Modification of Hexosaminidase Isozymes in Rat Hepatoma

Anne Weber, Livia Poenaru, Christiane Lafarge, and Fanny Schapira


SUMMARY
We have shown the existence of two molecular forms of hexosaminidase in rat tissues (liver, brain, muscle, lung, placenta, and red cells) and in rat serum. These isozymes have been separated by electrophoresis on cellulose acetate membrane and compared with isozymes of slow- and fast-growing hepatomas. In normal liver, as in slow-growing hepatoma, there is a preponderant band with slow anodic migration and another faint more anodic band. On the contrary, in two kinds of fast-growing hepatomas, the fast anodic band was constantly very strong while the slow band was much weaker or even disappeared. This pattern was similar to that of brain and principally to that of fetal liver (16th day). In brain the two bands possessed not only different charges but also different thermostabilities. The isozyme found in rapidly growing hepatoma had the same thermostability as the fast band of brain.

Experiments performed on regenerating liver after partial hepatectomy show that the pattern is almost normal; our findings indicate that the isozymic modifications are not a mere consequence of cellular multiplication.

The modifications of hexosaminidase isozymes give a new example of the resurgence of fetal enzymes in cancer.

INTRODUCTION
The resurgence [first described in our laboratory (34)] of molecular fetal forms of enzymes in cancerous tissues is now well demonstrated (30), especially in human and experimental hepatoma. On the other hand, isozymic forms of several lysosomal hydrolases have been described including β-2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase (EC 3.2.1.30) (hexosaminidase), which hydrolyzes both N-acetyl-β-glucosaminide and N-acetyl-β-D-galactosaminide.

Human tissues were known to contain 2 forms of N-acetyl-β-glucosaminidase, which can be separated by electrophoresis on various media (5, 10, 12) or by electrofocusing (27) into Form A (called acidic form) (26) and Form B (called basic form). Both migrate toward the anode in cellulose acetate electrophoresis at pH 6.5, B being the slowest form. More recently, a 3rd, faster isozyme has been discovered in brain by Hooghwinkel et al. (9) and then in most fetal and adult organs by Poenaru and Dreyfus (22); it was called “Isozyme C.”

β-Hexosaminidase deficiency is known to be responsible for the various forms of GM1 gangliosidosis and, more particularly, the A isozyme is related to the most frequent form of gangliosidosis, Tay-Sachs disease (21, 36).

In rat tissues, Robinson et al. (25) demonstrated the presence of hexosaminidase but did not show the different molecular forms of this enzyme; Goldstone et al. (7) separated 2 bands with hexosaminidase activity in kidney.

The question arose whether different isozymes exist in other rat tissues and, if they do, how they are distributed in hepatoma and whether they are similar to those in normal liver. Moreover, the problem of reappearance of enzymic embryonic forms and the significance of this phenomenon have not been solved yet. This mechanism could be due only to cellular multiplication or could express a more specific phenomenon.

The purpose of the present communication is to study the kinetic and electrophoretic properties of different normal rat tissues and to compare them with those of slow- and fast-growing hepatoma. In the same way the properties of rat regenerating liver after partial hepatectomy, which represents the best example of intense cellular multiplication in adult liver, have been compared with those of normal liver and hepatoma.

MATERIALS AND METHODS
Normal, hepatectomized, and cancerous rats were young albino Wistar rats. Rats over 1 year old and host livers from rats with Reuber hepatoma H.178 were also used.

Partial hepatectomies were carried out according to the method of Higgins and Anderson (8) adapted by Frayssinet (6); 60 to 70% of liver was removed. Rats were sacrificed at different times after operation (from 6 hr to 7 days).

Controls were either normal rats or rats of the same age submitted to sham surgery and sacrificed at the same times. (One lobe, the median one, was displaced and then replaced.) In some experiments, we have compared biopsy liver and the same liver 2 or 3 days after sham operation.

Regeneration was also induced by i.p. injection of CCl4 (17) (100 μl/100 g of body weight). The rats were sacrificed between 18 hr and 12 days after the injection.

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Hepatomas used were either primary hepatomas or solid transplanted hepatomas.

Reuber hepatomas (H.178), poorly differentiated and fast growing, were induced in ACI/N rats ingesting 0.025% N-2 fluorenylidacetamide in the diet and then were transplanted (24). The animals were killed at 52 weeks.

LF hepatomas, poorly differentiated and fast growing, were induced with 4-dimethylaminoazobenzene by Fraysinet and then transplanted i.p. Rats were sacrificed 1 week after transplantation.

Well-differentiated (slow-growing) primary hepatomas were obtained by De Nechaud by submitting 6-week-old rats to the Miller and Miller diet (18), which contains 600 mg of 3'-methyl-4-dimethylaminoazobenzene per kg of diet. Rats were sacrificed 5 months after.

Substrates (4-methylumbelliferyl-N-acetyl-β-glucosaminide or β-galactosaminide, or β-glucuronide) were obtained from Koch-Light Laboratories, Ltd., Colnbrook, United Kingdom.

Preparation of Tissue Extractions. Transplanted and primary tumors were carefully dissected free of normal tissues and necrotic areas.

Portions of residual hepatic tissue after partial hepatectomy and equivalent portions of liver from sham-operated animals and other tissue samples were homogenized in 4 volumes of an aqueous solution of Triton X-100, 0.2%, which allows the extraction of lysosomal enzymes. The homogenates were centrifuged at 37,000 × g for 15 min in the cold and the supernatants were decanted. Normally, samples were examined immediately; in some experiments, they were stored at −75°C. In some others, fresh tissues were homogenized in 4 volumes of 300 mM sucrose and centrifuged at 20,000 × g for 10 min.

Fetal livers were obtained between the 16th and 19th days of gestation.

Electrophoresis was performed on cellulose acetate according to the method of Fluharty et al. (5), with slight modifications, for 2 hr under 200 V and 3 ma/band. Three to 5 μl of extract or 10 μl of undiluted serum were applied near the anodic end with 40 mM potassium phosphate buffer, pH 6.5, in the vessels.

The staining substrate was glucopyranoside (4-methylumbelliferyl-N-acetyl-β-D-glucopyranoside) (500 μM solution in water). Strips of Whatman No. 3MM paper soaked with 40 mM citric phosphate buffer, pH 4.4, and with the substrate were pressed against the electrophoresis band on a glass sheet. After about 15 min of incubation at room temperature, a strip of Whatman paper soaked with 1 M glycine-sodium hydroxide buffer, pH 10, was substituted for the substrate paper. The alkaline reaction stops the enzymic activity and transforms the product 4-methylumbelliferone in a strongly fluorescent anion. The fluorescent spots were visualized in a Chromatovue cabinet and photographed immediately. Galactopyranoside and β-glucuronide were also used as substrates.

Determination of Activity of the N-Acetylglucosaminidase. The quantification of enzymic activity of tissue extracts was based on the fluorimetric measurement of 4-methylumbelliferone liberated from appropriated glycoside.

Sample tissues freshly removed were diluted 50 times for extraction in aqueous solution of 0.2% Triton X-100 or 300 mM sucrose.

Twenty μl of supernatant were incubated with 0.5 ml of 40 mM citrate phosphate buffer, pH 4.4, and 100 μl of 500 μM glucopyranoside.

Enzymic reaction was stopped after 30 min at 24°C by 1 M glycine-sodium hydroxide buffer, pH 10.

Blanks were obtained for each experiment by adding glycine buffer before extracts. The fluorescence was measured in an Aminco Bowman spectrofluorimeter using an excitation wavelength of 366 nm and an emission wavelength of 446 nm.

In some experiments the glucopyranoside substrate was replaced by galactopyranoside.

Methylumbelliferone liberated was estimated from the fluorimetric measurement by comparing with fluorescence of serial dilutions of a standard solution of 4-methylumbelliferone in the same conditions. Activity was expressed as nmol of substrate hydrolized per g of fresh tissue and by min at 24°C.

RESULTS

Electrophoresis. Fig. 1 shows the electrophoretic pattern obtained with several different normal tissues. In all tissues, a large band of slow anodic migration and variable intensity is visible. Another band of faster anodic migration, hardly visible in normal liver and lung, and that seems absent in muscle, is, on the contrary, intense in brain, placenta, and fetal liver. In red cells, 2 bands are visible after prolonged incubation.

Fig. 2 shows normal liver compared with brain, with fetal liver (16th day), and with hepatoma (slow and fast growing). Slow-growing hepatoma induced by 3'-methyl-4-dimethylaminoazobenzene possesses the same pattern as normal liver.

By contrast, in 12 different experiments with the 2 types of fast-growing hepatomas, we always find a diminution and sometimes even a disappearance of the slower band with considerable increase in the intensity of the faster one. This pattern is similar to the fetal pattern (16th day).

Chart 1 gives some examples of the pattern after hepatectomy and after sham operation (2nd and 7th days). It is identical; nevertheless, in both cases, the anodic band seems slightly strengthened. In order to confirm that sham operation is able by itself to modify the isozymic distribution, we have compared the electrophoretic pattern of the same liver before and 2 or 3 days after a sham operation.

Results were variable; in some experiments the faster band was strengthened, and in some others no difference
Modification of Hexosaminidase Isozymes

Chart 1. Hexosaminidase isozymes in liver of hepatectomized and sham-operated rats. Tissue samples were regenerating livers (after partial hepatectomies or after i.p. injection of CCl₄) and control livers (before and after sham surgery). Homogenates and electrophoresis were performed as indicated in Fig. 1.

In fast-growing hepatoma, we find a mean total activity of 72.2 ± 9.6 nmoles (glucopyranoside) and 16.5 ± 2.9 nmoles (galactopyranoside). No significant change can be noticed between sham-operated and hepatectomized animals. Nevertheless it seems that total activity is slightly lowered both in sham-operated and in hepatectomized animals.

We have found that hexosaminidase activity of cancerous serum was equal to that of normal serum and approxi-

Table 1

Total N-acetyl-β-D-hexosaminidase activity of normal and cancerous rat liver

<table>
<thead>
<tr>
<th>Substrate</th>
<th>nmoles hydrolyzed/g fresh tissue/min</th>
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<tbody>
<tr>
<td>β-D-Glucopyranoside, 500 μM</td>
<td>161.7 ± 13.3*</td>
</tr>
<tr>
<td>β-D-Galactopyranoside, 500 μM</td>
<td>33.7 ± 2.1</td>
</tr>
</tbody>
</table>

*Means of 4 different experiments ± S.E.

In normal liver, Band B is stable even after 30 min at 50°C; consequently, Isozyme A preponderant in brain and hepatoma is much more thermolabile than Isozyme B preponderant in normal liver and hardly visible in hepatoma; the thermostability of Isozyme B is similar in the 3 tissues.

The action on the 2nd substrate, N-acetylgalactosaminide, has also been tested after electrophoresis. The relative intensity of each band was not modified.

Kinetic Studies. Extracts of normal liver, fast-growing hepatoma, and liver after sham operation and after hepa-
tectomy were examined for total hexosaminidase activity toward both substrates: glucosaminide and galactosaminide. Table 1 shows the values obtained with normal liver and with fast-growing hepatoma.

In normal liver, we find a mean total activity of 161.7 ± 13.3 nmoles min/g fresh tissue with glucopyranoside as substrate. These values are comparable to those found by Verity et al. (38). With galactopyranoside as substrate, the mean activity is 33.7 ± 2.1 nmoles.

The activity of supernatant in normal liver after su-
crolose extraction is approximately one-tenth of the total activity after Triton X-100 extraction; this agrees well with the results obtained by electrophoresis of tissues extracted in sucrose and in Triton X-100.

In fast-growing hepatoma, we find a mean total activity of 72.2 ± 9.6 nmoles (glucopyranoside) and 16.5 ± 2.9 nmoles (galactopyranoside). No significant change can be noticed between sham-operated and hepatectomized animals. Nevertheless it seems that total activity is slightly lowered both in sham-operated and in hepatectomized animals.

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A is more thermolabile than the slower Isozyme B. After 30 min at 43°C the fast-moving band disappears in brain (we shall call it “A”) while the slow band persists (we shall call it “B”). A similar pattern has been obtained in hepatoma.

In normal liver, Band B is stable even after 30 min at 50°C; consequently, Isozyme A preponderant in brain and hepatoma is much more thermolabile than Isozyme B preponderant in normal liver and hardly visible in hepatoma; the thermostability of Isozyme B is similar in the 3 tissues.

Chart 2. Hexosaminidase isozymes in normal liver, brain, and fast-
growing hepatoma. Tissue samples were extracted either in 0.2% Triton X-100 or in sucrose (4 volumes). The homogenates were centrifuged at 37,000 x g or 2,000 x g, respectively, for 15 min at 4°C, before being submitted to electrophoresis on cellulose acetate (see Fig. 1).

was visible from normal liver. In any case, isozymes from regenerating and sham-operated liver were always more similar to normal liver than to hepatoma. Moreover the electrophoretic pattern of liver regeneration after injury induced by CCl₄ injection was identical to the normal liver.

Price and Dance (23) have shown that N-acetyl-β-glucosaminidase activity is distributed in bound and in soluble fractions. We have consequently compared the normal liver, brain, and hepatoma isozymes according to their subcellular distribution.

Chart 2 shows the results obtained after extraction in isosmotic sucrose and in Triton X-100.

In all cases, the slower band is weaker in sucrose, this isozyme appearing to be principally in bound fraction. On the contrary, the faster anodic form, which is a more soluble form, gives similar patterns in sucrose or in Triton X-100 except in some experiments where the anodic band of hepatoma appears sometimes feebler in sucrose. For comparison, we have also studied the subcellular distribution of another lysosomal enzyme, β-glucuronidase (EC 3.2.1.31).

A single band was visible in liver and in hepatoma; in both cases it was weak in isosmotic sucrose and strengthened in Triton X-100.

In order to compare the properties of the fast-growing hepatoma isozymes with those of the brain isozymes, we have studied the thermostability of these molecular forms, because we recall that in human tissues, the faster Isozyme
mately one-tenth of that of liver. Consequently, an eventual contamination was not able to influence our results.

**DISCUSSION**

The presence of hexosaminidase activity in rat tissues has been previously demonstrated by Price and Dance (23) and by Robinson et al. (25); Goldstone et al. (7) have found 2 bands in rat kidney. We have studied the distribution of hexosaminidase isozymes in different tissues of this species. Moreover, no hexosaminidase activity was noted in human or rat red cells by previous authors.

The most striking finding is the increase of the faster isozyme in LF and Reuber hepatomas with a concomitant decrease of the slower isozyme.

Experiments on normal liver, brain, and hepatoma after extraction in sucrose and Triton X-100 have demonstrated that the different subcellular repartition of hexosaminidase isozymes of normal liver and brain does not account for the modifications observed in hepatoma. These modifications seem to correspond to a true isozymic modification as proved by the different charge and different thermostability of these bands.

Our results on hexosaminidase show that during the cancerous process there is an increase of the free form of this enzyme; Lanzerotti and Gullino (14) have described a shift from bound to free form of 4 acid hydrolases during regression of an hormone-dependent rat mammary carcinoma. Nevertheless these authors have not separated the molecular forms of their hydrolases.

In fetal liver (16th day), we have found an isozymic pattern to a certain extent similar to the hepatoma one. At 19th day of gestation the relative intensity of Band A is feeble, and hexosaminidase isozymes appear to be intermediary between normal and cancerous ones. Moreover, there is generally a rough correlation between the growth rate of the tumors and the degree of isozymic abnormalities. The modification of hexosaminidase isozymes seems to give a new example of a general phenomenon, the resurgence of fetal molecular forms in cancerous tissues. We recall some other examples: aldolase (31, 33); hexokinases (35); glutaminases (11, 15); pyruvate kinase (3, 32, 37); uridine kinase (13); phosphorylase (29); and also the "brain pattern" of branched-chain amino acid transaminases (20). This modification confirms also the remark of Weinhouse (40) that "neither antigens nor isozymes are lost or gained absolutely but are altered in amounts."

In the livers of sham-operated and hepatectomized animals, stress may possibly explain the discrete and inconstant modifications that we found. Slight modifications have also been found for other enzymes, such as hexokinase (28) and aldolase (39) in regenerating liver, but not after sham operation. Nevertheless, there is a striking opposition between the almost normal regenerating liver pattern after hepatectomy or CCl4 injection and the strong isozymic modifications of fast-growing hepatoma. Consequently, it seems that the mechanism that causes the resurgence of fetal-type isozymes is not simply a consequence of the cellular multiplication.

The problem arises whether, in fetal liver and in hepatoma, the anodic form is synthetized by the same cells that synthetize the slow adult liver form. It is a very general problem, the solution of which is not perfectly clear at the present time. In some examples, hybridization between adult and fetal forms (for example between Aldolase B and Aldolase A) brings evidence in favor for the synthesis by the same cells (16, 19). In spite of an increasing number of experiments this problem has not been completely solved, even for α-fetoprotein (1, 2). If hybrids between the different types of hexosaminidase might be found in hepatomas, it would obviously be a strong argument in favor of the synthesis by the same cells. We recall the hypothesis formulated by Abelev (2) for the existence of a stem cell population already present in normal tissues, which would be the origin of abnormal synthesis (such as α-fetoprotein).

A last point must be mentioned. The fast anodic band could correspond to C band of human tissues because of their negative charges. But in this case, it is not known whether the slow band of rat tissue corresponds only to 1 or to 2 isozymes (A and B) closely related. Experiments performed to separate this band into 2 components have been unsuccessful; that is why we call the faster isozyme "A." We recall that, in human tissues, Isoenzymes A and C are simultaneously deficient in Tay-Sachs disease (9).

In conclusion, hexosaminidase offers a new example of fetal pattern in cancerous tissue; these findings strengthen the hypothesis of an impairment of the mechanisms that regulate the expression of genes in differentiated cells (40, 41), but the concepts of "repression" and "derepression" probably do no apply perfectly to eukaryotes (4, 41).

**ACKNOWLEDGMENTS**

We thank B. De Nechaud (Villejuif, France), Dr. C. Frayssinet (Villejuif, France), and Dr. M. D. Reuber (Bethesda, Md.) for the kind gift of hepatomas and Dr. F. Loisilier for the histological control of the tumors.

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1928 CANCER RESEARCH VOL 33
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Fig. 1. Hexosaminidase isozymes in rat tissues. Tissue samples were extracted in 4 volumes of an aqueous solution of 0.2% Triton X-100. The homogenates were centrifuged at 37,000 x g for 15 min at 4°C and then submitted to electrophoresis on cellulose acetate for 2 hr (see band) with 40 mM potassium phosphate buffer, pH 6.5, in the vessels. The staining substrate used for revelation was β-D-glucopyranoside (500 μM) in 40 mM citric phosphate buffer, pH 4.4.

Fig. 2. Hexosaminidase isozymes in adult and fetal liver in brain and in hepatoma. Hepatomas used were either transplanted, poorly differentiated, fast-growing hepatomas (Reuber and LF) or primary, well-differentiated, slow-growing hepatomas. Extraction and electrophoresis were performed as indicated in Fig. 1.
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