Studies on the Metabolism of Rat-Ascites-Tumors with Nitrogen Mustard Sensitive and Resistant Strains

IV. On the Distribution of Various Enzymes in the Cells of Normal Liver, Regenerating Liver and Hepatoma

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Since the development of the subcellular fractionation method, the fact that many enzymes are unequally distributed in the cells has now been firmly established by several investigators. Among them, C. de Duve and his group (1) have already reported intracellular distribution patterns of enzymes in normal rat liver tissues.

This paper is a comparative study on the intracellular distribution patterns of some enzymes with normal livers, regenerating livers and two strains of rat-ascites-hepatoma. These patterns were different for normal and regenerating livers as well as for two strains of DAB-hepatoma. However, the greatest difference was generally observed between normal liver and hepatoma even after taking account of the fundamental difference in the mass of the nuclei or mitochondria of those two tissues.

EXPERIMENTAL

The experiments were performed on the livers of albino rats weighing 100-120g. and on rat-ascites-hepatoma cells (AH 130 strain and AH 7974 strain) implanted in the abdominal cavity of albino rats. The hepatoma cells were harvested after 10-12 days implantation and homogenized with 0.25M sucrose and made 20% homogenates. Regenerating livers were made by the method of Higgins and Anderson (2) and used 48 hours after the surgical removal of a part of the livers. The subcellular fraction was carried out by the method described by Hogeboom (3). Nucleal, mitochondrial, microsomal and supernatant fractions were obtained by the differential centrifugation.

Enzyme Assays

Acid Phosphatase—The activity of this enzyme was measured by the method described by Ohmori and Fujita (4). To 1.0 ml. of substrate solution containing 0.001M p-nitrophenol phosphate and 0.1M acetate buffer (pH 5.0) was added 1.0 ml. of enzyme solution (in the form of homogenates or of separate subcellular fractions). The total volume of 2.0 ml. was incubated at 37°C for 30 minutes. The reaction was stopped by adding 2.0 ml. of 10% trichloroacetic acid. After centrifugation, 2.0 ml. of the supernatant solution was added to the same volume of saturated Na2CO3 solution. The color developed by p-nitrophenol liberated by phosphatase activity was measured by a Hitachi electrocolorimeter (Model FPW-4) with an S42 filter.

Aldolase—The activity of the enzyme was assayed by the method described by Sibley and Læhning e r (5). To 0.25 ml. of substrate solution containing 0.05M fructose-1, 6-diphosphate, 0.25 ml. of 0.56M hydrazine solution (pH 8.6) and 1.0 ml. of 0.1M Tris buffer (pH 8.6) was added. After thermal equilibrium at 36°C, 1.0 ml. of enzyme solution was added and kept at 36°C for 30 minutes. The reaction was stopped by adding 2.0 ml. of 10% trichloroacetic acid. After centrifugation, 1.0 ml. of the supernatant was transferred to a test tube containing 1 ml. of 0.75N NaOH solution. The mixture was kept at room temperature for 10 minutes. After the addition of 1.0 ml. of 0.1% dinitrophenyl hydrazine solution into the test tube, the mixture was incubated at 37°C for 10 minutes. Again 7.0 ml. of 0.75N NaOH was poured into the test tube. The developing brown violet color was measured by a Hitachi electrocolorimeter (Model FPW-4) with an S42 filter.

Alkaline Phosphatase—The procedure is essentially the same as that for acid phosphatase except that the substrate solution contains 0.001M p-nitrophenol phos-
phate, 0.01M MgCl₂ and 0.05M Veronal buffer (pH 9).

Arginase—Arginase activity was assayed in a modified Sakauchi reaction (6) to measure the residual arginine. The reaction medium was composed of 1.0 ml. of 0.001M arginine and 1 ml. of enzyme solution containing 0.005M MnSO₄ and 0.1M Veronal buffer (pH 9.0). The incubation was carried out at 37°C for 30 minutes, then stopped by the addition of 2.0 ml. of oxine-sulfosalicylic acid, 0.005M glycine and 0.05% oxine. The resulting precipitate was filtered off and 2.0 ml. of the filtrate was kept cold and added to 1 ml. of 2.5% NaOH. After allowing 15 minutes for chilling, 1.0 ml. of hypobromite (1% bromine in 5% NaOH) was mixed in and the total volume was made 10 ml. An orange color developed in several minutes and was measured by means of a Hitachi colorimeter (Model FPW-4) with an S₅₂ filter.

Catalase—For assaying the high activity of catalase in normal and regenerating livers, the following method (7) was adopted: To 20 ml. of cold 0.01N hydrogen peroxide in 0.007M phosphate buffer, 1 ml. of enzyme solution was added. 5 ml. of the aliquots were taken at 0, 3, 6, 9 minutes after the addition of the enzyme, and the aliquots were added to 5 ml. of 2N-H₂SO₄ and titrated with 0.005N potassium permanganate.

In the case of the low activity in tumor tissues, to 5 ml. of the aliquots were added 1 ml. of 5% KI and 0.5 ml. of saturated ammonium molybdate (8). The mixture was allowed to stand for 3 minutes, then titrated with 0.005N sodium thiosulfate. The calculation was carried out using the following equation.

\[ K = \frac{1}{t} \log \left( \frac{a}{a-x} \right) \]

K: first order velocity constant
a: initial concentration
x: concentration after t hours

Cathepsin—The activity of cathepsin was estimated by the method of Snöke, Neurath and Lowry (9). In 1 ml. of the substrate solution containing urea denaturated haemoglobin “Takeda” (0.25 g. of haemoglobin and 8.6 g. urea were dissolved in 14 ml. of 0.1N NaOH, incubated at 30°C for 30 minutes, and neutralized by 1N HCl (final pH 3.6)) was mixed 1 ml. of enzyme solution. (The solution was previously diluted with acetate buffer (pH 3.6) to the appropriate concentration. The final concentration of acetate buffer must be M/10.) The final concentration of acetate buffer was 0.6M trichloroacetic acid, the mixture was kept at 30°C for 30 minutes. The amino acid content in 1 ml. of the supernatant was measured by Folin-Ciocalteu’s method.

Cholin Esterase—The activity was estimated by the technique of Hestrin (10). 1 ml. of the substrate solution containing 0.2 ml. of 0.04M acetyl cholin-HCl and 0.8 ml. of 0.1M phosphate buffer (pH 7.2) and 1 ml. of the enzyme solution were mixed and incubated at 37°C. After 30 minutes, 4 ml. of 1M alkaline hydroxyamine solution was added to the incubation medium and soon after the medium was acidified by the addition of 2.0 ml. of 4N HCl, and the precipitates which occurred were filtered off. 4.0 ml. of the filtrate was mixed with 1 ml. of 0.37M FeCl₃. The brown-violet color developed by the reaction with the residual acetyl cholin was measured by means of a Hitachi colorimeter (Model FPW-4) with an S₅₂ filter.

Glutamic-oxaloacetic Transaminase—The assay method was that described by Caband, Leeper and Wrobleski (11). The total volume of 1.5 ml. medium contained 13.3 mg. Dl-aspartic acid, 3 mg. α-ketoglutarate, 10 mg. KH₂PO₄ and 0.5 ml. of enzyme solution. The pH was adjusted to pH 7.4. After incubation at 26°C for 20 minutes, the reaction was stopped by the addition of 2 gts. of 50% trichloroacetic acid. To the supernatant, 1 gtt. of anilin-citric acid mixture (5 ml. of water, 5 g. of citric acid and 5 ml. of aniline) was added and after 10 minutes, 0.5 ml. of 0.1% di-nitrophenylhydrazine-HCl was also pipetted into the supernatant. The resulting solution was kept at room temperature for 5 minutes, then 2.0 ml. of toluene was mixed with vigorous shaking. 1 ml. of toluene layer was added with 3 ml. of 2.5% KOH-alcohol and the brown color which developed was optically measured by a Hitachi colorimeter (Model FPW-4) with an S₅₂ filter.

Histidase—The activity of this enzyme was measured by the method described by Takeuchi (12) using the determination of the residual histidine. The reaction mixture was composed of 1 ml. of enzyme solution and 1 ml. of substrate solution containing 0.04 M histidine and 0.05 M phosphate buffer (pH 8.3). After incubation at 37°C for 1 hour, the reaction was stopped by 2.0 ml. of 16% trichloroacetic acid. 1 ml. of the filtrate was mixed with 1 ml. of cupric sulfate mixture (0.075% CuSO₄ 10 ml. + conc. H₂SO₄ 9 ml. + 1M glycine 10 ml. and sufficient water so as to make 100 ml.), 1 ml. of KBrO₃ and 0.5 ml. of saturated bromine water. Then the mixture was incubated at 37°C in the dark. After 1 hour, 3% arsenite solution was added to the mixture until the bromine color was disappeared. Then the mixture was extracted by 5.0 ml. of ethylacetate-xytol mixture (1:1). The aqueous layer and washings were combined and mixed with 2 ml. of 27.2% sodium acetate. The final volume
was made 10 ml. with water. After an incubation at
37°C for 10 minutes, a red color developed. The
optical measurement was carried out by a Hitachi
electrocolorimeter (Model FPW-4) with an S54 filter.

Peptidase—The enzymic activity was determined
by a method proposed by one of the authors (Furiya)
(13). 1 ml. of the substrate solution containing 0.001 M
Dl-leucyl-m-aminobenzoic acid and 0.05 M phosphate
buffer (pH 7.4) was mixed with 1 ml. of enzyme
solution and incubated at 37°C for 30 minutes. By
adding 1 ml. of 10% trichloroacetic acid, the reaction
was stopped and the protein fraction was filtered off.
To 1 ml. of the cold filtrate 5 gtt. of N·HCl was added.
Two minutes after the addition of 1 gtt. of 30% 
NaNO₂, 2 ml. of H acid-ammonium sulfite mixture
(0.05% H acid, 0.5% NaSO₃ and 2.5% ammonia) was
promptly mixed in. The total volume of the mix-
ture was made 10 ml. by adding water. After 10
minutes, the color of the diazotized dye was measured
by a Hitachi electrocolorimeter (Model FPW-4) with
an S53 filter.

Urocanase—The assay method of this enzyme was
that which Edlbacher (14) developed for the deter-
mination of the residual urocanic acid. 1 ml. of the
enzyme solution and 1 ml. of the substrate solution
containing 0.002 M urocanic acid and 0.02 M phosphate
buffer (pH 7.4) were mixed and incubated at 37°C
for 1 hour. The reaction was stopped by the addition
of 2.0 ml. of 16% trichloroacetic acid. The resulting
precipitate was filtered off, and 1 ml. of the filtrate
was neutralized by adding 1 ml. of 0.5 N NaOH. 1 ml.
of this solution was chilled, and then 5.0 ml. of 0.53%
Na₂CO₃ and 0.5 ml. of diazoreagent (2 volumes of
0.25% p-nitroaniline solution containing 0.03 N HCl
and 1 volume of 0.5% NaNO₂ were added). The
mixture was allowed to stand in the cold for 15
minutes. The resulting orange dye was extracted by
10.0 ml. of butanol. The optical density was measured
by a Hitachi electrocolorimeter (Model FPW-4) with
an S54 filter.

Units of Enzymes and Presentation of Results—To
simplify the construction of the table, the following symbols
will be used to designate the isolated fraction:
N = nuclear fraction; M = mitochondrial fraction; P =
particulates or microsomal fraction; S = final super-
natant.

The results of the enzyme distribution studies will
be expressed in units/mg. protein of enzyme contain-
ing fraction.

Except for cathepsin and catalase, one unit of
enzymic activity refers to the decomposition of 1 μmole
of substrate/hour under the conditions of the assay.
The molarity of the products of cathepsin action was
expressed conventionally in terms of tyrosine equiva-
lents of the color developed with the Folin-Ciocal-
teu reagent and one unit of catalase refers to the
hydrolysis of 1 μmole/min.

The distribution patterns of enzymes were expressed
by the method originally established by De Duve
(1). Fig. 1 is an example of such an expression.
Glutamic-oxaloacetic-transaminase of normal rat liver
(heavy line) and AH 7974 hepatoma (light line). Ordinate: mean relative specific activity of fractions.
The relative specific activity was expressed as the ratio
of the specific activity of each fraction per specific
activity of the original homogenate.

A unit is according by the specific activity of the
homogenate. Abscissa: fractions are represented by
their relative nitrogen content measured by the method
of Folin-Ciocalteu, in the order in which they
are isolated, i.e. from left to right: N, M, P, and S.

FIG. 1. The distribution patterns of glutamic-
oxaloacetic transaminase in normal liver and hepa-
toma (AH 7974).

The area of each block is thus proportional to
the percentage of activity recovered in the correspond-
ing fraction, and its height to the degree of purification
achieved over the homogenate.

RESULTS

General Survey of Quantitative Data—Specific
activities of the enzymes in homogenates of
normal and regenerating livers as well as hepa-
toma cells are recorded in Fig. 2.

In general, there are two groups of en-
zymes: those that show very weak activities
in cancer homogenates compared with the
normal or regenerating livers homogenates,
(e.g. arginase, catalase, glutamic-oxaloacetic-
transaminase, histidase and urocanase) and
those that have equal or sometimes more activi-
ity in cancer homogenates than in normal or regenerating livers homogenates. (e.g. aldolase, cathepsin and leucine amino-peptidase). In most cases, there are no distinct differences of specific activities between the AH 130 strain and the AH 7974 strain of hepatoma. However, in the case of alkaline phosphatase, the specific activities of this enzyme were quite low in AH 7974 homogenates whereas those in AH 130 homogenates were always high in every trial.

From the view point of the distribution patterns of enzymes (Fig. 3), one could divide the enzymes tested into the following three categories: Type 1, the distribution patterns are almost similar in hepatoma cells as well as in normal and regenerating liver cells. (e.g. aldolase, choline esterase, glutamic-oxaloacetic-transaminase and urocanicase). Type 2, the distribution patterns are similar in the two types of normal cells and in the two types of tumor cells, but entirely different patterns were observed if normal and tumor cells are compared to each other. (e.g. arginase, catalase and peptidase) Type 3, the distribution patterns are similar in normal and regenerating livers but different from strain to strain in tumors. (alkaline phosphatase).

**Detailed Analysis of Results Obtained for Each Enzyme**

**Aldolase**—There are several reports concerning the increased activity of this enzyme in cancer cells and in the serum of cancer patients. In our data also, normal liver homogenates showed the lowest activities compared with the proliferating tissues. However, the distribution patterns of this enzyme were rather similar in normal and neoplastic tissues, and everytime the supernatant fractions contained the highest activity of this enzyme. This fact might have some relationship to the high activity in the serum of cancer patients since the enzyme may leak from the cell-sap of cancer tissue by the change of permeability of cancer cells.

**Acid and Alkaline Phosphatase**—The specific activities of acid phosphatase in homogenates of tumor cells were comparatively lower than those in normal cells. The lower activities of the enzyme in particulates fractions are characteristic in neoplastic cells.

As noted before, the specific activities of the AH 130 homogenates were specifically high and the lowest activities were measured in the AH 7974 homogenates. The distribu-
FIG. 3. The distribution patterns of various enzymes in rat liver cells and hepatoma cells.
tion patterns of this enzyme are also characteristic in the AH 130 cells: very low activity was observed in the supernatant fraction whereas the highest activity was found in the microsomal fraction. This pattern is entirely different from that of AH 7974. Katayama already reported the higher activity of this enzyme in the nucleolar fraction of AH 130 compared with the same fraction of AH 7974 (17). Although there exists a general idea that different types of malignant tissues demonstrate rather similar protein and enzyme patterns (18), this observation may encourage our working-hypothesis that each strain of tumors has different metabolic patterns since they have a different number of chromosomes.

Arginase—The specific activities of these enzymes in cancer were about one hundredth of the normal tissues. The reason why the lower activity was observed in cancer was discussed by some authors (19).

The difference of the distribution patterns between normal tissues and cancer cells was striking. However, as Schneider, Laird and De Duve have already reported, the use of 0.25 M sucrose for the subcellular fractionation resulted the different distribution pattern of arginase (20, 21, 1). Carruthers adopted another fractionation method using glycerol and obtained rather different patterns compared to our results (22).

Catalase—Very low activity of catalase in cancer homogenates may be one of the characteristic features of neoplastic tissues. As the main localization of this enzyme is in the mitochondrial fraction, the weak activity of this enzyme may be attributed either to the decreased number of mitochondria in cancer cells or to the lower content of iron in cancer cells.

Cathepsin—There are no remarkable differences between the specific activities of the hepatoma homogenates and normal liver homogenates. Similarly the distribution patterns of cancer cells were fundamentally the same as those observed in normal livers.

Choline Esterase—There are conflicting reports on the activity of this enzyme in hepatoma: The high activities were observed in hepatoma in situ (23). In our case, comparatively lower activities were observed in ascites hepatoma homogenates. In the distribution pattern, the highest activities in microsomal fractions were characteristic in normal tissues.

Glutamic-oxaloacetic Transaminase—Although there are some reports that the activity of this enzyme in the sera of cancer patients increases according to the development of the tumor, our results show relatively lower activities in hepatoma homogenates. The distribution patterns were similar in normal and neoplastic cells.

Histidase—Like the previous reports by Masayama or by Kishi, the activity of this enzyme was scarcely detected in our experiment (24, 25).

Urocanicase—Voillier observed that an enzyme splitting imidazole ring was reduced in DAB hepatoma (26). As our method to measure urocanicase activity is based on the degree of splitting activity of the imidazole ring, both enzymes are presumably identical ones. This enzyme is located mainly in the supernatant fraction of normal and neoplastic tissues. However, small peaks were observed in cancer mitochondrial fractions.

DISCUSSION

De Duve proposed to divide the mitochondrial fraction into two subfractions. Applying this fractionation method, he proposed four groups of the distribution patterns of various enzymes. The first group comprises cytochrome oxidase, rhodanase and cytochrome reductase. All these enzymes have been found to be associated with the heavier cytoplasmic granules, and may therefore be considered as truly mitochondrial. In the enzyme studied, cathepsin might belong to this group. In the second group, which comprise the microsomal enzymes, are found glucose-6-phosphatase and special β-glucuronidase (opt. pH 5.2–5.3). Arginase and choline esterase in normal tissues seem to belong to this category. The third group of enzymes includes acid phosphatase, ribonuclease and deoxyribonuclease. The peak of specific activ-
Enzyme Distribution

1. The specific activities of a number of enzymes has been investigated in normal and regenerating rat liver homogenates as well as two strains of hepatoma homogenates. Arginase, catalase, glutamic-oxaloacetic-transaminase and histidase showed lower activities in cancer homogenates whereas aldolase was found more active in neoplastic tissue homogenates.

2. The intracellular distribution of the enzymes was compared and divided into three groups: Type 1 has a distribution pattern common to normal and regenerating liver as well as cancer cells (aldolase, choline esterase, glutamic-oxaloacetic-transaminase and uricase). Type 2 has a peculiar pattern for cancer cells (arginase, catalase and peptidase). Type 3 shows a common pattern for normal tissues but entirely different shapes will be observed in each strain of hepatoma (e.g. alkaline phosphatase).

3. In discussing these results, some conflicting evidence as compared with De Duve's work has also been brought to light.

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