Quantitative Stereological Analysis of the Effects of Age and Sex on Multistage Hepatocarcinogenesis in the Rat by Use of Four Cytochemical Markers

Yi-hua Xu, Harold A. Campbell, Gerald L. Sattler, Suzanne Hendrich, Robert Maronpot, Kiyomi Sato, and Henry C. Pitot

ABSTRACT

Altered hepatic foci (AHF) were analyzed by quantitative stereology on frozen serial sections stained sequentially for γ-glutamyltranspeptidase (GGT), canalicular adenosine triphosphatase (ATPase), glucose-6-phosphatase (G6Pase), and the placental isoenzyme of glutathione S-transferase (GST). Livers for these analyses were obtained from both male and female rats of different ages which had been subjected to initiation with a nonnecrogenic dose of diethylnitrosamine following a 70% partial hepatectomy with subsequent phenobarbital (PB) feeding. Different combinations of these four marker alterations (from single marker to four-marker combinations) were used to analyze the data, and the results were compared for their ability to detect AHF. In rats on the above protocol, GST was the single most effective marker, exhibiting a high sensitivity for scoring both number and volume of foci. There was a high degree of overlap with GGT. The combination of the four different markers, GST/GGT/ATPase/G6Pase, scored 80% more foci in number and 60% more in volume than the routinely used GST/GGT/G6Pase method.

When all four markers were used to score AHF, PB promotion was equally effective in both sexes at weaning and at 6 months of age, but at 1 year of age males showed a dramatic reduction in the effectiveness of PB as a promoting agent, both for number and volume percentage of liver occupied by AHF. On the other hand, initiation was more effective in the male at weaning and at 6 months of age, although by the 12-month point no distinction between the sexes could be made. When only GGT was used as a marker, promotion by PB appeared to be markedly less effective in males than in females at all ages. In the absence of PB administration, both the number and volume fraction of AHF in the livers of both males and females increased with age. Likewise, both the number of AHF per liver and their volume fractions increased with age in both sexes when uninitiated animals were fed PB, although only after a 6-month lag in females. These experiments demonstrate that the stages of initiation and promotion in hepatocarcinogenesis in the rat as monitored by the number and volume percentage occupied of AHF are altered by both the age and sex of the animal. The combination of GGT and GST identified all AHF scored with three markers to the use of four markers simultaneously in order to determine the effect of age and sex of the rat on the stages of initiation and promotion during hepatocarcinogenesis in the rat.

INTRODUCTION

The multistage nature of hepatocarcinogenesis in the rat has now been well established in a number of laboratories (1–3). As with multistage carcinogenesis in other tissues, multistage hepatocarcinogenesis consists of at least three distinct stages, those of initiation, promotion, and progression (4). The development and nature of these three stages can be monitored by the qualitative and quantitative analysis of focal lesions, termed altered hepatic foci, which develop following initiation during the stages of promotion and progression. During the stage of promotion, AHF* are the clonal progeny of cells initiated by one or more doses of an initiating agent and/or complete carcinogen (5–7). AHF may be identified, characterized, and quantitated by their morphology with routine histological stains, such as hematoxylin and eosin (8, 9), or by the altered expression of genes which are expressed in adult hepatocytes under certain conditions (1, 2, 10).

Methods for the enumeration and size determination of AHF have been reported from several laboratories (11–15). By these techniques based primarily on the mathematics of quantitative stereology (14), it is possible to determine both the number and volume percentage of the liver occupied by AHF during the early stages of carcinogenesis. The importance of utilizing the techniques of quantitative stereology rather than direct enumeration of focal lesions on histological slides has been previously emphasized (15). In addition, the effectiveness of specific markers, particularly GST, has also been reported (16, 17). In this paper, we have extended previous quantitative techniques allowing for the enumeration and size determination of AHF scored with three markers to the use of four markers simultaneously in order to determine the effect of age and sex of the rat on the stages of initiation and promotion during hepatocarcinogenesis in the rat.

MATERIALS AND METHODS

Chemicals and Diets. PB as the free acid was purchased from Sigma Chemical Co. (St. Louis, MO). DEN was obtained from the Eastman Kodak Co. (Rochester, NY). The crude cereal-based NIH-07 diet was obtained from the Teklad Corp. (Madison, WI).

Animals and Treatments. Male and female F-344 rats of different ages were obtained from the Harlan-Sprague-Dawley Co. (Madison, WI). The experimental groups and conditions for initiation and promotion by DEN and PB, respectively, are outlined in Fig. 1. DEN was administered by intubation in a single dose of 10 mg/kg 24 h following a 70% partial hepatectomy. Two weeks following this procedure, where indicated in Fig. 1, PB at a concentration of 0.05% was administered in the diet for the periods depicted. At the times indicated, rats were weighed and then sacrificed by decapitation, and their livers were removed and weighed. For each animal, two random 3-mm-thick slices of each of the three remaining lobes were taken, and two composite tissue blocks of three slices each were formed on a piece of filter paper. These tissue samples were frozen on dry ice and stored at −70°C. Additional samples from each lobe were removed and fixed in buffered 10% formalin for later histopathological examination.

Histological Methods. Four serial frozen sections of 10-μm thickness were cut from each composite tissue block (two per animal). The first

Received 6/15/89; revised 9/14/89; accepted 10/24/89.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Studies described in this paper were supported in part by grants from the National Cancer Institute (CA-07175, CA-22484, and CA-45700) as well as a contract from the National Toxicology Program (ES-82-12) and a training grant from the National Cancer Institute (CA-09451).

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* The abbreviations used are: AHF, altered hepatic foci; GGT, γ-glutamyltranspeptidase; ATPase, canalicular adenosine triphosphatase; G6Pase, glucose-6-phosphatase; GST, the placental isoenzyme of glutathione S-transferase; DEN, diethylnitrosamine; PB, phenobarbital.
were continued beyond the point of sacrifice for the periods designated in Table 4. •¿, date of birth; $, partial hepatectomy; |, DEN 10 mg/kg intubation; G, NIH-07, one month; 0.05% PB in NIH-07, six months total.

4. •¿, date of birth; $, partial hepatectomy; |, DEN 10 mg/kg intubation; G, NIH-07, one month; 0.05% PB in NIH-07, six months total.

section in each series was stained histochemically for the enzyme GGT; the second, for ATPase; the third, for G6Pase; and the fourth determined immunohistochemically for GST. The staining methods for GGT, ATPase, and G6Pase have been described previously (16). Immunohistochemical analysis of GST was performed according to the method of Hendrich et al. (16).

Stereological Methods. Quantitative stereological data for the individual serial sections were obtained with combination instrumentation modified from that previously described in this laboratory (14). In short, each serial section was projected, by means of an overhead projection apparatus built by the Physical Sciences Laboratory, Stoughton, Wisconsin, onto a Summagraphics Digitizer (area, 48 x 60 in). AHF identified on each tissue section in sequence were outlined with a cursor, and the values obtained were automatically transmitted to an HP-9872B plotter controlled by the computer. On the plotter the four serial sections stained with different markers were overlaid by aligning two anatomical markers. Data for each of the four serial sections obtained in this way were then edited directly on the video (19 in), and the area, the position of the centroid, and the tissue outline were recorded directly on an 8-in floppy disk. All data were stored on such disks for further analysis.

For the analysis of single alterations, focal transections of AHF were sorted by different size classes according to their diameters. The size classes used in these calculations were the same as those previously described (14). The number of foci per cm3 of liver was calculated according to the method of Saltykov (18), and the number of foci per liver was determined from the liver weight of each animal. The volume fraction of AHF was calculated by the method of Delesse (18, 19). When multiple alterations of AHF were determined from the overlay of serial sections, the appropriateness of the overlay of the focal transections was determined in each of the 15 possible combinations of phenotypic alterations based on the location of the centroid of the focal transection. Enumeration and determination of volume percentage occupied by AHF of all possible phenotypes was determined by the method previously described from this laboratory (14).

In order to determine the total number per liver and the volume percentage of the liver occupied by AHF, the "ANY" phenotype was used. The ANY phenotype is defined herein in the same way as that reported by Campbell et al. (14). With a single phenotypic alteration, the ANY phenotype of that alteration is equal to the total number of AHF scored by that marker. When multiple alterations in any AHF occur, the ANY phenotype includes AHF with a single alteration as well as any combination which includes that alteration. A simple example of these two conditions is depicted in Fig. 2 where, for simplicity's sake, two enzyme phenotypes in two marker combinations are depicted. With 2 markers, 3 phenotypes and phenotypic combinations are possible as shown. Expansion of this to 3 markers and 4 markers gives rise to 7 and 15 possible phenotypic combinations, respectively. When 4 marker enzymes are used, the 15 possible phenotypes include 4 single marker phenotypes as well as combinations of 6 2-marker, 4 3-marker, and 1 4-marker alterations, all of which are included in the phenotype ANY.

Fig. 3 shows for the four markers a plot of the summation of AHF per cm3 with a maximum diameter larger than the truncation diameter. The number of AHF per cm3 liver at different truncation values was calculated by the Saltykov method (see text). The number along the abscissa designate the smallest diameters in μm of the individual size classes analyzed (14).

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reliably observed. The Fullman method (20) results in low estimates of AHF per cm$^2$ compared to other methods, especially when the average diameter of the observed transactions is small (11, 15). When the focal transaction data are truncated at a diameter appropriate for the specific data being analyzed, the Nicholson method (21) and the Pugh et al. method (11) give an estimate of AHF per cm$^2$ with maximum diameters larger than the truncation diameter. In the Saltykov method (18), the number of AHF per cm$^2$ is determined for each size class, and the total AHF per cm$^2$ is determined by the summation of the AHF per cm$^2$ in all of the size classes. Only focal transactions having a diameter within the limits of the size class and not attributable to off-center focal transactions of foci in larger size classes are used to compute the AHF per cm$^2$. In the smaller size classes, the number of focal transactions observed can be less than the focal transactions attributable to foci of larger size classes. In such cases where there is a deficiency of focal transactions, it is not appropriate that the total number of larger AHF per cm$^2$ be decreased considering the limitations noted above of observing small focal transactions and in the context of our experiment, where a negative altered focal transaction has no physical meaning. Thus, the plot of total AHF per cm$^2$ plateaus at the small size classes as shown in Fig. 3.

If the AHF per cm$^2$ is to represent a condition of the liver rather than the characteristics of the histology procedures or the ease or reliability of observing the alteration, the focal transaction data must be truncated at the same diameter, which must not be smaller than the diameter where the AHF per cm$^2$ levels for all alterations are compared. In this experiment where the sensitivity of the various markers is emphasized, the method of Saltykov is used in the estimation of thenumber of AHF per cm$^2$, and all focal transaction data are truncated at 125-μm diameter.

RESULTS

The liver weights as well as the liver/body ratios of male rats were consistently significantly higher than those of female rats in all treatment groups (Table 1). In both sexes of each age group, rats in the DEN + PB group showed the highest liver weights. In the two control groups (DEN only and PB only), the liver weights of animals on PB only were significantly higher than those on DEN only. This finding is not remarkable in view of the known hyperplastic and hypertrophic effect of PB on rat liver (cf. Ref. 22). However, the age of the animal at the time of sacrifice did not significantly affect the liver weights of the weanling and 6-month groups, although liver weights in the weanling and 6-month groups, although liver weights in time of sacrifice did not significantly affect the liver weights of aging and thus could be scored with reasonable accuracy. Similarly, in those animals given PB, centrilobular areas showed a depression of staining by ATPase and G6Pase. This effect of the promoting agent made the scoring of small AHF marked by deficiencies in these two enzymes more difficult than in livers of those rats not receiving PB. However, since these two negative markers proved to be the least sensitive in scoring the greatest number of AHF, this effect was not a significant factor in evaluating the relative effectiveness of the four markers. On the other hand, as indicated by previous workers (26), no significant background effects were noted in sections stained immunohistochemically for GST. On review of the sections, however, the conclusions reached in this paper were not the result of the difficulties in reading GGT, ATPase, and G6Pase noted above but rather, as we and others have indicated (16, 17), were due to inherently greater numbers of AHF scored by GST and GGT.

In all groups, the number and volume percentage fraction of AHF were calculated for each of the 15 different possible combinations when using 4 markers. In order to demonstrate the sensitivity of the various marker combinations, the set of females from Group 4 (Fig. 1) was used to exemplify this result. It should be noted, however, that other groups tested both at earlier and later times showed essentially the same result. The 15 possible marker combinations and the average volume percentages and number of AHF per cm$^2$ are seen in Table 2. From the table, it is apparent that when the marker combination includes both GST and GGT, the maximal number and the near maximal volume percentage of AHF are scored. The ratios of the numbers of AHF per cm$^2$ of the two ANY designations is slightly higher for the SRG and SR as compared to the four-marker combination, SRYG. This can be explained on the basis that the SRYG combination scored has a slight increase in diameter over the SRG and SR diameters, resulting in less AHF per cm$^2$ computed. On the slides, as noted in the table, the lesser area in the SRG and SR categories indicates that some AHF are of smaller diameter and therefore may be counted as representing slightly greater numbers in three dimensions based on the Saltykov relationship (18). However, this does not alter the conclusion that the combination of GST and GGT scores as many foci as the SRYG combination. Furthermore, one must conclude that GST alone or in combination with the ATPase and G6Pase markers scores a greater volume percentage fraction and number of AHF per cm$^2$ than GST alone or in combination with ATPase and G6Pase.

Effect of Sex and Age on AHF. The number and volume percentage fraction occupied by AHF with the phenotype ANY (SRYG) for Groups 1–9 (Fig. 1) are seen in Fig. 4. Since previous studies from this laboratory (27) have demonstrated that the feeding of PB for 6 months would result in the maximal expression of all AHF initiated, those animals initiated with

Table 1 Liver weights and liver/body ratios of rats

<table>
<thead>
<tr>
<th>Age at initiation</th>
<th>Sex</th>
<th>Group</th>
<th>Liver wt (g)</th>
<th>Liver/body ratio</th>
<th>DEN only</th>
<th></th>
<th>DEN only</th>
<th></th>
<th></th>
<th>DEN + PB</th>
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<th></th>
<th>DEN + PB</th>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weanling</td>
<td>F</td>
<td>2</td>
<td>6.59 ± 0.19</td>
<td>8 2.897 ± 0.054</td>
<td>8</td>
<td>4.84 ± 0.21</td>
<td>8</td>
<td>3.563 ± 0.033</td>
<td>1</td>
<td>8.46 ± 0.31</td>
<td>9</td>
<td>3.675 ± 0.108</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>2</td>
<td>12.31 ± 0.27</td>
<td>8 3.046 ± 0.039</td>
<td>4</td>
<td>17.68 ± 0.60</td>
<td>4</td>
<td>4.166 ± 0.069</td>
<td>3</td>
<td>16.79 ± 0.29</td>
<td>9</td>
<td>4.012 ± 0.039</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 mo</td>
<td>F</td>
<td>5</td>
<td>7.00 ± 0.24</td>
<td>8 2.730 ± 0.047</td>
<td>6</td>
<td>9.05 ± 0.32</td>
<td>6</td>
<td>3.406 ± 0.025</td>
<td>4</td>
<td>9.13 ± 0.32</td>
<td>3</td>
<td>3.236 ± 0.046</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>5</td>
<td>13.38 ± 0.34</td>
<td>6 3.021 ± 0.084</td>
<td>8</td>
<td>18.70 ± 0.47</td>
<td>7</td>
<td>3.939 ± 0.054</td>
<td>7</td>
<td>18.97 ± 0.72</td>
<td>7</td>
<td>4.075 ± 0.067</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 mo</td>
<td>F</td>
<td>8</td>
<td>7.63 ± 0.13</td>
<td>6 2.335 ± 0.156</td>
<td>6</td>
<td>9.99 ± 0.27</td>
<td>7</td>
<td>3.287 ± 0.039</td>
<td>7</td>
<td>10.33 ± 0.43</td>
<td>7</td>
<td>3.329 ± 0.064</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>8</td>
<td>12.32 ± 0.63</td>
<td>6 2.835 ± 0.071</td>
<td>7</td>
<td>18.31 ± 1.69</td>
<td>7</td>
<td>3.772 ± 0.240</td>
<td>7</td>
<td>18.88 ± 0.50</td>
<td>6</td>
<td>3.956 ± 0.059</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values listed are the mean ± SEM; n, number of animals in each group. See text for further details.

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Table 2. Sensitivity of different marker combinations (ANY phenotype)
The 15 possible combinations resulting from the 4 markers used as well as the average volume fraction (%) of the liver occupied and the number of AHF/cm³ of the liver are denoted for each phenotype of enzyme markers, the symbol for each marker combination being designated on the left. Each value of volume % fraction and number of AHF/cm³ liver is calculated from the marker combination on that line to the left. The data in this table are derived from Group 4 (Fig. 1).

<table>
<thead>
<tr>
<th>Marker combinations</th>
<th>Vol. % fraction</th>
<th>No. of AHF/cm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>S GST</td>
<td>4.12 0.836</td>
<td>3311 0.945</td>
</tr>
<tr>
<td>R GG</td>
<td>2.37 0.480</td>
<td>1907 0.544</td>
</tr>
<tr>
<td>Y ATP</td>
<td>1.20 0.243</td>
<td>281 0.080</td>
</tr>
<tr>
<td>G G6P</td>
<td>1.03 0.209</td>
<td>178 0.051</td>
</tr>
<tr>
<td>SR GST-GGT</td>
<td>4.56 0.923</td>
<td>3541 1.011</td>
</tr>
<tr>
<td>SY GST-ATP</td>
<td>4.36 0.883</td>
<td>3258 0.930</td>
</tr>
<tr>
<td>SG GST-G6P</td>
<td>4.38 0.887</td>
<td>3287 0.938</td>
</tr>
<tr>
<td>RY GGT-ATP</td>
<td>2.75 0.558</td>
<td>1895 0.541</td>
</tr>
<tr>
<td>RG GGT-G6P</td>
<td>2.73 0.552</td>
<td>1894 0.541</td>
</tr>
<tr>
<td>YG ATP-G6P</td>
<td>1.60 0.323</td>
<td>357 0.102</td>
</tr>
<tr>
<td>SRY GST-GGT-ATP</td>
<td>4.74 0.960</td>
<td>3504 1.000</td>
</tr>
<tr>
<td>SRYG GST-G6P-ATP</td>
<td>4.78 0.969</td>
<td>3535 1.009</td>
</tr>
<tr>
<td>SYG GST-ATP-G6P</td>
<td>4.57 0.925</td>
<td>3250 0.928</td>
</tr>
<tr>
<td>RYG GGT-ATP-G6P</td>
<td>3.02 0.611</td>
<td>1899 0.542</td>
</tr>
<tr>
<td>SRYG GST-GGT-ATP-G6P</td>
<td>4.94 0.100</td>
<td>3503 1.000</td>
</tr>
</tbody>
</table>

* Ratio, value of ANY category of that group/value of ANY category of GST/GGT/ATP/G6P.

DEN and given PB reflect the total number of cells initiated. From this it can be seen that the numbers of cells initiated at 6 months in both males and females were significantly greater than those initiated at the time of weaning. However, this is likely to be due in part or entirely to the fact that the cell number in livers of rats at weaning is less than one-half the number in livers of animals at 6 months of age (23). In addition, the rate of DNA synthesis stimulated by the 70% partial hepatectomy is greater in younger than older rats (28). Therefore, if one corrected for the cell number at the time of initiation, the number of cells initiated as a function of the total number of cells present in the liver at the time of initiation would be roughly equal. In animals initiated at 12 months of age, PB promotion in the female resulted in no greater increase in the number of AHF than at 6 months of age, which would be expected. However, PB promotion of AHF in livers of male animals initiated at 12 months of age was dramatically less efficient than that seen in livers of male rats initiated at 6 months of age. It is likely that this is not due to a coalescence of the focal lesions since the volume fraction was also lower in those animals (Fig. 4). An alternative explanation is that PB promotion in the older animal is not as effective as in the younger animal, as has been described for tetradecanoylphorbol acetate promotion during multistage epidermal carcinogenesis in the mouse (29, 30).

Although the number and volume percentage fraction of AHF scored by the GST marker, as well as by ATPase and/or G6Pase (data not shown), reflected changes scored by the SRYG combination (Table 3), the volume fraction of GGT-positive AHF in livers of male animals initiated with DEN and promoted with PB was significantly less than in females (Fig. 5). Since PB has...
AGE AND SEX IN MULTISTAGE HEPATOCARCINOGENESIS

Table 3  Number and percentage fraction of AHF in uninhibited rats given 0.05% PB for 6 months

<table>
<thead>
<tr>
<th>Age at initiation</th>
<th>Sex</th>
<th>Tissue block</th>
<th>Group</th>
<th>No. of foci/liver</th>
<th>Vol. % of foci in liver</th>
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<tr>
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<td>3</td>
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<td></td>
<td>M</td>
<td>8</td>
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<tr>
<td>6 mo</td>
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<td>12</td>
<td>6</td>
<td>900</td>
<td>84</td>
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<td></td>
<td>M</td>
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<td>2140</td>
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</tr>
<tr>
<td>12 mo</td>
<td>F</td>
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<td>157</td>
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<tr>
<td></td>
<td>M</td>
<td>14</td>
<td>9</td>
<td>2751</td>
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Statistical analysis (Student's t test)\(^*\) Vol. %; \(^*\) = \(P < 0.05\)

<table>
<thead>
<tr>
<th>Group and sex</th>
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\(^*\) Refers to group as identified in Fig. 1.

\(^{\dagger}\) Symbols 3F, 3M, 6F, etc., refer to the group numbers (3, 6, 9) and sex (M, F).

been shown to induce GGT activity in AHF (31), these data suggest that such regulation may be less effective in the male. This conclusion is supported by the lack of increase in GGT-positive AHF in the absence of PB relative to that seen with other markers.

Previous studies from this laboratory (32) utilizing GGT only as a marker indicated that the spontaneous occurrence of AHF did not increase when the promoting agent was administered chronically after 3 months of age. In Table 3 may be seen the numerical values, depicted graphically in Fig. 4, for the numbers of AHF in livers of males and females after 6 months of feeding 0.05% PB in the diet beginning at weaning, 6 months, and 12 months of age. Unlike the earlier study, these data demonstrate that both the number and percentage volume of AHF in both males and females increase significantly over the first year of life, although the female appears to exhibit a lag period for the first 6 months of age. Similar findings are depicted with the GST-only and GGT-only markers (Fig. 5). These data then suggest that the spontaneous initiation of hepatocytes continues throughout at least the first 12 months of the life span of the animal, and in preliminary experiments, \(^5\) it has been observed to increase to 26 months of age in both male and female rats.

Although the number of AHF per liver is significantly lower in both male and female rats initiated at the time of weaning, there is no significant difference in the volume percentage of AHF in the livers of males initiated at any of the three time points with the exception of the group of males initiated at 12 months that show a slightly lower volume fraction. Since, however, the livers of female animals are roughly one-half the size of livers of male rats (Table 1), this result is to be expected if (a) the number of hepatocytes in the male is also roughly equal to that in the female, and (b) the size of hepatocytes in the female is about one-half that in the male. Since (b) appears to be correct (33, 34) and, at weaning, the number and size of hepatocytes are essentially identical (23), the numbers of hepatocytes in the adult male and female must be essentially the same. Thus, the volume percentage fraction of AHF, which is a percentage of the entire organ, indicates that there is no difference in the liver of male and female animals initiated at any of the times studied and promoted with PB, except for the anomalous finding of the male initiated at 12 months. On the other hand, the fact that there is not a difference in the volume percentage fraction within the same sex indicates that the rate of clonal growth of cells within AHF initiated at weaning and promoted for the following 6 months is significantly greater than in animals initiated at 6 months of age and promoted for the next 6 months. As can be seen in Table 1, the liver weights change little if at all within each sex during these time periods. Furthermore, the mean diameter of AHF in animals initiated at weaning was significantly greater than in animals initiated at 6 months of age (data not shown). This further argues, in concert with multistage carcinogenesis in mouse skin (29), that tumor promotion is less effective in the older animals than in the young.

Hepatic Histopathology at 14–26 Months. In Table 4 may be seen a summation of the histopathology of preneoplastic and neoplastic lesions in the livers of rats in Groups 1–9 (Fig. 1). All animals were maintained on the regimen of the individual group, as noted in Fig. 1, for 6–8 months beyond the time designated in Fig. 1 before sacrifice and examination of the histopathology of their livers. As can be seen from the table, virtually all animals possessed AHF within the liver parenchyma, as determined by light microscopy with hematoxylin and eosin staining. Neoplastic nodules (35) were present in the livers of almost all animals that were initiated with DEN and promoted with PB. However, those rats of either sex initiated with DEN at weaning or at 6 months of age showed a much lower incidence of neoplastic nodules, although the groups initiated at 12 months of age exhibited this lesion in almost all livers of animals of both sexes. Only an occasional rat receiving PB only exhibited neoplastic nodules. Hepatocellular carcinomas were by far most prominent in the males of Group 1, initiated at weaning. Although hepatocellular carcinomas were present in females in this group, the occurrence of carcinomas in either males or females of animals initiated at 6 and 12 months followed by PB promotion was much lower, suggesting that the complete carcinogenic process was more effective in the weanling animal than at 6 or 12 months of age. Occasional carcinomas were also seen in other groups but with no definite pattern.

DISCUSSION

Perhaps more than any other model of multistage carcinogenesis, hepatocarcinogenesis in the rat has been most extensively subjected to quantitative analytical procedures as a result of the demonstration of the importance of readily demonstrable and quantifiable focal lesions in histological sections, which are critical entities in the early stages of initiation and promotion during carcinogenesis in this tissue. An obvious goal of the use of AHF as a quantitative end point in determining parameters of the stages of hepatocarcinogenesis is that of identifying and quantifying the total and thus maximal number of such lesions during various experimental protocols. In the past, several markers have been identified as being exceptionally efficient in scoring more or most of the AHF induced by the various regimens used. Such markers include: iron staining deficiency (36), canalicular ATPase (37), GGT (38, 39), UDP-glucuronyltransferase (40), and GST (16, 17). Data presented in this paper (Table 2) confirm and extend the fact that under the initiation and promotion conditions of DEN and PB, respectively, GST is the most efficient marker of AHF enumerated...
The phenotypic distribution of AHF with the four markers is subject to some error due to the total thickness of the four serial sections used. The rationale for this conclusion is seen in Fig. 6, which indicates, in a graphical sense, the results of multiple sections through a focus of phenotype SRYG and diameter 120 μm. The sections are indicated to be 10 μm in thickness, showing an error of approximately 40% of a possible by quantitative stereological means. Furthermore, this marker is substantially better than GGT, but the combination of the two scored essentially all AHF marked by the four enzyme phenotypes monitored in these studies. Thus, with the exception of the carcinogenic peroxisome proliferating agents (41), the combination of GGT and GST may offer a reasonable compromise for scoring the greatest number of AHF induced by an agent without resorting to a multitude of various marker analyses. These findings, with quantitative stereological techniques, confirm our previous conclusions (16) wherein only three markers could be quantitated at a time. An advantage of the simultaneous determination of four markers is that a clear comparison between the individual markers, one with another, can be made without requiring relative comparisons. Furthermore, since two-dimensional analyses do not allow an accurate determination of the number of AHF when such lesions exhibit significantly different diameters, our data may be said to validate early studies leading to similar conclusions (17) but using number of AHF per cm² instead of the more accurate stereological analyses.

Fig. 6. Representation of the phenotypic distribution error resulting from the finite thickness of serial sections through an individual spheroidal focus. See "Discussion" for details.
15 correct combinations. Since, in the experiments described in our study, foci were truncated at 125 µm, and most foci actually occurred in size class 7 (200–251-µm diameters), the error would then be about 20%, as indicated earlier. We feel that such an error does not significantly alter the conclusions of our findings. Furthermore, this error can be substantially decreased, not only by decreasing the thickness of sections utilized, but also by using double staining techniques on the same slide. Obviously, if one could stain for GGT and GST and their combination on a single section allowing quantitation, most of the labor involved in obtaining the data reported herein could be eliminated without substantially altering the conclusions.

Because of the changes in liver size and cellularity with the age of the animal, our results suggest that there are no striking differences in the efficiency of initiation of hepatocytes capable of developing into individual AHF as noted in those animals of both sexes initiated with DEN and promoted with PB. Since, in order to measure the number of initiated cells capable of developing into AHF, it is necessary to promote the development of each clone to a sufficient size to be scored by the techniques utilized herein, a 6-month period of promotion was believed to be sufficient to accomplish this aim (27). Inasmuch as the hepatocytes of female livers are about 50% smaller than those of male livers (33, 34), it is likely that the dramatic differences in liver weights (Table 1) are primarily due to differences in absolute cellularity. Therefore, the volume percentage fraction per total liver is an appropriate comparison between males and females when the livers of each sex have the same numbers of cells. The lower level of initiation in males at 12 months relative to that of males at 6 months of age, which is consistent no matter how the data are expressed, is somewhat perplexing if one views the effect as that on initiation only. It is well known that the metabolism of xenobiotics decreases with aging in both male and female rats (cf. Réf. 42). Thus, it might be suggested that the lower level of AHF in the male is due to the lack of initiation. This seems unlikely, since there is no evidence that xenobiotic metabolism in the female is much more effective than in the male at age 12 months.

On the other hand, as suggested above, the liver of the 12–18-month-old male may be less sensitive to the action of PB on gene expression, as has been previously reported (cf. Réf. 42), which may reflect, in turn, its efficacy as a promoting agent. Again, however, the striking contrast between male and female animals at 12 months of age does not coincide with a comparable dramatic differential sex effect on the alteration of genetic expression by PB in liver. More substantial evidence that the stage of promotion is more effective in young rats than in older rats can be seen from the volume percentage parameters in weanling, 6-month-old, and 12-month-old animals wherein there is no significant difference whether the data are expressed per liver or per cm³, with the possible exception of the 12-month-old male (Fig. 4). With the dramatically lower number of foci per liver, there is a greater proportional increase in volume percentage fraction in the weanling animals, arguing strongly that these AHF have a greater growth potential in the presence of the same concentration of PB by dietary intake. Although, in multistage hepatocarcinogenesis, there is no quantitative evidence corroborating this finding, at least two studies of multistage carcinogenesis in the skin (29, 30) have presented evidence showing a decrease in the effectiveness of promotion with age. On the other hand, Loehrke et al. (43) have demonstrated that, when animals were initiated, not by direct application but systemically by intragastric administration of a poly-

cyclic hydrocarbon carcinogen, no effect of age on promotion by tetradecanoylphorbol acetate could be demonstrated. Therefore, as yet, one must conclude that there is insufficient evidence in the model systems of multistage carcinogenesis to demonstrate clearly an aging effect on either initiation or promotion, although there is some suggestion that there is no effect of age on initiation, but aging may inhibit tumor promotion.

Differential effects of sex hormones on hepatocarcinogenesis have been known for many years (cf. Réf. 44). Not only are there sex differences in the spontaneous incidence of neoplasms, especially in mice (45, 46), but with the case of specific chemical carcinogens, one sex or the other appears to be more susceptible. This is usually true of the male, although such a tendency toward that sex is by no means clear in all instances (cf. Réf. 44). In support of the data shown in Table 4, Reuber (47) reported that in castrated male rats given testosterone, 2-ace-
ylaminofluorene was a much more efficient carcinogen in rats 12 weeks of age than in those 52 weeks of age.

The data of Table 3 are of interest in relation to the possible role of spontaneous (fortuitous) initiation in the carcinogenicity of known hepatic promoting agents which lack significant direct DNA-damaging capacity, e.g., PB, 2,3,7,8-tetrachlorodibenzo-
p-dioxin, and peroxisome proliferating agents. Our results (Table 3), which confirm and extend those of other workers (48, 49), have demonstrated that AHF promoted in the absence of exogenous (experimentally induced) initiation occur in relatively large numbers, increase in number and focal volume with the age of the animal, and exhibit responses to sex and promoting agents which make them biologically indistinguishable from those AHF induced by a variety of protocols of multistage hepatocarcinogenesis in the rat (50). Furthermore, the effects of age and sex on parameters of AHF in the stage of promotion in hepatocarcinogenesis in the rat are analogous to similar effects seen in multistage epidermal carcinogenesis in the mouse, extending the basic biological similarities in these two model systems of multistage carcinogenesis.

ACKNOWLEDGMENTS

The authors are indebted to Jane Weeks and Jennifer Potter for expert histotechnological processing of tissues and to Kristen Adler for expert technical typing of the manuscript.

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Quantitative Stereological Analysis of the Effects of Age and Sex on Multistage Hepatocarcinogenesis in the Rat by Use of Four Cytochemical Markers

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