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Abstract

Activities of intracellular RNase of the liver cytoplasm, normal liver cells exposed to 3'-Me-DAB and hepatoma cells, have been studied in correlation with the contents of RNA and DNA and morphologic changes of the cells with or without treating RNase. The data showed that in hepatoma cells the intracellular acid RNase activity decreases with the decrease of RNA and unchanged DNA contents and alkaline RNase activity. Morphologic observation proved that hepatoma cells show a small low massed vesicular or vacuolar endoplasmic reticulum having ribosomes. For the exposure to RNase the hepatoma cells proved to be much less resistant comparing to normal liver cells. The former lost the granules and was destroyed in its endoplasmic reticulum, whereas the latter retained ribosomes and ER. From these experiments it has been speculated that acid RNase in the cell may be involved in RNA synthesis and alkaline RNase in RNA decomposition, though the effect of the difference in concentration in the case given RNase experimentally can not be neglected.

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ACID RIBONUCLEASE ACTIVITY OF HEPATOMA CELLS

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A correspondence of the activity of intracellular ribonuclease (RNase) with cell growth has been noted by several investigators (BRODY¹, GROTH², BAKER, and CANNON³, and the suggestion has been made by many workers that intracellular RNase may have a ribonucleic acid (RNA) synthetic activity (BRODY¹, REDDI³, HEPPEL, WHITEFELD, and MARKHAM⁴) as well as the well-known activity of disintegrating RNA, with which RNase may be functioning to regulate the metabolism of RNA keeping it in some level according to the intracellular environment. In the living cell, however, RNase is safely contained in cytoplasm without giving any abnormal disintegrating activity to RNA. Concerning the liver cells its cytoplasm contains at least two RNases, an acid and alkaline RNases (de LAMIRANDE., ALLARD., da COSTA⁵, CANTERO and ROTH^{6,7,8,9}). Cancer cells also have a quantity of two sorts of RNase like normal cells showing the close correlation with the nucleic acid metabolism as has been studied cytochemically by LEDOUX^{10,11,12,13}, BRODY¹⁴, REID, and LOTZ¹⁶, and DAoust.

Recently, it has been demonstrated that RNase given from outside penetrate into the cell disturbing the metabolism of RNA and inducing various morphologic and biochemical changes, like abnormal mitosis, destruction of endoplasmic reticulum and granules, disturbed desoxyribonucleic acid (DNA) and protein syntheses^{17,18}. And yet it has been demonstrated that cancer cells are especially susceptible to RNase given from outside and it is supposed that the permeability to RNase in each cell is due to the contents of RNase, i. e. the smaller the quantity of RNase in a cell the better is the permeability to RNase. (LEDoux *et al.*) From these observations it is reasonably expected that RNase decrease when the normal cells turn to cancer cells. From this view point the authors observed RNase activity in both of normal liver and hepatoma tissue, with referring to the amount of RNA and DNA.

MATERIALS AND METHODS

About 90 rats of Wistar strain weighing 200–350 g. were used. All these were fed on polished rice containing 0.06 % 3'-Methyl-4-Diaminoazobenzene (3'-Me-DAB) and each two animals were sacrificed at 90, 120, 150, 200, 280 and 310 days of dye feeding. More than 30 animals died on the way of dye feeding, some did not have tumor tissue and all these were discarded from the observation. Apparently normal, cirrhotic and tumor tissues were obtained separately from the liver of living animals by laparotomy under narcosis with ether.

Morphologic observations : In each sample a piece of fresh tissue is fixed in 10 per cent formol for paraffine section and hematoxyline-eosin staining. Besides these, a small piece, 5 mm in size, was fixed with 1 per cent osmic acid with or without treating with 1 per cent RNase in phosphate buffer pH 7.4 containing succrose for an hour. These were embedded in methacrylate by the routine method and observed under electron microscope.

Estimation of the activities of RNase :

One gram of tissue was added with 50 ml. of distilled water and homogenized and then added with 14 ml. of 1 per cent sodium deoxycholate to detach RNase from the organellae. The mixture was stirred and centrifuged at 8,700 g for 20 minutes to remove gross unhomogenized fragments. The supernatant is brought to 27.5 per cent saturation of ammonium sulfate, pH is adjusted to 4.5 by using 1 N HCl and centrifuged at 8,700 g for 20 minutes. The supernatant is added with ammonium sulfate to 55 per cent saturation at pH 4.5. The solution is stood over night in a cold room and centrifuged at 3,000 r. p. m. for 5 minutes. The resultant precipitate shows a strong activity of RNase and used for the estimation. The precipitate is dialyzed against one liter of glass distilled water in a cold room for 24 hours changing the water at 16th hour once. After dialyzation one part of RNase precipitates in distilled water and it was centrifuged at 60,000 g for 20 minutes. The precipitate contains alkaline RNase and the supernatant contains acid RNase.

Estimation of acid RNase :

The supernatant was eluated through the column, 0.9 × 30 cm of cation exchange resin IRC 50 by the method of HIRS by using 0.2 M phosphate buffer, pH 6.47. The fractionated eluates, each 0.5 ml and 200 samples as total, whose protein contents can be estimated at 2,800 Å by Beckman spectrophotometer, after diluting with 3 ml of distilled water, is mixed with 0.65 ml of 0.1 M acetate buffer (pH of mixture : 5.0), 0.2 M Tris buffer (pH of mixture : 6.0) or 0.2 M Tris buffer (pH of mixture : 6.2) as described by ROY^{7,8,9} and REID¹⁰, and added with 0.25 ml of solution of yeast RNA. (Yeast RNA 3. mg : distilled water 2.5

ml) The hydrolysis of mixtures was allowed to proceed for 30 minutes at 37°C. At the end of incubation the reaction was stopped by adding 0.25 ml of 0.75 per cent uranyl acetate in 25 per cent perchloric acid. The reaction mixture is centrifuged at 3,000 r. p. m. for 5 minutes, by which the undecomposed RNA is precipitated and the mono- or oligo-nucleotides are solved in the supernatant. 0.5 ml. of the supernatant was diluted with 3. ml of distilled water and the contents of the nucleotides are estimated by Beckman's spectrophotometer at 2,600Å.

Estimation of alkaline RNase :

The precipitant obtained by removing acid RNase in the supernatant is homogenized in a small glass homogenizing by adding about 5 ml. of ice-cold 0.25 M sulfuric acid and allow to stand over night in the refrigerator. Then the pH of the suspension is adjusted to pH 7.0 with 1 N NaOH, by which alkaline RNase is detached from the precipitant and solved in the supernatant. The activity of alkaline RNase can be measured with the supernatant similarly as in the case of acid RNase.

Estimation of RNA and DNA :

Nucleic acids were measured biochemically by the method of SHIBATANI, which is modified of SCHMIDT and THANNHAUSER, by using homogenate of 200 mg liver in 2 ml of physiologic saline solution. Nucleic acids were extracted by perchloric acid and the coloration of RNA has been made by phloroglucinol reagent, and estimated at 610 m μ . or 680 m μ . DNA was colored with diphenylamine reagent and estimated at 640 m μ .

Paper chromatography of ribonuclease digest of yeast RNA and RNA of hepatoma :

Ribonucleic acids of hepatoma cells were isolated from the cancer tissue of rat liver according to the method of Kirby's phenol method. Pancreatic ribonuclease used in these studies was a crystalline preparation obtained from Shimakyu Corporation, Osaka. Five mg of yeast RNA and hepatoma RNA were dissolved in 0.5 ml of 0.02 M phosphate buffer at pH 7.6. As standard solution, five mg of uridylic, guanylic, adenylic, cytidylic acids were dissolved in one ml of the same buffer solution. To these RNA solution, were added 125 γ of ribonuclease dissolved in 0.5 ml of 0.02 M phosphate buffer at pH 7.6, and the mixture was incubated at room temperature for 12 hours. At this interval of time 0.3 ml aliquot was withdrawn, placed on a Toyo filter paper No. 51. B (32 cm \times 4 cm) in a band at a distance of 3 cm from the top of the filter paper, and dried in a current of air. On the same paper a mixture of cytidylic acid, uridylic acid, guanylic acid and adenylic acid was run wide. The chromatography was developed (up) in ammonia sulfate-isopropanol and water (79 : 2 : 19) solvent system for 24 hours at room temperature. The developed chromatography was dried at room temperature and marked through ultra-violet light according to

the technique of SMITH, ALLEN¹⁹ and REDDI²⁰.

RESULTS

Morphologically the liver tissue adjacent to the cancer tissue showed actually the normal structure. In cirrhotic area there were a number of nests of adenofibrosis surrounding the apparently normal parenchymal cells showing nodular hyperplasia.

Most of the tumor appearing in later stages were of hepatocellular, type though some of them showed adenomatous arrangement. (Photos. 2, 3, and 5) Materials for chemical analysis were obtained from several parts in one liver, e. g. from apparently normal, cirrhotic and tumor tissues. (Photos. 2, 3, 4 and 5)

The normal liver tissue from control animal showed a strong activity of acid RNase at the elutes Nos. 15 and 40 (Fig. 1 : A).

The rechromatography on the samples of Nos. 15 and 40 revealed the distinguished activities at near Nos. 15, 40 and 50 (Fig. 1 : B). In the rat liver administered with DAB (for 36 days), electron microscope observations reveal degenerated liver with degenerated mitochondria. (Photo. 1). The activity of acid RNase at pH 5.0 and 6.0 decreased slightly but that observed at pH 6.4 proved to be in normal range.

In the liver tissue having no cirrhotic change acid RNase activity appeared at Nos. 10, 20, and 30 of eluates at pH 5.0, Nos. 15 and 40 at pH 6.0 and Nos. 10 and 20 at pH 6.4 (Fig. 2).

In cirrhotic liver acid at Nos. 10 and 15 and a small peak at No. 50 at pH 5.4 and at Nos. 10, 20, 40 at pH 6.2 (Fig. 3).

In liver tissue having small disseminated cancer tissue RNase activity appeared at Nos. 10, 20, at 30 and 40 at pH 5.0, at Nos. 10 and 40 at pH 6.0 and at Nos. 10 and 40 at pH 6.4 (Fig. 4).

In the hepatoma tissue having no liver tissue the activity of acid RNase was detected at Nos. 10, 20 at pH 5.0, No. 10 at pH 6.0 and Nos. 10 and 40 at pH 6.4 showing almost normal distribution but much less in activity comparing to the normal liver tissue, especially in the peak at No. 40 (Figs. 2 and 3), which is

EXPLANATION FOR PHOTOS

Photo. 1: Electron micrograph of the rat liver administered with 3'-Me-DAB (for 36 days). Note the degenerating mitochondria or vesicles of various irregular sizes. At this time acid RNase activities at pH 5.0 and 6.0 are decreasing but RNase activity at pH 6.4 is a little lower than normal liver. RNA contents is decreased.

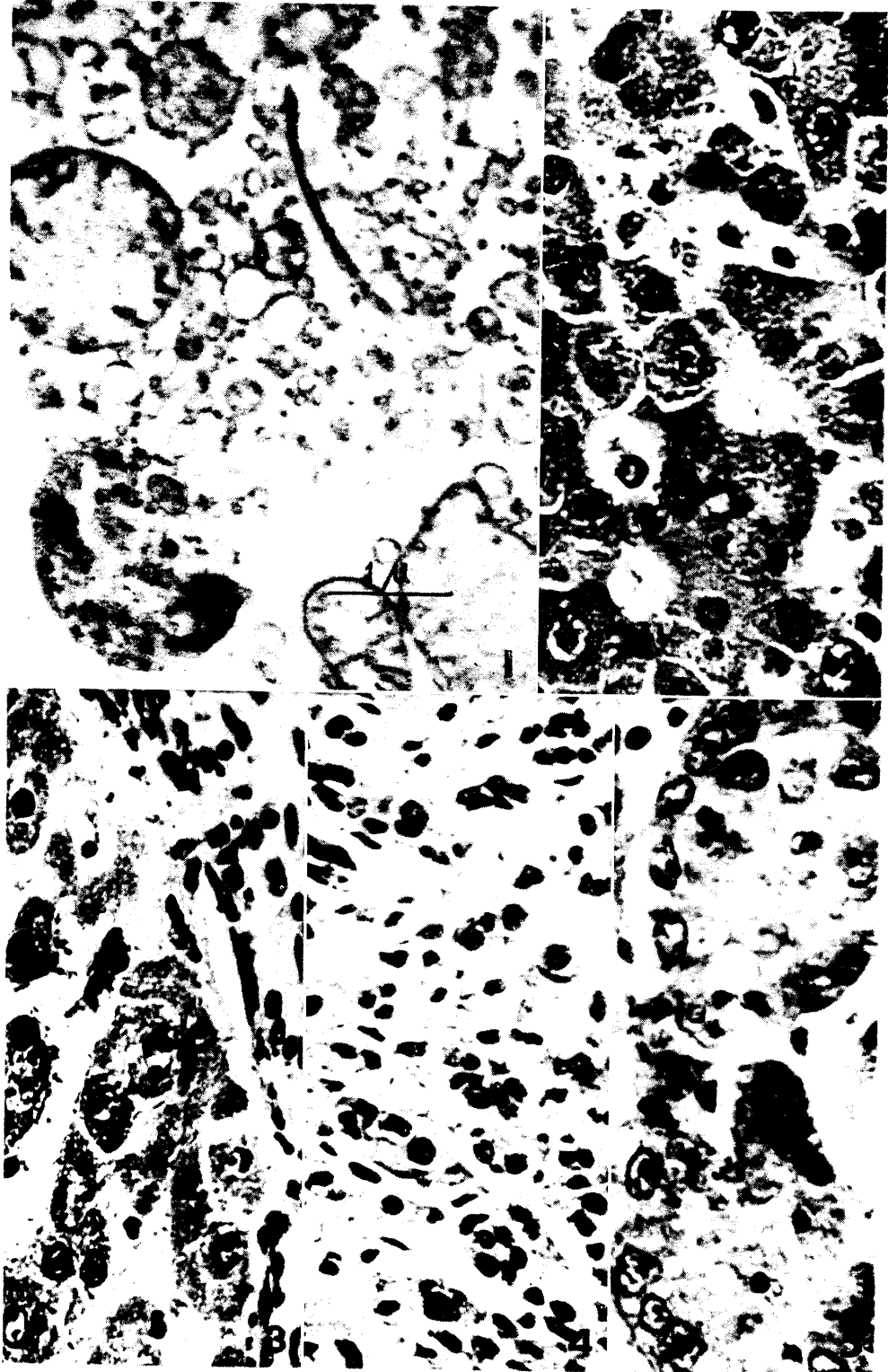
Photo. 2: Hepatoma Cells.

Cancer cells are hepatocellular type. Hematoxylin eosin staining.

Photo. 3: Hepatoma cells

Normal liver tissue is cirrhotic.

Photos. 4 and 5: Disseminated hepatoma in normal liver tissue (Photo. 4) and hepatoma cells (Photo. 5). Hematoxylin eosin staining.



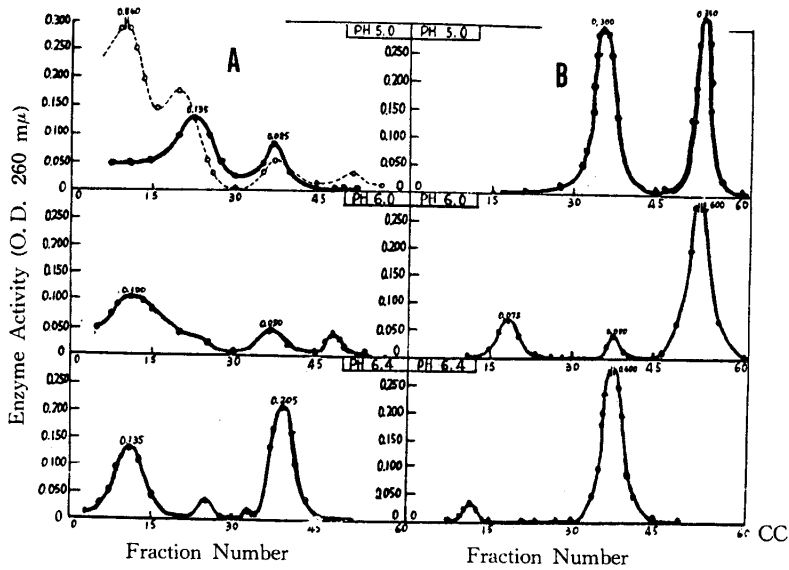


Fig. 1: Acid RNase activities of the normal liver tissue from control normal rat.
 A: The normal liver tissue showed a strong activity of acid RNase at the elute Nos. 15 and 40.
 B: The rechromatography on the A samples of Nos. 15 and 40 revealed the prominent activities at near Nos. 15, 40, and 50.

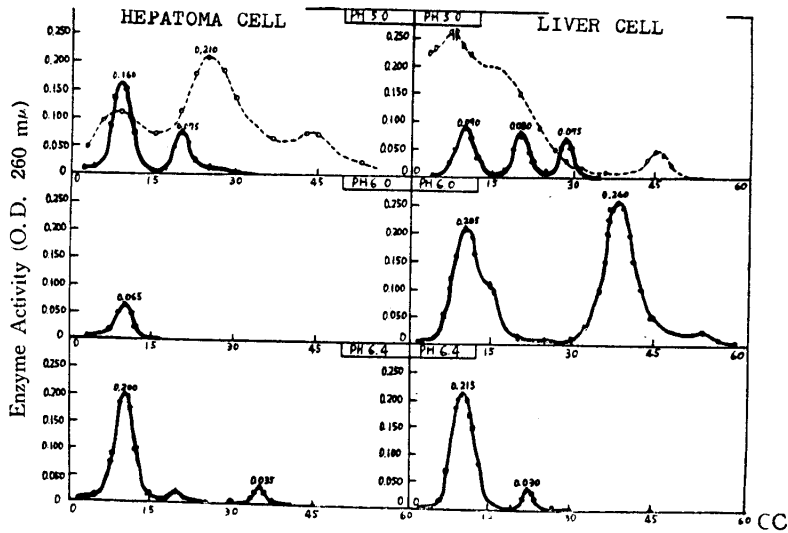


Fig. 2: Acid RNase activities of hepatoma cells and the liver cells having no cirrhotic change.

Acid RNase Activity of Hepatoma Cell

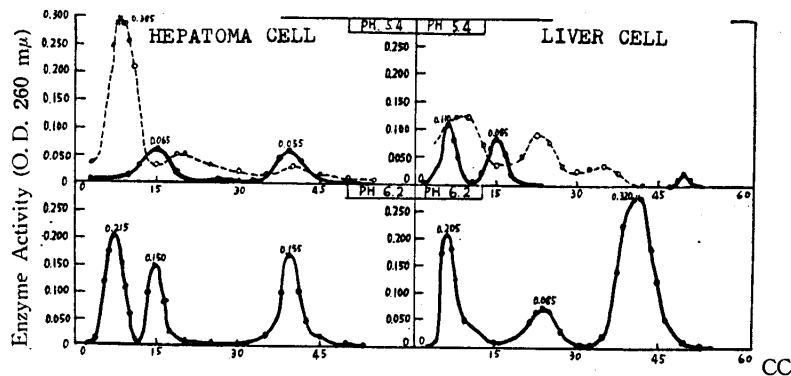


Fig. 3: Acid RNase activities of hepatoma cells and cirrhotic liver tissue.

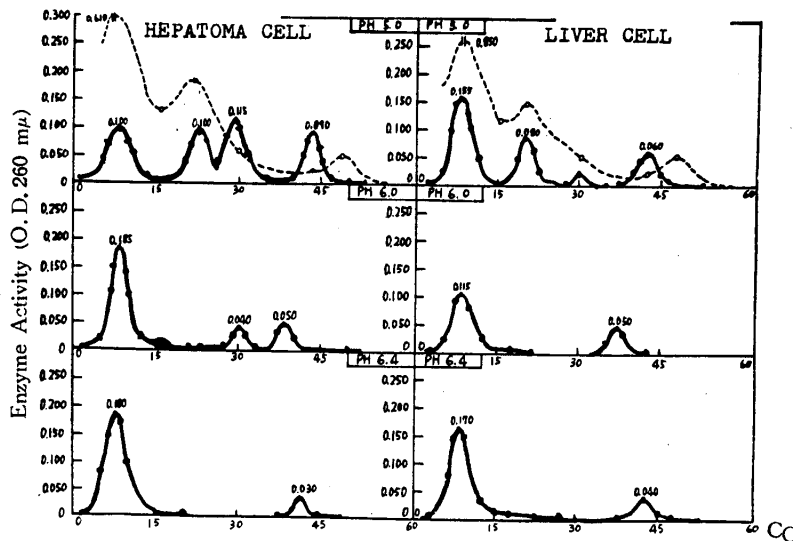


Fig. 4: Acid RNase activities of hepatoma cells and liver cells having small disseminated cancer cells.

In the hepatoma cells the activity of acid RNase was detected at Nos. 8, 10, 20 at pH 5.0, No. 10 at pH 6.0 and Nos. 10 and 40 at pH 6.4, showing almost normal distribution but much less in activity comparing to the normal liver tissue, especially in the peak at No. 40 (Fig. 2 and 3).

In the liver cell having no cirrhotic change acid RNase activity appeared at Nos. 10, 20 and 30 of eluates at pH. 5.0, Nos. 15 and 40 at pH. 6.0 and Nos. 10 and 20, at pH. 6.4 (Fig. 2).

In cirrhotic liver the activity of acid RNase was detected at Nos. 10 and 15 and a small peak at No. 50 at pH. 5.4 and at Nos. 10, 20, and 40 at pH. 6.2 (Fig. 3).

In liver tissue having small disseminated cancer tissue the activity appeared at Nos. 10 and 20, in traces at 30 and 40 at pH 5.0, at Nos. 10 and 40 at pH 6.0 and at Nos. 10 and 40 at pH 6.0 and at Nos. 10 and 40 at pH 6.4 (Fig. 4).

typical RNase activity.

Alkaline RNase showed elevated activities at Nos. 30 and 40 in tumor tissue and Nos. 10, 30 and 40 at pH 8.0, actually showing no marked differences between normal and tumor tissues (Fig. 5).

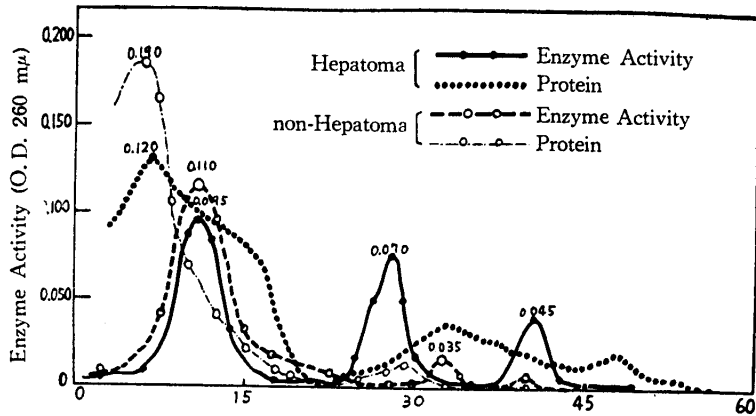


Fig. 5: Alkaline RNase activity of hepatoma cells and normal liver cells.

Alkaline RNase showed elevated activities at Nos. 30 and 40 in hepatoma cells and Nos. 10, 30 and 40 at pH 8.0 actually revealing no marked difference between normal and hepatoma cells.

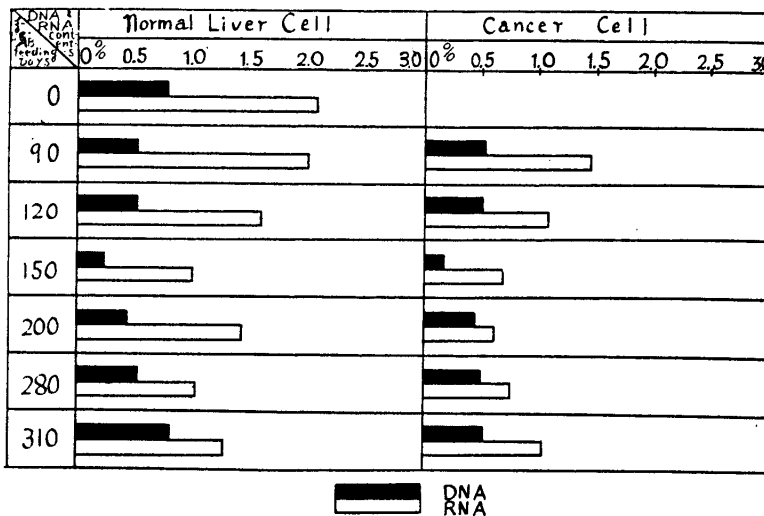


Fig. 6: DNA and RNA contents of normal liver cells and hepatoma cells.

RNA contents is reduced in DAB hepatoma cells comparing to normal liver cells, while DNA contents are revealed to be at the same level in both of normal and hepatoma cells, showing the reduced amount in RNA is not due to the reduction in the number of cells but to the reduction in RNA contents in each cell.

RNA contents is reduced in cancer tissue comparing to normal liver tissue, while DNA contents are to be at the same level in both normal and tumor tissues, showing the reduced amount in RNA is not due to the reduction in cell number but to the reduction in RNA content in each cell.

Morphologic observation of the hepatoma cells revealed the increased basophilicity of cytoplasm in some cells (Photos. 2 and 3), and electron microscope observation demonstrated a small number of masses of small vacuolar and vesicular endoplasmic reticulum with granules, though some glycogen deposit in cytoplasm and mitochondria provided with poorly developed cristae. (Photo. 6)

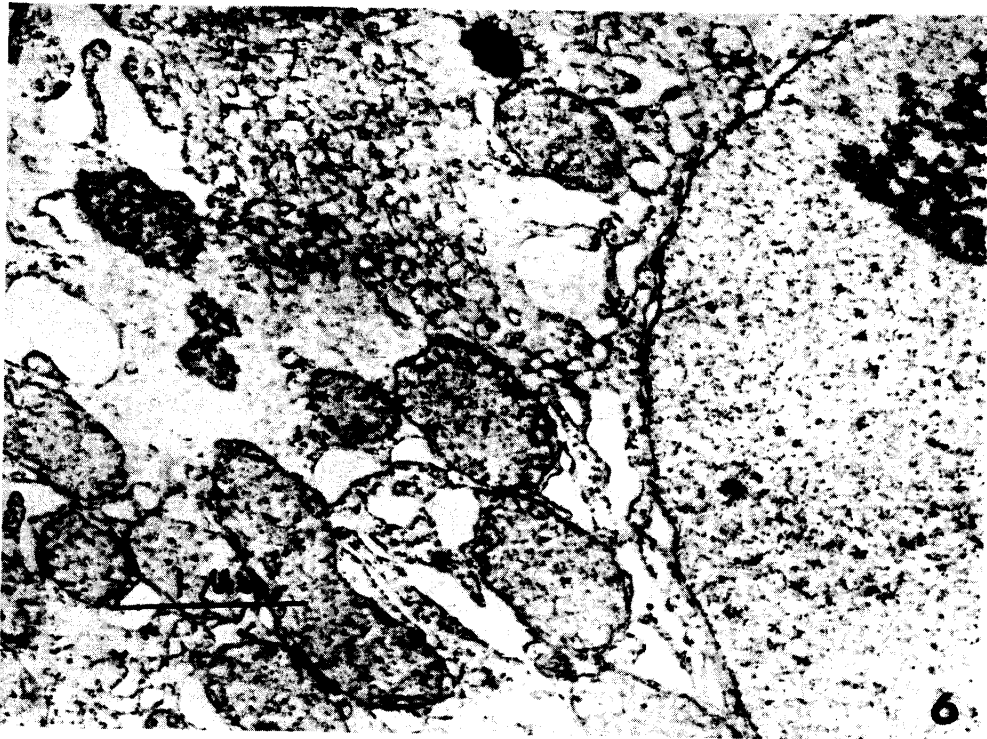
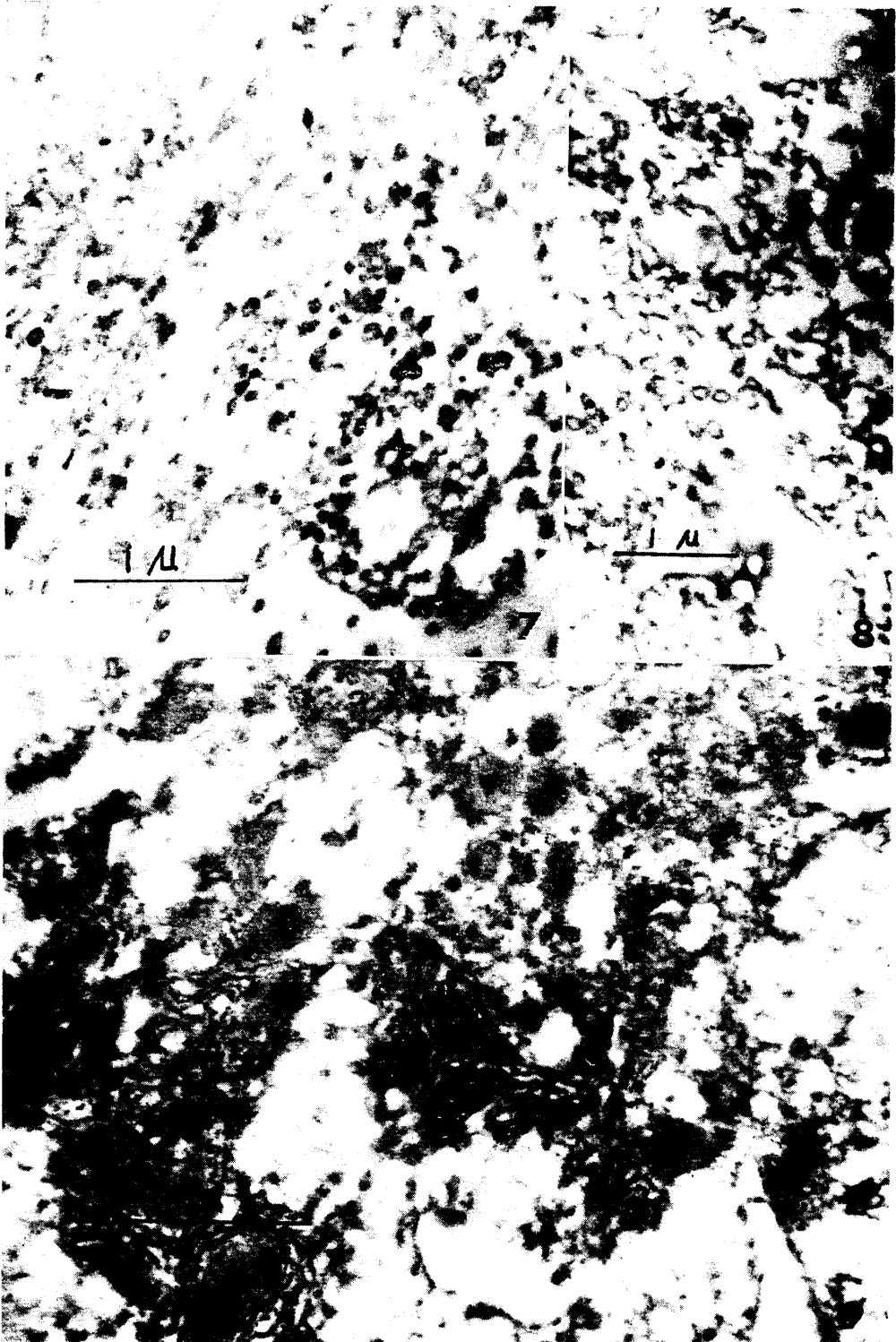


Photo. 6: Hepatoma cell of electron microscope findings

Note a number of masses of small vacuolar and vesicular ERs with disintegrating RNA granules, and some glycogen deposition in cytoplasm. Mitochondria have poorly developed cristae.

Electron microscope picture of the hepatoma cells exposed to 1 per cent RNase (pancreatic RNase) solution proved the broken granules of ER while normal liver cell proved to be more resistant than hepatoma cell to RNase showing the remnant granules and filaments of endoplasmic reticulum. (Photo. Nos. 7, 8 and 9).



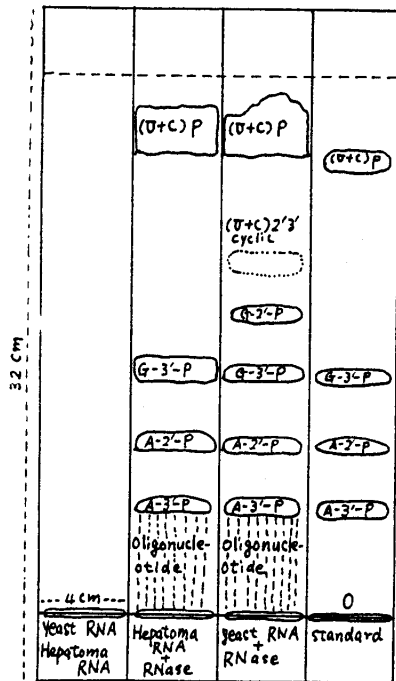


Fig. 7: Chromatography of pancreatic RNase digest of yeast RNA and RNA of hepatoma cell at 23°C and pH 7.6, developed in ammonia sulfate-water and isopropanol solution.

- O: Origin of chromatography
- P: Phosphate group
- A: Adenylic acid
- G: Guanylic acid
- C: Cytidylic acid
- U: Uridylic acid

ceptible than normal cells to RNase given from outside of the cell. However, it is uncertain whether the decrease in RNase activity in cancer cell is correlated directly to the increased penetration of RNase to cancer cell or not, because RNase decreased in cancer cell is acid RNase but RNase given to the cell from outside is mainly of alkaline RNase as pancreatic RNase.

And the appearance of mononucleotides and oligonucleotides in the alkaline

Fig. 7 is a schema of the chromatography of ribonuclease digest of yeast RNA and RNA of hepatoma cells. The appearance of mononucleotides and oligonucleotides in the alkaline RNase digests of yeast RNA and hepatoma RNA were almost the same and pancreatic ribonuclease is a highly specific phosphodiesterase which hydrolyses only secondary phosphate esters of ribonucleoside 3'-phosphates.

DISCUSSION

Recently it has been demonstrated that rat liver cells contain an acid RNase which is maximally activated at pH 5.0 or close to acidic range (Hirs and SCHMIDT *et al*²¹.) It is suggested that the acid RNase often remains in an inactive state in cells, associating with its specific inhibitors and also adhering to various membranes of intracellular particles. In the author's study, it was clarified that intracellular activity of alkaline RNase showed no variation during normal and neoplastic growth.

The biochemical and morphologic data obtained on liver cells exposed to DAB proved that acid RNase activity as well as RNA decreased when the cell develop to cancer cell, and became more sus-

Photos. 7 and 8: Hepatoma cells exposed to one per cent pancreatic RNase (pH 7.4).

Note the destructed granules of ER and disintegration of granules.

Photo. 9: Normal liver cell exposed to one per cent pancreatic RNase (pH 7.4)

Normal liver cell showed RNA more resistant than its of hepatoma cell to RNase showing the remnant granules and bilaments of endoplasmic reticulum

RNase digest of normal RNA and cancer RNA were almostly the same and normal RNA and hepatoma RNA seem to have no difference in chemical properties.

The possible speculation is that in the cell alkaline RNase might be mainly correlated to the decomposition of RNA, whereas acid RNase to the synthesis of RNA, because in hepatoma cells the decreased acid RNase activity was always accompanied by the decreased contents of RNA of the cell, and actually no change in the activity of alkaline RNase between normal and cancer cells.

But acid RNase, too, may act as to decompose the RNA when it is given to the cell from outside. This is the problem that requires further studies. However, concerning the acting mechanism of RNase contained originally in the cell and that given from outside, there is perhaps actually no difference. The RNase in the cell was originally supposed to be contained in mitochondria but it seems more likely to be contained in lysosome of DE DUVE. Lysosome means the vesicular bodies composed of cytoplasmic membrane and contains foreign bodies uncertain in characteristics (NOVIKOFF²²). RNase of the cell may be excreted into this vesicle or obtained from outside. RNase taken from outside will also be contained in these vesicles and it is preserved in the cytoplasm without showing its destructive effect on RNA. And it is supposed that as lysosome is formed by pinocytosis, RNase also is taken by the cell through pinocytosis, as pointed out by BRACHET and collaborators¹⁷.

Consequently, it is reasonably supposed that there will be no difference in acting mechanism of RNase between those contained originally in the cell and those ingested from outside. Only the difference between the case of RNase given experimentally and that of RNase originally contained in the cell is the concentration, which will be very important in enzymic reaction in general. RNase in a small quantity may act as to accelerate the syntheses of RNA and to destroy it when a large dose is given, as pointed out by EGAMI²³ or suggested by the experiment of LEDOUX *et al.* and the authors¹⁸. The problem is very important in the therapy of cancer and should be solved in near future.

CONCLUSION

Activities of intracellular RNase of the liver cytoplasm, normal liver cells exposed to 3'-Me-DAB and hepatoma cells, have been studied in correlation with the contents of RNA and DNA and morphologic changes of the cells with or without treating RNase. The data showed that in hepatoma cells the intracellular acid RNase activity decreases with the decrease of RNA and unchanged DNA contents and alkaline RNase activity. Morphologic observation proved that hepatoma cells show a small low massed vesicular or vacuolar endoplasmic reticulum having ribosomes. For the exposure to RNase the hepatoma cells proved to be much less resistant comparing to normal liver cells. The former lost the granules

and was destroyed in its endoplasmic reticulum, whereas the latter retained ribosomes and ER.

From these experiments it has been speculated that acid RNase in the cell may be involved in RNA synthesis and alkaline RNase in RNA decomposition, though the effect of the difference in concentration in the case given RNase experimentally can not be neglected.

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