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Intracellular Distribution of Proteolytic Enzymes in Rat Liver Tissue.* (23201)

JOHN T. FINKENSTAEDT (Introduced by C. N. H. Long)
Department of Biochemistry, Yale University, New Haven, Conn.

The presence of intracellular proteolytic activity in mitochondria isolated from rat liver homogenates has been demonstrated(1, 2). deDuve *et al.*(2) utilized a modified hemoglobin substrate that may have been attacked by many enzymes. Maver and Greco (1) used benzoyl-L-argininamide, but their mitochondrial preparations were contaminated with large cytoplasmic granules, probably nuclear material.

Two intracellular proteinases have been extensively purified and studied(3,4,5). These enzymes have been designated cathepsins B and C. Previous work has shown that a synthetic substrate suitable for the assay of cathepsin B is benzoyl-L-argininamide, while one for cathepsin C is glycyl-L-argininamide, while one for cathepsin C is glycyl-L-tyrosinamide. This paper offers additional information about localization of the intracellular proteolytic enzymes of rat liver obtained by using synthetic substrates and cellular fractionation procedures.

Materials and methods. Liver tissue from 170 g Wistar strain rats was used in all experiments. Four grams of liver were homogenized in 36 ml of sucrose solutions by the method of Potter and Elvehjem(6). The 0.25 M sucrose plus 7.5% polyvinylpyrrolidone (PVP) solution of Novikoff† and 0.88 M sucrose were used as fractionation media. Fractionation of the homogenates was obtained by differential centrifugation following the schemes presented by Schneider and Hogeboom(7) with minor modifications. Following fractionation, 5 components were available for testing: 1) homogenate, 2) nuclei and debris, 3) mitochondria, 4) microsomes, and 5) supernatant fluid. 0.88 M sucrose was added to bring the final volume of each fraction to 40 ml. Immediately thereafter, the enzyme activity was determined at 37°C in a reaction mixture with the following composition: 1) 0.4 ml of citrate buffer 0.04 M, 2) 0.1 ml of cysteine hydrochloride 0.04 M, 3) 0.2 ml of the above components. 4) 0.2 ml of substrate 0.05 M, and 5) sucrose to a final volume of 1 ml and a pH of 5. Samples were removed from the incubation mixtures at one and 2 hours for analysis in duplicate Conway vessels. The analysis was performed with the use of a glass electrode system and the addition of alkali by a microcapillary burette calibrated in lambdas(8). The substrates utilized were benzoyl-L-argininamide hydrochloride monohydrate (BAA), and glycyl-L-tyrosinamide acetate (GTAA). The rates of hydrolysis were linear in all instances. Results are listed as cathepsin units (C.U.). A cathepsin unit is defined as that amount of enzyme or enzyme-containing material that provides 1% hydrolysis per minute. Analysis for protein nitrogen was done by the micro Kjeldahl technic(8) or the biuret method(9).

Results. The data given in Table I indicate that the enzymes which hydrolyse BAA

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TABLE I. Cathepsin B and C Activity of Normal Rat Liver Fractions.

Fractions	N, mg/ml	Cathepsin B* A‡ C.U.	Cathepsin B* A‡ C.U./mg × 10^2	Cathepsin B* B§ C.U.	Cathepsin B* B§ C.U./mg × 10^2	Cathepsin C† A‡ C.U.	Cathepsin C† A‡ C.U./mg × 10^2	Cathepsin C† B§ C.U.	Cathepsin C† B§ C.U./mg × 10^2
Homogenate	16.0	.08	.5	.1	.6	.12	.7	.12	.7
Nuclei + debris	5.6	.04	.7	.04	.7	.03	.5	.03	.5
Mitochondria	3.4	.27	8.0	.29	8.5	.16	5.0	.17	5.0
Microsomes	2.4	.04	1.7	.03	1.3	.03	1.3	.04	1.7
Supernatant fluid	4.6	.04	.9	.03	.6	.03	.6	.03	.6
Mitochondria + microsomes		.26				.17			
Mitochondria + supernatant fluid		.04				.04			
Mitochondria + microsomes + supernatant fluid		.06				.05			

* Substrate, .05 M BAA. † Substrate, .05 M GTAA. ‡ .88 M sucrose fractionation.
§ .25 M–PVP sucrose fractionation.

and GTAA are localized in the mitochondrial fraction of liver homogenates. This hydrolytic activity is greater than the homogenate and/or any of the other fractions. This phenomenon may be the consequence of the removal of an inhibitor or inhibitors for the hydrolysis of GTAA and BAA present in the supernatant fluid of the same homogenates. Table I demonstrates this inhibition by showing the marked C.U. reduction produced by adding supernatant fluid to reaction mixtures containing mitochondria.

The inhibiting substance for the hydrolysis of BAA is stable to heat (90° for 10 minutes) and to freezing. Its activity is recovered in the dialysing fluid following dialysis against an equal volume of 1% sodium chloride for 24 hours at 0°C. This inhibiting activity is lost if the supernatant fluid is either treated with an equal volume of 12 N HCl at 110°C for 24 hours or reduced to an ash.

Additional studies indicate that the inhibitor present in the supernatant fluid is as effective in preventing the hydrolysis of BAA by partially purified cathepsin B as it is in preventing the hydrolysis of BAA by the mitochondrial fraction. It is far less effective in preventing the hydrolysis of BAA by crystalline trypsin. Crystalline soy bean trypsin inhibitor does not alter the hydrolysis of BAA by the mitochondrial fraction of rat liver homogenates.

The fractionation media utilized were selected because of their demonstrated ability to keep mitochondria as close to the intact state as possible. However, incubation media and conditions probably lead to disintegration of the mitochondria. Therefore, it is possible that hydrolytic activity for BAA and GTAA is associated with the mitochondrial fraction. It is not yet possible to postulate the exact position of this activity in or on the mitochondria.

Discussion. The results presented support the finding of deDuve showing that intracellular proteolytic activity is associated with the mitochondrial fraction of rat liver homogenates. Furthermore, the substrates utilized in these experiments are more specific for the cathepsins B and C of Fruton and others (3,4).

Studies on partially purified cathepsin B by Greenbaum and Fruton(3) have demonstrated that crystalline soy bean trypsin inhibitor had no significant effect on rate of action of cysteine activated cathepsin B on BAA. These investigators observed a nonlinearity between cathepsin B concentration and its activity. This was attributed to the presence, in spleen extracts, of some inhibitor.

The results here presented show that the mitochondrial substance acting on BAA has properties similar to cathepsin B as follows: 1) it hydrolyses BAA under the same conditions, 2) it is not inhibited by crystalline soy bean trypsin inhibitor, 3) it is inhibited by the supernatant fluid substance. The demonstration of this inhibitor for hydrolysis of

BAA may be an explanation for the findings of Greenbaum and Fruton.

Hydrolysis of GTAA by the mitochondrial fraction of liver homogenates suggests that cathepsin C is associated with this same particulate. Cathepsin C has an optimal hydrolytic activity at pH 5.2. From pH 6.6 to 7.8 extensive transamidation has been observed with progressively less hydrolysis(10). Using the preparations described in this article one will be able to test cytoplasmic particles for transamidation and other transfer reactions ascribed to the cathepsins. Their role in the nitrogen metabolism of normal cells may well be larger than the peptidase activity demonstrated by the results presented.

Summary. The mitochondria of rat liver homogenates contain proteinases similar to cathepsins B and C. An inhibitor for the hydrolytic activity of cathepsin B is present in the supernatant fluid of the same homogenates.

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In vitro Response of Functional Experimental Adrenal Tumors to Corticotropin (ACTH).* (23202)

ARTHUR I. COHEN, ERIC BLOCH† AND EVEMARIE CELOZZI
(Introduced by Jacob Furth)
Children's Cancer Research Foundation, Children's Medical Center, Boston, and the Worcester Foundation for Experimental Biology, Shrewsbury, Mass.

Two secretory adrenocortical tumors are being reported; one originated in an irradiated mouse and the other in a radiothyroidectomized rat(1). The animals bearing the tumor transplants secreted steroids with glucocorticoid and mineralocorticoid but no gonadal activities. The urine of tumor-bearing mice contained an increased amount of 17-ketosteroids, particularly the 11-oxygenated 17-ketosteroids (Bloch and Cohen, unpublished data). These tumors grew in hypophysectomized animals. The adrenals of unoperated hosts were markedly atrophic indicating inhibition of ACTH discharge. In the following experiments the *in vitro* responsiveness of mouse and rat adrenal tumors to ACTH was studied in comparison with normal adrenal tissues and the nature of the steroidal secretions was investigated.

Materials and methods. Adrenal tissues. The previously described mouse strain 2 and rat strain 1 adrenal tumors were used(1). Normal adrenals were obtained from 3-month-old animals of the same strains (LAF_1 mice and WR rats). All animals were killed by decapitation. Tumors measuring 2-4 cm in diameter were excised and placed in cold Krebs-Ringer bicarbonate buffer, pH 7.4, fortified with 200 mg % glucose(2). Tumor slices were cut free hand with a razor and placed in a Petri dish containing cold buffer.

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† Present address: Children's Cancer Research Fn., Boston, Mass.