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Glucose-6-phosphatase activity in regenerating cholangiole cells and hepatoma cells*

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Abstract

Histogenesis of hepatic cancer has been analysed by observing glycogen by PAS staining and the histochemically demonstrable G-6-Pase activity on the liver of rats fed with 3'-Me-DAB or 3'-Ni-DAB. By observations on normal hepatic tissue it has been revealed that these two reactions are specific to the cytoplasm of liver parenchymal cells. Observations on the liver from the early stage of dye feeding, up to 100 days, show a marked proliferation of cholangioles in 3'-Me-DAB feeding on polished rice but only a poor reaction of cholangioles in 3'-Me-DAB feeding with synthetic diet. After 15-16 weeks of 3'-Me-DAB feeding cancer develops, a great erpart of which is consisted of cholangiocellular carcinoma and a portion, hepatocellular carcinoma. Histochemical observations on G-6-Pase and glycogen reveal that regenerating cholangiole and adenomatous tissues seem to have poles, on one side, the cells differentiate to liver parenchymal cells and on the other side, they differentiate to bile duct cells. Cancers develop mainly from these regenerating adenomatous tissues and they develop to cholangiocellular cancer or to hepatocellular cancer. The histogenesis of the latter can be traced histochemically. In the cases fed with 3'-Ni-DAB, the activity of cholangiole cells and the development of adenomatous tissue are rather poor with the delayed cancer formation. However, in these cases the majority of cancers are of hepatocellular carcinoma and the developmental mode of hepatocellular cancer can easily be traced by the G-6-Pase activity.

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GLUCOSE-6-PHOSPHATASE ACTIVITY IN REGENERATING CHOLANGIOLE CELLS AND HEPATOMA CELLS.

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The histogenesis of primary liver cancer has not yet been settled, though it is generally believed that hepatocellular carcinoma originates from liver parenchymal cells and cholangiocellular carcinoma from bile duct cells. For example, EWING[1] and others claim that hepatocellular cancer originates from the regenerating liver parenchymal cells which show nodular hyperplasia, first developing to adenoma and finally to carcinoma.

Cholangiocellular carcinoma has been suspected to develop on the tissues of cirrhotic liver (MILNE[2], and YAMAGIWA[3] *et al.*) or of cholangio-fibrosis (OPIE[4] and FIRMINGER[5]) in which cholangioles are proliferating.

Recently, GRANT and REES[6] suggested that the lack of vitamin B_{12} induces the proliferation of cholangiole cells, which may have close correlation with the development of cholangiocellular carcinoma. This suggests that regenerating cholangiole cells have the potentials to differentiate to bile duct cells and to bile duct cell carcinoma, though there is no concrete evidence.

For the clarification of histogenesis of liver cancers it may be a promising way to check the enzyme activities of the cells of normal, transitional and cancer cells found in the animal liver exposed to some carcinogenetic substance, as several enzymes are specific to liver parenchymal cells (NOVIKOFF[7]). Especially, the activity of the histochemically demonstrable glucose-6-phosphatase (G-6-Pase) [8,12-17], which is concerned with the glycogen metabolism and expected to appear in the cells differentiating to parenchymal cells, will give a clue to know the mother cells of each type of cancer. In this paper the findings revealed by studying G-6-Pase activities of the rat liver histochemically at various stages of carcinogenesis to hepatoma formation induced by azo dye feeding are demonstrated.

MATERIALS AND METHODS

One hundred and twenty male Wistar rats, weighing 125—200 gm, were used. They were divided into 4 groups. The animals of the first group, 10

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animals, were kept on polished rice. Animals of the 2nd group, 50 animals, were fed on the same diet but containing 0.006 per cent 3′-methyl-4-dimethyl aminoazobenzene (3′-Me-DAB), which had been prepared by mixing 1 cc of 6 per cent alcoholic dye solution with 1,000 g of polished rice and drying at room temperature. Those of the 3rd group, 50 animals, were kept on synthetic diet composed of 160 g of acid casein, 50 g of cotton seed oil, one droplet of halibut oil and containing 0.006 per cent 3′-nitro-4-dimethylaminoazobenzene (3′-Ni-DAB). Other 10 animals were of the pair fed controls of the 3rd group. Of 100 animals kept on the diet containing dyes 80 animals died on the way to cancer development. In these dead animals histologic observations only were made but not histochemical observations. The 20 animals surviving were killed during the period of from 130 to 360 days of dye feeding with their pair fed control and of these animals histochemical observations have been done with histologic observation. Besides these, the materials from a case of human hepatoma and some of the normal human liver, whose autopsy was carried out within a few hours after the death, were also observed histochemically as well as histologically. Pieces of the liver of these animals and autopsy materials were fixed in 10 per cent formalin and 95 per cent ethanol, dehydrogenized and paraffin sections were prepared.

Morphologic changes were observed on the tissues fixed with formalin and stained with hematoxylin eosin, polysaccharides on tissue fixed with ethanol and stained with periodic acid-Schiff reagent.

For the histochemical demonstration of G-6-Pase a modified CHIQUOIEN'S[8] method was employed on the frozen sections of fresh tissue. The sections of 10—13 μ in thickness were brought into the substrate mixture of pH 6.7 and incubated at 32°C for about 5 minutes. After five-minutes' incubation the tissue slices were washed with distilled water and exposed to 1 per cent ammonium polysulfate solution for about five minutes, washed again and postfixed with 6 per cent solution of neutral formalin, referring to the Gomori's method for phosphatase. Mounting with glycerin observations were conducted.

The substrate, the solution of potassium salt of G-6-P, was prepared by mixing 250 mg. of the barium salt of G-6-P suspended in 10 ml. of distilled water acidified with one or two drops of 2 *N* HCl, with approximately 120 mg. of potassium sulfate. After standing the solution for two hours at room temperature and stirring frequently, freed barium was removed by centrifugation. The supernatant was added with a pinch of potassium sulfate to see if the solution contained any trace of barium. If the precipitation occurs, the solution should be treated with the potassium sulfate solution again to eliminate barium by repeated centrifugation. The supernatant thus obtained was used as the original solution of the substrate and kept in a cold place. Before use, the fluid was diluted

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to 30 ml. with distilled water and the pH was adjusted to 6.7 with 1 *N* KOH. Next, one volume of this diluted solution was mixed with two volumes of 0.006 *M* lead nitrate (0.2 per cent). This solution served as the incubation medium, a slight precipitation could be removed through filter paper, if needed.

For the histochemical detection of alkaline and acid-phosphatases the Gomori's methods have been applied.

RESULTS

3'-Me-DAB proved to have a very powerful carcinogenic activity on the rat fed on polished rice and water. Gross and histologic pictures of the livers of the animals died at varied stages of dye feeding but within 100 days showed the changes on the way toward the cancer development, with several focuses of cirrhosis in one slice. Of course, most of animals survived more than 100 days had cancer.

The sequence of changes in the liver of the animals fed with the dye was actually the same as that described by PRICE and others[9]. After 21 days of dye feeding minute oval cell proliferation from the portal areas was observed in some lobules. This hyperplasia increased with time, so that by 49 days later many liver lobules were involved with small regenerating nodules. In the later stages, however, greater numbers of the proliferated oval cells were arranged surrounding small lumina, forming hepatic ducts, cholangioles, or the cholangiole hyperplasia. By 3 to 5 months liver cirrhosis developed with the proliferated connective tissues surrounding cholangioles in hyperplasia which showed an adenomatous change, and some livers had actually cancer.

The histochemical observation revealed the negative activity of G-6-Pase on the regenerating cholangiole cells and strong positive activity in parenchymal cells, especially marked in the cells situated in the peripheral area of the lobules. Nuclei were negative in activity in all cells. In the human liver having normal structures, the appearance of G-6-Pase activity was completely the same as that in rat liver. These findings suggest that histochemically demonstrable G-6-Pase activity is specific to liver parenchymal cells (Figs. 1, 2 and 3).

In the later stages of dye feeding, there developed a number of adenofibrotic areas which seemed to promote the cirrhotic change of liver. In these areas one could see the adenomatous arrangement of the cells which were irregular in shape and showed sometimes a great difference from each other in their shape and size, i. e. some of them were small and thin but some others big and rather cuboidal. The former resembled cholangiole cells and the latter parenchymal cells, and it suggested a possibility that the regenerating cholangiole could differentiate to liver parenchymal cells as suggested by ELIAS[10] in rat liver fed with bentnite and by SENO[11] in liver of the rat fed 3'-Me-DAB. G-6-Pase activity appeared

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fairly strong in the big cuboidal cells and negative in the small and thin ones. Besides these, there were adenomas being composed of the annular arrangement of cylindrical cells, whose picture resembled bile duct. Some cells composing this adenomatous tissue were still small and thin resembling cholangiole cells. This suggested the differentiation of regenerating cholangioles to bile duct. All of these cylindrical and thin small cells were completely negative in G-6-Pase activity. After 100—130 days of dye feeding the liver cancer developed. Most of them were of cholangiocellular cancer and negative in G-6-Pase activity (Figs. 4 and 5). But at later stages of dye feeding or in many cases of 3′-Ni-DAB feeding hepatocellular cancer or hepatoma appeared (Fig. 6), the cells of which contained glycogen (Figs. 7 and 8) and G-6-Pase positive granules (Fig. 9) by which it could be distinguished from cholangiocellular carcinoma. (Figs. 9 and 10). The hepatoma may develop further to an undifferentiated type, which lost glycogen granules and the G-6-Pase activity. The same type of cancer may develop directly from the undifferentiated regenerating cholangiole (Picture I and II). These undifferentiated cancers, however, will be put aside the present problem, as we are dealing with the correlation of the differentiations of cholangioles hepatic into and bile duct cells with the development of hepatocellular and cholangiocellular carcinomas.

The activity of alkaline phosphatase tested was strong in the capillary of Glisson's capsule and in the lumen of regenerating cholangioles, making it easy to find the regenerating cholangioles. 3′-Ni-DAB feeding caused relatively severe degeneration of parenchymal cells comparing to the 3′-Me-DAB feeding but the repair process of liver by the proliferating cholangiole cells was rather weak. i.e. after 20 days of dye feeding a marked degeneration of liver parenchymal cells occurred but poor in regenerating processes. After 6 to 7 month dye feeding, however, a slight proliferation of cholangioles appeared, which disappeared again in the later stage without developing to adenomatic change partially.

In these cases the development of adenofibrosis, which occurred after the disappearance of cholangiole hyperplasia and prior to cancer formation, was rather poor comparing to the cases of 3′-Me-DAB feeding. Cancer developed rather late at about one year after initiation of dye feeding, and the development of hepatocelluar cancer was predominant (Figs. 6 and 7), though cholangiocellular cancer was not infrequently. In the hepatocellular cancer some characteristics of normal parenchymal cells were observed persisting more or less marked, i. e. glycogen granules and G-6-Pase positive granules were seen in hepatoma cells, though there were a marked variety in the reaction intensities from cell to cell. That is, some cancer cells showed a markedly reduced or negative PAS staining and some others gave nearly a normal staining, though an increased staining of the normal liver cells surrounding the cancer tissue was

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general. (Fig. 8).

The cells of reduced staining in PAS also showed a low activity in G-6-Pase, and these cells were supposed to be the undifferentiated hepatoma cells (Fig. 9).

Observations on the liver supposed to be in the initial stage of hepatoma formation suggest that the development of hepatocellular cancer cells seems to be closely correlated to the adenofibrosis as well as to the development of cholangiocellular cancer. In those areas having adenomatous cell proliferation some cells show clearly the characteristics of liver parenchymal cells giving positive G-6-Pase activity, and in some hepatoma the picture is very similar to the adenomatous proliferation in cell arrangement and histochemical reaction. That is, the proliferating adenoma cells show a marked variety even in both glycogen granules and G-6-Pase activity, the ones that resembled the cancer tissue (Figs. 10, 11, 12 and 13) suggest that cancer will come from adenofibromatous change of hyperplastic cholangiole cells.

DISCUSSION

The findings described above indicate that in liver tissue, positive G-6-Pase activity and PAS positive glycogen granules in cytoplasm are of the characteristics to liver parenchymal cells, and by the application of these histochemical reactions the parenchymal cells can easily be distinguished from other cells, bile duct cells, cholangiole cells and connective tissue cells, etc., even in the tissue of severely distorted structure.

Observations proved that the degenerated parenchymal cells by being exposed to the azo dye, 3′-Me-DAB or 3′-Ni-DAB, seem to be replaced by the regenerating cholangiole cells which differentiate to parenchymal cells as clearly be seen by alkaline phosphatase reaction of the tissue as pointed out by SENO[11]. In such case the cholangiole cells acquire the positive G-6-Pase activity and PAS granules, when they show a tendency to differentiate to parenchymal cells. These very characteristics are inherited to hepatocellular cancer cells, though there are generally marked differences in the reaction intensities between individual cancer cells, but these characteristics, especially histochemically demonstrable G-6-Pase reaction proves to be quite convenient to trace the histogenesis of hepatoma, as can be seen on the pictures presented. (Picture. I)

As mentioned in the above sections there can be seen G-6-Pase negative hepatoma, undifferentiated hepatoma, but there are generally gradual change from G-6-Pase positive cells to negative ones or they are irregulary mixed with positive cells, with which they can be easily distinguished from cholangiocellular carcinoma which is originally negative in the reaction and never turns to positive cells. In the cases of 3′-Me-DAB feeding, the cancer, both of hepatocellular

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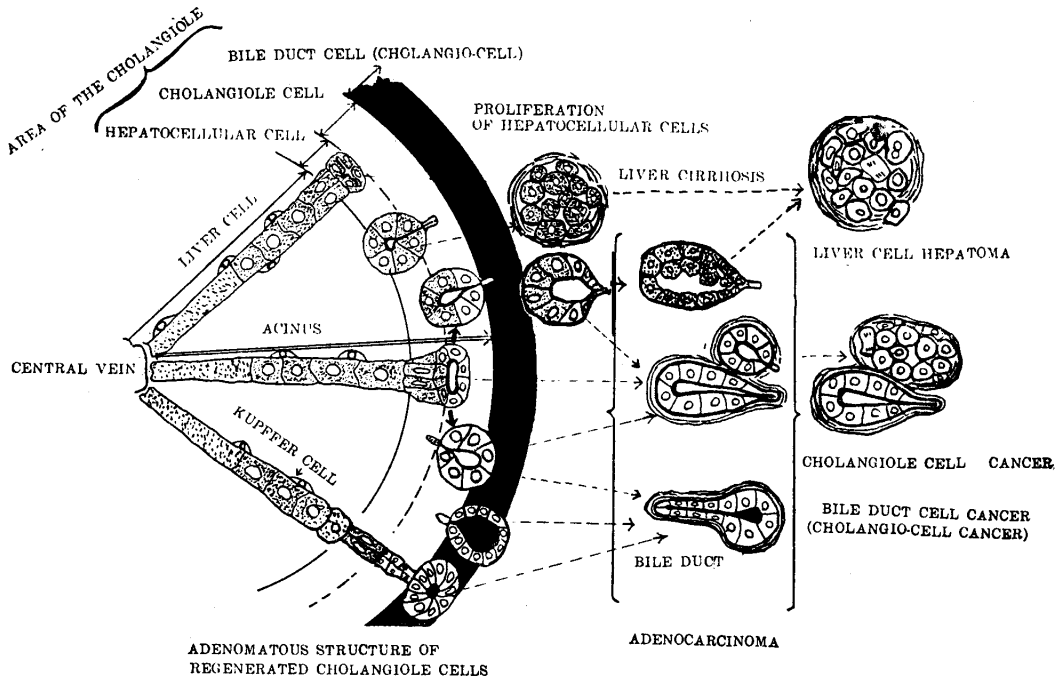


Picture I. Glucose-6-Phosphatase Activity of the Regenerating Cholangiole Cells at Various Stages of Development to Hepatoma and Cholangiole Cell Cancer.

: G-6-Pase Positive Cell: Liver Parenchymal Cell and Hepatocellular Cell of cholangiole.

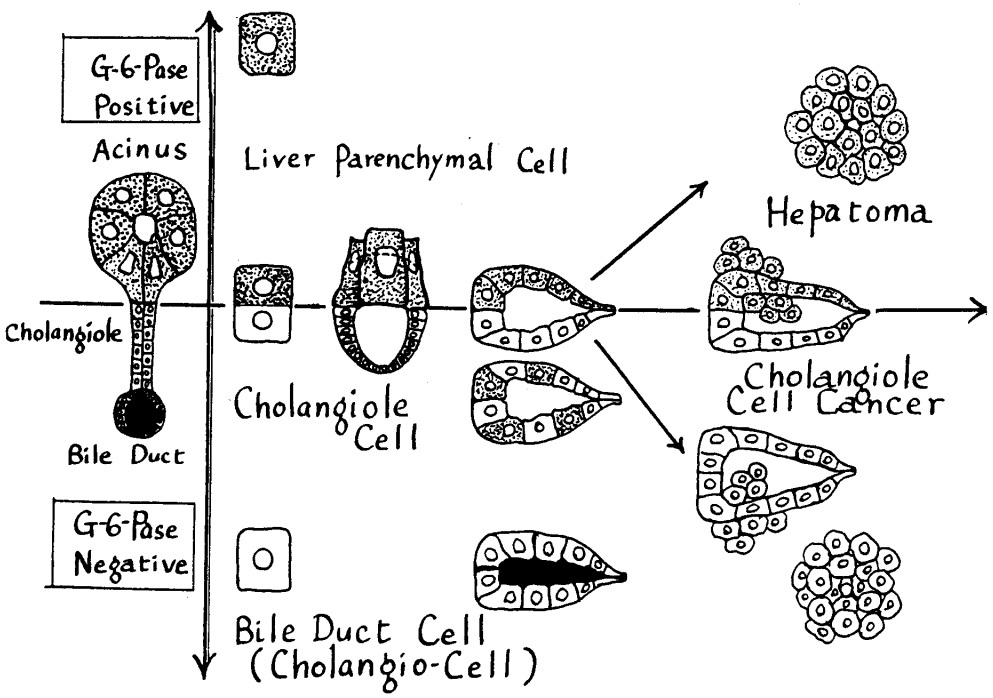
: G-6-Pase Negative Cell: Cholangiole Cell or Bile Duct Cell.

and cholangiocellular type, seems to develop from the adenofibrotic area or adenomatous proliferation. As mentioned by SENO it has been reconfirmed in this experiment that the regenerated cholangioles differentiate to parenchymal cells when they invade into acinus and those proliferate into Glisson's capsule and then into bile duct, forming adenomatous tissue with the proliferation of connective tissue.

Histochemical observations on G-6-Pase and PAS staining, however, reveal that some adenomatous cells, which can be found in the adenofibrotic area and look like those differentiated to bile duct cells, still have a tendency to differentiate to hepatic cells. Sometimes they increase in their size and show positive G-6-Pase activity and PAS positive granules. From these findings the authors are of the opinion that the regenerating cholangioles have always the potenciality to differentiate to hepatic cells even those grown to adenofibrosis area, though most of them seem to differentiate to bile duct cells. Differentiation of the adenomatous cells to hepatic cells seems to occur especially marked in those

lying in the peripheral area of adenofibrosis. Precise observation suggests that there seems to be the pole in a unit of regenerating gland just like in gastula stage of embryonic cell development as shown schematically in the picture. (Picture. II).

Thus the fact found by SENO, that is the differentiation of the regenerating cholangiole to hepatic cells in lobules and to bile duct cells in Glisson's capsule, may be based on regenerating cholangioles having poles, so that on one side the cells differentiate to hepatic cells and on other side to bile duct cells. The observations on the liver of rats fed with 3'-Me-DAB and 3'-Ni-DAB suggest both of hepatocellular cholangiocellular cancer can develop from the regenerating cholangiole. SENO stated other possibility that hepatocellular cancer may develop from the regenerating hepatic cells as well as from regenerating cholangioles. But he did not show any fact supporting his view that hepatocellular cancer should originate from regenerating hepatic cells. In the cases fed with 3'-Ni-DAB, cancer development



Picture II. Schematic Drawing of A Unit of Cholangiole and Its Differenciation to Liver Cell and Bile Duct Cell
arrow: Directions of Differentiation and Undifferentiation.

: G-6-Pase Positive Cell. Liver Parenchymal Cells

: G-6-Pase Negative Cell. Bile Duct Cells.

is delayed and most of the tumors developed are of hepatocellular type. It has been well ascertained that hepatoma cells will develop probably from regenerating parenchymal cells as well as from adenomatous tissue, especially from the hepatocellular cell of a unit of the cholangiole. In the cases fed with 3′-Ni-DAB as described precisely above, the activity of cholangiole cells are rather poor and almost all of them disappear before the development of cancer, even in the cases that have typical adenocarcinoma. It is obscure from this experiment whether this difference in tissue response is entirely due to the different characteristics of two dyes or due to the difference in diet in two group of animals, but the findings obtained on 3′-Me-DAB fed rats coincide well with those observed on rats fed 3′-Me-DAB with synthetic diet.

It must be briefly mentioned that the histochemically demonstrable G-6-Pase positive reaction will show only an extremely high activity of this enzyme, because any cells can not be free from glucose utilization as the energy source of the metabolism and should have a more or less amount of G-6-Pase, but such a slightamount of G-6-Pase can not be detected histochemically.

Consequently, in liver cells the histochemically demonstrable G-6-Pase activity will be that concerned with the storage and decomposition of glycogen. The existence of PAS positive granules in the hepatic cells and hepatocellular carcinoma coincides well with the positive reaction of G-6-Pase. Therefore, it is understood that these two histochemical reactions would offer an information on the differentiation of the cells to liver parenchymal cell.

CONCLUSION

Histogenesis of hepatic cancer has been analysed by observing glycogen by PAS staining and the histochemically demonstrable G-6-Pase activity on the liver of rats fed with 3′-Me-DAB or 3′-Ni-DAB. By observations on normal hepatic tissue it has been revealed that these two reactions are specific to the cytoplasm of liver parenchymal cells. Observations on the liver from the early stage of dye feeding, up to 100 days, show a marked proliferation of cholangioles in 3′-Me-DAB feeding on polished rice but only a poor reaction of cholangioles in 3′-Me-DAB feeding with synthetic diet.

After 15–16 weeks of 3′-Me-DAB feeding cancer develops, a great erpart of which is consisted of cholangiocellular carcinoma and a portion, hepatocellular carcinoma.

Histochemical observations on G-6-Pase and glycogen reveal that regenerating cholangiole and adenomatous tissues seem to have poles, on one side, the cells differentiate to liver parenchymal cells and on the other side, they differentiate to bile duct cells. Cancers develop mainly from these regenerating adenomatous tissues and they develop to cholangiocellular cancer or to hepatocellular

cancer. The histogenesis of the latter can be traced histochemically. In the cases fed with 3′-Ni-DAB, the activity of cholangiole cells and the development of adenomatous tissue are rather poor with the delayed cancer formation. However, in these cases the majority of cancers are of hepatocellular carcinoma and the developmental mode of hepatocellular cancer can easily be traced by the G-6-Pase activity.

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EXPLANATION FOR PHOTOS

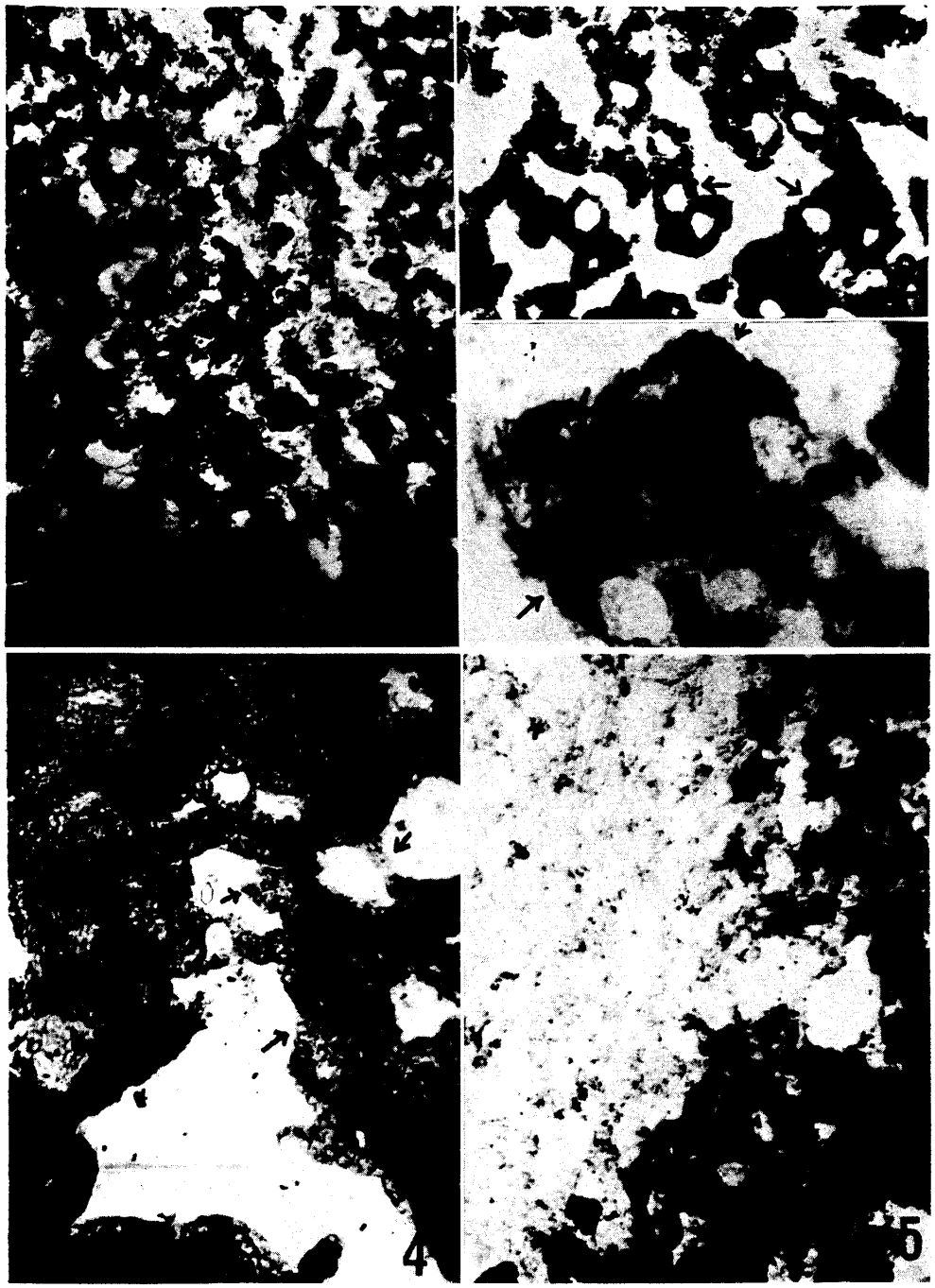
Fig. 1. G-6-Pase activity demonstrated in a human liver tissue having normal structure. A section from an autopsy case of hepatoma. Note the strong activity in the peripheral area of the lobules (P) and the weak reaction in the center. (C). 10×10

Fig. 2. G-6-Pase activity of the rat liver cells in the stage of liver cirrhosis. 14 weeks of 3'-Me-DAB feeding. 40×10

Fig. 3. An enlarged picture of G-6-Pase activity of liver parenchymal cells appearing almost normal. Cells are of those in the stage of liver cirrhosis. 13 weeks of 3'-Me-DAB feeding. Note the G-6-Pase positive granules (↓) densely distributed in the cytoplasma of liver parenchymal cells. 90×10

Fig. 4. Picture shows the G-6-Pase activity in an area of rat liver of 16 weeks of 3'-Me-DAB feeding. In this area the G-6-Pase negative adenoma tissues (cholangiocellular carcinoma. arrows) are invading into the liver tissue, whose parenchymal cells are of positive G-6-Pase activity and appear black in the picture. 10×10

Fig. 5. Negative G-6-Pase activity of cholangiocellular cancer from an autopsy case. 10×10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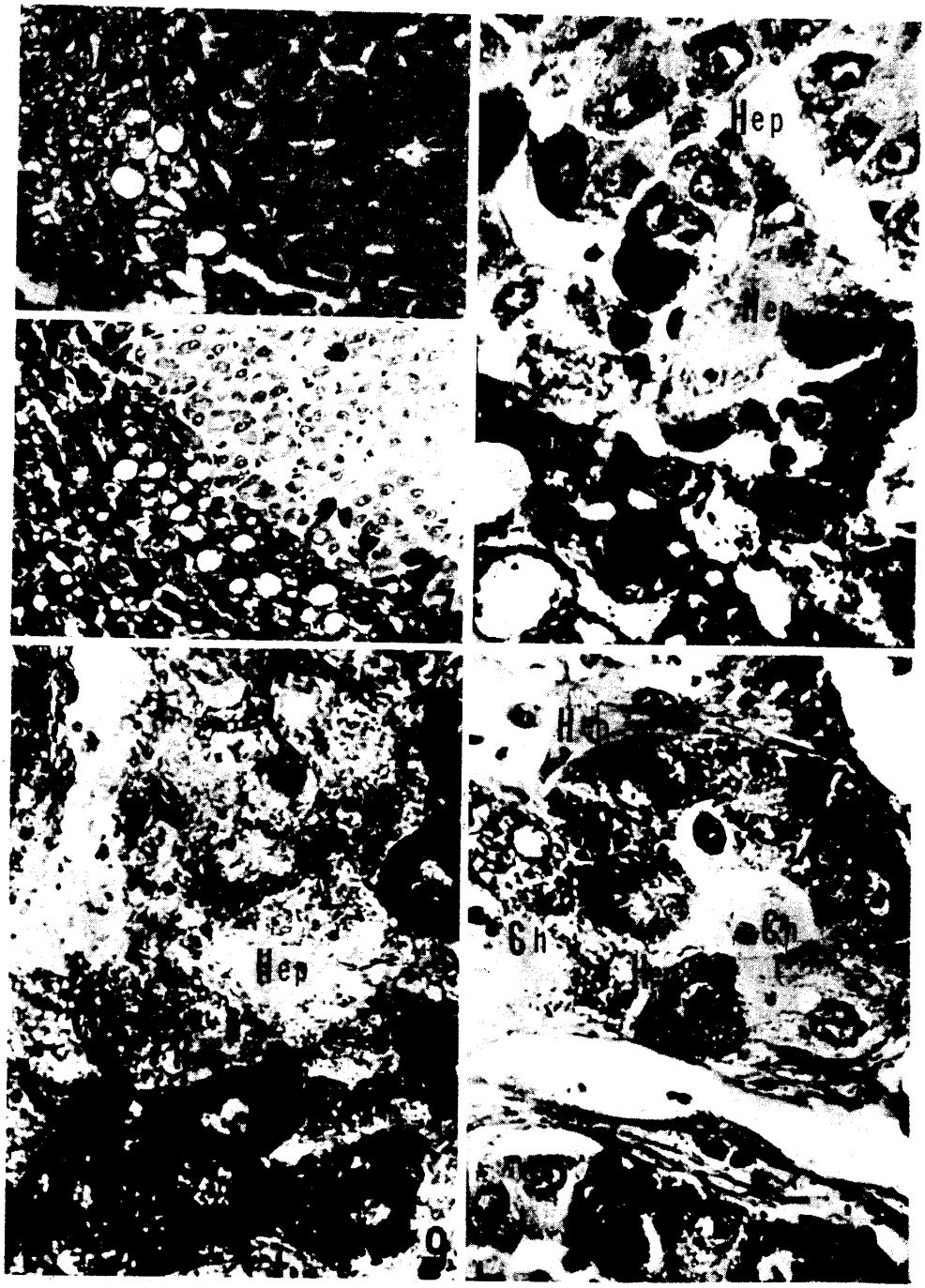
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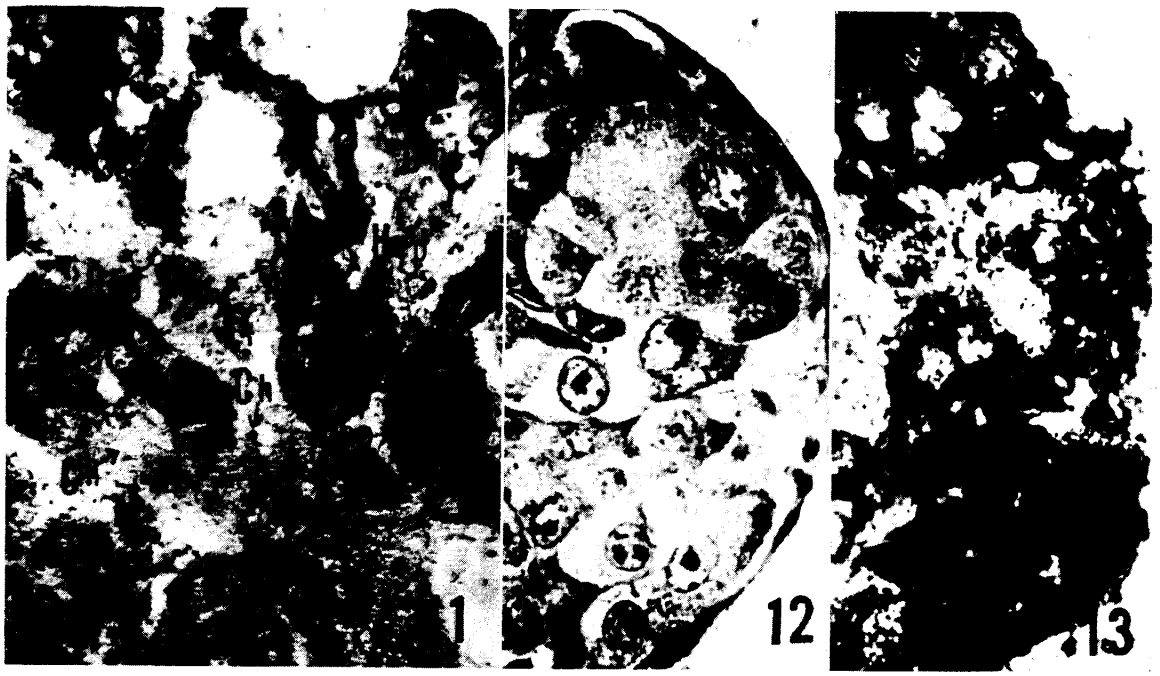

Fig. 6. Nodular proliferation of hepatocellular cancer surrounded by atrophic liver tissue. A tissue from 28 weeks 3′-Ni-DAB feeding. Hematoxylin eosin staining. 10×10

Fig. 7. PAS and hematoxylin staining of a section from the same tissue as appearing in Fig. 6. Note a marked decrease in glycogen in cancer cells. 10×10

Fig. 8. An enlarged picture of a part of Fig. 7. PAS and hematoxylin staining of hepatoma cells (Hep) just invading into normal parenchymal cells. Note positive but markedly reduced PAS positive substances in cancer cells comparing to the liver parenchymal cells surrounding hepatoma cells. 40×10

Fig. 9. G-6-Pase activity of hepatocellular cancer cells. From the same sample as in Figs. 7. and 8. Picture shows the border area of cancer tissue and normal tissue. Note a markedly reduced activity of G-6-Pase in cancer cells (Hep) comparing to that of normal cell. Cancer cells show a marked variety in the intensity of G-6-Pase reaction. 40×10

Fig. 10. 40 weeks of 3′-Ni-DAB feeding. PAS staining and slight hematoxylin staining. Cholangiole cells of adenomatous pattern.
PAS strongly positive cells are characteristic for the hepatocellular cells (Hep) in the cholangiole. PAS negative or weakly positive cells are characteristic of the essential cholangiole cells (Ch). 10×40

Fig. 11. G-6-Pase activity of cancer cells having hepatocellular character. Note G-6-Pase strongly positive cells are characteristic for the hepatocellular cells, (Hep) and reduced or negative activity cells are characteristic for the cholangiole cells (Ch). A section from the same sample in Fig. 10. 40×10

Figs. 12. and 13. Nodular development of hepatocellular cancer. The liver of 50 weeks of 3′-Ni-DAB feeding. Fig. 12 shows the picture of PAS staining and Fig. 13 is of G-6-Pase activity. They show the two sections obtained serially. The strong G-6-Pase activity in the peripheral area of the lobules becomes negative (in the center) according to the degree of undifferentiation. 10×10