carcinogen for 34 weeks and liver tumors induced by 2-acetylaminofluorene were analyzed in the same manner.

2. In general, the levels of protein, pentose-nucleic acid, and riboflavin, when expressed on a cell basis, fell to minimum values at 4 weeks. These minimum values were either maintained or there were slow increases toward the levels found in normal liver cells. In general, the tumor cells contained about half as much of each constituent as was found in the liver cells after 4 weeks of treatment. The damaged liver cells and the tumor cells contained the same amount of desoxypentose-nucleic acid as did normal liver cells.

3. At 4 weeks the number of cells per liver was essentially normal, but the cells were only about two-thirds as large as the usual liver cell. Therefore, the cells retained their small size, except for some very large cells which arose late in the experiment, but the number of cells per liver was nearly twice the initial level at 7 weeks, and nearly 3 times the normal level at 14 weeks.

**REFERENCES**


Studies on the Intracellular Composition of Livers from Rats Fed 2-Acetylaminofluorene

A. K. Laird and E. C. Miller