Distribution of Hydrolases among Anterior Pituitary Cell Fractions (Anterior Pituitary Hydrolases).*

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The existence of intracellular particles with sedimentation characteristics intermediate between those of mitochondria and microsomes has been demonstrated in liver by studies of enzyme distribution among subcellular fractions (1–3), and the postulated intermediary particles were named "lysosomes" because of their manifold hydrolytic properties (1). The electron microscope shows that this intermediary fraction is composed primarily of small mitochondria and large microsomes, but Novikoff *et al.* (4) have also encountered therein a few dense, polymorphic particles, less than 0.5 micron in diameter, which are assumed to be the lysosomes. The fraction under consideration is distinguished by the highest specific activity for such hydrolytic enzymes as acid phosphatase, ribonuclease, deoxyribonuclease, uricase, and cathepsin among all cell fractions. Bennett (5) has suggested that the lysosomes may be an essential component of the phagocytic cells of the liver.

We have studied several hydrolases, as well as enzymes which in liver are localized in mitochondria or microsomes, in the subcellular fractions of pig anterior pituitary glands and have observed their distinct intracellular distribution.

Materials and Methods

Anterior lobes from fresh pig pituitaries were homogenized in the cold in 0.25 M sucrose containing 0.02 per cent heparin to make a 10 per cent (*w/v*) homogenate. *Nuclei, unbroken cells, and debris* were removed by spinning for 15 minutes in a model QV-110 International centrifuge at 1200 R.P.M. (approximately 700 *g*). The supernate was centrifuged at 7000 *g* for 20 minutes in a Spinco model L refrigerated centrifuge to give a milk white pellet, the *acidophilic granules*. Centrifugation of the resultant supernate at 34,000 *g* for 15 minutes yielded a tan-colored *mitochondrial* fraction. This fraction consisted of a firm pellet with an overlying fluffy layer. In these experiments the fluffy material was kept separate, and the labels *heavy* and *light mitochondria* are used to denote the firm pellet and fluff, respectively. The *microsomes* were collected as a red, translucent gel by centrifuging the supernate from the previous centrifugation at 78,000 *g* for 3 hours. The final *supernate* was a perfectly clear, reddish solution. The granules identified as basophilic in electron micrographs of sectioned pituitaries could not be found in homogenates. In the preparation of each of the particulate fractions, each pellet was rehomogenized and respun two or three times at slightly increasing speeds. No washings were discarded. (The techniques of centrifugation and morphological examination will be presented in detail elsewhere.) Nitrogen was determined by Nesslerization (6). Succinic dehydrogenase was assayed by the method of Schneider and Potter (7) except that adenosine-5-phosphate (0.012 M) and adenosine-5-triphosphate (0.012 M) were added to the reaction medium. Acid ribonuclease was determined as described by de Duve *et al.* (1) and acid proteinase by the method of Adams and Smith (8). Alkaline and acid phosphatase were assayed by measuring the release of inorganic phosphorus from beta-glycerophosphate (min. 99.9 per cent beta Eastman). The medium for determining alkaline phosphatase contained 1.0 ml. of 0.1 M boric acid-KCl-NaOH buffer at pH 9.8, 0.5 ml. of 0.2 M beta-glycerophosphate, tissue, and water to 2.0 ml. The assay of acid phosphatase differed from that for alkaline phosphatase in that the reaction medium contained 1.0 ml. of 0.1 M acetate buffer at pH 4.5. The reactions for phosphatases were stopped by the addition of 2 ml. of 10 per cent trichloroacetic acid, the tubes were centrifuged, and 2 ml. of the supernate were taken for the determination of inorganic phosphorus (9). Assays for phosphatases, ribonuclease, and proteinase were carried out in test-tubes incubated at 37°C. for 10 minutes, with occasional shaking in a final dilution of 2 ml. with 2 blanks and triplicate samples for each fraction in each test. The blanks were treated in the same manner as the regular assays, with the exception that the substrate was added after the reaction had been stopped by the addition of acid. The subcellular fractions were routinely frozen before enzyme assays were performed, since it was observed that maximal activity occurred only after such treatment.

Results and Discussion

The nitrogen content and total enzyme activities for each subcellular fraction are presented in Table I. The recoveries for nitrogen, acid proteinase, alkaline phosphatase, and acid phosphatase

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TABLE I

Intracellular Distribution of Nitrogen and Enzymatic Activities

Absolute values are presented for Experiment 1. (The recoveries for Experiment 2 were very similar, although the distribution of activities differed slightly as indicated in Fig. 1.)

The percentage distribution of nitrogen or a given activity was calculated on the basis of the recoverable activity.

Fraction	Nitrogen		Succinic dehydrogenase		Alkaline phosphatase	
	mg.	%	Units	%	Units	%
Homogenate	286	—	12,300	—	63,800	—
Nuclei	27.3	9.8	368	5.2	990	1.6
Acidophilic granules	25.3	9.3	75	1.1	600	1.0
Heavy mitochondria	26.8	9.6	1,950	27.6	7,100	11.5
Light mitochondria	36.8	13.2	3,300	46.8	17,600	28.6
Microsomes	24.4	8.8	132	1.9	21,700	35.2
Supernate	138	49.3	1,230	17.4	13,600	22.1
	278.6		7,055		61,590	
Recovery (per cent)	97.5		57.3		96.6	

Fraction	Acid Proteinase		Acid Phosphatase		Acid Ribonuclease	
	Units	%	Units	%	Units	%
Homogenate	30,300	—	84,600	—	72,000	—
Nuclei	1,100	3.4	8,920	10.9	1,980	4.4
Acidophilic granules	3,100	10.0	5,100	6.2	4,700	10.5
Heavy mitochondria	7,800	24.2	17,000	20.7	11,100	24.8
Light mitochondria	6,240	19.4	21,800	26.6	11,300	25.2
Microsomes	1,130	3.5	11,000	13.4	2,260	5.1
Supernate	12,800	39.5	18,400	22.2	13,500	30.0
	32,170		82,220		44,840	
Recovery (per cent)	106		97.3		62.2	

were very satisfactory; but only a little more than half of the activity of the whole homogenate was recovered for both succinic dehydrogenase and acid ribonuclease. The possibility of a low recovery of succinic dehydrogenase activity from subcellular fractions has been discussed (1, 10), and ascribed to the presence of an activator in the cytoplasmic fraction from which mitochondria were removed. A similar explanation might be advanced for explaining the low recovery for ribonuclease, although de Duve *et al.* (1) have reported

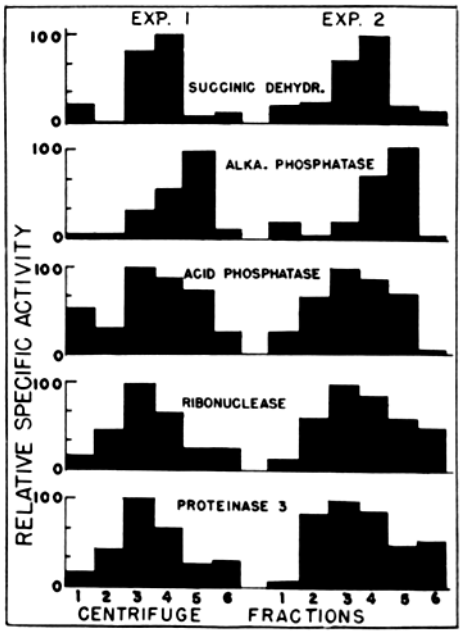

FIG. 1. Distribution of enzymatic activities among the subcellular fractions of pig anterior pituitary. Numbers 1 through 6 refer to *nuclear, acidophilic granule, heavy mitochondrial, light mitochondrial, microsomal,* and *supernatant* fractions, respectively. *Specific activity* was calculated as the number of activity units per milligram of nitrogen. For a given assay, the fraction with the highest specific activity was given a value of 100 and the other activities were referred to this value in determining the *relative specific activity.*

more complete recoveries in the case of this hydrolase from liver. It is possible that the low values for ribonuclease in the various fractions of pituitary represent an artifact in the assay method. Even with the addition of adenine nucleotides to the reaction medium, the recoveries of succinic dehydrogenase among the cell fractions are considerably lower than generally reported for this enzyme. It is possible that the low values reflect differences between pituitary and liver with respect to the reactivity of this enzyme among isolated cell fractions. It is interesting to note, however, that when the enzyme activity in each fraction is calculated on the basis of the recoverable activity, the percentage distribution of ribonuclease parallels quite closely that listed for acid proteinase, despite the low recovery of the former.

The enzymes studied demonstrate three patterns of distribution among the cytoplasmic fractions (Fig. 1). The specific activity of succinic dehydrogenase is highest in the light mitochondrial fraction; alkaline phosphatase activity is highest in the microsomal fraction; while acid phospha-

tase, acid ribonuclease, and acid proteinase show greatest activity in the heavy mitochondrial fraction. Succinic dehydrogenase activity is limited almost exclusively to the two mitochondrial fractions, while the hydrolases are spread over several fractions, indicating that they are associated with particle types which show considerable variation in size. Although we have been able to detect high uricase activity in liver, this enzyme could not be detected in the anterior pituitary. The present findings agree with the general opinion that, for liver at least, succinic dehydrogenase and alkaline phosphatase are localized in mitochondria and microsomes, respectively. The striking difference between anterior pituitary and liver is that in the latter organ the hydrolases, described above as components of the lysosomal elements, demonstrate highest specific activities in the lighter mitochondrial fraction rather than the heavier fraction as we have observed for the anterior pituitary.

Freezing and thawing, aging, or incubation in distilled water of subcellular fractions from anterior pituitary result in an increase in the activity of these hydrolytic enzymes, indicating that they are "bound" in the intact cell or that they are contained in a semipermeable sac as suggested by de Duve for liver hydrolases (1).

The variable enzyme activity seen in the two experiments in the nuclear fraction (Fig. 1) may be a direct reflection on the number of unbroken cells. The high activity of each enzyme in the supernate, usually 20 to 35 per cent of the total in the case of the hydrolases, is probably the result of rupture of cell particles during homogenization and centrifugation.

The gravitational forces employed for isolating mitochondria and microsomes from anterior pituitary are higher than those generally reported for separating liver fractions. When lower forces are used for preparing mitochondria, the time of centrifugation was significantly prolonged for complete sedimentation of succinic dehydrogenase activity. The greater centrifugal force resulted in an initially higher degree of microsomal contamination in the mitochondrial fraction, but this was considerably reduced by subsequent washings. The maximal centrifugal force of the available equipment was utilized in isolating the final particulate fraction merely to reduce the time for total recovery of the PNA-containing particles.

On the basis of the present findings, it appears that hydrolytic enzymes are segregated in distinct particles in other tissues besides liver.

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