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Rat Hepatocarcinogenesis Induced by N-nitrosodiethylamine and N-nitrosomorpholine Continuously Administered at Low Doses

From Basophilic Areas of Hepatocytes to Hepatocellular Tumors

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The development of hepatocellular tumors was investigated with histological, histochemical, and morphometrical methods in male Sprague-Dawley rats continuously administered N-nitrosodiethylamine (DEN) or N-nitrosomorpholine (NNM) in the drinking water at low doses (0.5 mg DEN / 100 ml; 1 mg NNM / 100 ml). Groups of control, DEN-, and NNM-treated rats were investigated at 5-week intervals. Similar results were obtained in DEN- and NNM-treated rats. Two types of areas composed of basophilic or glycogenotic hepatocytes were observed preceding the appearance of hepatocellular adenomas and carcinomas. Besides their cytologic differences, the basophilic and glycogenotic areas induced displayed distinct histochemical features. Both types of areas were detected simultaneously and increased in parallel with time to a similar incidence, but basophilic areas reached larger sizes than the glycogenotic ones. Furthermore, each type of area, which clustered around and along efferent veins, was differently linked to tumorigenesis. Basophilic areas frequently developed into basophilic adenomas and trabecular carcinomas through a characteristic sequence. Early basophilic areas consisted of hepatocytes with lamellar cytoplasmic hyperbasophilia and exhibited the normal laminar liver structure. With time, an increasing number of basophilic areas also contained hepatocytes with powdered diffuse hyperbasophilia, which frequently were arranged in thick trabeculae, showed abundant mitotic figures, and invaded efferent veins. Neither such signs of malignancy nor conversion into basophilic areas or tumors could be established for areas of clear and acidophilic glycogenotic hepatocytes. However, a few small glycogenotic adenomas probably developed from glycogenotic areas. Our data thus underline the central role of basophilic areas for hepatocarcinogenesis. Moreover, taking into account the data from other experiments, it seems likely that although glycogenotic areas may be associated with the application of some carcinogens at high doses, they are not obligatory precursors of hepatocellular tumors. (Am J Pathol 1991, 139:1157–1171)

Areas or foci of altered hepatocytes have been reported repeatedly in rats and other species preceding the appearance of hepatocellular tumors (adenomas and carcinomas) induced by chemicals. These areas of cellular alteration, generally considered as tumor precursors, have been characterized and classified as various types according to histological and histochemical data.[1,2] Three major types of area are usually distinguished with ordinary histological stains by their composition of basophilic, clear (glycogenotic), or acidophilic hepatocytes. These three types of areas are usually detected in livers of rats administered hepatocarcinogens, but the incidence of each one differs markedly in some experiments compared with others conducted with the same or different carcinogens. Areas of basophilic hepatocytes have

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been early observed in rats administered various carcinogens and have been suggested to represent sites of neoplastic transformation (see [3]). Furthermore, basophilic areas have been described as the predominant or unique focal lesions before rat hepatocellular tumors induced by substances such as aminoazo dyes,[3,4] N-2-fluorenylacetamide[5] and aflatoxin B1.[6] Basophilic areas are also the predominant or unique precursors of the naturally occurring hepatocellular tumors in Fischer-344 rats.[7] On the other hand, a predominance of acidophilic areas in rat livers exposed to N-2-fluorenylacetamide[8] or of clear (glycogenotic) cell areas in livers of rats administered DEN[9,10] or NNM[11,12] has been described.

The reasons for the morphologic diversity of the areas of cellular alteration and their varied incidence have not been clarified, but it is obvious that factors related to animals (strain and sex of rats) and carcinogens (nature, dosage) used in experiments are involved. Concerning the dosage of carcinogen in particular, Herranz and coworkers[13–15] have described changes in the incidence of basophilic and glycogenotic areas preceding rat hepatocellular carcinomas induced by DEN continuously administered at different doses. Whereas with a daily dose of 10 mg DEN/Kg body weight a high incidence of both glycogenotic and basophilic areas was noted,[13] after a low dose of 1.2 mg DEN/Kg body weight/day, the incidence of glycogenotic areas was clearly reduced and it was suggested that hepatocellular carcinomas developed directly from basophilic areas.[14,15] Another explanation for the morphologic diversity of areas of cellular alteration is a sequential conversion of one type of area into others. Such a hypothesis has been principally proposed by Bannasch and coworkers.[11,12] According to these authors, rat hepatocellular carcinomas induced by different doses of NNM (8, 12, 16, 20 mg/100 ml drinking water) administered for various periods of time (1, 4, 7, 14, 20 weeks) originated from areas of clear and acidophilic glycogenotic hepatocytes through a conversion into mixed cell areas (made up of clear, acidophilic and basophilic cells) and finally a progression to adenomas and carcinomas.[12]

To investigate the significance of the different types of hepatic areas of cellular alteration, we analyzed the development of rat hepatocellular tumors induced by continuous exposure to the carcinogens DEN and NNM at low concentrations: 0.5 mg DEN and 1 mg NNM/100 ml drinking water.

Materials and Methods

Experimental Design

Male Sprague-Dawley rats (purchased from Zentralinstitut für Versuchstierzucht, Hannover, FRG) of about 160 g were continuously administered DEN or NNM dissolved in the drinking water. The concentration of DEN and NNM was 0.5 and 1 mg/100 ml, respectively. Control rats did not receive carcinogens. Four to twelve rats of both the DEN- and the NNM-group were sacrificed periodically after the start of the experiment: DEN-treated rats after 10, 15, 20, 25, 30, 35, 40, 45, 50, and 55 weeks; NNM-treated rats after 20, 25, 30, 35, 40, 45, 50, 55, 60, and 65 weeks. Four control rats were also sacrificed at the same time points. Administration of carcinogens was stopped 4 weeks before sacrifice. Rats were killed between 9:00 and 11:00 AM, after a fast of 22 hours and a 2-hour meal as described by Babcock and Cardell.[16] Livers were removed rapidly from the animals under anesthesia, and adjacent slices of the left and median lobes and of tumors were processed for histological and histochemical studies. Livers of rats found dead were only studied histologically.

Light Microscopy

For histological studies, liver samples were fixed in Carnoy's fluid. Paraffin sections cut at 2 μm were stained with hematoxylin and eosin, cresyl violet, and the periodic acid-Schiff (PAS) reaction. From the left hepatic lobe of four rats (two DEN- and two NNM-treated rats) killed at the 35th week of the experiment a series of forty sections was made to determine the spatial disposition of the areas of altered hepatocytes (basophilic and glycogenotic areas) within the liver. Other organs than liver were also processed for light microscopy when they appeared altered at the autopsy.

Enzyme Histochemistry

For enzyme histochemical studies hepatic samples were frozen in isopentane at −150°C and stored at −80°C. Series of cryostat sections of 6 μm were stained by methods for cresyl violet, the PAS reaction, glucose-6-phosphatase (G6Pase),[17] glucose-6-phosphate dehydrogenase (G6PDH),[18] membrane bound adenosinetriphosphatase (ATPase) and γ-glutamyl transpeptidase (γGT),[19] and adenylate cyclase.[20] Other slices were exposed to glutathione S-transferase placental form (GST-P) immune sera (gift of Dr. K. Sato, Hirosaki University, Japan), raised, and purified as described by Satoh et al.[21] and stained by the avidin-biotin-peroxidase complex method (ABC Vectastain Kit, Vector Laboratories, Burlingame, CA). The histochemical pattern of basophilic and glycogenotic areas was investigated in livers of two rats of the control, DEN and NNM groups sacrificed at 20, 35, and 50 weeks after the start of the experiment. For

each experimental time point, at least 20 areas of each type per group were analyzed. A total of 22 tumors (12 from DEN- and 10 from NNM-treated rats) were studied for the same enzymes.

Morphometry

Quantitation of numbers and volume of basophilic and glycogenotic areas was performed in livers of four rats of the control, DEN, and NNM groups sacrificed after 15, 25, 35, 45, and 55 weeks. Two consecutive cryostat sections of 2 μm from the left lobe were stained with cresyl violet and the PAS reaction. These sections were projected through a microscope (Zeiss Ultraphot) and their outlines as well as those of areas of altered hepatocytes were digitized by means of a digitizer (Digicad, Kontron) connected to a microcomputer (Compaq Deskpro 386/20). The developed program realized the superposition of the two digitized consecutive liver sections with the aid of fixed landmarks (hepatic veins) in sections. From the two-dimensional parameters of sections, the program calculated the number of basophilic and glycogenotic areas per square centimeter (Table 3), and gave an estimation of the number of the two types of area per cubic centimeter (Figure 25) as well as the volume (mm^3/cm^3) occupied by them[22] (Table 4).

Means and standard deviations obtained at each time point were calculated as descriptive parameters. The number and volume occupied by basophilic and glycogenotic areas were logarithmically transformed by omitting cases with zero values and were analyzed for the effect of treatment with DEN or NNM compared with control, as well as for differences between the two treatment groups by ANOVA methods[23,24] with time x treatment interaction. Adjustment for multiple testing was achieved by the Bonferroni-Holm procedure.[25] A time effect was tested by a one-way ANOVA and by Tukey's studentized range test.[26] Excellence of fit was judged by R^2. Time-dependent differences between basophilic and glycogenotic areas were assessed by a linear regression with time as an independent variable.[27,28] In any case, a P value less than 0.05 was judged as being statistically significant.

Results

Macroscopic Findings in Liver

Gross hepatic changes were found in both DEN- and NNM-treated rats from the 35th week of the experiment. The first changes were small, round, whitish spots on the liver surface. These spots appeared multicentric and increased progressively in number and size with time. By increasing in size they gave origin to protuberant tumors, the first ones at the 40th week of the experiment. Tumors were solid masses of nodular appearance and exhibited grayish color with frequent hemorrhagic areas. They increased also in number (Table 1) and size with time. Intraperitoneal hemorrhages from hepatic tumors were rarely seen in rats which died spontaneously.

Histological Findings in Liver

Areas of Altered Hepatocytes

Areas of altered hepatocytes and hepatocellular tumors were the unique lesions observed in rat livers in the present experiment. Two kinds of areas of cellular alteration were distinguished in livers of control, DEN- and NNM-treated rats: basophilic and glycogenotic areas. Both types of area were arranged multicentrically and increased in number and size with time. Basophilic and glycogenotic areas were sometimes disposed face to face. Areas composed of an intermingled population of

Table 1. *Tumor Incidence in DEN- and NNM-treated Rats**

	DEN-treated rats				NNM-treated rats		
	Liver tumors			Rats with esophageal papillomas	Liver tumors		
Experimental weeks	Rats with tumors	No. adenomas	No. carcinomas		Rats with tumors	No. adenomas	No. carcinomas
10,15	0/8	0	0	0/8	ND	ND	ND
20,25	0/16	0	0	0/16	0/8	0	0
30,35	0/8	0	0	0/8	0/8	0	0
40,45	10/13	3	7	6/13	7/16	2	5
50,55	22/22	5	29	22/22	13/14	4	10
60,65	ND	ND	ND	ND	24/24†	5	40

* The number of rats with tumors was determined macroscopically. The number of liver adenomas and carcinomas was obtained by examination of one transversal section of both left and median hepatic lobes per rat. ND, no data.

† Two rats with lung metastases from hepatocellular carcinomas.

basophilic and glycogenotic hepatocytes were, however, never observed.

Basophilic areas consisted of hepatocytes with increased lamellar or diffuse cytoplasmic basophilia. Most areas were exclusively made up of hepatocytes with a lamellar hyperbasophilia. More precisely, this was the unique type of basophilic areas in livers of control rats as well as in livers of DEN- and NNM-treated rats killed at early time points (until the 30th week) of the experiment. The lamellar appearance of the hyperbasophilia in hepatocytes was caused by the parallel disposition of thick basophilic trabeculae (lamellae) arranged perpendicularly to the cell surface facing Disse's space (Figure 1). Hepatocytes with lamellar hyperbasophilia were similar in size or slightly smaller than normal hepatocytes. Areas of these hepatocytes did not show alterations of the normal laminar structure of the liver (Figures 1, 2a). A thickening of the basophilic lamellae was associated with the enlargement of basophilic areas (compare Figures 1, 2b). In addition to hepatocytes with dense basophilia of the lamellar type, large basophilic areas often showed other hepatocytes with a powdered diffuse basophilia filling the entire cytoplasm (Figure 3). The latter type of hepatocytes was also characterized by variations in cell and nuclear sizes. Hepatocytes with diffuse basophilia increased in number at the latest time points of the experiment (Figure 4). A retention of glycogen was observed in about 20% of basophilic areas of different size at any time of the experiment in both hepatocytes with lamellar and diffuse hyperbasophilia (Figure 5a, b). (According to the protocol of Babcock and Cardell[16] followed, after the 22 hours fasting the glycogen content throughout rat hepatic lobule was strongly reduced.) At late time points of the experiment, vacuolated hepatocytes but no typical clear glycogenotic cells were frequently observed in basophilic areas (Figure 6). In these areas some basophilic hepatocytes showed few vacuoles (Figure 6), which suggest the origin of vacuolated from basophilic cells.

Basophilic areas displayed both a characteristic topographic disposition and shape. All the areas were near efferent hepatic veins, in para- (Figures 1, 5a) and perivenous (Figures 2a, 4, 6) dispositions. Moreover, by a detailed examination of the spatial disposition of a large number of areas in serial sections, it could be established that basophilic areas were located around and along efferent veins, the para- and perivenous dispositions depending on the plane of the section at which areas were cut. The small basophilic areas were without exception made up of pericentral hepatocytes in the immediate neighborhood of efferent veins (Figures 1, 5a). Large basophilic areas extended to longer distances, both parallel and centrifugally to the course of the efferent veins, being sharply limited at the level of the portal septae. Thus, the basic shape of basophilic areas resembled that of a sleeve, centered by an efferent vein, rather than of a sphere. Some basophilic areas, mostly of large size, exhibited a more complex or composed appearance. Characteristic examples of such areas are illustrated by Figures 2a, 7, 8, and 9. From these figures (and from detailed observations in serial sections), it is clear that the shape of such areas resembles that of the pericentral liver parenchyma domain that itself follows the branching pattern of the efferent hepatic veins.[29,30]

Basophilic areas composed mostly of hepatocytes with diffuse hyperbasophilia showed a loss of the normal hepatic structure with formation of thick trabeculae (Figures 4, 9, 10), and increased number of mitotic figures. These phenomena were often accompanied by compression of the adjacent hepatic parenchyma and invasion of efferent veins (Figures 11, 12). One or a few basophilic areas invading efferent hepatic veins were detected in DEN- and NNM-treated rats sacrificed after the 40th and 50th week, respectively, being seen in almost all the DEN- and NNM-treated rats sacrificed respectively at 50–55th and 60–65th week.

Beside basophilic areas, areas consisting of a varied proportion of hepatocytes with clear (Figure 13a) and acidophilic (ground-glass) cytoplasm were observed in livers of control, DEN- and NNM-treated rats. These areas were termed glycogenotic because of the high content of glycogen in both clear and acidophilic cells (Fig. 13b). Clear and acidophilic hepatocytes were generally larger than the normal surrounding hepatocytes. At late time points of the experiment, a varied number of vacuolated large hepatocytes were frequently observed in glycogenotic areas (Fig. 14). These hepatocytes also stored glycogen. Hepatocytes with lamellar or diffuse hyperbasophilia were not seen in glycogenotic areas. Like basophilic areas, glycogenotic areas appeared closely related to efferent hepatic veins and enlarged along and around them (Figs. 13 and 15). In contrast to basophilic areas, glycogenotic areas frequently compressed the surrounding hepatic parenchyma (Fig. 15). Furthermore, signs of neoplasia such as thickened trabeculae, vascular invasion, and increased numbers of mitotic figures were absent from glycogenotic areas.

Hepatocellular Adenomas and Carcinomas

Liver tumors observed macroscopically in DEN- and NNM-treated rats were hepatocellular adenomas and carcinomas. Hepatocellular adenomas were small nodular neoplasms observed much less frequently than carcinomas (Table 1). Two different types of adenomas were distinguished according to their cellular composition. Seventeen of the 19 adenomas examined under the microscope were composed of basophilic cells, whereas

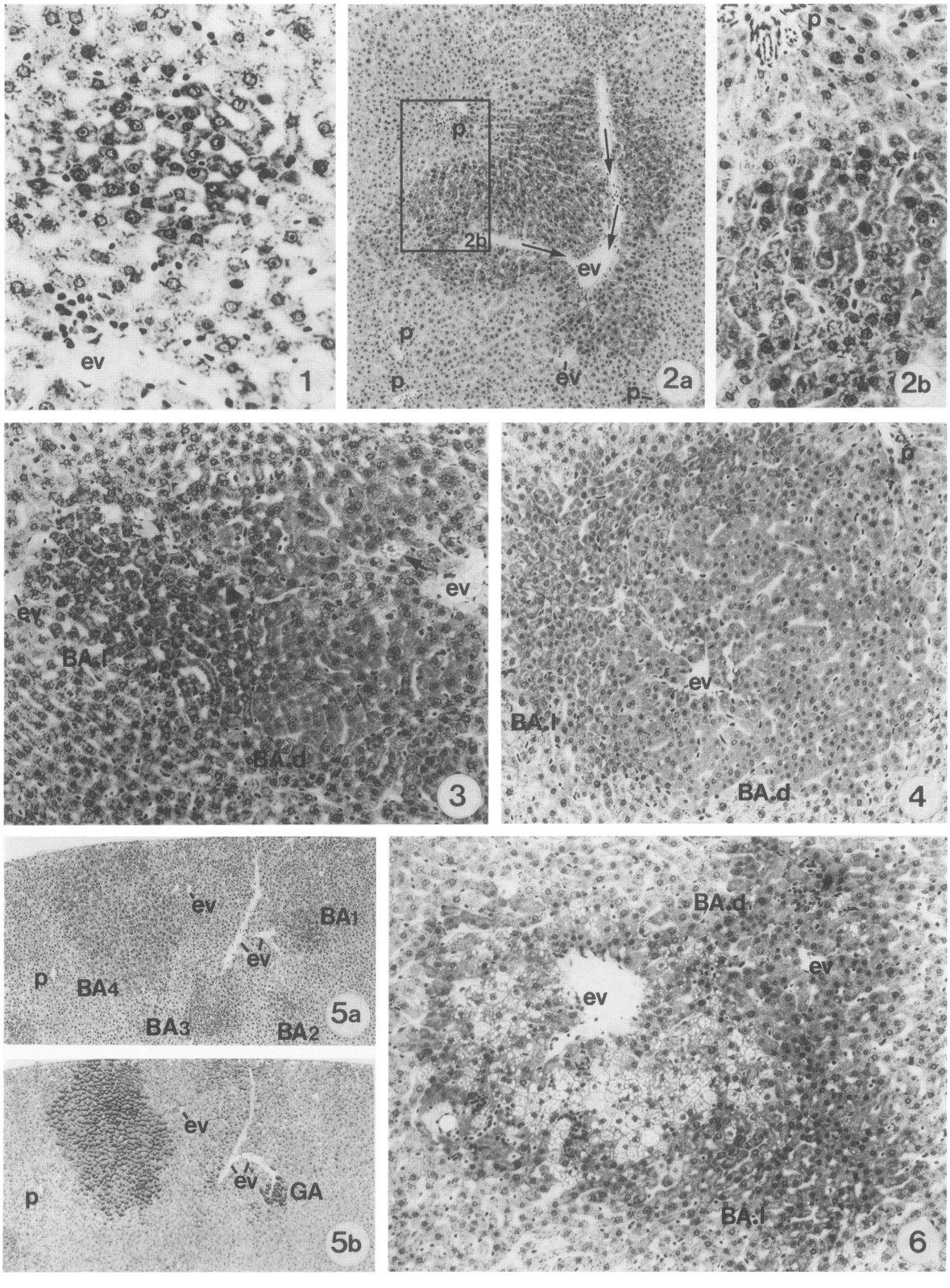


Figure 1. *Small basophilic area located in the vicinity of an efferent vein (ev). Note the ordered distribution in trabeculae (lamellae) of the basophilic bodies at the periphery of the cytoplasm. DEN-treated rat killed at the 25th week. Cresyl violet ×80.*

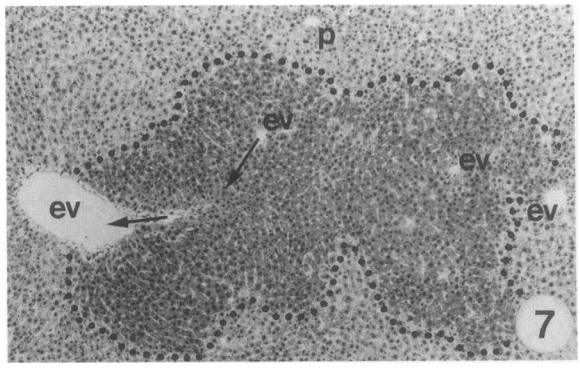
Figure 2. *Basophilic area at the confluence of two efferent veins (ev)* (**a**). *Note differences in thickness of the lamellar basophilia in the hepatocytes of this area* (**b**). *DEN-treated rat killed at the 35th week. Cresyl violet.* **a**, *×15;* **b**, *×70.*

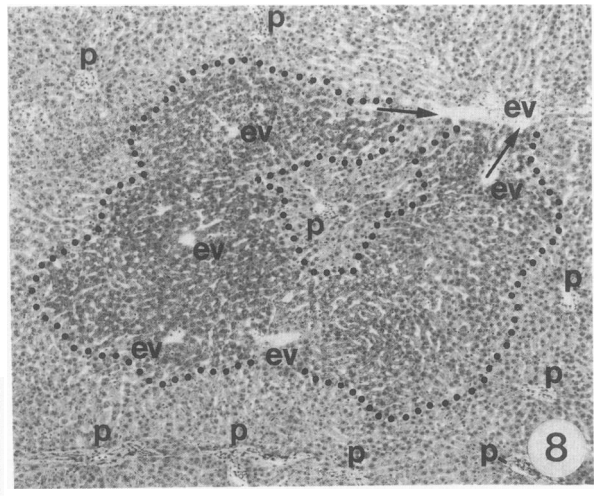
Figure 3. *Basophilic area composed of two portions of hepatocytes with lamellar (BA.l) or diffuse (BA.d) cytoplasmic hyperbasophilia. ev, efferent vein. Arrow, vacuolated cells. NNM-treated rat killed at the 35th week. Cresyl violet, ×50.*

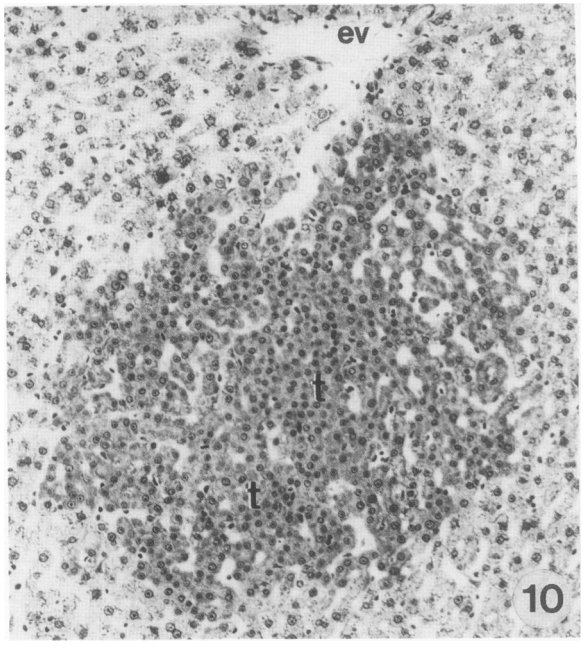
Figure 4. *Basophilic area around an efferent vein (ev) made up of hepatocytes with lamellar (BA.l) or diffuse (BA.d) basophilia. p, portal space. DEN-treated rat killed at the 40th week. Cresyl violet, ×40.*

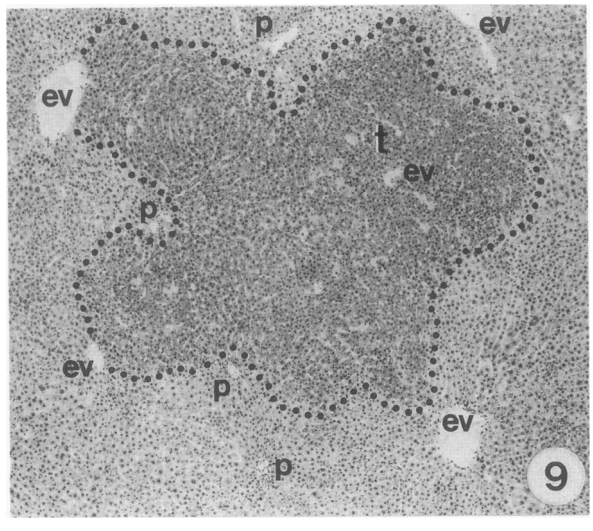
Figure 5. *Serial sections (***a**, *cresyl violet;* **b**, *PAS) showing four basophilic areas (BA1, BA2, BA3, BA4) and one glycogenotic area (GA). Note retention of glycogen in the largest basophilic area (BA4;* **b**) *composed of both hepatocytes with lamellar and diffuse basophilia, ev, efferent vein. p, portal space. DEN-treated rat killed at the 40th week, ×12.*

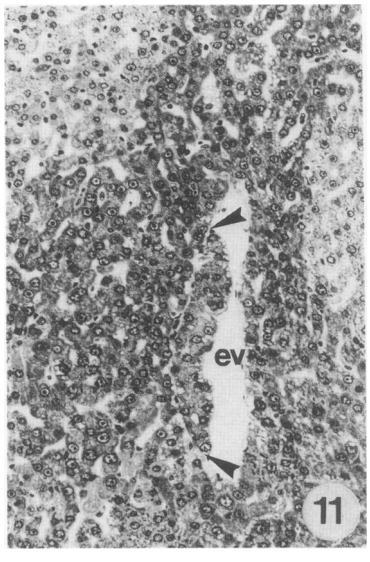
Figure 6. *Basophilic area consisting of hepatocytes with lamellar (BA.l) or diffuse (BA.d) basophilia and of vacuolated hepatocytes. Small vacuoles are also present in some basophilic hepatocytes. DEN-treated rat killed at the 45th week. Cresyl violet, × 45.*











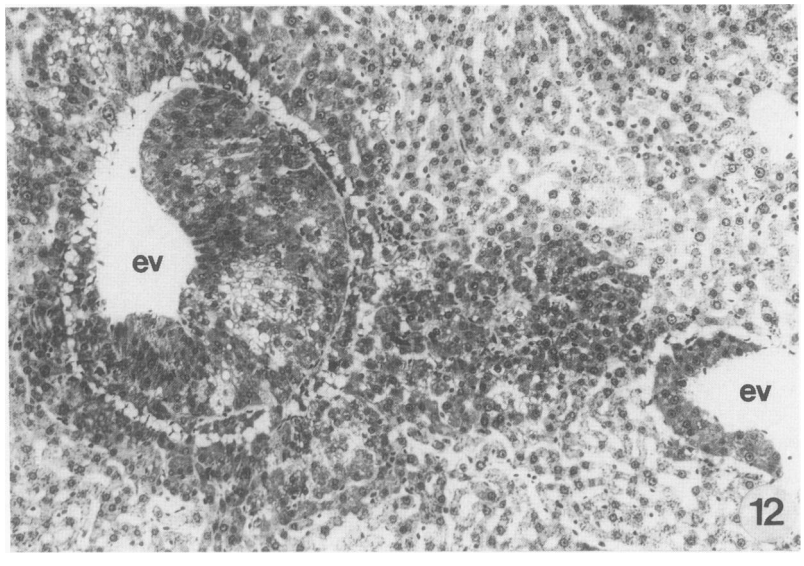


Figure 7. *Long basophilic area disposed as a sleeve around and along several efferent veins (ev) coming together (→). p, portal space. DEN-treated rat killed at the 40th week. Cresyl violet, ×15.*

Figure 8. *Basophilic area. The basophilic hepatocytes are located around and along small efferent veins (ev). Hepatocytes near portal spaces (p) do not show the basophilic change. NNM-treated rat killed at the 50th week. Cresyl violet, ×15.*

Figure 9. *Large basophilic area. Note that the area is sharply limited at level of several portal spaces (p), and that some basophilic hepatocytes are arranged in trabeculae thicker than normal (t). ev, efferent vein. DEN-treated rat killed at the 40th week. Cresyl violet, ×14.*

Figure 10. *Basophilic area near an efferent vein (ev) composed by both hepatocytes with lamellar and diffuse basophilia. Hepatocytes with diffuse basophilia are partly arranged in thick trabeculae (t). NNM-treated rat killed at the 45th week. Cresyl violet, ×45.*

Figure 11. *Basophilic area with a portion (→) within an efferent vein (ev). DEN-treated rat killed at the 50th week. Cresyl violet, ×50.*

Figure 12. *Basophilic area invading two efferent veins (ev). Note the presence among basophilic hepatocytes of some vacuolated cells. DEN-treated rat killed at the 45th week. Cresyl violet, ×50.*

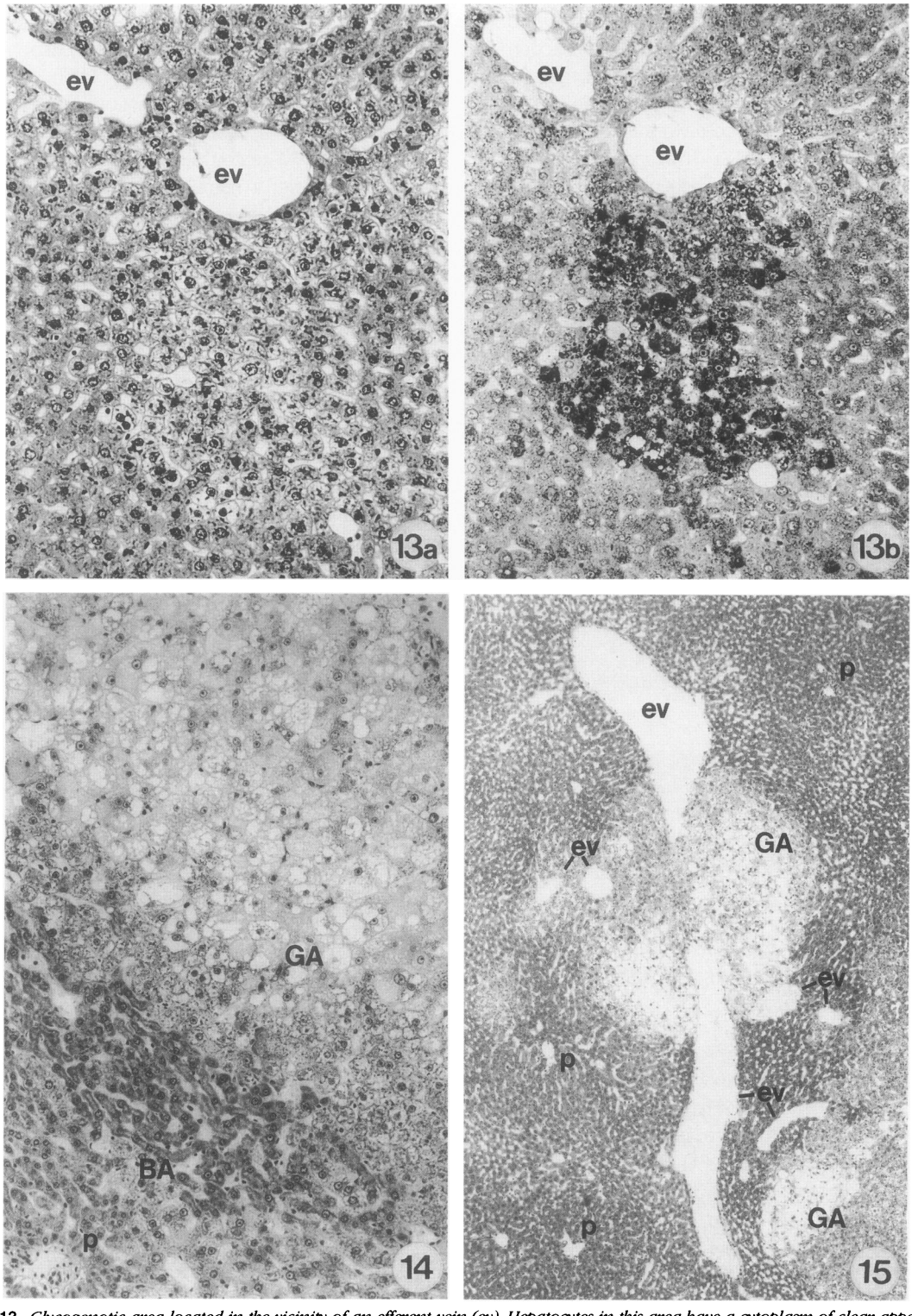


Figure 13. *Glycogenotic area located in the vicinity of an efferent vein (ev). Hepatocytes in this area have a cytoplasm of clear appearance after staining with cresyl violet* (a) *and show a strong retention of glycogen after the PAS reaction* (b). *NNM-treated rat killed at th 35th week, ×60.*

Figure 14. *Glycogenotic area (GA) adjacent to a basophilic area of the lamellar type (BA). Hepatocytes in glycogenotic area display a marked variation in size and appearance (clear, ground-glass, vacuolated cytoplasms). p, portal space, NNM-treaed rat killed at the 65th week. Cresyl violet, ×40.*

Figure 15. *Glycogenotic areas (GA) around efferent veins (ev). Note the compression of the adjacent normal hepatic parenchyma. p, portal space, NNM-treated rat killed at the 65th week. Cresyl violet, ×17.*

the remaining two adenomas were composed of clear and acidophilic cells. Basophilic adenomas consisted of both hepatocytes with lamellar and diffuse basophilia (Figure 16). A retention of glycogen after fasting was observed in some portions of 9 basophilic adenomas. In addition vacuolated large cells were frequent in basophilic adenomas (Figure 17). Thickened trabeculae of cells typical of carcinomas were found in portions of basophilic adenomas (Figure 18a, b, c). The two adenomas composed of clear and acidophilic glycogenotic cells lacked portions with signs of carcinoma. These two adenomas also contained vacuolated cells (Figures 19, 20). All carcinomas were of the trabecular type and consisted of basophilic hepatocytes (Figure 21). Marked differences in the thickness of trabeculae and in the size of cells were usually noted from one portion to the other within carcinomas (Figure 22). Portions with adenoid structure were occasionally seen in carcinomas (Figure 23). A retention of glycogen was observed in some portions of about 50% of these tumors (Figure 24). Frequently, such portions contained vacuolated cells. Blood vessels invaded by tumor cells were common in carcinomas.

Lung Metastases and Esophageal Papillomas

Lung metastases from hepatocellular carcinomas were found in two rats treated with NNM which died at 60 and 65 weeks of the experiment. A high number of DEN-treated rats killed after the 40th week of the experiment showed multiple papillomas in the esophagus (Table 1).

Enzyme Histochemical Profile of Basophilic and Glycogenotic Areas and of Hepatocellular Tumors

As shown in Table 2, basophilic and glycogenotic areas as well as hepatocellular tumors were characterized by specific changes in the activity of the enzymes investigated. The enzyme histochemical pattern of each type of area was similar in control, DEN- and NNM-treated rat livers examined at weeks 20, 35 and 50 of the experiment. The tumors investigated, adenomas and carcinomas, all made up of basophilic cells, also showed a similar enzyme histochemical pattern in DEN- and NMM-treated rats.

Number and Total Volume of Basophilic and Glycogenotic Areas

Basophilic and glycogenotic areas were seen for the first time in control rats at 15 and 20 weeks, respectively. In carcinogen-treated rats, both types of area were simultaneously detected, at the 15th week in DEN rats and at the 20th week in NNM rats. The number of basophilic and glycogenotic areas per surface (cm^2) (Table 3) in control rats was low and similar, and did not become higher with time. In comparison, basophilic and glycogenotic areas in DEN- and NNM-treated rats were much more frequent, and they increased in number with time. No difference in incidence between the two types of areas in DEN- or NNM-treated rats was noted. These results corresponded to the estimated number of areas per volume (cm^3) by Saltykov's method[22] (Figure 25). The curves clearly showed that the number of basophilic and glycogenotic areas increased in parallel with time both in DEN- and NNM-treated rats.

The volume of the liver occupied by basophilic and glycogenotic areas, estimated from the number and size of areas in histological sections, is shown in Table 4. In control rats, the volume occupied by the two types of areas was small and did not enlarge with time, with the exception of the higher volume occupied by glycogenotic areas at the 55th week. No difference in the volume of each type of area was noted. In contrast, the volume occupied by basophilic and glycogenotic areas in DEN- and NNM-treated rats increased progressively with time to high values. Moreover, basophilic areas occupied a larger volume than the glycogenotic ones. This was a marked difference in DEN-treated rats and a tendency in NNM-treated rats ($0.05 < P < 0.1$). The volume occupied by glycogenotic areas was greater in NNM- than in DEN-treated rats.

Discussion

Liver tumors induced by DEN or NNM at low doses were adenomas and carcinomas histologically similar to most hepatocellular tumors produced by DEN or NNM continuously administered at higher doses,[11,13] and by other carcinogens.[1] Carcinomas were trabecular structures of basophilic cells. Most adenomas were also basophilic but in two cases they consisted, as glycogenotic areas, of clear and acidophilic cells. In line with this finding is the uncommon variant of clear (glycogenotic) cell hepatocarcinoma observed in humans, a tumor type believed to derive from areas of clear (glycogenotic) hepatocytes.[31,32] The comparative low incidence of adenomas in the present study (Table 1) may be due to the direct development of carcinomas from basophilic areas, a number of which presented signs of malignancy. Signs of carcinoma, such as thickened trabeculae, were also present in portions of basophilic adenomas (Figure 18), suggesting the evolution of these tumors to carcinomas.

Two types of areas made up of basophilic or glycogenotic hepatocytes were observed in both control and

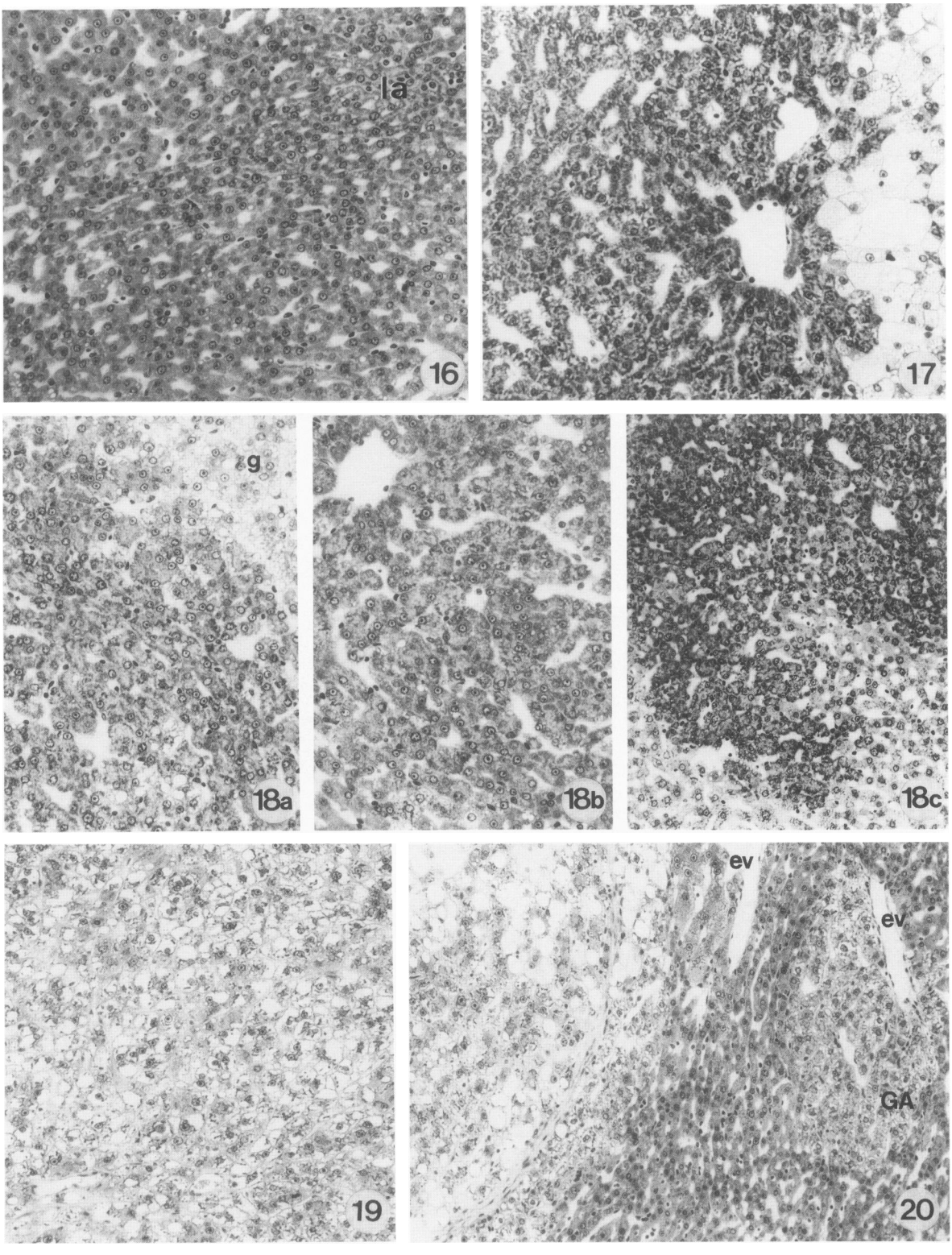


Figure 16. *Hepatocellular adenoma composed of hepatocytes with lamellar (la) or diffuse hyperbasophilia. Note presence of small vacuoles in some tumor cells. NNM-treated rat killed at the 60th week. Cresyl violet, ×65.*

Figure 17. *Hepatocellular adenoma made up mostly of cells with lamellar basophilia and, in addition, of large vacuolated cells. NNM-treated rat killed at the 60th week. Cresyl violet, ×70.*

Figure 18. *Three portions from one hepatocellular adenoma.* **a**: *Note cellular heterogeneity in central parts of the tumor. Beside the predominant tumor cells with lamellar or diffuse basophilia, other cells of clear appearance were observed. The latter (g) contain vacuoles and retain glycogen in their cytoplasm (observations from parallel sections).* **b**: *Thick trabeculae of basophilic cells.* **c**: *Marginal part of the tumor consisting of hepatocytes, with lamellar basophilia, arranged according to the normal laminar hepatic structure. NNM-treated rat killed at the 40th week. Cresyl violet, ×65.*

Figure 19. *Hepatocellular adenoma of the glycogenotic type composed of cells with cytoplasm of clear, ground-glass, and/or vacuolated appearance. NNM-treated rat killed at the 60th week. Cresyl violet, ×50.*

Figure 20. *Glycogenotic adenoma. GA, glycogenotic area. ev, efferent vein. NNM-treated rat killed at the 65th week. Cresyl violet, ×50.*

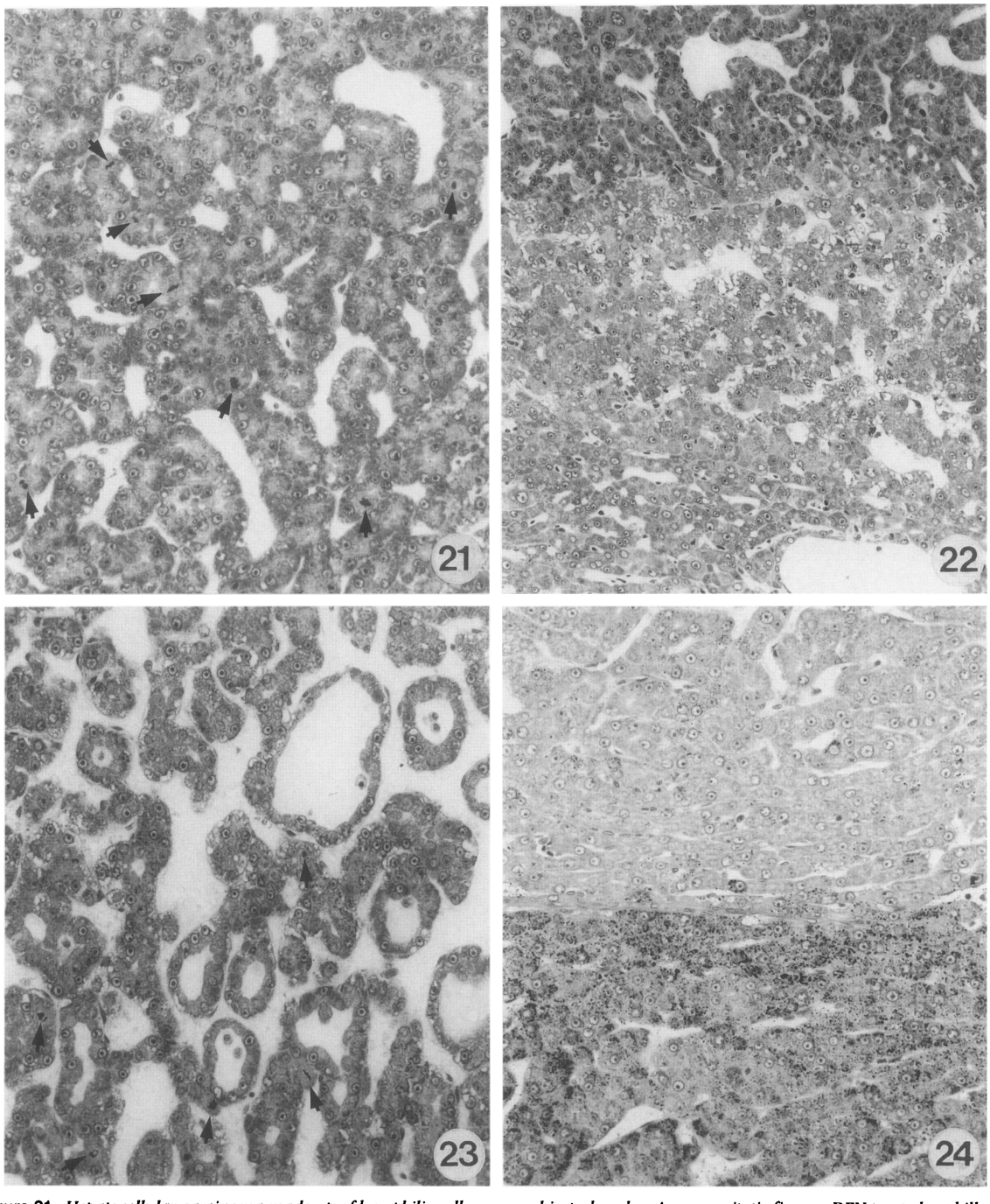


Figure 21. *Hepatocellular carcinoma made up of basophilic cells arranged in trabeculae. Arrows, mitotic figures. DEN-treated rat killed at the 55th week. Cresyl violet, ×65.*
Figure 22. *Hepatocellular carcinoma. Basophilic tumor cells display marked polymorphism, some showing cytoplasmic holes (glycogen storing places), and are arranged in irregular trabeculae. NNM-treated rat killed at the 60th week. Cresyl violet, ×45.*
Figure 23. *Hepatocellular carcinoma. Portion of tumor with an adenoid structure. NNM-treated rat killed at the 55th week. Cresyl violet, ×65.*
Figure 24. *Hepatocellular carcinoma. Variable content (retention) of glycogen in basophilic tumor cells. DEN-treated rat killed at the 50th week. PAS, ×45.*

carcinogen-treated rats. The presence of basophilic and glycogenotic areas in the control rats is in line with descriptions on spontaneous development of foci of altered hepatocytes, of various types, in aged rats of several strains, among them CD rats,[33] WAG/Rij rats,[34] BN/Bi rats,[34] (WAG × BN) F1 rats,[34] Wistar rats,[35] female Sherman rats[36] and Fischer-344 rats.[7,37] The incidence of the focal hepatic lesions is variable between these strains

Table 2. *Enzyme-histochemical Pattern of Basophilic and Glycogenotic Areas and of Hepatocellular Tumors**

Enzyme	Area†		Tumor‡ (22)
	Basophilic	Glycogenotic	
G6Pase	↓ ↓	NC, ↓‖	↓ ↓ (21), ↑ (1)
G6PDH	↑	↑ ↑	↑ + ↑ ↑ (22)
ATPase	↓	↓	↓ (22)
γGT	ND	↑	ND(13), ND + ↑ (9)
GST-P	ND, ↑¶	↑	↑ (10), ND + ↑ (12)
Adenylate cyclase	↑, NC#	↓ ↓	↑ (21), ↓ (1)

* The enzymatic reaction in areas of altered hepatocytes and tumors was evaluated as compared with that of control livers. Signs: ↑, ↑ ↑, ↓, ↓ ↓, moderate and strong increase (↑, ↑ ↑) or decrease (↓, ↓ ↓) reactions. NC, not changed. ND, not detected as in normal hepatic parenchyma, ND + ↑, tumor containing portions both with and without enzymatic reaction.

† Areas were investigated in livers of two rats from control, DEN and NNM groups killed after 20, 35, and 50 weeks from the start of the experiment. For each experimental time point, at least 20 areas of each type per two DEN- and two NNM-treated rats were examined.

‡ 12 tumors (2 adenomas and 10 carcinomas) from DEN-treated rats; 10 tumors (1 adenoma and 9 carcinomas) from NNM-treaed rats.

‖ Seen in some glycogenotic areas of DEN- and NNM-treated rats killed at the 50th week.

¶ Detected in some parts of large basophilic areas from DEN- and NNM-treated rats killed at the 50th week.

Some small areas in livers of rats killed at the 20th week.

and even within a single strain. In our (male Sprague-Dawley) control rats, basophilic and glycogenotic areas were already detected at 15th and 20th week, respectively, and their incidences did not increase significantly up to the last time points (45th and 55th week) of the experiment investigated. Aging rats of strains with high numbers of areas of cellular alteration are also affected by increased incidences of hepatocellular adenomas and carcinomas and a histogenetic relationship has been suggested.[7,34] Thus, basophilic areas have been proposed as beginners of both adenomas and adenocarcinomas in Fischer-344 rats.[7] The cause of the spontaneous development of areas of cellular alteration, as well as of hepatocellular tumors, in rats is unknown. However, it is evident that it partly depends on endogenous factors. Upon a specific genetic background and hormonal status, exogenous factors such as carcinogens contaminating rat diets[38] but also non-genotoxic natural compounds[39] might contribute to elicit both the initiation and growth of the focal hepatic lesions.

Both DEN and NNM given at low doses strongly increased the incidence of basophilic and glycogenotic areas. Each type of area was endowed, independently of the carcinogenic treatment, with typical cytologic features. In accordance with a previous description,[15] we could distinguish two kinds of hepatocytes with lamellar or diffuse cytoplasmic hyperbasophilia in basophilic areas of carcinogen-treated rats. Areas made up exclusively of hepatocytes with lamellar hyperbasophilia were the first basophilic areas observed in treated rats, as well as the unique type of basophilic area encountered in control rats. This type of basophilic area has also been observed, although not subclassified, by other authors, judging by descriptions and illustrations presented by them. Thus, these areas have been noted in rats administered aminoazo dyes,[3,4] N-2-fluorenylacetamide,[5] and aflatoxin B1,[6] as well as in untreated rats.[7,34] Under the term "tigroid cell foci," this type of area has been reported at increased incidence in rats treated with a single dose of aflatoxin B1.[40] Tigroid cells, rich in stacks of rough endoplasmic reticulum, composed not only areas but also a few small nodules; hepatocellular carcinomas were, however, not observed in these rats.[40]

At late experimental time points an increasing number of basophilic areas in both DEN- and NNM-treated rats, but not in control rats, consisted also of hepatocytes with

Table 3. *Number of Basophilic and Glycogenotic Areas per Square Centimeter in Livers of Control, DEN- and NNM-treated Rats at Different Time Points of the Experiment**

Weeks	Basophilic areas			Glycogenotic areas		
	Control	DEN†	NNM†	Control	DEN†	NNM†
15	1.2 ± 2.4	16.0 ± 5.2‡	ND	0	11.4 ± 6.0‡	ND
25	1.6 ± 2.0	23.0 ± 11.4‖	22.8 ± 18.1¶	1.1 ± 1.2	18.3 ± 9.1‖	21.6 ± 5.8#
35	12.3 ± 16.1	86.1 ± 38.4	57.5 ± 27.0	2.2 ± 2.6	49.9 ± 19.0	54.7 ± 30.5
45	4.4 ± 3.0	82.6 ± 27.5	76.3 ± 24.9	4.5 ± 2.5	75.0 ± 25.1	72.3 ± 12.0
55	2.4 ± 2.8	ND	100.4 ± 16.9	6.6 ± 6.0	ND	91.0 ± 24.5

* Results are expressed as mean ± SD of four rats. ND, no data.

† Overall differences in the number of basophilic or glycogenotic areas between DEN- or NNM-treated rats and control rats, $P < 0.05$.

‡ Differences in the number of basophilic or glycogenotic areas between 15 and 35, 45 weeks, respectively, $P < 0.05$.

‖ Differences in the number of basophilic or glycogenotic areas between 25 and 35, 45 weeks, respectively, $P < 0.05$.

¶ Differences in the number of basophilic areas between 25 and 45, 55 weeks, respectively, $P < 0.05$.

Differences in the number of glycogenotic areas between 25 and 35, 45, 55 weeks, respectively, $P < 0.05$.

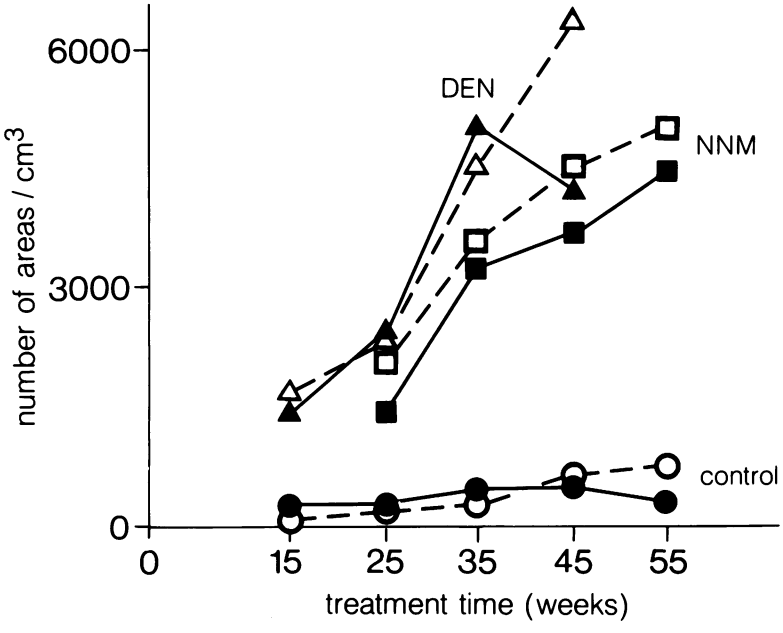


Figure 25. *Estimated number of basophilic (solid lines) and glycogenotic (broken lines) areas per cubic centimeter in control, DEN- and NNM-treated rats at different time points of the experiment (Saltykov's estimation). Each point represents the mean value of four rats.*

cytoplasmic hyperbasophilia. Interestingly, as reported by Reparaz[15] the appearance of these hepatocytes was frequently associated with other signs such as thickened trabeculae, increased numbers of mitotic figures, and vascular invasion, all which suggest the progression of these areas to hepatocellular adenomas and carcinomas. Of them, we believe that the development of thick trabeculae of basophilic cells is the main sign of progression to carcinoma. The vascular invasion by basophilic areas would constitute another expression of distorted growth and would partly depend on the development of the areas in the vicinity of efferent veins. The almost complete absence of metastases in DEN- and NNM-treated rats does not disprove the preneoplastic and neoplastic character of basophilic areas. Despite vascular invasion being a feature of hepatocellular carcinomas, only two cases of lung metastases were registered (Table 1). Likewise, Ward[7] reported the absence of distant metastases from spontaneous rat hepatocellular carcinomas which invaded blood vessels. Vascular invasion by basophilic areas has previously been observed in mice administered a single dose of DEN.[41,42] In these mice, in which basophilic areas were the only precursors of hepatocellular carcinomas,[41,42] the growth of areas into central veins of hepatic lobules has been submitted to a different interpretation. Whereas according to Goldfarb et al.[41] the malignant and premalignant character of the basophilic areas is also supported by the finding that some of them were found to invade terminal hepatic veins, Vesselinovitch and Mihailovich[42] suggested a hyperplastic rather than neoplastic nature for the growth (herniation) of basophilic areas into the veins.

Table 4. *Estimated Volume Occupied by Basophilic and Glycogenotic Areas in Livers of Control, DEN- and NNM-treated Rats at Different Time Points of the Experiment**

	Basophilic areas			Glycogenotic areas		
Weeks	Control	DEN†‡	NNM†	Control	DEN†	NNM†‖
15	0.05 ± 0.1	2.0 ± 0.6¶	ND	0	0.7 ± 0.4¶	ND
25	0.2 ± 0.2	3.2 ± 1.4≠	11.1 ± 14.8**	0.1 ± 0.1	1.6 ± 0.9≠	3.2 ± 1.7‡‡
35	0.6 ± 0.3	36.2 ± 25.5	32.2 ± 21.9	0.2 ± 0.3	8.8 ± 7.4	20.0 ± 17.0
45	0.6 ± 0.4	36.9 ± 14.8	69.6 ± 47.8	0.5 ± 0.4	11.0 ± 3.7	26.6 ± 12.8
55	0.5 ± 0.6	ND	78.7 ± 15.1	1.8 ± 1.5††	ND	40.9 ± 11.9

* Results represent the mean volume (±SD) in mm^3/cm^3 of four rats. ND, no data.
† Overall differences in the volume of basophilic or glycogenotic areas between DEN- or NNM-treated rats and control rats, $P < 0.05$.
‡ Overall differences between the volume occupied by basophilic and glycogenotic areas in DEN-treated rats, $P < 0.05$.
‖ Differences in the volume occupied by glycogenotic areas between NNM- and DEN-treaed rats at weeks 25, 35 and 45, $P < 0.05$.
¶ Differences in the volume of basophilic or glycogenotic areas between 15 and 35, 45 weeks, respectively, $P < 0.05$.
≠ Differences in the volume of basophilic or glycogenotic areas between 25 and 35, 45 weeks, respectively, $P < 0.05$.
** Differences in the volume of basophilic areas between 25 and 45, 55 weeks, respectively, $P < 0.05$.
†† Differences in the volume of glycogenotic areas between 55 and 25, 35, 45 weeks, respectively, $P < 0.05$.
‡‡ Differences in the volume of glycogenotic areas between 25 and 35, 45, 55 weeks, respectively, $P < 0.05$.

Glycogenotic areas consisted of a varied proportion of clear, acidophilic, and vacuolated cells, as described in detail by others.[11,12] In contrast to basophilic areas, no signs of malignancy were discovered in glycogenotic areas. Moreover, no evolution of glycogenotic areas to basophilic areas or carcinomas could be established. Nonetheless, our cytologic data give certain indications that glycogenotic adenomas were enlarged forms of glycogenotic areas.

From our data, basophilic and glycogenotic areas were initially small centrilobular areas of transformed hepatocytes which further enlarged centrifugally. The areas extended both around and along efferent hepatic veins, being sharply limited at portal spaces and from the unaltered or transformed hepatocytes of neighboring lobules. Such a location and pattern of growth indicates that the adequate unit of liver parenchyma for ordering topographically basophilic and glycogenotic areas is the hepatic lobule, as redefined by Matsumoto and Kawakami[29] and Lamers et al.,[30] but not Rappaport's acinus.[43] A centrilobular location has also been reported for basophilic areas observed in DEN-treated rats[10,13,15] and mice.[41,42] Concerning "tigroid cell foci," they have been suggested to develop predominantly in the second zone of Rappaport's acinus.[40] This location may be open to discussion because of the difficulties to identify the boundaries of Rappaport's zones in single liver sections.[30] Glycogenotic areas induced in rats by DEN at high and low doses have been reported to be in centrilobular regions[10,13,15] as is the case in the present study. By contrast areas of clear and/or acidophilic glycogenotic hepatocytes induced by NNM at high doses have been reported to be periportally located.[11] This discrepancy is probably due to NNM dose dependency. At high doses, this carcinogen intensively damaged the central parts of the lobule, leaving the periportal ones free from such toxic effects.[11] A periportal location has also been indicated for ATPase deficient foci induced in rats by a single dose of N-nitrosomethylurea after partial hepatectomy.[44]

Considering the characteristic topography and shape of basophilic areas and the fact that they developed multicentrically and up to high numbers, it seems likely that several areas might having come together to give origin to some of the large basophilic areas, particularly those exhibiting a composed or complex appearance (as those in Figs. 2a, 7, 8, and 9). Therefore, it is also probable that some basophilic tumors had developed from several rather than from single basophilic areas.

Basophilic and glycogenotic areas were characterized not only by specific cytologic features but also by distinct enzyme-histochemical patterns. Areas with intermediary or mixed histochemical profiles were never observed. Interestingly, the G6Pase was strongly reduced in basophilic areas but it was unchanged in the majority of glycogenotic areas. Basophilic areas with reduced G6Pase have also been observed in rats given a diet containing 5 p.p.m. aflatoxin B1 for 6 weeks[6] and mice given a single dose of DEN,[41,45] but an unchanged or slightly reduced enzyme reaction was seen in rats treated with a single dose of aflatoxin B1.[40] In contrast to the present results, clear (glycogenotic) cell areas induced by DEN[9] and NNM[46] at higher doses showed a reduced G6Pase. The increased G6PDH reaction in both basophilic and glycogenotic areas is in accordance with data in the literature.[6,40,45,46] The reduced ATPase in the two types of area has already been reported[6,9] but an unchanged or increased enzyme reaction has been noted in basophilic areas of rats and mice treated with a single dose of, respectively, aflatoxin B1[40] and DEN.[41] The γGT, an assumed marker for murine hepatocarcinogenesis[47] was only detected in glycogenotic areas. Another proposed marker, the GST-P[21] was detected in portions of large basophilic areas and in all glycogenotic areas. The adenylate cyclase reaction was increased in most basophilic areas and, as already reported by Ehemann et al.,[48] reduced in glycogenotic areas. In contrast to the histochemical demonstration of this enzyme, we have measured normal levels of adenylate cyclase activity in basophilic areas by means of a reliable biochemical method.[49] We therefore conclude that the histochemical method employed[20] is not reliably reflecting adenylate cyclase activity.[49]

As compared with basophilic and glycogenotic areas, hepatocellular tumors showed less homogeneity in the reaction of the studied enzymes (Table 2). This heterogeneity was, however, less marked than that of rat hepatocellular tumors induced by N-2-fluorenylacetamide.[50] Whereas γGT and GST-P were the enzymes with more variation from one tumor to the other, the G6Pase was strongly decreased in 21 of 22 tumors as occurred in basophilic areas. The same number of tumors showed increased adenylate cyclase activity but, as mentioned for basophilic areas, these results are in opposition to the sharply reduced adenylate cyclase activities we have measured biochemically in the same tumors.[49]

The results obtained in the present and other works on the cytologic and histochemical properties of areas of altered hepatocytes and hepatocellular tumors reveal the significance of the experimental design for hepatocarcinogenesis. Among other factors, the dose of carcinogen applied for tumor induction is of first importance in determining the nature and potency of tumor precursors. In fact, the role of the dose is clearly pointed out by our results. Two different substances such as DEN or NNM continuously given at very low doses induced an elevated number of two distinct types of areas made up of basophilic and glycogenotic hepatocytes. For basophilic

areas, the evolution to adenomas and carcinomas could be easily established, but for glycogenotic areas, in spite of their frequency, only an infrequent enlargement to glycogenotic adenomas could be inferred. The central role of basophilic areas for hepatocarcinogenesis has also been noted in other experiments, in which basophilic areas were the unique or predominant areas preceding hepatocellular tumors developed spontaneously in rats[7] or chemically induced in rats[4–6,15] and mice.[41,42] Our data contrast strongly with those reported from some experiments conducted with higher doses of DEN or NNM. After high dosages, glycogenotic areas have been described, as well as unspecific toxic changes, as the early and predominant focal hepatic changes and have been said to further suffer a conversion to basophilic areas and to hepatocellular adenomas and carcinomas.[9–12,46] Furthermore, Moore et al.[12] in a study conducted with NNM on the dose dependence and sequential appearance of focal hepatic lesions reported that the small basophilic areas described as early findings by Williams et al.[5] and Butler et al.[6] could be the result of treatments with highly toxic doses of carcinogens. This assumption is, however, refuted by the present data. We have used NNM at doses much lower than those of Moore et al.[12] and neither a predominance of glycogenotic areas nor their transformation into basophilic areas and carcinomas was observed. So it can be concluded that glycogenosis in tumor precursors is a dose-related phenomenon. Of course, the cytologic, histochemical, and biochemical changes of the areas of altered hepatocytes and tumors can be complicated by various factors such as the dose of carcinogen and the simultaneous or sequential administration of other substances such as carcinogens and hormones, but the new changes so elicited cannot be proposed as essential for or closely related to tumor development.

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